Role of Macroporous Matrix in Stem Cell Differentiation

and Tissue Engineering Applications

THESIS

Submitted by

DEEPTI SINGH

For the Award of the Degree

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DR.MGR UNIVERSITY
(Declared u/s 3 of the UGC Act, 1956)

CHENNAI 600 095

November-2010
APPENDIX – VII
(Ref: Regulations: VII b (i))
CERTIFICATION BY THE SUPERVISOR

I certify that the thesis entitled ‘Role of macroporous matrix in stem cell differentiation and tissue engineering application’ submitted for the degree of Doctor of philosophy by Miss. Deepti Singh, is a bonafide record of research work carried out by her during the period from August 2007 to November 2010 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associate – ship, fellowship or other titles of this University or any other university or Institution of Higher Learning.

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DEEPTI SINGH
DEDICATION

This Thesis is dedicated to my parents and my beloved sister Mitu. I know Mit you are there with me every time I look up at sky I see you shine for me.
The biophysical nature of the cellular microenvironment in combination with its biochemical properties can significantly modulate the outcome of three-dimensional (3-D) multi-cellular morphogenesis. This is critically important in the field of tissue engineering where the design of the scaffolds is intimately linked to the functional output of the cells. Three-dimensional polymeric biomaterials are often used as cell scaffolds in tissue engineering to construct specific tissue or organ via mimicking functions of the native extra-cellular membrane. The artificial extracellular matrix (ECM) is a functionally bioactive material that can spatially orchestrate cell location and temporally regulates cell phenotype. We have focused on developing cryogel scaffold which is blend of polymer of 2-hydroxyethylmethacrylate (synthetic polymer) – gelatin (natural polymer) (HG) and explored its potential in cartilage and cardiac tissue engineering. Cryogel are synthesized below the bulk freezing temperature of the reaction system. A macroporous structure in the final material appears due to the existence of ice crystals acting as template for the formation of the pores. This technique involves the cross-linking co-polymerization of the monomer and the cross-linkers mix in the presence inert diluents. To obtain Macroporous structure phase separation has to occur during the course of the network formation process. After the polymerization, the diluent is removed from the network, leaving a porous structure within the highly cross-linked polymer network. The mechanical strength of the scaffold eventually defines fate of cells as during development, cells are assembled into coherent grouping by virtue of cell-cell and cell-matrix interaction and the fate of the cells depends upon the metabolic and mechanical factors surrounding these cells. Exploring this principle, the surface charges of the synthesised HG cryogel were altered using high energy plasma polymers of allylamine and human mesenchymal stem cells (MSCs) were seeded on these surface modified 3-D matrices. The results showed chondrogenic differentiation of MSCs which was confirmed by gene and protein expression. The microenvironment in which stem
cells are cultured regulates the fate of stem cells and by altering the surface chemistry and charges showed specific or desired differentiation of the MSCs in the 3-D matrix. This is further explored by co-culturing the MSCs with foetal limb cells on HG cryogels and it was found that MSCs co-cultured in static and hypoxia environment expressed chondrogenic differentiation in both conditions. The matrix architecture along with effect of surrounding cells and cell-matrix interaction induces the differentiation cascade in the stem cells. The concept of differentiation pattern of the stem cells depending upon the microenvironment can be extensively used in the \textit{in-vivo} tissue regeneration. Stem cells incorporated scaffolds can be implanted \textit{in-vivo} and depending upon the site of implantation, the stem cells can be signalled for differentiation thereby leading to tissue regeneration. Investigating the potential of the synthesised HG cryogel in other tissue engineering field, myoblast skeletal cells were cultured for over three weeks and cells were seen to align in preferential direction leading to the formation of myotubes in the 3-D matrix whereas in 2-D culture, due to less surface area cells were seen to aggregate and de-attach. The premise is that to unlock the potential of multipotent stem cells, at least some aspects of the 3-D environments that are related with their renewal, differentiation and assembly in native tissues needs to be reconstructed. In the general context of tissue engineering, we have utilized the environments for guiding stem cell differentiation by an interactive use of biomaterial scaffolds and focussed on the interplay between cell and scaffold for development of bioactive material for tissue engineering purposes.

\textbf{Key Words:} Chondrocytes, Chondrogenesis, Differentiation, Macroporous cryogel scaffolds, Mesenchymal stem cells, Microenvironment, Myoblast skeletal cells, Plasma polymerization, Tissue engineering.
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<td>2-D</td>
<td>Two-dimensional</td>
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<tr>
<td>3-D</td>
<td>Three-dimensional</td>
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<tr>
<td>µ-CT</td>
<td>Micro-Computed tomography</td>
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<tr>
<td>µL</td>
<td>Microliter</td>
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<tr>
<td>a-KG</td>
<td>Alpha Ketoglutarate</td>
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<tr>
<td>Amp</td>
<td>Ampicillin</td>
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<tr>
<td>APS</td>
<td>Ammonium per sulphate</td>
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<tr>
<td>CO2</td>
<td>Carbon-dioxide</td>
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<tr>
<td>COL I</td>
<td>Collagen type I</td>
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<tr>
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<td>Collagen type II</td>
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<tr>
<td>COMP</td>
<td>Cartilage oligomeric matrix protein</td>
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<tr>
<td>DAB</td>
<td>p-Dimethylaminobenzaldehyde</td>
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<tr>
<td>DAPI</td>
<td>4’, 6-Diamidino-2-phenylindole</td>
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<tr>
<td>dH₂O</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DMSO</td>
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<td>DNA</td>
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<td>DNSA</td>
<td>Dinitrosalicyclic acid</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EDTA</td>
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<td>EtBr</td>
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<td>FBS</td>
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<td>hMSCs</td>
<td>Human mesenchymal stem cells</td>
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</tr>
<tr>
<td>ml</td>
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<tr>
<td>MSCGM</td>
<td>Mesenchymal stem cell growth medium</td>
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<tr>
<td>MTT</td>
<td>3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide</td>
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**Figure 5.46:** Cell attachment was visualized under fluorescent microscopy by using nuclear stain DAPI (A). The image was recorded after 48 h of cell seeding and (B) after 2 weeks. The fluorescent microscopy images show significant increase in cell number and proliferation of C2C12 on HG cryogel.
CHAPTER I

INTRODUCTION

1.0 INTRODUCTION
Stem cells has attracted an enormous amount of attention in recent times as it is hoped to prove a potent treatment for various incurable diseases via cell or transplantation therapy (Figure 1.1). Stem cells can be broadly divided into two category foetal/ embryonic and adult stem cells. Embryonic stem cells are totipotent and are involved in the formation of the entire organism (Brivanlou et al, 2003), but due to various ethical issues scientists have focussed on isolating and manipulating adult stem cells in the in-vitro conditions for further use in regenerative medicine. One important reason why stem cells caught imagination of researchers worldwide is the promise that understanding the inimitable properties of these cells may provide insight into the biological aspects of cells leading a path towards the treatments for variety of degenerative/ incurable diseases (Melton and Chow, 2004).

![The Promise of Stem Cell Research](Figure 1.1: The potential application of stem cells to differentiate into different lineages of cells that can be used in several incurable diseases either by generation of functional tissue or using stem cells for cell based therapy (http://stemcells.nih.gov/info/media/promise.html)).

1.1 Definition and classification of stem cells
Stem cells can be defined as “cells that has the capacity to self-renew as well as the ability to generate differentiated cells” (Weismann et al, 2001; Smith, 2001). Stem cells are undefined cells in body that retain the capacity of differentiating into specialized cells with new specific cell functions (Polak and Bishop, 2006). The remarkable capability of these cells to develop into any cell/tissue type during course of development has captured the attention of researcher world-wide (Papaioannou, 2007). These cells act as an internal repair system replenishing the tissue without limit through life term of the organism (Kooy and Weiss, 2000). One of the common example is bone marrow stem cells which remains unspecialized however, these cells differentiate into more specialized cells like, RBCs, WBCs and other blood cells with specific functions of supplying oxygen, producing antibodies and to combat infection acting as scavenging agents, respectively (Iscove and Nawa, 1997). One cell stems out from another and thus defined as ‘STEM CELLS’. Stem cells can divide into daughter cells which can be identical to mother cells (self-renewal) as well as generate progeny with more restricted potential which are differentiated cells. But this definition can break down when foetal and adult stem cells needs to be distinguished as adult stem cells have a reduced self-renewal capacity. This can be defined in simplest terms as cells that self-renew throughout one’s life span as mostly stem cells are defined as immortal or with unlimited capacity of proliferation which does not hold good as these cells after 160 passage reaches senescence (Sherr and DePinho, 2000; Houch et al, 1971). Second important parameter that is used in classifying stem cells is the differentiation potency of the cells. Stem cells that differentiate into all three germ layers (ecto, meso and endoderm) are known as ‘pluripotent’. Stem cells that differentiate into multiple cells are known as ‘multipotent’ (Figure 1.2) and stem cell that can differentiate into one linage are referred as ‘unipotent’ (Potten and Loeffler, 1990). Thus a complete description of stem cells includes potency, clonality and replication ability. The working definition of stem cells can be “stem cells are clonal, self-renewing entity that is multipotent and can generate several differentiated cell types” (Melton et al, 2004).
CLASSIFICATION OF STEM CELLS

Figure 1.2: Classification of stem cells based on their potency (Ref: www.stemcells.bajamx.com).

1.1.1 Embryonic stem cells

These cells originate from the primordial germ cells of the gonad ridge of 5-6 weeks old foetus and are pluripotent (Edward, 2001). However, the fertilized blastomere and oocytes cannot be termed as “stem cells” as there is limited number of cells and cannot self-renew even though it holds the capacity of forming entire organism. The inner cell mass (ICM) isolated from the blasotcytes (5-6 days old embryo) is rich source of pluripotent embryonic stem cells (ESCs) (Evans and Kauffman, 1981). During the development stage of embryo the ICM differentiate into two distinctive layers i.e., hypoblast and epiblast. The hypoblast forms the yolk sac and becomes redundant in humans and epiblast goes on to form the three germ layers (ecto, meso and endoderm) (Edward, 2004). A very small population of multipotent cells called ‘definitive endoderm’ goes on to form all the endoderm dependent organs in humans and these populations of cells are separated from the pluripotent ICM during gastrulation directly
after implantation (Papaioanou, 2001). There are wide number of applications that ESCs can be exploited using technologies like cell therapy, in-vitro modelling for diseases etc (Figure 1.3).

**APPLICATION OF EMBRYONIC STEM CELLS**

![Diagram showing applications of human embryonic stem cells (hESCs) (Ref: Cervera and Stojkovic, 2008).](image)

**Figure 1.3**: Applications of human embryonic stem cells (hESCs) (Ref: Cervera and Stojkovic, 2008).

1.1.2 *Adult stem cells*
In early 1950-60s all the cells with unlimited proliferative rate were referred to as stem cells until groundbreaking paper by Till and McCulloch (1961), showed first evidence that in bone marrow not all the proliferative cells were identical. Basic characteristic of these cells are single cells (clonal) that generates differentiated cells which are capable of self-renewal. The two most crucial concepts that moved adult stem cells to forefront of the research is the plasticity of these cells that helped in overcoming the idea of restriction of cell fate which is regarded as permanent phenomena but the fate of these adult stem cells are easily flexible and reversible. The model example of reversing the fate of differentiated cells comes from the experiment where the terminally differentiated somatic cells fashioned into another animal via cloning or nuclear transfer (Rideout et al, 2001). This experiment proved that even terminally differentiated cells can be returned to most primal state if provided with the appropriate signals (Liu et al, 2003; Blau et al, 2001).

1.1.3 Hematopoietic blood stem cells

The recent isolation of a wide range of somatically derived stem cells has confirmed the concept that homeostatic maintenance of most organs and tissues is mediated by tissue-specific progenitor and stem cells which has fuelled enthusiasm of using these cells in strategies aimed at replacing or repairing diseased, damaged or genetically deficient tissues and organs (Bryder et al, 2006). Hematopoietic stem cells (HSCs) are arguably the best-characterized tissue-specific stem cell, with basic researcher investing more than a decade for the evaluation and clinical application providing profound understanding of the principles of stem cell biology (Kovacic and Boehm, 2009). The hematopoietic stem cells could be defined after 5 decade of evaluation as stem cells retrospectively, as these are functional unit that give rise to 10 lineages of matured cells (Figure 1.4) (Rathinam and Flavell, 2008) and prospectively these are rare cells that can be purified by physical means (Spangrude et al, 1988). Haematopoiesis is defined as mechanism through which body produces and maintains the blood stem cells and also proliferate and differentiate.
cells of peripheral blood. Blood cells are responsible for maintaining and providing the immunity to every organ of the body (Davidson and Zon, 2000). Evidence of the stem cells presence in vascular system came from studies performed on patients affected with high dose of irradiation in 1945 (Umansky et al, 2006). But it was in 1960, studies performed by Till and McCulloch (1961) who analyzed the bone marrow to identify the component primarily responsible for regeneration of over 50 million new cells per day.

**LINEAGE OF HEMATOPOIETIC STEM CELLS**

![Image of lineage of hematopoietic stem cells](image)

**Figure 1.4:** The lineage of HSCs defining the self-renewal and differentiation capacity of the HSCs into specific functional blood cells (Ref: Metcalf, 2001).

1.1.4 *Mesenchymal stem cells (bone marrow stromal)*
Mesenchymal stem cells (MSCs) originate postnatally in the bone marrow (BM) stroma in non-hematopoietic region and are made of heterogeneous population of cells. MSCs were first described by Fridenshtein (1982) as ‘cells capable of creating fibroblast-like colonies that could differentiate into adipocytes and osteocytes’. After isolation of these MSCs from BM and bone tissue antigenic determinates has been defined which allows the selection of MSCs from human BM and rodent to almost homogeneity (Gronthos et al, 2003). Using various techniques like cell sorting and ring cloning, researchers have shown that MSCs can be differentiated not only to adipocytes and osteocytes (Figure 1.5) but also to skeletal myocytes, chondrocytes and smooth muscle myocytes (Pittenger et al, 1999; Wakitani et al, 1995). The in-vivo grafting of these MSCs to bone defects or cartilaginous lesions resulted in tissue specific differentiation of the MSCs (Gao et al, 2001).

**MESENCHYMAL STEM CELLS**

![Diagram of MSCs differentiation](image)

**Figure 1.5:** Lineage of MSCs and potential application of the adult stem cells in the field of regenerative medicine.

1.2 Potential applications of the adult stem cells
It is envisaged that one day stem cells will be used to replace defective parts of body by creating bioartificial tissues or in-vivo infusion. This may be possible in future but the immediate potential benefit of these stem cells is that they present powerful tools for studying self-renewal and differentiation properties of cells. Studying the progenitor cells and their intermediate progeny enables researchers to explore the conditions that are required in development of functional organs or tissues (Reyes et al, 2002). Stem cells and their progeny (differentiated cells) can be used in defining genetic program that can be activated or inactivated for cell differentiation to occur giving further insight into developmental cascade and thereby helping in creating proteins or molecules that can be used in activating the developmental program. Understanding the underlying genetic mechanisms which defines the stem cells plasticity properties like re and de-differentiation of cells can be invaluable (Lakhmipathy and Verfaillie, 2005). “Cell programming” has already proven of great importance and has made way to clinical setting in which the demethylation and histone deacetylases agents are used to reactive foetal haemoglobin of patients with hemoglobinopathies (Dover et al, 1983). Adult stem cells have greater potential and can be used in treating the degenerative and genetic diseases in any organ. Undifferentiated adult stem cells can also be used in-vitro condition to develop specific tissue as there is so far no evidence suggesting that undifferentiated stem cells can lead to tumour formation or any pathological disorders (Leri and Kajstura, 2005).

These adult stem cells might be used as autologous grafts but to use and manipulate these cells in lab conditions to generate a functional tissue requires other field of biology to be combined. Engineering the environment or providing these stem cells with right signalling molecules for differentiation can be achieved by using tissue engineering approaches.

1.3 Tissue engineering concepts and principles
Tissue engineering concept originated from the thought of engineering ‘tissue’ and was articulated by Y.C. Fung in 1985. In the first symposium held under name of tissue engineering in 1988, the working definition was proposed as “The application of the principles and methods of engineering and life sciences towards the fundamental understanding of structure-function relationships in normal and pathological mammalian tissue and the development of biological substitutes to restore, maintain, or improve tissue functions”.

![Figure 1.6: Step-wise explanation of the concept of tissue engineering (Image modified from http://www.centropede.com/UKSB2006/ePoster).](image)

Tissue is a derivative of french word which mean ‘to weave’ and basic components of the tissue “fabric” is cells and the matrix secreted by cells, called as extra cellular matrix
(ECM) and collectively these two components defines the functionality of the tissue (Figure 1.6). Cells constantly produce and degrade the ECM to give the basic geometric structure to tissues and provide many essential tissue properties (Palsson et al, 2004). To mimic this ECM in the laboratory conditions and provide the 3-D architecture to cells, biomaterials or scaffolds are being designed using various natural and synthetic polymers. Over the years, there have been various techniques that have been used in designing polymeric scaffolds that can provide the mechanical strength to support cells and also can be engineered for modifying the cellular fate and responses. Biomaterials have gained importance in biomedical applications and the interpretation of definition of biomaterial differs in clinical medicine and in material sciences (Roach et al, 2007). Biomaterial can be defined as “synthetic material that is used to replace part of living system or to function in intimate contact with living tissue” (Shi et al, 2006). Biomaterial is interdisciplinary area which requires the knowledge of 1) material engineering and sciences involves the inter-relationship of biological material (ceramic, metals composites and polymers) and synthetic materials, 2) physiology and biology of cells, anatomy and molecular biology and 3) clinical sciences (e.g. cardiovascular surgery, neurosurgery and histopathology). Rapid development of biomaterials is attributed to advancement in the field of tissue engineering (Donaruma, 2004). The basic focus of the researchers has been in developing biomaterial that is compatible, mechanically stable and bioactive. New challenge in this area is controlling microstructure, chemical, biological and physical behaviour of biomaterials as medical field places new demand in these aspects (Shi et al, 2006). Most fascinating development in the field of biomaterials is found in tissue engineering which involves direct usage of biomaterials in biological systems (e.g. skin tissue engineering grafts, temporary liver support, etc.). Fabrication of organ-scale construction with cellular resolution, incorporating functional cells yielding to 3-D structure for tissue function is the biggest challenge faced by tissue engineers (Shi et al, 2006).
Two typical tissue engineering components comprise scaffolds and cells. Scaffold provides the structural support whereas cells perform the biological functions and the interaction between these two components helps in successful integration of the construct to the native tissue with negligible immune-response, desired vascularisation by control signalling between the host and construct for maintenance or restoration of biological function of the tissue (Anselme, 2000). Since the scaffold plays an integral part in replacing or repairing of damaged or missing tissue, researchers over the years have developed different methodology for designing these 3-D structure and these can be developed with following properties (Figure 1.7).

![Ideal properties of 3-D scaffold for tissue engineering](image)

**Figure 1.7**: Property of an ideal 3-D scaffold for tissue engineering application.

With all these properties the ultimate aim of scaffold designing is not just providing structural support but also to facilitate exchange of physio-chemical signals from the environment of surrounding cells and host (Hubbell, 1995).
1.4 Definition and requirement of ideal scaffold in tissue engineering

A scaffold in an artificial 3-D frame that mimics the ECM for cell attachment, migration, proliferation and tissue regeneration, while architecture and microstructure of these matrices ultimately defines the shape of the regenerated tissue or organ. Tissue engineering scaffold is expected to have following characteristics irrespective of its application (Mayer et al, 2000; Atala, 2000; Peter et al, 1998; Hubbell, 1995)

i) Biocompatible: should not be detected as foreign body during the implantation which causes immunological response

ii) Porous structure: should have interconnected porous network for cell growth and migration and facilitates easy transport of media and exchange of gaseous

iii) Surface chemistry: the surface of the scaffold should help in cell attachment and signalling for proliferation and differentiation

iv) Degradable: rate of degradation or resorption should be in-tune with in-vitro/in-vivo/ex-vivo tissue regeneration

v) Mechanical stability: mechanical strength of the scaffold should match the strength of normal tissue or organ of interest

vi) Bioactive: should encourage tissue regeneration

vii) Shape: 3-D design should be capable of regeneration of tissue in normal physiological shape.

1.5 Methodology for scaffold designing

Researchers over the years have used different techniques for designing artificial matrix and some of the most popularly used techniques are
1.5.1 **Solvent casting**

This methodology exploits the evaporative property of some solvents for designing the scaffold by one or other way. One way is to dip a mould into the polymer solution and by allowing sufficient time to drain off the solvent leading to formation of polymeric membrane layer. Second approach is pouring polymer solution in mould and allowing the solvent to vaporize over sufficient time which leaves polymeric membrane adhering to the mould (Mikos, 1993). This is a very easy, inexpensive technique as it does not require any specialized equipments. The rate of degradation does not have effect on the tissue regeneration. Disadvantages of solvent casting are the use of highly toxic solvents that can denature the incorporated bioactive molecules and proteins. The possibility of solvent retention is high but it can be overcome by completely drying the membrane. However, this is highly time consuming and labour intensive method of scaffold fabrication (Mikos, 1993). Researchers have combined different techniques to overcome the disadvantage of this technique but it can be used successfully only in the fabrication of thin films.

1.5.2 **Particulate leaching**

This is one of most popular approaches used by tissue engineers for porous scaffold fabrication. The pores are created by porogens (salts, wax or sugar). In brief, the polymer solution is poured into the mold which has prearranged porogens with desired size, shape and orientation and as the solvent evaporates the polymer/porogen composite is formed (Sachlos and Czernuszka, 2005). Once these composites are formed the porogens are leached out (e.g. water can be used to leach sugar and salt out). Depending upon the size and shape of the porogen the different microstructure of the scaffolds can be fabricated and depending upon the usage of porogen three types of leaching technique are, particle leaching, fibre leaching and ball leaching (Mikos et al, 1993) (Figure 1.8). The major disadvantage of this system is similar to that of solvent casting: Organic solvents that are used precludes the possible addition of pharmacological agents during the fabrication of
scaffold. Also, the leaching step for water-soluble porogens considerably increases the scaffold preparation time (Freed et al, 1999; Ma et al, 1999).

![Figure 1.8](image-url): Overview of salt and particle leaching methods for fabrication of scaffold (modified from Skoplyak, 2008).

### 1.5.3 Fabrication of scaffold using textile technologies

Textile techniques have been employed in preparing biodegradable polymeric scaffolds. Non-woven and woven scaffolds can be formed by this technique using fibers. These fibrous scaffolds are highly porous with interconnected pores which are desired property in tissue engineering (Cima et al, 1991). In case of woven scaffold the pore sizes are too large for cell attachment, even though this may not be an issue in non-woven as the pore size and porosity is controlled by degree of compression of fibers. However, the mechanical strength is low and pore morphology is difficult to control and there are limited variations in fiber diameter (Hacker et al, 2007).

### 1.5.4 Electrospinning
This technique is known since 1934, when the first patent was filed by Foramhals, ever since then the technique has evolved to great extent with Freudenberg Inc commercializing an ultrahigh-efficiency filter prepared using electrospinning technology also known as ‘Hybrid Bicomponent Fibers (BCF)’ (Groitzsch et al, 1986). The diameter of fibers formed by wet or melt spinning is determined by size of nozzles of spinneret and volume of solution drawn and stretch applied prior to wind-up. Conventional fibers diameter falls in range of 10 µm for multifilament and around 500 µm for thicker filaments (Gentsch and Borner, 2010). Polymer solution is exposed to electrostatic field at high voltage (5-30 KV) that helps in overcoming the surface tension exerted by polymer and this accelerates fine flow of polymer solution to the target (Reneker et al, 2000). As this polymer jet cools or losses solvent there is a series of unstable loops that are generated which further solidifies and collected as interconnected mesh of fine fibers on special target or grounded rotating drum. Polymer chemistry, solution viscosity and uniformity of electric field plays very important role in deciding the fineness of the final fiber (Figure 1.9).

1.5.5 Hydrogel

Hydrogel is water-swollen structure of cross-linked polymeric chain containing either the covalent bonds which are produced by simple reaction between one or more co monomers. They can be physically entangled or linked by strong van der waals interaction or hydrogen bonds between the chains (Pappas, 1987), or the crystallites of the polymers can bring together two or more macromolecular chains (Hickey et al, 1995). Depending upon preparative methodology hydrogels can be classified as homopolymer hydrogel, multipolymer hydrogel, co-polymer hydrogel and interpenetrating hydrogels. Based on the ionic charges, hydrogels can be neutral hydrogel, cationic hydrogel, anionic hydrogel and ampholytic hydrogels (Ratner, 1976). Last depending upon the physical structures, the hydrogels can be amorphous or semicrystalline, hydrogen-bonded or
complexation structure. The hydrogen bond and complexation are majorly responsible for the final 3-D structure of hydrogels.

Figure 1.9: Schematic representation of steps involved in synthesis of electro-spinning fibers (modified from http://www.amyshah.com/tissue-engineering/electrospinning).

Hydrogels are prepared by swelling polymer/monomer solutions in biologic fluid or water and cross-linking of polymers can be done by chemical, photopolymerization or irradiation of the initial network of polymer units. Cross-linking indicates the connection points of long or several polymer chains (Wheeldon et al, 2007). Since the solvent used in preparing these hydrogels are water and biological fluid, the material exhibit high biocompatibility and a much desired property for tissue engineering and biomedical
application. However, the structural evaluation reveals that the ideal network is rarely observed and hydrogel with molecular defect can always be possible that is the unreacted functionalities with partial entanglement or chain loop formation, neither of two contributes to the structural stability or mechanical integrity of the hydrogel (Peppas, 1987).

1.6 Cryogels

Macroporous polymeric materials have gained interest in the biomedical and biotechnology applications. There are different fabrication methodologies through which macroporous structure can be obtained, 1) freeze-drying 2) porogenation 3) phase separation 4) cryogelation. Among all these methods cryogelation has emerged as most widely used approach for synthesis of macroporous polymeric matrix due to easy fabrication methods to obtain open porous structure with well controlled porosity (Lozinsky et al, 2002). Cryogelation technique provides possibility to prepare macroporous hydrogels with wide range of porosities compared to any gel-forming system (Lozinsky et al, 1998).

Cryo-gelation takes place via cryogenic treatment of the systems containing aqueous solvent and monomer/polymer precursors. The crystallization of the solvent is the critical feature of cryogelation, which differentiates cryogelation from freeze induced gelation. Depending upon the polymer precursor the pore size ranges from 0.1 μm to 200 μm. Cryogels are produced via a gelation process at sub-zero temperatures where most of the solvents freezes and dissolved polymeric precursors concentrates in small nonfrozen regions (nonfrozen liquid microphase), where the chemical reaction and gel formation proceeds over the set period of time. While all the reagents concentrate in nonfrozen liquid microphase some portion of solvent remains unfrozen and this provides space for solute to concentrate into nonfrozen part with sufficient segmental or molecular mobility for reactions to perform. After melting the solvent crystals (in case aqueous media it will be ice), a large continuous interconnected pores are formed. Shape and size of the pores
generated directly depends upon the shape and size of the crystals formed during freezing (Figure 1.10). Other factors that contribute to the pore size is rate of freezing, freezing temperature, initial concentration of macromers/monomers in solution, amount of cross-linker, sample size, nucleation agent and prehistory of the reaction (Tripathi et al, 2009).

**Figure 1.10:** Schematic representation of three steps involved in synthesis of cryogel biomaterial.

**Figure 1.11:** Different formats of cryogels synthesised by cryogelation (A-B). The scanning electron microscopy (C) showing interconnected network of pores. Confocal images of HEMA-gelatin cryogel (E-F) using different filters.
1.6.1 Application of cryogels

a) Bioseparation

Cryogels have found to be interesting matrix in the area of bio separation where supermacropores are utilized for protein, microbial and mammalian cell separations. Cryogels synthesised using polyacrylamide and polydimethylacrylamide are used for affinity based separation of various mammalian cells (e.g. blood cells). A model system of separating stem cells with CD34^+ antigen surface marker has been developed on cryogel affinity columns. Recently separation of stem cells from umbilical cord blood derived has been established (Kumar and Srivastava, 2010). Similarly, these columns can also be used for metal affinity chromatography or as gravity columns in high through-put screening system designed for protein analysis where crude homogenate is directly used for analysis. The major advantage of using cryogel based separation technique is that crude homogenates can be directly processed in the columns without any prior treatment thereby considerably reducing the cost and is applicable in both preparative and analytical scale. This gives an advantage to cryogel over the existing technologies for cell separation which are mainly used on analytical scale. The spongy and elastic nature of cryogels has led to development of a distinctive system used for elution of affinity bound bio-particles which can detach from the cryogel affinity column via elastic deformation. The uniqueness of this method is in the fact that the cells are eluted by low mechanical force. In addition, the same cryogel columns can be applied as scaffolds for cell immobilization (Tripathi et al, 2010, Jain et al, 2009).

b) Bioreactors for production of therapeutics

Cryogel based perfusion bioreactor is developed for production of protein therapeutics such as urokinase and monoclonal antibody (Jain et al, 2010; Nilsang et al, 2008; Bansal et al, 2006). The speciality of this system lies in the fact that it provides an integrated reactor where the cells are immobilized over a cryogel based bioreactor and the secreted
product can be purified using a different cryogel separation column. The reactor has been found to be three times more efficient than the T-flask culture and a cell density can increase more than $10^8$ cells/ml without blocking pores. The interconnected pore within these matrix forms a network of capillaries over which the cells can be immobilized and the media is perfused within them. These reactors have shown a very high potential as high density perfusion bioreactors and can also be used as disposable reactors. Due to the easier fabrication technique and easy upgradation makes it very economical for industrial usage (Jain et al, 2010).

c) **Cryogel for tissue engineering**

In the field of tissue engineering these matrices are relatively new and being explored extensively due to its ideal 3-D architecture. Cryogel having interconnected pores with large pore size facilitates easy nutrient and gaseous exchange. The surface chemistry can be easily modulated by varying the polymer concentration or amount of cross-linker used thereby providing ideal environment for specific cell type. Cryogel scaffolds have been used in cartilage (Kathuria et al, 2009) and skin tissue engineering (Dainiak et al, 2010) and presently been explored for neural regeneration and as bioartifical liver support system.

1.7 **Cartilage composition and function**

Cartilaginous tissues are found all through the body and perform a wide range of mechanical and structural functions. In diarthrodial joints such as the shoulders, knee, articular cartilage and fibrocartilage plays very important role in the free movement and load bearing capabilities of the joint. High levels and complex combinations of compressive, tensile, and shear forces are experienced by these tissues during normal activity. However, the unique compositions and structures of the extracellular matrix (ECM) in articular cartilage and fibrocartilage help the tissue to perform normal biomechanical functions.
1.7.1 *Articular cartilage*

Articular cartilage is cushion that is at the ends of long bones and serves to reduce friction of surface for smooth joint motion (Figure 1.12). In addition to joint lubrication, articular cartilage can withstand significant compressive and shear forces, often several times body weight (Ahmed et al, 1983).

![Figure 1.12: Detailed explanation of cartilage anatomy showing articular cartilage, patella, tibia and meniscus (Modified from Shah, 2010).](image)

The cartilage ECM is composed of water (68-85 %), collagen (10-20 %), and proteoglycans (5-10 %) (Mow et al, 1997), and the interactions between these components gives tissue its load bearing capabilities (Lai et al, 1991; Maroudas et al, 1976). In addition, the organization of the matrix molecules in three zones of the tissue (superficial, middle, and deep) results in a depth-dependent variation in material properties that reflect the complex loading environment within the joint (Wilson et al, 2007; Aspden and Hukins, 1981; Muir et al, 1970).

Type II collagen is the most abundant type of collagen found in articular cartilage and is primarily responsible for bearing of shear and tensile forces by tissue (Mow et al, 2005). Collagen II is organized in long fibres and is supported by several other fibril-associated collagens, such as collagen VI and IX (Muir et al, 1970). The collagen II
content is highest in the superficial zone, and the fibrils are organized tangentially to the articulating surface in order to withstand the higher levels of shear and tension experienced in this region. The collagen fibers are oriented randomly within the middle zone and perpendicular to the surface within the deep zone where they are anchored to the subchondral bone (Yarker et al, 1983; Aspden and Hukins, 1981; Eyre et al, 1975).

The second major structural moiety in articular cartilage is aggrecan, which consists of abundant sulfated-glycosaminoglycan (sGAG) chains which in turn is attached to a protein core (Haynesworth et al, 1987). The aggrecan molecules are linked to a hyaluronic acid backbone forming a large macromolecule. The highly negatively charged sGAG confer fixed charge to the cartilage (Gu et al, 1993; Maroudas et al, 1969), and interactions between proteoglycans. The mobile ions in the interstitial fluid create an osmotic swelling pressure which is resisted by the collagen network (Lai et al, 1991; Minnis et al, 1977). This resulting osmotic pressure and low hydraulic permeability allows articular cartilage to bear the large compressive forces that develop during normal activity. The proteoglycans and water contents are highest in the middle and deep zones which experience primarily compressive forces (Mow et al, 1997; Bayliss et al, 1983). Taken together, the zonal organization of collagen and proteoglycans within articular cartilage highlights key structure-function relationships between matrix organization and tissue mechanics.

Articular chondrocytes are the resident cells within articular cartilage and are responsible for the maintenance of the extracellular matrix. Articular chondrocytes express and synthesize the major ECM components, collagen II and aggrecan. In addition, the cells produce proteolytic enzymes that breakdown the matrix and contribute to the homeostatic turnover of the tissue (Hardingham and Fosang, 1995). Articular chondrocytes are fully differentiated cells and are normally non-proliferative in- vivo. Although generally considered a single cell type, there are regional variations in the expression levels of the matrix proteins that reflect the differences in matrix composition.
In-vivo, chondrocytes have primarily round morphologies, but rapidly adhere and spread in monolayer culture. These changes in morphology are accompanied by a steady loss of collagen II and aggrecan expression and reduced proteoglycan (PG) synthesis (Brodkin et al, 2004; Stokes et al, 2002). However, chondrocytes cultured in three-dimensional environments, such as hydrogels or cell aggregates, can maintain their differentiated phenotype for multiple weeks in the in vitro conditions (Kolettas et al, 1991).

1.8 Cartilage degeneration and repair

1.8.1 Osteoarthritis

Damage to articular cartilage and fibrocartilage represents a significant health problem world-wide and can arise from a traumatic injury or chronic degradation, such as osteoarthritis. Acute injuries, including focal defects and cracks in the cartilage, often result in impaired movement, pain, and inflammation (Wilson et al, 2007). The persistence of these injuries can lead to further degeneration of the tissue and the development of severe osteoarthritis (Wilson et al, 2007). Osteoarthritis is typically considered a chronic disease due to “wear and tear” of the articular cartilage. The condition tends to increase with age and it is most common in adults over the age of 40 (Imler et al, 2005). The symptoms range from mild pain and impaired motion to severe pain and complete debilitation.

Progression of osteoarthritis is characterized by a loss of proteoglycans and water from the tissue, followed by degradation of the collagen network (Imler et al, 2004; Kavanagh and Ashhurst, 2001). With time, the loss of ECM components results in impaired mechanical function. Specifically, the lower proteoglycans content reduces the osmotic swelling and compressive stiffness of the cartilage (Kavanagh and Ashhurst, 2001). In addition, damage to the collagen network decreases the tissue’s tensile stiffness and ability to resist deformation (Vanderploeg et al, 2004). The low cellularity of articular
cartilage and limited vascular supply significantly inhibit its intrinsic ability to repair damage. In addition, chondrocytes from older patients have a lower biosynthetic activity (Hunziker, 2002) which further hinders the ability to regenerate a cartilage matrix.

Damage to cartilage tissues, particularly the meniscus is most commonly due to acute injuries. Approximately one third of meniscal injuries are sports related (Setton et al, 1994) and associated with overloading or twisting of the joints (Guilak et al, 1994; Ratcliffe et al, 1992). Partial and full-thickness tears are the most common type of damage to the menisci and develop in several forms. Lesions in the vascularized, outer region of the meniscus can naturally heal, while those in the inner region rarely heal (Martin and Buckwalter, 2002). In past 20 years it has become clear that the cartilage is critical for joint stability and load distribution (Tran-Khanh et al, 2005; Drosos and Pozo, 2004). Removing the damaged tissue by total meniscectomy increases the contact stresses on the underlying cartilage and almost always leads to osteoarthritis (Drosos and Pozo, 2004; Hede et al, 1991). Unrepaired tears can also disrupt the joint’s biomechanics and increase the risk of further injury. Therefore, successful treatment of meniscal damage is important for protecting against future injuries and maintaining healthy joint function.

1.8.2 Current treatments

There are limited options for treating injuries or degeneration of cartilaginous tissues. Less severe cases of osteoarthritis can be managed by treating the pain with nonsteroidal anti-inflammatory drugs, while in the most severe cases of arthritis, total joint replacement is highly successful treatment for restoring joint function. Unfortunately, a major drawback with joint replacement is the limited lifespan of the implant. Artificial joints last an average of 10-15 years before requiring a second surgery and are only realistic options for patients over 60 years old (Walker et al, 1975), as the younger patients will need repetitive surgery for replacement of artificial joint. Like many types of surgery, joint replacement also includes risks of infection, blood clotting, and other complications.
Alternative treatments, such as microfracture and subchondral drilling, attempt to exploit the body’s own healing response by exposing the cartilage defect to the vascular supply in the subchondral bone and stimulating new tissue formation (Donahue et al, 2002). This strategy has had some success in promoting cartilage growth (Donahue et al, 2002), but the new tissue often has a disorganized, fibrous matrix and is mechanically inferior to native articular cartilage (Hede et al, 1991). Although these techniques have been shown to provide short term pain relief and improvements in joint function, they do not ultimately protect the cartilage from future degeneration (Kurtz et al, 2005). Autograft and allograft transplants of osteochondral tissue have had similar short term successes (Beiser and Kanat, 1990) but studies in animal models indicate that the implanted cartilage fails to integrate with the surrounding tissue (Hice et al, 1990). Clearly, the current strategies for repairing articular cartilage are insufficient, and there is a significant need to develop new therapies or devices that promote normal tissue regeneration and restore long term joint function.

The primary goal of treating cartilage injuries is to preserve the load distribution function of the menisci and cartilage thereby, maintaining the biomechanics of the knee (Wohl et al, 1998). Therefore, strategies that encourage healing or regeneration of the cartilage are preferred to total or even partial meniscectomy. While some minor tears can heal naturally (Hurtig et al, 2001), most injuries require some type of surgery. Common surgical techniques for repairing meniscal lesions involve improving the vascular supply to the damage site and the use of closure devices, such as sutures, arrows, or fibrin glue (McAndrews and Arnoczky, 1996). Together these treatments stabilize the tear, while enhancing the healing process. Surgical intervention is most successful in the outer one-third of the meniscus (DeHaven et al, 1989). However, repairing damage to the inner region is still a major challenge requiring additional investigation.
1.8.3 Limitation of current treatment

Therapies discussed above do not have limitation of availability as the material is artificial or metal based but these therapies do not restore all functionality of the tissue and in long term applications they often fails or need follow up surgeries. Creating neo-tissue for replacing lost or malfunction tissue completely is virtually impossible by current treatments. Due to these limitations, tissue engineering approach is used to enhance cartilage regeneration by combining cells and biomaterial of 3-D architecture.

Scaffold used for tissue regeneration should provide ideal environment for cell adhesion, proliferation and secretion of natural ECM components. This can be achieved by various methods either by synthesizing scaffold that is fabricated from native tissue components for cells to recognise them as native structure or scaffolds that can signal or instruct progenitor’s cells like MSCs to differentiate to chondrocytes in the *in-vitro* or *in-vivo* conditions.
CHAPTER II

REVIEW OF LITERATURE
2. LITERATURE SURVEY

A paradigm shift is taking place in medicine from using synthetic implants and grafts to a tissue engineering approach that uses degradable porous scaffolds integrated with biological cells or molecules to regenerate tissues (Hollister, 2005). Backbone of tissue engineering/regenerative medicine is cell based therapeutics and delivery system. The effectiveness of this system depends upon successfully delivering cells to the sites of degeneration where the injected live mammalian cells exert therapeutic effects to replace or repair the degenerated tissue (Wang et al, 2010). The biomedical and bioengineering field has advanced by use of various novel and modern techniques for designing an effective delivery system. Polymeric scaffolds have attracted a great deal of attention for applications in field of bioengineering like drug and protein delivery system, cell culture, tissue engineering, enzyme and cell immobilization (Pavlyuchenko et al, 2006). For tissue engineering applications, ideal scaffold needs to provide a biocompatible three-dimensional (3-D) architecture that mimics microenvironment of cells of particular tissue type. Besides being biocompatible, scaffolds degradation should be in tune with tissue regeneration without any toxic by-product.

2.1 Tissue engineering

Tissue engineering is broadly described as interface of engineering and biomedical science that uses cells or material capable of attracting endogenous cells to support tissue formation or regeneration, thereby producing diagnostic or therapeutic benefits. As discussed in the introduction about the emergence of tissue engineering and the importance in today’s medical field, briefly the central doctrine of tissue engineering is seeding cells on scaffold that are composed of natural or synthetic polymers and in-vitro tissue regeneration is allowed to proceed (Figure 2.1). The engineered construct is implanted in-vivo to the site of injury for repairing or replacing or restoring the affected organs (Fuchs et al, 2001; Langer and Vacanti, 1993).
Figure 2.1: Central doctrine of tissue engineering is cells and scaffold. The combination of these two components results in repair or regeneration of tissue in the *in-vitro* or *in-vivo* conditions.

### 2.2 Cells for tissue engineering

Cells are basic functional unit of life as stated by Robert Hook and are smallest unit that defines or governs all the functions of organism and is correctly referred as building blocks of the life. Cells used in tissue engineering can be obtained from autologous source which are patients own body cells, allogeneic cells from other donors and xenogeneic from animal source or can be progenitor stem cells which are undifferentiated (Figure 2.2). The premise of using cells as therapeutical agents needs the harnessing of
cell’s delicate biochemical machinery to perform functions that are not possible to mimic by exogenous agents such as drug or surgery (Skalak and Fox 1988).

Figure 2.2: Different types of cells used in tissue engineering are primary cells (chondrocytes, bone etc), immortalized cells (Hela cell line, picture from Wikipedia) and progenitor cells (embryonic stem cells).

2.2.1. Primary cells isolation and use in tissue engineering

Primary cells are defined as cells isolated from the organism directly and is used for in-vitro set up. Isolation of the primary cells is basically from the ex-plant or from the patient’s tissue. Tissue is enzymatically digested to liberate single cells which are further proliferated in laboratory conditions until desired cell number is achieved for further use. Primary cells present the best experimental model of in-vivo conditions and therefore have been extensively used in tissue engineering. Most important criteria for designing tissue-engineered medical device is replacement of damaged tissue with functional tissue
constructs and main facet of this is cell number and functionality (ECM production) being maintained in 3-D environment. The primary cells expanded in the in-vitro conditions, the number of division a primary cell can undergo is subjected to ‘Hay flick limit’ which is defined as number of doubling population cell can divide before it reaches senescence. All primary or terminally differentiated cells have fixed length of telomeres and once it reaches a critical length, cells stop dividing. Considering the number of cells required for tissue constructs Hay flick limit presents stifling limitation especially while using cartilage cells or liver cells (Finch, 1990).

### 2.2.2 Immortalized cells and use in tissue engineering

These cells continue to proliferate and divide indefinitely in the in vitro conditions for as long as the appropriate culture conditions are maintained. Immortalized cell lines are often referred as ‘transformed cells’ since the basic proliferative property of these cells are altered. However, it does not necessarily have to be "cancer" or "tumour" cells. Alteration in basic property is performed by transformation which is a complex process and can be performed by many different techniques, e.g. chromosomal changes or infection by transforming tumour viruses. These cells loose the contact inhibition mechanism and hence grow on top of each other making it difficult to generate a 3-D system using these cell lines (Quellette et al, 2000). Due to this limitation, stem cells have emerged as potential candidate for cell based therapy and tissue engineering applications.

### 2.2.3 Stem cells and tissue engineering

Stem cells building blocks of tissue: Commitment and lineage dependent differentiation of stem cells generally addresses the organ functions, formation and repair. The cellular-fate processes like cell growth, differentiation, motion and death together determines the fate of committed cells. Loss of stem cells or depletion of these cells can lead to partial or complete loss of organ function. For these reasons, stem cells can be starting material for many key processes in tissue engineering and is considered as an important foundation of
regenerative potential in the *in-vivo* and *ex-vivo* cell-culture processes (Bianco and Robey, 2001).

### 2.3 Basic concepts and properties

Stem cells are undifferentiated cells that differentiate into specialized cell types depending upon the signals provided by either surrounding cells or by exogenous factors. Universally, stem cells come from two main sources:

1. **Embryonic stem cells** are formed during the blastocyst phase of embryological development and
2. **Adult stem cells** are generally found in adult tissue like bone marrow.

Both types of stem cells are characterized generally based on their potency or potential to differentiate into specialized cell types (such as muscle, skin, bone, etc.) (Lonza, 2004).

#### 2.3.1 Potency

Stem cells are categorized majorly based on their potential to differentiate and form other types of cells. Embryonic stem cells are known to be the most potent since they go on to become every type of cell in the body. The full classification includes the following types (Potten and Wilson, 2004):

- **Totipotent** - the ability of stem cells to differentiate into all possible cell types. Classic examples of this are the zygote formed at egg fertilization and the first few cells that result from the division of the zygote holds the key for the formation of entire organism.
- **Pluripotent** - the ability to differentiate into most of the cell types but not all. Best examples include embryonic stem cells and cells that are derived from the three germ layers (meso, endo and ectoderm) of embryo formed in the beginning stages of embryonic stem cell differentiation.
• **Multipotent** - the ability of stem cells to differentiate into a closely related family of cells. Examples include hematopoietic (adult) stem cells that differentiate everyday to replenish the body with red and white blood cells or platelets.

• **Oligopotent** - the ability to differentiate into only few cell types. Example includes (adult) lymphoid or myeloid stem cells.

• **Unipotent** - the ability of stem cells to produce cells of their own type, but retains the property of self-renewal which is basic criteria required to be labelled as stem cell. Example includes muscle stem cells.

Embryonic stem cells are recognized as pluripotent instead of totipotent as they do not have the ability to form the part of the extra-embryonic membranes or the placenta.

**2.4. Types of stem cells**

**2.4.1 Embryonic stem cells**

Embryonic stem cells are derived from 4-5 days old blastocyst phase of human embryo. During sexual reproduction male's sperm fertilizes the female's ovum (egg) to form a single cell called a zygote. The single zygote cell undergoes a series of cell cycle divisions to form 2, 4, 8, 16 cells, etc. After 6th but before implantation of zygote in the uterus, a mass of cells are called blastocyst (Edward, 2001). The blastocyst contains an inner cell mass (embryoblast) and an outer cell mass known (trophoblast). The outer cell mass goes on to become a part of the placenta, and the inner cell mass group differentiate to become all the structure of an adult human. During course of normal pregnancy, the blastocyst stage continues till implantation of embryo in the uterus and at this point the embryo is referred as a fetus. This usually happens by the end of the 10th week of gestation after which all the major organs of the body are formed (Hansis and Edward, 2003). However, during the *in-vitro* isolation of embryonic stem cells, the blastocyst stage signals the "inner cell mass" in a culture dish containing a nutrient-rich broth to divide and replicate due to lack of the necessary stimulation to differentiate but also
maintains their ability to form any cell type of the body. Eventually, these undifferentiated cells can be signaled or stimulated to differentiate to more specialized cells (Edward, 2001). Although, these cells hold great importance in fundamental research but there are ethical concerns due to which scientist have not been able to use them in real applications. In advent of this, adult stem cells especially mesenchymal stem cells have emerged as forefront runner for researchers.

2.4.2 Adult stem cells

Somatic or adult stem cells are found throughout the body after embryonic development. These stem cells have been found in tissues such as the bone marrow, brain, blood vessels, skin, skeletal muscles, and the liver. They remain in a quiescent (non-dividing) state until activated by signals produced by other cells due to disease or tissue injury. Adult stem cell’s potential to divide or self-renew indefinitely, enables them to generate a wide range of cell types from the originating organ or even regenerate the entire organ with specific functions (Potten and Booth, 2000). It is generally believed that adult stem cells have limited ability to differentiate depending on their tissue of origin, but few experiments have shown evidence that suggests that these cells can differentiate to become other cell types. There are two types of adult stem cell; hematopoietic which differentiated to all the blood cells and mesenchymal stem cells which are generally responsible for musculoskeletal tissues (Lanza, 2005). MSCs have been explored extensively in the field of tissue engineering due to its self-renewal capacity and its immunogenicity. What is the factor that determines the self-renewal property of cells?

2.5 Telomeres and self-renewal of cells

A main reason that stem cells have captured the imagination of scientist world-wide is due to its self-renewal capacity. Do stem cells really self-renew? During the replication process the DNA’s lagging strand is synthesized in discontinuous fashion through the formation of “OKAZAKI fragment”. The synthesis of the last OKAZAKI fragment is
not initiated by DNA polymerase therefore; the lagging strand is shorter than the leading strand. At end of all the chromosomes there are non-coding regions/sequences that are called as telomeres. Human non-germline/somatic cells have telomeres that are generally shortened by 50-200 bp per replication which is primarily responsible for mitotic clock. Once the length of telomeres reaches 5-7 kbp, the chromosome becomes unstable and replication starts to cease (Dahlen et al, 2003). This mechanism is believed to contribute to the limited capacity of primary cells to proliferate or propagate in cell culture which is known as ‘Hayflick limit’. During cell culture the culture conditions can have drastic effect on the proliferation rate of cells (Holt et al, 1997).

Telomeres activity is found in hematopoietic stem cells but with very low activity level (Wang et al, 2005). There are evidences of shortening of telomeres with donor age and increase in doubling number is seen in immature hematopoietic cells but the rate of telomeres shortening is finite and slower in stem cells in comparison to other cells of the body (Holt et al, 1997).

2.5.1 Immunogenicity of MSCs

Human and mouse MSCs has shown the expression of major histocompatible complex I (MHC I) but the expression of MHC II remains controversial (Atoui et al, 2008). There are some reports suggesting low MHC II expression but overall MSCs have shown negative expression of MHC II which makes them ideal for the cell therapy and tissue engineering purposes as there is no or reduced immunogenicity. In one experiment where the MSCs were used as stimulator in mixed lymphocyte reactions (MLR), MSCs independent of its origin (Human, mouse or rat) did not provoke T-cell response in contrast to inhibited T-cell proliferation (Bang et al, 2006; Potian et al, 2003). Accumulation of studies have suggested not only does MSCs inhibit T-cell but can also modulate the effects of B-cells (antigen secreting cells), innate killer cells and antigen presenting dendritic cells (Uccelli et al, 2006, Chen et al, 2006). Studies performed on animal model where allogenic transfer was performed demonstrated MSCs were not
rejected by immune system of the animal and these cells retained their differentiation potential (Djouad et al, 2003). Siato et al, (2003) had administrated xenogenic MSCs and traced the fate of the implanted stem cells in the recipient animal; found that these cells underwent coronary artery ligation. Results showed that Lac Z labelled mice MSCs engrafted bone marrow of immunocompetent recipient rats for 13 weeks after transplantation and many cells that were isolated from myocardial infarcted area were absent before the coronary ligation. In human trail patients suffering from Hurler’s syndrome where infused with MSCs did not result in alloreactive T cells generation or graft vs. host response/rejection (Koc et al, 2002).

Induction of immune responses mainly depends upon the interaction between the antigen presenting cells (APC) and T cells. Most potent APC in mediating humoral or cellular immune responses against non-self or self antigens are dendritic cells (DC). Maturation of DC represents most important check point in increased immune response since immature DC promotes T cells tolerance. It is believed that MSCs modulated the generation and antigen presenting potential of DCs which in turn influences the stimulation of T cells (Beyth et al, 2005, Jiang et al, 2005). In fact, some studies have shown that MSCs interfere with DC differentiation (CD40, CD80 and CD86) expression, maturation (IL-2 and endocytosis), and activation (stimulation of allogeneic T cells) (Aggarwal and Pittenger, 2005; Groh et al, 2005). Its worthy to note that monocytes when exposed to maturing stimuli in presences of MSCs acquires tolerogenic phenotypes (Beyth et al, 2005) and DC isolated in these conditions have shown a significant reduction in TNF and IL-2 production, thus accounting their ability to inhibit T cell responses (Parijis et al, 1997).

MSCs have extensively proven the potential for suppressing immune response on virtually all the cellular components of immune system. However, the mechanism by which MSCs perform these powerful acts in not fully elucidated. There are hypothesis stating that MSCs selectively affects the proliferative capacity rather than the effector
function of immune cells. Whatever be the way MSCs modulates the immune response but this property have great impact in developing new cell based therapy and reason of MSCs being first choice in field of tissue engineering for the treatment of pathological conditions without generating unwanted immune response (Dazzi and Timoshanko, 2007).

2.6 Strategy for differentiating stem cells

Development of functional organ from single, unspecialized precursor cells is best understood in nematode Caenorhabditis elgans where Sulston et al, (1983, 1977) described complete lineages of this nematode and found that development of whole functional organism starts from single cells. However, humans have approximately 100 trillion cells (Hoffman and Merrill, 2007) is process is much more complicated and it is very unlikely that we will be able to mimic this complex system to recapitulate all signals and events that leads to formation of functional matured organ/tissue in course of human development. Many strategies have been developed to overcome this problem and science has modulated itself to regenerate a functional tissue by providing artificial analogues for differentiation (Kaigler et al, 2005).

To induce differentiation in more specific manners several different strategies have been formulated like culturing cells on specific three dimensional matrix that is functionally active or modified to induce differentiation or by co-culturing stem cells with other cells that provide cues for differentiation or using of soluble growth factors, cytokines and other non-proteinaceous compounds in growth media, Adjusting the physical parameters or combining two or more strategy can activate differentiation signalling cascade in stem cells (Kawaski et al, 2000).

2.6.1 Co-culturing stem cells for specific differentiation

Co-culturing of MSCs has been successfully shown to differentiate stem cells to neural (Lei et al, 2007) and bone cells (Rosa et al, 2010). One main principle that helps in
differentiation of stem cells is extracellular signalling. The extra cellular molecular signalling pathway is actively being investigated. Extracellular signalling is known to involve the interaction between cell surface receptors and soluble cytokines and growth factors along with the extracellular matrix (ECM), or with the surface proteins of the adjoining cells (Heng et al, 2004). The ECM along with maintaining the structural integrity of the tissues has important physiological functions, such as transport of nutrients to the cells, as reservoir for physiological mediators, and acting as mediator of cellular functions through interaction with cell surface receptors (Comper, 1996). The integrins is a major family of ECM receptors that helps in transmitting information from the matrix surface to cells, thereby playing a significant role in the regulation of cell proliferation, differentiation, survival, and matrix remodelling (Loeser, 2002). In order to induce chondrogenesis in MSCs requires factors that support strong cell–cell interaction, 3-D environment that helps to maintain of morphology of primary cells and growth factors are all important. Strong cell–cell interaction is governed by cell adhesion molecules such as N-cadherin and integrins that allows MSCs differentiation to pre-chondroblasts at the pre-cartilage mesenchymal condensation phase during limb development (Haas and Tuan, 1999; Hall and Miyake, 1995).

Various bioactive factors, such as transforming growth factors (TGF) β1 and β3, fibroblast growth factor, insulin-like growth factors, bone morphogenic proteins (BMPs)-2,6, and 9, have been reported to either induce or maintain chondrogenesis (Indrawattana et al, 2004; Sekiya et al, 2002; Muraglia et al, 2000). It is well known that cells must interact with the microenvironment, from where soluble and insoluble signals triggers the physiological responses in cells through contact with the cell surface to maintain homeostasis (Wayne et al, 2005; Qi and Scully, 2002). Cukierman and co-workers have also explicated that mode of matrix adhesion is unique to cells in 3-D culture system (Cukierman et al, 2001), suggesting that the effects of biological signal which are due to cell-matrix interactions should not be extrapolated from experiments set up for 2-D
culture, but should be re-examined or reconfirmed for the 3-D microenvironment. The importance of 3-D matrix is increasing over years as these are most important component of tissue engineering. The biomaterial sciences over the years have evolved with invention of more sophisticated technology of scaffold fabrication that has provided wide range of options to select most ideal scaffold depending upon the applications.

2.7 Invent of biomaterial sciences

By the dawn of 21st century, biomaterials are widely used in biotechnology, medicine and dentistry, but 50 years before the biomaterial word did not exist (not used). There were no manufactures or designer for biomaterial but it was used in some crude form without any formal recognition (Williams, 1987). The experiments and studies that laid foundation of biomaterial are from 1920-1980. The archeological department of US found pre-historical man commonly known as ‘Kennewick Man’ dated 9000 year old. He was described to be tall with active healthy life and had a spear embedded in his hip which had healed without any rejection problem. This spear point implant might not resemble the modern times biomaterials but it was well ‘tolerated’ by body without exhibiting any foreign body reaction or rejections (Lyman and Rowland, 2007). Early civilizations shows evidence of using implants for regeneration of lost tissue e.g., Mayan people of 600 A.D, had fashioned nacre teeth from the sea shells and have achieved what we know as seamless integration into bone (Bobbio, 1972). Similarly, from corpse dated 200 A.D an iron dental implant was found in Europe (Crubezy et al, 1998). This implant was also reported to have good bone integration. There might not be any material sciences or medical procedure involved in these procedures but the success and longevity is impressive and highlights two most important points, the tolerance or forgiving nature of our body and pressing need of restoring the lost tissue by implants even in prehistorical times. Sutures are probably best known biomaterial used from 32,000 years approximately (Natnews, 1983). Large wounds in ancient times were closed using cautery or sutures and evidences show usage on Linen sutures prevailed during early
Egyptians times. Gelen of Pergamon (130-200 A.D) had described the use of gold wires for stitching large wounds. In 1816, Prof. Philip Physick of University of Pennsylvania suggested the usage of lead wires as suture, had no noting reactions. J. Marion Sims of Alabama, in 1849 performed many successful operation using silver wires made by jewelers.

Post World War II, metal, ceramics and polymeric materials transitioned from restricted wartime items to peacetime available commodities (Kolff, 1979). The possibilities of using an inert material which is durable and novel immediately intrigued physicians with need to restore or replace the damaged organ or tissue. Surgeons utilized metals such as Teflon, polyurethanes, nylon, silicones, titanium and stainless steel which were mainly used for airplane manufacturing but was taken ‘off the shelf’ by surgeons (Clark et al, 1976). During the wartime the surgeons and physician collaborated with scientist and engineers to improvise or invent devices for the patients whose life were at stake (Ratner et al, 2004). Many materials were tried and some fortuitously succeeded in replacing or restoring the normal activity of patients. These were high-risk operations but the surgeons took chances only in cases where there were no other options left. The ‘surgeon hero’ era is justified as these surgeons took huge technological and professional leap to restore or repair damaged tissue in an individual (Yaszemski and Yasko, 1998). Thus, the foundation of biomaterial was laid by fiercely committed and creative individuals who took the world of health care with storm and changed the concept of dealing with the lost or damaged tissue.

The first generation of biomaterials used was largely off the shelf materials which were widely available industrial materials that were not intended to be used for medical usage (Hench and Polak, 2002). They were selected due to the desired combination of their physical properties which were specific to the clinical usage, and they were desired to be ‘Bioinert’. New evolved biomaterials belonged to second generation that was intended to elicit a nontrivial, controlled reaction with therapeutical advantages. Second
generation biomaterials included development of resorbable materials with varied degradation rates which in-tune with desired application (Griffith, 2002). Third generations of biomaterials were intended to evoke or stimulate more specific highly precise reactions with cells and proteins at the molecular level (Chiu et al, 2003) (Figure 2.3).

Figure 2.3: Evolution of biomaterials science and technology (adapted from Rabkin and Schoen, 2002).

2.9 Development of biomaterials based device

Thomas Edison once stated that he would only invent devices that people would buy and this seems to be central dogma for biomaterial sciences as the designing of biomaterial devices is primarily based on clinical need of patients or clinicians as shown in Figure 2.4 which illustrates the multidisciplinary interactions of biomaterials and the progression in development of biomaterial based medical devices (Saha and Saha, 1987). It provides the prospective on how researchers and physician from different discipline work together from identification of need and development biomaterial based devices to manufacture, implantation and ex-plantation from a patient (Lysaght and Laughlin, 2000).
Much of richness of biomaterial is due to its interdisciplinary nature and two pillars that supports structure of biomaterial sciences is material- sciences and biological-medical sciences (Papenburg, 2009). Biomaterials have evolved in three generation; presently ideal scaffold must be bioresorbable polymer with porous structure with desired geometry matching the tissue of interest to aid cell adhesion, proliferation and extracellular matrix (ECM) production. There are two phases that are involved in tissue engineering a functional organ. First the in-vitro tissue regeneration is achieved by placing the specific cells on 3-D scaffold and stimulated to enhance cell proliferation and ECM production either by help of bioreactor or providing a mechanically supportive environment. Second phase can aim for in-vivo tissue regeneration, in this approach the construct is loaded with progenitor cells and implanted to anatomical location where the remodelling occur in-vivo and recapitulate the normal functionality of the organ. The paradigm of tissue engineering is biomaterial – tissue interactions.

2.8 At the leading edge of medicine

The magnitude of biomaterial based medical device expresses both the importance of need and size of commercial market. Four commonly used biomaterial devices; hydrocephalous drainage stunt, hip joint, contact lenses and heart value and all have been successful in fulfilling the medical need (Enderle et al, 2005). The number devices used per year clearly indicates the human and commercial needs and medical significance ranges from cosmetic to life-saving. In year 2000 alone, more than 20 million people lives were either sustained, supported or restored by replacement with functional organ to normal using biomaterial devices. World-wide prostheses and organ replacement cost exceeds $300 billion US dollars and contributes to 7-8 % cost of total healthcare spending per year. These Figures explain why biomaterial devices are at forefront of medical research and the magnitude these devices present in healthcare system (Lysaght and Laughlin, 2000).
Figure 2.4: Disciplines involved in biomaterial sciences and path from demand to manufacture of medical device (reproduced from biomaterial sciences 2nd edition; An introduction to materials in medicine).
2.9 Important property of biomaterial devices

A) Biocompatibility
Measurement and understanding of biocompatibility is unique to biomaterial sciences. Unfortunately there are no precise definitions or measurement of biocompatibility and mostly is described as success of performing a specific task without leading to rejection in the in-vivo system. The operational definition is based on patient’s condition if patient survives the implant and returns to normal activity, the device is believed to be ‘biocompatible’ (Williams, 2008).

B) Toxicology
Biomaterial strictly should not be toxic until it is been designed to be performing that function e.g. smart drug delivery device that is engineered to be toxic to cancer cells. The non-toxic is most important norm; toxicology has evolved into sophisticated sciences for biomaterials (Figure 2.5). It identifies the molecule that migrates out of material like in case of polymers with low molecular weight. There can be molecules that leach out exhibiting cell toxic behaviour or leading to some physiological changes which are not required. Toxicology also deals with fineness of biomaterial and its fabrication technique when new material is in developmental stage. It is safest to state that biomaterial should not have any substances that leaches out from inner core mass of material in the in-vivo condition until unless specifically designed to do so (Anderson et al, 2004).

The key processes taking place during the in-vitro and in-vivo phase is

1) Cell proliferation and differentiation
2) Extracellular matrix production and organization
3) Degradation of the scaffold
4) Remodelling and potential growth of tissue.

In order to achieve all the above stated conditions designed biomaterials have evolved
**Figure 2.5:** Biomaterial-tissue interactions illustrate the importance of developing biocompatible matrix for implantation (reproduced from Schoen, 2002).

### 2.10 Designed biomaterials

Biomaterials used post world-war II were largely off the self materials but 1960’s saw development of materials specifically designed using biomaterials revolutionizing the science by using materials which commodity materials to engineered/synthesized biomaterial. Silcones, Teflon (LeVeen and Barberio, 1949), polyurethanes (Abuchowski et al, 1977), poly (ethylene glycol), poly (lactic-glycolic acid) (Akutsu et al, 1959) titanium, hydrogels and hydroxyapatite (Levitt et al, 1969) were mainly components explored in entire range of medical device invented during 1940-1980.

The methodology used for synthesizing scaffold in our laboratory is by cryogelation technology.
2.10.1 Cyrogel scaffold fabrication

Macroporous polymeric materials have gained interest in the biomedical and biotechnology. The different fabrication methodology through which macroporous structure can be obtained 1) freeze-drying 2) porogenation 3) phase separation 4) cyrogelation. For all the methods cryogelation is most widely used approach for synthesis of macroporous polymeric matrix due to easy fabrication methods to obtain open porous structure with well controlled porosity (Kumar et al, 2006). Cryogelation technique provides possibility to prepare macroporous hydrogels with wide range of porosities from practically any gel-forming system (Lozinsky et al, 2003).

Cryo-gelation takes place via cryogenic treatment of the systems containing aqueous solvent and monomer/polymer precursors. The crystallization of the solvent is the critical feature of cryogelation, which differentiates cryogelation from freeze induced gelation. Depending upon the polymer precursor the pore ranges from 0.1 µm to 200 µm. Cryogels are produced via a gelation process at sub-zero temperatures where most of the solvents freezes and dissolved polymeric precursors concentrates in small nonfrozen regions (nonfrozen liquid microphase/NLM) (Kumar and Srivastava, 2010). This is where the chemical reaction and gel formation proceeds over set period of time. While all the reagents concentrate in NLM, some portion of solvent remains unfrozen and this provides space for solute to concentrate into nonfrozen part with sufficient segmental or molecular mobility for reactions to perform. After melting the solvent crystals (in case aqueous media it will be ice), a large continuous interconnected pores are formed. Shape and size of the pores generated directly depends upon the shape and size of the crystals formed during freezing (Figure 9 shown in introduction). Other factors that contributes to the pore size is rate of freezing, freezing temperature, initial concentration of macromers/monomers in solution, amount of cross-linker, sample size, nucleation agent and prehistory of the reaction (Dainiak et al, 2006).
The scaffold fabrication and final outcome depends upon the choice of polymers used for synthesising scaffolds. These polymers give critical property to material and decide its future application. In this thesis work the polymers used for synthesizing the matrix are 2-hydroxyethylmethacrylate (pHEMA) and gelatin.

2.10.2 p (hydroxyethylmethacrylate): A synthetic polymer and its properties

2-Hydroxyethyl methacrylate (HEMA) (Figure 2.6) is one the most widely used and studied neutral hydrophilic monomer. The monomers of HEMA are water soluble, but the homopolymer is water-insoluble. The HEMA monomer forms the basic material for many hydrogel products such as soft contact lenses however, these are widely used as polymer binders for controlled drug release, lubricious coatings and absorbents for body fluids. As a co-monomer with other ester monomers, HEMA can be used to control hydrophobicity or introduce reactive site.

![Chemical structure of 2-hydroxyethylmethacrylate](image)

**Figure 2.6:** Chemical structure of 2-hydroxyethylmethacrylate (chemical formula: C₆H₁₀O₃ and the molecular weight: 130.14).

Hydrophilic polymers are promising biomaterials intended to replace conventional products in tissue engineering. Many hydrophilic polymers were low protein adsorption is attributed to high hydrophillicity is modified to improved cell attachment and proliferation on its surface. pHEMA being one of the widely used polymer however, the homo polymer of HEMA is not consider ideal polymer for tissue engineering application due to its hydrophilicity (Satin et al, 1996). The biocompatible hydrophilic polymer of 2-hydroxyethyl methacrylate (pHEMA), coupled with other polymers are being used in the tissue-engineering field (Chirila, 2010). pHEMA scaffold modified by coating or
polymerization in such a manner that the cells can efficiently adhere and proliferate like in natural polymers, e.g., in collagen (Pavlyuchenko, 2006).

2.10.3 Gelatin: Natural polymers and its properties

Gelatin, a hydrolytic protein substance is derived from collagen which is natural protein present in the tendons, ligaments, and tissues of mammals. It is produced by boiling the connective tissues, bones and skins of animals, usually cows and pigs. Gelatin's ability to form strong, transparent gels and flexible films that are easily digested, soluble in hot water, and capable of forming a positive binding action have made it a valuable commodity in food processing, pharmaceuticals, photography, and paper production. Gelatin gels are completely thermoreversible which is arguably its most interesting property. Structure and composition of gelatin gel depends upon the methodology of gel fabrication. Gelatin has high amino acid sequences which terminate with amino group at one end and carboxyl group at another end (Karim et al, 2009) (Figure 2.7).

![Chemical structure of gelatin showing amino terminal at one end and carboxyl terminal at the other end of the chain.](image)

**Figure 2.7:** Chemical structure of gelatin showing amino terminal at one end and carboxyl terminal at the other end of the chain.

Gelatin and other protein like laminin, fibronectin etc, contain the Arg-Gly-Asp (RGD) attachment site for the integrins, that serve as receptors for them and constitute a major recognition system for cell adhesion (Tritanipakul et al, 2009). The RGD sequence is the cell attachment site for a large number of adhesive extracellular matrix, cell surface
proteins and blood, half of the 20 known integrins recognize RGD sequence in their adhesion protein ligands. The integrin-binding activity of adhesion proteins can be reproduced by short synthetic peptides containing the RGD sequence. As the integrin-mediated cell attachment is known to influence and regulate cell attachment, migration, proliferation, differentiation, and apoptosis, the RGD peptides and mimics are frequently used in probing integrin functions in various biological systems (Waldeck et al, 2007).

Gelatin due to all above stated reason helps to overcome the limitation of pHEMA. pHEMA–gelatin (HG) composite scaffolds have been synthesized earlier using different approaches like gelatin coating on pHEMA gels, pHEMA–gelatin blend hydrogels, interpenetrating network (IPN), etc. (Satin et al, 1996; Rao et al, 1995). These composites have limitations like hydrogels which are not porous enough to facilitate cell migration and macromolecule transport within gel (Satin et al, 1996).

2.11 Alpha-ketoglutarate: Exogenous growth factor for tissue engineering

Second approach in tissue engineering is using exogenous growth factors and checking for the effect on cell-matrix interactions. There are various growth factors available that has been used in enhancing the cell growth and decreasing time of neo-tissue formation. For example, bone morphogenetic protein II (BMP II) has shown to enhance the cartilage formation and helps in accelerating cell proliferation.

The advancement in the field of biotechnology has opened up arena for bio-artificial synthesis of therapeutics which includes hormones, vaccines, antibodies and various immuno-adjuvants (Rudra et al, 2010). One of the most important components for production of these biological products is primary or immortal cells. The cells cultured in laboratory has specific requirements in which the cell media plays an important role (Nilsang et al, 2008) and frequent accumulation of toxic catabolic components like ammonia effects the overall cellular growth which directly has effect on the therapeutic production (Nilsang et al, 2008). Researchers have constantly been focussing in exploring molecules that shows positive effect on overall cellular growth,
metabolism and can also help in removing the toxic catabolites. One of such molecule is alpha-ketoglutarate (α-KG), which is a known Kreb’s cycle intermediate, and also plays critical role in the cellular energy metabolism (Son et al, 2007). MacKenzie and group indicated α-KG as component for new possible therapy in treating cancers associated with TCA cycle dysfunction. Fumarate hydratase (FH) and succinate dehydrogenase (SDH) which are important components of tricarboxylic acid and act as tumor suppressors and loss of either of these components activates a major tumor-supporting event as there is pseudohypoxia condition created in the cells. This event in-turn activates hypoxia-inducible factor (HIF) as HIF-α is stabilized by FH and SDH and excess or defiant TCA components inhibits HIF-α prolyl hydroxylase (PHD). Introduction of cell-permeating α-KG derivatives showed restoration in PHD activity and level of HIF-α to SDH-suppressed cells. This study showed potentiality of α-KG as new bioactive molecule (MacKenzie et al, 2007). α-KG is recognized as a scavenger for ammonia in hepatocyte culture (Rutten, 2005) as it serves as a precursor for non-essential amino acid like glutamate and glutamine (Sheu and Blass, 1999; Nissim, 1999). Glutamine is found to inhibit degradation of proteins in the muscles and along with transaminated alpha-ketoglutarate forms excitatory neurotransmitter glutamate (Gibson et al, 2000). It is also found to penetrate into the bone tissue and helps in its metabolic regulations (Berkich, 2005). Shuprisha et al, (2005) showed real-time assessment of effect of α-KG on organic anion secretion in proximal tubules of perfused rabbit and illustrated that in presences of α-KG, the luminal and basolateral Na-DC co-factors could support significantly the net FL secretion. It is also reported in few studies that alpha-ketoglutarate is involved in collagen metabolism by increasing the activities of prolidase, a known key factor in collagen metabolism (Son et al, 2007). Collagen being important connective tissue in the body which contributes largely towards the structural integrity of the tissues, thereby suggesting that α-KG can be used for controlling the metabolism of the collagen. Affect of α-KG on collagen can make it a helpful molecule in various therapeutical and cosmetic applications. Other reported beneficial activity of α-KG includes prevention against
ischemic injury in heart surgeries (Peuhkurinen et al, 1983) and prevention of protein depletion after trauma or surgery (Wernerman et al, 1990).

2.12 Bioactive scaffolds for stem cell differentiation

In the *in-vivo* system the tissue repair, engraftment and regeneration is performed by MSCs that are engaged by paracrine factors in the bloodstream in response to the microenvironment of the concerned tissue. This system is mimicked in the *in-vitro* condition by using matrix factors that can direct stem cell differentiation either by mechanical or chemical in nature (Engler et al, 2006; Ram et al, 2006). The proliferation and differentiation of MSCs can be regulated either by substrate stiffness (Saha et al, 2008; Engler et al, 2006), the presence of soluble factors (Engler et al, 2006) or ligand presentation (Hosseinkhani et al, 2008; Rowland et al, 2006; Yang et al, 2005). It is well proven that MSCs commit to neurogenic lineage when they cultured on soft brain-like substrates, and to osteogenic lineage when cultured on solid or rigid bone-like substrates (Engler et al, 2006). Many recent studies have shown the effect of matrix stiffness on the phenotype and differentiation lineage of mesenchymal stem cells (MSCs). MSCs differentiated into myogenic, neural, or osteogenic phenotypes depending weather it is cultured on 2-D substrates of elastic moduli in the lower range (0.1–1 kPa), higher range (34 kPa) or intermediate (8–17 kPa). Pek et al, (2010) cultured MSCs in thixotropic gels with varying rheological properties, and similar results were found for the 3-D culture as for 2-D culture. In the 3-D cell cultured on the thixotropic gels, the liquefaction stress (sy) that is the minimum shear stress that is required to liquefy the gel was used for characterizing the matrix stiffness. The highest expressions of neural (ENO2), myogenic (MYOG) and osteogenic (Runx2, OC) transcription factors were found for gels with sy value of 7, 25 and 75 Pa, respectively. Immobilization of the cell-adhesion peptide RGD was found to promote differentiation of MSCs along with proliferation, especially for the case of the gel with high stiffness (>75 Pa). This study demonstrated the effectiveness of matrix property that could instruct or induce MSC differentiation (Pek et al, 2010).
Another way of MSCs differentiation which is explored in this thesis work is by culturing MSCs on surface modified matrix like surface modification using plasma polymerization. Much effort has gone into design, fabrication and synthesis of biomaterials and devices to ensure appropriate mechanical durability, property and functionality. Few examples of this is hip-joint fabricated to withstand high stresses, pumping bladder in an artificial heart should flex for millions cycle without failure, a hemodialyzer is required to have requisite permeability characteristics and it’s the bulk property of the materials that governs all these properties (Ratner, 2004). The biological responses of biomaterials and devices, on the other hand is controlled largely by their surface chemistry and structure. The rationale of surface modifying of biomaterials is to retain the key physical properties of matrix while modifying the outermost surface to influence the bio-interactions. If surface modification is properly effected, the mechanical properties and functionality of the device will be unaffected, but the bio-response related to the tissue device interface will be modulated or improved. Surface modifications falls in two category 1) physically or chemically altering the atoms, compounds or molecules in the existing surface (chemical modification, mechanical roughening, etching) or 2) over-coating the existing surface with materials having different composition (coating, grafting and thin film deposition) (Figure 2.8).

**2.12.1 Plasma deposition for surface modification of scaffold**

Radio frequency glow discharge (RFGD) plasma used for surface modification of the scaffold are low-pressure ionized gas environments typically at ambient or slightly above the ambient temperature. These procedures are also referred as gas discharge or glow discharge deposition/treatments. Plasma can be used to modify surfaces by etching or ablation or in a deposition mode to overcoat the surfaces (Yasuda and Gazicki, 1982). Plasmas are atomically and molecularly dissociated gaseous environments that contains positive and negative ions, free radicals, atoms, electrons, molecules and photons (visible and UV). The conditions within plasma chamber includes an electron energy of 1-10 eV,
gas temperature of 25-60 °C, an electron density of $10^9$ to $10^{12}$/cm$^3$ and a operating pressure of 0.025-1.0 torr (Alexander et al, 2004).

**Figure 2.8**: Schematic representations of methods to modify surfaces of the scaffold.

There are many processes that take place during surface modification or deposition of scaffold. First, competition between the etching and deposition by high energy gaseous species also called as ablation (Yasuda, 1979). When the ablation is more rapid than the deposition, no deposition can be observed as due to energetic nature of ablation or etching process can cause substantial morphological and chemical changes to the surface of scaffold (Bible et al, 2009). There are few hypotheses on the mechanism of
deposition process; one stated that reactive gaseous environment and UV emission can create free radical and other reactive species on the surface of scaffold, which reacts with the polymerized molecules from the gas phase (Perez et al, 2010). Alternatively, reactive small molecules which are in gas phase can combine to form higher molecular weight units or particle that settles or precipitate onto the surface (Figure 2.9). There are most likely chances of both processes occurring in combination to result in modification of scaffold surface (Elsner et al, 2009). “When plasma interacts with organic molecules in a vapour phase, polymerisation may take place, and all surfaces of substrates inside the plasma chamber are coated with the polymers formed. This process is called plasma polymerization” (Inagaki, 1996). Application of RFGD plasma surface modification is increasing in biomaterials as such treatment or coatings have special promise for improved biomaterials.

Figure 2.9: The plasma treatment chamber with oxygen plasma (white glow) and allylamine plasma (ppAAm) (pink glow) used for modifying the HG cryogel scaffold.
Table 2.1: Biomedical application of glow discharge plasma-induced surface modification and its advantages.

<table>
<thead>
<tr>
<th>Plasma treatment steps</th>
<th>Advantages of plasma treatment</th>
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<tbody>
<tr>
<td>Etching</td>
<td>➢ Cleans, sterilize and cross links surface molecules</td>
</tr>
<tr>
<td>Etching and deposition</td>
<td>➢ Forms barriers films</td>
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<td></td>
<td>▶ Protective coating</td>
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<td>▶ Electrically insulating</td>
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<td>▶ Reduces absorption of materials from environment</td>
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<td></td>
<td>▶ Inhibits release of leachable</td>
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<tr>
<td>Deposition</td>
<td>➢ Modify cell and protein reaction</td>
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<tr>
<td></td>
<td>▶ Improves biocompatibility</td>
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<td></td>
<td>▶ Promotes selective protein adsorption</td>
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<td>▶ Improves cell growth</td>
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<td>▶ Forms nonfouling surfaces</td>
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<td>▶ For grafting or polymerizing</td>
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2.13 Myoskeletal tissue engineering

Cardiac and skeletal muscle tissue engineering provides a smart approach to overcome problems associated with organ transplantation and cardiac tissue and also lays a platform for superior alternative approaches in muscle regeneration. Tissue regeneration needs scaffold that balance temporary mechanical function with mass transport to aid biological delivery and tissue regeneration (Hollister, 2004). Development of the porous scaffold for sustainable three dimensional (3-D) growth of cells is of particular interest in the field of
tissue engineering and regenerative medicine (Wang et al, 1998) as these scaffolds are tailor-made to mimic the micro-environment or act similar to extracellular matrix which has defined role such as to provide suitable structural and chemical composition and the mechanical properties (Harrison and Atala, 2007; Partap et al, 2006; Lutlof et al, 2003; Shea et al, 1999). Due to limited ability of the cardiac cells to regenerate in-vivo, attempts are being made to restore the functionality of the heart affected from myocardial infarction by injecting myogenic cells into the affected myocardium. It has been proposed that the implantation of in-vitro cultured functional myocardial muscle could potentially enhance cell localization and efficiency of tissue repair (Bursac et al, 1999; Carrier et al, 1999). First material to be used for cardiac repair was combination of poly-lactic acid and poly-glycolic acid but these polymers and their derivatives failed to provide substantial mechanical strength to scaffold which is attributed along with elasticity as most important property while choosing materials for cardiac repair. For successful cardiac and skeletal repair a critical step involved is the creation of tailor made 3-D matrices that act host to the cells (defined as cardiac patches) and should help in maintaining cellular viability, proliferation, differentiation and support cell integration (Zammaretti et al, 2004).
CHAPTER III

MOTIVATION AND OBJECTIVE
3.0 MOTIVATION

Cartilaginous tissues, including articular cartilage and fibrocartilage, perform essential mechanical functions in joints throughout the body. Traumatic injuries and degenerative diseases, such as osteoarthritis, are major sources of damage to these tissues and can impair their mechanical function. Unfortunately, the limited healing ability of cartilage and the lack of effective treatments create significant health problems with economic and social implications. Osteoarthritis alone is estimated to affect over 20 million people in India by year 2020 according to reports from Govt of India and will result in an estimated $86 billion in indirect costs. In addition, these conditions can be extremely painful and debilitating. Therefore, new technologies or therapies that promote cartilage regeneration have the potential to restore long term joint function and significantly improve the quality of life for many patients.

The engineering of tissue replacements through the combination of cells, scaffolding materials, or growth factors is one potential strategy for repairing damage to articular cartilage and fibrocartilage structures. However, there are major challenges for recreating cartilage tissues with the necessary material properties to function in the mechanically demanding, *in-vivo* environment. Furthermore, the scarcity of autologous chondrocytes presents additional cell sourcing limitations. Mesenchymal progenitors are capable of undergoing chondrogenic differentiation and may be an alternative cell source for tissue engineering therapies, but it remains unclear how specific signals coordinate to promote the differentiation and maturation of these cells into articular chondrocytes and fibrochondrocytes. Therefore, a greater understanding of the regulatory mechanisms and designing matrix for governing cell-matrix interaction and mesenchymal stem cells (MSCs) differentiation will significantly improve the potential application in tissue engineering therapies.
3.1 RESEARCH OBJECTIVES

The objective of the research presented in this dissertation was to investigate the role of cell-matrix interaction in regulating the differentiation of MSCs. The goal of this study was to elucidate the fundamental mechanism of providing an ideal 3-D construct that regulates the chondrogenesis in progenitor cells. The central hypothesis for this work was that interactions between cell–matrix and 3-D architecture of the matrix can help in modulating the chondrogenic differentiation of MSCs. With this objective the specific aim of the thesis work have been:

Aim I: To design a supermacroporous scaffold that is ideal for tissue engineering or regenerative medicine and to evaluate the effect of alpha-Ketoglutarate on the growth and metabolism of chondrocytes cultured on cryogel matrix.

Aim II: Plasma polymerization of cryogel scaffold for modification of surfaces to induce MSCs differentiation.

Aim III: Characterize the accumulation, organization, and turnover of various ECM components by MSCs in synthesized cryogel during co-culture conditions.

Aim IV: To explore the use of synthesized cryogel matrix in other tissue engineering application such as cardiac skeletal development.

This study demonstrates the potential application of cryogel for cartilage and cardiac engineering and the effects of cell - matrix on MSCs. MSC differentiation to desired lineage highlights the importance of selecting appropriate biomaterials for tissue engineering applications. These findings can provide a framework for future prospect of using MSCs for articular cartilage engineering and cryogel as potent material for tissue engineering and regenerative medicine.
CHAPTER IV

EXPERIMENTATION
4.0 Materials

2-Hydroxyethyl methacrylate (HEMA, MW 141.09), gelatin (from cold water fish skin, MW approx. 60 000), poly(ethylene glycol) diacrylate (PEGDA), Dulbecco’s modified Eagle medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 98%) reagent, 4,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), PCR primers, Hoechst 342, all monoclonal antibody (COLL II, aggrecan and COLL I) and nystatin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The RNeasy mini kit was from Qiagen (CA, USA). Glutaraldehyde (25%v/v) was purchased from s.d. fine-chemicals limited (Mumbai, India). Fetal bovine serum (FBS) and streptomycin–penicillin antibiotic solutions were bought from HyClone (Utah, USA). Ammonia persulphate (APS) and formaldehyde was supplied by Merck (Mumbai, India). N,N,N,N-Tetraethylmethylene diamine (TEMED) was purchased from Sisco Research Laboratory (SRL) (Mumbai, India). Cell tracker- green fluorescent probe was purchased from Lonza (Walkersville, USA) and Alexa Phallodein 568 was obtained from Invitrogen (Paisley, UK). Alamar Blue was from AbD Serotec (Martinsried/ Planegg, Germany) and ds-DNA quantification kit, F02962 was from Molecular probes (Eugene, OR, USA). PCR reagents Taq DNA polymerase, Nucleotide mix, PCR grade water, RT buffer was purchased from Roche diagnostics GmbH (Mannheim, Germany). Histology staining solution like alcian blue, safarinin- O, von-kossa, masson trichrome and hematoxylin-eosin were obtained from local suppliers. Glass slides were purchased from Blue star, Polar industries corporation (Mumbai, India). All other chemicals used were of analytical grade.

4.1 Scaffold fabrication and characterization

4.1.1 Preparation of poly(hydroxyethyl methacrylate)-gelatin (HG) cryogel

Cryogelation technology was used for the synthesis of HG cryogel. For the best possible scaffold synthesis, different concentrations of polymers were used in the reaction mixture with the optimum concentration of crosslinkers required for efficient linking of the
polymeric precursors. The selected polymer composition comprises HEMA: gelatin (6:4) ratio, respectively was used for the further studies. For the above mentioned composition, gelatin crust (400 mg) with final concentration of 4 % (w/v) was weighed and dissolved in 9 ml degassed water. To this solution, HEMA (0.6 ml) was added with the final concentration of 6 % (v/v) followed by addition of poly (ethylene glycol) diacrylate (PEGDA) (0.3 ml) (ratio 2:1, HEMA: PEGDA) and vortexed for 2 min to ensure thorough mixing of both the polymer solutions. The 20 µl of glutaraldehyde (25 % v/v) was added in the ratio of 1:20 of the total polymer concentration. Finally, APS (0.1 ml of 10 % w/v) and TEMED (0.01ml) were added as initiator of free radical polymerization. Cryogelation reaction was allowed to proceed overnight at -12 °C in liquid cryostat (Lauda RP1840, Germany). The gels were removed after required incubation period and washed in de-ionized water at room temperature to remove un-polymerized monomers. After thawing, HG cryogels were immediately washed three times with deionized water and were vacuum-dried using lyophilizer (Martin Christ GmbH, Germany) and stored at room temperature.

4.1.2 Microstructure analysis of HG cryogel by SEM

Morphology of HG cryogel samples was studied with a scanning electron microscopy (FEI Quanta 200). HG (10 %) scaffolds were overnight dried in vacuum before gold coating. Samples were gold coated using a sputter coater (Vacuum Tech, Bangalore, India). The microscope was operated under high vacuum at 20 kV with sample spot size of 4.5 mm to image the samples. The pore range, pore diameter and interconnectivity of HG cryogel were determined using scanning electron micrographs associated image analysis software (Kumar et al, 2003).

4.1.3 Swelling kinetics and solvent absorption capacity

The rate of swelling of cryogel samples is an important parameter to address the solvent uptake capacity of cryogels from their surroundings. This study also demonstrates the
relative interconnectivity in the HG cryogels. The swelling kinetics of HG cryogel samples was carried out using conventional gravimetric procedure (Ceylan et al, 2007). This involved measurement of fluid uptake rate and capacity of macroporous HG cryogel in phosphate buffer saline (PBS) (0.1 M, pH 7.4) solutions at 37 °C. Cryogel samples were vacuum-dried and sectioned into 5 mm thick stubs, which were moulded in to 12 mm diameter. The weight of the test samples was noted before the experiment and then placed in PBS at 37 °C in a thermostatic water bath. Five samples of HG cryogel were examined of same size and diameter. Samples were taken out after regular time intervals and weighed and this was repeated until gels reached the equilibrium.

The water uptake capacity (Wu) (%) is given by-

$$W_u=100 \times \frac{(W_t-W_g)}{W_e}$$

Where, Wu is water uptake capacity, $W_t$ is weight at regular time interval, $W_g$ is weight of dry cryogel and $W_e$ is weight of water in swollen gels at swelling equilibrium at a particular temperature. The weight-swelling ratio was taken as a parameter to calculate solvent absorption capacity which was calculated as-

$$S.R=\frac{W_s-W_d}{W_d}$$

Where, S.R is swelling ratio, $W_s$ is weight of swollen gel and $W_d$ is weight of dry gel.

4.1.4 Measurement of flow resistance of cryogel samples

The flow resistance of cylindrical monoliths of HG cryogel (12 mm length, 13 mm diameter) was evaluated up to flow rate of 8 ml/min using peristaltic pump with hydrostatic pressure of 0.01 MPa and has shown a linear flow rate of 2,000–2,500 cm h$^{-1}$ (Kumar and Srivastava, 2010) which registered the flow rate at given pump settings. Flow of water across these cryogel monolith samples was calculated up to the point where no back pressure created by the porous structure of cryogel was observed. In a separate experiment as control, the pump settings were calibrated against flow rate with no sample connected between the paths (Adrados et al, 2001).
4.1.5 Study of in-vitro weight loss of HG cryogel

Dry cryogel samples of HG were weighed and then sterilized by incubating in 70 % ethanol for 2-3 h. Ethanol saturated samples were washed with sterile PBS (0.1 M, pH 7.4) to remove all the ethanol content from the samples followed by transferring these samples into 50 ml tightly capped plastic tubes filled with sterile PBS (0.1 M, pH 7.4). Test samples were incubated in a water bath set at 37 °C up to eight weeks under sterile conditions. Samples were taken out at predetermined time and thoroughly washed with de-ionized water. The samples were dried overnight by keeping in vacuum desiccators. The weight loss was determined by change in dry weight of test sample.

\[ W_L (\%) = \left(\frac{W_I - W_F}{W_I}\right) \times 100 \]

Where, \( W_L \) is degree of degradation, \( W_I \) is initial dry weight of sample before incubation and \( W_F \) is final dry weight of sample after incubation.

4.1.6 Unconfined compression test of HG cryogel

Unconfined compression tests were conducted on HG cryogels. Cylindrical cryogel samples (height 5 mm and diameter 12 mm) were saturated with PBS (0.1 M, pH 7.4) and were used for conducting the tests. Stress was given to the cylindrical shaped cryogel samples using (Zwick/Roell Z010 machine) with10 kN load cell under displacement control at rate of 1 mm/ min (Dainiak et al, 2006). The slope of the graph with stress measured in kPa was plotted on Y-axis and percentage deformation plotted on X-axis was measured at difference of 10 % deformation to calculate compressive modulus.

4.1.7 Rheology analysis for material characterization

The rheology analysis helps in measuring the flow of the material and deformation at given conditions (Vlachopoulos, 1981). Device (MCR301 SN824057, Malvern instruments, UK) was used for characterization of polymeric material as these scaffolds have their distinctive flow rate and behaviour in different temperatures. Sections (10 mm
thick) of the cryogel were cut and samples were placed on sample holder fitted with cone and plate geometry with gap width of 150 µm and cone diameter of 40 mm with 4° angle and applied force of 1N per sec for 15 min. The storage modulus ($G'$) and loss modulus ($G''$) was calculated using oscillatory logarithmic sweep at a frequency of 1Hz. Substance measurement points was kept at 1000, number of data points was set to 60 and all the conditions were kept constant for rest of the material analysis. Materials may act as liquid in short run but in longer run can transform to solid state and this dual nature of polymeric materials is known as visco-elastic behaviour. The visco-elastic property of cryogels can play a very important role in the skeletal muscle tissue engineering. HG cryogels were analysed at two different temperatures of 25 ºC and 37 ºC corresponds to room temperature and body temperature, respectively and cryogels were also checked for phase difference at dry and wet state. The dry state defines the scaffold lyophilized overnight and stored in vacuum to be completely dry and wet state of the scaffold describes saturation of scaffold with water. Dry cryogels were first tested and same sample were saturated with water for further analysis.

4.1.8 X-ray micro-computer tomography (micro-CT) for polymer distribution in cryogel

Cryogel section of approximately 2 mm sized was prepared and analysed using X-ray CT scanner (Skyscan1072, Aartselaar, Belgium) at 8.27 mm voxel resolution (50 magnification) on 30 kV with X-ray tube current 173 mA. Scaffolds were mounted on the sample holder with the help of sample glue. The scanning image was recorded every 0.3 degree of sample rotation. These scanning radiograph projections were used in standard cone-beam reconstruction software to generate a series of 625 axial slices. The resulting 3-D data sets were hence isotropic with voxel spacing of 15 mm over the entire 10243 spatial range. Three-dimensional reconstruction of the internal pore morphology was carried out using these axial images and analysed by CT Vol and CTan software provided by the manufacturer. For identifying distribution of each polymer in scaffold a sequential analysis of micro-CT images at 1000 mm intervals along the sample long axis enabled
detailed information to be congregate on the range of pore sizes and pore distribution within the cryogel scaffold. Number of layers scanned was 1000 with lower grey threshold 39 and upper grey threshold was 255 and threshold for both the scaffold was kept same in order to maintain the conditions for 2-D and 3-D construction. While performing 3-D analysis the threshold had to be chosen and the graph showed 2 distinct peaks. After choosing one peak, it showed vast difference in the 3-D structure of the cryogel scaffolds. The base to the tail end of the peak was chosen for each construction and while emerging of these images, the distribution of the polymers within the scaffold could be clearly observed.

4.2 IN-VIVO BIOCOMPATIBILITY OF HG CRYOGEL

*In vivo* studies (according the ISO 10993 regulations) were carried out in twelve “C57BL/6J” mice. Animals were divided in four groups (n=4 mice) for observation at three different time points. All mice had free access to food and water and similar living conditions were maintained for all (temperature and humidity controlled environment) at the animal centre of medical inflammation research, Karolinska Institute, Stockholm, Sweden. This study was completed with permission/approval from “N310-07 from ethical rights committee, Stockholm region, Sweden. The HG cryogel were implanted under sterile environment. The used cryogels were sterilized in gradient ethyl alcohol (20, 40, 60, 80, 100 %) and autoclaved to ensure cryogels were completely sterile at the time of implantation. Mice were anesthetized by 50:50 % mixtures of purfuran and air. The implantation regions were shaven and cleaned by 70 % alcohol before the surgery and the scaffold were implanted subcutaneously at back region of mice by making a horizontal incision. The length of incision was approximately 5-10 mm. After implantation of the cryogels, incision was closed by using resorbable sutures. After implantation, sutures were not removed and were found to have dissolved after few weeks of implantation. No inflammation was observed at the site of implantation.
4.2.1 Histological studies

Cryogel implanted mice were sacrificed after 2\textsuperscript{nd}, 4\textsuperscript{th} and 6\textsuperscript{th} week of implantation and skin tissues at/around the site of implantation were collected for histology. The excised tissues were fixed overnight in formaldehyde solution for histology and part of the tissues was fixed in tissue tek solution for immuno-histochemistry. Tissues were dehydrated in a gradient series of ethanol and xtrasol solutions before embedding in paraffin. Five micrometers thick sections were cut of tissues by microtome (HM 360) and cryomicrotome (Microm model HM 500 M) for immunostaining. Hematoxylin and eosin staining was performed for observing change in overall tissue morphology and tissue response to implanted material. For screening of infiltrated mast cells at site of implantation, safranin staining was performed on excised tissue sections. In safranin staining, first sections were dehydrated in absolute and 95 \% alcohol for 3 min. After dehydration, sections were incubated in Mayer HTX solution for 12 min. This step is followed by extensive washing of section done under running tape water up to 10 min. After washing, sections were stained with 0.1 \% fast green solution for 6 min. For reducing the pH sections were immersed in 1 \% glacial acetic acid followed by incubation with 0.1 \% Safranin. Finally, sections were dried in 95 \% and absolute alcohol for 2 min.

4.2.3 Immunostaining for macrophage and dendritic cells

Immunostaining was performed in nearby tissue at site of implantation. Skin tissues collected from site of implantation after different time intervals and immediately embedded in tissue tek solution at -20 °C. The 5 \µm thicker sections were cut by cryomicrotome and stored at -20 °C for staining. Immunostaining protocol of macrophage and dendritic cells was standardized at Medical Inflammation centre Karolinska institute, Sweden. For immunostaining, first sections were fixed in acetone for 5 min and then dried at room temperature for 5 min. The fixed sections were blocked by first blocking
solution (10 % Bovine serum albumin, 2 % rat serum, avidin) for 30 min. After incubation, sections were washed in Tris-buffer saline (TBS) for 3 x 5 min. Second blocking was done by biotin for blocking inherited avidin in tissue for 30 min. Blocked sections were washed 3-5 times and incubated with biotinylated M (1/70) monoclonal antibody (developed in medical inflammation research, Karolinska) for immunostaining of macrophage cells, biotinylated N-418 antibody for dendritic cells for 1h. Sections were washed 5 times for 5min and blocked by third blocking solution (3 % H₂O₂ in methanol) before incubation with DAB peroxidase substrate (DAB kit manufactured by Vector laboratories) for 12 min. After 5 min washing in TBS (pH 7.4), sections were stained in hematoxylin solution for 10 sec and extensively washing was done in running tape water up to 5 min. For dehydration, sections were dipped in 95% and absolute alcohol for 5 min before monitoring.

Moreover, immunostaining of implanted cryogels was performed on the excised tissue after every 2nd week of implantation. For macroscopic examination, the digital pictures were taken of the site of implantation after 2nd, 4th and 6th weeks.

4.3 CELL-MATRIX INTERACTION

4.3.1 Isolation of primary cells of chondrocytes

Articular cartilage was aseptically harvested from the knee joint of Indian Capra hircus (body weight: 4-6 kg, age: 3-6 months). Knee cartilage was minced in to 1-2 mm fine pieces and subjected to enzymatic digestion with 0.3 % collagenase type II at 37 °C for 12 h. The collagenase solution was prepared in serum free Dulbecco’s modified eagle’s medium (DMEM). The digested cartilage suspension was centrifuged at 150 g for 5 min in 15 ml centrifuge tube. The pellet was then treated with 0.25 % trypsin for 5 min to ensure release and separation of cells. Trypsin treatment was stopped by adding 10 % foetal bovine serum (FBS) in to the cell suspension and cells were centrifuged at 250 g for 10 min (Zhou et al, 2007). Cell pellet obtained were checked for cell number and
viability using haemocytometer and trypan blue vital dye, respectively. More than 90 % viable cells were used for cell culture experiments.

4.3.2 Chondrocytes culture on HG cryogel

To check the cell growth on HG scaffolds, cryogel sections (height 5 mm and diameter 13 mm) which were pre-saturated with serum free DMEM, chondrocytes were seeded with the cell seeding density of $1 \times 10^5$ cells/well with complete DMEM medium supplemented with 10 % foetal bovine serum, 1 % penicillin–streptomycin and 1 % nystatin. All cell culture experiments were setup in triplicates for authentication of results. Samples were analyzed by SEM from 3rd day onwards of cell culture. Before gold coating, the cells were fixed by glutaraldehyde (2.5 %) solution.

4.3.3 Cell-matrix interaction

4'-6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity for AT (adenosine-thymine), AU (adenosine-uracil) and IC (inosine-cytosine) clusters. The fluorescence of the DAPI bound to DNA is strongly enhanced. Chondrocytes were grown on 200 µm thin section for microscopic observation of cells and scaffold interaction. Cell seeding density $1 \times 10^5$ cells/well, the thin sections seeded with cells were fixed with 2.5 % glutaraldehyde for 2 h followed by 20 min incubation in nuclear stain DAPI (200 ng/ml) working solution prepared in PBS. After the incubation, sample section was gently washed twice with PBS. The morphology of the cell nuclei was observed under fluorescence microscope (Nikon, TE-2000 U) at excitation wavelength of 350 nm. The normal cell nuclei are considered to have bright fluorescence and homogeneous distribution of the dye.
4.3.4 Propidium iodide (PI) staining for proliferating cells on HG cryogel

Propidium iodide is an intercalating dye which binds by inserting itself between the strands of double stranded nucleic acid chains. Any change in the DNA which results in the nucleic acids being more tightly packed together (i.e. increased histone content and nucleosome formation) will reduce the "stainability" of the DNA. Thus there may be a slight difference in the $G_1/G_0$ peak of rapidly dividing cell cycling as opposed to slowly dividing cells even though they are both diploid (2C) cells of the same type can be clearly observed using PI stain (Riccardi et al, 2006). The 200 μm sections were used for analyzing the chondrocytes growth and their proliferation within the scaffold. Cell seeding density was same to that of DAPI and MTT assay. Cryogel sections were fixed in 2.5 % glutaraldehyde solution for 2 h. After the cell fixation, cells were permeabilized by the permeabilization buffer (0.1 % TritonX-100) for 30 min. Sections were then incubated for 15-20 min before observing under fluorescence microscopy.

4.3.5 Biocompatibility of scaffold examined by cell proliferation assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is indirect way of checking the cell growth, proliferation and viability, since the mitochondrial enzymes of the proliferating cells oxidizes the MTT solution, giving a typical blue-violet end product which can be quantified by spectrophotometric analysis (Berridge and Tan, 1993). Briefly, the HG cryogels (5 mm height and 12 mm diameter) were saturated in the DMEM medium supplemented with 10 % foetal bovine serum, 1 % penicillin–streptomycin and 1 % nystatin for 2 h into a 24-well tissue culture plate. Primary chondrocytes (0.5 ml) were seeded into each pre-saturated cryogel section with the cell density of $1.0 \times 10^5$ cells/ml. The control for the experiment was set up in 2-D conditions with cells seeded on the culture plates. The cell seeded sections along with controls were incubated up to 21 days at 37 ℃ in a humidified atmosphere with 5 % CO$_2$ environment. At each interval, the culture medium was removed from test wells and gently washed
with PBS (0.1M, pH 7.4). The serum free culture medium (0.5 ml) was added which contained thiazolyl blue (MTT) (0.5 mg/ml) solution into test wells and incubated at 37 °C for 5 h. The medium was aspirated carefully and 1.5 ml of dimethyl sulphoxide (DMSO) was added to each test well to dissolve intracellular formazan crystals to get blue–violet colored end product, which was measured by spectrophotometer absorbance at 570 nm.

**4.3.6 Functional chondrocytes analysis by glycosaminoglycan and collagen estimation**

The major anatomical structure of cartilage is collagen and proteoglycans which in turn are composed of glycosaminoglycans (GAGs) and structural protein (Xu et al, 2006). So, secretion of proteoglycans by chondrocytes in scaffold is important parameter and can be measured by indirect quantitative determination of GAG concentration by spectrophotometrically using 1, 9-dimethylmethylene blue (DMB) dye. The GAG content was analyzed from chondrocytes seeded samples along with the controls at every 4th day up to day 21st of cell culture. The amount of sulphated glycosaminoglycan (S-GAG) in chondrocyte culture was quantified by the digestion of cell culture samples in papain solution, pH 6.5 (2 mg/ml) taken in tubes and incubated overnight at 60 ºC. The whole digested samples were collected in 2 ml micro-centrifuge tube and centrifuge at 580 g for 20 min. The supernatant was mixed with DMB dye solution and allowed to react for 30 min. The solutions were then read for absorbance spectrophotometrically at 525 nm. The concentration of GAG in all the samples was calculated based on a standard curve plotted by using chondroitin sulphate (Peansukmanee et al, 2009). The GAG amount was measured in mg/ml which was reported as µg GAG per mg dry weight of the sample.

Collagen is the second most important component of the cartilage tissue. To determine the total collagen secretion by chondrocytes seeded on the HG cryogel matrix, collagen was estimated using hydroxyproline assay. The analysis of hydroxyproline indirectly determines the purity of the collagen as one mole of collagen is known to be composed of 7.36 hydroxyproline molecules (Srinivasan and Sehgal, 2009). In brief, the
HG scaffolds were lyophilized and subjected to papain (0.1%) digestion overnight at 60 °C. Digested samples were hydrolysed using 1M HCl at 120 °C for 10 h and the contents were transferred to glass scintillation vials and incubated uncapped at 90 °C until dry (brown residue at bottom of vial). Samples were cooled to room temperature and redissolved in 1 ml PBS (0.1M, pH 7.4). The dried hydrolysed samples were mixed with chloramines T and p-dimethyl amino benzaldehyde in equal proportion and incubated in a water bath at 60 °C for 30 min. The reaction mixture in vials was allowed to cool to room temperature for at least 10 min and read in a colourimetric plate reader at 540 nm (Stegeman and Stadler, 1967). Hydroxyproline standards were simultaneously used in different concentration and same protocol was followed to obtain standard curve to determine the unknown hydroxyproline concentration. The total collagen content was estimated using the following equation:

$$\text{Collagen content (\%) = } \frac{[\text{hydroxyproline}] (\mu g/ml) \times \text{dilution factor} \times \text{proportion hydrolysed} \times 100}{\text{dry weight of scaffold} \times 0.143 \times 10^6}$$

The term 0.143 is used because hydroxyproline forms approximately 14.3% of total collagen (Miyada and Tippel, 1956).

4.3.7 Reverse transcriptase (RT)-PCR for Coll I and Coll II from chondrocyte cultured on HG cryogels

Total RNA was isolated from chondrocytes cultured on HG cryogels using RNeasy mini kit and RT-PCR was carried out using primers for collagen type I (Coll I) and collagen type II (Coll II) for differentiating and identifying the total collagen content quantified by biochemical analysis and also to cross check the expression of Coll I. Polymerase chain reaction was performed by using a ThermoHybaid Px2 Thermal Cycler under the following conditions: 94 °C for 5 min for one cycle (initial denaturation), followed by 30 cycles of 94 °C for 30 sec (denaturing), 55-60 °C (depending on the melting temperature of the primers), for 30 sec (annealing) and 72 °C for 1 min (extension) and finally one
step of 72 °C for 10 min (final extension). The resulted product was analysed by gel electrophoresis.

**COL2A1 primer sequence:** 5`- AAGATGGTCCCAAAGGTGCTCG- 3`

3`- AGCTTCTCCTCTGTCTCCTTG- 5`

**COL1 primer sequence:** 5`- TAAAGGGTCACCGTGCT-3`

3`- CGAACCACATTTGGCATCA-5`

**4.4 USE OF ALPHA-KETOGLUTARATE IN CELL CULTURE**

**4.4.1 Seeding of NIH3T3 on HG cryogel**

To set up the experiment, NIH3T3 cells were seeded on HG cryogel scaffolds of 2 mm thickness. Scaffolds were sterilized using alcohol in stepwise gradient concentration (20 % -100 %) and equilibrated with PBS (0.1M, pH 7.4). The media was discarded before setting the experiment. NIH3T3 cells were seeded on HG cryogel sections with the density of 0.2× 10^5 cells/well. One set of the experiment was performed with 2 mM alpha-KG containing media and another without alpha-KG in the media. Similar protocol was followed for 2-D culture. Experiment was set up for 11 days and sample of media was collected at regular time intervals for glucose and ammonia estimation and also MTT assay for cell proliferation was performed on same wells. All the experiments were performed in duplicates.

Cellular growth and proliferation was checked using MTT assay and the protocol followed is as stated above.

**4.4.3 Estimation of ammonia**

Ammonia free water was used in the preparation of reagents and washing of glasswares for ammonia estimation. Solution I was prepared by dissolving 1 g phenol and 5 mg of sodium nitroprusside in 100 ml of degassed water. Solution II was prepared by weighing
250 mg of sodium hydroxide along with 2.16 g of disodium hydrogen-orthophosphate and 10 ml of sodium hypochlorite and the total volume was made upto 50 ml. All the above components were mixed well and were tightly capped until further use. The test sample (500 µl) was taken and to this 10 % sodium tungstate and 1N sulphuric acid was added to denature protein. The mixture of solution was centrifuged at 180 g for 15 min. The supernatant was immediately and carefully collected and mixed with 2.5 ml each of solution I and solution II (Murry, 1925). After thorough mixing, tubes were incubated at 37 °C for 35 min. The absorbance was recorded at 625 nm and same protocol was followed using ammonia as standard.

4.4.4 Estimation of glucose

Dinitrosalicylic acid (DNSA) weighing 10 g along with 500 mg of sodium sulphite and 10 g of sodium hydroxide were mixed in 1L of water. To this sodium potassium tartrate (40 %) prepared in distilled water was added along with 500 µl of DNSA and 500 µl of test sample solution and the tubes were kept at 90 °C for 15 min to develop red-brown colour complex. The coloured complex developed in the tubes was stabilized using 40 % sodium potassium tartrate (Rochelle salt). The samples were allowed to cool to room temperature and the absorbance was recorded at 575 nm using spectrophotometer.

4.4.5 Study of cell-matrix interaction

Cryogel scaffold seeded with chondrocytes were fixed with 2.5 % glutaraldehyde after completely removing the media. Air dried samples were sectioned for SEM and DAPI. The SEM of the cryogel scaffold was performed using same protocol as in case of microstructure analysis of scaffolds. DAPI is known for its affinity to bind with AT rich region of dsDNA and is used in regular basis for checking the distribution of cells on scaffolds. The fluorescence emitted upon binding with DNA is observed by Nikon, TE-2000 U at excitation wavelength of 350 nm.
4.4.6 Nuclei examination for cells cultured on HG cryogel matrix

Propidium iodide (PI) which is an intercalating dye binds to double standard nucleic acid by inserting itself between the strands of the DNA or RNA. So any change in the DNA which results in the nucleic acids being more tightly packed together (i.e. increased histone content and nucleosome formation) will reduce the "stainability" of the DNA. The sections of HG scaffold seeded with NIH3T3 cells were fixed in 2.5 % glutaraldehyde solution for 2 h. After the cell fixation, cells were permeabilized by the buffer (0.1 % TritonX-100) for 30 min and then observed under fluorescence microscope.

4.5 STEM CELL DIFFERENTIATION ON HG CRYOGEL

4.5.1 Surface modification of HG cryogel for stem cell differentiation

Surface modification experiments were done on 2 mm sections of HG scaffolds. The scaffolds were first etched with the oxygen plasma which leads to the etching of the scaffold surface making it more reactive towards the polyallylamine (ppAAm) deposition. The plasma chamber was first pumped to a base pressure of less than 20 mTorr. Thereafter, oxygen etching of the scaffolds was performed via oxygen plasma generated by oxygen gas flowing through the chamber at a pressure of 300-350 mTorr. Once oxygen etching is done, the scaffolds were exposed to the plasma of polyallylamine (ppAAm). Source of allylamine with an input power of 20 W was then used to generate polyallylamine plasma. The samples were exposed to this plasma for ~ 25 min and a layer of ~ 3.5 KA° thickness was deposited on the scaffolds. Once the plasma etching was complete the chamber was vented (Lakshmi et al, 2009). These plasma treated substrates were then sealed with parafilm and were used for further analysis like XPS, water contact angle, etc, after keeping them in a dark place for 48 h, such that the plasma coating formed on the surface are stabilized.
4.5.2 Surface analysis using water contact angle

A pico-liter sized droplets DSA 1000 (Krüss, Germany) was used to measure increment of water contact angles (WCA). The progression of the drop shape was captured over 1 s after deposition on the cryogel surface. The WCA was calculated from the very first stable image of the drop using a circular fit. WCA line profiles were obtained by averaging the WCA data for each channel. Acquisition of full raw data sets allowed for the retrospective construction of spectra from the imaged areas. Images are normalized to the total ion intensity (Perez et al, 2009).

4.5.3 X-ray photo spectroscopy (XPS)

XPS scans were performed at different locations on each of the test substrates and were used to determine the elemental composition at the surface. XPS spectra were recorded using a Kratos axis ultra spectrometer (Kratos Analytical Ltd., Manchester, UK). Employing a monochromatic Al Kα X-ray source (hν = 1486.6 eV), hybrid (magnetic / electrostatic) optics, hemispherical analyzer and a multi-channel plate and delay line detector (DLD) with a collection angle of 30º and a take off angle of 90º. During each XPS scan, the X-ray gun power was set to 150 W and all spectra were recorded using an aperture slot of 300 × 700 microns with pass energy of 80 eV for wide angle survey scans and 20 eV for high-resolution core level scans. All XPS spectra were recorded using the Kratos VISION II software; data files were translated to VAMAS format and processed using the CASA XPSTM software package (Version 2.3.2 and later). During experiments charge compensation was used (Kratos AXIS Nova charge neutralization system: a coaxial low energy electron source within the field of the magnetic lens) and samples were earthed via the stage using a standard BNC connector. All of the measured binding energies are referenced to the C 1s core level at 285.0 eV (Bible et al, 2010).

Biochemical and scanning microscopic analysis was performed using procedure stated before. Biochemical analysis involved measuring MTT and GAG. Immuno-histological
staining protocol followed throughout remains same until stated otherwise. RT-PCR for gene profiling was also done using same protocol as before. The primers used for gene profiling of differentiated MSCs are:

- **COL1**
  - 5'-AGCCAGCAGATCGAGAAGCA-3'
  - 5'-TCTTGTCCTGGGCTTTG-3'
- **COL2**
  - 5'-CACTCCAGGAAGCTGACTGA-3'
  - 5'-GCACCATTGGTAGGACACG-3'
- **SOX9**
  - 5'-CCCTTCAACCTCCACACTA-3'
  - 5'-TCAAGGTCGAAGTGCTGTG-3'
- **Aggrecan**
  - 5'-GGCACCTTTCACTGTCACC-3'
  - 5'-CTGCAGTGAAGACCTCACA-3'
- **COMP**
  - 5'-GCCTCAGCTTCTACCTGGGC-3'
  - 5'-ACCTGATGTCAGCCCACTTG-3'
- **GAPDH**
  - 5'-ACAGFACGCCGCATCTTTCT-3'
  - 5'-GACAAGCTTCCGGCTCTCA-3'

### 4.5.5 DNA quantification using Hoechst 33258 assay

The Hoechst 33258 working solution was prepared by adding 1 ml of TNE buffer (100 mM Tris; 2.0M NaCl; 10mM EDTA; pH 7.4) along with 9 ml of distilled water and the solution was filtered. To the filtrate 10 µl of Hoechst dye was added and covered with aluminium foil until further use. The test scaffolds were transferred into vials containing 250 µl of papin and were incubated at 60 °C overnight. The vials were allowed to cool to room temperature and the shredded tubes were used to centrifuge scaffold to ensure complete lysis of cells and to obtain clear supernatant free from any scaffold digest. Supernatant (100 µl) was mixed with 100 µl of Hoechst working solution and the fluorescence was measured using excitation and emission filters fixed at 360 nm and 460 nm, respectively (Maniatis et al, 1982).
4.5.7 Immunostaining for MSCs differentiation

The cell cultured onto the cryogel sections were fixed using formyl saline for 15 min and washed with PBS twice (0.1M, pH 7.4). The scaffolds were washed with blocking solution containing 10% normal donkey serum, i.e. 50 µl in 450 µl of PBS for 10 min. Scaffolds were rinsed using PBS containing 0.1 % (v/v) Triton X-100, 1 % (w/v) bovine serum albumin (BSA) for 30 min at room temperature. The primary antibody coll II 1:100 dilution was added in minimal PBS (containing 0.1 % BSA) to cover the sample. The negative control was taken without the monoclonal antibody. These sections were kept for overnight incubation at 4 ºC. The sections were rinsed with PBS (containing 0.1 % BSA) and the secondary antibody which was anti FITC or Rodamine labelled was added to all the sections in 1:200 dilutions. The sections were wrapped in aluminium foil for an hour and kept at room temperature away from light. After incubation, the sections were rinsed in PBS (containing 0.1 % BSA) and left for 10 min to wash away excess of secondary antibody. Before mounting the sections with vectashield mounting media the sections were rinsed thoroughly with 1×PBS. The monoclonal antibody used were for COLL II, aggrecan and COLL I.

4.6 Microenvironment dependent differentiation of mesenchymal stem cells to chondrogenesis

4.6.1 Acquisition and dissection of rodent joints for limb cells

Pregnant mice (18th day of gestation) were used for isolation of fetal limb cells (Biomedical service unit, university of Nottingham). The foetus was washed with warm sterile PBS/gentamicin and was placed into sterile 20 ml tubes. The limbs were placed in 20 ml tubes containing 0.25 % (v/v) Trypsin 0.02 % (w/v) EDTA solution and mixed for 30-45 min in an incubator at 37 ºC- 5 % CO₂ environment. Collagenase type II enzyme along with media was added to each tube after aspirating out Trypsin-EDTA solutions and agitated for 2-4 h in an incubator at 37 ºC in 5 % CO₂ environment. After this incubation period, cloudy single cell suspension was observed which was filtered via a 70
µm nylon filter into a tube in order to remove any large pieces of tissue. The cell pellet was centrifuged at 300 g for 10 min and washed with chondrocyte medium. The cells obtained were cultured in DMEM along with 10 % FBS and 1.5 % pencillin /gentamycin.

4.6.2 Human mesenchymal stem cell culture

Human MSC (bone marrow derived) (Cat no: 7500) from ScienCell Research Laboratories (UK) was used for the experiment. Cell density was >1x 10^6 cells in 1 ml volume. hMSC are characterized by immunofluorescent method with antibodies to CD73, CD90 and CD105. Cells were kept in water bath at 37 °C until completely thawed and cells were fed with mesenchymal stem cell medium and were incubated at 37 °C with 5 % CO₂ condition and medium was changed after overnight incubation. Fresh media was added every 48 h and after the desired cell number was obtained, cells were used further for experiments.

4.6.3 Seeding cells on HG cryogel

Human mesenchymal stem cells were seeded along with foetal mouse limb cells in 2:1 ratio on 2 mm and 200 µm sections of cryogels with seeding density of cells being 0.5 × 10^5 and fed with equal proportion of DMEM and mesenchymal stem cell media. Experiment was divided into two sets one set was kept in incubator with 5 % CO₂ at 37 °C and another in hypoxia condition. The experiment was carried in triplicates.

4.6.4 Reverse transcription – polymerase chain reaction (RT-PCR) analysis

The total RNA content was extracted using RNeasy mini kit (Qiagen, CA) using the protocol provided by the manufacturers. The total RNA content was determined using nanodrop (Nanodrop technologies, DE). The reverse transcription reaction was performed with 48 µg total RNA. The primer sequences were;

**SOX-9**

5′-GACTTCCGCGACGTGGAC-3′;

3′-CAGTACCTGCGCCCAAAC-5′.
Polymerase chain reaction was performed by using a Thermal Cycler (Thermohybaid Px2) under the following conditions: 94 °C for 5 min for one cycle (initial denaturation), followed by 30 cycles of 94 °C for 30 sec (denaturing), 55-60 °C (depending on the melting temperature of the primers), for 30 sec (annealing) and 72 °C for 1 min (extension) and finally one step of 72 °C for 10 min (final extension). The resulted product is analyzed by gel electrophoresis.

4.6.6 Determination of total cellular metabolic activity by alamar blue

The working solution of 10 % concentration was prepared by mixing 1 ml Alamar Blue stock solution mixed with 9 ml HBSS without phenol red and without serum mix and 0.2 µm filter was used for sterilization; the solution was kept in dark or wrapped in foil until further use. Media was aspirated from the wells and washed thrice with warm, sterile PBS. The scaffolds were transferred to a fresh 24 well tissue culture plate and 1 ml alamar Blue working solution was added and incubated at 37 °C in 5 % CO₂ environment for 90 min. Alamar Blue working solution 100µl was transferred (post-incubation with scaffolds) to a 96 microtitre well plate. The plate was wrapped in foil and fluorescence was measured (Ex 560 nm/ Em 590 nm) and absorbance read at 570 nm (Nasiry et al, 2008).

4.6.7 Quantification of extracellular matrix component- glycosaminoglycan (GAG)

Glycosaminoglycan (GAG) was measured by taking different sample dilution which was prepared in 1.5 ml tubes, using blank papain solution as the diluents. Blank papain solution (20 µl) was added to one of the wells along with 20 µl of standard followed by 20 µl of each sample dilution. 1, 9-dimethylmethylene blue (DMMB) solution (200 µl) was added to each well containing blank, standards or samples and the plate was read within 10 min of adding the DMMB solution at 540 nm (complex signal/absorbance) and 620 nm (uncomplex DMMB signal).
4.6.8 Quantification of extracellular matrix component- collagen

The sample for collagen estimation was prepared by acid hydrolysis. To 250 µl papain digest 250 µl conc HCl was added in a pyrex (acid resistant tube) and capped tightly with an acid resistant (phenolic PTFE lined) cap and incubated overnight at 120 °C on a heating block. The contents were transferred to glass scintillation vials and incubated uncapped at 90 °C until dry or until a brown residue is noticed at the bottom of the vials. The sample was cooled to room temperature and residue was re-dissolved in 1 ml sodium phosphate buffer.

4.6.9 Preparation of blanks and sample diluents

Hydrolysed blank heated papain solution aliquot, with an equal volume of conc. HCl at 120 °C, dry at 90 °C and re-suspended in an appropriate volume of sodium phosphate buffer. The hydrolysed papain solution was used for diluting standards and samples. Blank hydrolysed papain solution (50 µl) was added into the plate along with 50 µl of each standard in triplicate into the plate. Each sample (50 µl) and dilution in triplicate added along with 50 µl chloramine T solution and incubated at room temperature for 20 min followed by addition of 50 µl pDAB solution to each well and further incubated in a water bath at 60 °C for 30 min. The plates were allowed to cool to room temperature and read in a colourimetric plate reader at 540 nm.

4.6.10. Nuclear staining for co-culture set up

Double-stranded DNA and 4′6-Diamidino-2-phenylindole (DAPI) are known to form fluorescent complexes with fluorescence specificity for AU, AT and IC clusters, due to this complex the cell nuclei with normal phenotype appear glowing. This property of forming complex makes DAPI a useful tool in cell biology. Co-culture cells were grown on 200 µm thin section for microscopic observation of cells and scaffold interaction. Cell seeded thin sections were fixed with 2.5 % glutaraldehyde for 2 h followed by 20 min
incubation in nuclear stain DAPI (200 ng/ml) working solution prepared in PBS. After
the incubation, sample section was gently washed twice with PBS. The morphology of
the cell nuclei was observed under fluorescence microscope (Nikon, TE-2000U) at
excitation wavelength of 350 nm. Nuclei are considered to have the normal phenotype
when glowing bright and homogenously.

4.7 Myoskeletal tissue engineering

4.7.1 Designing of HG cryogel for skeletal tissue engineering

Gelatin (200 mg) was weighed and dissolved in 9 ml degassed water with final
concentration of 2 % (w/v). To this solution HEMA (0.8 ml) with the final concentration
of 8 % (v/v) was added and to ensure thorough mixing, solution was vortexed.
Ammonium persulphate (APS) (0.1 ml of 10 % w/v) and TEMED (0.01ml) were added
as initiator of free radical polymerization. Instantly, poly (ethylene glycol) diacrylate and
glutaraldehyde (25 % v/v) (ratio 1:20, glutaraldehyde: Gelatin) were added as cross
linkers. Cryogelation reaction was allowed to proceed overnight at -12 °C in liquid
cryostat (Julabo, Seelbach, Germany). The gels were removed after incubation and using
de-ionized water were thawed at room temperature. After thawing, HG cryogels were
immediately washed with deionized water and were vacuum-dried at -50 °C and stored at
room temperature.

4.7.2 Myoskeletal C2C12 on HG cryogel

HG cryogels were sectioned in 2 mm thickness and were sterilized stepwise by gradient
alcohol concentrations (20 %, 40 %, 60 %, 80 % and 100 %). Scaffolds were calibrated
with PBS (0.1M, pH 7.4) for 3 h followed by calibration with serum free DMEM
overnight. C2C12 cells were reviewed by spinning the cells down at 200 g for 10 min and
the cell pellet was re-suspended in 1 ml of DMEM media containing the foetal bovine
serum. Viability and cell count was performed before seeding cells on scaffold. The cell
seeding density was 1×10^6 cells/ml/per scaffold/per well. The culture plates were
incubated at 37 ºC with 5 % CO₂ for over three weeks and experiment was set-up in duplicates.

Total cellular metabolic activity assay using alamar blue and DNA quantification protocol followed is as stated previously.

**4.7.3 Cell differentiation**

Hoechst staining was performed by dissolving Hoechst 342 stock solution in distilled water at 1 mM concentration and final working concentration of 1 µM from the stock used for 15 min to stain the adherent cells. Hoechst 342 was visualized at excitation wavelength of 360 nm and emission is recorded at 480 nm. 4',6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with double-stranded DNA showing fluorescence specificity for AT, AU and IC clusters and because of this property DAPI is a useful tool in various cytochemical investigations. C2C12 cells cultured on cryogel scaffold were used for microscopic observation of cell-matrix interaction. Cell seeded onto the HG cryogel were fixed with 2.5 % glutaraldehyde for 2 h and were sectioned using microtome (Microm HM 560 CryoStar, Thermo) followed by 20 min incubation of sections in nuclear stain DAPI (200 ng/ml working solution prepared in PBS). After the incubation samples were gently washed with PBS (0.1M, pH 7.4). Sections were selected at different region of the scaffold (upper, middle and lower portion) to check penetration of cells in HG scaffold. The morphology of the cell nuclei was observed under fluorescence microscope (Nikon, TE-2000U) at excitation wavelength of 350 nm. Nuclei with normal phenotype were expected to appear glowing bright and homogenously distributed. Scaffold samples were fixed in 4 % paraformaldehyde for 10 min, and were then permeabilized with 0.1 % Triton-X 100 for 5 min and after each step through rinsing with PBS (0.1M, pH 7.4) at room temperature were performed. The working solution of cell tracker and phallodein stains were made up in 1:200 dilution with 1 % BSA and scaffolds were incubated for 15 min before imaging.
CHAPTER V

RESULTS & DISCUSSION
5.1 SCAFFOLD FABRICATION AND CHARACTERIZATION

In the initial studies, a series of cryogels were produced with different concentrations of polymer precursors and different cryogelation conditions were used for scaffold fabrication. With this optimization and with the concept of porous 3-D construct for tissue engineering applications with ideal properties (Nasir et al, 2007), 2-hydroxyethylmethacrylate-gelatin (HG) cryogel was synthesized with the final copolymer concentration comprising of 6 % HEMA and 4 % gelatin. The preparation of scaffold with high volume fraction porosity with soft, mechanically stable and high water absorbing ability was achieved in the optimized cryogel composite. The synthesized cryogel samples were light yellow in colour and have shown shape retaining property when dried at room temperature (Figure 5.1). The ice crystals play an important role in the cryogel formation, which can be formed into a defined 3-D structure during incubation at sub-zero temperature, which worked as a porogen to translate into resulting morphology of void volume (macropores) in the cryogel scaffolds (Kumar et al, 2003). It is important to explore the morphology of cryogel samples in the state in which the scaffold would exist either in the biological fluids or in the living tissues. These cryogels have shown stability and retain porous structure at dry state with slight decrease in diameter around 1 mm from the initial diameter of 13 mm of the scaffold. So, the ethanol dehydrated samples were examined using scanning electron microscopy (SEM) to evaluate the porosity (Figure 5.2). During the SEM analysis, HG cryogel samples with different polymer concentrations were analyzed (data not shown). It was observed that with higher concentration of pHEMA and/or gelatin, it resulted in constricted pores and very less porosity. While keeping very low polymer concentration, the scaffold was not stable enough to provide mechanical integrity for tissue engineering applications evaluated by physical examination. Thus the final concentration of HG cryogel (10 % gel) was found appropriate with respect to physical property and porous structure for further studies.
Figure 5.1: The digital image shows physical appearance of water saturated poly (hydroxyethyl methacrylate)-gelatin (HG) cryogel.

Figure 5.2: Scanning electron microscopy (SEM) images of HG-cryogel. (A) Shows the uniform pore distribution in the cryogel matrix (249X) and (B) shows the interconnected porous network within the cryogel at higher magnification (600X).
5.1.2 Swelling kinetics and solvent absorption

Synthesized cryogel sections (diameter 13 mm, height 2 mm) were analyzed to check the continuous porous network that might be needed to secure fluid connectivity inside the matrix during the cell culture (Hwang et al, 2010). To understand the complex architecture of pores it was evaluated by solvent absorption rate and capacity of scaffold. The vacuum dried cryogel sections were used for swelling study in PBS (0.1M, pH 7.4). The HG cryogels showed high swelling kinetics which swelled up to 80% of their original weight within one min, while these gels attain equilibrium within two min (Figure 5.3). Five sections were used to obtain the concurrent readings. Apart from that the average swelling ratio of HG cryogel was 8.39 ± 0.21. The high swelling kinetics and swelling ratio suggested large pore morphology and pore interconnectivity within the gel.

![Swelling kinetics of HG cryogels](image)

**Figure 5.3**: Swelling kinetics of HG cryogels. HG scaffolds swelled up to 80% of their original weight within one min and reached to equilibrium within two min. This experiment was carried out using 5 different samples and p value was <0.05.
5.1.3 Measurement of flow resistance of cryogel samples

In bioreactor culture, one of the most important characteristics is unhindered flow system that allows the good convective flow of nutrients and cell waste during the cell culture and even prevents cell death on high seeding density (Bansal et al, 2006). Since most of the cryogels synthesized are for biomedical and bioengineering applications, an important characteristic is to check adequate flow rate without any resistance (Lozinsky, 1998). Gelatin cryogel (4%) showed high back pressure against the solvent flow as shown in our previous work (Kathuria et al, 2008). This is because of soft nature of gelatin cryogels, where the soft walls of gelatin cryogel might be collapsing and resulting pores were blocked on high flow rate of solvent. However, during the flow rate analysis of synthesized HG scaffold, it was observed that these gels did not show any back pressure up to flow rate of 8 ml/min. Such a free flow of fluid suggests that the pores in the cryogel matrix are interconnected offering a negligible resistance against the solvent and this feature plays an important role in efficient gaseous exchange and unconstrained nutrient transport during cell culture. It can be inferred from that the optimized combination of pHEMA to gelatin is supporting to have better wall strength as well as interconnective macroporous system. Polymeric material is expected to be porous to allow high density of cells to be seeded, yet should be mechanically stable with well defined network of interconnected pores to allow growth into the implanted structure (Fang et al, 2005). The macroporous materials synthesised in our laboratory and by various other groups have been found to meet these requirements therefore, have been extensively used in tissue engineering (Jain et al, 2010; Tripathi et al, 2010).

5.1.4 In-vitro degradation study

Degradation of HG cryogels was examined up to eight weeks of incubation in PBS (0.1M, pH 7.4) at 37°C under sterile conditions. After eight weeks of incubation, substantial disintegration was observed in the cryogel samples which made handling of samples difficult. Therefore, degradation experiments were stopped after eight weeks.
During the whole experimental analysis, there was consistent weight loss observed up to fifth week (Figure 5.4). Thereafter, no significant weight loss was found. The degree of weight loss of HG was about 88% in two months. It is presumed that the macromolecules of the cryogel surface undergo preferential hydrolytic scission into low molecules (oligomeric units), which can be solubilized in PBS. The presence of hydrophilic groups such as amide and carboxyl allows gelatin to hydrolyze quickly in water. It manifests that the degradation of gelatin chains in the HG cryogel is quicker, which might degrade completely within five weeks (Figure 5.5A, B). Crosslinked pHEMA scaffolds are used in several biomedical applications and are shown to be biocompatible (Ushakov et al, 2000; Santin et al, 1996; Rao et al, 1995; Chirila et al, 1992). Thus, HG cryogels in this study provide biocompatible and degradable cryogel preparations for tissue engineering applications.

Figure 5.4: Weight loss study of the HG cryogel in the in-vitro conditions. HG scaffolds shows approximately 88% weight loss from its initial weight (degree of degradation) in eight weeks of incubation in sterile conditions.
Figure 5.5: Scanning electron micrographs (SEM) of degraded HG cryogels. The deformation in the cryogel walls after four weeks (A) time and (B) shows the collapsing of pores after a period of eight weeks.

5.1.5 Unconfined mechanical analysis

The mechanical analysis of HG cryogel was determined by unconfined compression test. Mechanical integrity and strength evaluation revealed a distinct upward trend, which was calculated from the slopes of graph formed between the stress and strain. The cryogel sections (12 mm diameter and 5 mm height) of HG showed considerable compressive modulus (Figure 5.6) along the whole column length. The average compressive modulus of HG cryogel was 32.73±2.36 kPa, calculated at 15% compression to their original length. However, the cryogels were compressed up to 90% of their original length without any permanent deformation. Even, after removing the load, scaffold reverted to their original shape when immersed in the reservoir water. In contrast to the matrix synthesised at room temperature that becomes brittle upon drying, cryogels have been found to be easily compressed to four to six folds of its original length without leading to any mechanical damage to the matrix (Dainiak et al, 2006). Cryogel matrix has been used in chromatography of cells in which the large interconnected pores enhances the chances of cell passage without clogging the column (Kumar and Srivastava, 2010). The critical
parameter in designing scaffold for cartilage tissue engineering is the maintenance of mechanical integrity during the application of mechanical strain since the cartilage is main load bearing tissue experiencing varying degree of mechanical stimulus so the mechanical stability of the designed scaffold should be at par with native cartilage. Here the HG cryogels have shown substantial mechanical strength which was further exploited by culturing the cells on these cryogels in 3-D environment.

Figure 5.6: Stress-strain behaviour of HG cryogel under uniaxial unconfined compression. This graph shows the mechanical stability of HG cryogel under stress-strain conditions. No deformation in cryogel morphology was noticed up to 80% compression of their original length.

5.1.6 Rheology study of HG cryogel

Rheology can be simply defined as a study of flow and deformation of materials. It is used in studying complex structure of substances including body fluid, suspensions, polymers, etc. The flow of these materials cannot be characterized by a single value of viscosity (at a fixed temperature). More theoretically rheology is the relationship between the flow/deformation, performance of material and its internal structure that cannot be
defined by classic fluid mechanics or elasticity. When material is subjected to a stress of different sorts (that is a force per area) (Macosko, 1994), material responds in various ways. The two important ways materials can change is either its elasticity or viscosity and in-between lie the third property of the material termed as visco-elastic behaviour of the material. Depending upon the processing time of the polymer, like if polymers are pumped or coated or sprayed the processing time can be different and with short processing time polymer may behave as a solid and in long processing time the material can behave as a fluid (Freidrich et al, 1995). This dual nature (fluid-solid) in rheology term is referred to as viscoelastic behaviour of materials. The solid behaviour of the material is depicted in rheology term as storage modulus \( (G') \) since it reflects relaxation time and elastic modulus and the perfectly elastic nature will develop stress that is in-phase with strain. The liquid behaviour is represented by loss modulus \( (G'') \) and it gives information about dissipation (viscous) of the flow. The equation to define storage and loss modulus is:

\[
G' = \frac{\text{In-phase stress}}{\text{Maximum strain}} \quad \text{STORAGE MODULUS}
\]

\[
G'' = \frac{\text{Out of phase stress}}{\text{Maximum strain}} \quad \text{LOSS MODULUS}
\]

The rheological measurement (such as \( G', G'' \), elongation viscosity and viscosity) can be used as excellent tools in material characterization and input data for computational simulation, etc (Macosko, 1994). Rheology study of the HG cryogel suggested that the scaffold were stable at different temperatures and phase angle remained constant in both
dry and wet state (Figure 5.7A-C). HG cryogel were able to bear increased stress without leading to deformation. Upon hydration (addition of water to the dry scaffold tested previously) of HG scaffold showed the shift from a stiff to a more pliable material which is seen in graph were the dry $>10^5$ to wet $<10^4$Pa value shifts (Figure 5.7A). The hydration is quick as observed and due to this there is rapid change and equilibrium position is attained within 30 sec of hydration. The time gap measurement was done between additions and levelling off in G’. By continuing hydration G’, G” (shear modulus) value remain constant with no further change in modulus. However, the change in phase angle $<0.24^\circ$ indicates a gradual increase in stiffness of the material over the time which may be due to hydration influencing changes in bonding and loss modulus which could be resulting in possible molecular re-arrangements (Figure 5.7 B). Upon further analysing HG cryogel shows steady state as there is no change in loss modulus and storage modulus, the scaling for the phase angle shows changes over 0.03° and from there on it is seen to be flat with no significant change in the phase angle indicating the equilibrium state of the cryogel as seen in Figure 5.7 C. The phase angle reduction in Figure 5.7 A shows the material exhibiting elastic characteristics. The rheology study of the HG cryogel showed the ability of the material to bear stress without leading to deformation and the mechanical strength along with elastic nature of scaffold. Friedrich et al, have tested pHEMA and co-polymers using rheology and found the pHEMA of high molar mass 500 KDa showed significant shear thinning and viscoelastic properties. Therefore, exploiting these material properties like stiffness of the scaffold, visco-elastic behaviour which are most important parameter for designing construct for cardiac tissue engineering. During the in-vivo conditions, the stress experienced by these tissues is probably the maximum as compared to other tissues or organs and the rheology studies of the HG scaffold illustrated the material properties that could be used in skeletal muscle regeneration.
Figure 5.7: The graph represents the rheology data of HG cryogels. The storage modulus, loss modulus and phase angle is plotted against the time at temperature 37 ºC in dry state (A) 37 ºC wet (B) and 25 ºC wet state (C). In the graph the storage modulus G’ is represented by (I), the loss modulus G” is depicted by (II) and phase angle by δ (III).

5.1.7 X-Ray micro-computer tomography (micro-CT) for micro structure and polymer distribution analysis in cryogel

X- Ray micro-computed tomography (micro-CT) study of the HG cryogel revealed the pore size and pore distribution in the synthesized scaffold. HG cryogel is scanned and 2-D construction is first obtained from the machine (Figure 5.8 shows the principle of the micro-CT). Using this 2-D constructs, 3-D structure is plotted. Two dimensional structure constructions allow the user to re-scan or re-set machine if the scan report is not up to the
mark e.g., if the voxel size is too large it means there is enormous background noise which could be due to the sample stage not fixed firmly or the sample itself rotates along the scanning period. Once the 2-D analysis report meets the standard criteria, 3-D construction is performed. While scanning HG cryogel the threshold graph showed 2 distinct peaks and trying to eliminate either of the peak resulted in change in pore size and porosity of the cryogel (Table 5.1). To confirm the peak shown is two distinct peaks as the polymer used in synthesizing the scaffolds were two different polymer units, gelatin cryogel was taken as control. Scanning of gelatin (Figure 5.9) cryogel showed only one peak and keeping all the scanning to threshold parameters, same HG cryogel was rescanned. The distribution of two polymers inside a scaffold was further confirmed by scanning other cryogels like alginate-gelatin (Figure 5.10), agarose-gelatin (Figure 5.11) along with HG cryogel (Figure 5.12) as the common polymer in all of these scaffold was gelatin. Choosing only one peak gave very drastic increase in pore size and porosity of the cryogels. The 3-D construction was done choosing both the peaks and then choosing only one threshold peak enabled us to locate the distribution of polymers in the closed system.

![Diagram of cone-beam high-lighted x-ray micro-CT apparatus](image)

**Figure 5.8:** The diagram shows the basic cone-beam high-lighted x-ray micro-CT apparatus schematic representation. Magnifications can be set between X 1.1 to over X100 by just moving the position of the X-ray camera and the rotation stage, X-ray energies is considered to be ideal for optimising a contrast between the different phases in scanned biomaterials and the dynamic range of the X-ray camera is ideal for collecting the data with a large signal-to-noise ratio (modified from Sakellariou et al, 2004).
Table 5.1: The pore size and porosity measurement using micro-CT. Choosing only one peak of threshold graph shows increase in the pore size and porosity of the scaffold. Results were validated using different cryogel scaffold which had one common polymer gelatin. Gelatin cryogel showed only one peak during scanning and was taken as control with the same scanning parameter followed for all the other scanned scaffolds.

<table>
<thead>
<tr>
<th>CRYOGE</th>
<th>AVERAGE PORE SIZE OF COMPLETE SCAFFOLD</th>
<th>AVERAGE PORE SIZE WITH ONE POLYMER PEAK</th>
<th>POROSITY OF COMPLETE SCAFFOLD (%)</th>
<th>POROSITY WITH ONE POLYMER PEAK (%)</th>
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<tr>
<td>Gelatin (control)</td>
<td>61.48 µm</td>
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<td>61.83%</td>
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<tr>
<td>Agarsoe-gelatin</td>
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<tr>
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<td>163.63µm</td>
<td>80.01%</td>
<td>81.33%</td>
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<tr>
<td>pHEMA-gelatin</td>
<td>72.47µm</td>
<td>200.72µm</td>
<td>81.41%</td>
<td>88.82%</td>
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</tbody>
</table>

Figure 5.9: Micro-CT analysis of gelatin cryogel. Image shown (A) maximum intensity projection (MIP) after scanning. Image (B) represents the binarised image showing pores of the scaffolds that are seen black dots and white dots depict the solid or non-porous part of the scaffold. 3-D reconstruction of gelatin cryogel (C) shows porous and homogenous distribution of gelatin in cryogel scaffold.
Figure 5.10: Micro-CT analysis of alginate-gelatin cryogel. Images shown in MIP (A) after scanning. Image (B) shows the 3-D reconstruction of Al- gelatin cryogel after choosing just one peak of thershold. Merging the peaks (C) showing 3-D porous and homogenous distribution of alginate and gelatin in cryogel scaffold.

Figure 5.11: Micro-CT analysis of agarose-gelatin cryogel. Images shown in MIP (A) after scanning. Image (B) shows the 3-D reconstruction of Ag- gelatin cryogel after choosing just one peak of thershold. Merging the peaks (C) showing 3-D porous structure and homogenous distribution of agarose and gelatin in cryogel scaffold.

Figure 5.12: Micro-CT analysis of HG cryogel. Images shown in MIP (A) after scanning. Image (B) shows the 3-D reconstruction of HEMA-gelatin cryogel after choosing just one peak of thershold. Merging the peaks (C) showing 3-D porous and prefential distribution of the HEMA and gelatin polymer in the HG scaffold.
5.2 IN-VIVO BIOCOMPATIBILITY OF HG

In-vivo studies were performed on mice which were divided into control and experimental groups. The vertical insertion was made on the back of the mice (Figure 5.13) and HG cryogel were implanted in every experimental animal and once implanted the site was sutured and all animals were kept in similar conditions. The in- vivo studies showed early angiogenesis around the implanted scaffolds (Figure.5.14 I). The implants were well accepted and mice showed no signs of discomfort. In particular, no effects on sleeping or feeding habits were observed even after six weeks post-implantation period. The in-vivo degradation of HG cryogel was slow and considerable amount of material was intact even after six weeks. Stereomicroscopic images indicated vascularization around the implanted cryogel. New blood vessels were seen in the host tissue adjacent to the surface of the implanted cryogel scaffolds. No signs of adverse reactions, such as necrosis, infection or granuloma, were observed around the implanted scaffolds even till six week post-implantation. Haematoxylin and eosin staining showed the infiltration of cells at the site of implantation (Figure 5.14 II). Large numbers of cells are found at the site of implant after 6 weeks of implantation demonstrating that implant can recruit cells surrounding implant. Furthermore safranin staining (Figure 5.14 III) was done to check the presence of mast cells. Even after six weeks the mast cells were not observed at the site of implantation signifying that material does not cause any immunological response which could lead to host vs. graft rejection mechanism. The implanted cryogel scaffolds showed mild inflammation at site of implantation and characterized by infiltration of macrophages, dendritic cells and proliferation of large population of endothelial cells. The localized inflammation reduced with formation of thin fibrous capsule around the cryogel. The tissue response was significantly higher after 2\textsuperscript{nd} week of implantation and in comparison to 4\textsuperscript{th} and 6\textsuperscript{th} week of implantation where insignificant amount of mast cells could be seen. In case of HG cryogel, connective tissue was found near the implanted material after 2\textsuperscript{nd} week. The material was found to completely integrate in to
host tissue after 4\textsuperscript{th} week of implantation. The material was found to constantly degrade in later week of implantation. At site of implantation the infiltration of phagocytic cells like macrophage was less even in 6\textsuperscript{th} week (Figure 5.14 IV). Moreover large infiltration of endothelial proliferative cells was observed at lower layer of dermis. Infiltration of few immune cells like dendritic cells was also observed in nearby tissue around material. An enormous amount of fine blood vessel formation was observed near the implanted material in the initial weeks of implantation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.13}
\caption{\textit{In-vivo} biocompatibility of HG cryogel. Experimental mice (C57BL/6J) (A) was given a vertical cut at the back (B) for creating the site of implantation for HG cryogel scaffold. The HG cryogel implanted (C) at the site of insertion was sutured (E) and closed. The control mice were anthesised and left without any insertion (D).}
\end{figure}
Figure 5.14: (I) Stereophotography of HG cryogel scaffolds implanted subcutaneously, (II) H&E staining of tissue surrounding cryogel implant, (III) safranin staining for mast cells infiltration surrounding scaffold, (IV) immunostaining of macrophage around implanted site.
5.3 CELL & MATRIX INTERACTION

5.3.1 Chondrocytes culture on HG cryogels

Cell growth and proliferation on HG cryogels were performed to check the biocompatibility by culturing primary cells of chondrocytes isolated from goat knee. Primary chondrocytes isolated were seeded onto HG cryogels pre-equilibrated in cell culture medium. The growth of chondrocytes on HG cryogel sections were carried out on 5 mm and 200 µm sections to check for the cell behaviour. The control experiments were set up by seeding cells on 2-D culture plates which were continuously monitored by microscopic examination. The pores of cryogel look large enough to allow cell to effectively migrate into the pores and also allow good nutrient supply and metabolic waste removal, essential for effective cell growth. The cells seem to be well adhered with uniform distribution and a single cell adhered on the wall of HG cryogel matrix releasing its own extracellular matrix (Figure. 5.15 A-D). The HG cryogel seems to provide native like environment for cell growth. Different regions in scaffolds were scanned during SEM analysis, which showed uniform cell distribution within the gel.

Chondrocyte adherence and proliferation on 200 µm section was observed under fluorescent microscope. The cell proliferation was examined using two dyes which stained cell nucleus i.e. DAPI and propidium iodide (PI). Cell cultured cryogel sections were incubated with DAPI and PI solutions for 20 min in separate wells and then gently washed with PBS (0.1M, pH 7.4). The morphology of the cell nuclei was observed after 7th day of cell culture at specific wavelength according to the applied stain. Cells adhered and proliferated adequately within the scaffold sections and increased cell population was observed (Figure. 5.16).
Figure 5.15: Scanning electron microscopic (SEM) image of chondrocytes on HG cryogel. The growth and proliferation of chondrocytes on the wall of HG cryogel supports matrix compatibility with cells. (A-D) is images taken at different magnification. ‘A & C’ showing homogeneous distribution of chondrocytes, while ‘B & D’ shows that chondrocytes have maintained their morphology along with secretion of ECM in HG cryogel scaffold.
Figure 5.16: Fluorescent image of cell-matrix interaction. Chondrocytes growth and uniform distribution within the HG cryogel sections was visualized under fluorescent microscope using (A) 4’6-diamidino-2-phenylindole (DAPI) and (B) propidium iodide (PI).

5.3.2 Cell activity and viability study

Chondrocytes proliferation and their metabolic activity in HG scaffolds were monitored at a regular time intervals up to day 21 of culture by MTT assay. The optical density of end product i.e., formazan crystals, reflecting the activity of cells was measured. The biocompatibility of HG cryogel was compared with the control. Initial readings showed the proliferation of cells in controls (2-D) and HG cryogels (3-D) culture system were equal but from the day 13, there was significant decline observed in control while HG scaffolds showed continuous increase in cellular metabolic activity with time (Figure. 5.17). An increase of cell metabolic activity measured by MTT assay indicates good cell adhesion and proliferation in the scaffolds. Chondrocytes are difficult to be maintained indefinitely in culture condition once they attain confluency (Lin et al, 2008) because of synthesis of sulphated-proteoglycans leads to a sharp decrease in the pH of the media and also due to lesser surface area in comparison to cryogel (3-D) system leading to metabolically inactive or dead cells in control (2-D) system. Much attention has been
diverted towards cartilage tissue engineering and there are many studies that have utilized different approaches for culturing chondrocytes in the *in-vitro* like using beads or encapsulating chondrocytes (Ceuninck et al, 2004) however, the cells are not found to proliferate well when embedded or encapsulated inside bead (Liu et al, 2009). In HG cryogel the ability of chondrocytes to retain its spherical shape confirms that scaffold is supportive for cells. It was also important to check biocompatibility of these cryogels to observe any toxic effect of using glutaraldehyde as cross linking agent for HG synthesis, which did not seem to exhibit any adverse effect on the cell growth and proliferation. The MTT assay confirmed that the transport of nutrients and gases in the HG cryogel were efficient for cells to remain viable and active. The cell growth rate in the HG cryogel opens its potential for tissue engineering application.

![Figure 5.17](image_url)

**Figure 5.17:** The relative viability of chondrocytes measured by MTT assay. Cells were grown up to 21 days in HG cryogels (3-D) (grey bar) and compared with the control (2-D) (black bar) as tissue culture plate. The absorbance of blue formazan was observed at 570 nm at different time intervals up to twenty-one days of culture.
5.3.3 Glycosaminoglycan (GAG) and collagen estimation

It is well known that GAGs have unbranched long chain and repeating disaccharide units and function as one of the major components of the extracellular matrix (ECM) of cartilage (Bayliss et al, 1983). In particular, it has been shown that GAGs linked to a protein core are organized to form proteoglycans, and these complex molecules are considered to play a key role in modulating cell morphology, proliferation, differentiation and function (Thanou et al, 2002). This suggests that the amount of GAG is the measure of cartilaginous tissue formation. During the chondrocyte cell culture, up to day 5, constant increase in the amount of GAG was noticed in both the cases i.e., control (2-D) as well as HG cryogel (3-D) with the slight but non-significant difference in the amount of GAG (Figure. 5.18A). After day 9, the HG cryogel showed continuous increase in the production of GAG, while in 2-D culture the GAG content was observed to slightly decrease at day 7 and after that remained constant. GAG concentration is found to increase in chondrocytes cultured in clusters and higher the cell seeding density, higher is the GAG production (Iwasa et al, 2003) however, culturing chondrocytes in 3-D system these cells tends to spread like fibroblast which affects the production of ECM components but in HG cryogels chondrocytes were found to have retained their original shape and showed enhanced production of GAG.

Collagen is estimated using hydroxyproline (Hyp) standard which is a post-translational product of proline hydroxylation and is catalysed by the enzyme prolylhydroxylase. As collagen is composed of 14.3 % hydroxyproline (Miyada and Tappel, 1956), the total collagen content of the samples can be calculated using hydroxyproline assay. The estimation method utilizes the ability to oxidize hydroxyproline to a pyrole compound and then to pyrole-2-carboxylic acid that reacts with p-dimethylaminobenzaldehyde (DAB) leading to the formation of chromophore that can be measured spectrophotometrically (Taskiran et al, 1999). The gene expression was checked to confirm the type of collagen produced in the in-vitro condition by the primary
chondrocytes. The collagen content in 3-D and 2-D system showed similar trend to that of GAG. The total collagen content is expressed per dry weight of the scaffold. From the day 9 of cell culture in HG scaffold, collagen content showed an upward increase and reached almost constant level from day 17 of the experiment (Figure 5.18 B). However, in 2-D culture the increase in the collagen content is seen up to day 9 and after that there was a decline. Collagen type I production was examined by using fluorescent collagen type-I antibody that was used to cross check the de-differentiation of primary chondrocytes towards osteoblasts and was found to be negative at every time point. The production of GAG and collagen measured in this study indicates the cellular production of extra cellular matrix and thus confirming that the scaffolds were supporting the chondrocyte proliferation and secretion of ECM components.

**Figure 5.18:** Production of glycosaminoglycan (GAG) and collagen by chondrocytes cultured on HG cryogel. The amount of glycosaminoglycan (GAG) (A) was continuously increasing in chondrocytes cultured in HG cryogel (grey bar) in comparison to the control (2-D) (black bar). Graph (B) total amount of collagen shows increasing upward trend in HG cryogels (grey bar) seeded with primary chondrocytes in comparison to the control (2-D) (black bar).
5.3.4 Reverse transcriptase (RT)-PCR for Coll-I and Coll-II from chondrocyte cultured on HG cryogels

It is known that the in-vitro culture conditions alter the mechanism of ECM production in chondrocytes (Waldman et al, 2003). As seen in the SEM analysis that the chondrocytes cultured on HG cryogels retained their spherical shape and established well 3-D connections with the neighbouring cells. The significant advantage of this is clearly seen in PCR analysis, where the Coll-II expression is seen from the 1\textsuperscript{st} week of the culture and continues to enhance till 3\textsuperscript{rd} week (Figure. 5.19). The PCR analysis clearly indicated the expression of Coll-II gene in chondrocytes culture on HG cryogels, whereas Coll-I gene expression was almost absent. This suggested that the collagen type II was the main component of the total collagen content estimated biochemically, which could be because the HG cryogels allowed re-acquisition of chondrocytes spherical morphology and thereby not allowing the chondrocytes to de-differentiate to fibrous structure. The concentration of HEMA polymer is relatively very high to that of gelatin in HG cryogels and pHEMA has shown to help in chondrocytes retaining its morphology (Olmedilla et al, 2006). This probably explains no expression of the collagen type-I which otherwise is produced either during culture of osteoblasts or the chondrocytes that de-differentiate to more fibrous structure (Lin et al, 2008).

![Coll-I and Coll-II gene expression](image)

**Figure 5.19:** Coll-I and Coll-II gene expression of chondrocytes cultured on the HG cryogel. The gene expression was checked for over 3 weeks showing the prominent expression of Coll-II suggesting the total collagen content can be collagen type II which is found as major anatomical structure in the native cartilage.
In conclusion, this study demonstrated the feasibility of using HG cryogel as a scaffold for cell carrier in cartilage tissue engineering. pHEMA- gelatin composites have been synthesized earlier by different approaches such as by preparing the poly(HEMA) sponges and saturating it with gelatin solution (Santin et al, 1996), but this study presents simple one step processing method employed for successfully synthesizing synthetic and natural polymeric blend cryogel scaffold. These scaffolds show good elastic and mechanical characteristics, and well controlled pore architecture, high porosity with relatively large pore size for better cell and waste transport. The cryogels do not show any failure during stress and strain procedure and their capacity to retain shapes after drying makes such scaffold materials more effective and easy for handling. Cell-matrix interaction observed while culturing chondrocytes, showed excellent cell attachment, proliferation and secretion of ECM, thus establishing the potential of cryogels for tissue engineering applications particularly for cartilage tissue engineering.

5.4 Effect of Alpha-Ketoglutarate for cartilage tissue engineering

Apart from the media and various growth factors, microenvironment plays important role in cell culture. Major difference in cell behaviour in the culture system and their counter parts in-vivo system is the dissociation of cells from 3-D geometry and their propagation on the 2-D substrate. The specific cell interactions characteristic of the histology of tissue is lost as cells spread out and starts to proliferate leading to de-differentiation of cells (Freshney, 2005). Culture environment also seems to lack the systemic components that are involved in the homostatic regulation in-vivo, as a result cellular metabolism can be more constant in the in-vitro than in-vivo conditions (Freshney, 2005). There are intrinsic factors that need to be added to obtain the cell functions that are true to the tissue aimed for regeneration (Alberts et al, 1994). Cells have shown to proliferate better in the 3-D environment due to greater available surface area and better transport of nutrients. Progression in the field of tissue engineering has lead to development of various scaffolds.
that are tailor made depending upon their applications. In our laboratory, scaffolds using natural and synthetic polymers are designed by special approach known as cryogelation (Tripathi et al, 2010; Srivastava et al, 2007) in which matrices are synthesized at temperature that is normally below the freezing temperature of the solvents. The ice crystals formed during synthesis melts in thawing procedure leaving behind a porous structure known as cryogels. The average pore size of these matrix lie between 50-200 µm (Jain et al, 2009) and the high porosity of the cryogel due to large interconnected pores ensure convective flow of nutrients and mass transportation of gases which is unhindered even in long –batch cultures. These cryogels have been successfully used in cartilage tissue engineering (Kathuria et al, 2009), monoclonal antibody productions (Jain et al, 2010) and cell separations (Kumar and Srivastava, 2010). In this study we have demonstrated the effect of α-KG on fibroblasts and chondrocytes on HG cryogel matrices in order to investigate that the effect shown by α-KG on cellular proliferation on the 3-D scaffold. We have also investigated how the ammonia accumulation and glucose consumptions differ in different cells in presence and absence of α-KG. Combining the effects of chemistry of these metabolites with tissue engineering applications would help in finding pathways that can finally yield better therapeutic products. This work was aimed to provide an insight for the role of α-KG as a bioactive molecule in the cell culture and tissue engineering applications.

5.4.1 Morphological analysis of cells seeded on HG scaffold in presence of α-KG

SEM was performed in order to validate the high rate of growth and proliferation observed in MTT for the cells seeded on α- KG containing samples. Scaffolds allowed uniform and highly dense growth of the cells on α- KG positive samples. In addition, high white ECM deposition was observed (Figure 5.20 A) on α- KG containing culture samples within three weeks of culture, whereas in the absence of α- KG, no such deposition was observed (Figure 5.20 B). These results were further confirmed by SEM which shows that the cells were embedded within the secreted ECM (Figure 5.20 C),
whereas the cells on scaffold without $\alpha$-KG showed good ECM secretion (Figure 5.20 D). Cells were found to be growing uniformly but ECM secretion was not observed in scaffold without $\alpha$-KG (Figure 5.20 D).

**Figure 5.20**: Digital image of ECM deposition on scaffold with $\alpha$-KG (A) and scaffold without $\alpha$-KG (B). Scanning electron micrograph image of HG cryogel scaffolds after 30 days. Cells found to be embedded within the ECM secreted by chondrocytes (C) over the period of 30 days in $\alpha$-KG containing scaffold. The appearance of white ECM deposition was clearly seen in this scaffold. In HG cryogel without $\alpha$-KG (D) after 30 days cells are seen secreting the ECM but was lesser as compared to $\alpha$-KG containing scaffold.

It is already reported that the *in-vitro* accumulation of ECM takes around 6 weeks (Freed et al, 2004). In addition, the ECM observed in HG which might be because it allowed
better cell adhesion, proliferation and growth when compared to 2-D where there is reduced surface area for cell proliferation.

5.4.2 Effect on cell -matrix interaction in presences of $\alpha$-KG

DAPI and PI staining of the cells cultured on alpha KG containing samples (Figure 5.21) clearly showed uniform and large number of cells growing throughout the HG scaffolds which is in accordance with the SEM results.

**Figure 5.21**: Digital image of DAPI and PI staining for cell-matrix interaction. Image visualized under fluorescent microscope shows stained nuclei of proliferating chondrocytes and homogenous distribution on the cryogel matrix HG (A& B). PI staining of the fibroblast seeded on HG (C &D) cryogel shows perfectly round shaped nucleic acid staining inferring no negative effect of $\alpha$-KG on fibroblast.
5.4.3 In-vitro cell proliferation assay for NIH3T3 and chondrocytes

The cell proliferation was studied using the MTT assay for both the cell lines seeded on two different kinds of scaffolds. NIH3T3 cells and chondrocytes were used in our work as previous reports have shown that addition of α-KG inhibits the catabolism of collagen in dermal fibroblasts as well as enhances collagen content via proline synthesis (Son et al, 2007). This could be used as an advantage for culturing chondrocytes as collagen is one of the most important ECM components of cartilage. The samples for MTT assay were collected every alternate day for 3T3 cells up to 11 days whereas, on every 5th day up to 30 days for chondrocytes. The optical density of these samples was compared with the control. The cells seeded on α-KG containing media showed a higher rate of proliferation compared to the cells seeded on both in 2-D and 3-D without α-KG containing media (Figure 5.22 A, B). Cells seeded on 2-D reached confluency faster than the 3-D culture due to the lesser surface area available for attachment and proliferation. In 2-D samples, as shown in Figure 5.22, fibroblasts achieved a higher cell density within 24 h as compared to chondrocytes because their faster doubling time and thus also reached confluency within 11 days of the experiment in the 24 well plate. The increment in the cell number for both the cell lines during the period of experiment was clearly observed (Figure 5.22 A, B). Alpha KG is an intermediate in the Krebs’s cycle and serves as a source of energy for the cells. Around 50% of the added α-KG is converted to energy whereas the rest of it is involved in the anabolic effects like synthesis of amino acids (Harrison et al, 2008). Moreover α-KG is recently reported to act through G protein-coupled receptors, which are very well known for signalling of the cells (Maio et al, 2004). Thus its addition might increase the cell’s metabolism and signalling which in turn brings about enhanced cell proliferation. Moreover α-KG acts as a scavenger for the toxic ammonia released by the cells in the media thereby allowing the α-KG containing samples to continue to proliferate for a longer period of time (Suthasinee et al, 2008).
Figure 5.22: The relative viability of NIH3T3 and chondrocytes by MTT assay. The bar diagram indicates an increase in cell proliferation rate in the culture containing α-KG as compared to those which were cultured without α-KG. The graph showing NIH3T3 (A) chondrocytes (B) cell proliferation exhibiting similar results.
5.4.4 Effect of alpha-KG on the ammonia scavenging in the media

Extended hyperammonaemia condition has been reported to result in coma and convulsions (Richard et al, 1973). Our results clearly shows reduction in the concentration of ammonia released in the media in the alpha-KG containing samples. Similar results were observed in our previous study (Suthasinee et al, 2006) in which α-KG showed to enhance cell proliferation by acting as scavenger for toxic ammonia, thereby increasing the monoclonal antibody production in the in-vitro system. The supernatant from both the 2-D and the 3-D samples was collected for ammonia analysis before performing MTT. The samples were then analyzed for presence of ammonia by the Indo-phenol reaction. The absorbance of the coloured product was read at 490 nm. Ammonia standard was taken in 0, 0.5, 1.0 mM concentration to obtain standard graph where concentration vs. absorbance was plotted to derive the $R^2$ value, which was further used in determining the ammonia concentration in test sample. The toxic concentration of ammonia for the cells has been reported to be 1-10 mg/dl at which cells enter the stationary phase and leading to apoptosis (Klucinski and Targowski, 1984). In both 2-D and 3-D culture, the alpha-KG containing samples showed decreased amount of ammonia in the media compared to the samples without alpha-KG (Figure 5.23). This might be because alpha-KG is known to maintain the N$_2$ balance. It does so by using the released ammonia from the medium to recycle amino acids like glutamine and glutamate. It serves as a precursor for glutamine, a non-essential amino acid. This conversion is well established (Haghighat and McCandless, 1997) and is as follows:

\[
\text{L-Glutamine} \rightarrow \text{NH}_4^+ + \text{C}_5\text{H}_7\text{NO}_3\text{(pyroglutamate)}
\]

\[
\text{Alpha-KG} + \text{NH}_4^+ + \text{NADH} \rightarrow \text{Glutamate} + \text{NAD} + \text{Glutamate} + \text{NH}_4^+ + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + \text{Pi}
\]

Glutamine is a known metabolic fuel for the cells. We observed here that alpha-KG helps in the enrichment of the media and at the same time causes reduction of the toxicity in the media leading to enhanced proliferation and survival of the cells over the period of time.
Figure 5.23: Ammonia accumulation in NIH3T3 and chondrocytes cultured with and without alpha-KG. Effect of α-KG on ammonia accumulation in NIH3T3 (A) and chondrocytes (B) culture shows decreasing trend in presence of alpha-KG in both 2-D and 3-D.
5.4.5 *Glucose consumption in the presence of alpha-KG*

Glucose is one of the main sources of energy for the cells to carry out various metabolic activities. So in order to check the effect of alpha-KG on cells and their proliferation, glucose assay using DNSA method was performed. The standard graph was used to determine the concentration of the glucose in the test sample as well as the control in milimoles. As shown in Figure 5.24, the glucose consumption increased for the alpha-KG positive samples which might be because alpha-KG undergoes transamination to glutamate and glutamine. Thus reducing the number of available carbon atoms for the production of the end products i.e carbon dioxide and water. It is already known that less amount of CO$_2$ production theoretically leads to higher glucose consumption (Haghighat and McCandless, 1997). Similar observation was found in this study in which glucose consumption is found to increase in presence of alpha-KG in both 2-D and 3-D culture (Figure 5.24 A, B). Addition of alpha-KG brings about reduction in ATP production via TCA cycle, which is a source of energy for the cells. So in order to compensate for this loss we hypothetically assumed based on the available literature, that the cell switches to anaerobic glycolysis and thus form lactic acid leading to higher glucose consumption (James et al, 1972).

Glucose $\rightarrow$ Pyruvate $\rightarrow$ lactic acid

![Graph showing glucose consumption over days](image)
Finally our finding addresses the positive effect of alpha-KG on different cells in 3-D constructs. In this experiment it was clear that enhanced cell proliferation could be achieved by adding alpha-KG in the culture media. In our previous study improved production of monoclonal antibody was achieved by addition of alpha-KG in media (Suthasinee et al, 2006). Macroporous cryogel matrix helped in the better nutrient transport and addition of alpha-KG prevented the accumulation of the toxic catabolite resulting in improved culture stability. In chondrocyte the ECM accumulation starting within three weeks of the experiment was well ahead than normal in-vitro ECM secretion which makes us understand that alpha-KG can be used as key ingredient in the cartilage tissue engineering. The reports on alpha-KG demonstrated that it can be used as component to prevent premature aging as it inhibits the collagen catabolism resulting in reduced wrinkle formation (Son et al, 2007). The effect of alpha-KG on both types of cells was positive with enhanced cell proliferation and increased ECM production. This again makes us to believe that alpha-KG can be used as growth factor especially in field of cartilage tissue engineering.

**Figure 5.24:** Graph explains the glucose consumption of NIH 3T3 (A) and chondrocytes (B) in presence and absence of α-KG.
5.5 Bioactive HG cryogel for stem cells differentiation

5.5.1 Surface modification of HG cryogel for stem cell differentiation

Plasma polymerization is effectively used for changing the surface chemistry of porous 3-D cryogel scaffolds to facilitate mesenchymal stem cell differentiation without use of any growth factors and differentiation media. The scaffold property such as mechanical strength along with plasma treatment which functionalizes the surface (Bible et al., 2009) of the scaffold with reactive groups is utilized for stem cell differentiation. The plasma polymerized surfaces of scaffolds can be more appealing for tissue engineering as these surfaces can acquire charges to support cell adhesion and later switch to differentiation of the proliferating cells (Howard et al., 2008). The cell proliferation and differentiation can be tuned to occur on the same scaffolds in different time phase and the differentiation can be controlled by changing the surface properties such as electrostatic properties (Malkov et al., 2008). Plasma polymers have been shown to be useful for modifying the surface chemistry of 3-D scaffolds (Siow et al., 2006) and other tissue engineering constructs such as micro particles and plasma etching of the surface makes scaffold more favourable for cell attachment (Dinelli et al., 2002) and in maintenance of the pluripotency of the stem cells. By combining cryogel and plasma polymerization techniques it can help in generating new matrices that can be used in not only inducing the differentiation but also maintaining the stem cells in long term culture. Developing an understanding of inter-relationship between cell behaviour and surface properties is needed in the development of more effective biomaterials and applications in tissue engineering can be realized. This novel combination of scaffold with altered surface charges has provided micro environment to mesenchymal stem cells that are signalled for chondrogenic differentiation.

5.5.2 Rationale of choosing allylamine plasma

An amine-containing surface has mainly been prepared by ammonia plasma treatment or by using plasma polymerization of allylamine monomers. The ammonia plasma treatment
approach suffers on some of the polymer substrates due to the short-lived nature of the treatment and the repetition can lead to the disappearance of some of the effects of treatment as treated chains repeat into the polymer bulk due to which the surface reverts partly towards the original untreated state (Hartley et al, 2000; Chatelier et al, 1995). The popularity of amine plasma polymerization stems from the ease of fabrication, even coatings can be achieved over a broad range of plasma parameters. Amine plasma polymers are reported to be useful surfaces for cell colonization (Griesser et al, 1994), but the large majority of reports utilize amine polymers as chemically reactive platform for covalent immobilization of biologically active molecules. Carbodiimide chemistry is so far the most popular approach. Reaction between the carboxy groups and surface amine groups on the molecules to be immobilized directs the formation of interfacial amide bonds. An alternative reaction scheme can be to make surface amine groups react with aldehyde groups on biomolecules via reductive amination. Aldehydes group is not normally found on biological molecules but can be produced on sugars by periodate oxidation (Dai et al, 2000). Few examples of bioactive molecules that have successfully been immobilized onto plasma-prepared amine groups are DNA (Zhang et al, 2005), immunoglobulin G (Hayat et al, 1993), protein A (Sano et al, 1993) and heparin (Yang et al, 2010).

5.5.3 Surface analysis using water contact angle

The wettability of the surface was mapped by water contact angle (WCA) measurements with picoliter sized droplets. The WCA map showed an increase in contact angle from hydrophilic HG (Figure 5.25 A) to more hydrophobic. This change in the contact angle is due to plasma polymer allylamine monomer coating (ppAAm). The angle reminded constant even down the channels in steepest on the 0.25 mm cross sectional channel. Areas that were in direct contact with the mask mostly maintained the lower WCA of the ppAAm surface (~ 66°) (Figure 5.25 B) whereas, no loss of pattern fidelity was observed.
The WCA profile was performed on five different scaffold samples and averaging the data of each sample was used to derive the WCA with standard deviation.

**Figure 5.25:** Water contact angle measurement using picometer software and WCA imaged and values derived from associated software.

**Figure 5.25 A:** Water droplet on the uncoated HG cryogel. Water contact angle of uncoated cryogel: 40.13 and SD value of ± 2.5.

**Figure 5.25 B:** Water droplet on the uncoated HG cryogel. Water contact angle of coated cryogel: 66.45 with SD value of ± 0.25.
5.5.4 Surface mapping using x-ray photospectroscopy (XPS) analysis

The chemical composition/atomic concentration of the surface of scaffold was analyzed by small area XPS (Figure 5.26 A, B) which is used to calculate the ppAAm thickness profile. Oxygen, carbon and nitrogen were detected on the surface. The oxygen contents incorporated in the plasma polymer due to post-oxidation was low and constant (<4 atom %), whereas the amount of nitrogen increased with increasing distance from the mask edge (from 10 to 16 atom % for the 5 mm thickness of scaffold). The ppAAm thickness \( t \) was calculated on the basis of the attenuation of the intensity of N 1s photoelectrons which is dependent on the thickness of the ppAAm over-layer using

\[
t = \lambda \ln \frac{I}{I_0},
\]

In this equation, the intensity of the N 1s photoelectrons from a ppAAm surface is denoted by “\( I_0 \)”, while \( I \) stand for the measured intensity after the photoelectrons traveled through the ppAAm layer. The inelastic mean free path \( \lambda \) of the N 1s photoelectrons originating from the buried ppAAm layer was calculated with the Tanuma, Powell, and Penn formula (IMFP-TPP2M) For this calculation it is assumed that the over-layer consisted of carbon only and that the N 1s photoelectrons have an energy of 1080 eV, giving an estimated value of \( (\lambda) = 2.8 \) nm for this system (Tanuma et al, 1993).
Figure 5.26: X-ray photo spectroscopy (XPS) analysis of coated and uncoated HG cryogel. The nitrogen peak in coated samples shows the penetration of plasma allylamine (ppAAm) inside the HG scaffolds. The y-axis is the atomic concentration and x-axis is thickness in mm of the scaffolds. The scaffolds were sectioned and six points on the surface were taken to check for the penetration of ppAAm. ppAAm ‘nitrogen’ peak is not seen in uncoated (A) where as the N penetration and peak is clearly visible in coated/surface modified cryogel (B) sample.

5.5.5 Biochemical analysis of the mesenchymal stem cells on surface modified cryogel

MTT graph showed proliferation of MSCs on coated and uncoated scaffold with no significant difference in the proliferative rate between the coated and uncoated HG cryogels (Figure 5.27 A). The DNA (Figure 5.27 B) quantification results were found to be in accordance to the MTT results further validating the HG cryogels favoured the cell growth and proliferation. However, MSC’s cultured on coated cryogels showed enhanced functionality which is evident by production of GAG (Figure 5.27 C) only on the coated cryogels. Scaffolds were further checked using SEM, which showed early ECM secretion on the coated cryogels and at the 5th week the scaffolds were found to have high deposition of ECM (Figure 5. 28 I). In the uncoated samples the cells were found to have round morphology on 3rd week and on 5th week the ECM depoistion was clearly noticd (Figure 5.28 II).
Figure 5.27: Biochemical analysis of MSCs on surface modified cryogel. The MTT graph (A) represents the MSCs growth and proliferation on 2-D, HG uncoated and coated scaffold. MSCs cultured for 6 weeks on HG cryogel shows increasing trend whereas the 2-D cells growth decreases drastically on 3rd week. The DNA quantification (B) results are in accordance to the MTT results. The GAG estimation (C) shows the secretion of chondrogenic ECM components in coated samples whereas, in uncoated there was no GAG found during biochemical analysis.
Figure 5.28: Scanning electron micrograph images on coated cryogel (I) and uncoated cryogel (II). The SEM images shows that in the coated surface modified cryogel the heavy ECM deposition is seen in 3rd week in comparison to the uncoated scaffolds and trend followed to 5th week. The control is coated and uncoated cryogel showing no difference of plasma polymerization on the pore morphology and scaffold architecture.

5.5.6 RT-PCR for gene profiling of differentiated MSCs

The gene expression of type I & II collagen, Sox 9 and aggrecan was examined by RT-PCR (Figure 5.29). The cell culture was set up for 6 weeks and gene expression was analyzed every alternative week. The MSCs gene profiling showed expression of COLL II, SOX-9 and aggrecan on ppAAm coated cryogels. The cartilaginous genes of COMP (cartilage oligomeric matrix protein) was expressed only by cells cultured on ppAAm
coated cryogel scaffold surfaces. Gene expression results were in accordance with biochemical and immunohistochemical results, which indicated that ppAAM modified cryogel surfaces promoted chondrogenic differentiation in the MSCs however, uncoated cryogels and 2-D surfaces does not show any differentiation of cells.

Figure 5.29: RT-PCR results of MSCs gene expression profile on ppAAM (coated) and uncoated cryogel. Second and fifth week of experiment COLL- II, Sox- 9, COMP and aggrecan all specific to chondrogenesis is expressed by MSCs cultured on coated surfaces and continues to show expression even until 5th week. But the chondrogenic expression was not observed at any time point. The data is normalized by GAPDH.

5.5.7 Immuno-histochemical examination for MSCs differentiation

Immuno-histochemical staining for analysing the differentiating MSCs cytoskeleton with non-differentiating cells was done using phallodein staining after observing change in phenotype of cells via phase contrast microscopy (Figure 5.30). The difference with cytoskeleton between the differentiating and non-differentiating cells was very clearly noticed (Figure 5.31 A-D). Cell tracker was used for observing cell-matrix interaction (Figure 5.31 E-F). The cells cultured on coated cryogels were stained positively for type II coll and aggrecan which is cartilage specific (ECM) proteoglycans (Figure 5.32). Staining was performed every alternative week showed increasing deposition of cartilage specific ECM components in coated cryogels however, the uncoated cryogel surfaces did not show any positive staining for chondrogenesis. MSCs cultured on the modified surfaces showing differentiation can be attributed to the fact that the plasma polymerization deposits certain charges that can affect the functionality of cells. Surfaces
with methyl, carboxyl, hydroxyl and amino groups have shown to affect the functionality of cells by modulating fibronectic structure and altering the available binding/receptor sites (Keselowsky et al, 2003). Curran et al (2010), showed that –NH₂ surfaces does not support the chondrogenic differentiation of MSCs in presence of serum media. However, the chondrogenesis could be achieved by using the differentiation media. In our study effect of –NH₂ is found to be different than above stated studies but similar results have been shown by Guo et al, (2008) where the cell pellets were used for culturing cells on ppAAm coated surfaces and the chondrogenic differentiation was reported only on these coated surfaces. HG cryogel used for this study has previous shown to help in retaining the morphology of primary chondrocytes. When primary chondrocytes were cultured on these matrices their original spindle shape was retained even until 3rd week of culture and this can provide an ideal 3-D microenvironment that facilitates chondrogenic differentiations in MSCs. Expression of COLL II and aggrecan along with COMP in PCR proves that 3-D microenvironment is most important parameter for differentiation of progenitor cells.

Figure 5.30 : Phase contrast imaging of MSCs cultured on uncoated (I) and coated coverslip (II). Difference in morphology and phenotype of stem cells on the coated coverslip stem cells more elongated structure whereas in the uncoated coverslip normal stem cells phenotype is observed.
Figure 5.31: Immuno-histochemical analysis for MSCs differentiation on coated and coated surface. Phallodein staining of MSCs cultured on uncoated cryogel and coverslip showing more of fibroblast phenotype (A & B). MSCs cultured on coated cryogel and coverslip showing elongated and differentiated phenotype (C & D). Cell tracker was used to confirm the adherence and penetration of cells on the cryogel scaffold. Both uncoated (E) and coated cryogel (F) showed even distribution and proliferation of MSCs.
Figure 5.32: Immuno-staining of MSCs cultured on coated and uncoated HG cryogels. The monoclonal antibodies used were specific to chondrogenesis and osteogenesis. These results showed the differentiation of MSCs to chondrogenesis on the coated cryogel. The slight expression of COLL I in uncoated cryogel would be noticed however, there was no expression of COLL I in the coated surfaces. The immuno-staining was performed every alternative week. Localization of ECM components was noticed to inter-cellular in the initial phase. During later stage the COLL II and aggrecan deposition could be seen outside the cytoskeletal of cells.
The electrostatic properties of the scaffold could affect functionality and proliferation of cells along with adhesion and differentiation (Verma et al, 2010). The positively charged –NH$_2$ from ppAAm modified surfaces of the HG scaffold to facilitate chondrogenic differentiation of MSCs. Uncoated and coated coverslip surfaces supported cell proliferation and adhesion but does not show chondrogenic differentiation. Cryogels have been emerging as most promising scaffold in field of bioengineering (Srivastava et al, 2007). These scaffolds are made of natural and synthetic polymers and mechanical strength, pore size and porosity can be modulated depending upon the desired applications. Ability to manipulate the surfaces of the scaffold to improve the cell adhesion and allows changing the cell intrusion phase leading subsequently to tissue development. This is achieved by surface modification by plasma polymerization. Plasma etching of the surface makes scaffold more favourable for cell attachment and helps in maintenance of the pluripotency of the stem cells. Combining these two techniques can help in giving new matrices that can be used in not only inducing the differentiation but also maintaining the stem cells in long term culture. With involvement of no cytokines and growth factors, differentiation of mesenchymal stem cells was achieved by modulating the surface of the cryogel scaffolds. The net overall positive charges generated by allylamine deposition and the mechanical strength of the cryogel provide a microenvironment for mesenchymal stem cell to differentiation to chondrogenesis. An inter-relationship between cell behavior and surface properties is exploited for the development of more effective biomaterials and applications in tissue engineering is achieved. Limited regenerating potential of primary chondrocytes is overcome by using mesenchymal stem cells and advantage of these mesenchymal stem cells in the in-vivo system would be that, these stem cells maintain low percentage population of cells that remain pluripotent and these would overcome the problems of limited regeneration as seen in primary chondrocytes. Controlled localized differentiation of stem cells by altering surface chemistry of the scaffold is observed first time where in
no expensive recombinant proteins are used. One step process to sterilize and modify the surface of the scaffold is utilized.

5.6 Microenvironment dependent differentiation of mesenchymal stem cells to chondrogenesis

The advanced approach in tissue regeneration is using of mesenchymal stem cells MSCs as these cells have proved to be far superior alternative to the primary cells especially to mature chondrocytes (Jorgensen et al, 2004; Johnstone et al, 1998; Mackay et al, 1998). MSCs are known for their multi-potency and these cells can easily be obtained, cultured and under specific condition can be differentiated into mesenchymal specific tissue type like cartilage and bone (Prockop et al, 1997). One major problem in this system is delivery of the mesenchymal stem cells to the cartilaginous lesion which has not yielded the desired results (Trippel et al, 2004). In order to modify this further, tissue engineering methods have developed delivering system like viral transfection or 3-D scaffolds. In this process the functional tissue is engineered by fabricating a scaffold that mimics the local \textit{in-vivo} environment of the desired cells and thereby help cellular proliferation and regeneration of the tissue which is mechanically and biologically similar to the native tissue (Sharma and Elisseeff, 2004).

5.6.1 Gene expression in co culture for chondrogenic differentiation of MSCs

MSCs differentiation was assessed by the expression of the SOX-9; which is special mesenchymal stem cell marker for differentiation into chondrogenic linage. With concentration of the RNA ranging from 30-59 ng/mL, the MSCs cultured along with limb cells showed clear expression of the SOX-9 but the MSCs cultured separately has not shown any gene expression at any given point of time. The 3$^{rd}$ week analysis of the culture showed faint or no expression but towards 5$^{th}$ week of the co-culture there was elevated expression of SOX-9 (Figure 5.33). The cDNA was mixed from both culture conditions and amplified and the expression was louder than the expression of the gene in single system. In our previous study we have found that the HG gels helped in the
maintenance of the primary chondrocytes and we presume that since scaffold were ideal for cartilage tissue engineering these scaffold might have helped in providing niche for the MSCs along with retaining the phenotype of the limb cells.

Figure 5.33: Gene expression of cartilage marker determined by RT-PCR in co culture. The Sox9 expression checked in 5\textsuperscript{th} week of co-culture shows clear band in both static and hypoxia culture conditions. MSC cultured without limb cell does not show expression whereas, other band indicating the differentiation of MSC to chondrogenesis in co-culture. Mix- cDNA from static and hypoxia, M- mesenchymal stem cell, S- static culture, LO\textsubscript{2} – low O\textsubscript{2} culture.

5.6.2 Immuno-histochemical analysis for expression of Coll II in the co-culture

After confirming the expression of genes by RT-PCR the cartilage specific marker coll II expression was checked by immuno staining. The scaffolds with co-cultured cells were stained and the staining after five weeks experiment showed positive expression of the Coll II antibody in the co-cultured conditions. Staining demonstrated MSC derived cartilage formation (Figure.5.34 A-C), the extracellular matrix of nodules consists of collagen II was also observed and formation of collagen II fibrils could be seen outside the nodules. It is well demonstrated in various previous studies the MSCs differentiation depends upon the cytokines, growth factors, cell-cell interactions and also cell-matrix dependent differentiation (Chen et al, 2005). In the present study, it is clear that inter cellular communication and cell-cell interaction has provided signals for differentiation or regulation of cell mechanism in the way that MSCs used these as a cue for differentiation. Similar studies by Chen et al, (2009) showed pre-conditioning of stem cells with human chondrocytes induced chondrogenesis in presence of chondrogenic growth factor. The cartilage specific Coll II expression in the static and hypoxia condition indicated the MSCs differentiation in the co-culture conditions.
Figure 5.34: Immunostaining of co-culture cells for Coll II expression. Staining performed after 5 weeks of co-culture show expression of Coll II as observed under fluorescent microscopy. Immunostaining for collagen II demonstrates MSC derived cartilage formation. (A) The extracellular matrix of nodules consists of collagen II. (B) The formation of collagen II fibrils could be seen outside the nodules. (C) Chondrogenic cells within the cartilage nodules showed a typical elongated phenotype. Magnification: 40X.

5.6.3 Scanning micrograph analysis for ECM secretion in co-culture

Cell growth and proliferation was checked by scanning electron microscopy along with the control scaffold which were blank and scaffolds with cultured MSCs. The scaffolds were preserved on the 3rd week and 5th week and analysed together. In the static and hypoxia co-culture the ECM secretion was large enough to cover the scaffold completely (Figure 5. 35 A, B) and by the 5th week the scaffold were hardly been seen as ECM had covered the entire area. Though the ECM in the hypoxia condition looked lesser dense (Figure 5.35 C, D) and more fibre kind then the static culture and the ECM in the MSCs (Figure 5.35 E, F) were even lesser than the hypoxia culture condition. The scanning micrograph clearly showed the difference in the ECM secretion and these results were well supported by the biochemical analysis in which both GAG and collagen content were higher in the static culture as compared to the low oxygen culture. The ECM in fifth week was seen to be uniformly distributed throughout the scaffolds. The amount of ECM secretion and the distribution was clearly different depending upon the culture conditions.
Figure 5.35: ECM secretion in co-culture seen by SEM micrograph. Static culture (A & B) and Low O₂ (C & D) conditions of mesenchymal stem cells in 3rd and 5th week (E & F), respectively. In the static culture the extracellular matrix was maximum as compared with hypoxia conditions followed by mesenchymal stem cells which shows very minimal productions. The last panel (G & H) are control cryogel image showing uniform pore distribution and interconnectivity of the HG cryogels.

5.6.4 Histological analysis for the chondrogenic expression by alcian blue and safranin-o

The formation of ECM in the co-culture condition was further evaluated by alcian blue and safranin-o staining for sulphated proteoglycan synthesis, which is an important ECM component in the cartilage (Chen et al, 2009). For MSCs cultured scaffold there was little to no staining observed but the static and hypoxia cultured scaffold clearly showed the patches of the positive staining in five weeks. The alcian blue (Figure 5.36 A) showed difference between ECM staining depending upon the cell culture conditions. The static culture scaffold was stained stronger by alcian blue then the hypoxia condition scaffolds (Figure 5.36 B). The intensity of the staining in both conditions was better than the staining of the MSCs cultured scaffold. Histochemical analysis for sulphate proteoglycan in the co-cultured scaffold was performed by staining with safranin-o- for sulphated proteoglycan as these form major part of the ECM and helps in maintaining structural integrity of the tissue (Figure 5.37 A, B). Static culture and hypoxia conditions culture shows positive staining with nuclei stained red and surrounding cellular matrix in
bright orange. (Figure 5.37 C, D) shows faint staining of the MSCs seeded scaffold and negative staining was observed for the blank scaffold. Strong alcian blue staining corresponds to collagen matrix secretion which is major component of articular cartilage being synthesised by the differentiated MSCs.

Figure 5.36: Histology staining of HG cryogels seeded with MSCs and limb cells for co-culture stained with alcian blue. Patches of the alcian blue stains positive in static (A) and low O₂ (B) MSCs seeded scaffold seen (C) similar to the blank scaffold indicating negative staining.
Figure 5.37: Histochemical analysis for sulphate proteoglycan in the co-cultured scaffold was performed. Proteoglycan forms major part of the ECM and helps in maintaining structural integrity of the cartilage tissue. A) Static culture and B) hypoxia conditions culture shows positive staining with nuclei stained red and surrounding cellular matrix in bright orange. Figure C & D shows faint and negative staining.

5.6.5 Cytoskeleton staining of the co-cultured cells by phalloidin

Phalloidin is used as the cytoskeletal staining and are known for very specific in binding between interphase of F-actin subunits and is useful tool in detecting the F-actin distribution in the cells. Immunofluorescence technique along with the fluorescence of phalloidin is used to evaluate the functions of cytoplasmic actin (Wehland et al, 1977). The morphological difference between the MSCs and co-cultured cells were observed under fluorescent microscope (Nikon, TE-2000 U). Cytoskeleton staining of the co-culture cells was performed to check the cell integrity being maintained in static and low O₂ conditions. (Figure 5.38.A, B). DAPI staining clearly shows intact nuclei confirming no adverse effect of static or hypoxia conditions on co-cultutred cells (Figure 5.38 C).
Figure 5.38: Cytoskeleton staining of the co-culture cells was performed to check the cell integrity being maintained in static and low O_2 conditions. A) Static co-culture cells stained with phalloidin, B) low O_2 co-culture cells with phalloidin, C) overlap image of phalloidin with nuclei counter stained by DAPI is seen in blue.

5.6.6 Total metabolic activity of the co-cultured cells by alamar blue

Alamar blue is used to check cellular proliferation and it works by detecting oxidation level during cellular respiration/metabolism. Being considered as best redox indicator when compared to any other components as alamar blue does not interfere between the electron transport chains of the cells. The midpoint potential being greater than MTT/MTS, it can detect the oxidation of all components involved in the electron
transport chain without effecting the redox reactions. The alamar blue was performed weekly for the co-culture, MSCs and the blank scaffold being kept as control. The alamar blue showed steady increase in the total metabolic activity of the cultured cells in five week of the experiments. The MSCs seems to proliferate well in the cryogel scaffolds and there was total of 69% increase (Figure.5.39) in the cellular proliferation in both static and hypoxia culture conditions. The static culture showed increase of 69±2% and hypoxia culture showed 65±3% in over five weeks of experiment.

![Graph](image)

**Figure 5.39:** Alamar blue performed for 5 weeks showed consistent increase in the total metabolic activity of cells in 3-D culture under static and low O2 conditions.

5.6.7 Quantification of extracellular matrix component - Glycosaminoglycan (GAG)

Cartilage aggrecan is highly complex macromolecule with core proteins and GAG as main components and it is the unique organization of GAG between inter and intra molecules of cartilage that provides strength to the cartilage tissue and resistance against the compressive or mechanical tension (Hunziker, 2002). The GAG estimation thus forms an important assay in cartilage study. The GAG assay was performed every week
for static and hypoxia culture, MSCs was excluded since there was no GAG synthesis found during analysis. It was noted that there was little or no difference between the amounts of GAG secreted by co-culture cells over period of 5 weeks and the difference between static and hypoxia conditions were negligible (Figure.5.40). GAG production in co-culture condition indicates the MSCs differentiation into chondrogenic lineage and production of extra cellular matrix signified that the scaffolds were supporting the co-culture cell proliferation and has helped in secretion of extra cellular matrix components.

![Glycosaminoglycan content](image)

**Figure 5.40**: Glycosaminoglycan content is analysed for static and low O₂ condition. Results are expressed in percentage dry weight of scaffold. Serial dilution of chondroitin sulphate was used as standard and the graph was plotted after obtaining value by plotting against the standard.

### 5.6.8 Quantification of extracellular matrix component-collagen

The architectural arrangements of collagen in the cartilage are well established and fibrous collagen is in linearly stacked to resist the tensile load that is experienced by the knee while in use. Collagen content was measured per week for 5 weeks and static culture showed constant increase and in comparison with hypoxia culture; the collagen content in static culture was higher in 5th week (Figure. 5.41) however, overall comparison between both culture conditions showed negligible differences.
Figure 5.41: Collagen content of co-culture is expressed as percentage dry weight of scaffold. The graph is plotted after obtaining reading from standard curve shows slightly higher collagen production in the static culture as compared to hypoxia culture conditions.

In this study, the principle of MSCs differentiation depending upon the microenvironment provided to these cells. Chondrogenesis is one of the earliest cellular events occurring in vertebrate skeletal development, the stages of differentiation are mesenchymal cell condensation, chondroprogenitors formation, chondrogenesis finally the terminal differentiation of progenitor cells and ossification (Goldring et al, 2006; Lefebvre and Smith, 2005). During mesenchymal cell condensation and aggregation, cell–cell interaction leads to cell adhesion and release of paracrine factors (Tuan, 2004; Johnsen et al, 1997). MSCs subsequent transition into chondrogenesis is controlled by the interplay of various factors, such as hedgehog signalling pathways, transcription factors, metabolites, and stress (Lefebvre and Smith, 2005; Tuan, 2004; Johnsen et al, 1997). Monroy et al, (2003) and Merino et al, (1998) delineated the events involved in chondrogenesis of cartilage and bone formation in-vivo, using immunohistochemical
analyses of whole-mount specimens. However, the exact molecular events that occur in chondrogenesis remain unclear (Chen et al, 2009). Co-culturing the human mesenchymal stem cells with foetal limb cells for differentiation of MSCs into chondrogenesis provided the cell signalling to MSCs for differentiating into specific lineage. Various biochemical and histological studies showed that MSCs had differentiated under the influence of the microenvironment provided as the MSCs cultured separately did not demonstrate the expression of various genes. HG cryogels were chosen as these scaffolds had previously shown to maintain the phenotype of chondrocytes for over 4 weeks time and these scaffold might have helped in the maintaining the limb cells due to which the cell-cell interaction could have led to differentiation of the MSCs.

5.7 Myoskeletal tissue engineering

5.7.1 Designing of HG cryogel for skeletal tissue engineering

The designing of cryogel scaffold with high volume fraction porosity within soft, mechanically stable and high water absorbing capacity was obtained in HG cryogels. The cryogel synthesized were light yellow (Figure 5.42 A) in colour and have shown to retain 3-D architecture while air drying. The SEM was performed to check the pore distribution and porosity of the scaffold and the pore size of HG cryogel were found to be in the range of 30 to 120 μm, while the average pore diameter range was lying in between 50- 80 μm (Figure 5.42B). The overview of cryogel (Figure 5.42C) showed uniform pore distribution and interconnected pore network. The interconnected pores allow convective flow of nutrients and facilitate gaseous exchange during cell culture conditions, which is considered to be a key characteristic for any tissue engineering scaffolds. These results suggest that the microstructure of HG cryogel might be a potential scaffold material for tissue engineering applications.
**Figure 5.42:** The digital image (A) shows physical appearance of water saturated HG cryogel. Scanning electron microscopy (SEM) image at magnification 400X of HG cryogel (B), showing the interconnected macroporous cryogel structure and an overview of the porous HG cryogel scaffold (C), showing even distribution of the pores.

### 5.7.2 Total cellular metabolic activity by alamar blue

To check the potentiality of the synthesized scaffold, C2C12 cells were seeded on the HG cryogels. The total metabolic activity of the cells was checked by using alamar blue. Alamar blue works on redox system which fluoresces and changes color in response to chemical reduction in the media resulting from the cell growth. The cellular growth has direct effect on the media provided for cell proliferation and alamar blue uses this chemical change in the media by either reduction or oxidation. The reduction which is related to cellular growth causes the redox indicator in the alamar blue to change from oxidized to reduced state. The normal color of alamar blue changes to the fluorescent red that depends upon the cellular growth and total metabolic activity. Figure 5.43 shows the rate of proliferation of C2C12 cells on HG cryogel scaffold and is compared with 2-D control. The cell proliferation and growth on the scaffold showed an increasing trend with 60% increase in the total cellular activity for over 3 weeks whereas in 2-D the diminished cell growth was noticed from the day 13th of cell culture. Cell-polymer construct has been used for transplantation of myoblasts using biodegradable polymer
strands to avoid transplantation buffer (Saxena et al, 2001), showing importance of matrix which acts as delivery system and also supports cellular proliferation. Three-dimensional engineered construct of cardiac muscle can provide a basic model system for cardiovascular research (Eschenhagen et al, 1997; Folkman and Haudenschild, 1973). Increase in the cellular viability and the total metabolic rate substantiates that these scaffolds provided good support for the cell growth and the positive effect of the cryogel matrix on the proliferation of the C2C12 cells.

![Graph](image)

**Figure 5.43:** The total metabolic activity of the cells was checked by alamar blue at regular intervals. The graph describes C2C12 growth on HG cryogel (black bar) which shows increasing trend in the metabolic activity but the 2-D culture (white bar) starts declining from 13th day which could be due to lesser surface area available for cells after attaining confluency. On the total cellular metabolic activity assay indicates the cryogels supporting the proliferation of C2C12 cells for over 3 weeks.

### 5.7.3 DNA quantification by Hoechst 33258 assay

To validate the alamar blue results and to check the metabolic activity being related to cellular proliferation, DNA quantification was performed. The bis-benzimidazole
derivatives of Hoechst 33258 exhibits fluorescence enhancement upon binding to A-T rich regions of double stranded DNA. The Hoechst assay results were in accordance to alamar blue. On HG scaffold the proliferation of C2C12 was observed to be better when compared to the 2-D system which results in higher DNA content being observed in C2C12 on HG scaffold (Figure 5.44). From the 1\textsuperscript{st} to 23\textsuperscript{rd} day, at intervals the scaffolds were weighed and subjected to papin digestion. The increase in the DNA content shows the HG cryogels have provided the C2C12 cells with native environment that supported better cellular growth and proliferation.

**Figure 5.44:** The DNA quantification was done by using the Hoechst assay. The proliferative cells are analyzed for total DNA content. The standard curve was used to derive the ng DNA per ml and further used for calculating DNA content in the test samples. Graph indicates C2C12 on the HG cryogels (black bar) which showed improved proliferation when compared with 2-D (grey bar) which resulted in the total DNA content increasing and this result can be used to validate the alamar blue results which shows similar trend.
5.7.4 Cell differentiation

To check the distribution and differentiation of C2C12 on HG scaffold, these were observed under fluorescent microscopy. Cells were seen to adhere and proliferate throughout the scaffold and tubular structure which were elongated and multinucleated was seen to penetrate and fill the void pores of the scaffold and were found to be highly aligned along channels of the scaffold pores as visualized under phase contrast, DAPI imaging and Hoechst staining (Figure 5.45 A, B), where 2-D cells were seen proliferating as monolayer (Figure 5.45 C, D) with delayed tubular formation. Cell tracker was used to check the cells on the scaffold (Figure 5.45 E) and the F-actin stained with rhodamine-phalloidin shows the myotubes aligning along the preferential directions on the HG cryogels (Figure 5.45 F), which show 3-D growth pattern of the cells on the scaffolds. The myoblast nuclei was stained using Hoechst staining and DAPI on 7th day and 14th days (Figure 5.46 A, B). It was clearly seen that the cells have been evenly distributed within the pores of the HG cryogel. The interconnected pores of the scaffold allows unhindered transportation of media and nutrient leading to enhanced proliferation of cells on HG cryogels which can be noticed by DAPI staining showing increased population in about 2 weeks time.
Figure 5.45: C2C12 cells observed by phase contrast microscopy (A) shows formation of tubular structure from day 6\textsuperscript{th} of culture. On further analysis by fluorescent microscopy using DAPI multinucleated tubular structure was seen to be formed by group of cells (B) along with background of cells spreading homogenously on the HG matrix. On 2-D culture the 6\textsuperscript{th} day cells are seen after Hoechst staining (C) and DAPI staining (D). Cell tracker showing the proliferation of cells on the scaffold (E), and the alignment of the C2C12 in preferential direction on the scaffolds (F).

Figure 5.46: Cell attachment was visualized under fluorescent microscopy by using nuclear stain DAPI (A). The image was recorded after 48 h of cell seeding and (B) after 2 weeks. The fluorescent microscopy images show significant increase in cell number and proliferation of C2C12 on HG cryogel.

This work investigates the potential use of supermacroporous cryogel scaffold for skeletal muscle tissue engineering, and on the basis of these findings we believe there is
suitable utility of cryogel scaffold for this application. For specific application scaffold was characterized mainly for degradation, elasticity and mechanical property and also cell attachment and proliferation was checked by measuring total metabolic activity of C2C12 on HG cryogels. We describe a simple inexpensive technique of designing scaffold for complex tissue engineering and conclude that the cryogel have shown promising cellular response by enhanced attachment and proliferation and can be potential scaffold for skeletal or cardiac tissue engineering. We aim to further tune the scaffold and characterize the engineered cardiac muscle construct by gene-level and cell-level electrophysiological studies. The ultimate aim will be to develop neo-cardiac tissue in the three-dimensional architecture.
CHAPTER VI

CONCLUSION
6.0 CONCLUSION

The macroporous cryogel as biomaterial has been advancing to be one of the most potential scaffolds for bioengineering applications. The main property that attributes to this advancement is interconnected network of pores which facilitates the movement of biomolecules in the *in-vitro* and the ease of procedure involved in fabricating these scaffold. The supermacroporous scaffolds synthesised using cryogelation technology can be fabricated in different formats like monolith, discs, sheets and beads using polymer precursor of choice.

Focusing on the specific aim this thesis work can be concluded as:

1) The HG scaffold synthesised showed the mechanical strength and precise control of the internal molecular architecture as in par with the musculoskeletal tissue so the two most important area cartilage and myo-skeleton were explored with success.

2) The usage of alpha-KG for enhanced cell growth and proliferation and negating the toxic effect of the ammonia to obtain faster deposition of ECM component leading to the formation of neo-cartilage. Alpha-KG can be explored in future as cost- effective substitute of the growth enhancer or factor available in market.

3) The surface modification that led to the differentiation of the MSCs without any growth factors or differentiating media proves the importance of the surface architecture and surface chemistry in the cell-matrix interactions. These scaffolds in future can be utilized as bioactive material that can be used for *in-vivo* differentiating progenitor cells for better tissue regeneration. The electrostatic properties of the scaffold could affect functionality and proliferation of cells along with adhesion and differentiation. The positively charged –NH$_2$ from ppAAm modified surfaces of the HG scaffold facilitated the chondrogenic differentiation of MSCs.

4) The co-culturing of the progenitor cells with completely committed cells (limb cells) and allowing differentiation of progenitor cells in this study demonstrates
the principle of MSCs differentiation depending upon the microenvironment provided to these cells. Co-culturing the human mesenchymal stem cells with foetal limb cells for differentiation of MSCs into chondrogenesis provided the cell signalling to MSCs for differentiating into specific lineage.

The overall outcome of this thesis is primarily a coherent set of qualitative and quantitative empirical study on new supermacroporous polymeric material. Application of the synthesised material in tissue engineering would be interesting to use this line of research into emerging biomedical field.

**Future work** could be mimicking these results in the *in-vivo* system, where cell-material interactions and cues provided by materials should be able to help in quick recovery of the injured tissues or help in regeneration of the desired organ/tissue. We predict that the scaffold can be utilized for other biomedical applications with some modifications to obtain desired results. Cryogel scaffolds with tailored functional properties could be of great use and may enable researchers to explore the interfaces of living tissues and biomaterials. The field of material engineering for various biomedical applications is definitely gaining deserved importance with promise to deliver products that will improve the basic lifestyle and prolong the life expectancy with mobility promised till the end.


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Park, Y.J. (1998) Controlled release of platelet- derived growth factor from porous poly (L-lactide) membranes for guided tissue regeneration. Controlled release J 51, 201-211.


Publication as outcome of this research


**Impact factor of Journal: 2.865**


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### ACADEMIC PROFILE

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<td>B. Sc. Microbiology</td>
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On hand experience (2005-2010)

- **2 years** experience in preparation of cryogel using different polymers for tissue engineering
- **2 years** of experience in stem cells isolation culturing and cryo preservation
- **4 years** of Tissue Culture (Chondrocyte, setting of primary culture and maintaining it for 6 months) and in fabrication of Collagen Scaffold for Chondrocyte
- Micro ct anyalsis of scaffold have checked the polymer distribution in cryogels beside the basic microstructure analysis
- Rheology testing of polymeric scaffolds
- Surface modification of scaffold using plasma polymerization for stem cell differeniation
- Training in stem cell culture and their clinical application
- Hands on experience with Scanning electron microscopy (SEM) and confocal microscopes
- Project on stem cells recovery with different concentration of density gradients.
- Cryo- preservation of Hela cell lines, Chondrocytes and Stem cells.
- Hands on training in Cytogenetics Cancer Institute (Adyar, Chennai, India)
- One week training in Flow Cytometry (Sri Chitra Thirunal)-Indo US Forum
- One week wet lab training in Electron microscopy at adyar cancer institute
- Genetic study of Sjögren Syndrome at CLRI, Adyar Chennai, India (M.Sc Project)
- Analysis of DNA and RNA
- DNA and RNA isolation from human and plasmid
- Cell culture worked on HEPA, HELLA cell lines, Fibroblast, Neuron and Cardiomyocyte (setting of primary culture)
• PCR
• Western Blotting
• SDS-PAGE
• Cell Sorting
• Apoptotic Index
• Spectroscopy
• Gelatin Zymography.

WORK / RESEARCH EXPERIENCE: (2005-2010)

Worked on stem cell isolation from umbilical cord blood and from bone marrow. Work involved isolation, characterization and preservation of stem cells. Worked on preparation of cryogel scaffold for tissue engineering applications.

Project A (2007- till date) - Cryogel scaffold fabrication for tissue engineering

Synthesis of Cryogel using natural and synthetic polymers like HEMA-gelatin in varying combination for cartilage and cardiac tissue engineering applications. Working on manipulating pcells towards chondrogenesis by using different biochemical and molecular signaling for eg Role of alpha keto glutarate on chondrocytes using Hema-gelatin cryogel scaffolds.

Project B (2009-2010) - Approach for Stem cell differentiation using bioactive scaffolds or by providing 3-D environment.

Have worked in Center for Biomolecular Science, University Of Nottingham under prof. Kevin shakesheff, project was human mesenchymal stem cell differentiation by co culturing with fetal limb cells on cryogel matrices. Worked involved culturing human mesenchymal stem cells, isolation of fetal rat limb cells and various gene expression anaylsis and confocal imaging.
Second project was surface modification along with Morgan Alxendar of supermacroporous cryogel for inducing differentiation of Mesenchymal stem cells. Work included plasma polymerizing the cryogel scaffold, performing XPS water contact angle and cell culture studies.

Project C (2005-2008)- Collagen based biomaterial synthesis and cell culture

Three years of experience in animal cell culture and collagen based scaffold preparation. Handling of cell lines and setting up primary chondrocyte culture from chick-embryo, chicken and bovine. Work included collecting of tissue from animal source, isolation of chondrocyte by enzymatic digestion and culturing them in lab. Passaging the monolayer cell, performing various histo-chemical and immuno-histochemical staining for identification and confirmation of chondrocyte and cryo-preserving the cultured cells.

Project D (2005-2008)- Collagen dressing for wound healing

Worked on Preparation of Triphala incorporated collagen dressing for wound healing. Work included isolation of anti-microbial compounds from Triphala testing their efficacy in wound healing. Creating wound in white albino mice, dressing it with Triphala anti-microbial isolates versus commercially available drugs for dressing. After proper interval tissue section was taken and histo-pathological and immuno-histochemical was performed.

PUBLICATIONS


INTERNATIONAL AND NATIONAL CONFERENCE PAPER/POSTER PRESENTATION

- Special approach for chondrogenesis of human mesenchymal stem cells on supermacroporous cryogel. Joint -E S A O – A S A I O Winter School 2010 Bioartificial Organs & Cell Therapy 21.-23.01.2010 ( Austria) abstract selected for Oral presentation

- An Overview On Cryogel Polymeric Scaffolds With Special Approach To Cartilage Tissue Engineering, Tissue and Cell Engineering Society Meeting 2009 (glassgow, Scotland)- Poster presentation

- Chondrocyte culture on collagen matrices- International conference in Bioengineering and Bioinformatics Kolapur, India- Oral presentation

- Using human mesenchymal stem cell for chondrogenesis on supermacroporous cryogel scaffold- "Emerging Paradigms in Biochemical Engineering" Banaras Hindu university- 9-11 oct,2009 - Oral presentation
• Chondrocyte culture on 3 d collagen matrices” BIND, IIT KANPUR, India on DEC – 2006 - Poster presentation

B. TECH PROJECT GUIDED:

• Prepare collagen bilayer dressing with anti-microbial compounds of Triphala at CLRI, India

• Triphala, An Antimicrobial Plant Extract Used With Cryogels In Tissue Engineering at IITK, India

OTHER SKILLS

Computer skills:

Knowledge on the following Software products:

• Windows XP, Windows 2000
• MS-Word, MS-Excel, MS-PowerPoint, MS-Access

PERSONAL DETAILS

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Date: 20-11-2010

DEEPTI SINGH