A THREE DIMENSIONAL HUMAN AMNIOTIC MEMBRANE/ FIBRIN SCAFFOLD FOR CARTILAGE TISSUE ENGINEERING APPLICATION

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF ENGINEERING SCIENCE

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ABSTRACT

In engineering a cartilage tissue, the survival of the chondrocytes is directly interdependent to the extracellular matrix production. Therefore, the choice of biomaterials for cartilage tissue engineering application is crucial in determining the survival of the chondrocytes. Natural biomaterials pose great advantages for tissue engineering applications due to their biocompatibility, biodegradability and ease of availability. These materials are not only sustainable but pose a lower risk of rejection by the body. Human Amniotic Membrane (HAM), for example, is a 2 dimensional (2D) biocompatible and biodegradable material. The main objective of this study is to develop a 3 dimensional (3D) scaffold made from HAM a well-known natural biomaterial to supports chondrocytes proliferation and matrix production with an ultimate aim to promote tissue regeneration in cartilage tissue engineering application. This thesis presents the development of a novel 3D HAM/ Fibrin scaffold that was produced from a crosslink of HAM extracts and fibrin to form a stable scaffold to ensure uniform cell dispersion throughout the scaffold. This thesis also outlines the parameters that validate the feasibility of the scaffold following the success of optimization of the developed 3D HAM/ Fibrin scaffold (1:1 (v/v) ratio of HAM/ Fibrin with a concentration of 5 million cells/ml set within 30 minutes). The results of total DNA and GAG content production of this novel scaffold supports the cartilage matrix production and cell survival *in vitro*. Predominantly, chondrocytes favoured the HAM/ Fibrin scaffold as results for both DNA and GAG content recorded a significant increase than the control group. Moreover, the histological evaluation also revealed chondrocytes favouring HAM/ Fibrin scaffold when it comes to proteoglycans synthesis which further supports the result from the DNA and GAG content production. The FESEM evaluation also affirms the DNA and GAG content production favouring HAM/ Fibrin scaffold with apparent signs of cell-cell connection, cell-ECM adhesion and

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cytoskeletal filaments. This study is a step forward in scaffold fabrication for cartilage tissue engineering as it is a known fact that HAM constituents are quite similar to native cartilage and the results from this study further support the use of natural biomaterial in mimicking the native microenvironment of the intended tissue replacement.

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ABSTRAK

Dalam kejuruteraan tisu tulang rawan, kemandirian sel kondrosit adalah secara langsung saling bergantung kepada pengeluaran matriks ekstraselular. Oleh itu, pilihan biobahan untuk aplikasi kejuruteraan tisu tulang rawan adalah penting dalam menentukan kemandirian sel kondrosit. Biobahan semula jadi memberi kelebihan yang besar untuk aplikasi kejuruteraan tisu kerana keupayaan bioserasi, biodegradasi dan mudah diperoleh. Bahan-bahan ini bukan sahaja mampan, tetapi menimbulkan risiko penolakan yang lebih rendah oleh tubuh badan. Sebagai contoh, Membran Amniotik Manusia (HAM) ialah bahan bioserasi dan biodegradasi 2 dimensi (2D). Objektif utama kajian ini adalah untuk membangun perancah 3 dimensi (3D) yang diperbuat daripada HAM yang merupakan biobahan semula jadi yang terkenal untuk menyokong proliferasi sel dan penghasilan matriks dengan matlamat utama untuk mempromosikan pertumbuhan semula tisu dalam aplikasi kejuruteraan tisu tulang rawan. Tesis ini membentangkan perkembangan perancah 3D HAM/ Fibrin yang dihasilkan daripada sambungan silang ekstrak HAM dan fibrin untuk membentuk hidrogel yang stabil dan memastikan penyebaran sel yang seragam di seluruh perancah. Tesis ini juga menggariskan parameter yang mengesahkan kebolehan perancah berikutan kejayaan pengoptimuman perancah 3D HAM/ Fibrin(nisbah 1: 1 (v/v) HAM/ Fibrin dengan kepekatan 5 juta sel/ ml yang ditetapkan dalam masa 30 minit masa). Hasil total produksi DNA dan GAG kandungan perancah novel ini menyokong produksi matriks rawan dan kemandirian sel *in vitro*. Keseluruhannya, sel kondrosit menggemari perancah HAM/ Fibrin sebagai hasil untuk kedua-dua kandungan DNA dan GAG mencatat peningkatan yang ketara daripada kumpulan kawalan. Selain itu, penilaian histologi juga mendedahkan sel kondrosit yang memihak kepada perancah HAM/ Fibrin apabila ia berkaitan dengan sintesis proteoglycans yang seterusnya menyokong hasil daripada pengeluaran jumlah kandungan DNA dan GAG. Penilaian FESEM juga mengesahkan pengeluaran jumlah

kandungan DNA dan GAG yang memihak kepada perancah HAM/ Fibrin dengan bukti kehadiran sambungan sel-sel, lekatan sel-ECM dan filamen sitoskeletal. Kajian ini adalah satu langkah ke hadapan dalam fabrikasi perancah untuk kejuruteraan tisu tulang rawan kerana ia adalah suatu fakta yang diketahui bahawa komponen membran amniotik manusia adalah agak sama dengan tulang rawan asli dan hasil kajian ini menyokong lagi penggunaan biobahan semula jadi dalam mencontohi alam mikro asli tisu bagi tujuan penggantian tisu.

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LIST OF SYMBOLS AND ABBREVIATIONS

ACI	:	Autologous Chondrocyte Implantation
ADL	:	Activities of Daily Living
ANOVA	:	Analysis of Variance
AC	:	Articular Cartilage
CaAM	:	Calcein-AM
CPD	:	Critical Point Drying
dH_2O		Distilled Water
DMB	:	Dimethyl Methylene Blue
DMEM	:	Dulbecco’s Modified Eagle Medium
DNA	:	Deoxyribonucleic Acid
εACA	:	ε-Amino Caproic Acid
ECM	:	Extracellular Matrix
EDTA	:	Ethylenediaminetetraacetic Acid
ER	:	Endoplasmic Reticulum
FBS	:	Fetal Bovine Serum
GAG	:	Glycosaminoglycans
GF	:	Growth Factor
HAM	:	Human Amniotic Membrane
HAM/F	:	Human Amniotic Membrane/Fibrin
HCl	:	Hydrochloric Acid
HA	:	Hyaluronic Acid
NaCl	:	Sodium Chloride
NaOH	:	Sodium Hydroxide
OA	:	Osteoarthritic

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PBE : Phosphate Buffer EDTA

PBS : Phosphate Buffer Saline

PENSTREP : Penicillin/Streptomycin

PE : Polyethylene

PGE : Polyethylene Glycol

PGs : Proteoglycans

TE : Tissue Engineering

TEN : Tris-HCL/EDTA

Tris-HCl : Trisaminomethane Hydrochloride

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Appendix A: The Fabrication of Human Amniotic Membrane Based Scaffold for Cartilage Tissue Engineering Applications: A Preliminary Study. 5th Kuala Lumpur International Conference on Biomedical Engineering, Malaysia, June 2011. 111

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CHAPTER 1: INTRODUCTION

1.1 Tissue Engineering

Human life expectancy is better than it was decades ago. The improvements in medical science have been contributing to this phenomenon. However, as we grow older, so is the need for our body to repair itself as tissue and organ wears-off and aging takes effect. Loss of tissues or organs due to pathological or trauma reasons often resulting in a need of a tissue or organ transplant for replacement. Although organ transplantation and artificial organ may be the best solution, it is not without morbidities. The scarce supplies in organ donation and the risk of immune rejection is still a conundrum in organ transplantation.

Remarkable advances in biomedical engineering field have given rise to advances in artificial organs, however, it still has issues in relation to biocompatibility and bio-functionality within *in vivo* environment. As an alternative, a new approach to tissue and organ reconstruction and replacement was introduced in 1993 by growing a human ear on the back of a mouse (Langer & Vacanti, 1993). This later was followed by other success such as trachea, bones and pulmonary arteries tissues cultured and engineered in laboratory through the technology now known as Regenerative Medicine.

Regenerative medicine can be classified into two categories as depicted in Figure 1.1. It involves two concepts with either the presence or absence of scaffold(s) to support tissue regeneration.

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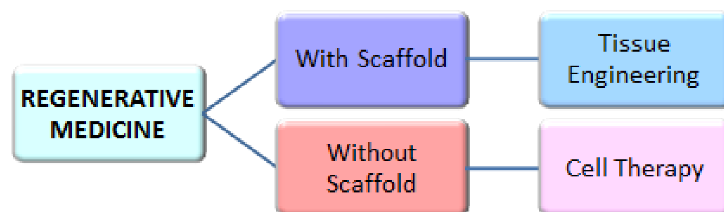

Figure 1.1: Classification of Regenerative Medicine based on the use of scaffold. Adapted from (Ikada, 2006).

1.2 Concept of Tissue Engineering

The basic concept of tissue engineering is to regenerate patient's own tissue and organ by using their own cells; be it blood, skin or muscle that would be biocompatible and bio-functional with almost zero immune rejection. It is often considered as an ultimate and ideal alternative of medical treatment.

In regenerating new tissues and organs, tissue engineering utilizes three basic ingredients; the cell, three dimensional (3D) scaffolds and growth factor as illustrated in Figure 1.2, all of which, interplay in reconstructing and regenerating a functional tissue or organ (Langer & Vacanti, 1993).

Obtaining adequate cells is a major challenge since many of the cell type taken from adult source has a very limited capacity for expansion. The presence of cells alone are questionable to form tissues. Although direct injectable was often used by using cell suspension, it has very little success in regenerating functional tissues. When cells are removed from their natural in vivo environment, they often lose their tissue-specific function, also known of phenotype. Cells must somehow adhere to a temporary structure or also known as scaffold which mimics the 3D environment it was used to for which it will adhere and organize into functional tissues.

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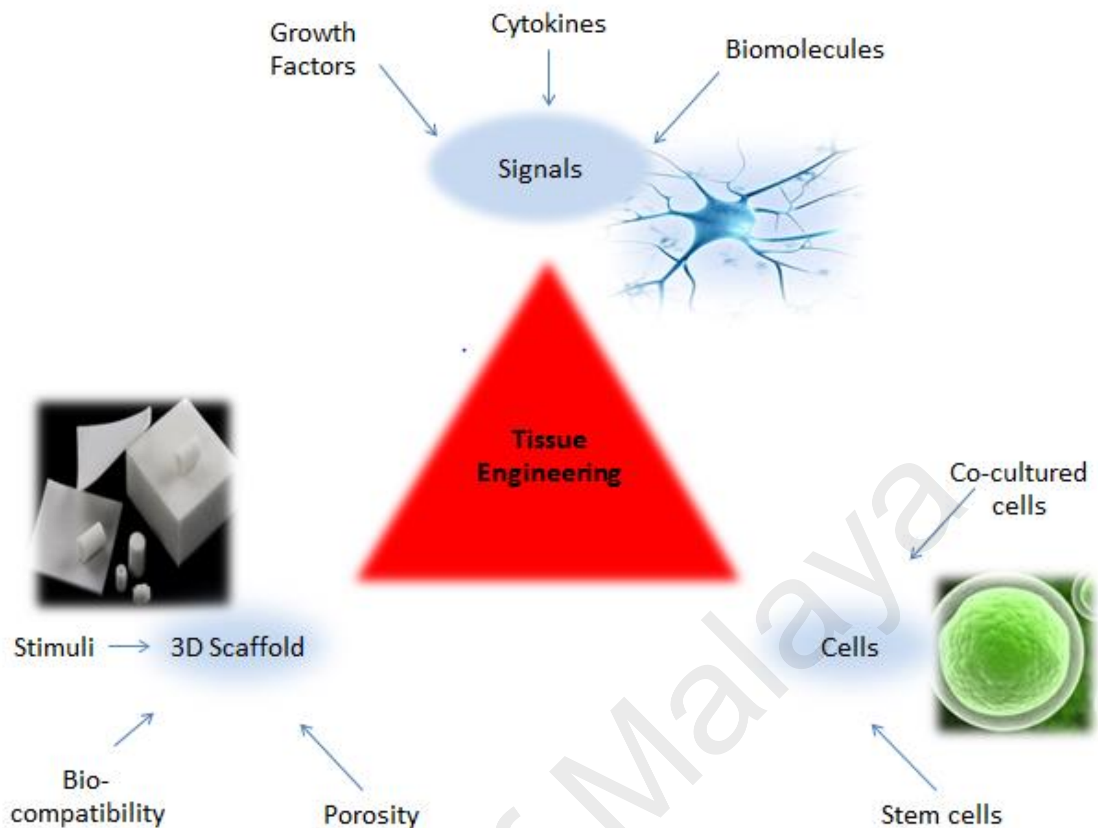

Figure 1.2: Tissue Engineering Triad. Adapted from (Partap, Plunkett, & O'Brien, 2010).

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A 3D biomaterial scaffold basically provides a temporary support for cells to grow and aid in cellular attachment. Meanwhile, the presence of growth factor cultivates the direction of which the cells would grow, proliferates, differentiates, migrates and even programmed cell death (apoptosis). Cells interact with soluble growth factors and cytokines as well as insoluble factors such as the extracellular matrix (ECM) and other cells in the cell-ECM interactions and cell-to-cell interactions.

The signaling cues to and from the neighboring cells and ECM plays an important role in regulating cell function. Cells are interconnected to each other with adhesion molecules called integrins which links intracellular to ECM. Most studies on engineering cellular microenvironment were performed in two dimensional (2D) while cells in the native environment are naturally in 3D organization. Studies have shown

that cells cultured in 3D environment present distinct phenotype from the same cells cultured in 2D settings (Benya & Shaffer, 1982)

Among many other strategies in fabricating functional tissue construct, scaffold based constructs offer the greatest potential for microenvironment control specifically in modifying the presence of bioactive ligands and proteolytic remodeling. The integration of these elements is important to fabricate the scaffold that can support the growth and maintenance of specific cell phenotype, ultimately the desired functional tissue construct.

1.3 Cartilage Tissue Engineering

Joint pain is a major cause of disability in middle-aged and older population. Damage to the articular cartilage may cause subsequent degeneration that may lead to osteoarthritis and other joint diseases. Millions of surgeries were done annually to treat this cartilage defects worldwide, caused either by trauma, osteonecrosis or osteochondritis. Approximately 70% of population aged 65 years and above are currently affected in some way by osteoarthritis (Buckwalter & Mankin, 1998). Until now, there is no current treatment or cure that has been proven to halt the progress of the disease. Although articular cartilage is a metabolically an active tissue, the cells within the matrix have a relatively slow turnover or self-healing capabilities. Its aneural property makes it rather impossible for it to support self-repair and remodeling. Due to this, injury to the tissue oftentimes leads to progressive damage and degeneration.

Treatment such as Articular Chondrocyte Implantation (ACI) which has been a cell-based approach in repairing articular cartilage injury is deemed better than joint replacement therapy which has its limitations (Alaminos et al., 2006; Redman, Oldfield, & Archer, 2005). The ACI treatment did not solve the problem as reports of sunken and

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further damage to the lesion were documented (Britt & Park, 1998; Brittberg et al., 1994; Chan & Gill, 1999; Marlovits, Zeller, Singer, Resinger, & Vecsei, 2006).

Tissue engineering has emerged as a new technology and approach in addressing the problem. The basic concept is using cells of the targeted tissue, scaffold as a support system and bioactive molecules to direct the tissue growth and repair in order to fabricate functional new tissue to replace damaged ones. The use of this approach in cartilage repair has promoted many researchers in improving joint function and preventing further joint degeneration (Chung & Burdick, 2008; T. Hardingham, Tew, & Murdoch, 2002; Nesic et al., 2006; Redman et al., 2005).

Tissue engineering approach involves the use of chondrocytes, 3D scaffold and growth factors that help in improving the repair of cartilage lesions. A variety of scaffold has been introduced and explored for cartilage tissue engineering applications. Throughout the years, various natural and synthetic materials have been investigated in exploring its functionality in cartilage repair. Scaffolds were regarded as promising materials due to its similarity in properties to the native cartilage and providing temporary support during cartilage repair. In addition, scaffold formulation can be crosslink *in situ* thus making it perfect to be utilized in a minimally invasive procedure (Cai, Liu, Shu, & Prestwich, 2005; C. D. Hoemann, Sun, Légaré, McKee, & Buschmann, 2005).

Naturally, sustainable biomaterial can be explored and investigated such as 2D Human Amniotic Membrane (HAM) into a 3D scaffold. A novel, injectable formula that can be crosslink *in situ* has been developed for the delivery of chondrocytes and growth factor to the area of cartilage defects. The scaffold made from HAM and Fibrin as a delivery vehicle is an ideal candidate for minimally invasive procedure of cartilage tissue repair.

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Previous work done by another group has shown the cytocompatibility and biocompatibility of 2D HAM in cartilage tissue engineering applications (C. Z. Jin et al., 2007). The properties of HAM that are high in collagen, hyaluronan, TGF-ß and cytokines, all of which can also be found in native cartilage tissues have made this sustainable materials a good candidate for cartilage tissue engineering scaffold.

However, HAM is a 2D material naturally and it has been well documented that chondrocytes are prone to alter its phenotype in monolayer setting (Benya & Shaffer, 1982). Due to this fact, we resorted in changing the HAM from a 2D form to a 3D form whilst still keeping its constituents and components intact in order to harvest its potential as a chondrocyte carrier/ scaffold.

1.4 Thesis Structure

This study is structured around the basis of understanding the tissue engineering concept. It is supported by previous research and findings and progressing alongside the evidence stated in Chapter 2.

The experimental work is reported in two different studies; the first is optimizing the methods of fabricating the novel 3D scaffold specifically in procuring and changing HAM in a 3D scaffold and optimizing the scaffold fabrication. The next study is a continuation of the first study, looking into the response and the effects of chondrocyte culture on the 3D scaffold, the biochemical changes occurring within the *in vitro* environment, morphological changes and histological evidence of this new scaffold.

This study is brought together in general discussion with previous results obtained and comparisons from other research. The present study ends with a general conclusion which was determined in the earlier part of the study and can be both used in clinical and research study purposes and as a ground for further future investigation.

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1.5 Aims and Objectives

1.5.1 Aim of the study

The main aim of this study is to fabricate a new 3D scaffold from sustainable Human Amniotic Membrane for Cartilage tissue engineering applications.

1.5.2 Objectives of the study

i. To evaluate a suitable method for the production of three dimensional scaffolds based on HAM and Fibrin; and

ii. To determine the feasibility of HAM/ Fibrin scaffold for chondrocyte proliferation and matrix production.

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CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

In this chapter, we discuss the previous related research on related subject matter/ current study in order to have a detailed understanding of the chosen study. This chapter focused on the cartilage itself with compact details on the anatomy, physiology, its pathology and current treatment. Next, we focus on the challenge of addressing suitable scaffold used in tissue engineering applications. Reviews of different scaffold materials with different properties and choosing what deemed fit and related to the current trend in this field.

2.2 Cartilage

Cartilage is a type of connective tissues that consists of chondrocytes and ECM. It can be classified as specialized connective tissues (Buckwalter & Mankin, 1998). The basic purpose of articular cartilage is to provide a surface covering of the articular ends of bones at the synovial joints (M. A. R. Freeman, 1973).

The main function of articular cartilage is being a flexible and resilient to compression that acts as a shock-absorber at the same time allowing a friction-free and smooth joint movement. The thickness of the articular cartilage may vary and usually can be found at around 2-4 mm depending on the area within the joints. The thickness remains unchanged throughout the human adult life (Fox, Bedi, & Rodeo, 2009). Therefore, it is unusual to become thin under normal physiological joint activity. This, however, differs in certain cases where a process called fibrillation occurs, where a localized lesion can be found on the healthy adult cartilage. This is the most common occurrence among the general population. Such lesion may usually lead to the thinning of the cartilage and finally expose the underlying covered joint ends, the subchondral

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bones (Buckwalter, Marsh, Brown, Amendola, & Martin, 2014; Makris, Gomoll, Malizos, Hu, & Athanasiou, 2015; Shapiro, Koide, & Glimcher, 1993).

Articular cartilage (AC) is devoid of nerves and is generally considered avascular. The tissue mainly consists of relatively small amount of chondrocytes with an abundance of ECM surrounding them. The cells and matrix are functionally interdependent, whereby the chondrocytes activity is necessary for the synthesis of matrix physiological degradation. In return, the matrix plays an important role in maintaining the cells' homeostasis environment (Buckwalter & Mankin, 1998; Getgood, Bhullar, & Rushton, 2009; T. E. Hardingham & Fosang, 1995). The matrix is a form of a stiff gel that comprises a large amount of water (80%), a meshwork of collagen fibers, glycosaminoglycan (GAGs) and proteoglycans (PGs). All of these substances if removed by experimental procedures or under pathological conditions may cause major effect to the cartilage rigidity.

The mixture of fluid and matrix allows the viscoelastic and mechanical properties necessary for the efficient function of the cartilage. In a healthy state, typically the articular cartilage has water, chondrocytes, proteoglycans and collagen (Hunziker, 2002). The collagen and PGs are solely responsible for the load of the articular cartilage. The primary constituent of PGs is aggrecans with type II as a majority. ECM of the AC is merely 5-7% that is slowly stimulated by a single cell chondrocyte. However, because of its limited self-repair ability, due to the avascular and aneural in nature, limited repair to that partial cartilage defects may lead to future degeneration of the surrounding tissue is inevitable (Frenkel & Di Cesare, 2004; T. Hardingham et al., 2002; Hunziker, 2002; Makris et al., 2015; Obradovic et al., 2001; Tuli, Li, & Tuan, 2003).

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The most common and abundant type of cartilage in the human body is hyaline cartilage. It has the ability to resist compression and tension. It mainly consists of water, which makes 80% of its wet weight constituent. Hyaline cartilage is abundant of type II collagen. It is located at the end of articulating surface joints, the nose, larynx, tracheal rings, bronchi and the epiphyseal plate of growing bones. Since the focus of this study is on articular cartilage, from this section onwards, we will only discuss on hyaline cartilage in particular.

2.3 Architecture of Hyaline Cartilage

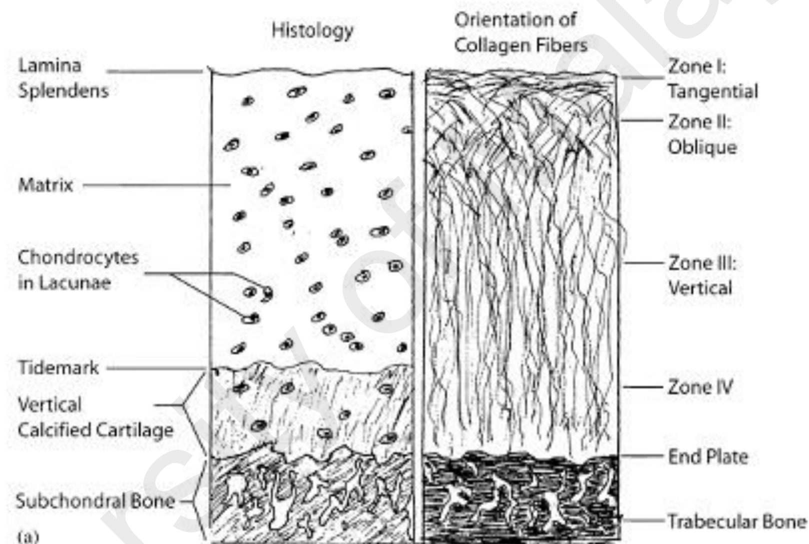

Figure 2.1: Architecture and zonal classification of articular cartilage. Adapted from (Seal, Otero, & Panitch, 2001).

AC *in situ* varies in shape, size and distribution according to the zone. In vertical sections, as shown in Figure 2.1, superficial cells appear as single and elongated oval outline. But in tangential to the surface, they are found to be round with a diameter of between 10-20 µm. In the intermediate zone, zone 2; the cells appear to be spherical with a diameter of 10 µm or more in singles and as it goes deeper, it occurs in groups of two or more. Meanwhile, in the deep zone, the cells tend to be grouped together vertically and resemble the cell columns (T. E. Hardingham, 1998).

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AC is found in the joint ends that provide smooth frictionless and stable movement that allows efficient load bearing and load distribution. The articular cartilage appears to be a simple white, avascular matrix. This highly specialized tissue comprises of a single cellular component the chondrocytes embedded in a highly hydrated and organized matrix of collagen type II and PG (T. E. Hardingham, 1998).

2.4 Cartilage Extracellular Matrix

The cartilage ECM plays a crucial role in maintaining the intracellular balance in cartilage. It is composed of a variety of molecules such as PGs, hyaluronan, fibronectin, laminin and is largely dominated by collagen fibrils. Other components that can also be found in the ECM of cartilage is integrins which mediate the attachment between cells and its surrounding and plays an important role in cell signaling within the cartilage matrix (Gao et al., 2014).

2.4.1 Collagen

The cartilage matrix is comprised of the GAGs, PGs and fibers which consists of collagen and elastin as depicted in Figure 2.2. Collagen can be found as the most abundant of the connective tissue fibers. It gives cartilage the flexibility function and high tensile strength properties. The collagen synthesis occurs within and outside of the chondroblasts. There are vast types of collagen found in the human body, from Collagen type I to XIX (Gentili & Cancedda, 2009).

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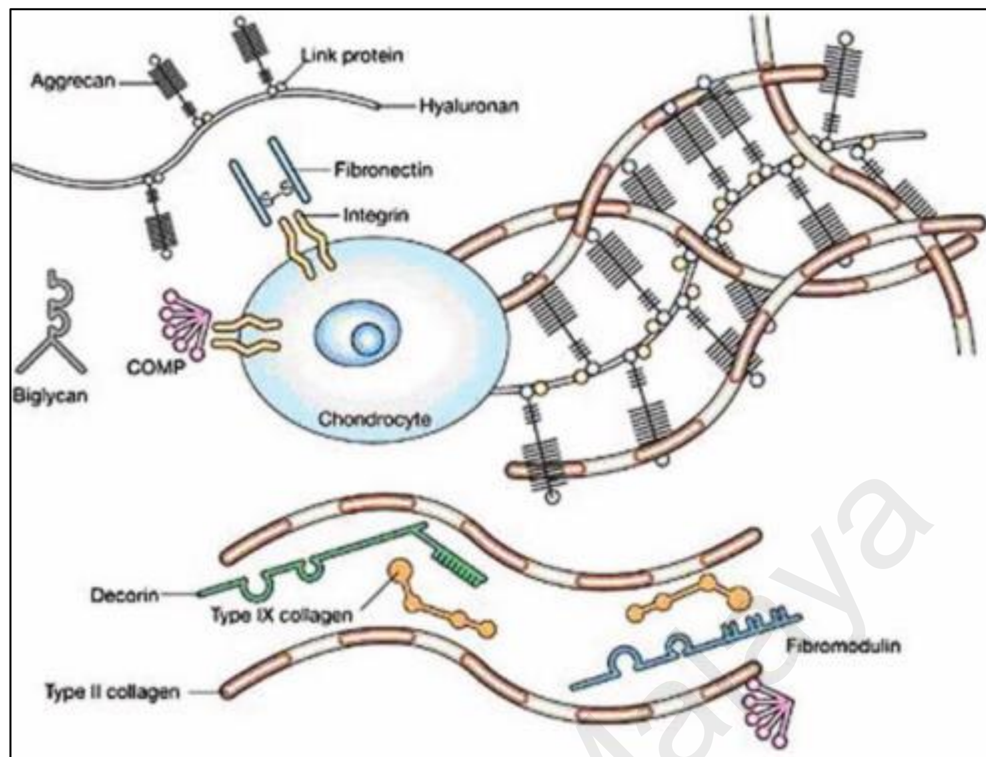

Figure 2.2: Extracellular matrix of articular cartilage. Adapted from (Fox et al., 2009).

Collagen fibrils are made up of aggregates of molecules known as Tropocollagen. Tropocollagen consists of three polypeptide chains wind around each other in a superhelix. In most mammalian, collagen appears to have two α1 polypeptide chains and one α2 polypeptide chain, which is rather different in amino acid composition although similar in molecular weight (Fox et al., 2009).

The basic biological unit of collagen is not protein in molecular form but in the association of molecules in the form of fibril. This ability of fibril formation in the structure provides stability by the formation of cross-links between molecules and the three polypeptide chains after secretion from the cell. The resulting network composed of fibrils, cross-link material, providing the material with high tensile strength. This unique structure makes collagen insoluble and resistant to attack by any but a specific enzyme so that it is metabolically inert in adult tissue (Fox et al., 2009).

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2.4.2 Proteoglycans

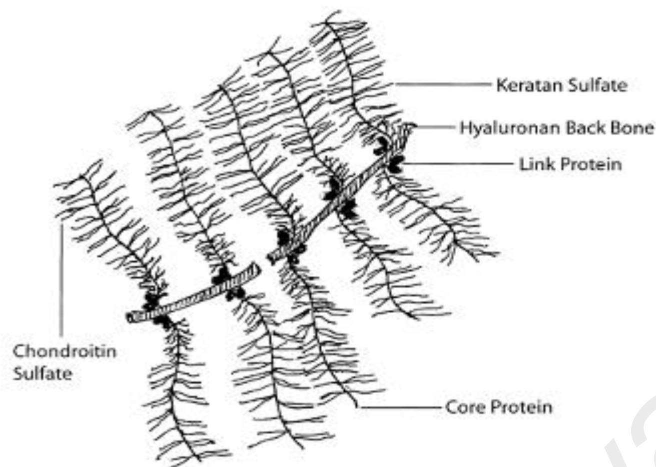

Figure 2.3: Structure of an aggregating proteoglycans. Adapted from (Seal et al., 2001).

Chondroitin sulphate is predominant GAGs of the cartilage proteoglycans. It consists of a protein core which has numerous chondroitin sulphate chains attach laterally to the hyaluronan core as shown in Figure 2.3. The behavior and degradation reaction of cartilage proteoglycans are consistent with this model, thus, the core protein holds together many GAGs chains should be made it less vulnerable to be attacked by enzymes. GAGs is a general term to the carbohydrate chain, does not occur free and unattached to a chain. Cartilage PGs consists of two types of GAGs chain, namely chondroitin sulphate and keratan sulphate (Fosang & Hardingham, 1996; Gentili & Cancedda, 2009; Lamoureux, Baud'huin, Duplomb, Heymann, & Redini, 2007; Olsen, 2007).

2.4.3 Water

In the intracellular and ECM, water accounts for approximately 70-80% of the AC (T. E. Hardingham, 1998). The amount of water content differs within the matrix as it gradually decreases from 80% in the surface to 65% in the deep zones. A temporary swelling of the AC attributed to the absorption of water from the joint space. The fluid flows from the synovial fluid transport and provides nutrients to the AC which functions

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as lubricant to the joint. PG is responsible for fluid entrapment within the cartilage matrix which enables the Ac to support high loads (Eyre, 2002; M. A. R. Freeman, 1973). Most of the water that exists in the ECM is in gel form. It navigates through the ECM by pressure gradient or by compression of the AC. Frictional resistance against the flow of fluid/ gel through the matrix is due to the low permeability of the tissue (Fox et al., 2009; Gao et al., 2014). The high content of PGs particularly aggrecans draws fluid into the AC creating a large osmotic pressure. It is the combination of this resistance and pressure across the ECM that forms the mechanism enabling the AC to withstand significant loads up to 5 times the body weight (Paul, 1976).

2.5 Chondrocytes

The chondrocytes are the mature cartilage cells and are the only resident cell within the cartilage tissue. It is a specialized, metabolically active cell with the main functions in the development, maintenance and repair of the cartilage matrix (Buckwalter & Mankin, 1998; Choi et al., 2013; Fox et al., 2009; T. E. Hardingham, 1998). The chondrocytes can be found trapped in its own matrix within the lacunae. These cells can be identified with features of round large nucleus. The number of cells per unit volume of adult articular cartilage is small. It accounts for approximately 2% of the total volume of the AC. The chondrocytes varies in shape, size and number depending on the anatomical region of the AC. Those that are in the deeper regions are densely packed and are rounder in shape as compared to those in the superficial region which are flatter and smaller.

The chondrocytes and the ECM are functionally interdependent. The chondrocytes activities are necessary for the synthesis of matrix physiological needs and in return, the matrix maintains a homeostasis environment feasible for chondrocyte activities. This process continues even after the completion of skeletal growth during the skeletal

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development and continues throughout adult life. It has a high metabolic rate associated with continuous ECM turnover (Fox et al., 2009).

2.5.1 Internal Structures

The only resident cell within the cartilage matrix is chondrocyte. It ranges from 10-20 µm in diameter depends on where it is located within the cartilage zones (M. A. R. Freeman, 1973). Like any other eukaryotic cells, chondrocytes are equipped with organelles such as the nucleus, rough endoplasmic reticulum (RER), golgi apparatus (GA) and mitochondria. The functions of these organelles are further explained as tabulated in Table 2.1.

Table 2.1: List of functions of organelles found in chondrocytes. Adapted from (I. Freeman & Cohen, 2009; M. A. R. Freeman, 1973; Stephen W. Schaffer, 2007).

Organelle	Functions
Nucleus	Produces mRNA and transmits signals to the cytoplasm
Rough Endoplasmic Reticulum (RER)	Involved in the production of protein required to be used within chondrocytes
Golgi Apparatus	Synthesize and transport chemical substances produced in the chondrocytes for secretion to the ECM
Mitochondria	The powerhouse of the chondrocytes and produces chemicals that fuel for activities within the chondrocytes
Lysosomes	Removal of intracellular materials and involved in the turnover of the ECM

Most chondrocytes contain cytoskeleton within their cytoplasm which can be identified as microfilaments, microtubules and intermediate filaments. The microfilaments contribute to the structural strength and contractile properties to the cytoplasm. It scatters within the cytoplasm and not encased by the membrane vacuole (M. A. R. Freeman, 1973). Meanwhile, the microtubules in the cytoplasm are responsible to transport the vesicle inside the cytoplasm. As adult chondrocytes neither divide nor it migrates, the roles of microtubules are in the cell-matrix interactions,

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intracellular transport and in the resiliency of the chondrocyte (Langelier, Suetterlin, Hoemann, Aebi, & Buschmann, 2000). It has been reported that microfilaments are responsible for the chondrocyte phenotype (Benya & Shaffer, 1982).

2.5.2 Chondrocyte Metabolism

Like any living cells, chondrocytes carry a complex chemical transformation in order to maintain the integrity of the cartilage tissue. As adult cartilage do not proliferate once matured even in the presence of growth factor, the chondrocytes are responsible for the sustenance of the ECM. The cartilage matrix is separated from the subchondral bone by the subchondral space and the nutrients for the cartilage matrix are gained through diffusion from the synovial fluid (Buckwalter & Mankin, 1998). Due to avascular and alymphatic in nature, chondrocyte has the ability to synthesize and secrete matrix in the avascular environment and depends primarily on anaerobic metabolism (Stockwell, 1978).

The chondrocytes synthesize ECM components such as the glycosaminoglycans. The metabolic activity of the chondrocytes can be altered significantly depending on the surrounding chemical and mechanical stimulation. There are pro-inflammatory cytokines that have an effect on the matrix synthesis and degradation such as interleukin-1 and tumor necrosis factor-α. Other than that, proteoglycans are also synthesized, maintained and secreted by the chondrocytes. These PGs are closely regulated by growth factors and peptides such as insulin-like growth factors, TGF-β, interleukin-1 and tumor necrosis factor-α (Fox et al., 2009).

2.5.3 Chondrocyte-ECM Interaction

The symbiosis relationship between the chondrocytes and the ECM in sustaining the integrity of one another is important. The interaction between these two regulates many biological activities crucial to the homeostasis of the cartilage. The dynamic reciprocity

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the ECM and chondrocytes to regulate cellular activities such as cell migration, differentiation, matrix production and breakdown is modulated by skeletal adhesion molecules (Helfrich & Horton, 2006).

Chondrocyte adhesion interaction plays a critical role in migration, proliferation and differentiation of cells. These adhesive interactions which are mediated by cell surface receptors bind to the ligands on adjacent cells or in the ECM. The intracellular signal transduction pathways are regulated by the integrins. There are 24 unique integrins dimers found in mammalian formed by 18α and 8β subunits. Integrins expressed by chondrocytes include fibronectin, laminin and collagen receptors. The most component of the chondrocytes integrins is the β1 chain (Gao et al., 2014). The β1 integrin family of cell surface receptors is crucial in mediating the chondrocyte –ECM interaction involved in cell-matrix adhesion, cell signaling, protein binding and receptor-mediated activities (Giancotti & Ruoslahti, 1999).

The fibronectin receptor; α5β1 integrin together with its ligand, fibronectin plays a role in dedifferentiation of chondrocytes. Meanwhile, IL-1 antagonist upregulates major components of ECM genes and is used in the anti-inflammatory and chondroprotective therapy of the cartilage (Gao et al., 2014).

The extracellular domain binds with low affinity to the ECM and the intracellular domain link to the cytoskeleton. These integrin molecules are freely diffuse within the cell membrane in their inactive state until there is an available binding domain in the ECM recognized. Upon ligand binding, the integrins will undergo conformational changes that lead to the recruitment of cytoplasmic proteins. Clustering multiple integrins means there will be more proteins recruited to the adhesion site with increment in size, adhesion strength and biochemical signaling activities (Boudreau & Bissell, 1998; García, 2005; García & Boettiger, 1999; Helfrich & Horton, 2006; Olsen, 2007).

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These largely clustered integrin and cytoplasmic proteins are focal adhesion and they act as sensors of ECM environment thus play an important role in signal transduction. The type of cell-matrix adhesion organized by integrin *in vitro* and *in vivo* and the signal that they transduce are strongly influenced by the architecture of the ECM; meaning the cell probes the stiffness of its environment. The stiffness of the ECM dramatically affects the cellular processes and cell differentiation. Whether a cell proliferates or dies, it is determined by a degree which a cell physically extends the amount of integrin-ligand interactions. Substrate flexibility affected the size of the focal adhesion and the strength of the adhesion (Boudreau & Bissell, 1998; García & Boettiger, 1999; Helfrich & Horton, 2006; Olsen, 2007).

2.6 Cartilage Disorder

The regeneration of cartilage is usually poor in adults due to its avascular nature. Disorders of cartilage may include:

i. Rheumatoid arthritis: chronic, systemic autoimmune disorders;
ii. Osteoarthritis: degenerative joint tissues due to trauma/ aging;
iii. Achondroplasia: Autosomal dominant disorders causing disproportionate dwarfism; and
iv. Chondroma: Benign tumor of the cartilage.

There are several causes of partial or complete loss of the articular cartilage. In deep fibrillation, the cartilage can thin or in the worst case, completely lost which in turn exposing the underlying bone. In an osteoarthritic cartilage, a section of the cartilage can separate from the joint surface leaving a crater-like defect as illustrated in Figure 2.4.

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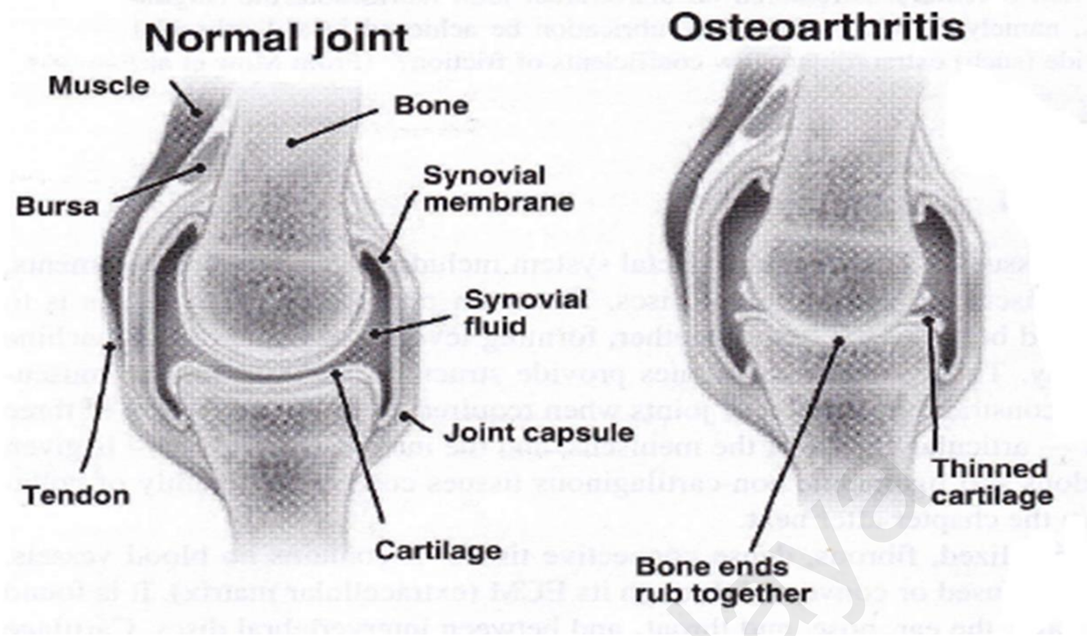

Figure 2.4: Comparison of a healthy normal joint and an osteoarthritic joint. Adapted from (C.Cowin & Doty, 2007).

Likewise, in rheumatoid arthritis, the thinning of the underlying bone occurs. This resulted in severe pain and loss of joint movement affecting the Activities of Daily Living (ADL) of the patient. As in an acute trauma, the cartilage can be fractured or fully disintegrated resulting in loss of joint movement due to excruciating pain (Boudreau & Bissell, 1998; Clouet et al., 2009; García & Boettiger, 1999; Helfrich & Horton, 2006; Hunziker, 2002; Martinek & Imhoff, 2003; Olsen, 2007; Pritzker & Aigner, 2010).

These defects can be further classified as partial thickness defects and full thickness defects. A partial thickness defect as illustrated in Figure 2.5 (a) represents a defect whereby there is still some cartilage intact at the base of the subchondral bone. Meanwhile, deep defect as illustrated in Figure 2.5 (b) extends completely to the underlying calcified zone, the subchondral bone (Ritsila et al., 1994; Steadman et al., 2003; Swieszkowski, Tuan, Kurzydlowski, & Hutmacher, 2007).

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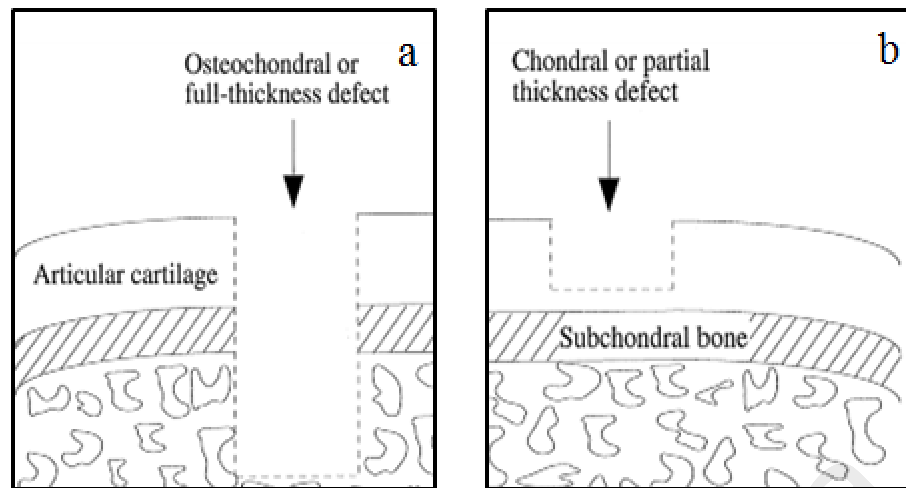

Figure 2.5: (a) Full Thickness defects of articular cartilage. (b) Partial thickness defects in articular cartilage. Adapted from (LeBaron & Athanasiou, 2000).

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The possibility of a cartilage defect actually depends to an extent on which type of defect is present. Potential repair mechanism:

i. Intrinsic repair

   Healing of the cartilage is entirely dependent on the activity of surviving chondrocytes adjacent to the defect. Under this circumstance, the healing is an exception in adults due to the limited capacity of mature hyaline cartilage to self-repair.

ii. Extrinsic repair

   In extrinsic repair, new tissue can be formed from the soft tissue spaces in the subarticular bone plate as new tissues can spread onto a joint surface from the juxta-articular region. This repair can be accompanied by the growth of new bone. This new cartilage often always is fibrocartilage rather than of the previous native hyaline cartilage.

Among other treatment options are debridement and lavage, arthroscopy method, abrasion therapy and the very last resort are a total knee replacement. New methods introduced in repairing cartilage lesion include Autologous Chondrocyte Implantation

(ACI). ACI was pioneered back in 1982 by isolating rabbit chondrocytes and propagate them *in vitro* (Benya & Shaffer, 1982).

2.6.1 Autologous Chondrocyte Implantation (ACI)

In ACI approach, the periosteal patch is sutured in place over the defect and the chondrocytes were introduced underneath them as shown in Figure 2.6. Despite the clinical results following ACI treatment, opportunities for an improvement for these strategies exist as there were reported cases of drawbacks which includes the "sinking" of the chondrocytes patients who have been treated with ACI do not typically return to full physical activity until one-year post surgery. The invasive nature of the procedure that includes an open arthrotomy and the time interval between chondrocyte implantation and production of functional repaired tissue has been responsible for the relatively long period of patient rehabilitation. In overcoming this, the latest and current trend is Tissue Engineering or more recently known as regenerative medicine has been introduced (Britt & Park, 1998; Brittberg et al., 1994; Giannoni et al., 2005; Hamby, Gillogly, & Peterson, 2002; Marlovits et al., 2006; Minas & Peterson, 2012; Peterson, Brittberg, Kiviranta, Akerlund, & Lindahl, 2002; Trattnig et al., 2005; Vasiliadis et al., 2010).

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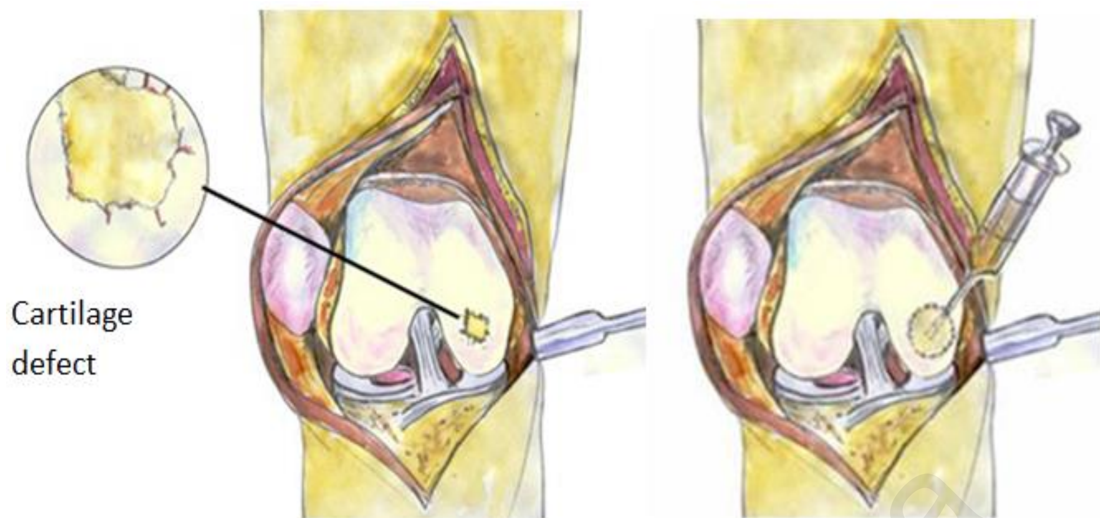

Figure 2.6: Autologous Chondrocyte Implantation procedure. Adapted from (Hamby et al., 2002).

2.7 Tissue Engineering

Tissue engineering can be as the structural or as functional reconstitution of cells, biomaterial and biological signals interplay. Challenges of this however are but limited to restoring the normal composition of the cartilage and restore the function of the damaged cartilage while avoiding future degeneration of the restored tissues (R. A. Brown, 2013; Chang et al., 2006; Vacanti & Vacanti, 2007).

The emerging of tissue engineering field may be the answer to the previous problems. The use of cells to regenerate damaged tissues with the aid of 3D substrate/material to organize the cells in order to maintain tissue specific functions once implanted. In choosing these materials, it is obligatory to choose one that exhibits good biocompatibility. The biomaterial must not elicit inflammatory response or immunogenicity (Drury & Mooney, 2003; Frenkel & Di Cesare, 2004; Luo et al., 2014; Pachence & Kohn, 2000; Rosso et al., 2005; Temenoff & Mikos, 2000a; Trachtenberg, Kasper, & Mikos, 2014; Zhang, Ortiz, Goyal, & Kohn, 2014).

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2.7.1 Principle Cell Sources Used In Tissue Engineering Studies

In engineering a new functional tissue or organ, the major challenges include the method of acquiring the cells. The golden standard would be to mass produce the desired tissue and organ so that off the shelf product can be reachable to many and not just to the exclusive few (Ikada, 2006).

The importance of cell source on the outcome of regenerating new tissue is very crucial. The cells that are applicable in tissue engineering applications may be classified into three different categories. The best and most ideal of cell source would be of course of autologous origin also known of being patient's own. Secondly, the cells can be of allogeneic source meaning it is harvested from other human being or rather than another subject within the same species. Lastly, we can have an option from an animal source being xenogeneic in origin (Nerem & Schutte, 2014; Redman et al., 2005).

The last two cell source, allogeneic and xenogeneic cell source in regenerating new tissue is considered to be less feasible than autologous cells due to immunologic reasons and the usage of these cells requires patients to undergo immunosuppressant therapy which is proven to be compromising the health outcome of the patient (B. N. Brown & Badylak, 2014; Ikada, 2006).

2.7.2 Cells and Their Extracellular Matrix

Monolayer culture has been the golden standard for rapid cell expansion and has been known to be the method used in obtaining a large number of cells of ACI purposes. However, the problem with cell expansion in monolayer setting is the loss of phenotype. The success of ACI repair was absolutely dependent on the ability of the implanted cells to maintain its phenotype and synthesize the cartilage matrix (Redman et al., 2005).

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In the 3D environment, chondrocytes have been reported to maintain its phenotypic stability because the culture environment is highly influential on the maintenance of chondrotypic phenotype (Holtzer, Abbott, Lash, & Holtzer, 1960). Cells have the ability to adjust their cytoskeleton organization, shape, motility and changes to their immediate environment. Integrin based adhesion complex which is associated with actin cytoskeleton which could recognize biochemical diversity of ECM surrounding also physical; and topographical characteristics. Integrin were able to sense environmental changes by the cytoskeletal network. Integrin are membranes that heterodimeric receptors that mediate communications between ECM and cells (Olsen, 2007).

2.7.3 Three Dimensional Scaffold

The growing needs in tissue repair and replacements have driven tissue engineering strategies to provide functional alternative biological solutions. Current strategies for articular cartilage regeneration and repair involve the potential use of chondrocytes. This requires the combination of cells, biodegradable scaffold and bioactive molecules. The key component surrounding tissue engineering, however, is in designing a suitable scaffold. Up until now, there is no evidence of an "ideal scaffold" design, but each tissue requires specific set of properties that they must possess and this "design" should start with a minimum requirement

A scaffold that is to be used in tissue regeneration must provide an environment necessary for cell attachment and promote adhesion, differentiation, allow nutrient and metabolic waste exchange and able to mimic and fulfill the final shape of the void space that is being replaced.

In the first phase of cartilage tissue engineering, the fabrication and design of a 3D scaffold must be taken into consideration. The 3D scaffolds should be made of a highly biocompatible material which will not elicit violent immunological reaction to the host

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tissue. The chosen material should be able to degrade at a controlled rate specifically to the nature of cell/ tissue regrowth onto the host tissue. The second phase would be the seeding of the viable chondrocytes onto scaffold in a static culture. This phase is usually done to introduce the cells to the biomaterial or 3D scaffold by incorporating the cells and allow attachment or adhesion to take place. Next, in phase 3, which is growing the cell culture in a dynamic environment, is the next phase although not all tissue regeneration approach requires this step. After which, mature tissue will be introduced in a clinical setting in phase 4, 5 and 6 via surgical transplantation to enable surviving cells in *in vitro* environment to accommodate the desired site of the intended tissue repair to further allow *in vivo* tissue remodeling (Hutmacher, 2000).

In order to create a 3D structure to occupy the desired cell in tissue engineering strategies, we need to choose the appropriate source of material for the intended application. Materials that are commonly used as 3D scaffold can be classified as organic and non-organic/ inorganic. This can be further classified as metals, ceramics and polymers.

Metal is inorganic material commonly used due to their ability to conduct electricity, strength and easily formed into complex shapes. This material is widely used in cardiology, dentistry and orthopedic applications as artificial heart valves, dental implants/ fillings and as hip and knee replacement.

Meanwhile, the inorganic ceramics are commonly used due to their hard properties and resistance to degradation in many conditions, unlike metals. It is widely employed as bone/ dental implant merited to its similarities to the chemistry of bone. Ceramics are usually chosen to cater applications acquiring small loads.

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Unlike the two classes of materials mentioned, the polymer is an organic material that is extensively utilized in biomedical applications. The vast range of physical and chemical properties of this material has accounted for its use in the medical industry. Polymers can either be synthetic or man-made or naturally sourced such as protein that is commonly found *in vivo*. The common uses for these types of polymers are listed in Table 2.2.

Table 2.2: Natural and synthetic polymer widely used in biomedical applications. Adapted from (Luo et al., 2014).

Polymer	Applications
Synthetic	
Poly(ethylene)	Orthopedic implants
Poly(ethylene glycol)	Wound dressing
Poly(propylene)	Sutures
Poly(2-hydroxylethyl methacrylate)	Contact Lenses
Natural	
Collagen	Orthopedic and nerve repair and tissue engineering matrices
Chitosan	Wound dressing
Fibrin	Hemostatic sealant
Hyaluronic Acid	Orthopedic repair matrices
Glycosaminoglycans	Orthopedic repair matrices

2.7.3.1 Scaffold Criteria

In natural tissue, the ECM that surrounds the cells in the body does not only support the cells but it also regulates cell proliferation, differentiation and morphogenesis. A scaffold should be evident to support proliferation whilst maintaining their dedifferentiated function. The scaffold can be from different types and variants. Numerous architecture shapes and texture all depending on the target tissue. Most feasible of the scaffold will be according to the scaffold criteria:

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i. 3D/ porous to enable cell growth and transport of nutrients and waste products;
ii. Biocompatible/ bioresorbable with controlled biodegradability while resorption rate match to the cell/ tissue growth *in vitro*/ *in vivo*;
iii. Able to allow nutrient exchange and byproduct transport across the constructs/ native tissue;
iv. Sufficient mechanical properties to substitute initial wound contraction forces, and later for the remodeling of tissues; and
v. Manufactured in a reproducible, controlled and cost-effective manner.

For the newly designed 3D scaffold, it is crucial for these scaffolds to be able to interact with the host cells in order to maintain cellular and molecular function in culture. It is well known, that cells respond differently to their environment as well as to the architecture of their environment be it 2D or 3D, resulting in different kinds of response. Cell-matrix adhesion mediates physiological response responsible for cell growth, survival, cell migration, differentiation, and tissue organization and remodeling (Hutmacher, 2000; Hutmacher, Sittinger, & Risbud, 2004). Cell signaling and metabolic cell activation may affect cell surface interaction. Cells can sense and respond to chemical and physical signals from biomaterials to identify the new biomaterial traits that could further alter the cell behavior *in vitro* and *in vivo* (Geiger, Spatz, & Bershadsky, 2009).

2.7.3.2 Scaffold Design

In designing functional cell-support system, it is essential for it to mimic the intended native tissues. It must be able to retain in the space that is designated for and provide adequate space for cells to grow into tissues replacing the scaffold (Hutmacher, 2000; Temenoff & Mikos, 2000). It must be able to provide a 3D matrix for tissue growth and

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in-growth whilst providing shape and support to the construct being ideally exhibited mechanical properties similar that of the host tissue. In designing a 3D scaffold, it is crucial to have a porous interconnected network that would allow tissue ingrowth.

This feature allows for nutrient delivery and byproducts to be expelled. The scaffold footprint should also be considered. Ideally, the resorption rate should be similar as the tissue regeneration. Other properties would be able to reduce fibrous tissue formation otherwise known as scar tissue. This is important as scarring at the site of implantation compromises the integrity of the host-construct interface. Lastly, the scaffold should be easily prepared, reproducible and sterilize while being relatively affordable for the patients to use (Frenkel & Di Cesare, 2004; Lu, Li, & Chen, 2013; Luo et al., 2014).

2.7.3.3 Natural vs. Synthetic Polymer Scaffold

The challenge in imitating nature which has the potential to be the answer to organ transplantation crisis and possibly address any unmet patient needs is far from reality. However, with recent discoveries in regenerative medicine, we are moving forward and closer to realizing that dream. In deeming what would be an ideal source for scaffold in tissue engineering application, one would have to consider the pros and the cons in each category and weigh the best possible outcome for the intended tissue and organ replacement.

Natural materials constituents and compositions are always in the center of concern in designing the scaffold for tissue engineering. The two natural groups that are known to be natural are proteins and polysaccharides. Natural materials can be degraded by the embedded cells and cells, in turn, will produce new matrix to fill in the spaces formed by this degradation. The general fiber forming and aggregating proteins that are well known are collagen, fibrin and silks. Meanwhile, polysaccharides which are another group of natural material that can give significant mechanical strength are starch,

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chitosan and hyaluronan (Kogan, Šoltés, Stern, & Gemeiner, 2007; Sell et al., 2010; Zhang et al., 2014).

Collagen is one of the most widely used materials in tissue engineering applications. Collagen has been used in many physical forms, most generally known in either gel or sponge form. It can be used either in its native form, crosslinked, in soluble form, in an insoluble form and aggregated form. Generally, it can be divided into 4 categories which are known:

i. Insoluble collagen
ii. Tropocollagen
iii. Atelocollagen
iv. Gelatin/ heat-denatured collagen

Insoluble collagen can be as raw as homogenized tissues, reconstitute and freeze-dried. The structural elements of these materials are made of shredded fiber bundles. Tropocollagen is the intact, monomeric, acid soluble form of collagen. Atelocollagen is a soluble monomer rich collagen but poor in gel formation abilities due to its protein digesting enzymes that remove the short non-helical end extension that cuts the cross-links. Finally, gelatin is the readily available and a more economy-friendly type of collagen. It is obtained by boiling the collagen hence the loss of all the triple helix structure and so are the tensile strength and resistance to enzyme degradation properties. Due to this, gelatins are poorly biomimetic, weak and bio-unstable (Boudet, Iliopoulos, Poncelet, & Cloitre, 2005; Pachence & Kohn, 2000).

Hyaluronan or Hyaluronic Acid (HA) is a linear polysaccharide formed from disaccharide units of N-acetyl-D-glucosamine and glucuronic acid. Hyaluronan can be found in almost all biological fluids and tissues. HA is the simplest form of

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glycosaminoglycan's group substances. It occurs primarily in the ECM and the pericellular matrix. HA influences the cell differentiation, and tissue repair in vivo (Toole, 2004).

The biological function of HA includes the elasticity and viscoelasticity of fluid connective tissues such as the synovial fluid, control of tissue hydration and water transport, molecular assembly of proteoglycans in the ECM and receptor mediated roles in cell mitosis, migration and inflammation (Balazs, 2009). Within the cartilage, HA plays a vital role in the development of cartilage the maintenance of synovial fluids and the regeneration of tendons. High concentrations of HA can be found in the ECM of adult joint tissues and in synovial fluids. HA is responsible for macromolecular assembly in the ECM due to the HA-protein interactions. In the synovial fluid, HA is responsible in providing lubrication and act as shock absorbers. It is also responsible in the event of signal transduction, and cell motility. Large matrix polymers of HA was found to be successful space fillers with properties of anti-angiogenic and immunosuppressive (Balazs, 2009; Kogan et al., 2007; Necas J BL, 2008).

The unique viscoelasticity nature of HA along with its biocompatibility and immunosuppressive properties has led its use in multiple clinical applications as shown in Table 2.3. Applications of HA in orthopedic surgeries and rheumatology has been successful since 1980's. The intra-articular applications of HA were reported to improve symptoms and decrease the use of non-steroidal anti-inflammatory drugs in patients suffering from osteoarthritis. A study has suggested that the usage of HA in viscosupplementation could exert a therapeutic effect by restoring the elasticity and viscoelasticity of the loss synovial fluid and that by injecting HA can induce synthesis of HA by the synovial cells itself, therefore, stimulate chondrogenesis and inhibit cartilage degradation. The anti-inflammatory action of HA used also reduce the

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inflammatory cell count in the synovial fluid and potentially reduce the amount of the reactive oxygen species content and there were also observed analgesic effect upon HA administration (Necas J BL, 2008).

Meanwhile, in surgery and in wound healing applications, HA preparations are usually applied topically to promote healing on fresh skin wounds. Due to its anti-oxidant properties, HA serves as an anti-inflammatory component in the wound dressing materials. HA has also been used to improve and promote biocompatibility used in drug delivery capsules by creating a cross-link scaffold for DNA entrapment. HA can be obtained from various natural sources. Therefore, it is crucial in preparing the right formula of HA-derived therapy to address appropriate biomedical procedures and tissue engineering applications. The attributes of HA in clinical applications are as in Table 2.3.

Table 2.3: The modalities of clinical applications of HA and its derivatives. Adapted from (Balazs, 2009).

Clinical Applications	HA modalities
Viscosurgery	Protect delicate tissues and provide space during surgical manipulations
Viscoaugmentation	Fill and augment tissue spaces, as in skin, sphincter muscles and vocal tissues
Viscoseparation	To separate the connective tissues surface traumatized by surgical procedures or injuries in order to prevent adhesion or excessive scar formation
Viscosupplementation	To replace of supplement tissue fluids such as synovial fluid in painful arthritis and to relieve pain
Viscoprotection	To protect healthy, wounded, or injured tissues surfaces from dryness or noxious environmental agents and to promote healing of such surfaces

While natural polymers have its advantages, it is mechanically weak and inconsistent in its sources. In designing functional cell-support system, the possibility of mass produced and controllable properties of materials are certainly desirable. Synthetic

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polymer has been widely explored in order to counter this downside of natural polymer materials. Synthetic materials such as Poly-(ortho ester) (POE), Poly-(vinyl alcohol) (PVA) and Poly-(ester amide) (PEA) are appealing to the scaffold design due to their controllable and reproducible properties and chemistry (Grishko et al., 2009). Synthetic materials have the ability to be incorporated with other water-soluble polymers and cross-link physically and chemically (Lu et al., 2013; Pachence & Kohn, 2000).

Although synthetic polymers are reproducible and the chemical composition of the material can be altered significantly in order to suit its intended use, it is still lacking in biomimetic features that natural materials possess. The ability of natural materials to work as an integral part of the cell-matrix remodeling process as compared to those that just degrade regardless to the cells activity is what sets the two apart (Luo et al., 2014).

2.7.3.4 Scaffold Fabrication

Scaffold remains the central concern of researcher in tissue engineering approach. Previous studies have shown that fabrication methods have been applied to process biodegradable and bioresorbable materials into 3D scaffolds with high porosity to surface area as shown in Table 2.4. Although there are many available methods and techniques for fabricating and producing scaffolds, there is no ultimate method to produce specific tissue regeneration. Scaffolds meant to be used in cartilage regeneration have to be designed to elicit biological and materials properties compatible to the need of the newly formed cartilage.

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Table 2.4: Scaffold fabrication techniques and studies.

Fabrication technology	Processing method	Material properties Required for processing	Scaffold design and reproducibility	Achievable pore size in µm	Porosity in %	Architecture	References
Solvent casting in combination with particulate leaching	Casting	Soluble	User, material, and technique sensitive	30-300	20-50	Spherical pores, salt particles remain in matrix	(Mikos, Sarakinos, Leite, Vacanti, & Langer, 1993)
Membrane lamination	Solvent bonding	Soluble	User, material, and technique sensitive	30-300	< 85	Irregular pore structure	(Widmer et al., 1998)
Fabrication of non-woven	Carding, needling, Plate pressing	Fibers	Machine controlled	20-100	< 95	Insufficient mechanical properties	(Mol et al., 2005; Tognana et al., 2005)
Emulsion freeze drying	Casting	Soluble	User, material and technique sensitive	< 200	< 97	High volume of interconnected micropore structure	(Whang, Thomas, Healy, & Nuber, 1995)
Thermally induced phase separation	Casting	Soluble	User, material and technique sensitive	< 200	< 97	High volume of interconnected micropore structure	(Baker, Brown, Casadio, & Chirila, 2009)

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Table 2.4, continued.

Fabrication technology	Processing method	Material properties Required for processing	Scaffold design and reproducibility	Achievable pore size in µm	Porosity in %	Architecture	References
Super-critical fluid technology	Casting	Amorphous	Material and technique sensitive	< 100	10-30	High volume of non-interconnecting micropore structure	(Harris, Kim, & Mooney, 1998)
Supercritical fluid technology in combination with particle leaching	Casting	Amorphous	Material and technique sensitive	Micropore (< 50) Macropore (< 400)	< 97	Low volume of non-interconnected micropore structure combined with interconnected macropore structure	(Whang et al., 1995)
Fuse deposition modeling	Solid free form fabrication	Thermoplastic	Machine and computer controlled	> 150	< 80	100% interconnected macropore structure (triangles, pentagons, honeycomb, etc.) Design and fabrication layer by layer	(Dunkelman et al., 1995)

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2.8 Human Amniotic Membrane

2.8.1 Introduction

Both natural and synthetic materials have been mass-researched throughout the use for tissue engineering scaffold. Concerns arise for the use of both have been discussed and agreed that materials that would be ideal must exhibit certain properties that may include good biocompatibility, biodegradable, easily obtained and demonstrates little to none inflammatory response to the host tissues with good mechanical properties depending on the placement of the scaffolds (Temenoff & Mikos, 2000b).

In recent years, Human Amniotic Membrane has been utilized in numerous applications in the medical field (Bilic et al., 2005; Branski et al., 2008; Bujang-Safawi, Halim, Khoo, & Dorai, 2010; Liang et al., 2009; Stoddart, 2008). Successful cell-scaffold integration depends on the type of scaffold and the ECM component of the scaffolds. One of the pioneers in biomaterial used as scaffold was the fetal membrane. Amongst the use of a fetal membrane includes; transplantation of skin, management of burns, reconstruction of the oral cavity, tympanoplasty, arthroplasty and bladder (Dell'aquila & Gaffney, 1982; Lin, Lai, Hou, & Yang, 1985).

Human amniotic membrane has gained its importance in tissue engineering field due to its ability to reduce inflammatory and scarring, we enhancing wound healing and this served well as a scaffold for cell proliferation and differentiation as an outcome to its antimicrobial properties(C. Z. Jin et al., 2007; Niknejad et al., 2008).

In addition, the ECM of the amniotic membrane such as its constituents and growth factors are similar to that of native cartilage thus suggesting it would be an excellent candidate for a 3D scaffold for this application. Moreover, this biomaterial is a sustainable material that can be easily attainable, transport and processed.

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HAM develops from the extra embryonic tissue. It forms a protective barrier for the fetus during the gestational period. It is the innermost layer of the placenta membrane that is thin, elastic and translucent devoid of vascular, nerves and lymphatic vessels (Wilshaw, Kearney, Fisher, & Ingham, 2006). It is made up of three layers which are the epithelial, basement membrane and stroma. The amniotic membrane is thickness ranges from 20 µm to 0.5 mm. Schematic structure of HAM is shown in Figure 2.7.

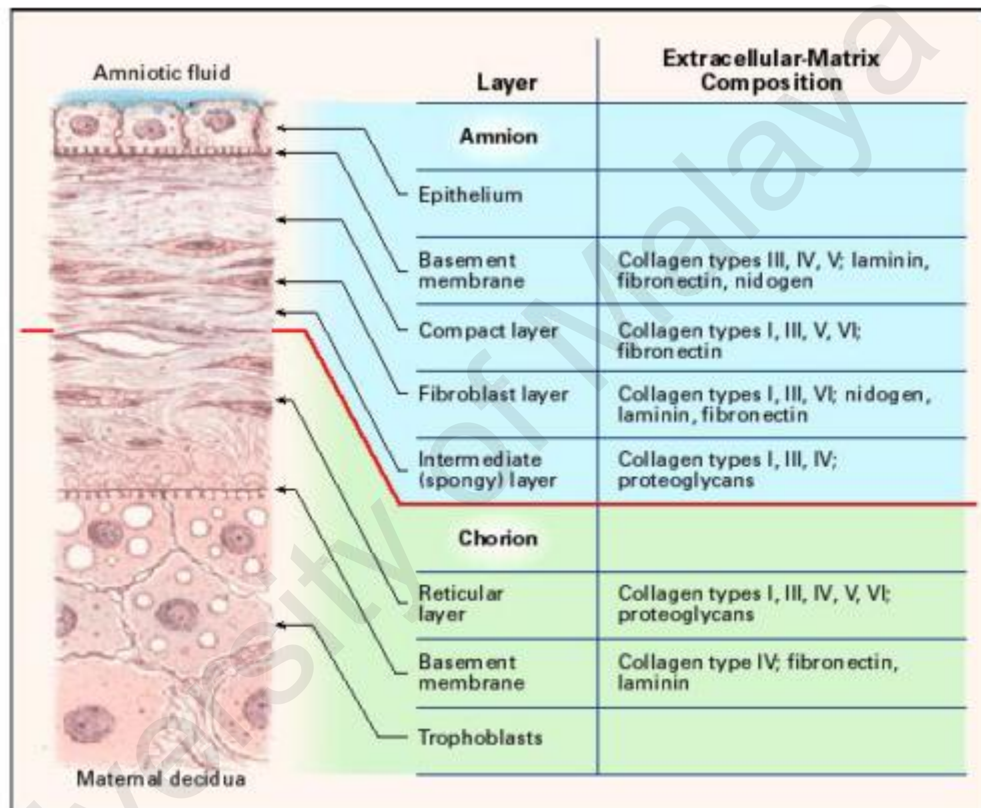

Figure 2.7: Schematic structure of the HAM. Adapted from (Niknejad et al., 2008).

This natural biomaterial content is high in Collagen type I, II, III, IV, laminin and fibronectin (C. Z. Jin et al., 2007; Niknejad et al., 2008) . It has been utilized in numerous medical applications such as skin transplant to treat burns, as a wound dressing, in the application of ophthalmology, gynecology and regenerative medicine. Among other benefits that this biomaterial possess is it has natural cartilage constituents such as collagen, proteoglycans, and HA (Chandra et al., 2005; Díaz-Prado et al., 2010;

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C. Z. Jin et al., 2007; Mamede et al., 2012; Niknejad et al., 2008; Wilshaw et al., 2006). Moreover, HAM has multiple benefits as a biomaterial that is not shared by any other material either natural or synthetic as shown in Table 2.5.

Table 2.5: Properties of Human Amniotic Membrane. Adapted from (Faulk et al., 1980; Stoddart, 2008).

Inexpensive	Easily Obtained	Wound Protecting	Reduce Pain
Anti-Inflammatory	Anti-Microbial	Anti-Fibroblastic	Anti Angiogenic
Reduce Scarring	Promote Healing	Anti-Tumorigenic	Light to no immunogenicity
Bacteriostatic	Protease Inhibiting	Anti Phlogestic	Anti-Apoptotic

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Studies have reported that this biomaterial is rich in cytokines further proving the study done by others for its wound protecting and scar reducing ability (Hao, Ma, Hwang, Kim, & Zhang, 2000; Wolbank et al., 2009). Predominantly, HAM constitutes of hyaluronan which is responsible for retaining fluid (Mamede et al., 2012; Meinert et al., 2001). HA chemical structure is as illustrated in Figure 2.8.

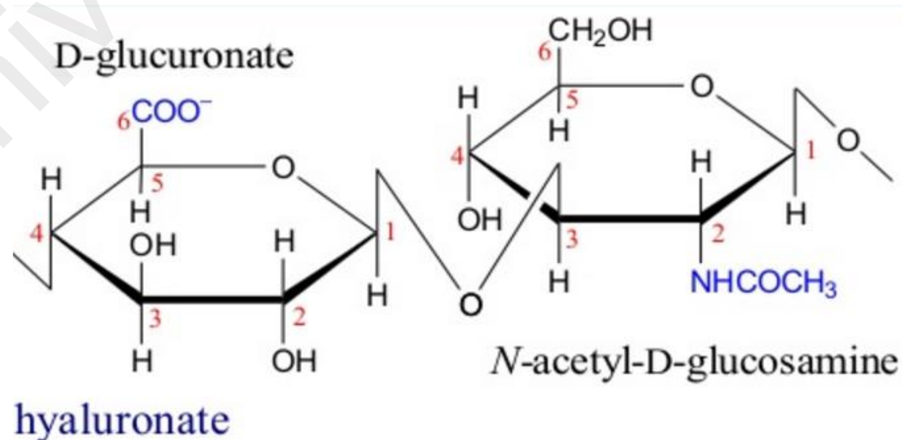

Figure 2.8: Structure of Hyaluronic Acid. Adapted from (Necas J BL, 2008).

The nutrients and by-products are being transported via diffusion in HAM (Toda, Okabe, Yoshida, & Nikaido, 2007). The proteoglycans; rich in heparin sulphate, actin, vimentin and laminin which furnish for cell survival, shape and maintenance of tissue phenotype are also in abundance in this biomaterial making it a potential for tissue engineering application (Wolbank et al., 2009).

Another important factor that HAM possesses is the resistance to proteolytic factors making it better at withstanding the protein breakdown. It is known that HAM expresses genes that are related to cartilage such as SOX and BMP and its receptors. HAM mainly consists of HA which is primarily D-glucoronate and N-acetyl-D-glucosamine that is capable of yielding large amount of fluid. It also plays a crucial role since it is a ligand for Cluster of Differentiation 44 (CD44) which is an antigen expressed by inflammatory cells (Wolbank et al., 2009).

HAM is suitable for allo transplantation due to its anti-inflammatory, anti-bacterial, anti-viral and low immunogenicity properties. A study has also reported that HAM reduces inflammatory mediators by entrapping the T lymphocytes. Moreover, it has also been reported that HAM secretes factors that inhibits both innate and immune cells making it the anti-bacterial and anti-bacterial features (Bonci, Bonci, & Lia, 2005; Bujang-Safawi et al., 2010; Díaz-Prado et al., 2010; Hao et al., 2000; Higa et al., 2007; C. Z. Jin et al., 2007; J. Kim, Kim, Na, Jeong, & Song, 2000; W. Li et al., 2006; Mamede et al., 2012; Meinert et al., 2001). This biomaterial also produces anti-inflammatory factors such as IL-1, IL-2 and IL-10 and endostatin that inhibits angiogenesis and tumor growth (Hao et al., 2000).

2.8.2 Current Applications of Human Amniotic Membrane

A summary of studies that looked into the effects of HAM as a biomaterial is as summarized as in Table 2.6.

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Table 2.6: Summary of Human Amniotic Membrane related research.

No.	Human Amniotic Membrane-related research	Findings	Reference
1.	Properties of Human Amniotic Membrane for potential use in tissue engineering. Human amniotic membrane has biological properties important for tissue engineering applications including anti-inflammatory, anti-scarring, anti-microbial, anti-fibrosis with low immunogenicity and reasonable mechanical property.	This study also found that the ECM component of human amniotic membrane creates an almost similar to that of the native scaffold for cell seeding in tissue engineering.	(Niknejad et al., 2008)
2.	Amniotic membrane extracts solution for ocular chemical burn.	Topical application of amniotic membrane extract is effective in reducing inflammation, promoting reepithelization in the treatment of ocular chemical burns especially in for mild to moderate acute cases.	(Liang et al., 2009)

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Table 2.6, continued.

No.	Human Amniotic Membrane-related research	Findings	Reference
3.	Topical management of facial burns.	It was found that healing time and amount of reapplication of topical amnion dressing was significantly shorter in patients with partial thickness burns of the face, head and neck and a TBSA burn size under 40%. It showed efficacy and safety of amnion in burn wound healing in an exclusively paediatric population.	(Leon-Villapalos, Jeschke, & Herndon, 2008)
4.	Human amniotic membrane as a deliver matrix for articular cartilage repair.	It was found that denuded human amniotic membrane could be one of the ideal cell carrier matrices for cartilage regeneration.	(C. Jin et al., 2007)

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Table 2.6, continued.

No.	Human Amniotic Membrane-related research	Findings	Reference
5.	Autologous amnion graft for repair of myelomeningocele: technical note and clinical implications.	A novel repair technique using autologous amnion graft and successfully treated myelomeningocele. The postoperative healing process of the wound was excellent. The amniotic tissue has no risks of rejection, foreign body reaction, or transmission of slow virus infection to reconstruct the lesion of newborn patient. The amnion autograft promote wound healing, being applied as a part of a variety of paediatric neurosurgical procedure.	(Hasegawa, Fujisawa, Hayashi, & Yamashita, 2004)

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Table 2.6, continued.

No.	Human Amniotic Membrane-related research	Findings	Reference
6.	Interleukin-1 receptor antagonist (IL-1RA) prevents apoptosis in ex vivo expansion of human limbal epithelial cells cultivated on human amniotic membrane.	This study found that human amniotic membrane may prevent apoptosis in ex vivo expanded human epithelial cultivated on amniotic membrane.	(Sun et al., 2006)
7.	Sterilized, freeze-dried amniotic membrane: A useful substrate for ocular surface reconstruction.	The sterilized and freeze-dried amniotic membrane retained most of the physical, biological and morphological characteristics of cryopreserved amniotic membrane and deemed useful as a biomaterial in ocular surface reconstruction.	(Nakamura et al., 2004)
8.	Proteoglycans and Hyaluronan in human fetal membrane.	The study found that the fetal membrane contains high concentration of collagen, proteoglycans and Hyaluronan.	(Meinert et al., 2001)

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2.9 Fibrin

2.9.1 Introduction

Fibrin is an organic polymer that has been studied for its many functional properties in the medical field. Fibrin is a natural polymer scaffold that has been thoroughly researched (Bensaïd et al., 2003; Bhardwaj et al., 2011; Dainiak et al., 2010; des Rieux, Shikanov, & Shea, 2009; Eyrich et al., 2007; Hall, 2010; Ho, Cool, Hui, & Hutmacher, 2010). It is the first known biomaterial to mankind, naturally occurring when there is an injury in the body, forming biodegradable matrix within the human blood at the site of contact. Its long list of use as a biomaterial, pose a great advantage as it attaches to most biological surfaces with no adhesion problem. Fibrin can be autologously-derived therefore it is highly reproducible. It is well known that it helps in wound healing and the fibrin clot characteristics can be altered significantly by adjusting and manipulating the concentrations of its components. The degradation rate of fibrin can be adjusted and controlled with the help of fibrinolysis inhibitor (Plug & Meijers, 2016). The polymerization can be significantly altered by its components to the desired physical structure either of thin or thick fiber (Akpalo & Larreta-Garde, 2010; Shaikh et al., 2008).

Fibrinogen is the precursor of fibrin matrix in every blood clot process. When blood clots, as shown in Figure 2.9, fibrin immediately self-aggregates to form a fibrillary gel, then it progressively crosslinks to form dense gel material. Polymerization of fibrinogen and thrombin as illustrated in Figure 2.10 with the aid of physiological enzyme, is rapid and convenient (can be injected directly into tissue spaces). Cells seeding can be done at the moment of fibrin fiber formation; therefore the cells are trapped inside the fiber meshwork as they would be in *in vivo* environment. This is a great advantage as the time needed for cell seeding or infiltration is zero and homogenous cell dispersion can be achieved (Eyrich et al., 2007; Harold A, 2004).

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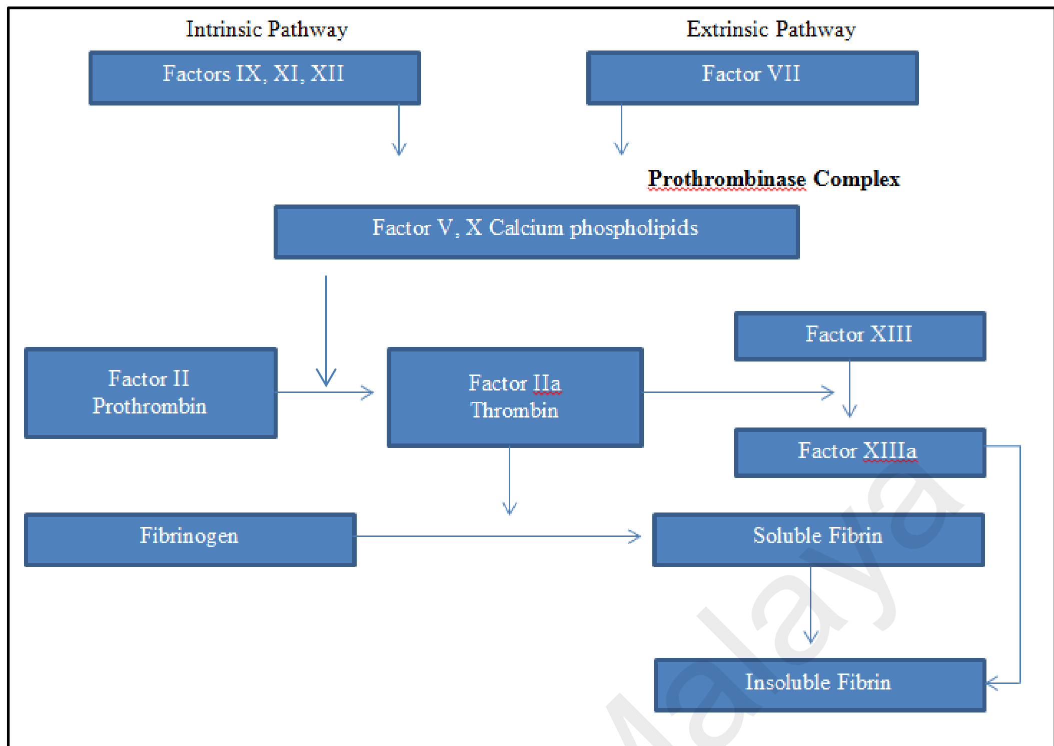

Figure 2.9: Simplified fibrin adhesion cascade. Adapted from (Blann, Landray, & Lip, 2002).

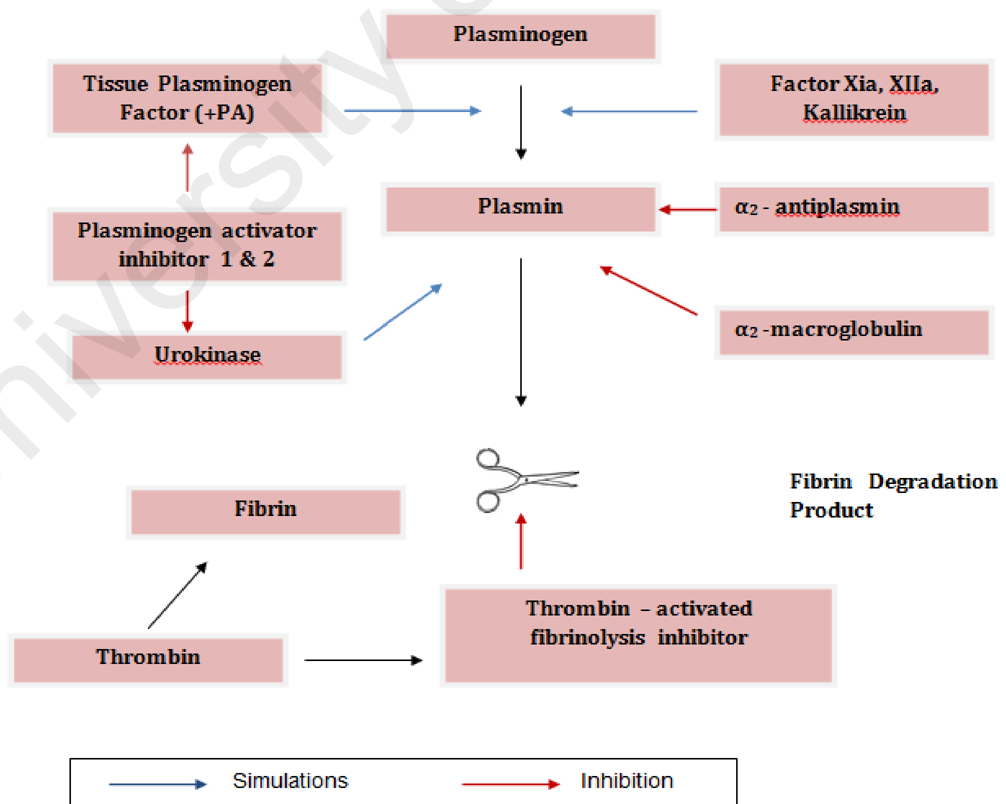

Figure 2.10: Signaling pathway of a fibrin clot. Adapted from (Blombäck & Bark, 2004; Harold A, 2004; Schwartz, Pizzo, Hill, & McKee, 1973).

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2.9.2 Current Applications of Fibrin in Tissue Engineering

Fibrin has long served as a biomaterial in numerous biomedical applications and is comparable to an ideal scaffold as shown in Table 2.7. Due to its vast availability and ease of procurement and manufacturing, it has been utilized in most scaffold research as tabulated in Table 2.8. Not only can it cater to patients own blood, it has amazing advantages among of which are major concerns with other biomaterials. Lacking in mechanical properties may be its major disadvantage but not all applications require such criteria. Currently, in this study, we are not focusing on the biomechanical aspect of this scaffold, as we are more concern on the feasibility of the scaffold that involves in supporting chondrocytes proliferation and ECM production.

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Table 2.7: Desirable features of an ideal scaffold comparable to fibrin gels scaffold.

Ideal Characteristics	Fibrin Characteristics	References
Biocompatible Non-Toxic Non-Allergic Non-Inflammatory	Fibrin-based biomaterials have high affinity to various biological surfaces and are already in clinical use	(Bensaïd et al., 2003; Wozniak, 2003; Ye et al., 2000)
Biodegradable Controlled and adjustable biodegradation allowing sufficient time for new tissue formation	Biodegradation can be easily controlled with the use of cross-linking of fibers or using inhibitors of fibrinolysis	(Mol et al., 2005; Wozniak, 2003)
Autologous Nature Non-immunogenic No foreign body rejection	Fibrin can be autologously harvested from patients' own blood, limiting immune and foreign body rejection	(Aper, Schmidt, Duchrow, & Bruch, 2007; Jockenhoevel et al., 2001; Ye et al., 2000)
Biological Nature	Fibrin is a provisional matrix in normal wound healing, its structural and biochemical properties make it a promising candidate as a scaffold in tissue engineering	(Rowe, Lee, & Stegemann, 2007)
Three-Dimensional Assisting cell growth Permitting easy diffusion of nutrition, gas transport and waste product	Offer excellent cellular growth and tissue development in 3D matrix structure	(Aper et al., 2007; Rowe et al., 2007; Ye et al., 2000)
Easily processable Manufactured in less time Reproducible	Can be easily produced from patients' own blood and are completely reproducible	(Aper et al., 2007; Rowe et al., 2007; Ye et al., 2000)
Economical Affordable to all	Being natural and autologous scaffold, the cost would be affordable to all	(Flanagan et al., 2007)
Variable in shape and size	Possible to construct fibrin scaffold to fill any desired shape and size Can also be used as an injectable scaffold	(Jockenhoevel et al., 2001)
Adjustable chemical, physical and mechanical properties	The compliance and polymerization rate of fibrin can be controlled by varying ionic strength and the concentration of fibrinogen and thrombin	(Eyrich et al., 2007; Kjaergard & Weis-Fogh, 1994; Rowe et al., 2007)

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Table 2.8: Summary of Fibrin scaffold related research.

No.	Fibrin scaffold-related research	Findings	Reference
1.	The effect of in vitro culture on a fibrin glue hydrogel embedding swine chondrocytes.	The chondrocytes survived in the fibrin glue gel and enhanced their synthetic activity.	(Scotti et al., 2010)
2.	The influence of fibrin based hydrogels on the chondrogenic differentiation of human bone marrow stromal cells.	The use of fibrin for cartilage repair facilitates Bone Marrow Stem Cells (BMSC) chondrogenesis and cartilaginous growth in an osteochondral environment.	(Ho et al., 2010)
3.	The effect of Gelatin-Fibrinogen cryogel derma matrices for wound repair.	*In vitro* studies demonstrates Gelatin-Fibrinogen as a good potential as matrices for wound healing.	(Dainiak et al., 2010)
4.	The study of Fibrin hydrogels for non-viral vector delivery in vitro.	The inclusion of non-viral vectors into fibrin-based hydrogels can induce transgene expression of encapsulated and infiltrating cells and may be employed with *in vitro* models for tissue growth to alter the intrinsic bioactivity of fibrin.	(des Rieux et al., 2009)

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Table 2.8, continued.

No.	Fibrin scaffold-related research	Findings	Reference
5.	Fibrin as a Delivery System for Therapeutic Drugs and Biomolecules.	In depth reviews of the advantageous biological aspects of fibrin, the history of the scaffold material, and its present role in the delivery of drugs, growth factors, cells, and gene vectors.	(Breen, O'Brien, & Pandit, 2009)
6.	The effect of chondrocytes implantation in Fibrin-Hyaluronan matrix.	The MRI findings showed good filling of the defect with tissue having the imaging appearance of cartilage in all patients. Level of Evidence: Level IV, Therapeutic study.	(Nehrer, Chiari, Domayer, Barkay, & Yayon, 2008)
7.	A fibrinogen-based microporous scaffold for cartilage tissue engineering.	The use of Acetone and genipin to improve the biomechanical strength of Fibrinogen based scaffold.	(Linnes, Ratner, & Giachelli, 2007)
8.	Resurfacing potential of heterologous chondrocytes suspended in Fibrin glue in large full-thickness defect of femoral articular cartilage.	Isolated heterologous chondrocytes can be used for transplantation in articular cartilage defects; however, fibrin glue does not offer enough biomechanical support to the cells to maintain its function as a three-dimensional scaffold as newly formed tissue was observed with subsequent replacement by fibrous tissue.	(Wood et al., 2006)

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Table 2.8, continued.

No.	Fibrin scaffold-related research	Findings	Reference
9.	The long term stable fibrin gel for cartilage engineering.	A coherent cartilaginous was obtained and was homogenously distributed throughout the construct suggesting stable fibrin gel are desirable for other tissue engineering applications.	(Eyrich et al., 2007)
10.	A study on the construction of a complete rabbit cornea substitute using fibrin-agarose scaffold.	All three types of corneal cells cultured, expanded and used in constructing a full thickness cornea substitute.	(Alaminos et al., 2006)

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2.10 Summary of Literature Review

Numerous studies related to HAM as mentioned in Table 2.6 have centered on the advantages of its many properties. Although a study has been done on denuded human amniotic membrane as a cell carrier for cartilage regeneration (C. Z. Jin et al., 2007), it has been well documented that monolayer culture of chondrocytes resulted in the loss of the chondrocytic phenotype and replaced by a complex collagen phenotype consisting predominately of type I collagen and reduction of proteoglycans production (Benya & Shaffer, 1982). However, there is yet to be a study to assess the feasibility of a 3D HAM scaffold for cartilage tissue engineering application. This study of HAM/ Fibrin scaffold incorporated the use of fibrin as a binding agent in order to assess the feasibility of 3D HAM/ Fibrin scaffold for cartilage tissue engineering application as this study progress in Chapter 3, 4 and 5.

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CHAPTER 3: METHODOLOGY

3.1 Introduction

In this chapter, the methods which were utilized in conducting the experiments are explained, starting off with the descriptions of medium and enzymes used, cell isolation method, procurement of amniotic membrane, processing of the amniotic membrane into powder form, fabrication process of HAM/ Fibrin scaffold, descriptions of biochemical assays, live/dead assay, scanning electron microscopy and histological evaluation. This chapter also explains the type of statistical analysis used to assess the results.

3.2 Culture Medium and Enzymes

In previous cartilage tissue engineering studies, there were a few different forms of chondrocyte culture medium preparation. The method in the present work was first optimized and then utilized in all of the experiments. Table 3.1 shows the detailed composition of chondrocyte culture medium. In this study, chondrocyte culture medium is known as Dulbecco's Modified Eagle Medium (DMEM) + 20% Fetal Bovine Serum (FBS).

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Table 3.1: Composition of chondrocyte culture medium.

Medium / Chemical	Quantity	Concentration	Catalogue No
Dulbecco's Modified Eagle Medium (D5901)	500 ml	Stock	D5921, Sigma Aldrich
Fetal Bovine Serum (FBS)	100 ml	Stock	F9665, Sigma Aldrich
Hepes-Buffer	10 ml	16 mM	H0887, Sigma Aldrich
Penicillin/ Streptomycin	10 ml	16 mM	P4333, Sigma Aldrich
ε-Aminocaproic Acid[1]	Stock	20 mM	A2504, Sigma Aldrich
L-Glutamax	5 ml	1.6 mM	35050-061, Invitrogen
L-Ascorbic Acid	0.075 g	0.068 mM	Duchefa Biochemie
ε-Aminocaproic Acid	Stock	5 mM	Sigma Aldrich

Enzymes used for pre-digestion of cartilage matrix are as shown in Table 3.2.

Table 3.2: Enzymes used in pre-digestion of cartilage matrix.

Enzyme	Quantity	Concentration	Catalogue No
Protease	10 ml	20 U/ml prepare in DMEM + 20% FBS	P8811, Sigma Aldrich
Collagenase Type II	30 ml	200 U/ml prepare in DMEM + 20% FBS	Worthington, USA

[1] ε-Aminocaproic Acid is supplemented solely for the HAM/Fibrin and fibrin scaffolds culturing medium.

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3.3 Chondrocyte Isolation

Bovine joints of a year old *Bhramal Cross* were obtained from a local abattoir and the cartilage was harvested from the metacarpal-phalangeal joint within the same day under aseptic conditions as in Figure 3.1 (a) and (b). The finely-minced cartilage was temporarily kept in DMEM + 20% FBS as in Figure 3.1 (c) before being subjected to enzymatic digestion. The process was then continued to the pre-digestion of the harvested cartilage with 20 U/ml Protease for an hour at 37°C and 5% CO_2 and subsequently in 200 U/ml collagenase type II for 16 hours as in Figure 3.1 (d).

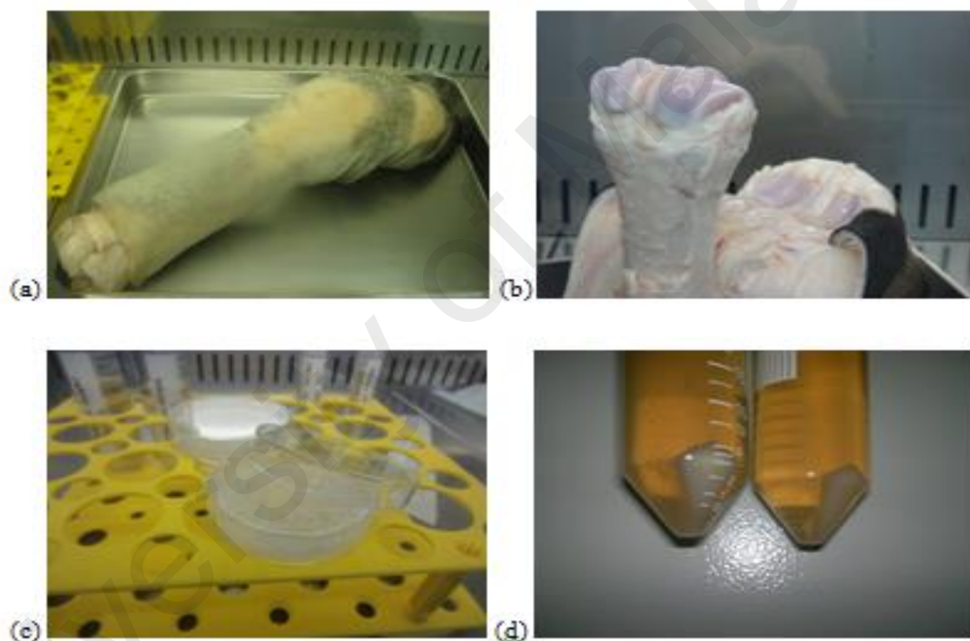

Figure 3.1: Bovine chondrocyte isolation method. (a) Metacarpal-phalangeal joint. (b) Exposed metacarpal-phalangeal joint. (c) Minced cartilage chips in DMEM + 20% FBS. (d) The resultant supernatant containing chondrocytes.

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3.3.1 Cell Count and Viability

For the cell count and cell viability, the method used was the standard Trypan blue dye exclusion assay with the aid of a haemocytometer. A sample of 20 µl of cell suspension from the isolated cells was mixed with an equal amount of prepared Trypan

blue solution at a ratio of 1:1 (v/v). A standard method to equate the number of cells in 1 ml cell suspension from the 20 µl that we tested is as Equation 3.1.

$$\textbf{\textit{Number of cell per ml}}$$
$$= \textit{Number of cell per } mm^3 * \textit{dilution factor} * 10^4$$

Equation 3.1: The Naubauer Ruling

3.4 Procurement of Human Amniotic Membrane

Human placentas were obtained immediately after birth in accordance with the tenets of the Declaration of Helsinki and with approval from the Ethical Committee, University Malaya (Reference No: 751.26) (Appendix B) from ten elective Caesarean-sectioned mothers who were tested seronegative for HIV, Hepatitis B and C and Syphilis. The placentas were processed under stringent sterile conditions. The HAM was peeled carefully from the chorion part of the placenta and was washed several times in Phosphate Buffered Saline (PBS) supplemented with 100 U/ml Penicillin and 100 µg/ml of Streptomycin to remove blood clots. Remaining blood clot and mucus was gently scraped off by using a Techno-Plastic-Products (TPP) cell scraper (Trans-Techno Enterprise). The HAM was then washed several times by gentle agitation on a shaker platform in sterile normal saline as shown in Figure 3.2 (a) – (e). The membranes were stored in a gradual increment of glycerol every day up to 85% from 20%. They were then stored in 85% glycerol at 4°C until further use (Ravishanker, Bath, & Roy, 2003).

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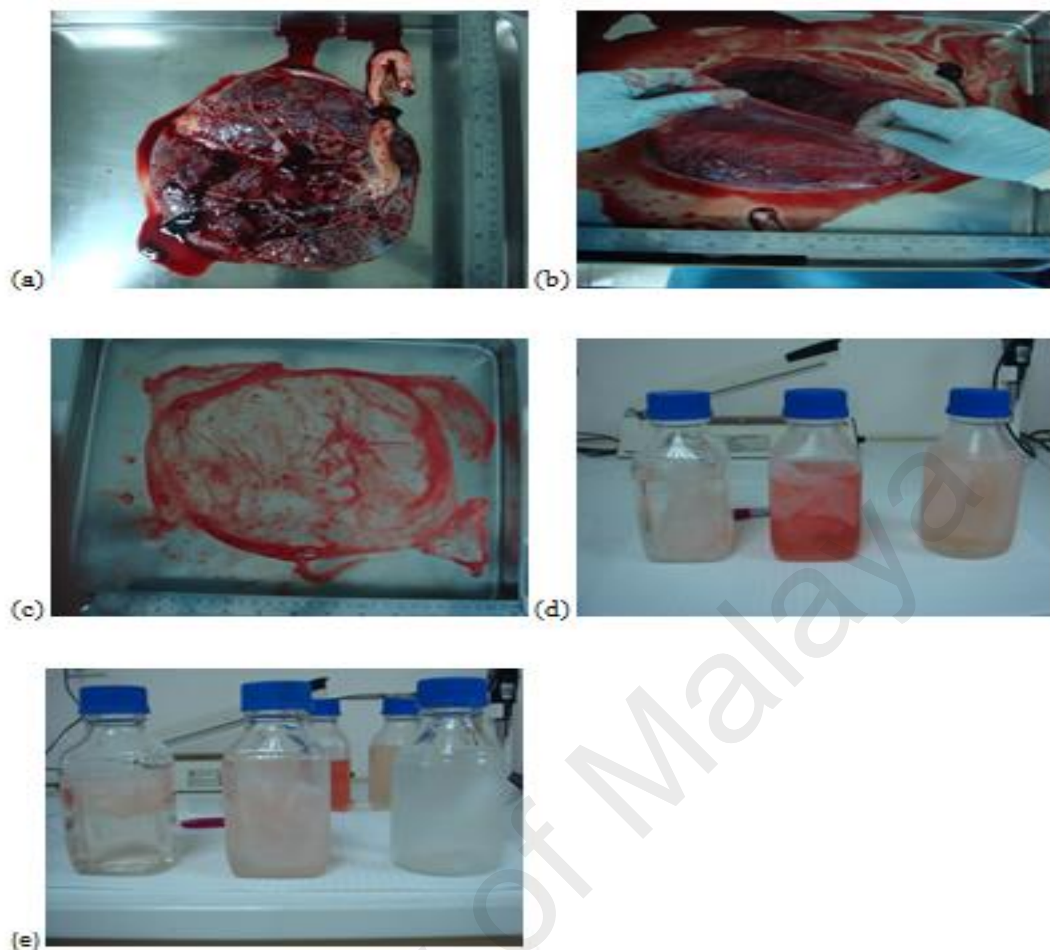

Figure 3.2: Procurement of HAM. (a) The procured placenta. (b) Peeling/ separation of HAM from the chorionic membrane. (c) Procured HAM. (d) Irrigation of HAM with PBS supplemented with Penicillin/ Streptomycin. (e) HAM in glycerol for storage.

3.5 HAM Extracts Production

Following meticulous irrigation, the HAMs were then cut into smaller pieces ~1 cm^2 each and then frozen in liquid nitrogen. These HAMs were then pulverized in the Biopulverizer (Bio Spec Products Inc.) before being homogenized in the Homogenizer (Bio Spec Product Inc.) in a homogenizing medium that consists of PBS and Protease Inhibitor cocktail (P8340 - Sigma Aldrich). The homogenized HAMs were then further frozen at -20°C, and then -80°C, before being lyophilized at -50°C at 85 Pa overnight. The resultant extracts were pre-weighed (0.5 g) and packed before they were sent for γ-sterilization (25 kGy) at the Malaysian Institute of Nuclear Technology, Bangi, Malaysia. Figure 3.3 summarizes the HAM extraction process.

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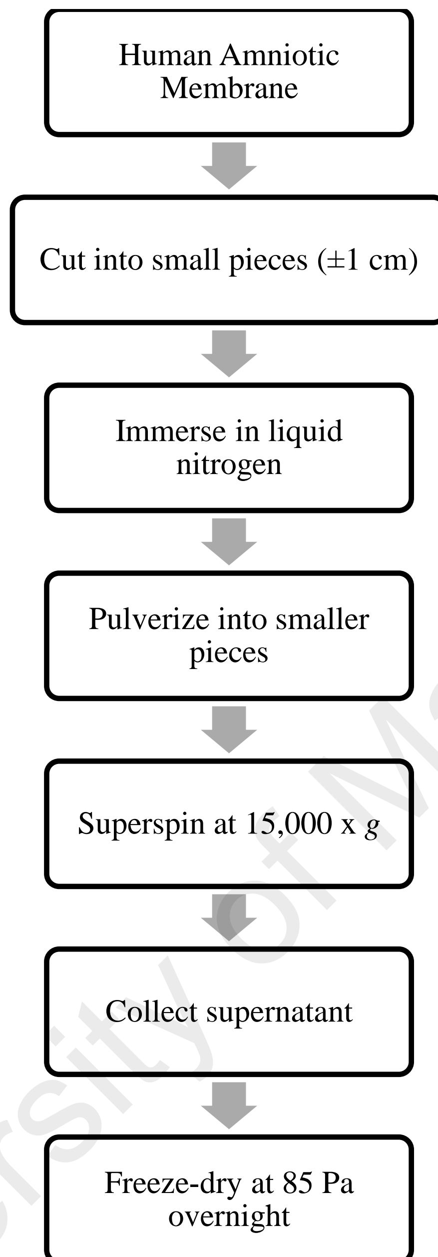

Figure 3.3: Workflow of HAM extracts production.

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3.6 Fabrication of HAM/ Fibrin scaffold

The HAM/ Fibrin scaffold has never been explored before by anyone, therefore several protocols have been developed in order to explore the optimum concentration for the HAM/ Fibrin scaffold. The protocol for HAM/ Fibrin scaffold optimization is as shown in Table 3.3.

Table 3.3: HAM/ Fibrin scaffold optimization protocol.

Cell number (millions/ ml)	HAM : Fibrin (v/v)	Setting Time (minute)
1	0.5:1	30
		45
		60
3	1:1	30
		45
		60
5	1:0.5	30
		45
		60

Following the cell count and cell viability tests, a final concentration of approximately 10 million cells per ml was prepared in suspension. These cell suspensions were mixed carefully with the aid of 21 G needles. Bovine fibrinogen of Type I-S (P8630, Sigma Aldrich) was dissolved in 0.9% NaCl at 37°C for 2 hours at 200 mg/ml. The prepared fibrinogen solution was mixed carefully with cells and HAM according to Table 3.3, and then it was mixed simultaneously with thrombin at a final concentration of 2.5 U/ml in 20 mM $CaCl_2$. After optimization, the HAM/ Fibrin scaffolds were prepared at a concentration of 1:1 (v/v) ratio. The scaffolds were then allowed to polymerize in a custom-made 316L stainless steel mould with an inner diameter of 5 mm and 5 mm thickness at 37°C and 5 % CO_2 as shown in Figure 3.4.

The preparation procedure resulted in an optimized HAM/ Fibrin scaffold with a final concentration of 50 mg/ml with a thrombin concentration of 2.5 U/ml, $CaCl_2$ at 20 mM, and ~5 million/ml chondrocytes. Fibrin scaffolds, as the experimental control, were fabricated from the same concentration but without any presence of HAM. The 3D scaffolds were transferred into 24-well plates (NUNC Brand, Thermo Fisher) with 1 ml of chondrocyte culture medium per well as prescribed earlier, but supplemented with 5 mM of ε-Aminocaproic Acid that was refreshed every other day.

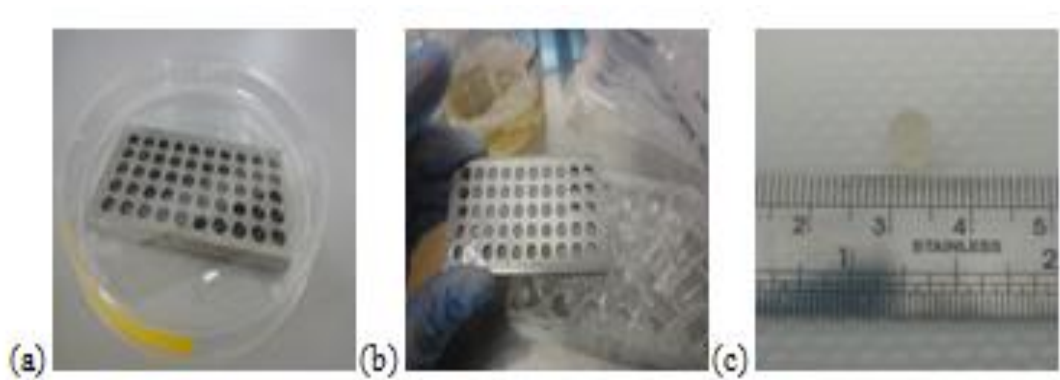

Figure 3.4: (a) 316L Stainless steel custom made mould. (b) Polymerization of scaffolds in the 316L stainless made mould. (c) HAM/ Fibrin scaffold.

3.7 Biochemical Assay

3.7.1 Digestion of HAM/ Fibrin Scaffold

Assessments of the scaffolds were done at 4 different time-points, beginning at day 7, followed by day 14, 21 and 28. The 3D construct was removed from the static culture and then were transferred to the Biosafety Cabinet for further characterization and analysis. The 3D construct had to undergo an enzymatic digestion prior to total GAG and DNA quantification assessment. The protocol used was adapted (C. D. Hoemann, 2004).

The 3D construct was cut into halves (transversely) and transferred to a microcentrifuge tube. Then 250 µl of digestion enzyme with a concentration of 2.5 U/ml of Papainase (Sigma Aldrich) in 20 ml of sterile Phosphate Buffer EDTA (PBE) supplemented with 63 mg of L-Cysteine Hydrochloride was added. The scaffolds were left in the oven overnight at 60°C. Next the digested scaffolds were then centrifuged at 10,000 x *g* for 5 minutes at room temperature before being assessed for DNA and GAG contents (C. D. Hoemann, 2004).

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3.7.2 Deoxyribonucleic Acid (DNA) Assay

DNA content was assessed immediately following overnight enzymatic digestion of scaffolds. This is due to depletion in DNA content over time and sample freezing (C. D. Hoemann, 2004). Post scaffolds digestion, the DNA contents were assessed by using the Hoechst 33258 assay. The Hoechst 33258 dye is DNA specific and exhibits fluorescence upon binding with bovine DNA. A DNA stock standard solution was made of Deoxyribonucleic Sodium Salt from Calf Thymus (Sigma Aldrich) with a concentration of 2 mg/ml, which was prepared (C. D. Hoemann, 2004). The DNA working solutions were prepared with TEN Buffer (10 mM Tris-HCl, 1 mM EDTA and 100 mM NaCl, pH 7.5) (Caroline D. Hoemann; Y. J. Kim, Sah, Doong, & Grodzinsky, 1988).

The DNA of the digested scaffolds was analyzed by further diluting the stock standard solution to the initial standard concentration of 12.5 μg /ml. Then serial double-dilutions were prepared with PBE until the final concentration of 0.391μg/ml achieved. In triplicate in the first 3 columns, 10 μl of each blank, standards and samples were subjected to 200 μl working solution with the aid of multichannel pipette in the 96 well (NUNC Brand) with a layout as indicated in Table 3.4. PBE buffer was used as blank. The DNA fluorescence reading was accessed immediately by using a microplate fluorometer (FLUOstar Optima, BMG Labtech) at 360 nm excitation and 460 nm emission wavelength (C. D. Hoemann, 2004).

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Table 3.4: Orientation of Blanks (B), Standards (S) and Samples (X) for DNA/ GAG analysis.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	B		X1	X1	X1		X8	X8	X8	
B	S1	S1	S1		X2	X2	X2		X9	X9	X9	
C	S2	S2	S2		X3	X3	X3		X10	X10	X10	
D	S3	S3	S3		X4	X4	X4		X11	X11	X11	
E	S4	S4	S4		X5	X5	X5		X12	X12	X12	
F	S5	S5	S5		X6	X6	X6		X13	X13	X13	
G	S6	S6	S6		X7	X7	X7		X14	X14	X14	

3.7.3 Glycosaminoglycans (GAG) Assay

The amount of GAG produced inside the scaffold matrix was assessed by using the Dimethyl-Methylene Blue (DMB) assay, a strong metachromatic dye for histochemical detection of sulphated GAGs. The digested scaffolds can be assessed immediately or up to a week from the day that it has been fully digested. The DMB assay was prepared prior to the GAG quantification. 16 mg of Dimethyl Methylene Blue (DMB) was dissolved in 5 ml of ethanol (Sigma Aldrich,) in a dry vial, kept under foil and stirred with a magnetic stirrer (WiseStir MSH10, Daihan Sci.) for 2–16 hours until fully dissolved (C. D. Hoemann, 2004).

In a separate Duran bottle, 2.37 g of NaCl and 3.04 g of Glycine were added to 900 ml double distilled water. Then, the dissolved DMB was added to the NaCl-Glycine solution. The remaining DMB was washed with 200 µl ethanol rinse. The pH of the final solution was adjusted by using 1N HCl to pH 3.0. The solution was brought to a final total volume of 1 liter with double distilled water. The final solution was then

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filtered before being stored in a foil-wrapped bottle at room temperature (C. D. Hoemann, 2004).

The GAG contents from the digested samples were quantified by comparing the fluorescence values from the Chondroitin-6-sulphate standard. The Chondroitin-6-sulphate was mixed at a concentration of 1 mg/ml in a 50 ml conical tube with distilled water. In order to generate a series of standard curves, the initial standard concentration of 200 µg/ml was set, followed by serial dilution until the final concentration of 6.25 µg/ml. The serial dilution was achieved by diluting the standard stock solution of 1 mg/ml with PBE. Triplicate 10 µl samples of each standard solution, blanks and samples were subjected to 250 µl of DMB solution in accordance to the layout as mentioned in Table 3.3 in a 96 well plate (NUNC Brand). dH_2O and DMB were used as blank. The Fluorometer microplate reader (FLUOStar Optima, BMG Labtech) was used to read the absorbance level. The microplate fluorometer reader was set to read absorbance between 520 and 590 nm wavelength (Caroline D. Hoemann).

3.8 Live/ Dead Assay

The 3D scaffolds were pre-washed with PBS before being cut sagittally in halves and incubated at room temperature for about 15 - 45 minutes in 1 ml of DMEM (Sigma Aldrich) supplemented with 4 µM of Calcein-AM (17783, Sigma Aldrich, Malaysia) and 1 µM of Ethidium Homodimer (46043, Sigma Aldrich) before proceeding with the scanning (Benjamin Gantenbein-Ritter, 2008). The scaffolds were then scanned from their top to bottom surface at ~50 µm depth at random locations with a confocal laser microscope (Leica TCS SP5 II) with a magnification of 500X.

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3.9 Histological Staining

The samples were also subjected to histological staining to view the microstructure of the scaffolds. The samples were fixed in neutral buffered saline for 24 hours prior to processing. After that, the samples were subjected to a series of dehydration and rehydration processes, in order to further fix the cells in the scaffold and avoid the possibilities of the cells from being washed off during the staining processes. The processed samples were then embedded in paraffin. The embedded samples were sectioned thinly (~5 μm) with a microtome. Prior to the histological staining of the sectioned samples, the Safranin-O stains were prepared as according to Table 3.5. The sectioned samples were then pretreated with serial alcohol dehydration and rehydration followed by a staining process as detailed in Table 3.6.

Table 3.5: Safranin-O staining preparation. Adapted from (Schmitz, Laverty, Kraus, & Aigner, 2010).

Reagent	Composition
Wiegert's Hematoxylin solutions	Solution A: 1% Hematoxylin in 95% Alcohol
	Solution B: 30% Ferric chloride (anhydrous) – 4 ml
	Concentrated HCl – 1 ml Distilled water – 95 ml
	Add equal parts of A + B mix and use immediately
0.001% Fast green (FCF) solution	Fast green : 0.01 g Distilled water: 1000 ml
1% Acetic Acid	Acetic acid glacial: 1 ml Distilled water: 99 ml
0.1% Safranin-O Solution	Safranin-O: 0.01 g Distilled water: 100 ml

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Table 3.6: Safranin-O staining protocol. Adapted from (Schmitz et al., 2010; Tran et al., 2000).

Reagent / Chemical	Time (minutes)
Xylene	4
Xylene	4
Xylene	4
96% Ethanol	1
96% Ethanol	1
70% Ethanol	1
Tap water rinse	1
Weigert’s working solution	10
Running tap water	10
Fast green (FCF) solution	5
1% acetic acid solution	10-15 seconds
0.1% Safranin-O solutions	5
100% Ethanol	5
100% Ethanol	5
100% Ethanol	5
Xylene	5

After the staining process, the samples were held onto the slides by covering them with a coverslip, with the aid of DPX mountant (Sigma-Aldrich), before viewing them with a standard light microscope.

3.10 Field Emission Scanning Electron Microscopy (FESEM)

Both the structure and morphology of the scaffolds were observed using FESEM. The samples were processed prior to FESEM viewing as tabulated in Table 3.7.

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Table 3.7: FESEM sample preparation protocol.

Chemicals / Reagents	Time (minutes)
Glutaraldehyde	15
Osmium Tetroxide (2%)	15
Dilute with H_2O	15
10% alcohol	15
20% alcohol	15
30% alcohol	15
40% alcohol	15
50% alcohol	15
60% alcohol	15
70% alcohol	15
80% alcohol	15
90% alcohol	15
100% alcohol	15
100% alcohol	15
3 parts ethanol: 1 part acetone	15
1 part ethanol: 1 part acetone	15
1 part ethanol : 3 part acetone	15
Absolute acetone (3X's)	20

These processes were followed by a process called Critical Point Drying (CPD) where the samples were mounted on a stub and were held under vacuum conditions in a CPD machine (Biorad) to eliminate any traces of water before being coated with gold in the gold sputter machine (Polaron). Finally, samples were viewed with the FESEM machine (Qanta FEG 450) with a magnification of 500X.

3.11 Statistical Analysis

The results are expressed as mean ± standard error. A two-sample t-test was used to determine the significance of the number of day's culture between the two scaffolds. Statistical significance was assessed by ANOVA and the Scheffe post hoc test at a level of $p < 0.05$ using SPSS 20.0 software to determine the cell proliferation of the two scaffolds for different days.

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3.12 Summary of Research Methodology

Throughout this chapter, the study revolves around numerous methods that have been simplified as depicted in Figure 3.5.

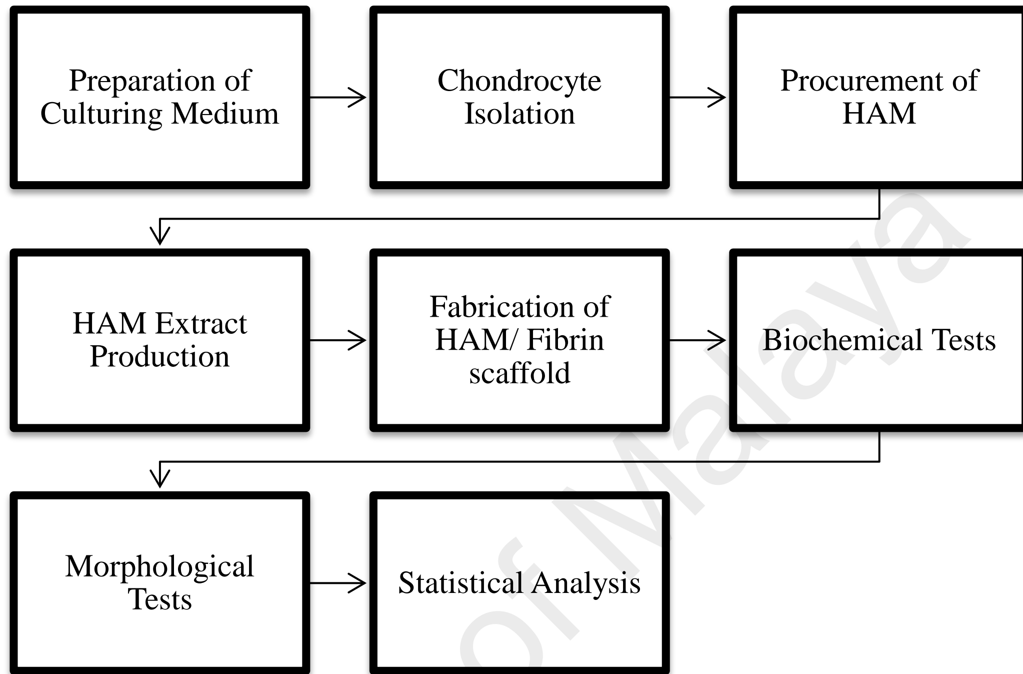

Figure 3.5: Summary of Research Methodology

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CHAPTER 4: STUDY 1 - HAM/ FIBRIN CONSTRUCTS FABRICATION & OPTIMIZATION

4.1 Introduction

Due to the limited capacity of self-repair, minor injury to the cartilage often progress further, damaging the surrounding healthy tissues and leads to degeneration. The lack of effective treatments to address this matter leads to new approaches in articular cartilage repair. Engineering functional cartilage tissue involves the combination of cells, scaffold and bioactive molecules. The choice of scaffold used is crucial in determining the outcome of the engineered cartilage. Numerous studies have been done on different types of materials to access the feasibility for various tissue engineering applications.

In our current study, HAM has been chosen due to its many credentials. Other than being widely available at 4.2 births per seconds worldwide, HAM is usually discarded following childbirth hence the ease of availability. Furthermore, HAM was not only feasible due to its tremendous list of advantages as a biomaterial in tissue engineering applications (Branski et al., 2008; Bujang-Safawi et al., 2010; He et al., 2009; Oxlund, Helmig, Halaburt, & Uldbjerg, 1990; Ravishanker et al., 2003; Rodríguez-Ares et al., 2009; Sun et al., 2006) , it also comprises of similar constituents of that in native cartilage which made it more appealing in cartilage tissue engineering.

The HAM is a 2D film like sheet that has been used in many research fields as scaffold mainly in plastic surgery and ophthalmology. The fact that chondrocytes tend to change its phenotype in 2D architecture has tempted this research to change the 2D environment of HAM to cater chondrocytes to this amazing biomaterial without compromising its phenotype (Benya & Shaffer, 1982).

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In this chapter, we were evaluating the optimized concentration of HAM/ Fibrin constructs in order to proceed with other evaluations. One of the main aspects that revolved around Tissue Engineering is the 3D scaffolds. The main issues concerning this scaffold are its biocompatibility, biodegradability and cytotoxicity. The 3D scaffolds act as a matrix that sends signaling cues to the surrounding cells on how to behave and organize. It is important that these scaffolds meet certain criteria while at the same time, optimized with the right amount of cells in order for it to play its part *in vitro* before we could proceed in evaluating them *in vivo*.

Currently, there are no studies being done on the effect of turning 2D HAM into 3D forms, let alone optimizing it. We are looking into studying the 3D form of HAM incorporated with fibrin and optimizing it in return of studying the effects of these constructs on cartilage tissue engineering applications.

Optimization of this newly developed construct involves both the material and the isolated cells simultaneously. This will be elaborated in details of the parameters used. The optimized concentration of HAM/ Fibrin construct is used in further studies involving the feasibility of the construct in maintaining and supporting chondrocytes proliferation and maintenance of matrix production. Incorporation fibrin in this scaffold is as an integral part to HAM in order to hold the two materials together, thus forming what we called the HAM/ Fibrin scaffold.

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4.2 Methods

Primary cells from adult bovine joints as explained in Chapter 3 were obtained and used in optimizing the HAM/ Fibrin constructs.

4.2.1 Different concentrations of HAM to Fibrin ratio

After successful cell digestion, cell count and cell viability check (~90% alive), the chondrocytes were suspended in 10 ml of culture medium. The different concentration of HAM and fibrin were prepared as mentioned in Table 3.3. First, the pre-weighted sterilized HAM was mixed with fibrinogen in a cell suspension in accordance to Table 3.3. Then, the HAM-cell suspension was injected simultaneously with thrombin supplemented with $CaCl_2$ into a custom-made 316L stainless steel mould using a multi-channel pipette as previously described in section 3.6.

4.2.2 Incubation Time

The duration of incubation time to set the HAM/ Fibrin scaffold was evaluated. The incubation time was at 30, 45 and 60 mins in 5% CO_2 and 37°C.

4.2.3 Cell Concentration

The amount of cells used in the optimization of HAM/ Fibrin scaffold was also evaluated. The numbers of cells were as stated in Table 3.3.

4.3 Results

All of the scaffolds had cells incorporated into them as part of the process, as direct injection moulding method is being used instead of the surface seeding method. As a result, we can directly monitor the physical appearance of the scaffold as shown in Table 4.1.

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Table 4.1: Result of optimization of HAM/ Fibrin scaffold.

Cell number (millions/ ml)	HAM : Fibrin (v/v)	Setting Time (minute)	Observations
1	0.5:1	30	Turbid, fail to polymerized
3		45	Turbid, fail to polymerized
5		60	Turbid, fail to polymerized
1	1:1	30	Clear & complete polymerization
3		45	Clear & complete polymerization
5		60	Clear & complete polymerization
1	1:0.5	30	Turbid, polymerization occurs on half outer diameter
3		45	Turbid, polymerization occurs on half outer diameter
5		60	Turbid, polymerization occurs on half outer diameter

The optimization protocol resulted in an optimized HAM/ Fibrin scaffold with a final concentration of 50 mg/ml (1:1 HAM to fibrin ratio) with a thrombin concentration of 2.5 U/ml, $CaCl_2$ at 20 mM, and ~5 million/ml chondrocytes set within 30 minutes time.

4.4 Discussion

A tissue engineering scaffold has the main function to provide adequate cellular attachment and adhesion. It should allow cell migration, proliferation, nutrient and waste exchange whilst providing space for tissue development. The stability of the shape is also an important factor whereby it is necessary for the scaffold to provide sufficient time for cells to produce their specific matrix while the scaffold slowly degrades as the occupying cells replace the scaffold.

Optimizations of the constructs are to assess the best concentration of HAM to fibrin ratio. This procedure is vital before we proceed with other assessments to ensure that the constructs are at its optimum condition; cell to scaffold ratio, attachment and its physical appearance.

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Cross-linking HAM with fibrin as stated in Table 4.1 has varied in results. However, there are similarities to all the three different cell number in the same 1:1 (v/v) HAM to fibrin ratio. In all of these three conditions; 1, 3 and 5 million in a final concentration of cell number in the construct, it appears to show complete polymerization with no change in size and shape after being released from the custom-made mould. The constructs appear to be clear with the shape according to the mold (D: 5 mm, H: 5 mm). As for the other two HAM to fibrin concentration; the results showed negative outcome as one fails to achieve complete polymerization [0.5:1(v/v)] and other showed partial polymerization [1:0.5 (v/v)] after suggested incubation time and after being released from the mould.

Variation of the fibrin gel appearance has been mentioned in many studies (Kjaergard & Weis-Fogh, 1994; Sidelmann, Gram, Jespersen, & Kluft, 2000). The gels with a concentration of 0.5: 1 of Ham to fibrin ratio (v/v) appeared to be turbid and did not polymerize completely and almost completely dissolved when being released from the mould following sufficient incubation time. This result was reported as the constructs has large fiber diameter and large pore size in the fibrin clot formation or the construct formation in this case.

In contrary, the gels with a concentration of 1:0.5 of HAM to fibrin ratio also appear to be turbid and had partial polymerization at the outer diameter of the constructs. Releasing the constructs from the mould resulted in partial gelation of the construct with some contraction as the middle whereby it appears to be hollow and this cannot be accepted to proceed as an optimum concentration of HAM to fibrin ratio for further investigation.

It is known that the variation of fibrinogen concentration may affect the gel appearance and stability (Kjaergard & Weis-Fogh, 1994; Sidelmann et al., 2000). At a

low concentration of fibrinogen, the fibrin may appear to be turbid which attributed to the large fiber diameter within the fiber mesh network. And in contrary, it has also been reported that increased fibrinogen concentration resulted in finer and thinner fiber network thus more rigid and appear clear and less turbid. In our case, however, the increased of fibrinogen concentration has no effect on the gel appearance as the incorporation of HAM should somehow play a role in the lagging the polymerization of the fibrin.

From this study, we found the best way to optimize HAM to fibrin concentration in order to proceed with other tests to determine the feasibility of this new cell-scaffold construct for cartilage tissue engineering application. It is evident that the best concentration is at 1:1 (v/v) ratio of HAM to fibrin concentration with 30 minutes incubation time. The highest amount of cells was chosen as reports of higher viable cells seeded supports matrix production (Provin, Takano, Sakai, Fujii, & Shirakashi, 2008; Solchaga et al., 2006). As stated earlier, the optimized concentration will be the basis of the HAM/ Fibrin construct for all of the tests and investigations.

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CHAPTER 5: STUDY 2 - FEASIBILITY OF THE HAM/ FIBRINCONSTRUCTS IN CARTILAGE TISSUE ENGINEERING APPLICATION ON CELLULARITY AND EXTRACELLULAR MATRIX PRODUCTION

5.1 Introduction

In order to evaluate the scaffold properties, the feasibility of the scaffold for chondrocytes growth and production of ECM, an *in vitro* study was conducted. We managed to fabricate and optimized the HAM/ Fibrin scaffold in the first part of the study. This chapter is a continuation of the previous chapter explaining the biochemical and histological evaluation of the optimized HAM/ Fibrin construct covering the aspect of evaluating the rate of ECM production, cell proliferation and looking into the cells morphology and histological evaluation. This chapter will generally describe the experimental study that has been done to access the feasibility of this newly fabricated construct with the influence of chondrocytes. As previously described, this study will not include nor discuss the biomechanical properties of the scaffold.

5.2 DNA Production

Upon constructing the HAM/ Fibrin scaffold seeded with chondrocytes, we managed to evaluate the total DNA content of the constructs within a period of 4 weeks according to the protocol mentioned in section 3.8. The constructs were subjected under physiological condition and were under static culture. Throughout the culture, the scaffolds seeded with chondrocytes were maintained at 37°C and in a 5% controlled humidity at all times.

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5.2.1 Result

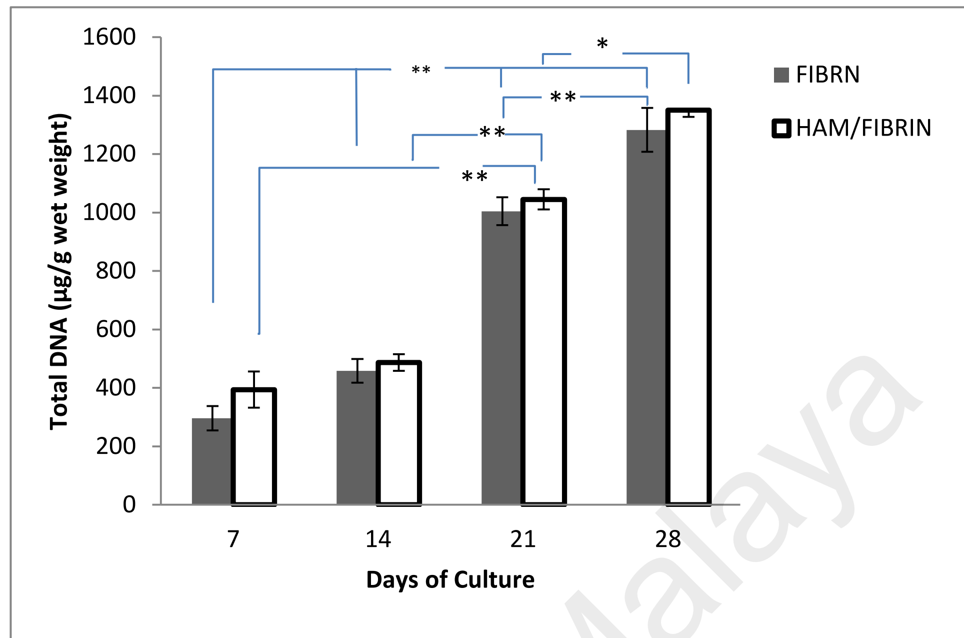

Figure 5.1: Total DNA over 4 weeks of culturing period. The data shown are as mean±standard error mean (SEM), where n=12, **represents *p*<0.01, and *represents *p*<0.05.

The influence of chondrocytes within the HAM/ Fibrin scaffold on the DNA content is presented as in Figure 5.1. Following protocol under section 3.8, specimens were immediately removed and undergone Papainase digestion and were subjected to DNA measurement. The DNA content within the 4 weeks culturing period increased substantially from week 1 to week 4. Although there was no significant difference over control in each week, there was a slight difference in HAM/ Fibrin DNA content throughout the entire experiment.

5.3 GAG Production

Under the 3D influences, chondrocytes-seeded scaffolds resulted in GAG synthesis and retention within the ECM. Another study that was also performed to evaluate the feasibility of the scaffold in *in vitro* setting was the GAG production. The constructs were subjected to the processed as described in section 3.8. Throughout the culture

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period, the scaffolds seeded with chondrocytes were also maintained at 37°C and 5% controlled humidity at all times.

5.3.1 Result

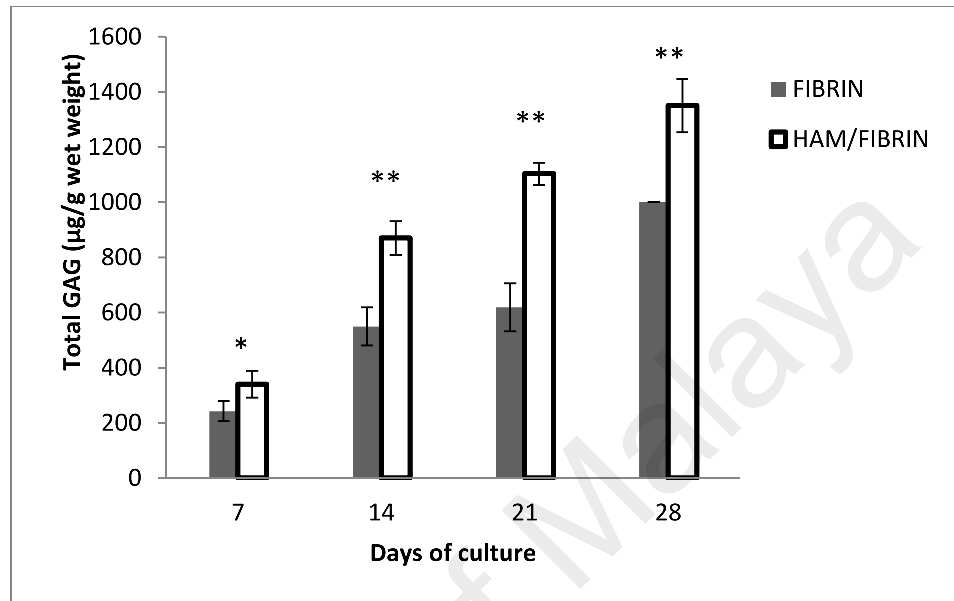

Figure 5.2: Evaluation of GAG content production. Data represent mean and standard of mean, where n=12, ** represents *p*<0.01, and * represents *p*<0.05 when compared between the 2 constructs across 4 different days of culture.

The influence of chondrocytes within the HAM/ Fibrin scaffold on the GAG content is presented as in Figure 5.2. Upon timely manner, the constructs were subjected to Papainase digestion following protocol as described in section 3.8 for GAG measurement. The total GAG content increased significantly as compared to control between weeks. Although initially, at the beginning of the study, the GAG content was $p<0.05$ as compared to control it increased significantly in the following week with the significance of $p<0.01$ and were 3 folds higher in HAM/ Fibrin constructs as compared to initial week 1. At the end of week 4, GAG content in HAM/ Fibrin constructs were 30% higher than control.

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5.4 Confocal Microscopy

Assessing the viability of cells inside 3D scaffolds is an important parameter in tissue engineering study. It is used to evaluate the survival of the cell in *in vitro* culture. In this study, the cell viability was performed on the construct with the aid of a confocal microscope with the Live/Dead assay. The principle behind the Live/Dead assay is the dimeric-dyes used; Calcein-AM and Ethidium Homodimer. Ca-AM is enzymatically hydrolyzed into Calcein in living cells, turning it green upon contact. Meanwhile, Ethidium Homodimer will only be able to stain the nuclei red by entering compromised cell membranes. The scaffold seeded cells were subjected to a dimeric-dye as described in section 3.8. Throughout the culture period, the scaffolds seeded with chondrocytes were maintained at 37°C and 5% controlled humidity at all times and were subjected to the Live/Dead assay on day 7 and 28 of culturing period.

5.4.1 Result

At the end of week 1 of culture, both HAM/ Fibrin scaffold as shown in Figure 5.3 (a) and control in Figure 5.3 (c) were subjected to the Live/ Dead assay in order to evaluate the cellular viability. It is notable that both groups displayed homogenous cell dispersion throughout the scaffold. The initial cell dispersion appears to be sparse due to the initial cell seeding of approximately five million cells. Both groups displays high cell survival with no red staining to indicate cell death. At the end of the culturing period, at week 4, both groups were again subjected to the same protocol and results are as shown in Figure 5.3 (b) and (d). HAM/ Fibrin scaffold displayed to have higher cell survival as compared to the control group. Fibrin scaffold has been well recognized to support cell survival *in vitro* and the macroscopic observation clearly supports this (Hunter, Mouw, & Levenston, 2004; Munirah, Samsudin, Aminuddin, & Ruszymah, 2010).

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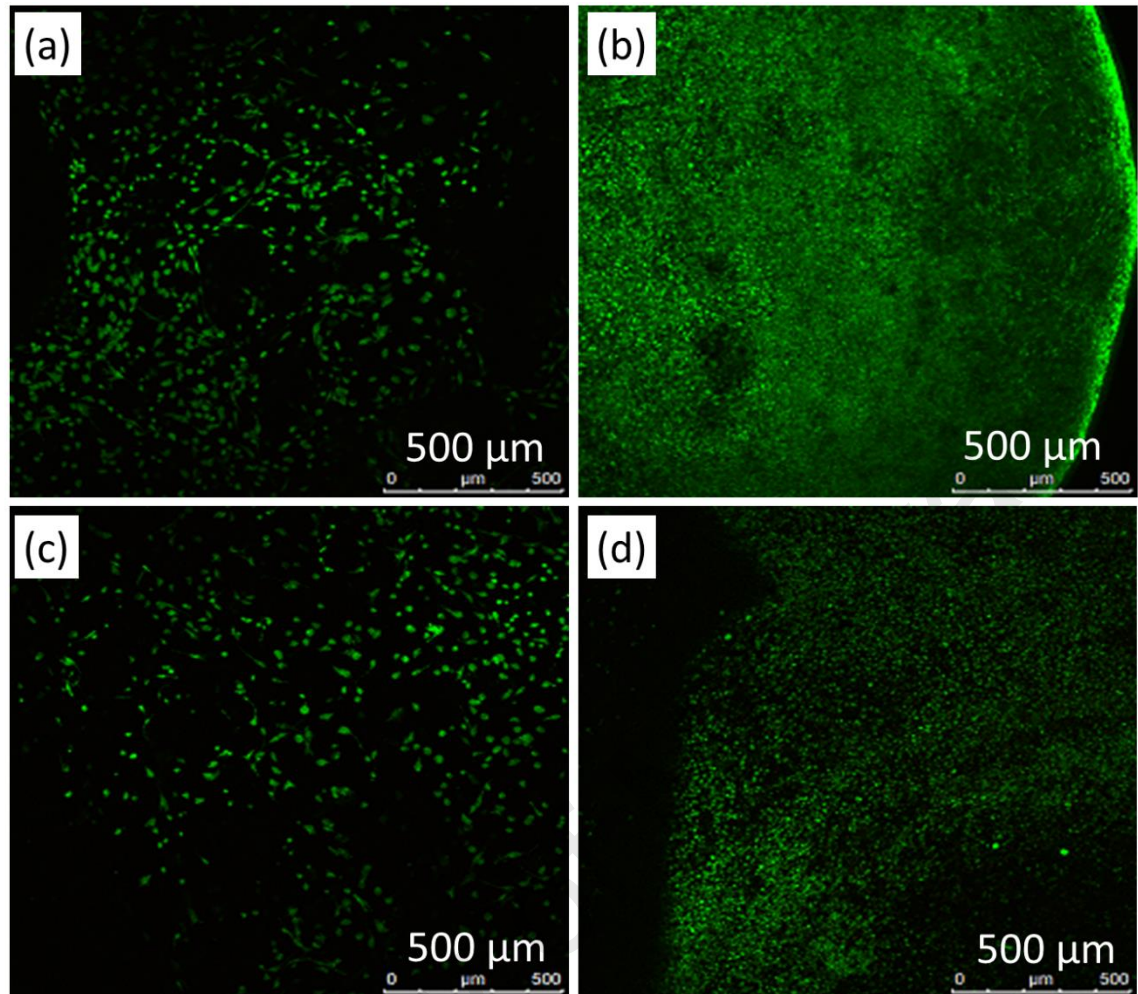

Figure 5.3: The morphology and viability of the HAM/ Fibrin construct cultured for (a) 7 and (b) 28 days. The morphology and viability of the fibrin construct cultured for (c) 7 and (d) 28 days.

Although both groups were negative of Ethidium Homodimer stain identifying the dead cells, HAM/ Fibrin scaffold appears to be fully occupied by the chondrocytes as evidently the scaffold is densely stained with the green dye of Calcein-AM as shown in Figure 5.3 (b). The results concur to be relatively comparable to the previous findings in GAG and DNA content productions presented in section 5.2.1 and 5.3.1.

5.5 FESEM

FESEM is an excellent method to determine the biocompatibility of materials in Tissue Engineering applications. It has been widely utilized in assessing the surface morphology and the architecture of 3D scaffolds. By using FESEM micrograph, observations of the interface between cells and the materials, the homogeneity of the

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surface layer and the cellular attachments and adhesion behavior can be done. Both groups of the scaffold was subjected to FESEM processing as mentioned in section 3.10.

5.5.1 Result

The FESEM images of the constructs at day 1 are as shown in HAM/ Fibrin construct appeared to be porous in nature as displayed in Figure 5.4 (a) and (b) respectively. The surface morphology is evidently different as compared to the control group fibrin as shown in Figure 5.4 (c) and (d). Fibrin scaffolds have been well documented to have fiber-oriented surface and morphology (Collet et al., 2000; des Rieux et al., 2009; Noailly, Van Oosterwyck, Wilson, Quinn, & Ito, 2008). This surface morphology appear to be the same for all scaffold made from fibrin (Bensaïd et al., 2003; Bhardwaj et al., 2011; Blombäck & Bark, 2004; Eyrich et al., 2007; Garcia-Fuentes, Meinel, Hilbe, Meinel, & Merkle, 2009; Hokugo, Takamoto, & Tabata, 2006).

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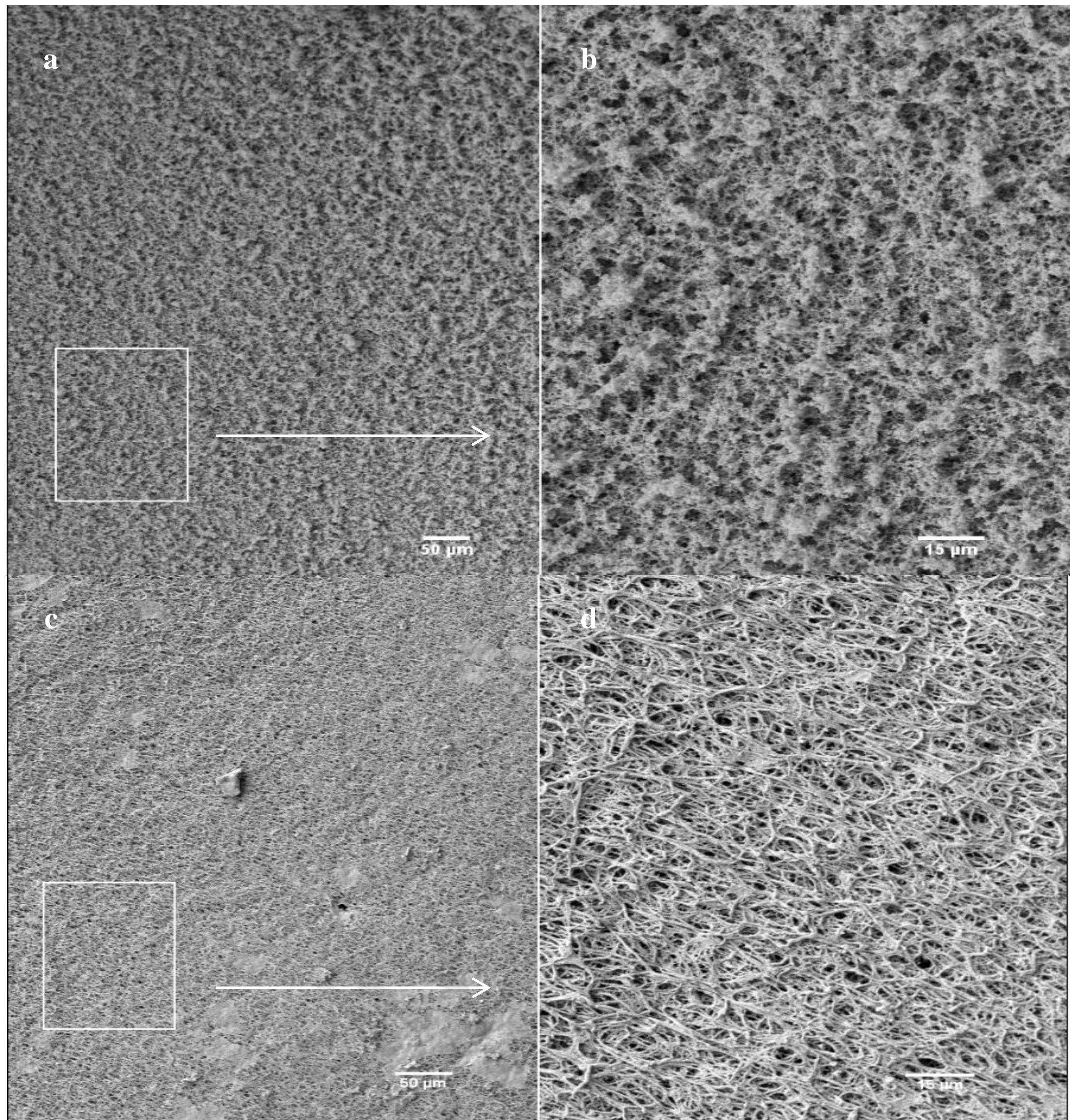

Figure 5.4: FESEM image of HAM/ Fibrin construct at Day 1 of culture (a) 50 µm, (b) 15 µm and Fibrin scaffold at Day 1 of culture (c) 50 µm, (d) 15 µm.

The porous surface and morphology of HAM/ Fibrin scaffold in Figure 5.5 (a) resulted in good chondrocyte attachment and adhesion as observed in Figure 5.5 (c). The control group with fiber surface and morphology in Figure 5.5 (b) also displayed good chondrocyte attachment as indicated in Figure 5.5 (d). The matrix production was evident as there are signs of cell-cell, cell-ECM adhesion and cytoskeletal filaments. Both groups maintained apparent chondrocyte phenotype as the cells appear to be round in shape as evident in Figure 5.5 (c) and (d). The chondrocytes appear to be dissipating homogenously throughout the scaffold in both groups.

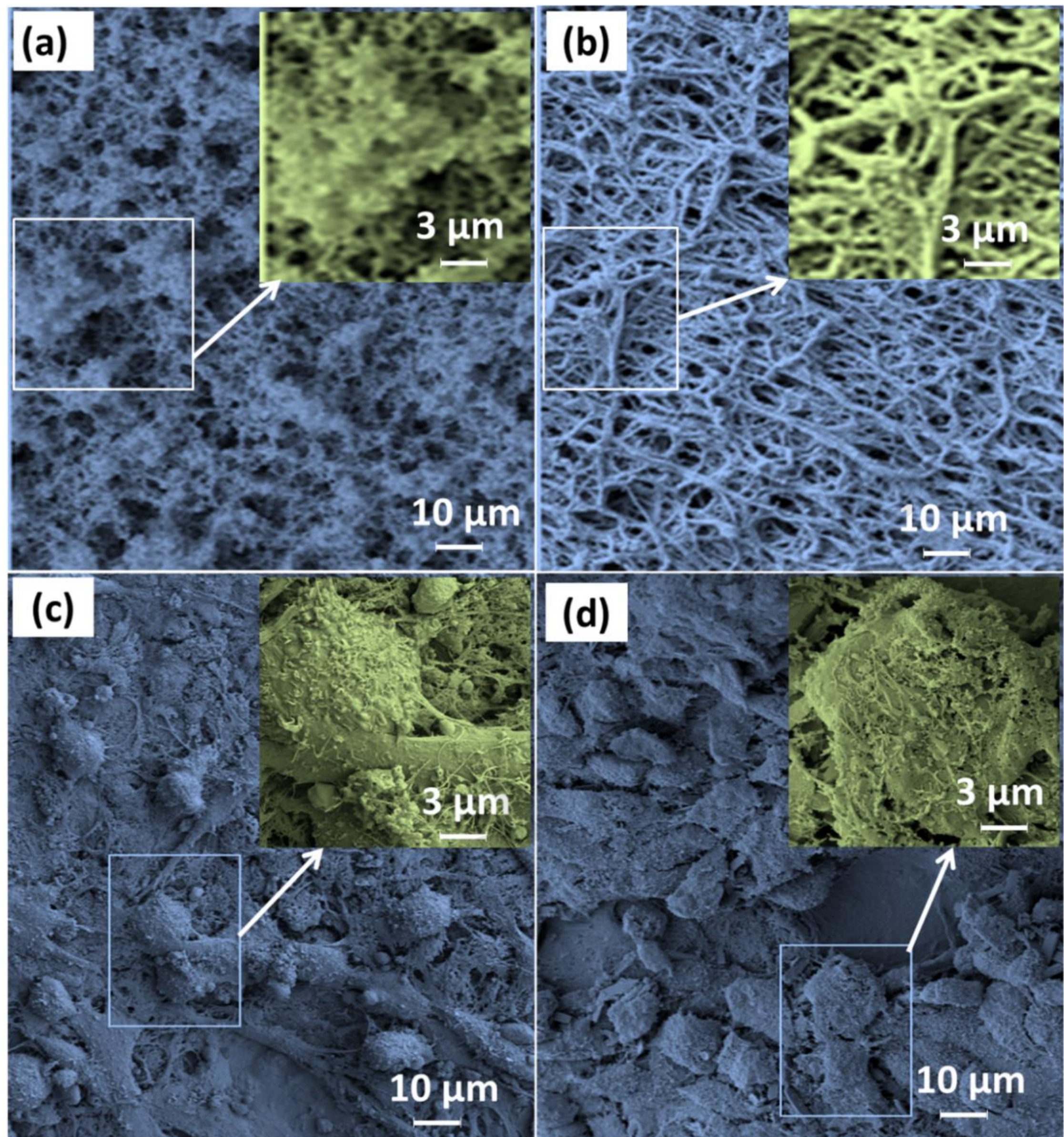

Figure 5.5: FESEM micrographs of (a) HAM/ construct at day 7 of culture. (b) Fibrin construct at day 7 of culture. (c) HAM/ Fibrin construct at Day 28 of culture. (d) Fibrin construct at day 28 of culture. The magnified feature of each micrograph (blue accent) is presented as an inset image (olive green).

5.6 Histology

Histological and histochemical protocols are an important tool in evaluating tissue morphology. The most basic histological staining for general assessment of cell, tissue morphology and distribution is Hematoxylin and Eosin (H&E) staining. Hematoxylin stains the cells nuclei purple and cartilage ECM pinkish in color. The H&E staining was

performed in accordance with the protocol mentioned in section 3.9. The constructs were assessed at day 7 and day 28.

Following the basic Hematoxylin and Eosin staining, Safranin-O staining was performed in order to further assess the PG contents of the construct. As a cationic dye, Safranin-O stains PG as well as glycosaminoglycan. Safranin-O is a proteoglycans indicator as the staining intensity is directly proportional to the PG content. The Safranin-O staining was done at day 7, 14 and 28 according to the protocol as in section 3.9.

5.6.1 Result

The H&E staining of the HAM/ Fibrin and Fibrin constructs are as shown in Figure 5.6 (a) and (c), respectively. The initial staining was at week 1 of culture. Both groups of HAM/ Fibrin and Fibrin appear to be mildly stained with H&E indicating lower cellular component within the construct. During the final week, at day 28, the staining appeared to be deeper in color and in HAM/ Fibrin construct (Fig. 5.6(b)), the stain became apparent and more distinct as compared to the control group (Fig. 5.6(d)) which appears to be less apparent in H&E stains. These results are parallel to the findings in section 5.2.1 and 5.3.1.

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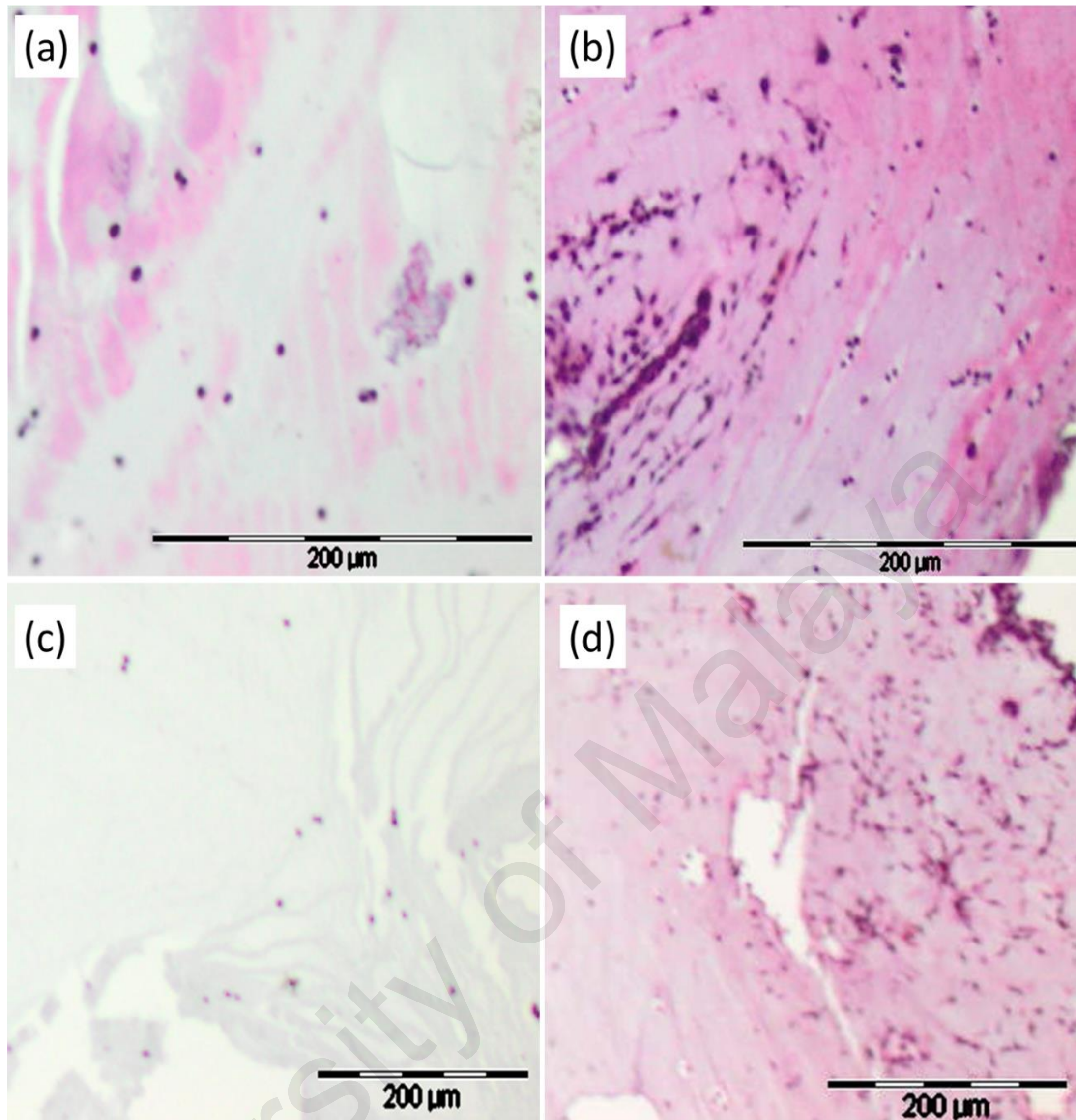

Figure 5.6: Hematoxylin & Eosin staining on HAM/ Fibrin construct at (a) Day 7 and (b) Day 28. Hematoxylin & Eosin staining on Fibrin construct at (c) Day 7 and (d) Day 28.

The staining evidence of Safranin-O staining for both groups is as shown in Figure 5.7 - Figure 5.9. During the initial week, the staining appears to be rather weak with a slight appearance of pinkish stains in HAM/ Fibrin constructs as well as control group as indicated in Figure 5.7(b) and 5.7(a) respectively. The cells appear to be sparsely located and low in number. At day 14, the staining appears to show a slight increase in cell number in both groups with HAM/ Fibrin construct (Fig. 5.8(b)) showing no significance to control (Fig. 5.8(a)). In the final week, the staining appears to have streaks of mild red staining across the specimen indicating cartilage matrix production

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as shown in the respective Figure 5.9 (a) and (b). Both groups displayed this mild staining and results can concur to the results from section 5.2 - 5.5 accordingly. The indication of bluish stain represents the accumulation of proteoglycan contents in that area, being deeper in color representing higher content in PG. This finding further supports the GAG content production in the previous result as mentioned in section 5.3.1. The control group was indicating results as reported previously by other studies (Scotti et al., 2010; van Susante et al., 1999).

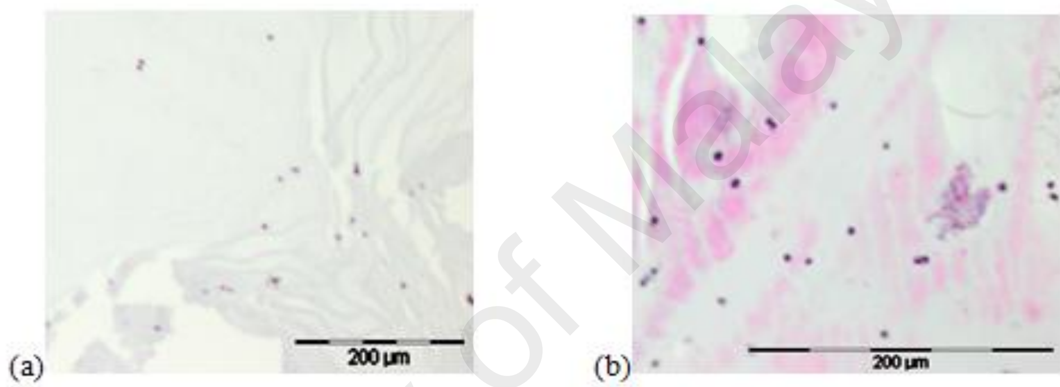

Figure 5.7: Safranin-O staining at Day 7 of (a) Fibrin and (b) HAM/ Fibrin constructs.

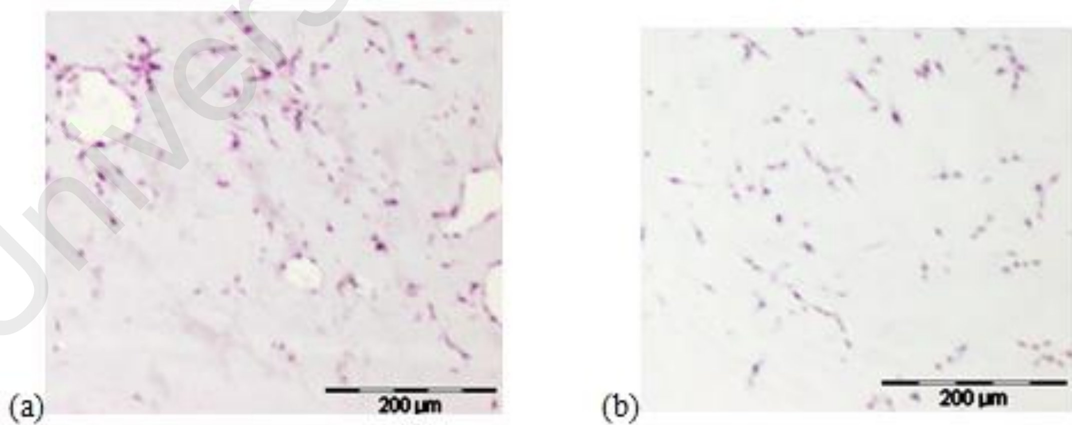

Figure 5.8: Safranin-O staining at Day 14 of (a) Fibrin and (b) HAM/ Fibrin constructs.

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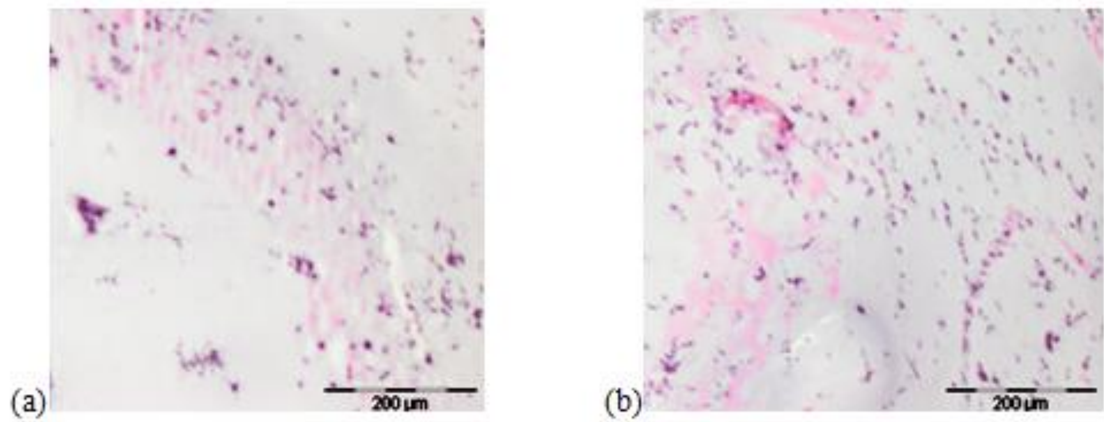

Figure 5.9: Safranin-O staining at Day 28 of (a) Fibrin and (b) HAM/ Fibrin constructs.

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CHAPTER 6: DISCUSSION

6.1 Introduction

This chapter draws together all the work that has been done in the previous chapters. This chapter discusses in depth the choice of sustainable biomaterials involved, fabrication techniques, and methodologies. Furthermore, the mechanism that is responsible for the modification of the architecture is discussed. This chapter also discusses how HAM is responsible for the up-regulation of cell viability and the key findings of the entire study. We first touch on the fabrication of the scaffold, and on how optimizing the scaffold plays an important role in order to continue with assessing the feasibility of the scaffold for cartilage tissue engineering applications. Next, we will be discussing the investigation of the feasibility of an optimized scaffold to support cartilage cell proliferation.

Furthermore, we discussed the results and discussions in depth to draw an understanding of the findings in this study in addition to cartilage tissue engineering scaffold/ field.

6.2 Suitability of Biomaterials

The present study utilized HAM and fibrin for three dimension scaffold fabrication both known to be sustainable biomaterials. HAM has been clinically utilized in numerous clinical studies (Branski et al., 2008; Leon-Villapalos et al., 2008; M. Li et al., 2006; Sheridan & Moreno, 2001). HAM availability is virtually limitless with 4 births recorded per second worldwide. The ECM components of HAM which includes collagens, fibronectin, laminin, proteoglycans and hyaluronan are almost as similar to the ECM that is found in the native cartilage.

HAM possesses several clinical advantages as a biomaterial. Its properties of bacteriostatic, anti-microbial, anti-fibrosis, anti-angiogenic, anti-inflammatory and also

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the ability to inhibit scarring hence enhance wound healing *in vivo*. None of these characteristics are shared by any other biomaterials be it natural of origin or synthetic. It has been documented that HAM poses almost no immunogenicity although the transplantation risk is always of concern (Branski et al., 2008; Bujang-Safawi et al., 2010; Hasegawa et al., 2004). Through strict screening of HAM donor, this biomaterial is an excellent candidate for cartilage tissue engineering application. The challenge in culturing chondrocytes using HAM for tissue engineering applications is in culturing it in a 3D setting. It has been well established that monolayer culture of chondrocyte resulted in loss of chondrocytic phenotype (Benya & Shaffer, 1982). Hence the need to turn HAM into a 3D scaffold would be more suitable in reaping the benefits that it has for tissue engineering applications. The other challenge in utilizing HAM in a 3D form or setting is that it does not set itself after freeze-dried processes. The need for an adhesive material to set and crosslink it together is inevitable. Fibrin has long been studied and applied in tissue engineering applications. Fibrin gel which is a naturally occurring biodegradable biomaterial has high affinity to various biological surfaces. Being naturally occurring physiological scaffold in our body, it supports angiogenesis and tissue repair. Fibrin naturally contains sites for cellular binding and adhesion and has been shown to have excellent cell seeding affinity and good tissue development. The most appealing factor for using fibrin as the hybrid polymer for this study is the fact that fibrin gels can be autologously derived from patients' own blood, eliminating the potential risk of immuno-rejection. Scaffolds made from natural biomaterials have advantages such as innate biological ligands that helps induce cell recognition to promote cellular remodeling (Munirah et al., 2010; Scotti et al., 2010; Wozniak, 2003).

6.3 Optimization of HAM/ Fibrin Scaffold

In assessing this newly fabricated scaffold, optimization ensures the best composition in maintaining chondrocyte phenotype, maintenance and production of cartilage matrix

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components. Optimization is crucial to evaluate the scaffold capacity to retain its shape and form without sudden failure whilst retaining the cells inside. The optimization study was essential before other studies could proceed. There are many methods that can be utilized in fabricating a 3D scaffold as described in detail in section 2.7.3.4. The method that was used in fabricating this 3D HAM/ Fibrin construct is via *in situ* crosslinking through direct injection moulding method. The absence of high heat influx, which is a common mode of 3D scaffold fabrications, eliminates the need of time-consuming scaffold preparation. The results from optimizing the scaffold fabrication are beneficial to be used in clinical applications. In terms of practicality, the HAM/ Fibrin construct would be a great advantage for the non-invasive procedure as it can be directly injected into various shapes and spaces within the suggested time period. The HAM/ Fibrin construct can also be assessed optically based on the results in section 0.

6.4 Cell Seeding Density

It is well known that fibrin set after a certain period of time into a solid form. Due to this, cells are incorporated into the HAM/ Fibrin scaffold before solidification occurs. The need for higher cell density as previously reported resulted in the synthesis of functional matrix (Mauck, Wang, Oswald, Ateshian, & Hung, 2003). This present study also used high cell density at 5 x 10^6 cells/ ml and was proven to be the optimized concentration that follows in all tests evaluated. The cell density of 5 X 10^6 cells/ ml was sufficient to access characterization and analysis of the 3D scaffold as reported in Chapter 5.

6.5 Feasibility of HAM/ Fibrin Scaffold for Cartilage Tissue Engineering Application

In this present work, the feasibility of the HAM/ Fibrin scaffold can be measured and assessed by analyzing the total DNA and GAG content production. The total DNA

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content production assessment can be related directly to the cell density inside the constructs. Cell density can be calculated with the assumption of DNA/ cell to be at 7.7 pg as previously reported (Y. J. Kim, Sah, Doong, & Grodzinsky, 1988). The result shown in section 5.2.1 represents the total DNA content production. Throughout the whole study, HAM/ Fibrin scaffold appears to have slight differences in DNA content compared to the control group. The presence of fibronectin, laminin, proteoglycans and hyaluronan that was reported in HAM could contribute to this result.

The total GAG content production correlates directly to the ECM production by chondrocytes. The result shown in section 5.3.1 represents the total GAG content production. This result represents the feasibility of the constructs in supporting and maintaining chondrocyte metabolism. Throughout the whole study, HAM/ Fibrin evidently resulted with higher GAG production. The presence of HAM in the construct may be contributing to this result by having similar ECM to the native cartilage in particular proteoglycans and hyaluronan. This suggests that GAG production is higher in HAM/ Fibrin scaffold although it is apparent that the amount of cells was not much different to the control group as in section 5.2.1. HAM/ Fibrin scaffold could encourage the higher GAG content production by inducing ECM production. The microenvironment within the scaffold must be feasible for the chondrocytes to excrete higher GAG production as compared to control.

The Live/Dead assay further supports the previous results of DNA and GAG content production. Both groups were able to retain viable cells. The cell viability was apparent at day 7. Both groups showed homogenous cell dispersion throughout the scaffold. This is due to the incorporation of cells together with fibrin before solidification as described in section 3.6. The chondrocytes were dominating and occupying the HAM/ scaffold by day 28. This result further supports the findings in section 5.2.1 and 5.3.1, namely the

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DNA and GAG content production. The presence of HAM in the scaffold may have contributed to the high cell survival. The components that can be found in HAM have certainly had effects on this as well. As compared to fibrin, if HAM has no significant effect at all, then the results of both groups should be the same. It has been well documented that HAM supports cellular survival (Gruss & Jirsch, 1978); (J. Kim et al., 2000). It is apparent that the presence of HAM in HAM/ Fibrin scaffold enhances the survival of cells *in vitro* and induces the ECM production.

The FESEM images as shown in section 5.5, represents HAM/ Fibrin and fibrin scaffold. The morphology and architecture of the scaffold can be observed in detail. HAM/ Fibrin scaffold was observed displaying porous structure and surface morphology, a very distinct feature from a fibrin hybrid scaffold. Previous study that involves fibrin, have always been documented to display fiber orientation when it comes to its surface morphology and architecture. This result is due to the presence of HAM that has totally changed the normal display of fibrin based scaffold. We do not know exactly how this happened, but by incorporating HAM, the scaffold appears to be more porous. This is a good characteristic for a scaffold meant for cell attachment and adhesion molecules to cling onto. This may explain the higher GAG content production and cell survival as indicated previously in section 5.3.1.

The FESEM images at day 28 also showed both groups displayed good cell attachment and adhesion. The cytoskeletal filaments were apparent in both groups indicating the presence of cell-to-cell and cell-to-matrix communication. The cells appear to be round in shape, indicating that chondrocytic phenotype was attained in both micro-environments. This showed that either fiber orientation or porous surface were able to retain chondrocytic phenotype for the chondrocytes.

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Histological staining was also viable for both groups. Both H&E and Safranin-O staining further support the findings on section 5.2 to section 5.5. This indicates that HAM/ Fibrin scaffold was feasible to support chondrocyte survival and metabolism. It was apparent that the histological evidence indicates the cellular proliferation and ECM production by the colors it displayed.

Hyaluronic acid (HA) is widely used in treating OA due to its chondroprotective effects. AC is known to actively produce reactive oxygen and nitrogen species capable of causing chondrocyte dysfunction and apoptosis (Grishko et al., 2009; Jahn, Baynes, & Spiteller, 1999; Rahman & MacNee, 2002; Ray, Huang, & Tsuji, 2012). It has been documented that Hyaluronic Acid inhibits reactive oxygen species and nitrogen species (Cirillo et al., 2015; Soltes et al., 2006). We hypothesize that the HA in the HAM/ Fibrin scaffold contributed to the survival of the chondrocytes as reported in Chapter 5 as there was increased proliferation shown by the DNA results.

6.6 The Advantages of 3D HAM/ Fibrin Scaffold

Fibrin is a well-documented biodegradable and resorbable natural biomaterial (Bensaïd et al., 2003; des Rieux et al., 2009; Eyrich et al., 2007; Janmey, Winer, & Weisel, 2009b; Shaikh et al., 2008), however by supplementing the medium with ε-Amino Caproic Acid (εACA), we were able to controlled the fibrinolysis of the scaffold hence aiding the chondrocytes proliferation (Adelman, Rizk, & Hanners, 1988; Collet et al., 2000; Kupcsik, Alini, & Stoddart, 2008; Ries & Zenker, 2003; Soter, Austen, & Gigli, 1975). The presence of HAM in the scaffold resulted with higher GAG production was probably due to better ECM production with better cell-ECM interactions. The presence of known integrin in HAM could also be contributing to this hence higher proliferation inside the scaffold as compared to the control group.

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As fibrin initiates cellular attachment, as seen in both groups during the first week as can be seen from the total GAG and DNA results, the HAM/ Fibrin scaffold definitely resulted in higher GAG and DNA content towards the third and fourth week. Similarly, collagen in HAM has been known to help in promoting and enhancing GAG synthesis, and maintenance of chondrocyte phenotype (Díaz-Prado et al., 2010)

The HAM/ Fibrin constructs displayed a different surface morphology than the control and any other fibrin crosslink constructs. As multiple studies done previously, none reported in these changes (Bensaïd et al., 2003; Bhardwaj et al., 2011; Blombäck & Bark, 2004; des Rieux et al., 2009; Eyrich et al., 2007; Harold A, 2004; Janmey, Winer, & Weisel, 2009a; Sha'ban, Kim, Idrus, & Khang, 2008). The fiber orientation of the fibrin matrices in the HAM/ Fibrin scaffold were transformed to porous-like architecture, which is favored in scaffold fabrication for better cell attachment.

It is known that HA enhanced cell attachment, proliferation and differentiation of chondrocytes in cartilage tissue engineering applications. The HA presence in HAM was observed to enhance attachment of chondrocytes with significant GAGs and DNA content production. The presence of HA has been reported to induce cartilage tissue engineering in terms of tissue morphological characteristic (Yoo, Lee, Yoon, & Park, 2005). This could be the reason for the change in the morphological structure of HAM/ Fibrin that differs from other fibrin-based scaffold.

6.7 The Effect of HAM to Chondrocyte Behavior

It has been reported that IL-1 cytokines have clinical effects on the cartilage erosion (Westacott & Sharif, 1996). HAM is known to have anti-IL-1 cytokines that could possibly reverse or halt the progress of cartilage degeneration (BrunoFautrel, 2012). Moreover, another study has also reported that the presence of HAM suppresses the activity of IL-1 cytokines in cell culture (Solomon et al., 2001). These reports are in line

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with the results that have been documented in section 5.2.1 - 5.4.1. Cell viability was apparent in all these tests which are parallel to previous studies. This scaffold might be suitable for cartilage tissue engineering applications considering the multiple properties it possesses that would be an advantage if it is able to advance into clinical trials.

6.8 Possible contribution in a clinical setting

In a clinical case scenario whereby if the patient has a full thickness defect as shown in **Error! Reference source not found.**, the subchondral bone is exposed and the injectable HAM/ Fibrin scaffold could plug the area/exposed site. The exposed underlying subchondral bone would allow blood to bind/ interact with the scaffold due to the presence of fibrin in the scaffold. Even though there are concerns of fibrous formation at the "border" of the scaffold subchondral bone; the interconnection between the newly formed scaffold and the native tissue site, reports of HAM within the scaffold poses a great advantage of an anti-inflammatory, anti-angiogenic, and anti-scarring agent could possibly halt the fibrous tissue formation at the site of scaffold-subchondral bone margin/ periphery/ boundary. This could be a possible injectable scaffold that clinicians could use in clinical settings for smaller defects in the articular cartilage region. Although the biomechanics of the scaffold has not been assessed in this study, the use of this scaffold in a clinical setting for defects of less than 1 cm^2 should not induce further damage to the surrounding tissues. It is more likely to have successful cell-cell integration to the adjacent tissue/ neighboring tissue due to the presence of HAM and fibrin with almost similar ECM in that of native cartilage.

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CHAPTER 7: CONCLUSION

7.1 Introduction

This chapter concludes the work that was discussed in the previous chapters and suggests some possible direction of future work.

7.2 Summary of Key Findings

Generally, we can conclude that this study has:

i. successfully fabricated a 3D HAM/ Fibrin scaffold optimized at a concentration of 1:1 (v/v) ratio with 5 million cells/ml set within 30 minutes;

ii. demonstrated that the 3D HAM/ Fibrin scaffold supports chondrocyte proliferation and cartilage matrix production; and

iii. demonstrated that the 3D HAM/ Fibrin scaffold may serve as a potential cell delivery vehicle and a structural basis for *in vitro* tissue engineered articular cartilage.

7.3 Knowledge Contribution from This Work

i. Development of HAM extract from two dimensional Human Amniotic Membrane.

ii. Development of 3D HAM/ Fibrin as a cell delivery vehicle in simultaneous injectable form for potential use in minimally invasive surgical procedure.

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7.4 Future Work and Recommendations

Although the studies that were reported in this dissertation has proven a few new details on the HAM/ Fibrin scaffold, further studies can be carried out to better understand this newly developed scaffold for Tissue Engineering applications.

7.4.1 Biomechanical Property Evaluation

As tissue-engineered cartilage is meant to be utilized in a load bearing area, a biomechanical study needs to be done in order to verify the biomechanical properties of this HAM/ Fibrin scaffold. The study may include static and dynamic biomechanical testing in a bioreactor to determine the influence of mechanical load on cell proliferation, ECM production and maintenance of this novel scaffold.

7.4.2 Measurement of Other Biochemical Parameters

This study has shown that the 3D HAM/ Fibrin scaffold supports matrix production, based on the analysis of DNA and GAG content production. Nevertheless, delivering similar objectives to further study other biochemical parameters, different tests can be conducted to verify the presence of collagen type II production and phenotypic expression via gene expression. The tests may include the SOX 9, Aggrecans, and COMP test, in order to further confirm the role of HAM/ Fibrin scaffold in cartilage tissue engineering application.

7.4.3 Animal/ *In vivo* testing

It has become apparent that following a successful *in vitro* culture of cartilage engineered tissue, an *in vivo* test or animal test/ transplantation should be done to further evaluate the effects and reaction of the HAM/ Fibrin scaffold in a native environment. Moreover, *in vivo* tests could also investigate the theory of whether there is a possibility of a good HAM/ Fibrin to subchondral bone fusion and whether there are a good ECM attachment and coherent chondrocyte proliferation to induce better cartilage repair.

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