BIOFUNCTIONALISED ELECTROSPUN SCAFFOLDS
FOR
CARTILAGE TISSUE ENGINEERING
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Abstract

The field of tissue engineering has advanced and evolved to focus on biomimetic strategies to meet the rise in demands of tissue replacements for surgical reconstruction. One of the key strategies focuses on developing growth factor delivery systems, by incorporating growth factors into tissue scaffolds. While growth factors are crucial cell-inducing components, their limitations such as short half-lives and dose related adverse effects remain a challenge. To overcome these challenges, this thesis is focused on the development of a novel biomimetic tissue scaffold concept incorporating cell-mediated activation of growth factors for cartilage regeneration. The latent transforming growth factor-β1 (TGF-β1) was selected as a model latent protein due to its well established effects on cartilage as well as its ubiquity in many other tissue types. The thesis first focused on the development and characterisation of the tissue scaffold. A non-woven fibrous scaffold was fabricated by electrospinning, surface modified using ammonia plasma, followed by scaffold surface biofunctionalisation with the latent TGF-β1. Physiochemical characterisation revealed that: (1) the scaffold architecture closely resembled that of the native cartilage extracellular matrix; (2) the scaffold surface was chemically modified for subsequent biofunctionalisation reaction; and (3) the latent TGF-β1 was incorporated onto the scaffold and the active TGF-β1 was detected upon acid and enzymatic activation. Biological effects of the biofunctionalised scaffold were assessed using human nasal chondrocytes in a serum free environment and compared with conventional TGF-β1 supplementation on non-biofunctionalised scaffolds (as control). The biofunctionalised scaffold group induced a lower cell metabolic activity and significantly higher gene expression of cartilage specific transcription factor Sox9 after 14 days. The second part of the thesis evaluated the chondrogenic efficacy of the biofunctionalised scaffolds, using the aforementioned chondrocytes and human mesenchymal stem cells (MSC), in an in-vivo rat model. Cell-scaffold constructs were implanted into subcutaneous pockets of athymic rats for six weeks. Gene expression and immunohistochemistry showed that the biofunctionalised group induced significant chondrogenic differentiation in chondrocytes and type II collagen production when compared to controls. Interestingly, a converse response was observed in MSC where the control group induced relatively higher chondrogenic potentials that the biofunctionalised group. This thesis demonstrates that the latent TGF-β1 biofunctionalised scaffolds induced chondrocytic differentiation in chondrocytes and more importantly the proof of concept of cell-mediated activation of growth factors as a novel approach for functional tissue regeneration.
Preface

Author’s Declaration
This thesis is a record of the work carried out by me in the Department of Materials, the Institute of Biomedical Engineering and the Central Biomedical Services Unit at Imperial College London. The work described herein is my own with the exception of: (1) scaffold surface chemical analysis with X-ray Photoelectron Spectroscopy which was performed by Ms. Emily Smith, Nanotechnology and Nanoscience Centre, University of Nottingham; (2) biochemical evaluation (collagen content) of the \textit{in-vivo} cultured implants was carried out by Mr. Trevor Sims, the Department of Cell and Molecular Medicine, University of Bristol.

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<th>Description</th>
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<tr>
<td>DAPI</td>
<td>4’6-diamindino-2-phenylindole</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTAF</td>
<td>Diaminotriazinylaminofluorescein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>HMDS</td>
<td>Hexamethyldisilasane</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency-associated peptide</td>
</tr>
<tr>
<td>LLC</td>
<td>Large latent complex</td>
</tr>
<tr>
<td>LTBP</td>
<td>Latent TGF-b1 binding protein</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>PDLGA</td>
<td>Poly(D-lactide-co-glycolide)</td>
</tr>
<tr>
<td>PDLLA</td>
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<td>PLGA</td>
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<td>PLLA</td>
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<td>pLTGF</td>
<td>Plasma treated electrospun scaffolds with immediate random immobilisation of latent TGF-b1</td>
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<td>ptPLLA</td>
<td>Plasma treated electrospun scaffolds</td>
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<td>RGD</td>
<td>Arginine-glycine-aspartate</td>
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<tr>
<td>RNA</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase - polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>SLC</td>
<td>Small latent complex</td>
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<tr>
<td>SMCC</td>
<td>Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate</td>
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<tr>
<td>TGF-b1</td>
<td>Transforming growth factor -b1</td>
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Chapter 1
Introduction

1.1 Background and Motivation

Adult cartilage is predominantly an avascular, aneural and alymphatic tissue. It inherently lacks the ability to regenerate following destruction by disease or degeneration. Significant cartilage defects are seen clinically in congenital anomalies (e.g. microtia, cleft palate), following facial trauma and excision of tumours of the respiratory tract and in degenerative osteoarthritis (Fig. 1.1). However, current reconstructions based on either autograft, allograft, implants or prostheses - are less than ideal.

Although autologous rib and ear cartilage are widely used due to their biocompatibility and transplant viability with minimal resorption phenomenon(1), autologous soft tissue harvest is often aesthetically imperfect. It involves multi-stage surgery and is associated with significant donor site morbidity(2) as well as surgical complications. The availability for autologous cartilage may also be limited when the defect requiring cartilage reconstruction is too large or when multiple revision surgeries are required(2). Allogeneic and xenogeneic transplantation carry the risks of transmission of infection and transplant rejection(1;3). Furthermore, due to the texture and shape of the native cartilage, it is difficult to surgically mould the native graft into the desired three dimensional shapes for transplantation. Donor availability is often an issue for allogeneic transplantation and this shortage worsens every year with the increasing demands(4). Artificial prostheses may be associated with infection, implant extrusion and adhesive breakdown at the host-prostheses interface(4). They also lack
the ability to adapt to environmental stresses and changes as does native cartilage(4). Due to the deficiencies of current available treatments, the field of cartilage engineering has emerged with the aim of generating a functional cartilage replacement to meet specific patient needs.

Fig. 1.1. Examples of conditions requiring cartilage tissue for surgical reconstruction. (a) Congenital cleft lip and palate(5). (b) Congenital microtia. This is an intra-operative image with a cartilage framework graft pre-fabricated from the 6\textsuperscript{th}, 7\textsuperscript{th} and 8\textsuperscript{th} autologous costal cartilage(6). (c) A complex facial gunshot injury involving the entire nasal, maxillae and mandibular bone as well as soft tissue(7). (d) Anteromedial compartment degenerative osteoarthritis of the knee(8).
1.2 Principles of Tissue Engineering

Tissue engineering is a multidisciplinary field that applies the knowledge of engineering and biological sciences in the development of biological substitutes to restore, maintain or improve health and tissue functionality(4). It consists of four fundamental components: (i) cells, (ii) scaffolds, (iii) bioactive cues, and (iv) environmental factors(9).

In cartilage engineering, all four components contribute to the regeneration of functional tissue. Either chondrocytes or mesenchymal stem cells are key to the production of cartilage extracellular matrix (ECM). A biocompatible three dimensional (3D) scaffold would provide the foundation for the cells to adhere, proliferate and then be delivered to the recipient site. Bioactive cues such as growth factors and arginine-glycine-aspartate (RGD) peptide sequences are essential in signalling and inducing cells to proliferate and produce the appropriate ECM. Last but not least, chondrocyte survival and function will be largely influenced by environmental conditions such as pH, oxygen tension, and dynamic mechanical induction(9-11).

Current challenges facing the tissue engineering of cartilage include the difficulty in maintaining chondrocytic differentiation and supplying the appropriate environmental cues to produce a functional cartilage tissue replacement. For many tissue engineering strategies expansion of cell numbers is vital. As a result, chondrocytes rapidly dedifferentiate leading to the expression fibroblastic markers (e.g. Col1A1) whilst losing cartilage specific transcription factors and genes such as Sox9 and Col2A1(12;13). In developing strategies to overcome these challenges, many studies have investigated the effects of various important parameters in scaffold fabrication such as the type of biomaterial(14;15), three dimensional scaffold architecture, effects of mechanical influence(16), as well as the incorporation of various growth factors(17-19) and cell adhesion motifs(20).

To date, detailed cartilage regeneration studies of human hyaline cartilage have been predominantly focused on articular cartilage rather than nasoseptal, auricular or costal
cartilage. This was largely driven by the volume demand of new cartilage tissue to treat degenerative osteoarthritis(9;21). Articular cartilage samples have been more widely available to science due to the prevalence of joint replacement surgery. Nonetheless, the fundamental principles and advances of cartilage regeneration derived from articular cartilage studies provide a template for engineering of head and neck cartilage. This chapter reviews the native cartilage composition and structural architecture as the first fundamental step towards designing and producing the scaffold for tissue regeneration. This is followed by an overview highlighting the evolution and recent advancements in scaffold technology. Emphasis will also be given to state of the art strategies in biomimetic nanotechnology scaffold development for cartilage regeneration.
Chapter 1: Introduction

1.3 A Review of Cartilage Biology

1.3.1 Types of Cartilage

There are three different types of cartilage in the body: hyaline, elastic and fibrous cartilage. Each can be found at specific sites and with different characteristic properties and functions.

Hyaline cartilage can be found in joints, nose, trachea and ribs(22). In adults, its firm yet pliable character provides shock absorbent effect to mechanical loading in joints, and stent-like support to the nose and trachea, whilst allowing flexibility and recoil to the respiratory system during breathing(23). Its matrix contains collagen fibres, proteoglycans, and glycosaminoglycans.

Elastic cartilage as its name implies, provides elastic properties to the epiglottis, external acoustic meatus, Eustachian tubes and ears(24). In addition to the matrix composition of hyaline cartilages, elastic cartilage also contains elastic fibres and lamellae which contribute to its resilience, pliability and elasticity(24).

Fibrous cartilage is characterised by the abundance of collagen type I fibres in its matrix in addition to that of hyaline cartilage. It is present in intervertebral discs, menisci of knees, symphysis pubis, intra-articular discs of sternoclavicular and temporomandibular joints(24).

1.3.2 Composition of Cartilage

Cartilage is an avascular, aneural and alymphatic connective tissue which derives its nutrients and oxygen supply through diffusion from its surrounding tissue. It consists of cells, matrix macromolecules and water. Individual constituents have their own functions which are integrated and crucial for the survival and integrity of cartilage.
1.3.3 Chondrocytes

The chondrocytes occupy an approximate average volume of 1-2% of the total cartilage volume in the adult human\(^{25,26}\). However, the density of cells per cm\(^3\) depends on the site of the cartilage. Human nasal cartilage contains approximately 24.9 million cells per cm\(^3\), whereas the human femoral condylar and humeral head cartilage contains 14 and 15 million cells per cm\(^3\), respectively\(^{21}\). The rate of in-vitro expansion of cells also depends on the site of origin of the primary chondrocytes. For example, human nasal septal chondrocytes have a shorter doubling time of 2.6 days as compared to 3.4 days for human auricular chondrocytes\(^{27}\). Cell density is also age dependent. It increases with increasing age but has been found to be inversely proportional with the GAG content\(^{28}\). Cell density and donor site have their implications when considering tissue harvest for cell expansion.

Although it is known that the typical shape of a chondrocyte is spheroidal\(^{25}\), cells from different zones within the cartilage differ in shape and metabolic activity. For example, in the adult human articular cartilage, chondrocytes at the superficial zone are aligned parallel to the surface in the direction of shear stress and appear flat or discoid\(^{29,30}\). As we proceed from the superficial to the deep zone, the cells gradually become more spherical then ellipsoid\(^{26,30}\). Although chondrocytes reside in an environment of low oxygen tension, ranging from 10% at the surface to less than 1% in deeper layers, they are still metabolically active as the cells derive most of their energy requirements through the glycolytic pathway\(^{29}\).

Chondrocytes express a number cartilage specific genes i.e. for type II collagen, the \(\alpha_1\) (II) collagen gene (Col2A1); type IX collagen, \(\alpha_2\) (IX) collagen (Col9A2); \(\alpha_2\) (XI) collagen (Col11A2); aggrecan and link protein gene\(^{31}\). Two of these genes in particular, Col2A1 and Col11A2, have been shown to exhibit a similar pattern of expression from as early as precartilaginous mesenchymal condensations\(^{31,32}\) to the fully differentiated chondrocytes\(^{32}\). Therefore the expression of these genes indicates a high level of chondrocytic differentiation. The expression of tissue specific gene is regulated by positive or negative transcription factors. The activation of the positive factors is often coupled with suppression of negative regulators in the control of the specific gene. Both Col2A1 and Col11A2 are regulated by the Sox protein.
group Sox5, Sox6 and Sox9. Sox9 binds to and directly activates the cartilage-specific promoter sites of both Col2A1 and Col11A2. It also activates positive regulatory activities of Col9A1 and aggrecan gene. Sox5 and Sox6 are also vital for chondrogenesis as they bind to tissue-specific element of Col2A1 and aggrecan gene({31;32}). Therefore, Sox5, Sox6 and Sox9 are also essential in determining the degree chondrocytic differentiation.

Analyses of Col2A1, Col1A1 and Aggrecan gene expression have been used in numerous studies to determine the effects of various parameters in the maintenance of differentiation and induction of redifferentiation(10;33;34). Col1A1 on the other hand is an osteogenic marker as well as being a gene expressed by fibrocartilaginous and fibroblast-like cells(35;36). This gene is commonly used to characterise the degree of dedifferentiation of chondrocytes, which have undergone serial passage in-vitro(34;37). Evaluation of gene expression in cells on matrices often correlates to the production of extracellular matrix components.

### 1.3.4 Cartilage Extracellular Matrix

The cartilage extracellular matrix consists of interstitial fluid and an integrated framework of structural macromolecules which are produced and organised by the chondrocytes. The interaction between the interstitial fluid and structural macromolecular framework determines the mechanical properties of the cartilage as well as protecting the chondrocytes from mechanical changes. Water contributes to as much as 80% of the wet weight of cartilage while the remainder consist of the structural macromolecules i.e. collagen, proteoglycans, and non-collagenous proteins(25).

**Interstitial Fluid**

It acts as a solvent to gases, proteins, metabolites and a high concentration of cations which are attracted by the highly negatively charged proteoglycans and glycosaminoglycans. The volume and behaviour of water is influenced by its interaction with the matrix macromolecules which carry negatively charged sulphate and carboxylate groups. These anionic groups attract positively charged ions such as sodium and result in an influx of water which leads to an increase in osmotic pressure.
This increase in osmotic pressure, within the collagenous network, confer to the mechanical properties of the native cartilage(25).

**Structural Macromolecules**

Collagens constitute the major portion of the dry weight of cartilage, representing 60% of the dry weight of cartilage, while proteoglycans and non-collagenous proteins contribute 25-35% and 15-20%, respectively(25). The ability of cartilage to withstand tensile and compressive forces is primarily derived from the organisation of collagen fibrillar network and its interaction with proteoglycans and water. Type II, VI, IX, X and XI are found in the native cartilage.

**Collagen Type II**

Type II collagen is the most abundant collagen type in hyaline cartilage, accounting for up to 90-95% of the collagen content(25;26). These fibrils have a range of diameters which vary in the different regions of the cartilage. For example, in articular cartilage, they are approximately 20nm in diameter in the superficial zone. The diameter increases from 70-120nm, with increasing depth into the cartilage(26). The collagen type II molecule is composed of three identical $\alpha_1$(II) chains. Each $\alpha$-chain consists of repeating Glycine-X-Y sequences, where X and Y can be any amino acid but commonly proline and hydroxyproline respectively. The arrangements of these amino acid residues exhibit characteristic cross-striation periods of 60-70nm(38) which reflect the regularly staggered arrangements of amino acids, designated D1, D2, D3 and D4(38;39), of individual collagen fibrils(38). In the human collagen II, D3 and D4 periods have been shown to favour chondrocytic attachment, and migration due to the presence of one and two RGD sequences(39). The repeating units of hydrophobic (e.g., glycine and proline) and polar (e.g., hydroxyproline) groups containing amino acid residues also contribute to the ability to self-assemble and allow the formation of tightly packed final collagen triple helix confirmation(38;40). At the end of each triple helix, there are very short amino and carboxyl telopeptide domains. In this region, intra- and intermolecular crosslinks form between lysine residues with adjacent chains(26). An understanding of the molecular structure of collagen is important as this enables potential manipulation of chemical interactions when considering the potential functionalisation of synthetic biomaterials.
Other Collagen Types
Collagen Type VI can be found mainly in the pericellular matrix, which is also known as the chondron. This highly branched filamentous network binds decorin and hyaluronan. There is also a high concentration of proteoglycan aggrecan associated with this network. Collagen Type VI has been used as a marker for localisation of chondrocytes(41).

Collagen Type IX has been found to contribute 2% of the total collagen in the hyaline cartilage(26). It is crosslinked to the surface of the Collagen Type II and may serve to form interfibrillar connections within the collagen network(26). Type XI collagen can be found within and on the surfaces of Type II fibrils. Its linkage with collagen type II restricts the lateral growth of the fibrils(26;42). The existence of the intra- and interfibrillar crosslinks of collagen may have implications when considering re-enforcing the structural integrity of engineered fibrillar scaffolds.

Non-collagenous Proteoglycans
Decorin, which is present in abundance at the superficial zone and pericellular region, carries similar function like other leucine rich proteoglycan in limiting the diameter of collagen during fibril assembly. This small proteoglycan is present in similar concentration to the larger aggrecan.

Aggrecan is the predominant proteoglycan in cartilage, accounting for 35% of its dry weight as well as constituting to approximately 90% of the total proteoglycan content(25;43;44). This large aggregating proteoglycan consist of a long 300kDa core protein “backbone” which contains several different binding sites and functional regions(45). Aggrecan can bind non covalently with hyaluronan to form high molecular weight aggregates of greater than 200MDa(46). This association however is stabilised by the link protein(45;47). Aggrecan also provides an extensive region for the binding of polyanionic glycosaminoglycan (GAG) chains. Majority of the GAG chains on aggrecan are composed of chondroitin sulphates which are repeating disaccharide units of glucoronic acid and N-acetyl-galactosamine. Each of these GAG chains are anionic and highly hydrophilic, producing high intermolecular electrostatic repulsion, osmotic properties and swelling pressure. These aggregating proteoglycans when assembled onto hyaluronan form a highly hydrated gel which is entrapped
within the reinforcing network of collagen Type II fibrils. This provides the native cartilage tissue the ability to withstand compressive forces(26;45). Other proteoglycans and proteins with known functions include perlecan and chondroadherin which contribute to cell-matrix adhesion(26).

Every component of the ECM contributes to the integrated dynamic structural and functional complexity of the native tissue – providing the ideal environment for cells to perform specific functions. Therefore, an understanding of the composition and structural organisation of native cartilage tissue is essential for producing a successful three dimensional scaffold for cartilage tissue engineering.
1.4 Development of Tissue Scaffolds in Cartilage Tissue Engineering

1.4.1 Principles of Scaffold Development

Tissue engineering requires a scaffold as an intermediate vehicle which offers support and provides cues to simulate the native environment for cell proliferation, differentiation and matrix production(48). This will form the basis for tissue replacement at the required site. Ideally, the material used should include the following properties(9;49;50):

1. **Biocompatible**. The scaffold should naturally integrate with the surrounding host tissue without the activation of immune response as well as being non-cytotoxic.

2. **Biomimetic**. The scaffold provides cell inducing cues and carry features which resemble the native tissue environment therefore promotes new tissue formation.

3. **Biodegradable**. The scaffold is metabolised in the body and excreted without a trace after fulfilling its purpose.

4. **Porous**. This allows cell infiltration and diffusion of nutrients and waste.

5. **Mechanical support**. Ideally the mechanical strength of the scaffold should match that of its native counterpart until sufficient support is generated by the neotissue.

6. **Shelf life**. It is important to ensure that the scaffold does not lose its desired supportive features prior to the implantation *in-vivo*.

7. **Easy to sterilise**. This is important as infected implants are notoriously difficult to treat.

8. **Surgically easy to handle**. The scaffold should be physical easy to handle to allow surgeons to manipulate the scaffold into the desired shape prior to implantation.

Generally, a polymer is selected, for the use as a biomaterial scaffold, based on these criteria. Many research studies have been conducted on the usage of natural and synthetic polymers as scaffolds for tissue engineering. Natural or native materials which have been investigated include alginates(33;51), chitin(52-54), collagen(34;55-
59), elastin(55;57;58) and hyaluronan(20;60-62) etc while synthetic polymers such as poly(glycolides) and poly(lactides)(15;49;63;64) have been commonly tested and even utilised for biomedical applications.

Natural polymers have the advantage of being biocompatible and that they contain information that facilitates cell attachment or maintenance of differentiated function(4). However, many natural materials suffer batch variability, problems of availability of materials in larger scale(4), high cost and immunogenicity. Synthetic materials on the other hand have the advantage of being versatile in that their physical and chemical properties can be tailored to allow precise control over molecular weight, degradation time, hydrophobicity and other attributes(65). Recently, the advantages of both natural and synthetic materials have been combined in strategies in the production of scaffolds for tissue engineering, where critical amino acid sequences or components from natural polymers were grafted onto synthetic scaffolds(52;56;66-69). A well established example of this in Plastic and Reconstructive surgery is the Integra® Dermal Regeneration Template, where bovine type I collagen and chondroitin sulphate has been crosslinked to a poly(dimethyl siloxane) (PDMS) membrane to form a bilaminar silastic construct for the treatment of burns and scars(70;71). The PDMS layer acts as an epidermal barrier analogue to control fluid loss and prevent pathogen invasion while the co-type I collagen-chondroitin sulphate layer serves as an intermediate matrix for soft tissue and vascular integration. Several US Food and Drug Administration (FDA) approved synthetic biomaterials have been reviewed, in the following section, as potential scaffold materials for this project.
1.4.2 Synthetic Biomaterials for Tissue Engineering

To date, many synthetic polymers have been approved by the FDA(15). Currently, suture materials, craniomaxillofacial and orthopaedic fixtures which are commercially available such as the Bio-Anchor, Lactosorb Screws and Plates and Biologically Quiet Interference Screw, are made of Poly(L-lactide), 82/18 Poly(L-lactide-co-glycolide) and 85/15 Poly(D,L-lactide-co-glycolide), respectively. These biocompatible polymers and their chemical and physical properties will be discussed as follows.

1.4.2.1 Poly (glycolide) (PGA)

PGA is a linear aliphatic polyester (Table 1.1). The first synthetic absorbable suture, Dexon, was made of PGA. The intermediate dimer, glycolide, is used to synthesise PGA by ring opening polymerisation. This produces a high molecular weight material, which is 45-55% crystalline. The high degree of crystallisation is associated with high tensile strength, modulus and lower degradation time. In addition, due to its high crystallinity, PGA is not soluble in most organic solvent with the exceptions of highly fluorinated organic solvents such as hexafluoroisopropanol. With high tensile strength and modulus, PGA fibres are too stiff to be used as suture material except when it is braided. Sutures made from PGA have been reported to lose approximately 50% of their strength after two weeks and almost 100% loss of mechanical properties between 4 – 6 weeks, and are completely absorbed in four to six months(49;72). However, the stiffness and degradation time can be manipulated by copolymerisation with other materials such as lactides in poly(lactide-co-glycolide) (PLGA). PGA has been shown to be biocompatible for chondrocytic culture(73-75).

1.4.2.2 Poly(lactide) (PLA)

Lactide, which is the cyclic dimer of lactic acid, exists in two optical isomers - dextrorotary and levorotary which are signified by a D and L, respectively. L- lactide is the naturally occurring stereo-isoform, while DL-lactide is the mixture of D-lactide and L-lactide. Lactides undergo ring opening polymerisation process which is similar to that of PGA (Table 1.1). With the addition of a methyl group, PLA is more
hydrophobic than PGA but soluble in common organic solvents such as chloroform, dichloromethane and dimethylformamide.

Poly L-lactide (PLLA) is semicrystalline at 37%. Its hydrophobicity accounts for its slow degradation time of more than 6 months. It has been shown that PLLA exhibits superior ability in maintaining structural integrity and in supporting chondrocytic proliferation when compared to other members of its poly α-hydroxy ester family such as PGA, PDLGA 50:50, PDLGA 85:15, and PDLLA(15). This is a promising factor for cartilage tissue engineering as structural stability is required and is associated with better cellular proliferation.

Poly(DL-lactide) (PDLLA) is amorphous due to its random distribution of both the isomers. Therefore, it lacks the ability to arrange itself into a crystalline organised structure. This polymer has lower tensile strength and higher elongation. It degrades rapidly as soon as it comes into contact with water. Its application will therefore be more relevant as a drug delivery vector.

1.4.2.3 Poly(lactide-co-glycolide) (PLGA)

Copolymers of glycolides with lactides, regardless of their subtypes, are amorphous and tend to degrade rapidly (Table 1.1). Both L- and DL-lactide have been produced as devices and drug delivery systems. Interestingly, the half-lives and mechanical properties of the copolymer do not follow the linear relationship of the copolymer composition ratio (Fig. 1.2). For instance, PDLLGA 50:50 which consists of 50% lactide and 50% glycolide degrades faster than either of the homopolymer alone. The ratios 85:15 and 82:18 have been used to manufacture interference screws, suture anchors, plates and screws for craniomaxillofacial surgery. Degradation starts as soon as the material comes into contact with water(15). It has been shown that electrospun PDLLGA 85:15 and PDLLA fibres swell and occlude the pores of the scaffold as early as day 3. This may obscure the migration of cells.
Fig. 1.2. Half-lives of implanted PLA and PGA homopolymers and copolymers (49). The half-life of a polyester decreases when it is co-polymerised with another polyester. The half-lives are at their lowest when the biomaterials are co-polymerisation at equal ratios, as illustrated above at the midpoint of the curve.

1.4.2.4 Poly(ε-caprolactone) (PCL)

The PCL has a degradation time of about 2 years although this can be adjusted to degrade more rapidly by copolymerising with other materials such as PDLLA. PCL alone, like PLLA, has been shown to maintain its three dimensional structural integrity whereas other polymers such as PGA, PDLLGA 85:15 and PDLLA have degraded (Table 1.1). PCL homopolymer has been shown to be less effective in supporting chondrocytic proliferation as the cells have been found to attach poorly to the polymer (76). However, the copolymer of PCL and glycolide has been manufactured as a monofilament suture, Monocryl® by Ethicon.

1.4.2.5 Poly(dioxanone) (PDS)

The poly(dioxanone) is a polyether-ester family which is polymerised from p-dioxanone by the ring-opening step (Table 1.1). It has been known clinically as the monofilament suture, PDS®, which is marketed by Ethicon. The PDS® polymer typically has a high crystallinity of 55%. PDS® has been commonly used for its strength, non-reactive and non-toxic properties as compared to Vicryl and Dexon. It typically loses 50% of its strength in 3 weeks and is completely absorbed in six months.
In view of the physical properties and the biocompatibility of the above materials, PLLA has been selected, for its biocompatibility and long degradation time, as the biomaterial of choice for the fabrication of a three dimensional scaffold in this project.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Degradation Time (months)</th>
<th>Glass Transitional Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA</td>
<td>6 to 12</td>
<td>35 to 40</td>
</tr>
<tr>
<td>PLA</td>
<td>12 to 24</td>
<td>55 to 65</td>
</tr>
<tr>
<td>PLGA</td>
<td>1 to 6</td>
<td>45 to 55</td>
</tr>
<tr>
<td>PCL</td>
<td>&gt;24</td>
<td>-65 to -60</td>
</tr>
<tr>
<td>PDS</td>
<td>6 to 12</td>
<td>-10 to 0</td>
</tr>
</tbody>
</table>

Table 1.1: The chemical structures and materials properties of common FDA approved synthetic biomaterials for tissue engineering (49, 72).
1.4.3 Evolution of Scaffold Fabrication Technology

Traditionally, many engineered biomaterial scaffolds were designed and produced to support cell culture and to match the properties of the desired native tissue at a macroscopic level. For example, in the 1980s, the type I collagen-chondroitin sulphate-PDMS scaffold developed for Integra® Dermal Regeneration Template was produced by a freeze-drying technique(77) to achieve a highly porous scaffold architecture. Scaffolds which were produced by this method were considered to be microporous (Fig. 1.3a). In 1997, in pioneering work by Cao and Vacanti et al., bovine articular chondrocytes seeded on a PLA coated PGA fibrous scaffold moulded into the shape of a human auricle were evaluated using an in-vivo mouse model (Fig. 1.3b)(78). The fibre diameter of the PGA scaffold was 15µm which was almost equivalent to the size of a cell (10-20µm), and two orders of magnitude larger than a natural ECM fibrils (Fig. 1.3c). As the majority of native ECM (cartilage included) consists of a three dimensional, complex nanoscaled fibrillar network of not only structurally but also functionally integrated nanoscaled structures – the current trend in engineering tissue scaffold is to mimic the nanofibrous architecture of ECM proteins(9;79). This is the key difference from the preceding strategies which lack the nanoscaled features of the native ECM. The tissue engineered allogeneic airway transplantation reported by Macchiarini et al., in 2008, further emphasises this argument. The donor airway was decellularised leaving behind the ECM “skeleton” for subsequent recolonisation with the recipient cells(80) (Fig. 1.3d). This ECM “skeleton” may have provided the recipient cells with a near ready-made environment for cell survival, as indicated by the immediate successful clinical outcome.
Fig. 1.3. Pioneering tissue engineered scaffolds for skin and cartilage regeneration. (a) Scanning electron microscopy image (at 100× magnification) of the type I collagen-chondroitin sulphate scaffold which was fabricated with a freeze drying technique, which formed the basis of the Integra® Dermal Regeneration Template(77). (b) Human ear-shaped PLA coated PGA scaffold seeded with bovine articular chondrocytes and implanted into the dorsum of an athymic nude mouse(81), and (c) Scanning electron microscopy of the cell-scaffold construct prior to implantation(78). Note the micron size PGA fibres. Scale bar = 50µm. (d) Pre-implanted segment of tissue engineered allogeneic airway which was decellularised and reseeded with recipient’s bronchial epithelial cells and mesenchymal stem cell derived chondrocytes(80).
1.4.4 Implications of Nanoscale Architecture of Scaffolds

The cartilage ECM naturally consist of a three dimensional complex integrated network of not only structurally but also functionally interlinked nanoscaled structures. Based on this knowledge, many studies have investigated the role of nanoscale fibrous scaffolds (NFS) in tissue regeneration. The scaffold architecture governs how cells spread and bind, leading to changes in intracellular signalling pathways which ultimately result in a modification of gene expression and cell behaviour (82) (Fig. 1.4). Cells seeded on microscale scaffolds tend to flatten as though they were cultured on flat two dimensional (2D) surfaces. This 2D culture condition clearly differs from the natural three dimensional environment of the ECM which is vital for cell behaviour and function (83). Nanoscaled fibrous scaffolds on the other hand, being one to two orders of magnitude smaller than the cells, offer a closer simulation of the natural nanofibrous matrix for cartilage cells, increasing cell binding sites hence directing the desired cell behaviour and functions, as illustrated above. Scaffolds with nanoscale architecture also have a higher surface area to volume ratio for the adsorption of proteins and the binding of ligands, and this means that a higher number of binding sites and guidance cues are presented to cell receptors (82). This has strong implications to biomimicking the functions of native ECM in scaffold production.
Fig. 1.4. Scaffold architecture influences cell binding hence cell behaviour and function. This figure illustrates that nanoscaled fibrous scaffolds provide a three dimensional environment for cells which better resemble the fibrous ECM of cartilage. Nanoscale scaffold architecture also increases the surface area for the adsorption of signalling molecules which in turn increases the binding sites for cells [figure adapted from Stevens et al., Science 2005(82)].
1.4.5 Nanoscaled Fibrous Scaffold Fabrication Techniques

As illustrated above, one of the prime components for structural integrity of cartilage is the collagenous fibrous network. Therefore when designing tissue scaffold systems for the first instance, it is vital to consider a three dimensional fibrous scaffold structural architecture in the nanoscale. Currently, three well established techniques are used in producing nanoscale fibrous scaffolds, namely: self assembly, phase separation and electrospinning(82;84).

1.4.5.1 Self-assembly

Self-assembly is a process of autonomous self-organisation of molecules into patterns and structures without human intervention(85). It occurs commonly throughout nature and technology, mediated by non-covalent interactions such as van der Waals, electrostatic and hydrophobic interactions, hydrogen and coordination bonds(85). For instance, amphiphilic molecules possessing both hydrophilic and hydrophobic segments, may self-assemble into well ordered structures when dispersed in aqueous solvents(86). A naturally occurring example and commonly used biomaterial for tissue culture scaffold is collagen. Its propeptides self-assemble into procollagen and ultimately nanoscaled fibrils.

Synthetic polypeptide based systems have been found to be capable of fabricating nanoscale scaffolds with fibre diameter typically around 10nm(87;88), and have been shown to support the growth of various cell types(89-92). In cell culture, these scaffolds physically behave like hydrogel systems engulfing the cells in a three dimensional manner(89;91;92). This technique produces fibres with diameter which are typically smaller when compared to that of the native ECM (Fig. 1.5). An advantage of using peptide self-assembly is that it has the potential to carry many more biologically compatible motifs on its surface as compared to other synthetic polymeric biomaterials such as polyesters. However, its low yield in productivity and the complexity of its fabrication procedure may limit its use for tissue engineering at a larger scale(84).
Fig. 1.5. An example of self-assembled peptide used in a study of wound healing. A single peptide is shown and then thousands of peptides self-assemble to form a single nanofibre; trillions of peptides or billions of nanofibres form the scaffold (figure adapted from Schneider et al., PLoS ONE 2008)(92). Positive and negative charges are labelled in blue and in red, respectively. Scale bar = 1 μm

1.4.5.2 Phase Separation

Phase separation is a technique of separation of a polymer solution into polymer-rich and solvent-rich domains, which is either induced thermally or by the addition of a non-solvent of a polymer to form a gel, followed by a cooling process to fix the morphology and a freeze drying process to remove the solvent. This technique produces three dimensional fibrous network with fibre diameter ranging between 50nm to 500nm and with porosity as high as 98% (93) (Fig. 1.6). PLLA scaffolds produced by phase separation were shown to favour cell attachment, have higher adsorption of proteins such as fibronectin and vitronectin, as well as promoting differentiation when compared to solid walled porous scaffolds with equal porosity (94;95). Scaffolds fabricated from synthetic and natural polymers as well as composites of both, have been shown to support chondrocytic proliferation with desirable mechanical properties (96-99). Phase separation offers the ability to control the fibre diameter and porosity of the scaffold, as well as tailor its mechanical properties by manipulating several processing parameters such as polymer concentration, type of solvent, solvent exchange, gelation temperature (84;93;100). In addition, since this process of scaffold fabrication takes the shape of its moulds, it enables the scaffolds to be fashioned to any desired anatomical shape (100). Batch to batch consistency can be achieved with this technique but it requires experienced
laboratory skills. Although scaffold production with phase separation requires few specialised equipments it lacks the ability to align the fibres as seen in the electrospinning technique, when considering guiding the direction of growth of certain cell types.

**Fig. 1.6.** SEM images of PLLA scaffolds produced using phase-separation technique(93). (a) at 500x, (b) 20000x magnification (scale bars are 50µm and 1µm, respectively).

### 1.4.5.3 Electrospinning

Electrospinning is an electrostatic technique which produces non woven polymer fibres with controllable diameters ranging from a few microns down to a few nanometres. The technique employs the use of an electrical field that draws the polymer solution into ultrafine jets from the tip of the spinneret towards the grounded target collector (Fig. 1.7). Such a technique has numerous and diverse applications owing to its ability to produce nanoscaled fibres, technical simplicity and cost effectiveness(84;101). Since the first patent by Formhals in 1934, electrospinning has found wide spread application in textile industries, air filtration, biomedical applications including wound dressing and drug delivery(101). It has also become a powerful tool in producing non woven nanofibrous biomaterial scaffolds in tissue engineering application for numerous reasons(15;48;55;102).
Fig. 1.7. Schematic representation of a typical electrospinning system. The polymer solution is delivered, at a constant rate, to the tip of the charged spinneret. Electrospinning initiates from the Taylor Cone (depicted top right) when the charge overcomes the forces of surface tension. The electrospun fibres are then collected on the grounded target. (Figure adapted from an image courtesy of Dr. Julian George, Imperial College London).

The technique of electrospinning provides the ability to tailor-design the desired three dimensional nanoscaled architecture of the scaffold(48) and requires relatively simple equipment. It has the advantage over self-assembly and phase separation methods as it allows fibres to be either randomly orientated or aligned in parallel by rotating the grounded target collector at various speed(103). Aligned natural and synthetic electrospun fibres, such as collagen and polyesters, have been shown to provide topographical guidance to cell attachments orientating cells along direction of fibres(55;63;102;104;105). This is one of the strengths of electrospinning in mimicking the natural structural architecture of many tissue types such as neural, musculoskeletal, dermal and vascular tissues, where direction of tissue growth is crucial in determining the correct physiological function of the organ. It is known that scaffold three dimensionality and fibre diameter are associated with cellular differentiation and proliferation, which are important crucial points in cartilage tissue engineering(106). These properties mimic the three dimensional native environment and nanoscale natural collagen network. In addition to this, as mentioned above, with
the presence of nanoscaled fibrous scaffolds, very high surface area to volume ratio can be achieved for protein adsorption (82), cell incorporation and proliferation (63). The 3D architecture of electrospun scaffolds have interconnected interfibrillar space or “pores” which are vital for diffusion of nutrient and gaseous exchange as well as waste disposal (63). In electrospinning, the ability to control the scaffold fibre size, morphology and architecture is governed by several processing parameters as described in the following section.

For cartilage tissue engineering, various types of biomaterial scaffolds have been fabricated with electrospinning (Table 1.2). In these studies, human mesenchymal stem cells (hMSC), bovine and human chondrocytes have been used to characterise the scaffolds as summarised in Table 1.2.

As described in the above sections on scaffold fabrication techniques, electrospinning has been selected for the following reasons:

1. The electrospinning set up is simple, easy and relatively cheap to build. It is technically easy and simple to perform. Therefore it is a cost effective way of producing nanoscale fibrous scaffolds.
2. As compared to self-assembly, its high yield in scaffold productivity means that it is suitable for scaffold fabrication at a large scale.
3. It has the ability to produce and tailor the scaffold fibre diameter to the desired size in order to mimic the natural cartilage ECM structural framework, when self-assembly may produce fibrous scaffolds with fibre diameters that are typically an order of magnitude smaller.
4. It is also the only technique capable of controlling the alignment and orientation of scaffold fibres. This is known to guide cell attachment hence influencing cell behaviour.
<table>
<thead>
<tr>
<th>Material</th>
<th>Solvent</th>
<th>Scaffold modification</th>
<th>Scaffold Morphology</th>
<th>Fibre Diameter (nm)</th>
<th>Cell Type</th>
<th>Tests</th>
<th>Results and Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>THF, DMF 1:1</td>
<td>Random</td>
<td></td>
<td></td>
<td>Human</td>
<td>proliferation, histocytokinesis, gene expression</td>
<td>Cells were able to infiltrate the scaffold. Uniaxial tensile strength, strain of unswollen were 3.3 ± 0.3%Pa, 0.02 ± 0.005mm/mm.</td>
<td>Li WJ 2003(1)</td>
</tr>
<tr>
<td>Collagen Type II (Chick)</td>
<td>HEP 3% Glutaraldehyde vapor</td>
<td>Random</td>
<td>500 uncrosslinked, 1440 crosslinked</td>
<td>Human</td>
<td>Articular Chond</td>
<td>SEM, mechanical</td>
<td></td>
<td>Shields WJ 2004(2)</td>
</tr>
<tr>
<td>Chitosan-PEO 9:1</td>
<td>HEP TrisX, 10% DMF</td>
<td>0.5M acetic acid/DMF</td>
<td>Random</td>
<td>40</td>
<td>HTB15</td>
<td>SEM, material viscosity, cell morphology, cell proliferation</td>
<td>By adding TrisX &amp; DMF, fibrous scaffolds were electrically. Materials ratio of 9:1 maintained structural integrity. Chondrocytes maintained round morphology.</td>
<td>Bhattachary N 2005(3)</td>
</tr>
<tr>
<td>PGA, PDLLA</td>
<td>HEP (POA), CFM DMF 5:1 (PDLLA), THF DMF 1:1 (all others)</td>
<td>-</td>
<td>Random</td>
<td>300 - 1500</td>
<td>B. Chon</td>
<td>SEM, mechanical, cell proliferation</td>
<td>PLLA &amp; PCL maintained structural integrity. PGA, POLLA, PLLA, PLOA500, PLLA500 degraded upon incubation. PGA, PLOA had stiffer properties. PCL more compliant. Degradation and mechanical properties were a function of M W PLLA reported significantly lower proliferation. Proliferation: PLLA &gt; PCL &gt; PLLA &gt; PLLA &gt; PLLA500 &gt; PLLA500 &gt; PGA.</td>
<td>Li WJ 2006(4)</td>
</tr>
<tr>
<td>PCL</td>
<td>THF, DMF 1:1</td>
<td>-</td>
<td>Aligned</td>
<td>-</td>
<td>BMSC</td>
<td>SEM, mechanical</td>
<td>Aligned-fibrous scaffold can be produced with electrospinning. Alignment of scaffold may provide structural and functional mimic of the natural ECM.</td>
<td>Li WJ 2007(5)</td>
</tr>
<tr>
<td>PCL</td>
<td>CFM DMF 1:1</td>
<td>-</td>
<td>PCL nanofibres spun onto PLLA microfibre</td>
<td>400 - 500</td>
<td>Human</td>
<td>Chon</td>
<td>Porosity, SEM, Histo Alcan Blue/ von Gieson</td>
<td>Electrospinning nanofibres increased the surface area for cellular attachment and infiltration.</td>
</tr>
<tr>
<td>PCL</td>
<td>CFM DMF 1:2</td>
<td>-</td>
<td>Aligned</td>
<td>500 - 3000</td>
<td>hMEC</td>
<td>Cell viability, DNA content, protein content, Gene expression</td>
<td>Aligned-fibres guided initial cell orientation which may have contributed to hMEC differentiated into chondrogenic lineage. Fibre diameter of 500nm enhanced chondrogenic differentiation.</td>
<td>Wise JK 2009(7)</td>
</tr>
<tr>
<td>PLLA</td>
<td>HEP</td>
<td>-</td>
<td>Random</td>
<td>-</td>
<td>hMEC</td>
<td>SEM, porosity, Mechanical, Histo Collagen, Bone expression</td>
<td>Undifferentiated hMSC were not influenced by inductive factors. Micron scale fibres with large pore structures and mechanical properties comparable to cartilage enhanced chondrogenic.</td>
<td>Shamsuzzaman 2011(8)</td>
</tr>
</tbody>
</table>

Table 1.2. Various previously reported studies of electrosyn tissue culture scaffold for cartilage regeneration. Abbreviations: B – bovine, Chon – chondrocytes, CFM – chloroform, Histo - histology, TCP – tissue culture plastic, THF – tetrahydrofuran.
1.5 Electrospinning

1.5.1 Formation of Nanofibres

Two major forces involved in the formation of electrospun nanoscaled polymer fibres are surface tension and self-repulsive electrical forces. When an electrical field is applied across a polymer solution, ions are generated. For a fixed amount of fluid, the surface tension tends to retain the fluid in a spherical shape, while molecular self-repulsion between the charged ions encourages not only an alteration to its shape, but the formation of a jet towards a field of opposite or zero charge(107). In electrospinning, a drop of polymer solution suspended from a conducting spinneret, receives an electrical charge. When the electrical charge is increased to a point where the self-repulsive charge overcomes the forces of surface tension of the polymer solution - an accelerating jet is formed towards the grounded target(108).

This initial segment of the polymer jet increases in length along its axis as a result of the self-repulsive forces. As the jet travels in mid air, the solvent evaporates, leaving behind a charged polymer fibre which continues to be elongated under the influence of the self-repulsive forces. The increase in length of a given segment of the jet is accompanied by gradual but enlarging lateral arching and bending of the segment. This lateral arching of the jet has been observed to increase in magnitude and displace radially from the axis of the initial straight jet. This is related to the self-repulsive charges acting from segments above and below, forcing the jet path to bend and radiate outwards (Fig. 1.8)(107;108). This bending phenomenon has been referred to as the bending instability of electrospinning. Despite the bending instability, the overall flow of direction of the jet remains on course towards the grounded target. The bending instability causes the overall jet to “whip” in mid-air, thereby encouraging further evaporation of solvent as well as thinning and elongation of the polymer jet – ultimately forming nanoscaled polymer fibres(109).

Knowing the importance of self-repulsive electrical forces and surface tension in the formation of electrospun fibres, several key processing parameters further influences the interplay between these two forces. These electrospinning parameters are discussed in the following section.
**Fig. 1.8.** Schematic illustration of the bending instability mechanism of electrospinning. (a) depicts the initiation of the bending instability in a segment of electrospun polymer jet. Self-repulsive electrical forces arising from a segment below induces an upward and outward motion ($F_1$) while the repulsive charges from on-coming segments bring about a downward and outward motion ($F_2$). The consequence of these forces ($F_1$ and $F_2$) is a radial exponential expansion of the electrospun fibre path, deviating away from the original straight axis ($F_3$). (b) illustrates the directions of the resultant forces ($F_3$) of two adjacent electrospun fibre segments [figure adapted from Reneker et al., Polymer 2008(107)].
1.5.2 Electrospinning Parameters

There are several important parameters which influence and control the scaffold fibre diameter, morphology, and alignment(101;110-112). They include:

1. Type of material including its molecular weight
2. Type of solvent
3. Solution concentration/viscosity
4. Ionic charge modification of the solution
5. Feed rate of the solution
6. Accelerating voltage
7. Target distance
8. Type of collector

1.5.2.1 Type of Materials

Natural and synthetic polymers such as collagen and poly(α-hydroxy) esters are common materials used in studies for tissue engineering. The biomaterials which have been utilised in electrospinning were commonly polymers of high molecular weight. The molecular weight of the polymer used affects the fibre diameter i.e. higher molecular weight polymer yields fibres with larger diameter due to the increase of number of chain entanglements of the polymer molecules. The choice of material will also determine the type of solvent as certain polymer such as PGA only dissolves in fluorinated solvents.

1.5.2.2 Type of Solvent

Synthetic polymers require organic solvents while some natural polymers may also be water soluble. For example, chloroform is commonly used to dissolve poly(α-hydroxy) esters(113;114) while hexafluoro-isopropanol (HFIP) has been used as a solvent for both synthetic and natural polymers such as PGA, PLGA(52;63;115), collagen and elastin(58).

The choice of solvent is important. The volatility of the solvent will allow the fibres to dry in mid air prior to its deposition on the grounded target collector. This is very
important as any remaining solvents on deposition will re-dissolve the underlying fibres leading to formation of fused fibres, junctions, bundles or even the collapse of the entire scaffold. Fused fibres, junctions and bundling effects(101)(Fig. 1.9) may effect cellular migration, proliferation and mechanical properties of the scaffold. Conversely, utilisation of a highly volatile solvent such as dimethylchloride may result in intrafibrillar pores (Fig. 1.9) which may lead to a shorter degradation time and a change in scaffold mechanical properties. The formation of these pores is thought to be due to the rapid phase separation effects during the electrospinning process(110). Different types of solvents also carry different levels of electrical conductivity which will influence the fibre diameter and formation of beaded fibres(112). Therefore a precise balance of biomaterials and its solvents are crucial starting points to electrospinning.

Fig. 1.9. SEM images illustrating various fibre morphologies produced with electrospinning. (a) fusion of adjacent fibres (white arrows), scale bar shows 10µm(116); (b) & (c) intrafibrillar pores, scale bar shows 1µm and 500nm respectively(110); (d) beaded fibres, scale bar shows 5µm(117).
1.5.2.3 Solution Concentration and Viscosity

The solution concentration or its corresponding viscosity is an effective parameter to control fibre morphology and diameter\(^{(101;112;118)}\). At low concentrations, more beaded structures are formed instead of smooth fibres as the forces of surface tension predominate over self-repulsive forces. It is also difficult to produce fibres at the higher end of solution concentration as the solution becomes too viscous to be electrospun. Therefore there is an optimal window of solution concentration to fibre formation. Within this processing window, a common trend of fibre morphology change has been reported. At lower concentration, the fibres are irregular and beads-on-strings (Fig. 1.9) as well as junctions and bundles are produced. As we increase the solution concentration, the fibres gradually become more uniform, cylindrical with fewer beads and junctions but with the increase in fibre diameter\(^{(112;118)}\).

1.5.2.4 Ionic Charge Modification of the Polymer Solution

The addition of salt to the solution yields uniform, beadless fibres with smaller diameter. In the presence of salt such as sodium chloride, sodium or potassium phosphate, the charge density of the polymer solution is increased. This increase in charge density on the surface of the solution increases the elongation forces of the jet, as the overall tension in the fibres depends on the self-repulsion of the excess charges on the jet\(^{(118)}\).

Apart from the change in the charge density of the solution, the sizes of the ions also play an important role in determining the fibre diameter. Ions with smaller atomic radii carry a higher charge density and thus confer higher mobility under the influence of an external electric field. For example, electrospinning with the addition of sodium chloride instead of potassium phosphate yields fibres with smaller diameter\(^{(118)}\).

1.5.2.5 Rate of Delivery of Polymer Solution

To obtain different fibre morphologies, a certain amount of polymer solution in the shape of a droplet which is suspended at the end of the needle should be maintained in order to form a Taylor cone which is at equilibrium\(^{(118)}\). Varying the rate of delivery
of the polymer solution will in turn affect the size of this droplet. At lower feeding rate, the diameter of the fibres are relatively smaller as compared to spinning at higher feed rate. With a higher feed rate, more polymer solution is being carried away by the jet therefore yielding fibres with thicker diameter. However, Tan et al have reported that the solution feed rate has no significant impact on fibre diameter especially at lower polymer concentration(112).

1.5.2.6 Accelerating Voltage

When an electric potential is applied to the spinneret, the polymer solution within becomes charged. The surface of the droplet at the tip of the spinneret also becomes charged via the motion of ions. This charged solution reservoir is separated from an oppositely charged grounded target. This creates a static electric field difference. When the strength of the electric field is increased to a certain threshold level, the forces associated with surface tension of the polymer solution are overcome by the repulsion forces of the charged ions. This manifests itself to the naked eye as the Taylor Cone (Fig. 1.10) where the hemispherical shaped droplet is distorted into the shape of a cone pointing towards the grounded target(118). If the voltage is further increased, a jet of the solution is formed towards the target.

![Taylor Cone](image)

**Fig. 1.10.** A Taylor Cone in electrospinning. This image demonstrates the typical conical shape of a charged pendant drop of polymer solution at the tip of a spinneret. A polymer jet can be seen at the bottom of the image indicating the initial process of electrospinning [figure adapted from Loscestales et al., Science 2002(119)].

With the solution concentration or viscosity and feed rate being constant, an increase in the applied voltage leads to an increase in the jet velocity. As a result, more
solution is being removed from the tip of the droplet. For this reason, fibres spun at higher voltage exhibit larger average diameter(101). High voltage also produces smaller but non uniform fibre diameter when the self repulsive forces of the charged polymer ions causes multiple smaller jets to occur. However, care must be taken not to increase the applied voltage such that the droplet is reduced completely. This is because once the electrospinning process occurs within the spinneret, fibre morphology will be changed yielding beaded structures(101).

1.5.2.7 Target Distance

The choice of target distance involves taking various factors into consideration. An adequate distance is needed between the spinneret and the collector to allow the whipping effect of the polymer jet. This facilitates the evaporation of the solvent from the polymer as well as elongation of the jet which leads to further thinning of the fibres. However, care must be taken not to exceed the distance between the spinneret and other surrounding structures such as the wall of the electrospinning container. This is because the charged polymer deposition will find the path of least resistance i.e. the fibres will be deposited on to other structures in closer proximity other than the collector despite being the only object of high conductivity.

1.5.2.8 Type of Collector

The type of collector strongly influences the alignment of fibres. To collect random fibres, a flat conducting plate can be used(102) (Fig. 1.11a). To collect aligned fibres, various set-up have been reported and they include parallel rods, dual rings, rotating discs and mandrels(48;102;120) (Fig. 1.11b). Fibre bundles can also be formed by winding a conducting wire as an electrode on an insulated rod or cylinder(53). Fibre diameter and alignment can also be affected by the speed of the rotation of a mandrel. The degree of fibre alignment increases initially with the increase of the speed of rotation. The highest attainable alignment is obtained up to a certain rotating speed, after which no further improvements will be achieved(53).
Fig. 1.11. Examples of types of collectors. (a) A flat aluminium collector with mounted glass cover slips. (b) A rotating mandrel with aluminium rods.

An understanding of the intricate interplay between each electrospinning parameters is essential for the production of a fibrous scaffold which mimics the structural architecture of the native ECM. The parameters presented above are the key governing parameters which determine the final electrospun fibre morphology, size, and alignment. In engineering a biomimetic scaffold design, apart from fabricating the scaffold physical structure with physical and chemical properties to match the desired native tissue, it is also essential to consider designing the scaffold to possess as close as possible the natural physiological supportive function. The incorporation of bioactive molecules into a scaffold design is crucial to provide the appropriate signals for cell guidance.

1.6 Overview of Cell-Instructive Signals of the Extracellular Matrix

Cells in the native tissue constantly receive multiple signals from and interact with their surrounding ECM. These signals can be classified into either biophysical or biochemical. Both types of signals govern a wide range of cell behaviour and are crucial for cells to perform specialised functions. Initially, these stimuli bind to or are sensed by the cell surface receptors which transduce the signals across the cell membrane. These signals then activate various intracellular signalling pathways which then congregate to regulate gene expression – ultimately determining cell fate, proliferation as well as differentiation. Once the cells are isolated from their natural environment, they are removed from these vital signals – resulting in the lost of specific cell phenotype. Therefore, in tissue engineering, it is crucial to recapitulate
the dynamic signalling complexities of the tissue-specific ECM. The biophysical and biochemical signals from the ECM surrounding a cell can be derived from four sources: (1) structural macromolecules; (2) non-structural bioactive molecules; (3) environmental mechanical stresses; and (4) cell-cell interactions(121;122).

Structural macromolecules such as fibronectin, collagen, GAGs and proteoglycans have a common role in establishing cell adhesion and anchorage, as well as providing the key structural properties to accommodate specific cell types. Structural macromolecules largely transmit biophysical type signals and are detected by cell surface receptors such as integrins(122). When integrins bind to the macromolecules, specific intracellular signalling pathways are activated resulting in the regulation of cell migration, proliferation, differentiation, alteration in cell cycle and apoptosis. Fibronectin is a large glycoprotein which consists of three repeating modules – type I, II, and III, separated by connecting sequences(123). The type III module carries several RGD sequences which mediate cell adhesions via integrins(124;125). It also plays a role in the assembly of certain ECM proteins (e.g. collagen and latent transforming growth factor binding protein) as well as the regulation of growth factor incorporation into the ECM(126;127). As outlined in section 1.3.4, the nanoscaled collagenous framework provides the structural foundation for the assembly of ECM as well as the integration of signalling motifs for cells. GAG and proteoglycans, being highly hydrated macromolecules, provide a means for the diffusion of free and soluble non-structural bioactive molecules(121).

Mechanical stresses from the environment can be transmitted to cells via structural macromolecules as well as from neighbouring cells. These mechanical signals, which act at the cell-ECM/ cell-cell interfaces, are converted to intracellular signalling cascades which modulate gene and protein expression(128). In cartilage tissue engineering, mechanical induction of chondrocytes have been known to be one of the favourable method to modulate chondrogenesis in-vitro and in-vivo(10;16;129).

Non-structural bioactive molecules (bioactive molecules in short) have also been classified as “soluble factors” in other review articles(122). Bioactive molecules that are commonly involved in tissue engineering consist of predominantly growth factors and certain hormones. Although the latter is generally related to the endocrine,
gastrointestinal, reproduction and immune systems, hormones do play a role in tissue regeneration. For example, hormones (natural or synthetic) such as insulin and dexamethasone promote mitosis and protein synthesis. They are frequently used to induce chondrogenic differentiation *in-vitro* in conjunction with growth factors(130-132). Growth factors are polypeptides which are secreted by cells and bound to the ECM. The association of growth factors with the ECM prevents unnecessary degradation or loss of important information. The release or activation of the growth factors can be initiated by cells as required at a later stage. In the native ECM, multiple growth factors act synergistically and are required in minute amounts. They are also required at appropriate times, concentration as well as ratio(122). This dynamic spatial and temporal balance of growth factor delivery remains a challenge to tissue engineers. In order to recreate the multidimensional as well as multidirectional integration of information for cells, the incorporation of non-structural bioactive molecules into nanoscale scaffold designs would be highly beneficial to promote specific tissue regeneration. This process has been referred to as the biofunctionalisation of scaffolds. Scaffolds of synthetic materials such as poly(α-hydroxy) esters often require this process as they often lack functional surface chemical properties for biochemical and cellular interaction.
1.7 Biofunctionalisation of Scaffolds with Growth Factors

Biofunctionalisation often involves scaffold surface modification followed by physical and/or chemical linkage of certain biologically active compounds onto the scaffolds. Scaffold surface modification techniques commonly involve the use of either plasma or chemical treatments e.g. concentrated hydrochloric acid, diamine compounds(133). These surface modification techniques introduce reactive chemical groups such as amine, hydroxyl or carboxyl, which are recognisable by cells as well as form a platform for subsequent biochemical reaction with natural ECM components and bioactive compounds. These bioactive compounds include several native ECM components such as RGD peptide sequences for cell attachment, collagen and chondroitin sulphate, as well as cell-inducing growth factors e.g. transforming growth factor-β1 (TGF-β1) and basic fibroblast growth factor (bFGF)(56;66;67;134-136).

Growth factors play a vital role in the induction, promotion and maintenance of differentiation as well as the function of cells - particularly in the regeneration of cartilage tissue, which inherently lack the capacity to regenerate. Nonetheless, the use of growth factors in tissue regeneration is associated with several drawbacks. In general, growth factors have short half-lives, e.g. TGF-β1 (<30min), insulin-like growth factor (10-12min)(50). This means that a constant supply is required manually in *in-vitro* culture. Similar approach to supply growth factors may be manually impractical for *in-vivo* studies. Combined with the potential uncontrolled dose related adverse effects, these drawbacks of growth factors deter the translation of its application to the *in-vivo* or clinical setting. These challenges have persuaded researchers to incorporate growth factors into scaffold designs with controlled release systems. The controlled release of growth factors can also be tailored into biomimetic scaffold design(137).

Several methods of growth factor incorporation have been described(50): (i) by direct blending or emulsion of the growth factors with the biomaterials during the scaffold synthesis and fabrication stage, (ii) by physical adsorption through the immersion of scaffold in the growth factor solution, and (iii) by pre-chemical linkage to or
encapsulation by a carrier prior to step i. Currently numerous studies have “immobilised” or “incorporated” growth factors such as TGF-β1, FGF and IGF in tissue scaffold systems with sustained release properties(134;136;138-141). Although the results of current studies have been encouraging, further refinements need to be made.

The majority of these scaffold release systems are hydrogel based, where release of growth factors is by diffusion or degradation of the scaffold matrix(50;140). This often results in a “burst” release as well as a loss of scaffold mechanical and supportive properties required for cartilage tissue engineering(142). In addition, these studies utilised the active form of the growth factors and inevitably involved various processing steps which may result in a reduction of the bioactivity. At some point during the process of incorporation into delivery systems, growth factors may come into contact with chemicals such as crosslinking agents, various solvents as well as scaffold materials which may alter their true potential as well as bioactivity(143). For these reasons, our main aim is to incorporate the latent form of the TGF-β1 onto electrospun scaffold system for tissue regeneration. The growth factor remains immobilised on the scaffold and protected by its latent protein until its activation by cells.
Chapter 2
Scope of the Thesis

The main objective of this research is to develop a structurally and functionally biomimetic scaffold for cartilage tissue regeneration. By designing and producing a scaffold system that mimics the nanoscale architecture as well as the functional aspects of the cartilage extracellular matrix, the outcome of this project may provide a platform technology to overcome the well known poor regenerative capacity of cartilage.

To achieve this, specific aims of this project are to:

1. Examine and characterise the nanoscale architecture of the native cartilage extracellular matrix;
2. Fabricate a nanoscale fibrous scaffold by electrospinning;
3. Biofunctionalise the electrospun scaffold by incorporating the latent TGF-β1;
4. Biological evaluation of the biofunctionalised scaffold by in-vitro and in-vivo culture.

The purpose of Chapter 1 was to provide a background review of the field of cartilage tissue engineering, including the biology of cartilage, recent advances in scaffold fabrication and biofunctionalisation. Chapters 3 - 5 discuss the main experimental results of this research, which also include related literature reviews, experimental methodologies and suggestions for further work. Final conclusions and a general overview for further investigations are presented in Chapter 6. The following sections highlight the scope and aspects of each results chapter.
2.1 Structural Analysis of Native Hyaline Cartilage and Fabrication of Nanostructured Fibrous Scaffold by Electrospinning

In order to design and produce a tissue scaffold which structurally mimics the native cartilage architecture, the first step was to examine and understand the structural morphology and nanoarchitecture of the native cartilage ECM. In Chapter 3, human articular hyaline cartilage and reconstituted bovine nasal septal collagen were examined under scanning electron microscopy. The outcome of the analysis formed the basis for the subsequent scaffold fabrication step by electrospinning.

Electrospinning is a technique capable of producing three dimensional nanostructured polymer scaffolds that closely mimic the structural architecture of the native ECM. Chapter 3 then describes the parametric characterisation of the newly custom-built electrospinner. The optimal parameters to produce uniform nanostructured electrospun fibrous scaffolds were determined for further scaffold production in this thesis.

2.2 Biofunctionalisation of Electrospun Scaffold with Latent TGF-β1

Apart from designing the scaffold structural architecture to mimic the native ECM, the functional aspects of tissue scaffolds are equally essential. Bioactive cues such as growth factors play a vital role in guiding cell behaviour and have been used to promote the appropriate cell responses. The TGF-β1 is a well known chondrogenic growth factor. However, its use in the in-vivo setting may be impractical due to its limitations such as a short half-life and potential dose-related adverse effects. To address these challenge, the proposed concept detailed in Chapter 4 focused on developing a cell-mediated activation of growth factor scaffold system, using the latent form of the TGF-β1 as the model growth factor. Chapter 4 also included the physicochemical characterisation as well as the short term in-vitro biological assessment of the biofunctionalised scaffolds using primary human nasal chondrocytes.
2.3 *In-vivo* Assessment of Latent TGF-β1 Biofunctionalised Scaffold

Chapter 5 is focused on determining the long term outcome of the latent TGF-β1 biofunctionalised scaffolds in an *in-vivo* setting. The scaffolds and their controls were seeded with primary human nasal chondrocytes and human mesenchymal stem cells, prior to the implantation in the dorsal subcutaneous layer of athymic rats. Chondrogenic effects of the scaffolds were determined using gene expression studies and immuno-detection of cartilage matrix production.
Chapter 3
Structural Analysis of Native Cartilage and Scaffold Fabrication by Electrospinning

3.1 Introduction

In hyaline cartilage tissue, the type II collagen is one of the main components that provides structural integrity and confers the ability to withstand external and internal mechanical forces. The knowledge and understanding on the structural organisation and architecture of the native cartilage ECM is vital for the production of a successful biomimetic tissue scaffold for cartilage regeneration. Scaffolds in tissue engineering provide the foundation for cell growth as well as instructive cues for functional tissue regeneration. The initial aim of this chapter is focused on examining the structural architecture of the native hyaline cartilage. The hyaline cartilage analysed in this chapter was of human articular source due to its availability. Bovine nasoseptal collagen was also evaluated as a comparison. After the structural evaluation of the native hyaline cartilages, the following aim of this chapter is focused on producing a non-woven fibrous scaffold with electrospinning.

Electrospinning is a technique capable of producing polymer fibres with diameters ranging from 5µm to 5nm by accelerating a jet of polymer solution which is electrically charged in an electric field towards a grounded target of opposite polarity(101;112;117). Due to the inherent capability of producing nanoscaled fibres which mimic the structural features of the native extracellular matrix, the
electrospinning technique has become a major tool in the production of scaffold for the study of tissue engineering(15;52;53;55;63;102;115;144).

Our in-house built electrospinner was constructed early in the year 2006 (Fig. 3.1). The set-up consists of an electrospinning chamber, a syringe driver, a high voltage power supply, a motor to rotate mandrel collectors, gastight glass syringes, stainless steel needles and Teflon tubing. The electrospinning chamber was grounded to adjacent steel grounding pipes.

![Fig. 3.1. The electrospinning set up. On the right is the in-house built electrospinning chamber. It receives the polymer solution at a constant rate which is controlled by the syringe pump (Cole-Palmer, UK) situated in the centre. The high voltage supplier (Glassman High Voltage Inc.) on the left creates the electrical field difference necessary for electrospinning. The entire system is set up in a fume hood.](image)

The set-up required extensive characterisation in order to determine optimal settings and parameters for the consistent control of electrospinning. This is important considering the electrospinner will be used to fabricate three dimensional nanoscaled fibrous scaffolds for tissue engineering studies within the department. Numerous studies have reported the important governing parameters in electrospinning(101;112;117). However, these parameters may be set-up dependent; therefore this chapter sought to design a study to determine the optimal processing parameters and their consistencies for the production of nanoscaled fibres from biopolymers.

Poly(L-lactide) (PLLA) was the biopolymer of choice for this thesis as it has been shown to maintain structural integrity while the rest of its counterparts such as PGA,
PDLGA 50:50, PDLGA 85:15, and PDLLA degrade in aqueous solution. It has also been reported to be superior in promoting chondrocytic proliferation when compared to other members of its poly α-hydroxy ester family(15).

### 3.2 Materials and Methods

#### 3.2.1 Structural Analysis of Human Native Hyaline Cartilage and Reconstituted Bovine Nasal Collagen

##### 3.2.1.1 Preparation of Human Native Hyaline Cartilage for Scanning Electron Microscopy

Adult human articular hyaline cartilage was obtained with informed consent from a female patient undergoing hip hemiarthroplasty after a femoral neck fracture. Strips of the cartilage were sectioned perpendicular to its perichondrial surface down to the underlying bone. The strips were immediately fixed in 2.5% glutaraldehyde followed by the stepwise dehydration and critical point drying process (Appendix 1.1). The processed cartilage strips were then analysed with scanning electron microscopy (SEM, see below).

##### 3.2.1.2 Preparation of Bovine Collagen Films on Cover Slips

Bovine collagen solution of 1%(w/v) (Collagen from bovine nasal septum, Sigma-Aldrich, UK) was prepared by dissolving the collagen in 5%(v/v) acetic acid (pH 3.0). It was magnetically stirred until fully dissolved (5 minutes). 12.5µL of the collagen solution was pipetted onto the centre of 10mm diameter glass cover slips (Agar, UK). They were then left in the sterile hood overnight to dry.

**Non Self-assembled Collagen**

To study the structural features and self-assembly of collagen, three of the above coated cover slips were sputter coated for SEM (see following section on SEM). These samples were not subjected to incubation in culture media, the step-wise dehydration and critical point drying in order to elucidate the effects of immediate drying on collagen after sputter coating the cover slips.
Self-assembled Collagen

A separate group of collagen films were subjected to incubation in Dulbecco’s modified Eagle’s medium (DMEM – Invitrogen, Paisley, UK) at pH 7.5, 37°C for 12 hours to allow the self-assembly process to occur. The culture media was then removed before step-wise dehydration and critical point drying was performed on the samples (Appendix 1.1).

3.2.1.3 Comparison of Collagen Fibre Diameters of Human Native Hyaline Cartilage and Reconstituted Bovine Collagen

Three different SEM images from three different samples of native human hyaline cartilage and reconstituted bovine nasal septal collagen were obtained. Five collagen fibre diameters were measured on each image. A total of 45 collagen fibre diameters of both groups were measured using Image J software (National Institute of Health, USA). The means and standard deviations of both groups were calculated using SPSS 12.0 software package (SPSS Inc., Chicago, USA).

3.2.2 Parametric Study of Electrospinning

3.2.2.1 Preparation of Solution and Determination of Type of Solvents for Electrospinning

The polymer PLLA (PURAC Biochem, UK) with an average molecular weight of 300kDa, was used in this study. To determine the type of solvents to be used in this parametric study, several combinations of polymer solutions were prepared by dissolving the PLLA in dichloromethane (DCM, AGTC Bioproducts, UK) only, or in DCM and dimethylformamide (DMF, AGTC Bioproducts, UK), at three different weight ratios of 9:1, 8:2 and 7:3, at the polymer concentration of 5.0%(w/w). The polymer solutions were electrospun and analysed with scanning electron microscopy as described below.

For the parametric study, PLLA solution was prepared by dissolving the PLLA in DCM and DMF at the weight ratio of 7:3, at the polymer concentrations of 1.0% - 7.5%(w/w). As discussed in the results section later, the weight ratio of DCM to DMF of 7:3 was chosen because it produced fibres which were smooth and without pores.
DCM and DMF were initially measured according to the weight ratio of 7:3. The two organic solvents were kept separate. The weight of PLLA was then measured, added to the measured DCM and stirred magnetically until fully dissolved. DMF was then added to the solution and magnetically stirred until the two solvents were mixed thoroughly. The polymer solution was then ready to be electrospun.

3.2.2.2 Target Distance

The target distance which is the distance between the needle and the collector was set at 10cm. As the distance between the tip of the needle to the side walls were 12-15cm depending on the position of the needle, the target distance of 10cm was chosen due to the fact that the closest object regardless of its material will be the site of first deposition of the fibres. On the other hand, sufficient distance was required for the jet to travel, the evaporation of solvent as well as the thinning and elongation of the polymer jet.

3.2.2.3 Types of Collector

Two types of collectors were used – parallel aluminium rods of a mandrel and 1×1.5cm² glass cover slips mounted on a flat aluminium plate (Fig. 1.10). The latter was chosen for the lower polymer concentration 1.0 and 1.5%(w/w) when the formation of the initial jet was not observed. This ensured the collection of any fragmented fibres or electrosprayed droplets.

3.2.2.4 Accelerating Voltage

The high voltage was applied to the tip of the needle and this was regulated by the high voltage supply (Glassman High Voltage Inc., UK) to generate the polymer jet. The voltages used were 6, 10, 15, 20 and 25kV. The lower limit of the accelerating voltage parameter was set at 6kV as this has been found in this study to be the voltage where the initiation of the polymer jet occurred. Voltage of 25kV was chosen as the top limit because above this limit, static discharge occurred around the grounding wires. When investigating the effects of accelerating voltage at each polymer concentration, the rate of polymer solution delivery was set constant at 0.2mL/hr. This
rate of polymer solution delivery was selected as it was found to be the initial rate where the polymer solution droplet at the tip of the spinneret could be maintained during the electrospinning process. At a rate less 0.2mL/hr, where the removal of polymer solution by electrospinning exceeds the rate of delivery to the spinneret tip - the droplet was found to recede into the spinneret. This was consistent with previously reported studies(101;118).

### 3.2.2.5 Rate of Delivery

For each concentration, 5mL of the polymer solution was fed into a gastight glass syringe (Hamilton, USA) which was controlled by a syringe pump (Cole-Parmer, UK) at a constant feed rate of 0.2, 0.4, 0.6, 0.8 and 1.0mL/hour. Teflon tubing was used to connect the syringe to the needle which inner diameter of 0.51mm which was set up horizontally. Qualitative observations such as the subjective rate of fibre production during electrospinning were recorded (see raw data presented in Appendix 1.5). When investigating the effects of rate of delivery at each polymer concentration, the parameters were set constant at target distance of 10cm and accelerating voltage of 10kV.

### 3.2.3 Scanning Electron Microscopy

The native hyaline cartilage, bovine collagen and the electrospun fibres were mounted directly onto carbon tabs on aluminium stubs for SEM. They were then sputter coated with 20nm thickness of gold using an Emitech K550 sputter coater (EM Technologies Ltd., UK) operating at 20mA for 2 minutes. The morphology of the fibres was then observed under scanning electron microscopy (FESEM; Gemini LEO1525) at accelerating voltage of 4kV and working distance of 6-8mm. Triplicate samples were taken and more than three fields of view were obtained.

### 3.2.4 Electrospun Fibre Diameter and Bead Measurements

Three samples of electrospun fibres were collected from three different areas of the target collector, for every change of parameter. For every sample collected, three images were obtained from three different areas using SEM. Approximately 5 fibres
per image were measured using Image J software (National Institute of Health, USA) (Appendix 1.4). This equated to a total of 45 electrospun fibre diameters for every change of parameter. For fibres which were not circular but ribbon shaped, measurements of widths and heights were taken and an average was calculated. In order to determine whether sample sizes (i.e. number of fibre diameters measured per parameter change) were sufficient to establish, with a confidence of 95% ($z = 1.96$), mean parameters (fibre diameters) with an error margin of $\pm$ 5%, the following formula was used:

$$\frac{z_{\alpha/2} \times \sigma}{E}$$

(3.1)

where, $z_{\alpha/2}$ is the positive value that is at the vertical boundary for the area of $\alpha/2$ in the right tail of the standard normal distribution, $\sigma$ is the population standard deviation, $n$ is the sample size, and $E$ is the error.

The margin of error, $E = \text{critical value (z)} \times \text{standard error of the statistic}$,        (3.2)

where $z = 1.96$, standard error (SE) = $\frac{\sigma}{\sqrt{n}}$

For example, at a polymer concentration of 2.5%, rate of delivery of 0.2mL/hr and 6kV, the mean = 0.312, SD = 0.02 and $n = 45$ (see Appendix 1.5). Thus, $\text{SE} = \frac{\sigma}{\sqrt{n}} = 0.02 / \sqrt{45} = 0.0030$

Therefore, the margin of error = 1.96 x 0.02 / $\sqrt{45} = 0.005$

The number of beads and circularity were also measured using Image J. Circularity is defined as $4\pi \times (\text{area}/\text{perimeter}^2)$. A value of 1 indicates a perfect circle. As the value approaches 0, it indicates an increasingly elongated polygon (Appendix 1.4). The edges of the beads were traced and circularity was measured using Image J (Fig. 3.2). The data were processed and results were represented as graphs and bar charts using Microsoft Excel software.
Fig. 3.2. A typical SEM image of showing the analysis of beads in electrospun PLLA fibres. This image illustrates the method employed for the measurement of fibre bead numbers and circularity using the Image J software. The black lines encircle the beads, while the numbered labels indicate the number of beads that has been included for analysis.

3.2.5 Statistical Analyses

The means and standard deviations of the native human collagen, bovine collagen, the electrospun fibre diameters and bead measurements were calculated using the SPSS software package SPSS 12.0 software package (SPSS Inc., Chicago, USA). The Mann-Whitney U Test for two independent samples was performed using SPSS to determine statistical significance between the fibre diameters of the native human and bovine collagens. Statistical significance between various electrospinning parameters was also determined. The $p$ value of $<0.05$ was considered to be significant.

3.3 Results

3.3.1 Structural Analysis of Human Native Hyaline Cartilage and Bovine Nasal Collagen

The structural analysis of human hyaline cartilage with SEM confirmed that the native cartilage ECM consisted of densely packed fibrous collagenous network. These complex networks of collagen engulf lacunae which house chondrocytes and exhibit randomly aligned architecture (Fig. 3.3a and b). The lacunae typically measured 20 –
25µm. On high magnification, the characteristic cross-striated pattern of collagen was observed along the fibres (Fig. 3.2c).

Fig. 3.3. Representative SEM images of human hyaline cartilage. (a) The centre of the image shows a lacuna which is surrounded by dense fibrous collagen matrix. The scale bar shows 10µm. (b) This image illustrates the random alignment of the surrounding structural collagenous framework. (c) The staggered arrangements of amino acids within each collagen fibre manifest as the characteristic periodic cross-striations throughout each fibre. The scale bars of (b) and (c) show 1µm.
Initially, the reconstituted collagen exhibited fragmented grain like appearance on SEM, when dried immediately upon coating the cover slips and no collagenous fibres were observed (Fig. 3.4a). After being incubated in culture media at 37°C overnight, the collagen was seen in fibrillar form and exhibited a continuous interwoven network (Fig. 3.4b). These networks of collagen appeared randomly aligned but lack the interconnecting interfibrillar space found in the native hyaline cartilage. The fibre diameters of the bovine collagen (0.100 ± 0.020µm) were observed to be significantly lower ($p<0.05$) than that of human native collagen (0.171 ± 0.049µm).

**Fig. 3.4.** Representative SEM images of reconstituted collagen from bovine nasal septum. (a) A sample of collagen solution [in 5%(v/v)acetic acid] subjected to the effects of immediate drying. SEM analysis demonstrated grain-like fragments of the collagen. (b) A sample of collagen solution [in 5%(v/v)acetic acid] subjected to further incubation in cell culture media, prior to the drying step. The samples which were further incubated with media exhibited continuous interconnecting network of collagen fibres. The typical cross-striated pattern can also be seen on the collagen fibres. The scale bars show 500nm.
3.3.2 Parametric Study of Electrospinning

The important processing parameters of our custom-built electrospinner have characterised. The effects of these parameters on electrospun fibre size and morphology are described in individual sections as follows.

3.3.2.1 Effects of Type of Solvent

The fibres electrospun from PLLA dissolved in pure DCM were found to contain pores along the fibres (Fig. 3.5a). These fibres also contained beads and were not uniform in size. A gradual progression of reduction in pores and beads were found with increased DMF content. With the initial addition of DMF to the solvent (DCM:DMF 9:1), a reduction of pores along fibres were observed while beads were still present (Fig. 3.5b). At a DCM:DMF solvent ratio of 8:2, the pores on fibres were no longer present however the beads were still observed on fibres. Interestingly, the fibre morphology at this solvent ratio appeared to be relatively uniform compared to the fibres electrospun at a DCM:DMF solvent ratio of 9:1 (Fig. 3.5c). Finally, fibres electrospun at a DCM:DMF solvent ratio of 7:3, appeared smooth, uniform in fibre size, beadless and contained no pores (Fig. 3.5d). This final solvent ratio was used for all subsequent electrospinning parametric experiments of this study.
Fig. 3.5. Representative SEM images of electrospun PLLA fibres showing a trend of reduction of pores and beads on fibres with differing solvent ratio of DCM to DMF: (a) PLLA in pure DCM; (b) PLLA in DCM:DMF 9:1 weight ratio; (c) PLLA in DCM:DMF 8:2 weight ratio; and (d) PLLA in DCM:DMF 7:3 weight ratio. All of the above were electrospun at polymer concentration of 5.0% (w/w), accelerating voltage of 10kV, rate of delivery of 0.2mL/hr, target distance of 10cm and collected on parallel aluminium rods. “B” indicates a beaded fibre whereas the arrows (in figure b) point towards the locations of finer pores on the electrospun fibres.
3.3.2.2 Effects of Polymer Concentration

The effects of various accelerating voltages as well as rates of polymer solution delivery on fibres electrospun at various polymer concentrations are represented in Fig. 3.6 and 3.7. The results showed that the fibre diameters were significantly increased with increasing polymer concentration ($p<0.05$). Both graphs in Fig. 3.6 and 3.7 exhibited a sigmoid trend where the rise in fibre diameters were gradual and largely under 0.5µm, below a polymer concentration of 4.0%(w/w). The fibre diameters then increased rapidly between polymer concentrations of 4.0 and 5.0%(w/w) – reaching predominantly micron scale at a polymer concentration of 5.0%(w/w) and above.

![Graph showing the effects of various accelerating voltages on electrospun fibre diameter at different polymer concentration.](image)

**Fig. 3.6.** The effects of various accelerating voltages(kV) on electrospun fibre diameter at different polymer concentration. The rate of delivery of polymer solution was set constant at 0.2mL/hr while the target distance was 10cm. Below a polymer concentration of 4.0%(w/w), fibre diameters were submicron, while a polymer concentration of 5.0%(w/w) produced fibres largely in the micron scale. Fibre diameter was observed to increase with increasing polymer concentration. The trend was statistically significant for $p<0.05$ at each accelerating voltage.
The effects of various rate of polymer solution delivery (mL/hr) on electrospun fibre diameter at different polymer concentration. The accelerating voltage was 10kV while the target distance was 10cm. At a given rate of delivery of polymer solution, the increase in polymer concentration significantly increased the fibre diameter ($p<0.05$).

The minimum polymer concentration to produce fibres of any morphology was found to be at 1.5%(w/w). Below this concentration, few fibres were produced and the effects of electrospraying were observed especially at higher accelerating voltage of 20kV and above. These were seen as nanoscaled circular shaped deposition on the collector when examined under the SEM (Fig. 3.8a). At polymer concentration of 1.0 - 1.5%(w/w), fibres were produced but were found to be in fragmented (Fig. 3.7a and b). There was a gradual rise in fibre diameter with the increase of polymer concentration from 2.5 – 4.0%(w/w) (Fig. 3.6 and 3.7). A steep rise in fibre diameter (approximately two to four fold) occurred between polymer concentrations of 4.0 – 5.0%(w/w). However, few fibres were produced at a polymer concentration of 7.5%(w/w). At polymer concentration of 5.0 and 7.5%(w/w), excess polymer solution was frequently observed to plug the tip of the needle, clogging the flow of delivery. This required frequent removal of the polymer plugs to facilitate electrospinning (approximately 3-4 times in a 5 minute period). This problem did not occur when the electrospinning process was performed at a polymer concentration of 2.5%(w/w) and below.
**Fig. 3.8.** Representative SEM images of PLLA fibres electrospun at polymer concentration of 1.0 and 1.5\%(w/w). (a) The electrosprayed droplets of polymer can be seen in three areas as indicated by the arrows [polymer concentration 1.0\%(w/w), 0.2mL/hr, 20kV]. (b) By increasing the polymer concentration from 1.0 to 1.5\%(w/w), fibres were produced instead of electrosprayed droplets. However, these fibres were short and fragmented [polymer concentration 1.5\%(w/w), 0.2mL/hr, 6kV].
3.3.2.3 Effects of Accelerating Voltage

The effects of various accelerating voltages at a given polymer concentration are illustrated in Fig. 3.6. Alterations in accelerating voltage did not significantly influence the diameter of electrospun fibres, except at several parameters which will be discussed as follows.

At a polymer concentration of 1.0%(w/w), the fibre diameters produced at various accelerating voltage were essentially less than 0.100µm, except at an accelerating voltage of 20kV where the fibre diameter of fibre were significantly increased by approximately 1.5-folds (0.130 ± 0.049µm). The electrospraying effects were also observed at high accelerating voltages of 20 and 25kV (Fig. 3.8a).

The fibres electrospun at a polymer concentration of 1.5%(w/w) yielded diameters within the range of 0.100 – 0.200µm. Interestingly, the trend of increased of fibre diameter at a higher accelerating voltage observed at 20kV, polymer concentrations of 1.0, where also observed at a polymer concentration of 1.5%(w/w) – where the fibre diameters were significantly increased (30%, \( p<0.05 \)) at an accelerating voltage of 25kV (0.173 ± 0.047µm). Beads were observed in all samples at polymer concentration of 1.0 and 1.5%(w/w).

At a polymer concentration of 2.5%(w/w), the fibre diameters ranged from 0.220 ± 0.013µm to 0.312 ± 0.020µm, with the exception of fibres electrospun at 10kV. At an accelerating of 10kV, the fibre diameters increased by approximately 56% (0.501 ± 0.023µm), which was also considered statistically significant within the range of accelerating voltage (\( p<0.05 \)).

At a polymer concentration of 3.0%(w/w), the fibre diameters electrospun at 6kV (0.242 ± 0.058µm) were the smallest within the range of accelerating voltages. For fibres electrospun at 10 – 25kV, the fibre diameters ranged between 0.309 ± 0.063µm to 0.362 ± 0.098µm with no significant differences.

There were no significant effects observed at various accelerating voltages (from 6 - 25kV) on the fibre diameters electrospun at a polymer concentration of 4.0%(w/w).
The fibre diameters ranged between $0.515 \pm 0.110\mu m$ to $0.631 \pm 0.203\mu m$. Interestingly, although no statistical significance were found, at polymer concentrations of 3.0 and 4.0%(w/w), an increase in the fibre diameters was observed at an accelerating voltage of 15kV. The trend was also observed in fibres electrospun at a polymer concentration of 2.5%(w/w) and accelerating voltage of 10kV as mentioned above.

At a polymer concentration of 5.0%(w/w), the fibre diameters fell within the range of $2.493 \pm 0.106\mu m$ to $2.702 \pm 0.121\mu m$. The increase in accelerating voltage did not influence the fibre diameter significantly, except at an accelerating voltage of 25kV where the average fibre diameter was significantly reduced by approximately 75% - from $2.489 \pm 0.115\mu m$ (accelerating voltage 20kV) to $0.608 \pm 0.035\mu m (p<0.05)$. At an accelerating voltage of 15kV, the fibre diameter was found to be the largest within its group ($2.702 \pm 0.121\mu m$), even though no statistical significance was observed. This was consistent with the trends observed on fibres electrospun at accelerating voltages of 10 – 15kV and polymer concentration 2.5, 3.0 and 4.0%(w/w).

At a polymer concentration of 7.5%(w/w), fibre diameters was observed to range between $2.031 \pm 0.112\mu m$ (accelerating voltage of 6kV) to $1.448 \pm 0.333\mu m$ (accelerating voltage of 25kV). Interestingly, at an accelerating voltage of 10kV, the average fibre diameter was significantly larger at $2.982 \pm 0.156\mu m$ while at accelerating voltage of 25kV the fibre diameter was significantly smaller by approximately 40 - 50% ($p<0.05$).
3.3.2.4 Effects of Rate of Delivery of Polymer Solution

At a polymer concentration of 1.0 – 1.5%(w/w), the rate of delivery of the polymer solution did not significantly change the diameter of the fibres (Fig. 3.7). The fibre diameters fell within the range of 0.065 ± 0.022µm to 0.081 ± 0.016µm at a polymer concentration of 1.0%(w/w) while at polymer concentration of 1.5%(w/w) the diameter of fibres ranged between 0.093 ± 0.029µm and 0.127 ± 0.033µm. However, at polymer concentration of 1.5%(w/w), the rate of delivery of 0.2mL/hr produced significantly larger (0.127 ± 0.035µm) by 20-30% when compared to fibres spun within its group (rate of delivery of 0.4 - 1.0mL/hr). Interestingly, at a polymer concentration of 2.5%(w/w), the rate of delivery of 0.2mL/hr also produced significantly larger fibre diameters (0.501 ± 0.023µm), which were 2 – 3 folds increase relative to its group (at rate of delivery of 0.4 - 1.0mL/hr). In contrast, the fibres electrospun at a rate of delivery of 1.0mL/hr (0.154 ± 0.008µm) were significantly smaller than fibres produced between the rate of delivery of 0.2 – 0.8mL/hr.

The fibre diameters electrospun at a polymer concentration of 3.0%(w/w) were within the range of 0.242 ± 0.058µm to 0.362 ± 0.098µm, whereas at a polymer concentration of 4%(w/w), the range of fibre diameters produced were approximately twice the size at 0.538 ± 0.110µm to 0.631 ± 0.203µm. At a polymer concentration of 4.0%(w/w), rate of delivery of 0.8 and 1.0mL/hr yielded significantly smaller fibre diameter (0.542 ± 0.163µm and 0.538 ± 0.193µm, respectively) within its experimental group (p<0.05).

Although no statistical significance was observed in diameters of fibres electrospun at a polymer concentration of 3.0%(w/w) between 0.2 – 0.6mL/hr, the fibre diameters were significantly reduced by approximately 40 – 50% when the rate of delivery was increased to 0.8 – 1.0mL/hr (0.168 ± 0.045µm and 0.177 ± 0.039µm, respectively). Similar trends in fibre diameter were observed in fibres electrospun at polymer concentration of 4.0%(w/w). The fibre diameters produced at a rate of delivery of 0.2 – 0.6mL/hr ranged between 0.530 ± 0.244µm to 0.631 ± 0.203µm, which decreased significantly with increased rate to 0.8 – 1.0mL/hr, yielding smaller fibre diameters within the range of 0.386 ± 0.181µm to 0.437 ± 0.107µm.
At a polymer concentration of 5.0%(w/w), the effects of varying the delivery rate of the polymer solution were similar to that seen in polymer concentration of 2.5 and 5.0%(w/w), where the fibre diameters were the largest at rate of delivery of 0.2mL/hr (2.493 ± 0.106µm) and smallest at 1.0mL/hr (0.808 ± 0.055µm). There were no beads found at a polymer concentration of 5.0%(w/w) (Fig. 3.9a) but micron sized fibres were observed at this concentration. With the increase of the rate of delivery of polymer solution, the production of fibres was found to be quicker and the morphology of the fibres became more ribbon in shape (Fig. 3.9b).

Fig. 3.9. Representative SEM images of electrospun PLLA fibres. (a) Electrospun fibres are grossly uniform without beads. The electrospinning parameters were set at a polymer concentration of 5%(w/w), 0.4mL/hr, 10kV and target distance of 10cm. (b) An example of the ribbon-like morphology of the fibres. The electrospinning parameters were set at a polymer concentration of 5%(w/w), 0.2mL/hr, 10 kV and 10cm target distance.
At a polymer concentration of 7.5%(w/w), the rate of delivery of 0.4 – 1.0mL/hr produced fibre diameters ranging from 0.787 ± 0.059µm to 1.152 ± 0.067µm. However, the differences in fibre diameter at this range of rate of delivery (0.4 – 1.0mL/hr) were not significant. At a rate of delivery of 0.2mL/hr, the fibre diameter was found to be significantly larger by 3 - 4 folds when compared to the rest of the rate of delivery at this polymer concentration. The accelerating voltage did not influence fibre production but the increase of rate of delivery increased the rate of fibre deposition. Fibres were also ribbon in shape and there were no beads on the fibres (Fig. 3.10).

![SEM image of electrospun PLLA fibres. Fibres spun at this concentration yields micron sized fibres. Note the ribbon-like appearance of the fibres. Electrospinning parameters were set at a polymer concentration of 7.5%(w/w), 1.0mL/hr, 10kV and 10cm target distance.](image)

**Fig. 3.10.** SEM image of electrospun PLLA fibres. Fibres spun at this concentration yields micron sized fibres. Note the ribbon-like appearance of the fibres. Electrospinning parameters were set at a polymer concentration of 7.5%(w/w), 1.0mL/hr, 10kV and 10cm target distance.
3.3.2.5 Characterisation of Beads on Fibres

The production of beads on fibres was also observed to be associated with the increase in the rate of polymer solution delivery. For this reason, fibres spun at various rates for polymer concentration of 2.5 – 4.0%(w/w) were examined for the possible correlation with the production of beads. This range of polymer concentration was selected to be analyse primarily because below a polymer concentration of 2.5%(w/w), fragmented fibres have been found; while above a polymer concentration of 4.0%(w/w), the fibre diameters were largely micron scale which were beyond the desired range of fibre diameter for this thesis. The number of beads produced, increased with the increase in the rate of polymer solution delivery up to a rate of 0.6mL/hr and then plateau thereafter (Fig. 3.11). An apparent trend was observed on the circularity of beads with the increase in polymer concentration. The circularity of beads decreases with increasing polymer concentration, however, this effect is statistically insignificant as indicated by the standard errors in the data (Fig. 3.12). Further investigations will involve examining a larger sample size of electrospun fibres within this range polymer concentration.
Fig. 3.11. The effects of rate of delivery on beaded fibre production of electrospun PLLA at polymer concentrations: (a) 2.5%(w/w), (b) 3%(w/w) and (c) 4%(w/w). The number of beads
on fibres has been found to increase with increasing rate of polymer solution delivery up to a rate of 0.6mL/hr, and then appeared to plateau from 0.6 – 1.0mL/hr.

**Fig. 3.12.** The effects of polymer concentration 2.5, 3.0 and 4.0%(w/w) on circularity of beads on fibres electrospun at various rates of delivery of polymer solution. Blank, striped, and black bars represent polymer concentration 2.5, 3.0 and 4.0%(w/w) respectively. There is an apparent trend in the circularity of beads with an increase in polymer concentration. However, the effect was not statistically significant as illustrated by the error bars in this figure. (n = 3 – 280, as the number of beads produced were associated with the rate of delivery. See Fig. 3.11)
3.4 Discussion

The first part of this chapter emphasised on the importance of recognising and understanding the nanoscaled fibrous collagenous architecture of the hyaline cartilage extracellular matrix. The structural characteristics of the cartilage ECM would form the “reference point” for the fabrication of a structurally biomimetic scaffold. The natural cartilage ECM has porosity in the range of 0.75 – 0.8. Its interfibrillar pore size has been reported to be approximately 2.0 – 6.5nm. This together with the presence of the proteoglycan network contributes to a very low permeability value in the range of $10^{-15} – 10^{-16}m^4/Ns$(145). The second part of this chapter successfully characterised the key processing parameters of the custom-built electrospinning system, which are necessary for the fabrication of nanoscaled fibrous scaffolds for tissue regeneration. These processing parameters are also important for the control of scaffold fibre size, morphology and architecture.

3.4.1 Structural Analysis of Human Native Hyaline Cartilage and Bovine Nasal Collagen

The human hyaline cartilage provided insight into the morphology and fibre diameters of the collagenous framework in the natural ECM. The native cartilage collagen fibres have been reported to exhibit diameters ranging from 20nm to 300nm(26). Although both native human hyaline cartilage and reconstituted bovine collagen fibre diameters fall within the range of diameters reported in the literature, the difference in species may account for the difference in the fibre diameters. Another explanation to the difference in the diameters of the collagen fibres observed here is that the native human cartilage contains extracellular matrix materials which are predominantly polysaccharides that affect the average fibre diameter(146). The presence of polysaccharides in the native ECM may concentrate the collagen and promote their collision-induced interaction with subsequent fibril growth, resulting in larger diameters. The lack of polysaccharide macromolecules to inter-disperse the collagen molecules during self-assembly may have accounted for the lack of interfibrillar spaces (Fig. 3.4b). Nonetheless, the ability of collagen molecules to self-aggregate into fibrils has been observed(42). The typical cross striated appearance of collagen can be clearly seen upon self-assembly (Fig. 3.4b), which was not present on the
fragments of non-assembled collagen (Fig. 3.4a). The effects of changes in pH and temperature on self-assembly of collagen observed here confirmed the findings reported in other studies, where a neutral pH and temperature above 35°C would favour the self-assembly of collagen molecules into polymeric fibres(147). As outlined in Chapter 1, the nanoscaled features of the structural collagenous framework of the extracellular matrix are essential in promoting chondrocytic proliferation, differentiation and function(15;82). Thus, in order to produce a tissue scaffold which mimics the structural morphology and architecture of the native cartilage ECM, the electrospinning technique was employed.

3.4.2 Parametric Study of Electrospinning

To date, several studies have investigated the effects of various electrospinning parameters(53;111;112;117;118;148). However, our custom-built electrospinner required a systematic parametric study to characterise the effects of important governing parameters as our set-up may differ from that of other research centres. The electrospinning technique was successfully used in producing polymer fibres at nanoscale. Several parameters have been held constant relating to the set-up, i.e. the spinneret inner diameter is 0.51mm while the target distance was held at 10cm. Although the electrospinning process is a complex interplay between various parameters, the effects of the various parameters are discussed independently in the individual sections below.

3.4.2.1 Effects of Type of Solvent

The choice of solvent(s) for a given polymer is the first parameter to be considered for electrospinning. Several solution properties of a solvent will need to be considered, including its volatility, electrical conductivity and potential cytotoxicity. A highly volatile solvent with high electrical conductivity is generally required for electrospinning (Chapter 1). The DCM is a highly volatile solvent for PLLA, while pure DMF is a non-solvent. However, intrafibrillar pores were observed on the fibres that were electrospun using pure DCM. This observation was consistent with the results reported by Bognitzki et al. (Fig. 3.5a)(110). The formation of intrafibrillar pores is related to a rapid phase separation which is followed by a rapid evaporation
of volatile solvents and then a subsequent rapid solidification of the fibres, during the electrospinning process(110). The phase separation phenomenon, as its name implies, separates solvent rich regions from polymer rich regions. The solvent rich regions, on evaporation, result in the formation of pores. The addition of DMF, a less volatile solvent, reduced the rapid phase separation of the polymer and solvent as seen in the reduction in pores on fibres of electrospun PLLA (Fig. 3.5b and c). The rapid phase separation created pores on the fibres which will lead to an increase in surface area. An increase in surface area of the electrospun PLLA scaffolds can potentially contribute to an increased rate of fibre degradation by hydrolytic attack when immersed in culture media. However, the ability to generate pores may prove useful in protein adsorption, as well as other studies investigating the effects of surface properties and nanotopography on cell behaviour.

Apart from the gradual reduction of pores, further addition of DMF up to a weight ratio of 7:3 resulted in fibres which were more uniform, smooth and beadless (Fig. 3.5d). This can be explained from the fact that an addition of DMF to a polyester-DCM solution increases the electrical conductivity as well as decreases the surface tension of the polymer solution(112;149). An increase in the electrical conductivity of a polymer solution during electrospinning induces a rise in self-repulsive charge within the electrospun polymer – therefore resulting in the elongation of the beaded segment. An increase in the solvent conductivity also yields fibres with smaller diameter due to a prolonged elongation of the electrospinning jet. A reduction in surface tension of the polymer solution further prevents the formation of beads. Thus, this may account for the reduction of beaded fibre formation with the addition of DMF in this study. This finding is consistent with previously reported studies(112;117;149).

A low conductivity solution results in insufficient elongation of a jet by electrical force which is necessary to produce uniform fibres. When polymer macromolecules are dissolved in a solvent, they are orientated into an entangled network during the elongation flow of the electrospinning jet. This jet elongation persists as the fibres solidify. In a polymer solution with low conductivity, the contraction of the radius of the electrospinning jet, which is driven by surface tension causes the remaining solution to form beads(117). The addition of DMF to a DCM-based polymer solution
increases the conductivity therefore facilitates the electrospinning process in the production of fibres with smaller diameters and without beads. However, the potential cytotoxic effects of solvents need to be considered as a further increase in non-volatile organic solvents such as DMF may be detrimental to cells. The DCM:DMF weight ratio of 7:3 as a solvent for PLLA has been found to be safe for cell culture(102). Excessive non-volatile solvent which remains in the fibres may also dissolve the underlying fibres yielding fused fibres and junctions resulting in a more rigid or collapsed scaffold(101;107).

The solvent combination weight ratio of 7:3 of DCM to DMF has been selected for subsequent studies in this chapter, as it produces fibres without pores. Nonetheless, the production of smooth, uniform, beadless and nanoscale fibres also depend on the interplay of several other processing parameters of electrospinning as discussed below.

### 3.4.2.2 Effects of Polymer Concentration

Apart from the choice of solvents, two other important parameters of an electrospinning polymer solution to be considered are the molecular weight of the polymer and the polymer concentration. Both parameters have been considered to be the most effective parameters in controlling the electrospun fibre diameter and morphology(64;101;112;118;150). The molecular weight reflects the length of a polymer. In the case of a poly(α-hydroxyester) such as the PLLA, the linear molecular structure resembles the configuration of a chain. At a given polymer concentration, the number of chain entanglements increases with the length of the polymer (i.e. the molecular weight); alternatively, the same number of entanglements can be achieved at a fixed molecular weight by increasing the polymer concentration. Chain entanglements have been described as the physical overlapping between polymers, thus resulting in the interlocking of chains. In electrospinning, sufficient chain entanglements are required to overcome the effects of surface tension for the formation of fibres(112;151;152). For the formation of a stabilised electrospinning jet, it has been reported that more than 2.5 entanglements per chain is required(151). The molecular weight of the PLLA selected for electrospinning in this study was set at 300kDa, similar to the “high molecular weight PLLA” studied by Tan et al.(112).
The results described above (Fig. 3.6 and 3.7) showed that the diameters of electrospun fibres increased with increasing polymer concentration. The minimum polymer concentration to effectively electrospin uniform fibres was found to be at a polymer concentration of 2.5%(w/w) with an average diameter of 220 ± 13nm, which was comparatively lower than that reported by Tan et al. at polymer concentration of 3.5%(w/w) with fibre diameter of 322nm(112). Fibres electrospun below a polymer concentration of 2.5%(w/w), appeared fragmented and contained electrosprayed droplets. These fragmented fibres may not be suitable for use as tissue scaffolds despite its nanoscaled mean diameter ranging from 70 - 150nm. The transition observed between the production of fragmented fibres and electrospraying droplets to the formation of uniformly smooth fibres with increasing polymer concentration, demonstrated the role of chain entanglement with the increase in polymer concentration.

The production of beaded fibres was observed at polymer concentration of 2.5, 3.0 and 4.0%(w/w) but the amount of beads was also dependent on the rate of delivery of the polymer solution (see discussion below on the rate of delivery). The formation of electrospun beaded fibres largely depends on solution viscosity, net charge density and surface tension of the solution(117). Changing the polymer concentration alters the solution viscosity. A higher polymer concentration and net charge density would reduce the surface tension as well as encourage the elongation of a polymer jet – thus favouring the production of beadless fibres. At a low polymer concentration, surface tension on the other hand predominates – favouring the formation of beaded fibres. Thus, in this study, the predominance of surface tension may account for the beaded fibre morphology at polymer concentration of 4.0%(w/w) and below; while above a polymer concentration of 4.0%(w/w), uniform bead-free fibres were observed at polymer concentrations of 5.0 and 7.5%(w/w). In the study reported by Tan et al., the minimum polymer concentration to achieve bead-free fibres was at a polymer concentration of 4.5%(w/w) which was lower but relatively in agreement with our findings[polymer concentration of 5.0%(w/w)](112). The surface tension at a polymer concentration of 2.5%(w/w) can also account for the higher bead circularity (closer to 1, indicating a circular-shaped bead) as compared to the lower bead circularity (closer
to 0, indicating a more spindle-shaped bead) at a higher polymer concentration of 4.0% w/w, at a given rate of delivery (Fig. 3.11).

The fibre morphology has been found to change from circular to ribbon-shaped as seen with the increase in polymer concentration from 2.5 to 5.0% (w/w). The fibres electrospun at a polymer concentration of 7.5% (w/w) consisted entirely of ribbon-shaped fibres which may account for its plateau or dip as well as the larger standard deviation in diameter measurements on Fig. 3.6 and 3.7, as averages of the widths and heights of the fibres were taken. Ribbon-like fibre morphology is caused by the rapid solvent evaporation during electrospinning, resulting in the collapse of the polymer fibre with intact polymer “skin” as described by Koombhongse et al. (153) (Fig. 3.13). As a whole, the effects of polymer concentration on the fibre diameter and morphology presented in this chapter were in agreement with previously reported studies (101; 112; 117; 150; 151; 154).

![Fig. 3.13 Schematic illustration of formation of ribbon-shaped fibres during electrospinning. Rapid phase separation during electrospinning results in collapse of the polymer fibre (107; 153).](image)

### 3.4.2.3 Effects of Accelerating Voltage

The accelerating voltage is a crucial factor for the initiation of electrospinning of a polymer solution. A high voltage supply is required to induce the necessary coulomb self-repulsive charges in the polymer solution and to generate an external electric field, in order to overcome the surface tension forces of the electrospinning solution. In general, an accelerating voltage of approximately 6 kV is sufficient for the formation of a polymer solution Taylor Cone as well as jet initiation (101; 152).
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initiation of electrospinning jets in this study was observed at a similar accelerating voltage of approximately 6kV. In theory, after the initiation of the electrospinning jet, further increase in the accelerating voltage would increase the coulomb repulsive forces at the surface of the initial polymer solution, thus resulting in more polymer solution being removed in the electrospinning jet, leading to an increase in jet diameter(150;152). This may explain the initial increase in fibre diameter as observed when the accelerating voltage was increased to 10 - 15kV at polymer concentration of 2.5, 3.0, 4.0, 5.0 and 7.5%(w/w).

A further increase in the accelerating voltage would result in a further amplification in the charges on the electrospinning jet, leading to a further enhancement of the coulomb self-repulsive force. This coulomb self-repulsive force further accentuates the whipping instability as well as the elongation of the jet, hence thinning the electrospun fibre as it “whips” through mid-air en route to the collector(155-157). This may account for the decrease in fibre diameters observed from accelerating voltage of 15 to 20kV, at polymer concentrations 3.0 – 7.5%(w/w). At this point, when additional increase of accelerating voltage causes the coulomb self-repulsion forces to greatly exceed the forces of surface tension - multiple jets of fibres split from the main electrospinning jet, yielding fibres with smaller diameter but may lack uniformity(112;150). This may account for the smaller fibre diameters observed at polymer concentrations of 5.0 and 7.5%(w/w) electrospun at an accelerating voltage of 25kV. The findings of the effects of accelerating voltages on electrospun fibre diameters were consistent with previously reported studies(150;155;156;158).

As mentioned earlier, at a lower polymer concentration of 1.0 - 1.5%(w/w), higher accelerating voltages of 20 to 25kV result in the electrospaying of the polymer. Electrospraying occurs when the polymer concentration is too low to allow sufficient polymer chain entanglement, coupled with the dominance of surface tension as a result of a relatively high solvent to polymer content. Consequently droplets of the polymer break away from the initial electrospinning jet when a sufficient accelerating voltage is received(101). Several electrospinning studies have reported a positive correlation between accelerating voltage and the production of beads. This may be due to an increased instability of the jet when the Taylor Cone is receded into the spinneret as a result of excessive removal of polymer solution during
electrospinning(101;118;150). However, the increase in accelerating voltage in this study did not significantly influence the production of beads on fibres, which was consistent with data reported by Zuo et al.(157).

As a whole, although alterations in fibre diameters were observed with a change in accelerating voltage, the effects on fibre diameters were less significant compared to that of polymer concentration. This was in agreement with the findings of several previous studies(112;158-160).

3.4.2.4 Effects of Rate of Delivery of Polymer Solution

The rate of delivery of the polymer solution governs the amount of solution available to maintain the Taylor Cone for electrospinning, hence the production of fibres. The interplay between the supply of polymer solution to the tip of the spinneret (governed by the rate of delivery) and the removal of polymer solution during electrospinning (controlled by the accelerating voltage) will need to be balanced for the production of uniform beadless fibres. At a given accelerating voltage, an increase in the rate of delivery during electrospinning would result in an increase in the volume of polymer solution drawn towards the target collector. Consequently, there may be a corresponding rise in fibre diameter and bead production(118;157). Nonetheless, some studies have reported the insignificant effect of the rate of delivery on the final fibre size and morphology(112). In this chapter, several observations were made on varying the rate of delivery of the polymer solution.

First of all, the diameters of fibres of polymer concentration of 1.5, 2.5, 5.0 and 7.5%(w/w), produced at 0.2mL/hr were significantly high compared to those electrospun between 0.4 – 1.0mL/hr. As described in the results section, at polymer concentration of 2.5 – 7.5%(w/w), the diameter of fibres electrospun at 1.0mL/hr were significantly decreased compared to the rate of delivery between 0.2 – 0.8mL/hr. No significant changes in fibre diameters were observed between the 0.4 – 0.8mL/hr rates of delivery. Contrary to the findings reported in previous studies(112;118;152), the increase in the rate of delivery primarily decreased the fibre diameter. This could be related to the fact that at higher rate of delivery, more polymer solution was carried by the electrospinning. Initially, the solvent on the surface of the jet evaporates leaving
behind a “polymer skin” which still holds some polymer solution within its core. As the jet travels further in mid-air, more solvent from the core is evaporated therefore causing a greater collapse of the entire jet leading to a further contraction in the fibre diameter.

By increasing the rate of delivery, an increase in the rate of fibre production was observed but at the expense of producing beaded fibres. This can be explained by the fact that an increase in the rate of polymer solution delivery would “dilute” the charge density applied at a constant accelerating voltage(161). This would lead to a reduction in self-repulsive forces necessary for the elongation of the jet during electrospinning. As a result, surface tension would predominate, causing the formation of beads. This proportional increase in beaded fibres with the increase in the rate of delivery is consistent with the currently reported studies(118;157). The effects of the surface tension on the morphology of the beads can be seen from the decreasing trend in circularity with increasing polymer concentration. The trend in bead circularity demonstrated in this chapter is in keeping with the results reported by Lee et al.(154).

In this study, an increase in the rate of delivery from 0.6mL/hr also increases the size of material plug accumulated at the tip of the needle. The accumulation of polymer plug was observed especially at polymer concentration of 5.0%(w/w) and above. These plugs frequently clogged the flow of the polymer solution and required frequent manual removal of plugs. This resulted in wastage of the polymer material and disruption to the electrospinning process. The rate of delivery at 0.2 - 0.4mL/hr may be an ideal parameter for the fabrication of electrospun PLLA scaffold for further tissue engineering studies in this thesis.

3.4.2.5 Effects of Type of Collector

The aluminium rods of the mandrel have been used to collect fibres spun at a PLLA concentration of 2.5%(w/w) and above. The space between the rods makes the collection of the fibres for SEM simple without disrupting the fibres. Materials spun at a polymer concentration below 2.5%(w/w) were collected directly onto glass cover slips mounted flat aluminium plate. This was to ensure that fibres as well as electrosprayed droplets were collected. The aluminium rods aligned the fibres
perpendicular to the rods while the fibres deposited on the glass cover slip were randomly aligned. Although both types of collectors ensured adequate collection of fibres in this parametric study, further modifications to the collector will need to be made in order to produce scaffolds for tissue culture at a larger scale. To produce sufficient scaffolds, the diameter of the rotating mandrel will need to be increased (from the current 12cm to approximately 30cm) to increase the catchment area.
3.5 Conclusion

The first aim of this chapter was to examine the structural features of the hyaline cartilage extracellular matrix. The human hip articular cartilage, together with the reconstituted bovine nasoseptal collagen provided insight to the nanoscaled architecture of cartilage tissue. The understanding of the structural organisation and self-assembly properties of the native cartilage gathered in this chapter formed the basis for the fabrication a structurally mimetic tissue scaffold for cartilage regeneration. The electrospun nanofibrous scaffold produced in this study appear to resemble the structural features seen in the native human cartilage (Fig. 3.14).

![Fig. 3.14. A comparison between the structural architecture of the native human hyaline cartilage and an electrospun PLLA scaffold fabricated from this chapter. (a) A representative SEM image of the native human hyaline cartilage demonstrating the ECM structural architecture. (b) A representative SEM image of an electrospun PLLA scaffold fabricated at polymer concentration 2.5%(w/w), 0.2mL/hr, 10kV and target distance of 10cm. The resemblance of the scaffold morphology and architecture to that of the native hyaline cartilage was demonstrated following an extensive characterisation of the key processing parameters of our custom-built electrospinner.](image)

The electrospinning parametric study described in this chapter characterised the first in-house built electrospinning setup within our group. The effects of various processing parameters of electrospinning were investigated including polymer concentration, types of solvents, accelerating voltage, rate of delivery and type of collectors. With the use of the high molecular weight PLLA of 300kDa, the parameters which were observed to have the most profound effect on the electrospun fibre morphology and size were the polymer concentration and type of solvents. The accelerating voltage and rate of delivery of the polymer solution affected the ease of
fibre production and to a certain extent the fibre size. An increase of rate of delivery was also correlated with the production of beaded fibres. The target distance of 10cm ensures adequate deposition and collection of fibres while the type of collector affects the alignment of fibres. From this study, the optimal range of parameters required to produce uniform, and bead-free nanoscaled electrospun PLLA fibrous scaffold have been determined (Table 3.1). With the use of our electrospinner, operated at the optimal range of parameters determined in this chapter, a structural biomimetic tissue scaffold can be fabricated for further tissue engineering studies in this thesis.

<table>
<thead>
<tr>
<th>PLLA molecular weight</th>
<th>300kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of solvents and ratio</td>
<td>DCM:DMF, 7:3</td>
</tr>
<tr>
<td>Polymer concentration</td>
<td>2.5 - 4.0%(w/w)</td>
</tr>
<tr>
<td>Target distance</td>
<td>10cm</td>
</tr>
<tr>
<td>Rate of delivery</td>
<td>0.2 - 0.4mL/hr</td>
</tr>
<tr>
<td>Accelerating voltage</td>
<td>10 – 15 kV</td>
</tr>
<tr>
<td>Type of collector</td>
<td>Dependent on the desired fibre alignment</td>
</tr>
</tbody>
</table>

**Table 3.1.** Current optimal electrospinning parameters determined for the fabrication of PLLA fibrous scaffolds using the in-house built electrospinner. This set of parameters will form the key processing parameters in the fabrication of PLLA scaffold for subsequent chapters of this thesis.
Chapter 4

Biofunctionalised Electrospun Scaffolds with Cell-mediated TGF-β1 Activation: In-vitro Study

4.1 Introduction

Growth factors are one of the most vital bioactive cues in providing the appropriate regulatory signals to promote proliferation, maintain differentiation and induce matrix production(19;162). However, in-vivo applications of growth factors for tissue regeneration is still impractical due to their short half-lives and uncontrolled-dose related adverse effects(142) (Chapter 1, Section 1.7). As a result, attempts in tissue engineering have been made to incorporate growth factors into scaffold delivery systems to overcome these challenges(50;79;142). Growth factors such as fibroblast growth factor-2 (FGF-2), transforming growth factor-β1 (TGF-β1), platelet-derived growth factor (PDGF) and insulin-like growth factor 1 (IGF-1) are well known for their proliferative and anabolic effects in cartilage tissue engineering(17;19;162-165). Among these growth factors, TGF-β1 has been selected for this study for its broad spectral chondrogenic multi-regulatory roles such as inducing cell proliferation, maintaining chondrogenic differentiation, and promoting extracellular matrix (ECM) production(166;167). TGF-β1 is a growth factor that is secreted by chondrocytes and most cell types. Therefore successful TGF-β1 research outcomes may be transferable to other fields of tissue engineering.

In the native cartilage ECM, the TGF-β1 is secreted by chondrocytes in its latent form(168). The latent TGF-β1 consists of a mature (active) TGF-β1 and its pro-
peptide which is also known as the latency-associated peptide (LAP) (166;169) (Fig. 4.1). The LAP consists of two 249-residue polypeptide chains, dimerised by two disulphide bonds at cysteine-223 (Cys-223) and Cys-225 positions (170;171). The LAP associates with the mature TGF-β1 by non-covalent interactions, forming the latent TGF-β1 complex, also known as small latent complex (SLC). The SLC is then bound to the latent TGF-β1 binding protein (LTBP) forming the large latent complex (LLC) which is covalently anchored to the ECM. This complex formation between SLC and LTBP is mediated by an intramolecular disulphide exchange between the third 8-cysteine (8-Cys-3) domain of LTBP with a pair of Cys-33 residues of LAP (170;171) (Fig. 4.1).

The LAP-TGF-β1 association preserves the bioactivity of the growth factor. The half-life of the latent TGF-β1 of rats has been reported to be greater than 100 minutes as opposed to the short 2 - 3 minutes of the active form (172). Therefore in the native ECM, the latent TGF-β1 complex acts as a “storage” for the growth factor (173). The activation of TGF-β1 involves the disruption of the non-covalent interaction between the LAP and TGF-β1. There are several mechanisms known to activate the latent TGF-β1. These include proteases, thrombospondin-1, low pH and reactive oxygen species (166;169;174). The LAP also possesses two RGD sequences which when attached to a cell, activates the TGF-β1 (169).

In general, TGF-β1 has a growth stimulating effect on mesenchymal cells, whereas in epithelial and endothelial cell origin the effects on growth have been found to be inhibitory (174). Although the use of TGF-β1 in various studies has been shown to positively regulate osteogenesis, angiogenesis and chondrogenic proliferation and differentiation, its multipotency has its own drawbacks. The cell response varies depending on the dosage level, length of exposure and the target tissue (174). A prolonged exposure and excessively high dose of TGF-β1 may result in pathological conditions such as extensive fibrosis, hypertrophic scarring and formation of osteophytes (174-176). These adverse reactions may be counter productive in the development of a functional biological tissue replacement. Therefore, it would be highly beneficial to design a system capable of inducing cell-mediated TGF-β1 activation. The novel tissue engineering strategy proposed in this thesis is to incorporate the latent TGF-β1 onto an electrospun scaffold system, therefore allowing
cells to sense and activate the TGF-β1 as required. For this reason, the proposed biofunctionalisation strategy is to anchor the entire latent TGF-β1 complex onto the electrospun scaffold. To date, little is known about the effects of using the latent TGF-β1 complex as a cell-mediated bioactive cue.

In this chapter, two techniques of scaffold biofunctionalisation are described. The techniques involved initial scaffold surface modification using ammonia plasma, with or without the addition of a heterobifunctional crosslinking agent, sulfo succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC). The combined effects of the nanoscale scaffold fabrication and subsequent biofunctionalisation with latent TGF-β1, on cell behaviour are reported here using primary human nasal chondrocytes for cartilage regeneration applications.

**Fig. 4.1.** Schematic representation of the natural occurrence of the latent TGF-β1 complex in the native ECM. The latent TGF-β1 (LTGF) is attached to the latent TGF-β binding protein (LTBP) which is anchored to the ECM(169). The latency associated peptide (LAP) offers protection and “stores” the active TGF-β1. The 3D protein structure of the active TGF-β1 was obtained from the RCSB Protein Databank (PDB 1KLC). The visual molecular dynamic (VMD) model of the TGF-β1 molecule (yellow, white, turquoise) were rendered using VMD software(177) (University of Illinois, USA).
4.2 Materials and Methods

4.2.1 Scaffold Fabrication by Electrospinning

The non-woven PLLA fibrous scaffolds were electrospun using our custom built electrospinner as described in Chapter 3. Briefly, 3.0%(w/w) PLLA (Mw~300kDa, Purac Biochem, UK) dissolved in dichloromethane/dimethylformamide [70:30(w/w)] (AGTC Bioproducts, UK) was electrospun at 0.4mL/hr, 10kV and 10cm target distance.

The electrospun scaffolds were then sectioned using a sterile scalpel (Blade No.22, Swann-Morton, UK) into 1.5 × 1.5cm² and 4 × 4cm² squares prior to the sterilisation procedure. They were sterilised by ultraviolet irradiation in a sterile laminar flow hood for 30 minutes, followed by rinsing in 70%(v/v) ethanol solution. A portion of the scaffolds were rinsed in sterile PBS, and used as a control for physical and biological characterisation of scaffolds (see below) while the remaining portion were allowed to dry in the sterile cell culture hood. The scaffolds were then ready for subsequent surface modification using ammonia gas plasma.

4.2.2 Biofunctionalisation of Scaffolds

4.2.2.1 Surface Modification of Electrospun Scaffolds by Plasma Treatment

The electrospun PLLA 3.0%(w/w) scaffolds were treated with ammonia gas (NH₃) plasma using a plasma instrument (Plasprep 5, Gala Instrumente, Germany). The treatment process was performed at a range of exposure times (2, 5 and 10 mins) and powers (10 - 100W), while the pressure was kept constant at 2.0mbar. Surface chemical analyses were performed using X-ray photoelectron spectroscopy (XPS) and ninhydrin assay to identify incorporated surface amine groups.

4.2.2.2 Biofunctionalisation of Scaffold with Latent TGF-β1 Complex

In order to evaluate the growth factor bioactivity of as a result of the orientation of the latent TGF-β1, two different immobilisation strategies were performed to incorporate the latent complex onto the electrospun scaffolds. The first technique anchors the
latent TGF-β1 at non-specific sites of the LAP (random immobilisation) while the second crosslinks the latent TGF-β1 complex at a specific predicted site, cysteine-33 (Cys-33) position of the LAP (Fig. 4.1), using sulfo-SMCC (orientated immobilisation).

For the random immobilisation of the recombinant human latent TGF-β1 (R&D Systems, UK) onto the PLLA scaffolds, freshly prepared NH₃ plasma-treated PLLA scaffolds were immediately immersed in latent TGF-β1 solution (0.75µg/mL in PBS) for 2 hours at room temperature (Fig. 4.2). The supernatant was removed and the scaffold rinsed three times with sterile PBS.

For oriented immobilisation of the latent TGF-β1 (0.75µg/mL), the surface amine groups of the scaffolds were covalently linked to the cysteine residues of the latent TGF-β1 using sulfo-SMCC (Sigma, UK) as a heterobifunctional crosslinker (Fig. 4.2). The N-hydroxysuccinimide (NHS) end of this reagent typically couples to molecules containing primary amines, corresponding to the surface amine groups on plasma treated PLLA scaffolds, by formation of stable amide bonds at pH 7. The maleimide end of sulfo-SMCC couples specifically with free sulfhydryl groups by thioether linkage at pH 6.5 - 7.0. The latter reaction was predicted to occur between the cys-33 residue of LAP and the maleimide end of sulfo-SMCC.

Briefly, plasma treated PLLA samples were rinsed gently three times with sterile PBS to ensure the removal of unwanted free radicals generated from the plasma treatment. The scaffolds were then immersed in a 3mg/mL sulfo-SMCC solution in distilled water and left to react for 2 hours under agitation (orbital shaker) in a sterile cell culture cabinet. The supernatant was discarded and the scaffolds were rinsed three times with sterile PBS. The maleimide treated scaffolds were then immersed in a solution of recombinant latent TGF-β1 (0.75µg/mL) in sterile PBS for 2 hours. The supernatant was removed and the scaffold rinsed three times with sterile PBS. Finally, both types of biofunctionalised scaffolds were then placed in respective cell culture well plates for cell seeding.
Fig. 4.2. Schematic illustration of both random and orientated biofunctionalisation of PLLA scaffolds with latent TGF-β1, following ammonia plasma surface modification and chemical modification with sulfo-SMCC.
4.2.3 Characterisation of Biofunctionalised Scaffolds

4.2.3.1 Surface Chemistry Analyses using XPS\(^a\) and Ninhydrin Assay

X-ray photoelectron spectroscopy (XPS) is a quantitative spectroscopic technique for surface chemical analysis. It can be used to characterise elemental composition and chemical of a polymer surface. The depth of analysis has been reported to be typically less than 10nm from the surface.

In an ultra-high vacuum environment, the XPS utilises monochromated x-rays to excite core electrons of atoms in a sample. This leads to the ejection of electrons (photoelectrons) which have characteristic kinetic energy that are unique to the elements of the sample. The photoelectrons are then received by a detector and their characteristic kinetic energies are analysed. Since it is the binding energy of electron in the sample which is the important physical parameter, the spectrometers are programmed to convert the kinetic energies of emitted electrons and present the results in the form of binding energies. The binding energy can be derived from the emitted kinetic energy using the equation below:

\[ E_K = h\nu - E_B - \phi_{SP} \]  

Where \( E_K \) is the measured kinetic energy of the emitted electron, \( h\nu \) is the energy of the exciting X-ray photon, \( E_B \) is the electron binding energy in the sample and \( \phi_{SP} \) is the work function of the spectrometer (~5eV)(178;179).

In the field of biomedical and tissue engineering, the XPS has been used to characterise various surface modified biomaterials and protein adsorption on surfaces(180-182). By providing the essential information on elemental composition and chemical state, approaches to surface engineering technologies can be optimised. In this thesis, the XPS technique was performed to characterise the effects of ammonia plasma surface modification of the electrospun scaffolds.

\(^a\) The XPS analysis and data processing was performed by Ms. Emily Smith at the Centre for Surface Chemical Analysis, University of Nottingham. The processing protocol and results described in this chapter are based on the report of analysis provided by Ms. Smith (report no.:08-015).
XPS spectra of the surface modified samples and control were acquired on a Kratos–AXIS ULTRA DLD X-ray Photoelectron Spectroscopy Instrument using a monochromated Al kα X-ray source (1486.6eV) operated at 10mA emission current and 10kV anode potential – 100W. A photoelectron analyser take-off angle of 90° with respect to the sample surface was used. All measurements were taken under vacuum (3 x 10⁻⁹ Torr). The high resolution scans were charge corrected to the main C 1s peak (285eV) and then quantified to compare the amounts of each element present, using Kratos sensitivity factors(183). Components were fitted under the peaks to give chemical information. Data analysis was carried out using CASAXPS software with Kratos sensitivity factors to determine atomic percentage (%) values from the peak areas.

Ninhydrin (2,2-Dihydroxyindane-1,3-dione) is a chemical used to specifically determine the presence of primary amine groups. The reaction of ninhydrin with primary amine groups produces a dark blue (Rheumann’s Blue) colour. The assay was performed as previously described(184;185) to quantitatively detect the amount of amine groups on the ammonia plasma surface modified PLLA scaffolds. Briefly, the scaffolds and controls were immersed in 1M ninhydrin solution for 1 minute in glass vials. The vials were then heated up to 80°C for 15 minutes to accelerate the reaction between ninhydrin and the amine groups on the scaffolds. The ethanol component was allowed to evaporate under reduced pressure for a further 15 - 20 minutes. After the removal of the adsorbed ethanol, the scaffolds were dissolved in 5mL of 1,4-dioxane. The absorbance was measured at 570nm on a UV-vis spectrophotometer (Lambda 25, Perkin Elmer, USA). A calibration curve was obtained using glycine standards (Fig. 4.3, please see Appendix 1.6 for protocol).
Fig. 4.3. Glycine standard curve for ninhydrin assay. The $R^2$ value was 0.9984.

4.2.3.2 Quantification of Latent TGF-β1 on Scaffolds using an Immunoassay

The quantification of the immobilised latent TGF-β1 was performed on biofunctionalised scaffolds and compared to controls (Table 4.1) using a modified procedure described previously(168). The latent TGF-β1 biofunctionalised scaffolds were initially digested with 0.3U/mL of plasmin (Sigma-Aldrich, UK) in phenol free DMEM for 3 hours at 37°C to release the latent TGF-β1 from the scaffold. The reaction was stopped by the addition of aprotinin (Sigma-Aldrich, UK) to a final concentration of 5µg/mL. The supernatant was then collected and subjected to acidification with subsequent neutralisation. This was performed to release the active TGF-β1 from the latent complex. Briefly, 20µL of 1N HCl was added to every 100µL of supernatant and incubated at room temperature for 10 minutes. The acidified samples were then neutralised (pH 7.2 - 7.6) by adding 1.2N NaOH/0.5M HEPES. Subsequently, quantification of the activated TGF-β1 using Quantikine Human TGF-β1 Immunoassay (R&D Systems, UK) was performed as per manufacturer’s protocol. The standard curve for the quantification of TGF-β1 was generated as per
manufacturer’s protocol (Fig. 4.4). The amount of TGF-β1 released from the latent TGF-β1 biofunctionalised scaffolds were calculated using this standard curve.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLLA</td>
<td>Untreated electrospun scaffolds as control</td>
</tr>
<tr>
<td>ptPLLA</td>
<td>Plasma treated electrospun scaffolds as control</td>
</tr>
<tr>
<td>TGF</td>
<td>Plasma treated electrospun scaffolds with media supplementation of 10ng/mL of TGF-β1 for up to 7 days</td>
</tr>
<tr>
<td>pLTGF</td>
<td>Plasma treated electrospun scaffolds with immediate random immobilisation of latent TGF-β1</td>
</tr>
<tr>
<td>SMC</td>
<td>Plasma treated electrospun scaffolds modified with sulfo-SMCC</td>
</tr>
<tr>
<td>sLTGF</td>
<td>Plasma treated electrospun scaffolds modified with sulfo-SMCC and conjugated with latent TGF-β1 (orientated immobilisation)</td>
</tr>
</tbody>
</table>

*Table 4.1.* Experimental groups and their sample identification (ID). These sample ID will be used to refer to respective groups throughout the study.

![Standard curve of Quantikine TGF-β1 immunoassay with an R² value of 0.9889. The curve was achieved as per manufacture’s protocol.](image)

**Fig. 4.4.** Standard curve of Quantikine TGF-β1 immunoassay with an R² value of 0.9889. The curve was achieved as per manufacture’s protocol.
4.2.3.3 Cell Culture

Human primary nasal septal chondrocytes used in this study were kindly provided by Dr. Reza Ghazanfar (Imperial College London, UK). The chondrocytes were expanded up to third passage in chondrocytic growth media (CGM) consisting of DMEM (41966, Gibco, UK) supplemented with 2mM L-glutamine (Gibco, UK), 1%(v/v) non-essential amino acid (Gibco, UK), antibiotics-antimycotics (Gibco, UK) (100 U/mL penicillin, 1000U/mL streptomycin, 0.25µg/mL AmphotericinB), 50µg/mL L-ascorbic acid (Sigma, UK) and 10%(v/v) fetal bovine serum (Gibco, UK). The cells were maintained at 37°C in a humidified 5% CO₂ atmosphere. The culture media was replaced every 3 days. Cells were then harvested and seeded on to 1.5 × 1.5cm² scaffolds (see Table 5.1) for all biological assays at a seeding density of 9000 cells/cm². For the gene expression studies, samples were seeded at 3.125 × 10⁴ cells/cm² on 4 × 4cm² scaffolds. Approximately 5 × 10⁵ cells were snap-frozen and used as day-0 specimen for gene expression analysis.

In order to evaluate the true effects of the latent TGF-β1 biofunctionalised scaffolds in chondrocytic cell culture without the influence of any other growth factors, serum-free media(165;186) was used throughout the experiment. The serum-free media consisted of CGM supplemented with 100nM dexamethasone (Sigma, UK), ITS+ premix (BD Bioscience, UK) (diluted for final concentrations of 6.25µg/mL insulin, 6.25µg/mL transferrin, 6.25µg/mL selenious acid, 1.25mg/mL bovine serum albumin, 5.33µg/mL linoleic acid), but without fetal bovine serum. Serum-free media was replaced every 3 days. Cells cultured on plasma treated scaffolds (TGF experimental group) received an additional 10ng/mL of human recombinant TGF-β1 (R&D Systems, UK) supplementation for up to 7 days and were used as a control. The cell-scaffold constructs were collected at 1, 7 and 14 days for evaluation of cell viability, metabolic activity and gene expression.

4.2.3.4 Cell Viability Assay

Cell viability was assessed using the LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Netherland). The assay was performed on days 1, 7 and 14. At each of time point, samples were rinsed gently with warm sterile PBS (Gibco, UK)
prior to the incubation with the Live/Dead solution (2µM calcein-AM and 4µM ethidium homodimer-1) at room temperature in the dark for 30 minutes. The cell-scaffold constructs were analysed by fluorescence microscopy using an Olympus BX-51 reflected fluorescence microscope equipped with Olympus DP070 colour digital camera and Olympus DP Controller software (Olympus UK Ltd., UK) for analysis. Intracellular esterase activity in live cells converts calcein AM into fluorescent green calcein (495 - 515nm), which is then retained within the cells. Ethidium homodimer-1 only enters dead cells with ruptured cell membranes, and undergoes a 40-fold enhancement of fluorescence upon binding to their DNA causing the nuclei of the dead cells to fluoresce red (495 - 635nm). The dyes are virtually non fluorescent before interacting with the cells. The fluorescence microscopy images were taken from two samples with triplicate images. The number of viable cells (green) and dead (red) cells was counted. Cell viability was then calculated as a percentage average of live cells over total number of cells (green + red) and presented as mean percentage ± standard deviation.

4.2.3.5 Cell Metabolic Activity Assay

The effects of the various scaffolds on cell proliferation was assessed using the CellTiter 96™ Aqueous One Solution Cell Proliferation Assay (Promega, UK) (186). The cell proliferative activity is determined colorimetrically using tetrazolium compound MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] which is bio-reduced in mitochondria of metabolically active cells into coloured formazan product that is soluble in tissue culture medium. A higher absorbance indicates a higher metabolic activity may be associated with proliferation of cells. The assay was performed on days 1, 7 and 14. Briefly, the samples (cell-scaffold constructs) were transferred to new well plates and rinsed gently in sterile PBS (three times). The samples were then incubated with 400µL phenol red free DMEM plus 80µL of CellTitre 96 reagent, for 4 hours, at 37°C in a humidified 5% CO₂ environment. Aliquots of the supernatant (100µL) were transferred to 96-well plates in triplicate and optical density was measured at 490 nm using a microplate reader Anthos 2020 (Biochromo Limited, UK). A sample incubated under the same conditions in the absence of cells was used as a blank. After
the readings were obtained, all samples were rinsed gently with PBS (three times), and then resuspended in 400µL sterile deionised water. All samples were then subjected repeated freeze-thaw cycles for DNA extraction. DNA content was quantified using the ND-1000 UV-Vis Spectrophotometer (Nanodrop®, USA). The experiment was performed in triplicate and the results, normalised to DNA content, were presented as percentage averages ± standard deviation.

4.2.3.6 Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

PCR is a technique used to replicate and amplify any nucleic acid sequences. It consists of repetitive cycles of initial thermal separation of DNA chains (also known as denaturation, typically at 94°C) to provide templates for subsequent enzymatic propagation of complimentary deoxynucleotides binding. These complimentary binding of deoxynucleotides are initiated at an annealing temperature (typically 40 - 72°C), by a short synthetically produced DNA sequence called a primer. A DNA polymerase then attaches specific nucleotides to the primer according to base pairing rules(187). This step replicates the DNA segment of interest. The entire process is then repeated to thermally separate the replicated segment from the template DNA, which subsequently becomes a template itself. This leads to an amplification of the desired segment of DNA following several repetitive cycles.

Since the invention of PCR in 1983, the technique has undergone a series of evolutionary development which gave rise to the RT-PCR. The advent of RT-PCR meant that the identification and quantification of a specific RNA transcript can be achieved with a small amount of starting material. As its name implies, a reverse transcriptase (also called RNA-dependent DNA polymerase) synthesises a complimentary chain of DNA using the same process described above(187;188). The complimentary DNA (cDNA) is then used for subsequent amplification and quantification of genes.

The increasing importance of genetic quantification and profiling in scientific research and medical diagnostics has led to the development of quantitative real-time RT-PCR technologies - in terms of chemistry and automated instrumentation. The
term real-time refers to the technique of simultaneous data collection and display throughout the PCR process, hence combining amplification and detection into a single step. This is accomplished using different fluorescent chemistries. As the chemicals only fluoresce when bound to the PCR product, the fluorescence intensity is directly proportional to the concentration of product of amplification. As the fluorescence emission is monitored and recorded over time, the point in time or PCR cycle where the fluorescence signal significantly increases over a set threshold or background intensity can be indentified. This point in the PCR cycle is known as the cycle threshold ($C_T$). The $C_T$ can be correlated with the initial starting amount of cDNA template. Thus, the higher the initial amount of template, the earlier a significant fluorescence signal is detected(189;190). The real-time RT-PCR uses the same principles as conventional RT-PCR. However there are two fundamental differences: (1) The PCR product of real-time RT-PCR is detected and quantified using a fluorescent marker while the conventional method uses gel electrophoresis, and (2) The real-time PCR product accumulation is measured at every PCR cycle whereas in conventional RT-PCR only the end point of intensity is measured(187).

In a real-time RT-PCR study, variations may arise from both reverse transcriptase reaction and the PCR process itself which may lead to unreliable results. Differences in the efficiencies of the reverse transcriptase and PCR amplification will result in differences in the cumulative amount of the final PCR product. Although the quantification in real-time RT-PCR is based on the $C_T$, the potential variation in reverse transcriptase and PCR efficiencies must be corrected for. This is achieved by normalisation of the target gene to a housekeeping gene. A housekeeping gene is a gene which is expressed at a constant level, in all tissues and at all phases of development. The commonly used housekeeping genes include: β-actin, a cytoskeletal protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme and ribosomal RNA 18S (18S) which is responsible for protein synthesis(187;191). The 18S has been found to be the most stable gene and least affected by exogenous factors, thus most suitable for normalisation for various cell types including cartilage(191-193).
The aim of tissue engineering is to produce an alternative substitute that carries the structure and function unique to tissue it is designed to replace. As mentioned in Section 1.2, specialised cells or stem cells are often required to regenerate tissues with specific function. In the first instance, autologous cells are an appropriate source to avoid risks of transmittable diseases. However, the isolation and expansion of cells often lead to a process of dedifferentiation which results in the loss of specific cell function. Various strategies in attempt to recover the differentiated states of the tissue engineered cells have been reported. To evaluate the success of their strategies, gene expression studies using the reverse transcriptase polymerase chain reaction (RT-PCR) technique have been central in providing the essential information at genetic level. In this thesis, the RT-PCR technique has an important role in the assessment of cell behaviour. It is therefore essential to have an understanding of the basic principles of real time RT-PCR.

In this thesis, total RNA was extracted from the chondrocytes by the addition of 10µL –mercaptoethanol in 1mL RLT-Buffer (QIAGEN, UK). Total RNA was isolated using the RNeasy mini kit, treated with RNase-free DNase 1 (both QIAGEN), according to the manufacturer’s protocol and quantified using the ND-1000 UV-Vis Spectrophotometer (Nanodrop®, USA). 1000ng of total RNA was reverse transcribed (RT) into cDNA and a mastermix prepared for each reaction containing: 10µL Taqman™ universal mastermix (Applied Biosystems, USA), 7µL 0.1%(v/v) diethylpyrocarbonate (DEPC) water (Invitrogen Ltd, UK), 2µL extracted cDNA and 1µL Taqman probe (Applied Biosystems, USA). Taqman Gene Expression Assays used to amplify genes are detailed in Table 4.2. 18S was used as a control gene. Each reaction was carried out in triplicate. The PCR reaction was initiated by a 2 minute 50°C and 10 minute 95°C step to optimise thermal cycling conditions for the ABI Prism 7700 sequence detection system (Applied Biosystems, USA) used to detect relative quantification of gene expression. This was followed by PCR amplifications performed for 40 cycles in a Corbett Rotor-Gene 6000 (Corbett Life Science, Australia) at 95°C for 15 seconds and 60°C for 1 minute. The target signal was plotted against the number of cycles and the threshold level was set at 0.05. Comparison of all data was taken at the intercept, where sample reactions crossed this phase of amplification. Our results were correlated using the comparative CT
method(194). Fold changes in gene expression were presented as mean ± standard deviation change relative to day 0 cells.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Catalogue no.</th>
<th>Accession no.</th>
<th>Chromosome Location</th>
</tr>
</thead>
<tbody>
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<td>SRY (sex determining region-y)-box 9</td>
<td>Sox9</td>
<td>Hs00165814</td>
<td>NM000346</td>
<td>Chr.17</td>
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<tr>
<td>Collagen Type 1</td>
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<td>Hs00164004</td>
<td>NM000088</td>
<td>Chr. 17</td>
</tr>
<tr>
<td>Collagen Type 2, alpha 1</td>
<td>Col2A1</td>
<td>Hs00156568</td>
<td>NM001844.4</td>
<td>Chr.12</td>
</tr>
<tr>
<td>Eukaryotic 18S rRNA</td>
<td>18S</td>
<td>Hs99999901</td>
<td>X03205</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Table 4.2.** Taqman™ gene probes used in this study with details of their sources and specificities.

### 4.2.4 Statistical Significance

The means and standard deviations of the results were calculated using the SPSS 12.0 software package (SPSS Inc., Chicago, USA). The Mann-Whitney U Test for two independent samples was performed to determine statistical significance between various scaffolds. A $p$ value of $<0.05$ was considered to be significant.
4.3 Results

4.3.1 Characterisation of Scaffolds

4.3.1.1 Surface Chemistry Analyses using XPS and Ninhydrin Assay

Surface chemical analysis using XPS demonstrated that nitrogenous species were incorporated onto the surfaces of PLLA following ammonia plasma treatment. In the XPS spectra (Fig. 4.5), the intensity of surface nitrogenous species (400.0eV) on PLLA surfaces increased proportionally with the increase of both plasma treatment exposure time and power when compared to untreated PLLA scaffolds. The surface elemental compositional analysis (Table 4.3) revealed that at a plasma treatment power of 50W, the surface nitrogenous species increased with an increase in the treatment time – with the highest being at an exposure time of 10 minutes (3.0 ± 0.1 atomic %). When the scaffold plasma treatment was operated at a power of 100W for 2 minutes, an amount of surface nitrogenous species comparable to that of 50W for 10 minutes was observed (2.9 ± 0.2 atomic %). The highest amount of surface nitrogenous species was detected on the plasma treated scaffolds when the plasma treatment was operated at a power of 100W for 10 minutes (5.2 ± 0.3 atomic %). In other words, at an exposure time of 10 minutes, a treatment power of 100W would incorporate approximately 70% more nitrogenous species than at a treatment power of 50W.
Fig. 4.5. XPS spectra of ammonia plasma surface modified PLLA scaffolds at various exposure power (W) and time (min). (a) Non plasma treated control, (b) 50W 2min, (c) 50W 5min, (d) 50W 10min, (e) 100W 2min, (f) 100W 5min, (g) 100W 10min.

Chemical quantification using the ninhydrin assay demonstrated that the density of primary amine groups on the PLLA scaffold surfaces increased with longer exposure time and higher power during plasma treatment. The findings of the ninhydrin assay
were consistent with the results of the XPS analysis. The highest density of primary amine groups (66.4 ± 4.3nmol/mg PLLA) was found in scaffolds subjected to a plasma treatment power of 100W and 10 minutes exposure time (Table 4.3). At an exposure time of 10 minutes, an increase in plasma treatment power from 50W to 100W, increased the amount of primary amine groups incorporation by approximately 75%. The combined techniques of surface chemical analyses confirmed that the incorporation of amine groups onto the surface of PLLA electrospun scaffolds using ammonia plasma treatment were successful.

<table>
<thead>
<tr>
<th></th>
<th>Atomic % (N 1s)*</th>
<th>Amount of NH₂ (nmol/mg PLLA)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLLA control</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>50W - 2 min</td>
<td>0.6 ± 0.1</td>
<td>27.9 ± 0.3</td>
</tr>
<tr>
<td>50W - 5 min</td>
<td>1.2 ± 0.2</td>
<td>33.2 ± 0.6</td>
</tr>
<tr>
<td>50W -10 min</td>
<td>3.0 ± 0.1</td>
<td>37.9 ± 0.8</td>
</tr>
<tr>
<td>100W - 2 min</td>
<td>2.9 ± 0.2</td>
<td>44.8 ± 1.2</td>
</tr>
<tr>
<td>100W - 5 min</td>
<td>4.2 ± 0.3</td>
<td>57.8 ± 1.4</td>
</tr>
<tr>
<td>100W – 10 min</td>
<td>5.2 ± 0.3</td>
<td>66.4 ± 4.3</td>
</tr>
</tbody>
</table>

Table 4.3. Amount of nitrogen and primary amine groups detected on the surface modified PLLA scaffolds at various plasma treatment power (W) and exposure time (min). (*) determined by XPS; (**) determined by ninhydrin assay.

4.3.1.2 Quantification of Latent TGF-β1 on Scaffolds using Immunoassay

The results of the TGF-β1 immunoassay showed that the latent TGF-β1 complex was successfully grafted onto the electrospun scaffold surfaces, using both the random and orientated latent TGF-β1 immobilisation approach. An average of 195.4 ± 34.3pg/cm² of TGF-β1 was released from the pLTGF scaffold group (random immobilisation), which was significantly higher than sLTGF and control (p<0.05). Interestingly, an average of 14.1 ± 1.7pg/cm² of TGF-β1 was released from the sLTGF scaffolds (orientated immobilisation), i.e. approximately 10-fold less than the pLTGF group. No TGF-β1 was detected on the rest of the control scaffolds.
4.3.2 Biological Characterisation of Scaffolds

4.3.2.1 Cell Viability Assay

Primary human chondrocytes cultured on the various scaffolds were evaluated for cytotoxicity using Live/Dead assay on days 1, 7, and 14 (Fig. 4.6). This qualitative method showed a high live to dead ratio across experimental groups indicating that all plasma treated scaffolds were non-cytotoxic to primary human chondrocytes and maintained cell viability for up to 7 days (>80%). At day 14, cell viability declined in all experimental groups to the range of 70 - 80% except for the TGF group (57.7%).
### Chapter 4: Biofunctionalisation of Electrospun Scaffolds – In-vitro Study

<table>
<thead>
<tr>
<th></th>
<th>PLLA</th>
<th>ptPLLA</th>
<th>TGF</th>
<th>pLTGF</th>
<th>sLTGF</th>
</tr>
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<tr>
<td><strong>Day 1</strong></td>
<td>75.0±3.8%</td>
<td>92.8±8.3%</td>
<td>81.9±6.6%</td>
<td>95.1±5.7%</td>
<td>93.4±9.0%</td>
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<tr>
<td><strong>Day 7</strong></td>
<td>62.1±10.8%</td>
<td>88.5±4.7%</td>
<td>83.3±9.9%</td>
<td>86.3±2.0%</td>
<td>86.8±8.5%</td>
</tr>
<tr>
<td><strong>Day 14</strong></td>
<td>71.0±5.6%</td>
<td>77.0±8.8%</td>
<td>57.7±19.0%</td>
<td>75.2±7.8%</td>
<td>73.0±13.6%</td>
</tr>
</tbody>
</table>

**Fig. 4.6.** Cell viability of primary human chondrocytes cultured on various scaffold types at 1, 7, and 14 days (Scale bar = 200μm). Numbers shown in each images indicate the mean percentage ± standard deviation. Average number of cells per image on day 1, 7 and 14 were approximately 30, 50 and 30, respectively.
4.3.2.2 Cell Metabolic Activity Assay

The effects of the various scaffold types on cell metabolic activity of the primary human chondrocytes were evaluated using MTS assay at 1, 7 and 14 days. At day 1, cell metabolic activity of both latent TGF-β1 functionalised scaffold groups were significantly lower than other groups (Fig. 4.7). Cell metabolic activity remained significantly low in the pLTGF group at day 7. The results here show a general declining trend in cell metabolic activity with time. No significant differences between experimental and control groups were found at day 14.

Fig. 4.7. MTS assay of primary human chondrocytes cultured on various scaffold types at 1, 7 and 14 days. Results were normalised to amount of DNA (see Appendix 1.7) and presented as mean ± standard deviation. Symbols (○) and (▲) indicate significant differences when compared to the pLTGF and sLTGF groups, respectively ($p<0.05$).
4.3.2.3 Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The mRNA expression of Sox9 and Col1A1 from the chondrocytes cultured on the various scaffold types were analysed using real-time RT-PCR (Fig. 4.8). The results showed that latent TGF-β1 biofunctionalised scaffolds (pLTGF group) significantly up-regulated Sox9 expression by approximately 10-fold when compared to day 0. This level of expression was also significantly higher than all other scaffold groups in this experiment. Col1A1 was significantly higher in the TGF group, as compared to other scaffold groups.

![Real-time RT-PCR data showing mRNA expression profiles of primary human chondrocytes cultured on various scaffold types for 14 days.](image)

**Fig. 4.8.** Real-time RT-PCR data showing mRNA expression profiles of primary human chondrocytes cultured on various scaffold types for 14 days. Expression levels of Sox9 and Col1A1 were normalised to the 18S housekeeping gene and day 0 expression levels. Average change ± standard deviation of repeated experiments (n=3) are presented. The following symbols represent significant differences with (♦◊) TGF, (●○) pLTGF and (▲) sLTGF groups. Solid symbols denote Col1A1 gene whereas open symbols indicate Sox9 gene. The expression of both genes were significantly higher in the TGF and pLTGF groups when compared to the rest of the groups (p<0.05).
4.4 Discussion

The TGF-β1 has long been used as a growth inducing factor in tissue engineering. Faced with the need to overcome its drawbacks, various growth factor delivery systems have been previously explored(134;138;141;143;195). The key concept behind the scaffold system developed in this study was to guide cell-mediated activation of growth factor. This allows the cells to determine their own proliferation and function as necessary, preventing uncontrolled TGF-β1 effects which may lead to pathological conditions. To achieve this, the latent TGF-β1 complex was selected as a concept latent protein model for the development of a novel biomimetic tissue regenerative scaffold. Our results confirmed that the latent TGF-β1 biofunctionalised scaffold design successfully influenced chondrocytic behaviour and induced the highest level of Sox9 expression in this experiment. To our knowledge, this is the first study to report cell-mediated activation of growth factor for tissue engineering applications. In addition, the key principle of this research is in-line with the current trends in tissue engineering of inducing regenerative ability of engineered constructs by mimicking the structural and biological functions of the native tissue(82;84). The biological and physicochemical characterisations of the latent TGF-β1 functionalised PLLA scaffold were performed to verify the hypothesis of this study.

PLLA is a US Food and Drug Administration (FDA) approved biomaterial for biomedical applications. It was the polymer of choice for this study as it has been shown to exhibits superior ability in maintaining structural integrity and in supporting chondrocytic proliferation when compared to other members of its poly α-hydroxy ester family such as PGA, PDLGA 50:50, PDLGA 85:15, and PDLLA(15;49). In this study, PLLA fibrous scaffolds were produced by electrospinning. Electrospinning is a scaffold nanofabrication technique often used for its ability to mimic the fibrous structural architecture of tissues, such as the collagen type II network of cartilage ECM(82;84;196). In this study, the electrospun fibres produced and used for subsequent plasma surface modification were homogenous, non woven, randomly aligned, non beaded, smooth and uniform.
Since the PLLA backbone lacks reactive functional groups necessary for chemical conjugation, scaffold surface modification was performed using ammonia plasma treatment. The plasma treatment technique is a powerful, yet simple, fast and cost effective method for biopolymer surface modification without alterations to the polymer bulk properties. This technique introduces reactive functional groups and alters the surface chemistry of biomaterials commonly used in tissue engineering such as PLLA, PGA and polycaprolactone (PCL), rendering them more hydrophilic for tissue engineering applications(113;114;197). Oxygen and ammonia gas plasma treatments have previously been shown to graft reactive functional groups such as hydroxyl, carbonyl and amine onto polyester material surfaces(113;114). These functional groups were used for further surface adsorption and immobilisation of peptides and proteins such as arginine-glycine-aspartate (RGD) sequences, gelatine and collagen onto polymer surfaces and scaffolds(113;197-199). The results from the surface chemical analyses showed that the PLLA scaffold surface was functionalised with primary amine groups necessary for the subsequent conjugation with the latency associated peptide component of the latent TGF-β1 complex. The incorporation of these amine groups allowed further maleimide functionalisation of the scaffold, for subsequent conjugation with the thiol group of the latent TGF-β1 complex.

The TGF-β1 immunoassay revealed that the latent TGF-β1 complex has been successfully anchored onto the plasma treated electrospun scaffold surface. The direct linkage (random immobilisation) of the latent TGF-β1 to plasma treated scaffolds (pLTGF) was more efficient in anchoring the latent TGF-β1 complex, as compared to the orientated immobilisation approach that required the additional heterobifunctional crosslinker sulfo-SMCC (sLTGF). This could be due to the following reasons:
(1) the LAP has limited free thiol groups for cross linkage;
(2) the location of the free thiols may be sterically hindered; and
(3) the short spacer in sulfo-SMCC further limits its scope to react with thiol groups.

A combination of these three reasons may have contributed to the lower binding capacity observed in the sLTGF group. The immunoassay also demonstrated that the scaffold surface-bound latent TGF-β1 complex remained available for enzymatic and chemical activation. The bioavailability of the TGF-β1 on the latent TGF-β1
biofunctionalised scaffolds was further confirmed by primary human chondrocytic culture.

Expansion of cell number was carried out in serum containing media. However, to assess the efficacy of the various scaffold systems in promoting cell proliferation and chondrogenic differentiation, the primary human chondrocytes were cultured in serum-free media(SFM)(165;186) to avoid the influences of other growth factors. The TGF group was the only exception, receiving SFM supplemented with 10ng/mL of active TGF-β1.

The results of Live/Dead assay demonstrated that the latent TGF-β1 biofunctionalised electrospun scaffolds were non cytotoxic and sustained cell viability of over 70% throughout the experiment. The TGF group, despite receiving TGF-β1 (10ng/mL) supplementation for up to 7 days, failed to sustain the level of cell viability seen in the latent TGF-β1 group. The general declining trend in cell viability over time may be related to the use of complete serum-free media.

The serum-free environment is a well established in-vitro tool, which is used to revert the mitotic cell cycle induced by serum, into the interphase cycle(200). It is used when cells are required to re-express the differentiated function after being induced to proliferate to obtain the required cell numbers. It is also important to note that cells in an intact organ or tissue can only express specific function while they remain in the interphase. Chondrocytes naturally arrest in an infinite interphase cycle in the native cartilage(200). In this study, the serum-free environment together with other chondrogenic components have been used to revert the mitotic cell cycle (proliferation) induced by serum, into the interphase cycle to promote differentiation. A decrease in cell proliferation has been shown to occur when dedifferentiated chondrocytes redifferentiate on three dimensional culture systems in-vitro(201). Therefore, the significantly lower cell metabolic activity in the latent TGF-β1 biofunctionalised scaffold groups, from day 1 (both pLTGF and sLTGF) and in the pLTGF group on day 7, may suggest that a similar redifferentiation process may have taken place in the chondrocytes.
Chondrocytes are well known to dedifferentiate and lose their chondrocytic functions with every passage number in-vitro(33). In this study, the primary human chondrocytes were cultured up to third passage prior to seeding onto the various scaffold groups. Col2A1 was not detectable at day 14. This could be due to the use of highly passaged chondrocytes. The recovery of chondrogenic differentiation has been described as a slow process(202). Studies have also shown that passaged chondrocytes only expressed Col2A1 gene after 21 days of in-vitro culture(11;186). Therefore, a longer term study is required to evaluate the chondrogenic potentials of the biofunctionalised scaffolds.

Despite this relatively highly passaged cell culture in-vitro, our RT-PCR results demonstrated that both TGF group and the latent TGF-β1 biofunctionalised scaffold from pLTGF group significantly up-regulated the cartilage specific transcription factor Sox9 at day 14, as compared to the rest of the groups. Sox9 is a key transcription factor for chondrogenic differentiation as it directly activates and up-regulates the expression of Col2A1(203-205). This transcription factor is expressed in all chondrocytes (except hypertrophic chondrocytes)(205), however its expression declines with increasing passage(206). The up-regulation of Sox9 induced by TGF-β1, seen in the TGF group, was consistent with a previously reported study(207). Interestingly, the up-regulation of Sox9 in the pLTGF group was significantly higher (by approximately 30%) than the TGF group.

It is also important to highlight that the highest Sox9 expression level was induced by the amount of latent TGF-β1 available from the pLTGF scaffold (195.4pg/cm² scaffold) which was approximately 150-fold lower than the standard TGF-β1 supplementary dose received by the TGF group (10ng/mL)(17;163;208;209). The results from the pLTGF group demonstrated that the technique of immobilisation of latent TGF-β1 described in this study preserved the bioavailability of the growth factor to cells. Although the anchorage of the latent TGF-β1 on the scaffold surfaces may not resume the intended configuration as seen in its native environment [latent TGF-β1 complex bound at cys-33 to the latent TGF-β1 binding protein(170;171)], the latent TGF-β1 remained protected and available for activation by primary human chondrocytes.
The TGF group on the other hand expressed the highest level of Col1A1 which marks the pattern of dedifferentiation towards a fibroblast-like phenotype. TGF-β1 is well known to have positive effects on matrix production and chondrocytic differentiation(50;210-212). However, studies have reported the suppressive effects of TGF-β1 on collagen type II synthesis and down-regulation of chondrocytic differentiation(162;213-215). The high Col1A1 gene expression results of the TGF group were consistent with the studies that have reported the inhibitory role of the growth factor on chondrogenesis. The sLTGF group did not significantly up-regulate Sox9 gene expression. This may reflect the small amount of TGF-β1 present on the sLTGF scaffolds.

From our studies, we can deduce that there are two possible aspects of the TGF-β1 which influenced the behaviour of the chondrocytes. Firstly, the dosage of the growth factor and secondly, how the growth factor was presented to the cells. A high dose and frequent supplementation of growth factor does not necessarily result in the desired response (as seen in the low Sox9 and up-regulation of Col1A1 in TGF group). Conversely, a dosage 150-fold lower, coupled with the presentation of the growth factor in its latent form to cells, resulted in a change in the pattern of gene expression of the chondrocytes.

4.5 Conclusion

The concept behind the scaffold design presented in this chapter is that the biofunctionalised scaffold directs cell-mediated activation of growth factor, thereby allowing the cells to control their own growth, function and environment. This is the key distinction from the current studies in bioactive factor delivery. The latent growth factor remains protected on the biofunctionalised scaffolds until released by cells when required. Cell-mediated activation implies controlled activation or release of a desired signal at a physiological rate when necessary, as well as reducing unwanted adverse effects. The results and outcomes of this study may mean a step closer to guiding cells towards regenerating functional cartilage tissue, as opposed to allowing extensive reparative fibrous scarring to occur as a result of uncontrolled cytokine induction mentioned above. Although the results of this study are preliminary and
time points are relatively short, it is proof of concept of cell-mediated activation of growth factor can be achieved by the strategies proposed. However, further experiments to assess the tissue regenerative capacity in a “physiological culture” condition is required. In order to evaluate the efficacy of the biofunctionalised scaffolds in a “physiological culture” condition without any other supplementation of serum or growth factors, *in-vivo* studies were performed. This is described in the following chapter.
Chapter 5
Biofunctionalised Electrospun Scaffolds with Cell-mediated TGF-β1 Activation: In-vivo Study

5.1 Introduction
Research in Medicine often requires experiments to be conducted on animal and/or human subjects (in-vivo studies) for the evaluation of a therapeutic intervention. At times, moral dilemmas related to the experiment may be encountered. These moral issues must be thoroughly considered when planning an experiment and approved by the appropriate ethics committee. There are two main ethical theories which form the basis for moral justification in research, and they are the utilitarian theory (teleological), where the moral justification is based on the scale of the perceived advantage, while the deontological theory consists of judging the nature of the research regardless of its outcomes(216). These ethical perspectives in conducting experiments with animals are essential to a researcher and should always be acknowledged to minimise ethical dilemmas.

In the development of a successful tissue engineered product, the use of experimental animals is often necessary for the assessment of product safety and efficacy – prior to the translation into clinical applications for human. In the UK, the use of animals is strictly regulated by the Home Office.

In 1987, the Animal (Scientific Procedures) Act 1986 (ASPA 1986) was enforced to protect animals used for experimental and other scientific purposes in the United
Kingdom. The ASPA 1986 determines which animals are considered “protected”, which procedures are considered to be regulated, defines specific responsibilities of staff in an animal facility establishment [e.g. project and personal licence holder, Named Animal Care & Welfare Officer (NACWO), Named Veterinary Surgeons (NVS), animal technicians] as well as provides detailed guidelines for protocols and procedures (e.g. Schedule 1 method of humane killing). Experiments on animals are only permitted legally when no other means of investigations are capable in achieving the specified research objectives [Animal (Scientific Procedure) Act 1986]. Even so, the researcher is required to minimise the number of animals and their suffering during experiments, by conducting preliminary in-vitro studies, careful planning, and refining laboratory techniques to an optimal level(217).

In order to legally conduct an in-vivo experiment, a researcher must undergo an extensive application process which consists of two parts:
(1) for a project licence - an extensive justification for the use of animals with detailed experimental protocols is proposed and subjected to a ethical review; and,
(2) for a personal licence – the candidate is required to attend a four day training course with successful completion of all course assessments.

Only then, can a fully trained personal licensee be permitted to perform a specific procedure on a specific type of animal, as detailed in the project licence. For example, initially the athymic mutant rat strain was not included in the description of the project licence (PPL 70/6600). Thus, for the purpose of utilising this mutant strain in this thesis, an amendment to include them in the project licence was submitted to the Home Office which was subsequently approved.

The four day training course was absolutely essential in providing an overview of experiments with animals, to a researcher new to the field. Apart from gaining an understanding in the ethical issues and legislation behind animal studies, the course also provides insight into the steps necessary to ensure that the quality of animal welfare is maintained. The standard of animal welfare at an animal laboratory is crucial in providing an environment with the least possible stress levels to animals. This not only addresses ethical related issues, but also minimises experimental
variability as a result of poorly controlled environment. There are several key aspects of animal experiments essential to a new licensee/researcher, and they include:

1. health and safety awareness to staff
2. animal husbandry
3. clinical signs of pain, stress and suffering
4. technical handling of animals
5. peri-operative management of animals – analgesia, anaesthesia, surgery, post-operative recovery and potential complications.

Although animal husbandry is generally managed by NACWO, every personal licence holder is responsible for the care of all animals, submitted under his or her licence. This forms the foundation to utilising animals in research and starts from when the animals are first obtained. First of all, the immediate animal accommodation requires the appropriate type and size of cages, non-toxic and non-absorbent nesting materials which provides insulation, environmental enrichments to encourage natural animal behaviour, the appropriate diet and water supplementation. These are considered as micro-environmental requirements. In, addition, the macro-environmental factors will need to be adjusted to provide as close as possible a simulation of the natural habitat of the animals, and these include: temperature (approximately 19 – 23°C for rats), relative humidity (55 ± 10%), ventilation (15 – 20 changes of fresh filtered air), light to dark cycles (12:12 hours of alternating light and dark cycles for most laboratory animals) and general background noise level (below 50dB).b

Once the animals are acclimatised to their new environment, observations of their natural behaviour and normal activities should be noted and acquainted to. This will form the baseline for comparison of clinical features for the recognition of signs of pain and distress. The common signs of pain and distress include: un-groomed appearance, unusual posture, reduced food and water intake, reduction in body weight, abnormal bladder and bowel function and so on. These clinical features may be present after surgical intervention and at time distress signs can only be detected as a change in the normal pattern of behaviour and activities.

b Information from Imperial College London/Central Biomedical Services Pre-Licence Training Course under the Animal (Scientific Procedures) Act 1986, Module 1-4 2008 manual.
In-vivo experiments in Tissue Engineering may involve a breach of skin and tissue disruption in a surgical intervention. The extent of acute phase response and inflammation is proportional the scale of tissue damage. Therefore, in addition to pain-control and anaesthetic considerations, surgical tissue handling must be precise and kept to a minimum. Although the personal licensee training course included an overview of anaesthesiology and surgery, the detailed peri-operative management requires considerable thought and pre-planning in addition to the background knowledge of physiology and clinical experience in surgical skills. For instance, different stages of a surgical intervention require different but integrated considerations:

Pre-operative stage:
1. number of procedures/animals in a given period of time
2. availability of operative theatre and staff assistance
3. pre- and post-operative recovery environment in separate accommodation
4. designation of pre-, intra- and post-operative areas
5. pre-emptive analgesia ± prophylactic antibiotics
6. the need for fluid resuscitation
7. type of anaesthesia, route of administration, supplementary oxygen, related equipments and their availability
8. hair clipper, type of skin cleanser and skin marker
9. mode of transport of cell-scaffold constructs between laboratories
10. types of surgical instruments, sutures, sterile gloves, gowns and drapes
11. considerations for batch surgery – bead steriliser and the number of sets of surgical instruments required
12. the sequence of events from pre- to post-operative recovery with anticipation of potential complications
13. pre-operative sterilisation of operation theatre/ equipments/ instruments
14. pre-operative weight as a baseline

Operative stage:
1. the need of an assistant, anaesthetist
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2. knowledge of the anatomy of the body part of interest
3. knowledge of the potential physiological changes as a result of the surgical intervention
4. marking of surgical sites, skin preparation and ear tagging
5. assessment of the depth of anaesthesia
6. the need for fluid resuscitation
7. application of principles of surgery – sterile technique, minimal and gentle tissue handling, adequate haemostasis, non-tension wound closure

Post-operative and recovery stage:
1. a clean and separate accommodation/cage for recovery in a dim environment
2. readily available supply of food and water ± further fluid resuscitation/prophylactic antibiotic supplementation/analgesia
3. sterilisation of instruments/change of instruments, drapes etc.
4. preparation for the next procedure
5. observations for post-operative complications such as pain, bleeding, haematoma, wound re-opening/dehiscence with anticipation for re-anaesthetisation and revision surgery
6. post-operative “ward-round” inspection of wounds and observation of animal well-being on the following day
7. daily wound inspection and general clinical observation
8. plan for suture removal

An in-vivo experiment thus requires an exhaustive amount of time and preparation from applications for licences, to an in-depth understanding of animal welfare and science, to the detailed considerations of operative surgery.

In this thesis, the aim is to develop a “cell-mediated activation of growth factor” scaffold system by incorporating the latent TGF-β1 onto the electrospun PLLA scaffolds. As described in the previous chapter, in order to assess the efficacy of this system in guiding cells towards chondrogenesis – the passaged chondrocytes were cultured in a chondrogenic serum-free environment. The chondrogenic serum-free environment was used to induce chondrogenic differentiation as well as to avoid the
masking effects from uncharacterised bioactive factors present in the serum. However, the long term culture in a serum-free environment may effect the survival of the chondrocytes as seen in the reduction of cell viability at 14 days (Chapter 4). Therefore, to evaluate the long term effects of the latent TGF-β1 biofunctionalised scaffolds on chondrogenesis without serum or additional growth factor supplementation - *in-vivo* culture of the cell-scaffold constructs was performed.

*In-vivo* subcutaneous implantation cell-scaffold constructs was performed in athymic rats. This immunocompromised mutant strain is a well established model for the investigation of xenogenic transplantation as they are congenitally T-lymphocyte deficient(218). As a result of the T-cell deficiency, the athymic rats are incapable of mounting transplant rejection. Thus, they provide a physiological environment necessary to support cell survival for the investigations of the biofunctionalised scaffolds in this study. However, T-like cells have been found in increasing numbers after 4 - 6 months of age with some indications to skin allograft rejection(218). For this reason, the rats used in this study were obtained at 5 - 6 weeks old with the final post-implantation age being approximately 3-months to avoid any possibilities of transplant rejection. This chapter describes the *in-vivo* sequel to the evaluation of the latent TGF-β1 biofunctionalised scaffolds in promoting chondrogenesis.
5.2 Materials and Methods

5.2.1 Scaffold Fabrication and Biofunctionalisation

The non-woven PLLA fibrous scaffolds were electrospun, sterilised and subsequently biofunctionlised with latent TGF-β1 as described in Chapter 4. The scaffolds (1 × 1cm²) were divided into three experimental groups (Table 5.1).

<table>
<thead>
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<th>Sample ID</th>
<th>Description</th>
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<tr>
<td>ptPLLA +/-</td>
<td>Plasma treated electrospun scaffolds as control</td>
</tr>
<tr>
<td>pLTGF +/-</td>
<td>Plasma treated electrospun scaffolds with immediate immobilisation of latent TGF-β1</td>
</tr>
<tr>
<td>sLTGF +/-</td>
<td>Plasma treated electrospun scaffolds with sulfo-SMCC crosslinked latent TGF-β1</td>
</tr>
</tbody>
</table>

Table 5.1. Experimental groups and their sample identification (ID). The symbols (+) or (-) indicate scaffolds seeded with cells and their cell-free controls, respectively. These sample ID will be used to refer to respective groups throughout this chapter.

5.2.2 Cell Culture and Expansion

Human primary nasal septal chondrocytes used in this study were a gift from Dr. Reza Ghazanfar (Imperial College London, UK). The chondrocytes were expanded up to fourth passage in chondrocytic growth media (please see Chapter 4 for details of the media composition). As an alternative cell source with chondrogenic potential, human mesenchymal stem cells (MSC, Lonza Wokingham Ltd, UK) were used and expanded up to third passage in MSCGM™ Mesenchymal Stem Cell Growth Medium Bullet Kit® (Lonza Wokingham Ltd, UK). The cells were maintained at 37°C in a humidified 5% carbon dioxide atmosphere. The culture media was replaced every 3 days.

Cells were then harvested and seeded on to the scaffolds (see Table 5.1) at a seeding density of 8 × 10⁵ cells/cm² scaffold, in a serum-free media (see Chapter 4 for media composition). Cell pellets (5 × 10⁵ cells) were snap-frozen and used as day-0 specimen.
for gene expression analysis. After 24 hours, the cell-scaffold constructs were subjected to the following in-vivo implantation.

5.2.3 In-vivo Implantation of Cell-Scaffold Constructs

Eight 5 to 6-week-old female athymic rats (rnu/rnu, Harlan, UK) were allowed to acclimatise for 7 days. They were maintained in individually ventilated cages (four rats per cage) at 22°C, 50% humidity and 12 hourly night-day cycles. Sterile solid food and water were provided. Cell-scaffold constructs and their no-cell controls were implanted into individual dorsal subcutaneous pockets of the rats. This procedure was performed under UK Home Office approved project licence (PPL 70/6600).

The rats were anaesthetised by resident veterinary surgeon, Mr. Francisco Dias, with inhalation of isoflurane and oxygen. Pre-emptive carprofen (5mg/kg, Pfizer Animal Health, USA) and prophylactic enrofloxacin (5mg/kg, Bayer plc, UK) were administered by subcutaneous injection before any skin incisions were made. The dorsa were shaved and cleansed with chlohexidine (Hibrisrub, Regent Medical Ltd., UK). Subsequently, three 1cm midline skin incisions were made at 1cm intervals. Two subcutaneous pockets were created by blunt dissection, on either side of and away from each incision. The cell-free control scaffolds were inserted into the left pockets, followed by the insertion of cell-scaffold constructs into the corresponding pockets on the right. All cell-scaffold constructs were inserted with the cell-seeded side facing upwards and away from the underlying muscular layer. The approximate anatomical landmarks of various scaffold groups are as illustrated in Fig. 5.1. The skin incisions were closed with interrupted 4/0 polypropylene sutures (Ethicon Ltd., UK). Post-operative prophylactic oral enrofloxacin was administered as a supplement in the water (0.1mg/mL) for seven days. At 2 weeks, all sutures were removed with the animals placed under secure manual restraint (with assistance from an experienced animal technician).

After 6 weeks, all rats were sacrificed by neck dislocation and all implants together with the surrounding tissue were retrieved immediately. Each implant was divided into four equal quarters. The first quarter was embedded in optimum cutting temperature embedding compound (Bright Cryo-M-Bed, Bright Instrument Company
Ltd., UK) then snap-frozen in liquid nitrogen and stored at -80°C until further use for cryosection. For the following quarters, surrounding tissues were gently removed, as much as possible, from the implants. The second quarter was snap-frozen in liquid nitrogen for gene expression analysis. The third quarter was stored in -20°C for biochemical analysis. The fourth quarter was fixed in 2.5%(v/v) glutaraldehyde and processed as per protocol in Chapter 3 for SEM.

Fig. 5.1. The approximate anatomical landmarks of implant placements in the dorsal subcutaneous layer of the rats. The positions of scaffold groups were rotated through the three levels: subscapular, low thoracic and lumbar. Dashed line indicate the dorsal anatomical midline as located by palpation of the spinous processes. In this figure, P, L and S represent ptPLLA, LTGF and sLTGF groups respectively.
5.2.4 Characterisation of Implanted Specimens

5.2.4.1 Gene Expression Analysis

The frozen implants were homogenised in a 350µL lysis buffer which consist of 10µL β-mercaptoethanol in 1mL RLT-Buffer (QIAGEN, UK), at room temperature, using TissueLyser II (QIAGEN, UK) operated at 30Hz for two 3 minute cycles. The following procedures for gene expression analysis were performed as described in Chapter 4. Taqman Gene Expression Assays used to amplify genes are detailed in Table 4.2 of Chapter 4 with the addition of Runx2 gene for the MSC groups (Table 5.2). Rat subcutaneous tissue and cell-free implants were used as controls. The overall degree of chondrogenic differentiation can be drawn by obtaining the Col2A1/Col1A1 gene expression ratio as both are considered as the differentiation and dedifferentiation markers of chondrocytes respectively(19;219-221).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Catalogue no.</th>
<th>Accession no.</th>
<th>Chromosome Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runt-related transcription factor 2</td>
<td>Runx2</td>
<td>HS00298328</td>
<td>NM001015051</td>
<td>Chr.6 45,622,542 – 45,626,797</td>
</tr>
</tbody>
</table>

Table 5.2. Taqman™ gene probe (Applied Biosystems, UK) used in this study with details of their sources and specificities.

5.2.4.2 Gross Appearance of Implants

The quarters allocated for histology were observed using a Panasonic DMC-FX36 digital camera (Osaka, Japan), prior to being set in the embedding compound.

5.2.4.3 Cryosection of Implants

Full depth sections of 7µm thickness from all scaffold groups, were cut using a cryostat (Bright Instrument Company Ltd., UK) and fixed in ice-cold acetone for 5 minutes. Sections were then used for SEM, histology and immunofluorescence analyses as described below.
5.2.4.4 Scanning Electron Microscopy of Implants

Sections of all scaffold groups were processed and analysed using SEM as according to the protocol described in Chapter 3. Three fields of view from four samples with triplicate sections were analysed.

5.2.4.5 Histology and Immunofluorescence Analyses of Implants

Full depth sections of 7µm thickness were cut using a cryostat (Bright Instrument Company Ltd., UK) and fixed in ice-cold acetone for 5 minutes. Separate sections were stained with: (1) haematoxylin (Gill’s Formula, Vector Laboratories, USA) and eosin (Sigma-Aldrich, UK) to evaluate general tissue-implant morphology and cellular organisation; (2) 0.001%(w/v) fast green (Sigma-Aldrich, UK), 0.1%(w/v) safranin-O (Sigma-Aldrich, UK) and haematoxylin to assess GAG distribution; and for MSC groups only (3) 2%(w/v) alizarin red S (Sigma-Aldrich, UK) to identify calcium as a result of bone tissue formation. (For detailed histology protocol, please see Appendix 1.8)

All solutions and dilutions used in the immunofluorescence staining procedure were prepared from a ‘solution-A’ buffer which consists of 1:2000 dilution of 1M MgCl₂ (Sigma-Aldrich, UK) in Dulbecco’s phosphate buffered saline (PBS, Invitrogen, UK). The sections were initially incubated in solution-A with 2%(w/v) bovine serum albumin (BSA, Sigma-Aldrich, UK) for 30 minutes at room temperature to block non-specific binding sites. The sections were then rinsed (3×) in solution-A. All primary and secondary antibodies were incubated in a humidified chamber at 37°C for 1 hour. To reveal the presence of donor cells, sections were incubated with a mouse monoclonal antibody specific to a human nuclei antigen (Millipore, UK) – at a 1:50 dilution with 2%(w/v) BSA. For the detection of human type I and II collagen production, separate sections were incubated with a rabbit anti-collagen I polyclonal and mouse anti-collagen II monoclonal antibodies, at a dilution of 1:80 and 1:100, respectively (both from Abcam, UK). The sections were then rinsed (3×) in solution-A and incubated separately with: (1) Texas Red® conjugated sheep polyclonal to rabbit antibody for the detection of collagen I (596 - 620nm); and (2) diaminotriazinylaminofluorescein (DTAF) conjugated goat monoclonal to mouse
antibody for the detection of human nuclei and collagen II (both at a dilution of 1:1000 and from Abcam, UK) (495 - 528nm). Following the incubation period, all sections were rinsed (3×) in solution-A, blotted, and then a small drop of VECTASHIELD mounting medium containing 4’6-diamindino-2-phenylindole (DAPI) (Vector Laboratories, USA) was added to the sections to reveal all DNA (358 - 461nm) and cover slips were placed on the sections. The cell-free sections were used to evaluate species cross-reactivity of the antibodies. A human nasal septal cartilage and sections stained only with the secondary antibodies were used as positive and negative controls respectively. Immunofluorescence (IF) microscopy was then carried out using an Olympus BX-51 fluorescence microscope equipped with an Olympus DP070 colour digital camera and Olympus DP Controller software (Olympus UK Ltd., UK). Experiments were performed on four samples with triplicate sections.

5.2.5 Statistical Analysis

The means and standard deviations of the results were calculated using the SPSS 12.0 software package (SPSS Inc., USA). The Mann-Whitney U Test for two independent samples was performed to determine statistical significance between various scaffolds. The p value of <0.05 was considered to be significant. A post-hoc power analysis was performed with the two-sample t-test using the PASS 2008 software (NCSS, USA) to evaluate the power of the experiment as well as to determine the sample size for further studies. The results of the power analysis and sample size estimation are discussed presented in Table 5.3 in the discussion of this chapter.
5.3 Results

Post-operative recovery of all rats was unremarkable. All wounds healed without any complications. All implants were well tolerated throughout the experiment. All rats remained healthy and well throughout the 6 week period.

5.3.1 Gene Expression Analysis

The mRNA expression of Sox9, Col2A1, and Col1A1 for the implants together with their cell-free controls were analysed using real-time RT-PCR (Fig. 5.2). Runx2 expression was analysed in addition to the three genes for the MSC seeded constructs. No signals were detected on all cell-free implants and rat subcutaneous tissue for all gene probes except 18S.

The Sox9 was expressed across all experimental groups (Fig. 5.2a). The Sox9 expression observed in pLTGF+ groups which was approximately 1.2-fold and 3.9-fold higher when compared to sLTGF+ groups. Col2A1 expression was expressed in both latent TGF-β1 biofunctionalised scaffolds (pLTGF+ and sLTGF+) while it was undetectable in the control scaffold group (ptPLLA+) (Fig. 5.2b). The Col1A1 expression was detected in all scaffold groups with no significant differences between groups (Fig. 5.2c). The Col2A1/Col1A1 gene expression ratio of the sLTGF+ group was approximately 1.5-fold higher than the pLTGF+ group, although no statistical significance was found.
Fig. 5.2. Real-time RT-PCR quantification of gene expression of in-vivo cultured primary human chondrocytes on various scaffold types for six weeks. Expression levels of (a) Sox9, (b) Col2A1, and (c) Col1A1 were normalised to the 18S housekeeping gene and day 0 expression levels. (d) Col2A1/Col1A1 gene expression ratio indicating the degree of chondrogenic differentiation. Average fold changes ± standard deviation are presented (n=4). The following symbols represent significant differences (*p<0.05) with (*) ptPLLA+, and (●) sLTGF+ groups.

In the MSC culture, Sox9 expression was detected in all experimental groups with no significant differences between them (Fig. 5.3a). Interestingly, Col2A1 expression was detected in all experimental groups (Fig. 5.3b). However, no Col2A1 was detected in the initial hMSC day 0 samples. As a result, no baseline Col2A1 expression at day 0 was available for further normalisation. Consequently, only the \( \Delta C_T \) results were analysed and presented below, instead of the complete comparative \( \Delta \Delta C_T \) of Col2A1 (Fig. 5.3b). Although no statistical significance were found in Runx2 and Col1A1 expressions, an up-regulated trend can be seen in the ptPLLA+ group when compared to the two latent TGF-β1 biofunctionalised groups (Fig. 5.3c and d).
Fig. 5.3. Real-time RT-PCR quantification of gene expression of in-vivo cultured human MSC on various scaffold types for six weeks. Expression levels of (a) Sox9, (c) Runx2, and (d) Col1A1 were normalised to the 18S housekeeping gene and day 0 expression levels. In (b) Col2A1 expressions were only normalised to the 18S housekeeping gene because no Col2A1 was detectable in the human MSC day 0 culture, as indicated by the symbol ($\Delta C_T$). Average fold changes ± standard deviation are presented ($n=4$).
5.3.2 Gross Appearance of Implants

The gross appearances of the scaffold and implants were observed between experimental groups and between the seeded cell types (Fig. 5.4). In general, the post-implantation scaffolds carried a yellowish hue (compared to the original white appearance of the preimplanted scaffold as indicated by “C” in the Fig. 5.4) and were found to be encapsulated with fibrous tissue. No differences in appearance were noted between experimental groups as well as between chondrocytes and MSC.

Fig. 5.4. Representative gross appearances of implants. In this figure, P, L and S represent ptPLLA, LTGF and sLTGF groups respectively while “-” and “+” indicates the cell-free control and the cell-seeded scaffolds respectively. Scaffold “C” represents a pre-implanted scaffold. The differences in shapes of the implants may be related to the natural fibrous encapsulating reaction and/or the site of implantation where movements of the site may have altered the origin square shape. Scale bar = 1cm.
5.3.3 Scanning Electron Microscopy of Implants

SEM images of sections from all scaffold groups and both cell types indicated that tissue products were integrated into the scaffolds (Fig. 5.5)

![SEM images of sections from all scaffold groups and both cell types](image)

**Fig. 5.5.** Representative SEM images of sections of *in-vivo* implanted scaffold groups for both chondrocytes and MSC. Images were taken at low (250×, scale bar = 200µm) and high (6500×, scale bar = 10µm) magnification.
5.3.4 Histology and Immunofluorescent Staining of Implant Sections

In order to confirm the presence of the donor human cells at 6 weeks, immunofluorescence detection of a specific human nuclei antigens revealed that the human nasal chondrocytes and MSC were located adjacent to the scaffolds (D-I of Fig. 5.6 and 5.7). The cell-free implants (A-C of Fig. 5.6 and 5.7) and human nasal cartilage (Fig. 5.8) were used as controls to confirm the specificity of the anti-human nuclei antibody.

![Images of immunofluorescence detection](image)

Fig. 5.6. Immunofluorescence detection of donor cells using an antibody specific to a human nuclei antigen. The three columns represent the three scaffold groups as indicated by their experimental ID. Images A-C represent the respective cell-free control groups, while D-F were seeded with primary human chondrocytes. Images G-I are higher magnification images of D-F. The silhouette of the scaffolds can be seen traversing these representative images horizontally (A-F). The presence of donor cells (green) were distinguished from the surrounding rat cells (blue) as indicated by the arrows in images D-I. Scale bars: A-F, 500µm; G-I, 200µm.
**Fig. 5.7.** Representative images of immunofluorescence detection of donor human MSC using an antibody specific to a human nuclei antigen. The three columns represent the three scaffold groups as indicated by their experimental ID. Images A-C represent the respective cell-free control groups, while images D-F were seeded with MSC. Images G-I are higher magnification images of D-F. The presence of donor cells (green) were distinguished from the surrounding rat cells (blue) as indicated by the arrows in images D-I. Scale bars: A-F, 500µm; G-I, 200µm.
Fig. 5.8. Representative images of control specimens for immunofluorescence detection of the human cell nuclei. Human nasal cartilage was used as a control to show positive staining (A, C) for the antibody specific to human nuclei (DTAF labelled, green), which was co-localised using DAPI (blue). The human nuclei can be seen to stain both green and blue within well defined lacunae (C). Negative controls were stained with DAPI only (B, D). Scale bars: A-B, 500µm; C-D, 200µm.

Haematoxylin and eosin (H&E) examination revealed that all scaffolds were integrated with the surrounding tissue on both surfaces of the scaffolds (Fig. 5.9 and 5.11). Cells can be found within the thickness of each scaffold. Histostaining with safranin-O on both cell types and alizarin red S on MSC were negative and non-specific respectively (Fig. 5.9 and 5.11). Human nasal cartilage was used as a positive control for safranin-O, type I and II collagen immunofluorescent stains (Fig. 5.13). In chondrocyte-seeded implants, human type II collagen was detected in pLTGF+ group only (O of Fig. 5.9 and 5.10), while type I collagen was found in ptPLLA+ scaffold (H of Fig. 5.9 and 5.10). There was no type II collagen detected across all scaffold groups in the MSC-seeded implants (Fig. 5.11). However, type I collagen was found in both ptPLLA+ and pLTGF+ (J and S of Fig. 5.11)
Fig. 5.9. Representative histochemical and immunofluorescence images of haemotoxylin and eosin (H&E, first column), safranin-O (second column), Type II collagen (third column), and Type I collagen (fourth column) staining of implant sections. All experimental groups are as indicated by their IDs in respective rows. H&E images show tissue integration onto the scaffolds. The representative images in the second column revealed negative staining for safranin-O (stains red for proteoglycans) across experimental groups. In the third and fourth column, cell nuclei stain blue, type II collagen stains green and type I collagen stains red. Type II collagen was only detected on pLTGF+ group (O) while type I collagen in ptPLLA+ group (H) as indicated by arrows. The enlarged images of O and H are shown in Fig. 5.10. Cell-free implants acted as controls for type I and II collagen immuno-localisation, showing no cross-reactivity of the antibodies to rat tissue. Scale bar: 500 µm.
Fig. 5.10. Enlarged images O and H from Fig. 5.8 to illustrate the production of type II and I collagen by the implanted human chondrocytes (arrows). Scale bars: 500µm.
Fig. 5.11. Representative histochemical and immunofluorescent images of haemotoxylin and eosin (H&E, first column), safranin-O (second column), alizarin-red (third column), type II collagen (fourth column), and type I collagen (fifth column) staining of implant sections. All experimental groups are as indicated by their IDs in respective rows. General tissue integration onto the scaffolds is demonstrated by H&E staining. Images of safranin-O and alizarin red staining showed negative and non-specific signals respectively. Type II collagen was not detected as shown on the images of the fourth column. Type I collagen is stained red and shown in ptPLLA+ and pLTGF+ groups (J, S, arrows). (See Fig. 5.12 for enlarged images of J and S). Scale bars: 500µm.


**Fig. 5.12.** Enlarged images J and S from Fig. 5.11. These images show that only type I collagen production was detectable in both ptPLLA and pLTGF groups by the MSC. Scale bars: 500µm.

**Fig. 5.13.** Positive controls of cartilage extracellular matrix with safranin-O staining and immunofluorescence labelling of human type I and II collagen. Images were taken at the chondro-perichondrial interface from cryosections of human nasal cartilage with intact perichondrium, to differentiate cartilage specific matrices (proteoglycan and type II collagen) from the fibrous perichondrial tissue (type I collagen). (A) Cartilage matrix stained red for proteoglycan with safranin-O. (B) Type II collagen fluoresced green while type I collagen in the perichondrium was labelled red. Scale bars: 500µm.
5.4 Discussion

The results of this study demonstrated that the latent TGF-β1 biofunctionalised scaffolds induced significantly higher chondrogenic differentiation with type II collagen production in the in-vivo cultured primary human nasal chondrocytes, as compared to the control group. The findings of this in-vivo experiment also confirmed that the concept of cell-mediated activation of growth factors has been achieved by both scaffold biofunctionalisation methods described in Chapter 4. Interestingly, a converse effect was observed, on both of the latent TGF-β1 biofunctionalised scaffold groups, in the MSC implants - where the control group encouraged relatively stronger pattern of expression of Col1A1 and Runx-2 expression.

For the evaluation of gene expression, the absence of signals on all gene probes on cell-free control implants and rat subcutaneous tissue suggested that the gene probes used in this study were specific to human genes - with the exception of the 18S housekeeping gene. This suggested that the housekeeping gene may have cross-reacted with the rat tissue and resulted in a “dilution effect” on the rest of the genes, when the relative expressions were evaluated - as the 18S would have been derived from both human and rat origins. Despite this “dilution effect”, the expression of human genes was detectable, amongst a larger pool of rat genes. The detection of human specific genes with RT-PCR also indicated that both human chondrocytes and MSC survived the entire period of implantation within the rat subcutaneous layer. The presence of the donor cells were further confirmed with the anti-human nuclei immunofluorescence staining.

Sox9 is well known to be a transcription factor that directly regulates the type II collagen (Col2A1) gene expression(204;222). The Sox9 gene is one of the earlier markers of mesenchymal cells undergoing condensation and its expression has been reported to parallel Col2A1 during embryonic chondrogenesis(223). It is expressed in all chondroprogenitor cells (prechondrocytes, early chondroblasts, columnar chondroblasts) as well as chondrocytes, except hypertrophic chondrocytes(223-226). Once normal chondrocytes are isolated from its natural environment, a dedifferentiation process occurs. This process of chondrocytic dedifferentiation is
classically characterised by the down-regulation of cartilage-specific genes (e.g. Sox9 and Col2A1) with the gradual rise of Col1A1, a fibrogenic or osteogenic marker which is also considered to be a chondrocyte dedifferentiation marker(227). These changes are typically seen when chondrocytes are cultured and passaged *in-vitro*(206;228;229). Chondrocytes lose their differentiated functions as early as the first passage. Although this process is reversible, the capacity to redifferentiate reduces rapidly with increasing passages. A near total lost of this capacity has been reported to occur from the third passage onwards(13).

The chondrocytes used in this experiment have been expanded up to fourth passage. The dedifferentiated characteristics have been indicated in the previous chapter where the Col2A1 was undetectable at two weeks of *in-vitro* culture. Remarkably, at week 6 of this *in-vivo* study, Col2A1 expression was induced in the same batch of chondrocytes (fourth passage) which were seeded on to the latent TGF-β1 biofunctionalised groups (pLTGF+ and sLTGF+). This confirmed that the dedifferentiated chondrocytes have been induced to redifferentiate by the latent TGF-β1 biofunctionalised scaffolds. The expression of Col2A1 was undetectable in the implanted controls (ptPLLAl+) despite the presence of Sox9. Although Sox9 is required for the activation of Col2A1 gene, the RT-PCR results demonstrated that the Sox9 expression in the ptPLLAl+ group was not sufficient in activating the Col2A1 gene in the absence of the latent TGF-β1 biofunctionalised scaffolds. Although the Sox9 expression in the sLTGF+ group was significantly low compared to the ptPLLAl+ group, the Col2A1 was activated. Interestingly, the level of Sox9 expression seen in the pLTGF+ group, which was 3.9-fold higher than that of sLTGF+, was not accompanied by a proportional rise in the Col2A1 expression when compared to the sLTGF+ group. This “anti-parallel” or inverse proportion between Col2A1 and Sox9 in the chondrocytic group may be a gene profile characteristic of *in-vitro* passaged adult human chondrocytes, where the expression of Sox9 correlates to a lesser extent with the presence of Col2A1(206;222;229). This may be due to a decrease in Sox9 binding activity to the Col2A1 gene enhancer site with every increasing passage(222).

Although no statistical significance was observed, the degree of differentiation can be seen at 1.5-fold higher in the sLTGF+ as compared to pLTGF+ (Fig. 5.2d). This was surprising considering the quantified amount of anchored TGF-β1 on sLTGF was one
order of magnitude lower than pLTGF (Chapter 4). This may be related to the orientation of the incorporated latent TGF-β1 as it may have governed how the appropriate signals were presented to the cells. Theoretically, the orientation of the latent TGF-β1 in the sLTGF may be a closer simulation of the natural latent TGF-β1 complex in the native environment as illustrated in the introduction of Chapter 4. This chondrogenic differentiated state of the latent TGF-β1 biofunctionalised groups was evident in the immunofluorescence detection of type II collagen. The human type II collagen was detected in sections of pLTGF+ group which further confirmed the chondrogenic efficacy of the biofunctionalised scaffolds (Fig. 5.9). Unfortunately, the quantitative determination of type II and I collagen contents were inconclusive using an inhibition ELISA technique(230;231) (see Appendix 1.9). This was because the antibodies used in the assays cross-reacted with rat tissue as demonstrated by the positive signals in rat tissue controls. Further work will involve the determination of a suitable alternative method of quantification of Type I and II collagen. Preliminary assessment of species cross-reactivity of the quantification methods (with rat tissue) will need to be verified prior to the commencement of a full scale in-vivo experiment.

The integration of tissue products can be seen on haematoxylin and eosin staining as well as SEM images (Fig. 5.5). The negative staining for proteoglycan with safranin-O in the chondrocytic group, could reflect the slow recovery of the differentiated function in dedifferentiated chondrocyte(202). In addition, the TGF-β1 has been reported to reduce the glycosaminoglycan synthesis in mature chondrocytes and increase the degradation of aggrecan by up-regulating aggrecanase activity(232;233). This may explain the negative staining of safranin-O in both latent TGF-β1 biofunctionalised groups. The type I collagen on immunofluorescence, found in the ptPLLA+ group, was consistent with the Col1A1 gene expression and lack of Col2A1 expression - further confirmed that the chondrocytes remained in their dedifferentiated state. The latent TGF-β1 biofunctionalised scaffolds induced the highly passaged human nasal chondrocytes to re-express differentiated chondrogenic genotype as well as type II collagen production. However, the latent TGF-β1 did not evoke a similar chondrogenic response in the MSC group, when compared to the chondrocytic group.
The TGF-β1 has been reported to induce chondrogenesis in adult human MSC in-vitro (165;234;235). The TGF-β1 has been reported to be less effective in inducing chondrogenesis in human bone marrow derived MSC as compared to the TGF-β2 and TGF-β3 isoforms(234). In this chapter, although no statistical significance was observed in the gene expression data between experimental groups in the MSC culture, the RT-PCR results presented above suggested that the latent TGF-β1 of both biofunctionalised groups may have down-regulated the Runx2 and Col1A1 gene expression in the MSC, by approximately 50%, when compared to the ptPLLA+ group (Fig. 5.3c and d). It is likely that the presence of the latency associated peptide of the latent TGF-β1 may be a contributing factor. Both Sox9 and Runx2 have been reported to be co-expressed during mesenchymal condensation, in chondrocytic precursor cells and chondrocytes(225;226;236). Runx2 is also an essential transcription factor that guides mesenchymal precursors towards osteogenic lineage as well as promotes osteoblastic differentiation. Nonetheless, its expression has been reported to be inhibited by TGF-β1 in osteoblastic cells(237). Therefore, the latent TGF-β1 may have evoked a similar inhibitory response on osteogenesis in the MSC as seen in the non-specific alizarin red S stained sections and the relatively lower Runx2 and Col1A1 gene expression results in both biofunctionalised scaffolds.

The Col2A1 expression was detected in all experimental groups but not in day 0 MSC. This was as expected with the use of undifferentiated MSC. However, the absence of the Col2A1 in the day 0 MSC meant that the conventional comparison of the expression of the gene of interest (relative to the internal control – 18S), could not be made with the “untreated control” as according to the full equation of comparative CT method. The comparative CT method is a mathematical model that calculates changes in gene expression as a relative fold difference between experimental and calibrator samples. The following equations show the full formula to the comparative CT method(238;239):

\[
\text{Fold change} = 2^{\Delta\Delta CT} \tag{5.1}
\]

\[
2^{\Delta\Delta CT} = \left[(C_T \text{ gene of interest} - C_T \text{ internal control})_{\text{sample A}} - (C_T \text{ gene of interest} - C_T \text{ internal control})_{\text{sample B}}\right] \tag{5.2}
\]
where the \( C_T \) is the threshold cycle, the quantitative endpoint which is defined as the “PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold”; the internal control is usually a housekeeping gene used as a reference; the sample A may be the treated sample and the sample B, the untreated control/the calibrator sample(239). According to Schmittgen et al., RT-PCR data may be presented in the form of \( 2^{-\Delta CT} \) as they are considered as normalised individual data points which are unrelated to a calibrator(239). Therefore, in this study, comparisons of the Col2A1 expression of the MSC group can only be made between the experimental groups without reference to the fold change from the commencement of the experiment. Although no statistical significance were observed between experimental groups with low levels of expression, the Col2A1 expression of the pLTGF+ group were approximately 30% higher than both ptPLLA+ and sLTGF+ groups.

The presence of Sox9, Runx2, and Col1A1, together with the detectable Col2A1 may indicate that some of the MSC may have been guided towards: (1) a chondrogenic lineage or (2) a co-existence of both chondrogenic and osteogenic subpopulations as many growth factors that promote chondrogenesis in MSC, are also implicated to a certain extent in the process of osteogenesis(240). Nonetheless, the negative staining of safranin-O, alizarin red S together with statistically inconclusive gene expression data showed that additional cues are required for an effective induction of the MSC. Further investigations using MSC may require a longer \textit{in-vitro} culture of approximately 1 week, in the chondrogenic serum-free media to ensure adequate induction of the MSC towards a chondrogenic lineage, prior to the \textit{in-vivo} implantation(240;241). As mentioned earlier, the TGF-\( \beta \)2 and TGF-\( \beta \)3 have been shown to be more effective in inducing chondrogenesis in human bone marrow derived MSC when compared to the TGF-\( \beta \)1(234). Since TGF-\( \beta \)1, TGF-\( \beta \)2, and TGF-\( \beta \)3 are synthesized in a similar way, associated with its latency-associated peptide(169) - a scaffold biofunctionalised with a latent form of the TGF-\( \beta \)2 or TGF-\( \beta \)3 may be a more suitable chondrogenic scaffold system for the bone marrow derived MSC. Nonetheless, at present time, a recombinant form of the latent TGF-\( \beta \)2 or TGF-\( \beta \)3 is yet to be commercially available.
This *in-vivo* study represents a pilot study to gain an initial insight into the potential tissue regenerative outcomes of the latent TGF-β1 biofunctionalised scaffolds in an *in-vivo* setting. A pilot study was also necessary to identify the potential technical and logistical difficulties that might occur throughout the experiment. Several aspects of this *in-vivo* experiment posed as potential impediments and they include:

1. the limited familiarity with various peri-operative equipments such as the use of a bead-steriliser and an anaesthetic machine;
2. the distance between the cell culture laboratory and the animal facility meant that additional precaution was necessary for the safe and sterile transportation of the cell-scaffold constructs;
3. the spatial and anatomical feasibility of implanting six 1cm² scaffolds (the three experimental groups together with their cell-free controls) interspaced at 1cm on the dorsum of a rat;
4. the tolerance of the rats to the implants as well as the surgical procedure (in both short and long term);
5. the limited familiarity with tissue handling (surgically) in rats;
6. the tolerance of the implanted human cells to its host environment;
7. the potential adverse effects of pharmacological agents e.g. enrofloxacin on the donor human cells and their chondrogenicity;
8. unforeseen obstacles related to sample processing, for example, species cross-reactivity of detection probes of assays and antibodies.

For these reasons, a pilot study was essential to identify potential problems and uncertainties which may arise from various aspects of a new *in-vivo* study. This prevented unnecessary wastage of scientific resources and is also considered ethically more humane to the use of laboratory animals. This chapter also established the foundation for a further definitive *in-vivo* study with a larger sample size. With a sample size of 4 rats per cell type, the post-hoc power of the experiment has been calculated based on the means and standard deviations of the chondrogenesis endpoint gene of interest – the Col2A1. However, only the chondrocyte group was selected for analysis due to their statistical significance relative to the control. The power of an experiment is the probability of detecting a specified effect at a specified level of significance. The powers of the pLTGF and sLTGF groups were 6.1% and 83.9% respectively (Table 5.3). Conventionally, the power of an experiment is set between
80 – 90% in order to have a high chance of detecting a significant effect (242). This means that the higher the power, the larger the number of animals that will be needed for the experiment. In this chapter, the sample size of four rats were sufficient to achieve a power of 83.9% in the sLTGF group of the chondrocyte culture, while the sample size of pLTGF group will need to be doubled in order to achieve a power greater than 80% (Table 5.3). Since both biofunctionalised scaffolds and their controls were implanted in the same experimental unit (rat), the sample size required for further substantive in-vivo assessment of the scaffolds will be a minimum of 8 rats per time point to achieve a power of greater than 80%.

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<thead>
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<th>pLTGF</th>
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<tr>
<td>Power of experiment</td>
<td>6.1%</td>
<td>83.9%</td>
</tr>
</tbody>
</table>

Minimum sample size required:

<table>
<thead>
<tr>
<th></th>
<th>pLTGF</th>
<th>sLTGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>For power &gt; 90%</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>For power &gt; 80%</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

**Table 5.3.** Post-hoc power analysis of experiment on the chondrocyte group and determination of sample size required for further in-vivo studies with the biofunctionalised scaffolds. Based on the power analysis calculated on the Col2A1 expression of the chondrocytic groups, the minimum number of rats (sample size) for further in-vivo experiments for a given time point can be estimated according to the desired power. The calculations were performed using the PASS 2008 software (NCSS, USA), with \( \alpha = 0.05 \).
5.5 Conclusion

This *in-vivo* sequel to the *in-vitro* development of latent TGF-β1 biofunctionalised tissue scaffold has demonstrated and confirmed the proof of concept of a system capable of inducing cell-mediated activation of growth factor. The highly passaged human nasal chondrocytes (fourth passage) have been shown to re-express the Col2A1 gene as well as produce type II collagen on latent TGF-β1 biofunctionalised scaffolds. The results of this chapter suggested that the presentation of signalling cues to cells may be as important as the dosage, in guiding the appropriate cell behaviour. The outcome of its application also depends on the type of cells as well as the appropriate cell signalling factor used. Although the scale of this *in-vivo* study is preliminary, the proof of concept of cell-mediated activation of growth factors may provide a platform technology for further development of a growth factor delivery scaffold system for tissue regeneration.
Chapter 6
Conclusions

The field of tissue engineering has advanced remarkably in recent decades, to meet the rising demands for functional tissue replacements in treating injuries and diseases. In cartilage tissue engineering, the majority of the research studies have been driven by the orthopaedic surgical specialty in search of an ideal articular cartilage substitutes. Nevertheless, the advancements made may be applicable to cartilage regeneration for facial plastic reconstruction.

Even so, few tissue engineered products are clinically available for medical treatments. Although current trends in tissue engineering have evolved to focus on biomimicking the structure and function of the native tissue, its size and complexity remain an enormous challenge to the scientific and medical community. For this reason, the first fundamental step in tissue engineering research begins at the molecular and cellular level. An in-depth understanding of the intricate interactions between the cells and their extracellular matrix is vital to substantiate the advancement of tissue engineering.

In this thesis, a multidisciplinary approach has been applied in the development of a scaffold system which mimics the structural architecture as well as the function of the native cartilage tissue. The first study (Chapter 3) examined the structure of the native articular hyaline cartilage architecture comparing human to bovine origins. With the advent of micro-characterisation techniques, great insight into the nanoscale framework of the native hyaline cartilage extracellular matrix was achieved. The
human articular cartilage was more readily available for this initial study due to the prevalence of degenerative osteoarthropathy. However, a further in-depth nanoscale structural characterisation of the human nasoseptal and auricular cartilage would benefit the advancement of cartilage tissue engineering for facial reconstructive surgery, should specimens become available. It is likely that a full in-depth regional nano-structural characterisation of facial cartilages may need to be performed on cadaveric specimens because the availability of normal complete facial structures from the clinical practice that are suitable for scientific studies, are extremely rare.

In order to recreate the structure architecture of the cartilage, an FDA approved biopolymer poly(L-lactide) was selected and electrospun into non-woven nanofibrous scaffolds. The fibre diameter and general scaffold morphology resembled the native cartilage. On achieving scaffold architectural resemblance by electrospinning, the subsequent study focused on developing a functional scaffold by incorporating the latent TGF-β1. Amongst a range of known growth factors, TGF-β1 was selected for its broad spectral regulatory effects on chondrogenesis. Nonetheless, the shortcomings of the TGF-β1 such as a short half-life and potential dose-related adverse systemic effects may limit its use in an in-vivo setting.

In facing this challenge, the subsequent study developed a cell-mediated activation of growth factor system by incorporating the latent TGF-β1 onto the electrospun scaffolds. Based on the knowledge that the TGF-β1 is secreted in its latent form by chondrocytes in the native extracellular matrix and is protected by its latent peptide hence prolonging its half-life - the latent TGF-β1 was utilised as the model latent growth factor. Physicochemical characterisation techniques confirmed that the latent TGF-β1 was successfully incorporated onto the scaffolds and activated by means of acid as well as enzymatic treatment. The biofunctionalised scaffolds were shown to be non-cytotoxic and induced in-vitro dedifferentiated human primary nasal chondrocytes to express significantly higher cartilage-specific transcription factor Sox9 compared to the conventional media supplementation method of active TGF-β1 at 14 days. These initial results established that the concept system of cell-mediated activation of the latent TGF-β1 had been achieved. As the cell culture assessments of latent TGF-β1 biofunctionalised scaffold required a serum-free environment, the
potential longer term effects of the biofunctionalised scaffolds would necessitate further evaluation in an *in-vivo* physiological condition.

The six weeks *in-vivo* study demonstrated that the latent TGF-β1 biofunctionalised scaffolds significantly up-regulated cartilage-specific markers and re-differentiated the dedifferentiated primary human nasal chondrocytes. This further confirmed that the cell-mediated activation of growth factor had been successful and transference to the *in-vivo* setting. In addition, the outcome induced by this system will depend on the cell types used. Although these results were promising in promoting chondrogenic differentiation, further investigations are required to determine ways to increase the production of cartilage matrix by the cells.

First of all, further studies may need to focus on a larger expansion of cells for a higher seeding density *in-vivo*. A seeding density of greater than $1 \times 10^6$ cells/cm$^2$ may be required for a more effective promotion of chondrogenesis(74,243-245). However, this may mean a further increase in the number of passage which will inevitably result in further loss of chondrogenic capacity. In addition, a longer pre-implantation *in-vitro* culture period with chondrogenic serum-free media may be required to allow the cells to undergo the process of chondrogenic differentiation. The exact length of time for this will need to be determined, to allow sufficient time for the cells to re-differentiate in the *in-vitro* serum-free environment without compromising cell survival. Together with a higher seeding density, an implantation period beyond 6 weeks, possibly extending up to or greater than 3-6 months, may also contribute to chondrogenesis(246-249).

The cartilage tissue, like any other specialised tissue in the body - requires specific culture conditions, mechanical stimulation and multiple signalling molecules to maintain its differentiated state. Thus, the incorporation of these essential requirements is likely to further induce and promote chondrogenesis from multiple angles. For instance, expanded chondrocytes seeded onto the biofunctionalised scaffolds may benefit from a medium term (approximately 4 – 6 weeks) *in-vitro* culture in a dynamic condition provided by a bioreactor. This is to simulate the physiological flow of interstitial fluid which not only encourages nutrient-waste exchange but also stimulates cartilage matrix production(250-252). The bioreactor
culture system also enables the precise control of oxygen tension which will further encourage chondrogenic differentiation(11).

The chondrogenic serum-free media described in this thesis may also benefit from an additional combination of anabolic growth factors – namely insulin-like growth factor and bone morphogenetic protein-2. These growth factors have been shown to synergistically enhance the effects of TGF-β1 in chondrogenesis(19;143;253). These growth factors, coupled with the employment of dynamic mechanical induction during culture will further encourage the synthesis of cartilage matrix. The mechanical properties of the resultant constructs will also be determined. The novel concept of this research may also be applicable to other forms of scaffold systems. Currently, a member of our research group is investigating the possible methods of incorporating the latent TGF-β1 in a hydrogel scaffold for the purpose of developing an injectable system.

As this thesis aimed to establish the proof of a novel concept in growth factor/protein activation, the work described here was focused on the conception process and its characterisations. First, the anchorage of the latent protein onto the electrospun scaffold was established. Then, biocompatibility, cytotoxicity and the ability to produce cartilage matrix were determined. As mentioned above, further work to increase the production of cartilage matrix from the tissue engineered constructs is the next essential step. Once sufficient matrix production has been established, the possible in-vivo inflammatory effects will be determined. This will involve using substituting human chondrocytes with murine autologous chondrocytes harvested from auricular cartilages of immune competent specimens such as Sprague-Dawley rats (Harlan Hsd:Sprague-Dawley®TM). Blood C-reactive protein, white cell count analyses(254) together with histological comparison with immunocompromised athymic rats will demonstrate the immunogenicity of the resultant tissue engineered construct. The inflammatory response may also be influenced by the degradation products of poly(α-hydroxyesters) such PLLA and PGA which are essentially polymeric chains of lactic acid and glycolic acid. As scaffold surface modification inevitably results in polymeric chain scission, the degradation products as a result of hydrolysis would create an acidic environment which may be detrimental to cell
growth. Evaluation of degradation would involve structural analysis with SEM as well as XPS scaffold surface chemical analysis at various time points. Both techniques would demonstrate physical and chemical changes of the scaffolds. Degradation of scaffolds will also affect the mechanical properties of the construct. Therefore, the mechanical properties of the resultant constructs will also be determined. As mentioned previously, the natural structure and composition of cartilage confer the tensile strength, compressive stiffness and elastic modulus(26;251). Different zones of cartilage possess different mechanical properties due to the variation in structural organisation as well as its composition. Therefore, the final tissue engineered cartilage construct will require mechanical properties that reflect its native counterpart. This will be evaluated at various pre- and post-implantation time points.

In conclusion, the proof of concept presented in this thesis may form a technological platform for further development of a scaffold system equipped with cell-mediated growth factor or protein activation – ultimately towards a structurally and functionally biomimetic scaffold for optimal tissue regeneration.


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Appendices

1.1 Step Wise Dehydration and Critical Point Drying for SEM

1. Grow the cells in flasks
2. Follow sub-culturing protocol to get the cells in suspension
3. Count the cells using trypan blue protocol
4. Workout the density of cells needed to seed on the materials
5. Seed the cells and allow them to grow for desired period of time
6. Make sure that you do not pipette or pour the solutions on top of the cells
   (there is a very high chance of washing the cells off)
7. Wash the cells with PBS twice
8. Fix the cells with 2.5% glutaraldehyde in PBS for 40 minutes at 4°C
9. Rinse with PBS twice
10. Dehydrate with graded series (increasing concentration) of ethanol
    a. 25% - 5 minutes
    b. 50% - 5 minutes
    c. 70% - 5 minutes
    d. 90% - 5 minutes
    e. 100% - 5 minutes
    f. 100% - 5 minutes
11. Incubate in hexamethyilsilasane (HMDS) for 5 minutes
12. Incubate in fresh HMDS for 5 minutes
13. Sputter coat samples with gold
14. View under scanning electron microscope
1.2  Cell Culture Protocols

Most commercially available cells are purchased in frozen form. Upon receipt these frozen cells must be immediately transferred to liquid nitrogen. The protocol below describes how to resuscitate frozen cells stored under liquid nitrogen.

1.2.1  Resuscitation of Frozen Cells

1. Prepare growth media
2. Pre-warm media
3. Label flask and 15ml centrifuge tube
4. Add 8 ml of media to the centrifuge tube
5. Take the ampoule containing cells out from the liquid nitrogen bank (wear thermal gloves and mask)
6. Leave the ampoule at room temperature for approximately 1 minute (not more than 1 minute)
7. Transfer the ampoule to the water bath at 37ºC and allow it to thaw for approximately 4 minutes
8. Wipe the ampoule with a tissue soaked in 70% alcohol and transfer immediately to the safety cabinet
9. Carefully pipette the cells into the centrifuge tube
10. Rinse the ampoule with 1 ml of fresh media and add to the centrifuge tube
11. Centrifuge the tube at 200xg for 5 minutes
12. Take the supernatant out without disturbing the pellet
13. Flick the tube to disturb the pellet
14. Add 1 ml of fresh growth media and make an even suspension of cells by repeated pipetting
15. Add required amount of growth media to flask
16. Add the cell suspension to flask and mix well
17. Transfer the flask to the incubator

When the cells reach sub-confluence levels (70-80% confluent) they must be sub-cultured and seeded onto materials or into flasks at a lower density. This procedure is also known as passaging or trypsinisation. Every time the cells are sub-cultured they increase in passage number. For experiment reproducibility cell passage number
should be similar in different cell culture experiments. Increased passage may result in phenotypic changes in cells.

1.2.2 Sub-Culture of Adherent Cells

1. Prepare growth media
2. Pre-warm media, trypsin- ethylenediaminetetraacetic acid (EDTA), and phosphate buffered saline (PBS) without Ca$^{2+}$ and Mg$^{2+}$
3. Aspirate the media and discard
4. Wash the cells with PBS
5. Add trypsin-EDTA (approximately 1ml per 25 cm$^2$)
6. Rock the flask to cover the monolayer
7. Incubate the flask at 37ºC for 4 minutes
8. Check under the microscope to make sure the cells are detaching from the surface and floating
9. Gently tap on the sides of the flask to detach the remaining attached cells
10. Add growth media (twice the volume as trypsin) to the flask and mix well
11. Transfer the cell suspension to a centrifuge tube
12. Centrifuge the tube at 200xg for 5 minutes
13. Take the supernatant out without disturbing the pellet
14. Flick the tube to disturb the pellet
15. Add 1 ml of fresh growth media and make an even suspension of cells by repeated pipetting
16. Count the cells using trypan blue protocol
17. Add required amount of growth media to labelled flask
18. Add the required amount of cell suspension to flask and mix well
19. Transfer the flask to the incubator.

Cryopreservation of cells is very important in cell culture. It enables stock of cells to be stored for extended period of time (e.g. many years). It reduces the risk of contamination, risk of phenotypic changes, cost and need to have the cell lines in culture at all time. It also enable for the experiments to be carried out using cells at a consistent passage number.
1.2.3 Cryopreservation of Adherent Cells

1. Prepare freezing media (10% dimethyl sulfoxide (DMSO) + 90% FBS)
2. Pre-warm freezing media, media, trypsin-EDTA, and PBS (without Ca\(^{2+}\) and Mg\(^{2+}\))
3. Perform steps 3 - 14 of the sub-culture protocol
4. Add 1 ml of freezing media and make an even suspension of cells by repeated pipetting
5. Perform cell count and dilute the cell suspension in freezing media to achieve 2 – 4 \( \times \) \(10^6\) cells per ml
6. Add 1 ml of cell suspension into labelled ampoule (cryotube)
7. Place ampoule in a freezing flask (containing isopropanol) and leave it in a –80ºC freezer overnight (cells freeze at a rate of 1ºC/minute)
8. Transfer the ampoule to liquid nitrogen bank and record the position on the logbook

1.2.4 Seeding Cells on Materials

For reproducible results it is important to determine the number of cells/cm\(^2\) of material. Only by keeping the cell number constant from experiment to experiment can the bioactive material properties such as cell growth, attachment or the production of cellular factors be accurately assessed.

To determine the seeding density the trypan blue exclusion assay is routinely used. Trypan blue is a vital dye and does not interact with the cell unless the membrane is damaged. Cells that exclude the dye are viable. The proportion of viable and dead cells can then be determined with a haemocytometer.

Trypan Blue Staining of Cells

1. Make a cell suspension according to sub-culture protocol
2. Prepare haemocytometer and coverslip by spraying with 70% alcohol and drying with a clean tissue
3. Fix coverslip on haemocytometer by moistening the coverslip with water or exhaled air, slide the coverslip over the chamber, and move back and forth exerting slight pressure until Newton’s rings (rainbow coloured rings) appear
4. Place 0.2 mL of a suitable cell suspension (in complete medium) in a sealed container
5. Add 0.2 mL of 0.4% trypan blue stain and mix thoroughly (dilution factor of 2)
6. Allow to stand for 2-3 min at 15 to 30°C (room temperature). Prolonged exposure to trypan blue kills the cells.
7. With a pipette fill both chambers of the haemocytometer. Do not over or under fill the chambers. Make sure that there are no air bubbles
8. Under a microscope count the number of viable (unstained) and non-viable (stained blue) cells in eight - ten 4 × 4 squares or 0.1cm² area (Fig. A.1). Count the cells in the squares and touching left and top middle line
9. Calculate the number of viable and non-viable cells/ml using the formulae given below

Number of viable cells/mL

= The average number of viable cells per 0.1cm² area

  \times 10^4 \text{ (correction factor for volume of area indicated by brace)}

  \times 2 \text{ (dilution factor, 0.2ml of cell suspension in 0.4mL)}

Fig. A.1. Picture showing one 4 × 4 corner square (0.1 cm²) on one of the haemocytometer chambers.
1.3 Live/Dead Assay

Molecular Probes (L-3224): LIVE/DEAD® Viability/Cytotoxicity Kit *for animal cells* *1000 assays*

Materials:
Reagents:
- LIVE: Calcein AM (Component A), two vials, 40µL each, 4mM in anhydrous DMSO
- DEAD: Ethidium homodimer-1 (Component B), two vials, 150µL each 2mM in DMSO/H2O 1:4 (v/v)
Cell culture plates and coverslips, PBS, Assay Reagents.

Protocol:

Preparation
1. Culture cells on coverslips
2. Thaw LIVE/DEAD assay constituents.
3. Wash cells with PBS prior to assay to remove/dilute serum esterase activity.
4. Treat cells with cytotoxic agents if required.

Concentration Determination
5. Prepare controls of both live and dead cells on coverslips.
   Kill the cells using preferred method (0.1% saponin, 10 mins; 0.1-0.5% digitonin, 10 mins; 70% methanol for 30 mins or complement and appropriate IgG for 30 mins).
6. Using the dead cell sample, select an EthD-1 concentration that stains the dead cell nuclei bright red without staining the cytoplasm significantly: from 0.1 to 10µM EthD-1, typically 4µM. (20µL of 2mM EthD-1 added to 10mL PBS = 4µM EthD-1).
7. Using the dead cell sample, select a calcein AM concentration that does not give a significant fluorescence in the dead cell cytoplasm: from 0.1 to 10µM calcein AM, typically 2µM. (5µL of 4 mM calcein AM to the 10mL EthD-1 Solution = 2µM calcein AM)
8. Using the live cell sample, check that the chosen calcein AM concentration produces sufficient fluorescence in the live cell sample.

**The Assay**

9. Add 100-150 µL of the combined LIVE/DEAD assay reagents to the surface of a coverslip.

10. Incubate for 30 - 45 mins at room temperature in a covered dish to prevent drying.

11. Add 10µL drop of fresh LIVE/DEAD reagent or PBS to a clean microscope slide, then invert the coverslip and mount on the slide. Seal slide with clear nail polish to prevent evaporation.

12. View labelled cells under the fluorescent microscope.
1.4 Image J Manual

1.4.1 Measuring Circularity

1. To open an image, go to File/Open/ My pictures to select image to be analysed.

2. The images chosen were of red/green/blue stack (RGB stack) as seen on Live/Dead images.

3. Next go to Images/Type and select RGB stack. This converts all images to grey scale but this enables the program to identify red, green and blue spectrum on the picture.

4. We then select the “green” picture as indicated from the top left corner of the image.

5. To outline each cells, the images will need to be converted into binary form. Go to Process/Binary/Threshold/. Then go through the same route and select Outline to outline the cells.

6. From the tool bar, select Wand (tracing) tool.

7. Next go to Analyze/ Set Measurements to select the parameters to measure. Decimal places is set at 3 while Redirect to None. Then press OK.

8. We can now select the cells by just clicking on the image and a window displaying the results of the measurements will appear.

9. Do not forget to label the cells everytime we have selected them for measurement. Analyse/ Label.

10. Finally to save the images and results, click on File/ Save As. This can then be opened using another program for example Excel Analyse-it and ACDsee.

An alternative method of measuring cells which are too confluent to assess, we can avoid step 2-6 from above. Instead, we can trace the cells manually by selecting Freehand Selections from the Image J tool bar on the selected image. Set 7 onwards should then be followed.

A full detailed manual can be found on http://rsb.info.nih.gov/ij/
1.4.2 To Measure Diameter of Fibres

11. To open an image, go to File/Open/My pictures to select image to be analysed.
12. Go to Analyse, Set Measurements, and select features of interests. Also make sure the decimal places are correct.
13. Click on Straight Line Selection. Then click and drag on the scale bar for calibration.
14. To calibrate the pixels to the scale bar, go to Analyse, Set Scale. Change the scale and units (e.g. for microns, enter μm and for nanometers, enter nm) as accordingly. Select Global only if subsequent images carry the same scale bar or magnification.
15. We can now start to measure the diameter by selecting one edge of the fibre and dragging it to the other edge (Fig. A.2). Go to Analyse then Measure.
16. To label, it is important to immediately go to Analyse then Label.
17. At the end, go to Analyse, Summarise to obtain the mean and Standard deviation. Finally save as described in step 10.

Fig. A.2. An SEM image of electrospun PLLA fibres. This image illustrates the method employed for the measurement of fibre diameter using the Image J software. The black lines are where fibre diameters were measured, while the numbered labels indicate the number of fibres that has been included for analysis.
1.4.3 Beads measurements

Bead circularity measurements were performed as according to Appendix 1.4.1 (Fig. A.3).

Fig. A.3. A typical SEM image of showing the analysis of beads in electrospun PLLA fibres. This image illustrates the method employed for the measurement of fibre bead numbers and circularity using the Image J software. The black lines encircle the beads, while the numbered labels indicate the number of beads that has been included for analysis.
<table>
<thead>
<tr>
<th>Polymer Concentration (%)</th>
<th>Rate (mL/hr)</th>
<th>kV</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
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<td>6</td>
<td>0.088</td>
<td>0.024</td>
</tr>
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<td>1.5</td>
<td>0.2</td>
<td>10</td>
<td>0.074</td>
<td>0.021</td>
</tr>
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<td>0.085</td>
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<td>10</td>
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<td>6</td>
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<td>10</td>
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</tbody>
</table>

Table A1. Electrospinning parametric study data. Means and standard deviations (SD) of fibre diameters electrospun at different polymer concentrations 1 – 3% (w/w), rate of delivery of polymer solution and accelerating voltage are presented here.
<table>
<thead>
<tr>
<th>Polymer Conc [\text{%}(w/w)]</th>
<th>Rate [mL/hr]</th>
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<th>Mean</th>
<th>SD</th>
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Table A.2. Electrospinning parametric study data. Means and standard deviations (SD) of fibre diameters electrospun at different polymer concentrations 4 – 7.5\text{%}(w/w), rate of delivery of polymer solution and accelerating voltage are presented here.
1.6 Glycine Standard Curve for Ninhydrin Assay

1. 10mL of 0.1M Glycine (Sigma, UK) was serially diluted with 50\%v/v ethanol to achieve 0.075, 0.05, 0.025, 0.01, 0.0075, 0.005 and 0.0025mM.
2. 1mL of each concentration was placed in a 5mL glass vial.
3. 100\(\mu\)L of 1M Ninhydrin (Chapter 4) was added to each vial. Immediate colour change was observed.
4. All standards were heated up to 80\(^\circ\)C, for 15mins.
5. Absorbance was measured at 570nm using UV-Vis spectrophotometer.
6. The optical densities (570nm) were plotted against the concentration of glycine which is represented by NH\(_2\) concentration. Fig. 4.3.
1.7 DNA content from MTS assay samples

Fig. A.4. DNA concentration at day 1, 7 and 14. DNA concentration of cells from various scaffold types were obtained as described in Chapter 4. The DNA concentration was used for normalisation of MTS assay absorbance to obtain the metabolic activity of the cultured chondrocytes. Results are presented as mean ± standard deviation (n=3).
1.8 Histology Protocols

1.8.1 Haematoxylin & Eosin Staining

(Modified from Meyer’s Protocol from http://www.ihcworld.com/histology.htm)

Materials

Haematoxylin solution – Gill’s Formula, Vector Laboratories

Eosin Y Stock Solution (1%):
Eosin Y (Sigma-Aldrich, UK)------------- 10 g
Distilled water ---------------------------- 200 mL
95% Ethanol ----------------------------- 800 mL
Mix to dissolve and store at room temperature.

Eosin Y Working Solution (0.25%):
Eosin Y stock solution ------------------- 250 mL
80% Ethanol ----------------------------- 750 mL
Glacial acetic acid (concentrated) ------- 5 mL
Mix well and store at room temperature.
DPX Mountant (Low Viscosity) – Bios Europe Ltd., UK

Procedure:

1. Stain acetone fixed slides in haematoxylin solution for 1-2 minutes.
2. Rinse gently in running tap water for 10 minutes (until water runs clear).
3. Counterstain in eosin solution for 30 seconds to 1 minute.
4. Dehydrate through 50%, 70%, 95% alcohol, 2 changes of absolute alcohol, 2 minutes each.
5. Clear in 2 changes of xylene, 5 minutes each.
6. Mount with xylene based mounting medium.

Results:

Nuclei ---------------------------------- blue
Cytoplasm ----------------------------- pink to red
1.8.2 Safranin-O and Fast Green Staining

(Modified from http://www.ihcworld.com/_protocols/special_stains/safranin_o.htm)

Materials

0.001% Fast Green (FCF) Solution:
- Fast green, FCF (Sigma, UK) -------------- 0.01 g
- Distilled water -------------------------- 1000 mL

1% Acetic Acid Solution:
- Acetic acid, glacial --------------------- 1 mL
- Distilled water -------------------------- 99 mL

0.1% Safranin O Solution:
- Safranin O (Sigma, UK) --------------- 0.1 g
- Distilled water ------------------------- 100 mL

DPX Mountant (Low Viscosity) – Bios Europe Ltd., UK

Procedure:

1. Stain with haematoxylin solution for 1-2 minutes.
2. Wash in running tap water for 10 minutes (until water runs clear).
3. Stain with fast green (FCF) solution for 5 minutes.
4. Rinse quickly with 1% acetic acid solution for no more than 10 –15 seconds.
5. Stain in 0.1% safranin O solution for 5 minutes.
6. Dehydrate and clear with 50%, 70%, 95% alcohol, 2 absolute alcohol and 2 xylene changes, 2 minutes each.
7. Mount using resinous medium.

Results:

Nuclei ---------------------------------------- black
Cytoplasm ------------------------------------ gray green
Cartilage, mucin, mast cell granules ---------- orange to red
1.8.3 Alizarin Red S Staining

(http://www.ihcworld.com/_protocols/special_stains/alizarin_red_s.htm)

Description: Alizarin Red S, an anthraquinone derivative, may be used to identify calcium in tissue sections. The reaction is not strictly specific for calcium, since magnesium, manganese, barium, strontium, and iron may interfere, but these elements usually do not occur in sufficient concentration to interfere with the staining. Calcium forms an Alizarin Red S-calcium complex in a chelation process, and the end product is birefringent.

Fixation: Neutral buffered formalin or alcoholic formalin fixed, paraffin embedded tissue sections.

Positive Control: mouse embryo or a known calcium containing tissue sections

Solution and Reagents:

Alizarin Red Solution:

Alizarin Red S (C.I. 58005) -------------- 2 g
Distilled water ------------------------ 100 mL

Mix well. Adjust the pH to 4.1–4.3 with 10% ammonium hydroxide. The pH is critical, so make fresh or check pH if the solution is more than one month old.

Acetone (100%)

Acetone-Xylene:

Acetone (100%) ------------------------ 50 mL
Xylene ------------------------------- 50 mL

Procedure:

1. Stain slides with the Alizarin Red Solution for 30 seconds to 5 minutes, and observe the reaction microscopically. Usually 2 minutes will produce nice red-orange staining of calcium.

2. Shake off excess dye and blot sections.
4. Clear in xylene and mount in a synthetic mounting medium.

**Results**
Calcium deposits (except oxalate) -------- orange-red
This precipitate is birefringent.
1.9 Biochemical Characterisation of *In-vivo* Cultured Cell-scaffold Constructs

This section was performed by Mr T. Sims (Department of Cellular and Molecular Medicine, University of Bristol) as part of the collaboration for the *in-vivo* study (Chapter 5). Type I and II inhibition ELISA were carried out to determine the quantity of matrix produced by the human chondrocytes and MSC.

1.9.1 Materials and Methods

1.9.1.1 Proteolytic Digestion of Explants for Biochemical Analysis

Dry weights of the implants were determined after freeze-drying process. The implants were then digested with trypsin and processed for collagen content analysis. The samples were initially incubated for 15 hours at 37°C with 250µL tosylphenylalanylchloromethane-treated bovine pancreatic trypsin prepared at 2mg/mL in Tris Buffer, pH7.5, containing 1mM iodoacetamide, 1mM ethylenediaminetetraacetic acid and 10µg/mL pepstatin A (all from Sigma, UK). An additional 250µL of trypsin was added to all samples with a further incubation of 2 hours at 65°C. All samples were boiled for 15 minutes at the end of the incubation to stop any remaining enzyme activity (230;255;256). The dry weights of the trypsin-digested extracts were determined by obtaining the difference between dry weights of the initial implants and the remaining undigested materials.

1.9.1.2 Determination of Type I Collagen Content by Inhibition ELISA

The extracts were assayed by inhibition enzyme linked immunosorbent assay (ELISA) using a rabbit antipeptide antibody to type I collagen (230). Peptide SFLPQPPQ (Serine - phenylalanine - leucine - proline - glutamine – proline – proline - glutamine) synthesised by Dr A. Moir (Kreb’s Institute, Sheffield University, UK) was used as a standard in all immunoassays.

Round-bottom 96-well microtiter plates (Costar, UK) were precoated with Dulbecco’s phosphate buffered saline (PBS; Invitrogen, UK) containing 1% (w/v) bovine serum
albumin (PBS-BSA; Sigma-Aldrich, UK) 100µL/well for 30mins at room temperature, then rinsed once with PBS containing 0.1% (v/v) Tween 20 (PBS-Tween; Sigma-Aldrich, UK). To minimise the effects evaporation, the outer most wells were not used. The 8-residue peptide was used at a concentration of 4 to 1000ng/mL to produce a standard curve for each plate. The standards and samples were diluted in 0.8%(w/v) sodium dodecyl sulphate (SDS; Bio-Rad, UK) in tris(hydroxymethyl)methylamine (Tris; BDH Laboratories, UK). The SDS was used to solubilise type I collagen and to dissociate any interactions with other proteins present within the sample. Each pre-incubated plate contained six non-specific binding wells, containing SDS-TRIS (100µL/well). All other wells contained 50µL of the rabbit anti-peptide antibody diluted 1:1000 in Tris containing 4% (v/v) Triton X-100 (BDH, UK). The antibody in six of the wells was mixed with 50µL of SDS-Tris, to indicate maximum binding in the absence of inhibitory epitope. The antibody in all other wells was mixed with 50µL of either standard or sample, in triplicate.

After an overnight incubation at 37°C in a humidified atmosphere, all samples were transferred to the equivalent wells of an Immulon-2 ELISA plate (Dynex Technologies, UK), coated with immunising peptide (40µg/mL). After 30mins at room temperature, the plates were rinsed three times with PBS-Tween and incubated for 1 hour at 37°C with alkaline phosphatase-conjugated goat anti-rabbit antibody (Southern Biotechnology, USA) diluted 1:1000 in PBS-BSA containing 0.1%(v/v) Tween 20. The plates were then rinsed three times in PBS-Tween and once with distilled water. Subsequently, 0.5mg/mL alkaline phosphatase substrate disodium p-nitrophenyl phosphate (Sigma-Aldrich, UK) in 8.9mM diethanolamine (Sigma-Aldrich, UK), 0.25mM MgCl₂ (pH9.8) for 30 minuntes. Absorbance was measured at 405nm using a microplate reader Anthos 2020 (Biochromo Limited, UK). The mean absorbance from the six non-specific binding wells was subtracted from the absorbance value of all wells from the same plate.

As the 8-residue peptide was used as the standard in all assays, a conversion factor was used to calculate the amount of type I collagen present in all samples. The molecular weights of the α1-chain of human type I collagen and the 8-residue peptide were calculated from their amino acid sequences – 96962 and 999 respectively. As
type I collagen consists of two $\alpha 1$ (I) chain and one $\alpha 1$(II) chain, the concentration of type I collagen (ng/mg) extracted from the implants was calculated by multiplying the concentration of extracted peptide by 146 [i.e. $(96962 \times 3)/ (999 \times 2)$].

### 1.9.2 Determination of Type II Collagen Content by Inhibition ELISA


Round-bottom 384-well microtiter plates were blocked and rinsed as described for type I collagen. To minimise the effects evaporation, the outer most wells were not used. 20µL of 50mM Tris, pH7.6 were added to 6 wells of each plate and considered as non-specific binding wells. 10µL/well of COL2-3/4m antibody diluted 1:600 in Tris was added to each well of the preincubated plates, except the non-specific binding wells. This was to provide a detectable but inhabitable level of binding. 10µL/well of Tris was added to 6 wells, which already contained 10µL/well of COL2-3/4m antibody - to indicate maximum binding in the absence of inhibitory epitope. 10µL of peptide CB11B was used at a concentration of 0.5 to 6µg/mL to produce a standard curve for each plate. Subsequently, 10µL of samples were added to each remaining wells in triplicate.

After an overnight incubation at 37°C in a humidified atmosphere, 10µL of all samples were transferred to the equivalent wells of an Immulon-2 ELISA plate (Dynex Technologies, UK), precoated with bovine type II collagen (40µg/mL) (Sigma-Aldrich, UK).

After 30mins at room temperature, the plates were rinsed three times with PBS-Tween and incubated with 10µL of alkaline phosphatase-conjugated goat anti-mouse
antibody (Zymed Laboratories, USA) diluted 1:1000 in PBS-BSA containing 0.1% (v/v) Tween 20, for 1 hour at 37°C. The plates were then rinsed three times in PBS-Tween. Subsequently, 10µL of alkaline phosphatase substrate solution consisting of disodium p-nitrophenyl phosphate 0.5mg/mL (Sigma-Aldrich, UK) in 8.9mM diethanolamine (Sigma-Aldrich, UK), 0.25mM MgCl₂ (pH9.8) for 30mins. Absorbance was measured at 405nm using a microplate reader Anthos 2020 (Biochromo Limited, UK). The mean absorbance from the six non-specific binding wells was subtracted from the absorbance value of all wells from the same plate. The percentage inhibition of binding by samples and standards was calculated relative to the mean absorbance from the six maximum binding wells on the same plate, which represented 0% inhibition (100% binding).

The molecular weights of the human type II collagen α1-chain and of peptide α1(II)-CB11B were each determined from their amino acid sequences(231). It was assumed that there are 99 hydroxyproline and 20 hydroxylysine residues of the α1(II) chain. Based on this, the molecular weights of the α1(II) chain and α1(II)-CB11B peptide were calculated as 98291 and 2231 respectively. Therefore, the concentration (µg/mg) of type II collagen extracted from cartilage was calculated by multiplying the concentration of extracted α1(II)-CB11B by a factor of 44. Type I and II collagen contents were presented as mean ± standard deviation (n=4).

1.9.3 Results and Discussion

The cell-free implants and rat subcutaneous tissue obtained distant from the dorsal implantation sites were used as controls. Both controls showed positive signals to both which strongly suggest that both antipeptide antibodies for type I and II collagen detection cross-reacted with the rat tissue (Fig. A.7 and Fig. A.8). This meant that matrices produced by human cells were indistinguishable from rat tissue. Further work utilising antibodies which are highly specific to human type I and II collagen will need to be performed.
Fig. A.7. Determination of collagen contents using the inhibition ELISA for the primary human chondrocytic group. (a) Type II collagen content, (b) Type I collagen content. For sample group ID, see Chapter 6. Mean ± SD are presented (n=4).

Fig. A.8. Determination of collagen content using the inhibition ELISA for the MSC group. (a) Type II collagen content, (b) Type I collagen content. For sample group ID, see Chapter 5. Mean ± SD are presented (n=4).