

Development of Scaffolds Incorporating Zonal Complexity for Articular Cartilage Tissue Engineering

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Abstract

Articular cartilage is an anisotropic tissue composed of compositional and functional layers. One clinical approach to the regeneration of articular cartilage defects incorporates a porous polymer scaffold to support and direct cartilage formation in full-thickness defects. These scaffolds are regularly isotropic in structure, unlike the tissue they aim to regenerate.

A number of scaffold production techniques were combined to produce porous anisotropic scaffolds with zonally-biomimetic microarchitecture and mechanical properties. The final scaffold design featured a combination of an electrospun fibrous superficial zone, isotropic foam intermediate zone and directionally frozen deep zone. The zonal scaffold microenvironments influenced cellular distribution, gene expression, and extracellular matrix deposition *in vitro* without requiring chemical modification or culture under dynamic loading.

The scaffold development work culminated in a porcine *in vivo* study, currently on-going. Initial data from the 3-month preliminary surgical trial suggests full cellular infiltration of acellular scaffolds, no immunological response, and improved articular surface morphologies relative to empty defect controls.

Variations on the polymer poly(ϵ -caprolactone) (PCL) were investigated for use in osteochondral tissue engineering applications. The incorporation of alternate monomers was found to modify the biological and mechanical properties of the resulting materials and scaffolds.

The work contained within this thesis has expanded the field of anisotropic scaffold design, with implications for articular cartilage engineering. The combination of electrospun fibres and anisotropic foams for scaffold engineering was the first in the field when published in Steele *et al.* [1]. The design of the third-generation scaffold is new to the field, as is the order-of-magnitude increase in stiffness in a porous polymer scaffold while maintaining interconnectivity and polymer composition. The observation of differentially aligned ECM within a single multi-layer scaffold without zonally distinct materials or surface functionalisation is also believed to be the first in the field.

Declaration of Originality

I, Joseph A.M. Steele, declare that the original scientific content contained within this thesis was acquired and analysed by myself at either Imperial College London, UK or Queensland University of Technology, Australia, unless otherwise stated. All collaborative efforts are noted by name and affiliation in the text.

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List of Abbreviations

α -MEM	Modified Eagle's medium
BL	Generation 2 bilayer scaffold
cDNA	Complementary DNA
CMFDA	5-chloromethylfluorescein diacetate
D	Diameter
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCM	Dichloromethane
DF	Directionally frozen
DF100	Generation 3 multilayer scaffold with 100-300 μ m diameter pores in the PLZ
DF300	Generation 3 multilayer scaffold with 300-500 μ m diameter pores in the PLZ
DFZ	Directionally frozen zone
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DMMB	1,9-dimethyl-methylene blue
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E*	Dynamic or complex modulus
E'	Storage modulus
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
F*	Dynamic force
FBS	Foetal bovine serum
FDA	United States Food and Drug Administration
FDM	Fused deposition modelling
FZ	Fibre zone
GAG	Glycosaminoglycan
h	Height
H&E	Haematoxylin and eosin
HAc	Hyaluronic acid (commonly referred to as 'HA')
HAp	Hydroxyapatite (commonly referred to as 'HA')
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
i.m.	Intramuscular
IgG	Immunoglobulin G
IP	Intellectual property
ISO	International Organization for Standardization
ITS	Insulin-transferin-selenium
K*	Dynamic stiffness
NSAID	Nonsteroidal anti-inflammatory drug
MERF	Medical Engineering Research Facility
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
OCT	Optimal cutting temperature medium
p.o.	Per os (by mouth)
P2	Passage 2
PBS	Phosphate buffered saline

PCL	Poly(ϵ -caprolactone)
PCR	Polymerase chain reaction
PDMS	Poly(dimethylsiloxane)
PLCL	Poly(L-lactide-co- ϵ -caprolactone)
PLL	Poly(L-lactide)
PLZ	Porogen-Leached Zone
PVC	Poly(vinyl chloride)
qRT-PCR	Quantitative real-time reverse-transcription polymerase chain reaction
QUT	Queensland University of Technology
RH	Relative humidity
RNA	Ribonucleic acid
SEM	Scanning electron microscope/microscopy
SEC	Size exclusion chromatography
sGAG	Sulphated glycosaminoglycan
SZP	Superficial zone protein
TGF- β 3	Transforming growth factor β 3
TL	Generation 2 trilayer scaffold
UTS	Ultimate tensile strength
X*	Dynamic displacement amplitude

1 Articular Cartilage: Structure, Disease and Current Repair Strategies

“When a bone, cartilage, nerve, the slender part of the jaw, or prepuce, are cut out, the part is neither restored, nor does it unite.”
– Hippocrates, Aphorisms, 400 BC

1.1 An Introduction to Articular Cartilage

Articular cartilage is a tissue which covers the articulating surfaces of all synovial joints, including the knee and hip (Figure 1-1), allowing for near-frictionless joint articulation, while cushioning shock and absorbing dynamic and static loading [2]. The articular cartilage lining the bony surfaces of the knee is a focus of a significant amount of research due the high prevalence of damage and disease.

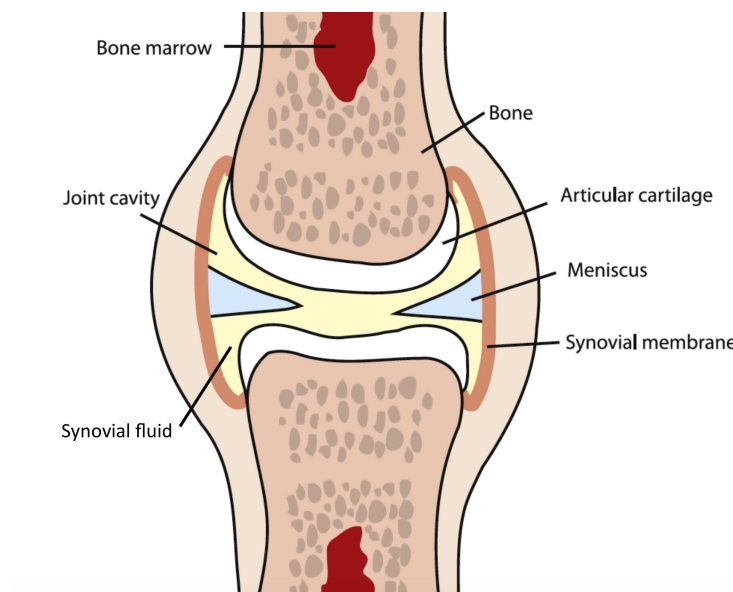


Figure 1-1. Schematic of a generalised synovial joint, such as the knee, modified from Hunziker *et al.* [3].

Cartilage is a deceptively simple tissue, with only one resident cell type, the chondrocyte, which produces, maintains, and resorbs the extracellular matrix. The tissue is 2.4 ± 0.5 mm thick in the adult human knee, with regions of the patella exceeding 5 mm in thickness [2,4]. Chondrocytes comprise only 2% of the tissue volume; the remaining 98% of cartilage is composed primarily of

water (70-85% (w/w)) and the extracellular matrix (ECM). The bulk of the ECM is composed of type II collagen and proteoglycans (Table 1-1) [2,5].

Table 1-1. Approximate ECM concentrations in articular cartilage sources [2,6–8]

Source	Collagen ($\mu\text{g}/\text{mm}^3$)	sGAG ($\mu\text{g}/\text{mm}^3$)	Chondrocytes (Cells/ mm^3)	Collagen ($\mu\text{g}/\mu\text{g DNA}$)	sGAG ($\mu\text{g}/\mu\text{g DNA}$)
Adult Human (superficial)	200	20	24 000	1080	108
Adult Human (deep)	200	50	7 700	3370	843
Foetal Bovine	50	25	150 000	40	22
Adult Bovine	100	25	30 000	400	110

Proteoglycans contain sulphated glycosaminoglycans (sGAG), primarily chondroitin-6-sulphate and keratin sulphate, bound to an aggrecan protein backbone – forming brush-like proteoglycan monomers (Figure 1-2A). The negatively charged sulphate groups in sGAG attract cationic molecules that increase the osmotic pressure of the tissue and swell, a process termed the Donnan effect [9]. The composition and variations in proteoglycans are comprehensively reviewed by Roughly [10]. The PG monomer backbones are in turn attached via a link protein to hyaluronan, in a brush-like fashion (Figure 1-2A) [11]. Type II collagen forms a fibrillar network that interacts with proteoglycan by binding to the bases of aggrecan monomers at their keratin-sulphate domains (Figure 1-2B) [12]. The interplay between the tensile and compressive properties of type II collagen and PG result in a very resilient tissue [9,13].

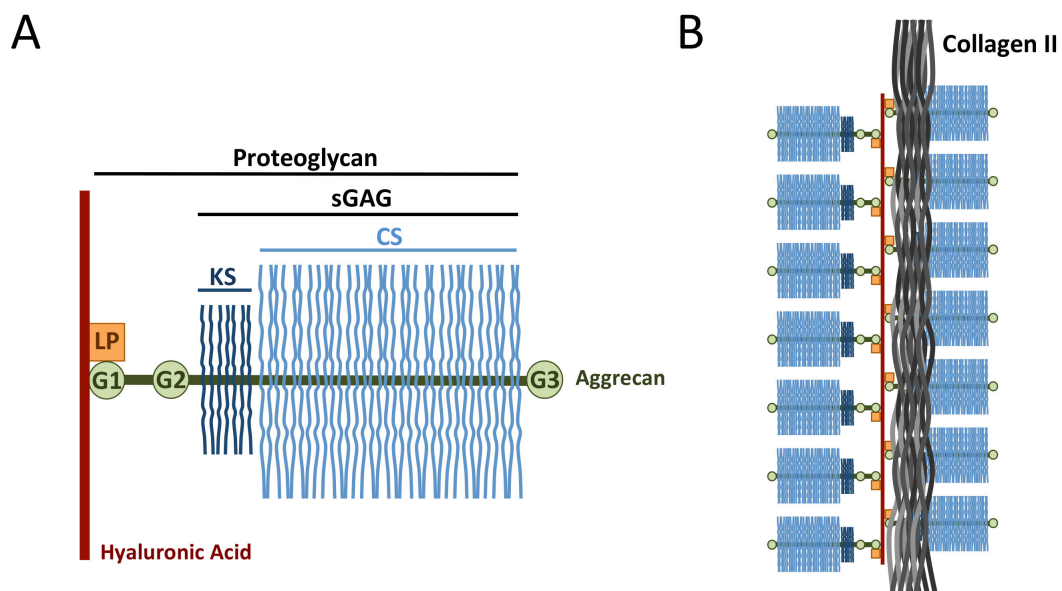


Figure 1-2. A) Structure of proteoglycan brushes. sGAG - sulphated glycosaminoglycans, CS - chondroitin-6-sulphate, KS - keratin sulphate, LP - link protein, G - aggrecan globular domain. B) Multiple PG subunits on the hyaluronic acid backbone and a type II collagen fibril interacting with the KS-rich domains.

The remaining components of cartilage perform a variety of roles, both mechanical and biochemical. Collagen VI accounts for less than 1% (w/w) of the collagens in articular cartilage but is an important minor component as it delimits the pericellular matrix, acting as the primary connection between the chondrocyte and its environment, imparting diffusional restrictions, and influencing the joint structure and development [14]. Collagens IX and XI account for 1% and 3% of the collagen in mature articular cartilage, but approximately 10% each in young growth cartilage [5]. Their functions are believed to be type II collagen fibril crosslinking and fibril formation, and they are found primarily within the chondron surrounding the chondrocytes. Type X collagen is associated with chondrocyte hypertrophy and is found in the ECM of the chondrocytes at the interface with the calcified cartilage [5]. Other minor components of the cartilage also include collagens III, XII and XIV which aid in the repair of minor matrix damage and cartilage homeostasis [5,15].

Cartilage is avascular and aneural, which greatly reduces the ability to sense damage and initiate repair cascades, relative to other tissues. The lack of vasculature also limits oxygen, nutrient, and waste transfer in the tissue [2]. The primary mechanism of nutrient and waste transfer is diffusion between the vascularised synovial capsule and the articular surface via the synovial fluid that fills the joint cavity (Figure 1-1). Diffusion throughout the cavity and into the depths of the cartilage is facilitated by joint movement and compression cycles, pumping fluids into and out of the tissue. Some evidence of diffusion from the subchondral bone interface has been observed, but predominantly in association with disease states [16].

1.2 Anisotropy of Articular Cartilage

The modern scientific study of cartilage began in the mid-eighteenth century with the publication of *'Of the Structure and Diseases of Articulating Cartilages'* by William Hunter, in which he outlines many of the basic anatomical features of articular cartilage [17]. As a skilled anatomist Hunter prepared a patellar specimen for an anatomical drawing by removing the 'perichondrium' covering the articular surface, allowing Hunter to depict and discuss the vertical fibres that composed the articular cartilage (Figure 1-3). In hindsight, articular cartilage does not possess a perichondrium and therefore the layer removed, with difficulty, by Hunter was the distinct superficial zone. This misunderstanding is the first recorded observation of the anisotropic nature and basic structure of articular cartilage.

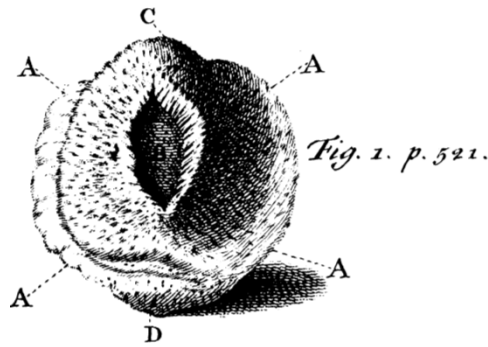


Figure 1-3. Articular cartilage of the patella, as observed by Hunter in 1742 [17]. A) – ‘The surface of the cartilage, appearing, when the perichondrium is removed, like velvet’.

Articular cartilage is anisotropic in structure, stiffness, chemistry, and cellular density from the articular surface through to the calcified cartilage layer that interfaces with the subchondral bone. This zonal organisation creates a load-bearing structure capable of absorbing cyclic high-impact forces and transitioning the energy from the smooth articular surface through to the rigid subchondral bone [18]. Articular cartilage can be subdivided into four distinct zones: superficial, intermediate, deep, and calcified cartilage, as shown in Figure 1-4.

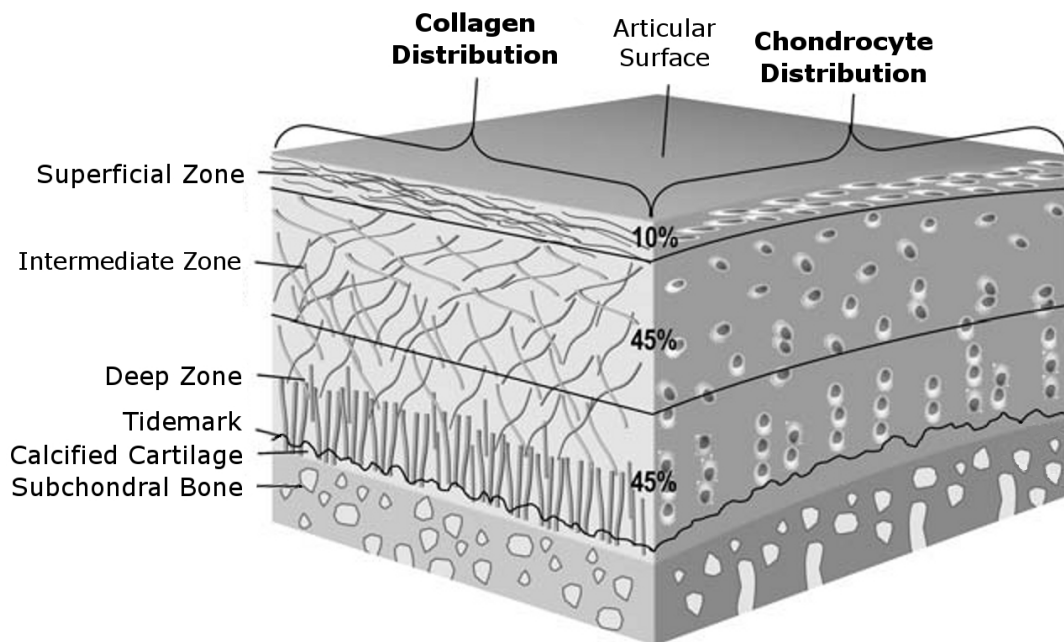


Figure 1-4. Osteochondral gradient illustrating the anisotropic nature of the type II collagen fibrils as well as the chondrocyte density and distribution. Modified from Woodfield *et al.* [18].

The orientation of type II collagen fibrils changes throughout the thickness of cartilage, as observed by Hunter in 1742 and depicted in Figure 1-3. The superficial zone has fibres aligned parallel to the articular surface, serving to increase the tensile strength of the articular surface, avoiding fissures when subjected to high shear. The intermediate zone is composed of randomly aligned collagen forming a tissue that is able to cushion loads. The deep zone of the cartilage has collagen fibres aligned perpendicular to the joint surface, forming a stiff interface with the underlying calcified

cartilage and subchondral bone. This transition from parallel through to perpendicular collagen alignment is integral to the mechanical properties of articular cartilage, as are the cellular distribution, density, and shape [2]. To complement the type II collagen structural anisotropy there is a depth-dependent zonal organisation of PG, increasing with depth, providing a gradient of osmotic pressure and matrix stiffness [2].

The zones also have distinct cellular concentrations and geometries (Figure 1-4). The articular surface contains the highest density of chondrocytes, with $24\,000 \pm 3\,000$ per mm^3 ($2.6 \pm 0.3\%$ (v/v)), which are elongated parallel to the surface, with volumes of $416 \pm 121\ \mu\text{m}^3$ (Figure 1-5). Through the thickness of the tissue, the cellular density decreases by greater than 70% to a minimum of $6\,900 \pm 800$ per mm^3 ($1.2 \pm 0.2\%$ (v/v)) in the deep zone, while chondrocyte morphology changes to a larger ($1117 \pm 287\ \mu\text{m}^3$) more rounded or vertically extended appearance with multiple chondrocytes grouped into chondrons [19].

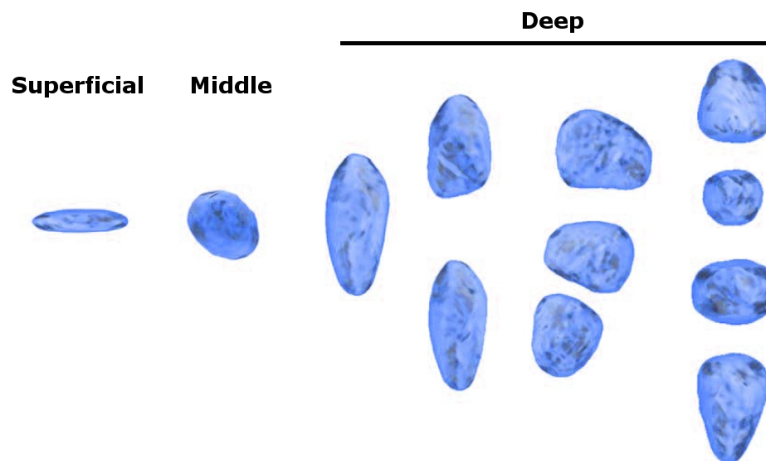


Figure 1-5. Representative chondrocyte morphologies in the superficial, intermediate and deep zones. Modified from Youn *et al.* [19].

Finally, in the deep zone leading into the tidemark (the calcified cartilage interface) the chondrocytes become hypertrophic, increasing type X collagen expression [2]. The calcified cartilage interface with subchondral bone is dendritic, with projections of interlocking calcified and uncalcified tissue. This geometry was initially thought to be a stress-transfer mechanism, but it may also have a role in diffusion between tissues [2,16].

1.3 Anisotropic Mechanical Properties of Cartilage

The mechanical properties of articular cartilage vary with zone according to the interplay between collagen alignment, fibril size, density, and PG concentration. The tissues in the system are variably viscoelastic living systems, storing energy in strained bonds and electrostatic interactions, while losing energy from stress relaxation due to fluid flow. Therefore, depending on the loading regime, strain rates, and experimental setup, the stated properties can be highly variable. Additionally, the properties fall across a distribution amongst the human population and change with age, location and a number of environmental and genetic factors [20].

The mechanical properties of articular cartilage, and mechanical testing protocols are reviewed by Little *et al.* [13], with the relevant properties summarised in Table 1-2.

Table 1-2. Mechanical properties of articular cartilage, modified from [13].

Mechanical Property	Range
Aggregate Modulus (Confined)	0.1-2.0 MPa
Hydraulic Permeability	10^{-16} - 10^{-15} m ⁴ /Ns
Compressive Young's Modulus (Unconfined)	0.24-0.85 MPa
Poisson's Ratio	0.06-0.3
Tensile Young's Modulus	5-25 MPa
Tensile Strength	0.8-25 MPa

Little *et al.* provides a range of bulk values for each parameter but the biochemical zonal organisation of the tissue is known to impart a significant mechanical anisotropy, as illustrated in Figure 1-6A, with the compressive modulus increasing 26-fold with depth. There is a non-linear deformation of the tissue, as shown in Figure 1-6B where a bovine cartilage explant is compressed 32% and the articular surface experiences > 60% strain, while the deep zone experienced < 5% strain [20]. This gradient of stiffness permits the cushioning effect of cartilage while avoiding a strain discontinuity at the subchondral interface and should be replicated in tissue engineered solutions in order to recreate a system with the proper microenvironmental cellular cues and to maintain construct integrity from implantation through regeneration.

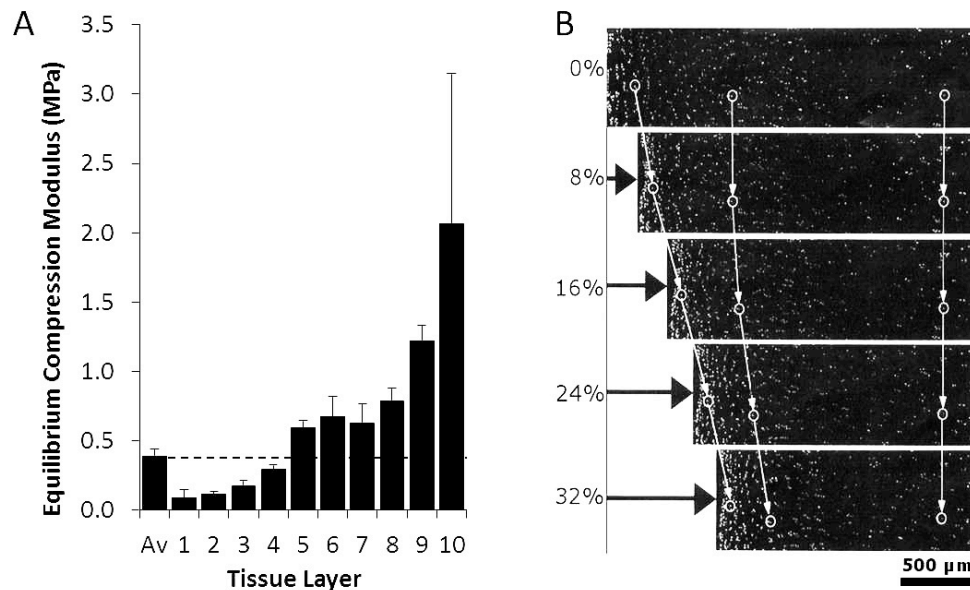


Figure 1-6. A) Distribution of equilibrium compression moduli of articular cartilage in 125 μm sections from the articular surface (1) to the interface with the subchondral bone (10) modified from Schinagl *et al.* [20]. The left-most bar and dotted line represent the full-thickness modulus, assuming the construct is homogenous. **B)** Epifluorescent images tracking the non-linear displacement of bovine chondrocyte nuclei under a series of compressive strains from Schinagl *et al.* [20]. The articular surface, to the left, experiences a greater strain than the deep zone, to the right. Circles indicate distinctive nuclei groupings tracked from the reference state throughout the compression regime.

The lack of agreement on a testing regime for cartilage constructs makes direct comparison of compressive moduli difficult. The establishment of equilibrium stresses can take over 16 minutes, which makes the determination of the equilibrium modulus time consuming and risks sample dehydration. For this reason, and the fact that joints are physiologically loaded at rates greatly exceeding equilibrium relaxation rates, dynamic compression testing regimes are regularly used. Dynamic testing protocols range from 0.0075% to 90% compressive strain at strain rates of 0.02% to 60% per second, under confined, unconfined, and probe-indentation protocols, with macro, micro, and nano-scale equipment; all of which produce different moduli for identical constructs [13,21,22]. There is some consensus in the field that 10% strain at 1Hz is optimal for mechanotransduction, and therefore this rate and strain are commonly used as a benchmark for mechanical characterisation.

1.4 Zonal Chondrocyte Sub-Population Phenotypes

The superficial, middle, and deep zones of articular cartilage possess distinct gene and microRNA expression profiles, as thoroughly summarised by Grogan *et al.* [23]. When chondrocytes are expanded *in vitro* to obtain sufficient cell quantities for implantation, they dedifferentiate, losing their characteristic gene expression profile as characterised by significant down-regulation of type II collagen, aggrecan and Sox9 by over an order of magnitude at the first passage, and upregulation of the expression levels of type I collagen [24].

Commonly investigated genes for the determination of chondrogenicity and zonal subpopulation of primary chondrocytes and differentiated MSC are COL1A1, COL2A1, COL10A1, ACAN, PRG4, SOX9, VCAM1, and CILP [1,23,25]. COL1A1, COL2A1, and COL10A1 all code for the pro-alpha-1 chains of type I, II and X collagen, and upregulation suggests increased collagen synthesis, specific to the type. Increased type II collagen synthesis is indicative of chondrocytes in a chondrogenic environment, whereas increased collagens I and X are indicative of dedifferentiation and hypertrophy, respectively [26–28]. The COL2A1/COL1A1 ratio is a commonly reported metric for cartilage tissue engineering as it relates to the degree of cellular differentiation. ACAN codes for aggrecan, the primary protein backbone in proteoglycans, which binds sGAG in healthy cartilage [29]. Sox9 codes for a transcription factor that is the primary regulator of chondrogenesis [30]. PRG4 codes for proteoglycan 4, also known as lubricin and superficial zone protein, a natural lubricant which is expressed mainly in the superficial zone of the tissue [31]. However, in the gene array study by Grogan *et al.* PRG4, while the most upregulated gene in bovine superficial zone relative to the deep zone (165-fold), was only upregulated 2.3-fold in the human superficial zone, less than 8 other genes, with the strongest being CHI3L1 – cartilage glycoprotein 39 [23]. VCAM-1 codes for the inflammatory mediator vascular cell adhesion protein 1, indicative of the inflammatory response found in rheumatoid and osteoarthritis [32]. CILP codes for cartilage intermediate layer protein, a rare indicator indicative of the transitional intermediate zone, and exhibits increased expression with age and the onset of osteoarthritis [33].

1.5 Cartilage Damage

At the time of Hunter's publication in 1742, the outcomes for an individual with advanced cartilage damage ranged from the lucky few who formed bony calluses, fusing their afflicted joints in place, to amputation and death from infection [17]. Despite incredible advances in medicine, osteochondral damage, caused by osteoarthritis or traumatic injury, is still a debilitating condition and currently the leading cause of chronic disability in many Western countries [34].

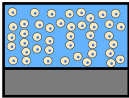
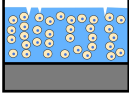
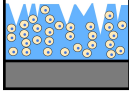
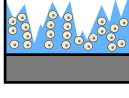
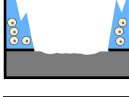
1.5.1 Focal Lesions

As articular cartilage lacks vasculature, innervation, and an active remodelling process, damage to the articular surfaces through sports or trauma seldom heals. The disease often progresses to the extent of exposed bone before the onset of discomfort. In an otherwise healthy and young individual small tears may heal by limited cellular migration from the surrounding tissues, but most injuries remain unhealed, except when blood is introduced through trauma to the bone. MSC from the blood promote the formation of fibrocartilage, rich in type I collagen and characterised by inferior mechanical properties [3]. Unhealed or poorly healed lesions can degenerate and eventually lead to the symptoms of joint locking, reduced function, and joint pain. The presence of lesions are often indicative of the onset of osteoarthritis (OA) and may accelerate the onset of secondary OA [3,35].

1.5.2 Osteoarthritis

Osteoarthritis is a broad disease of the whole joint, with a complex aetiology comprised of genetic, environmental, and age-related factors, outlined in a comprehensive review by Lories and Luyten [16]. It is characterised by the inflammatory destruction of articular cartilage, causing pain, swelling, and reduced mobility [36]. The progression of OA is divided into six distinct grades by the Osteoarthritis Research Society International, as outlined in Table 1-3 [37].

Table 1-3. Progression of osteoarthritis as outlined by Osteoarthritis Research Society International (OARSI). Adapted from Madry *et al.* [37]

Visualisation	OARSI Grade	Pathological Features
	Grade 1 Surface intact	Chondrocyte apoptosis, necrosis
	Grade 2 Surface discontinuity	Loss of superficial extracellular matrix
	Grade 3 Vertical fissures	Vertical clefts in mid-zone
	Grade 4 Erosion	Deep erosion
	Grade 5 Denudation	Denudation subchondral bone exposed
	Grade 6 Deformation	Deformation of subchondral bone, some fibrocartilage repair

Osteoarthritis of the knee affects nearly 1 in 2 adults by age 85, resulting in a conservative estimate of 27 million Americans and 8 million Britons currently afflicted with osteoarthritis [38–40]. In the United States osteoarthritis has an annual direct surgical cost of over \$42 billion (in 2009), with additional indirect costs, both economic and societal, of suffering and reduced productivity [41,42]. The financial impact of osteoarthritis and associated care, treatment, and lost-time costs is increasing quickly as increased life expectancies and obesity, among other factors, contribute to a larger population with joint disorders [16,43]. Obesity has shown to increase prevalence of OA by 4-fold in men and 5-fold in women [44].

The current pharmaceutical therapy landscape is limited to analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs), as OA is a progressive disease with no regenerative intervention or prophylactic measures currently available [45]. However, new research into lipid-lowering statins and the mood-stabiliser lithium, as well as the small molecule kartogenin have shown promise in the prevention of cartilage degradation [46,47].

An alternate joint pain-alleviating therapy is viscosupplementation, where hyaluronic acid is injected into the synovial capsule to replace hyaluronic acid lost to degeneration, restoring the viscosity of the synovial fluid. In a meta-analysis of 89 trials with over 12 000 patients by Rutjes *et al.*, viscosupplementation in the knee was reported to result in a small and clinically irrelevant benefit with an increased risk of serious adverse events [48]. The moderate therapeutic effect relative to

placebo is significant for 8 to 10 weeks after the last injection [49]. Investigations into high molecular weight and covalently modified hyaluronic acid hope to improve and prolong the therapeutic effect of viscosupplementation.

1.6 Traditional Surgical Interventions

The poor self-repair properties of articular cartilage and lack of pharmacological intervention often lead to the need for one or more surgical interventions over the lifetime of a patient. As early cartilage damage and OA is relatively pain-free, the joint often deteriorates substantially before surgical intervention. While the joint can be debrided to remove loose tissue and temporarily improve mobility, the most common treatment for advanced OA remains total joint replacement (Figure 1-7A) [39]. In a joint replacement, the articulating surfaces of the joint and underlying bone are removed and replaced with metallic and polymeric surfaces, in an invasive but routine and effective procedure. Total joint replacement was performed on 76,274 hips and 82,267 knees in England, Wales, and Northern Ireland in 2013 [39]. Partial joint replacements (Figure 1-7B) can be used if the damage is localised and can also be used to realign the load bearing surfaces to prolong the life of the remaining tissue [50]. One variant on total or partial joint replacement with promising results is HemiCAP® resurfacing, a polished metal implant for the repair of focal lesions or entire articulating surfaces of the knee or hip which does not remove the subchondral bone [51].

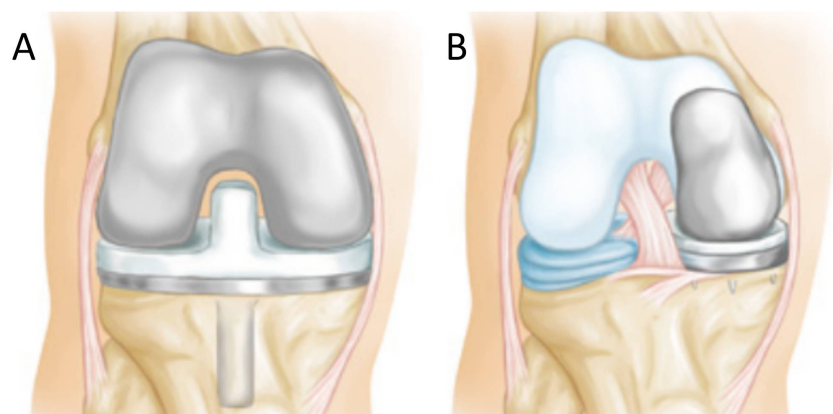


Figure 1-7. Total (A) and unicompartmental (B) joint replacement in the knee, modified from [52].

Despite advances in joint replacement design, all implants have limited life expectancies, and revision surgeries remain increasingly destructive to the joint. As the probability of revision decreases proportionally with age and activity, any procedure that delays joint replacement can improve long-term patient outcomes and decrease the cost to health care providers [39].

If a lesion does not warrant immediate full or partial joint replacement there are a number of current clinical techniques for surgical reduction of discomfort and repair. The most basic surgical

approach is debridement; however, this technique alone does not initiate repair. Cartilage self-repair is only possible when blood is introduced into the defect, for example in abrasion chondroplasty [53] where the lesion is debrided and then the surface is scraped down to bone with a mechanical burr. The introduction of blood from the subchondral bone marrow can also be achieved by the drilling/fracturing techniques of Pridie drilling [54], microfracture [55] and microdrilling [56], in chronological order of development and decreasing bore hole diameter (Figure 1-8).

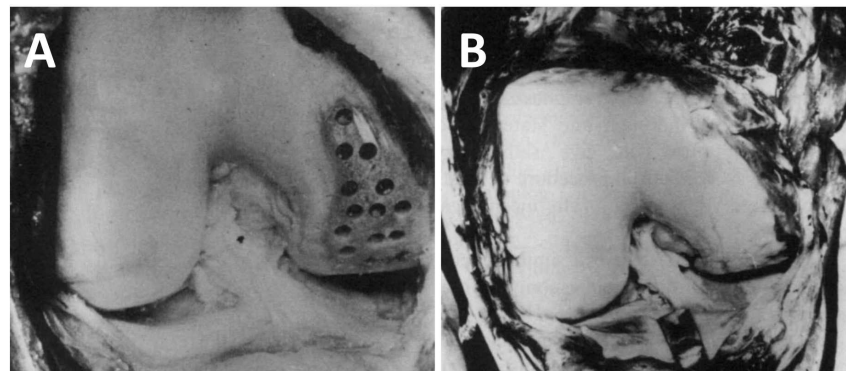


Figure 1-8. Pridie drilling of the femoral condyle showing A) large diameter drill bores and B) fibrocartilage repair 1 year later, modified from [54].

All techniques introduce blood into the lesion site, an idea originated by Pridie in 1959 [54]. When chondral lesions are simply shaved down without exposing the subchondral bone and associated vasculature, the remaining cartilage does not repair, instead it degrades in a manner mimicking OA [53]. The blood, originating from the subchondral bone marrow, initiates repair by producing a fibrin clot and provides a population of cells for regeneration. However, the tissue that is produced by these techniques is scar-like fibrocartilage, rich in type I collagen, with varying levels of type II collagen and proteoglycan, depending on the age and health of the patient [57]. This type I collagen-rich fibrocartilage is less resilient than hyaline cartilage, degrading in as little as a few months under dynamic mechanical loading, resembling early stage OA [58,59]. Despite the poor tissue properties, a 7-year follow-up study on microfracture found that over 80% of patients rated their condition as “improved”, with patients under 45 without meniscal or tendon pathology showing statistically significant improvement [60]. However, in patients over 45, ICRS scoring of microfracture repair dropped significantly between 18-36 months following the procedure. Increased success in younger patients (under 40) is attributed to the greater concentration and activity of regenerative cell populations in the marrow [61]. Microdrilling is very similar to microfracture but goes deeper than the 2-3 mm in microfracture, down 6-9 mm to the marrow, and has been shown to generate more hyaline-like repair tissue, due primarily to the drill depth [62]. Microdrilling It is now the most common bone-marrow-stimulation technique [3].

The quality of the repair tissue in these blood-initiated repair approaches can be further improved by the transplantation of an flap of autograft periosteum across the surface of the joint [63]. These flaps physically contain, concentrate, and stabilise the blood clot while the natural population of pluripotent cells within the grafted tissue produces growth factors to initiate progenitor cell migration into the clot and improve the quality of the repair tissue [63]. However, there is donor-site morbidity from periosteum harvest, and issues with hypertrophic growth on top of the periosteum, producing an uneven articulating surface [64].

Mosaicplasty autograft bypasses the regeneration of articular cartilage by rebuilding a new articular surface from cylinders of tissue from non-load-bearing surfaces of the patient's joint [65]. While rebuilding the load-bearing surface with mature cartilage tissue, mosaicplasty has the issue of severe donor-site morbidity, depending on the size of the joint surface and the defect to be filled. Additionally, the quantity and quality of autogenic tissue for mosaicplasty is dependent on age and disease state [66]. Despite over a century of use, issues with mosaicplasty beyond donor site morbidity including extensive post-operative bleeding, hyaline cartilage degeneration, and graft misalignment have led to decreased use, especially in North America [3,67,68]. Attempts to improve mosaicplasty through the regeneration of cartilage at the donor sites have provided unique opportunities to test tissue engineering strategies *in vivo*, leading to the development of advanced therapies [69].

1.6.1 Cell-Based Therapies

Surgical approaches rely on the introduction of a regenerative population of cells from the host tissues, i.e. blood, periosteum or osteochondral plugs. Additional approaches have been developed that introduce exogenous cell populations to supplement the endogenous repair mechanisms. These cellular therapies often use expanded chondrocytes from a biopsy of the afflicted joint. As small numbers of cells are collected, the primary cells are generally expanded *ex vivo* from hundreds of thousands to 10-30 million for implantation [70]. While this expansion permits greater numbers to elicit repair, the selected protocol has to balance cell number with dedifferentiation. Tissue produced by over-expanded chondrocytes is generally more similar to fibrocartilage, rich in type I collagen instead of type II collagen, similar to microfracture repair [58].

The injection of autologous expanded chondrocytes under a periosteal flap, termed Autologous Chondrocyte Implantation (ACI) was first performed on humans in 1994 with some success [71]. A random comparative study performed by Bentley *et al.* in 2002 found that 82% of ACI patients had 'excellent' or 'good' repair by arthroscopic assessment at 1 year in contrast to 34% for mosaicplasty

[65]. Clinical examples of this technique are the expanded autologous cell products Carticel® by Genzyme and ChondroCelect® by TiGenix (Table 1-4). The standard Carticel treatment vial contains 12 million expanded chondrocytes, for defects under 7 cm² [70]. While ACI has been successful in many patients, issues include hypertrophy on the periosteum and periosteal donor site pain and morbidity [72]. In Carticel® patient trials, 49% underwent subsequent surgical procedures within 4-years, with the most serious adverse events including adhesions, graft hypertrophy, chondromalacia, graft delamination and OA, each occurring in more than 5% of patients [70].

Whole blood and cell-based surgical techniques produce weak clots and cell aggregates with little mechanical strength, resulting in the need for patient immobilisation following the procedure, otherwise risking poor repair tissue quality and organisation [53]. Immobilisation also prevents flap delamination, which can occur, even following suturing [73].

To circumvent the aforementioned issues, the field required a clinical product that provided initial support to the joint surface while regenerating integrated hyaline-like cartilage with minimal donor-site morbidity and degrading when no longer required by the regenerated tissue. This regenerative engineering solution would restore the articular surfaces to their natural healthy state, eliminate complications of long-term implants, wear debris, and multiple surgical revisions, improve the quality of life for patients, and ultimately reduce long-term costs to healthcare providers.

1.7 Tissue Engineering Articular Cartilage

1.7.1 Tissue Engineering

The field of tissue engineering, defined in the seminal 1993 paper by Langer and Vacanti [74], endeavours to develop functional substitutes for damaged tissues using a combination of cells, engineered materials and biological cues. Since antiquity it has been understood that there are some materials which are bioinert, such as gold, and some natural materials which elicit bioactive properties, including nacre and animal bone [75]. However, the modern study of biomaterials, a key component of tissue engineering, began in earnest during World War II. Physicians examining the eyes of pilots following trauma observed that small shards of poly(methyl-methacrylate) shrapnel from Spitfire cockpits did not illicit an immune response, unlike the glass used in other models of aircraft [76]. These polymers were classified as bioinert and soon used to produce rigid intraocular lenses and bone cements, still in use today. The first purpose-engineered bioactive material, discovered in 1969, was a non-crystalline glass composition able to bond with bone and soft tissues through natural tissue remodelling pathways [77]. Used clinically since 1985, bioactive glasses are used to coat the surfaces of implants for improved bone fixation, and as powders, plates and

composites for non-load bearing bone repair applications [77]. In addition to ceramics, advances in biodegradable polymers produced absorbable polymer sutures and fixation devices for orthopaedic reconstruction that reduced the need for secondary procedures to remove metallic implants.

The success of these clinically-implemented biomaterials, and a parallel success in the *in vitro* culturing of primary human cells spawned the field of tissue engineering, the next step towards tissue regeneration. Tissue engineering scaffolds are developed to reproduce some key aspects of the physiological structure, micro-architecture, mechanical environment, and surface chemistry of a tissue or tissue-inductive microenvironment. These scaffolds are then seeded with cells with the goal of retaining or inducing the correct phenotype, resulting in functional tissues.

While the field has faced many challenges and unforeseen complexities over the past three decades, tissue engineering solutions for skin, bladder, vascular grafts, and cartilage provide examples of therapies that have been developed and transitioned to the clinic, improving the quality of life for patients, and in some cases, saving lives.

The engineering of skin benefits from the planar nature of the natural tissue, much like cartilage, and the proven functionality of full- and partial-thickness skin grafts, widely used in the clinic to treat burns and diabetic ulcers. Advances in culturing human cells in the 1960s lead to interest in a cell-based therapy to replace painful autograft procedures and temporary allograft procedures. Autogenic therapy using expanded sheets of keratinocytes was established and is still widely used today, however it requires over 3-weeks of preparation, and produces a very fragile sheet with less than 60% acceptance [78,79]. Through a series of refinements, Dermagraft® was developed as a human dermal equivalent, containing dermal matrix and viable cell populations of neonatal fibroblasts in an immunologically inert tissue, reinforced with a bioabsorbable poly(glycolic acid) mesh [80,81]. Apligraf® is a further advance on the technology, featuring a dermal and epidermal layer of viable cells in a bovine collagen matrix [81]. While both products failed to meet their initial high expectations and have faced bankruptcy and re-organisation, they have been a proving ground for the tissue engineering industry.

Two examples of more complex tissues that have passed into successful long-term clinical trials are tissue engineered bladder produced by the Atala group [82] and vascular grafts for dialysis produced by the Niklason group [83]. The engineered bladders successfully restored urodynamic function in seven young end-stage bladder disease patients, assessed at a mean of 46 months, equivalent to the standard gastrointestinal tissue transfer method but free from the accompanying morbidity [82]. The most successful bladders were composed of a poly(glycolic acid) (PGA) hernia mesh embedded within collagen, seeded with expanded autologous urothelial and smooth muscle cells, and cultured

for seven weeks before implantation beneath an omental wrap. The combination of a polymer mesh and collagen allowed for cellular attachment, proliferation, and eventual remodelling throughout the 2 mm-thick collagen construct while also benefitting from the initial tensile strength of the synthetic polymer mesh.

The artificial vasculature engineered by the Niklason group used a similar materials-based approach, but with a twist, facilitating commercialisation and scale-up [83]. The vascular grafts were composed of a tubular PGA felt seeded with human smooth muscle cells. The cells were cultured *in vitro* for 7-10 weeks under pulsatile flow to build a mechanically robust ECM [83]. The novel development was the subsequent decellularisation of the scaffold, producing an ECM-based construct that could be validated and stored for extended periods of time. The ECM graft could then be implanted off-the-shelf, directly into the patient, avoiding the time, expense, and logistical complexity of autologous cell seeding, while remaining patent. In unpublished data, the grafts have now been transplanted successfully into patients in a clinical trial to restore vascular access for haemodialysis and are being commercialised by Humacyte, Inc.

In addition to these tissues, materials-based approaches have also been developed and commercialised for cartilage regeneration.

1.7.2 Scaffold-Based Cartilage Engineering

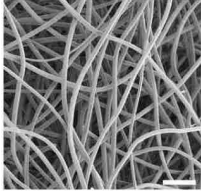
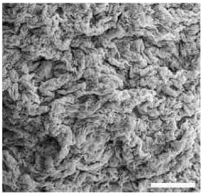
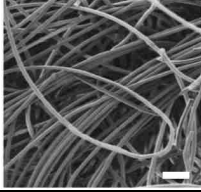
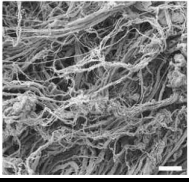
In a survey of cellular and engineered tissue therapies in Europe in 2012, it was found that 34% of all therapies were delivered on a tissue engineering scaffold, with more (19%) on a 3D scaffold than membranes and gels combined (15%) [84]. Cartilage repair was the most frequently reported indication with 518 procedures reported in 2012, accounting for 24% of all cellular and engineered tissue therapies in Europe. Within the cartilage field specifically, 56% of therapies involved a 3D scaffold, while 22% utilised a membrane or gel, and the remaining 22% were simply injected as a cell suspension.

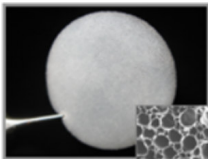
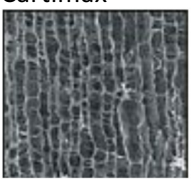
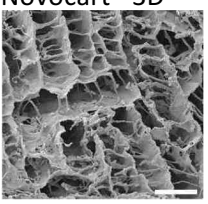
Collagen membranes were developed by Steinwachs and Kreuz to replace the periosteum flap in ACL, addressing the issues of hypertrophy and donor-site morbidity, while also reducing pain, surgical time, cost, and recovery time [64]. The replacement of periosteum with collagen, while simple, completely prevented symptomatic graft hypertrophy while improving ICRS and Cincinnati patient scores at 6, 18, and 36 months ($p < 0.01$). This is an example of a simple solution with clinical significance.

Beyond hypertrophy, the lack of clot and cell stability beneath the flap continued to be an issue. The solution, termed Matrix-induced Autologous Chondrocyte Implantation (MACI) implants a tissue engineering scaffold to fill the defect, stabilise the clot, and support tissue formation by deposited cells in a three dimensional environment [85]. Articular cartilage scaffolds come in many different forms, including hydrogels [86–89], fibre mats [90,91], random foams [92], oriented foams [92], open latticework [18], microparticle aggregates [93,94] and decellularised tissues [95,96].

The simplest forms of tissue engineering scaffold used clinically are sponges and gels implanted without cells to assist in the stabilisation of the clot. Commercial products for the stabilisation of the clot beneath the periosteal flap include sponges of modified hyaluronic acid (Hyalofast™ and Chondrotissue®) and injectable fibrin glue (CartiFill™) (Table 1-4). Commercial regenerative matrices for cartilage include Hyalograft®C, BioSeed C™, MACI™, NovoCart®, and others, summarised in Table 1-4. While the electrospun meshes, freeze-dried foams, and gels, provide support to the regenerating cartilage, many lack the ability to anchor the new tissue into the subchondral bone. The exceptions to this are TruFit CB® and MaioRegen®, both of which have a subchondral bone scaffold in addition to the chondrogenic zone.

Table 1-4. Summary of commercial cartilage regeneration products

Company	Product	Description
Anika Therapeutics www.anikatherapeutics.com	Hyalofast™ 	A non-woven pad of HYAFF®, a benzyl ester of HAc Implanted acellularly
[97] Scale = 100 µm	Hyalograft® C	Expanded autologous chondrocytes seeded on Hyalofast™ More effective tissue formation at 5 years relative to microfracture
Arthro Kinetics www.arthro-kinetics.com	CaReS® 	Type I collagen gel No <i>in vitro</i> chondrocyte expansion Mini-arthrotomy surgery No suturing
[97] Scale = 100 µm		
BioTissue Technologies www.biotissue.de	Chondrotissue® 	Cell-free cartilage implant Used after debridement and microfracture Resorbable (6 months) hyaluronan and polyglycolic acid fleece combined with autologous serum Arthroscopic implantation
	BioSeed C™	Autologous chondrocytes seeded on Chondrotissue® Attached with a fibrin glue >1,000 patients successfully treated Arthroscopic transplantation possible No periosteum patch
[98] Scale = 100 µm		
FinCeramica www.finceramica.it	Maioregen® 	Lyophilised equine type I collagen cartilage zone, transitioning through a 60:40 type I collagen:Magnesium-enriched HAp (Mg-HAp) tide mark to a 30:70 type I collagen: Mg-HAp subchondral layer Implanted acellularly
Geistlich Pharma www.geistlich-pharma.com	ChondroGide® 	Bilayered fibrous collagen membrane used with microfracture (AMIC) and autologous chondrocyte implantation (ACI) 7 years in clinic
Genzyme (Sanofi) www.carticel.com www.maci.com	Carticel®	Autologous chondrocytes injected beneath periosteal patch sealed with fibrin glue First and only FDA-approved cell therapy product for articular cartilage injuries
	MACI™ 	ACI-Maix® scaffold seeded with 10M autologous chondrocytes Type I/III collagen membrane
[97] Scale = 100 µm		

Company	Product	Description
Histogenics	NeoCart® 	Chondrocytes expanded 2-3 weeks, seeded on an open-pored collagen type I matrix Cultured dynamically for 1 week and statically for 2-4 weeks before implantation. Currently in Phase III trials.
Matricel GmbH. www.matricel.net/	Cartimax® 	3D collagen membrane produced by directional freezing type I/III collagen from aqueous solution.
RMS www.rmsbio.net	CartiFill™	Atelocollagen and fibrin glue for injection following microfracture or as a cellular therapy, seeded with chondrocytes or autologous stem cells
Smith & Nephew	TruFit CB® 	Poly(D,L-lactide-co-glycolide) and calcium sulphate osteochondral scaffold for acellular implantation, Developed to fill mosaicplasty donor sites. No longer on the market.
TETEC (B Braun) www.tetec-ag.com	Novocart® 3D 	Bilayered type I collagen/chondroitin sulphate matrix with a porous open-celled foam for seeding and a surface membrane for tensile properties Sutured to the surrounding tissue For sue with autologous chondrocytes
[97] Scale = 100 µm	Novocart® Inject	Autologous chondrocytes In situ hydrogel polymerisation
TiGenix www.tigenix.com	ChondroCelect®	Suspension of expanded autologous chondrocytes Sealed under a membrane after debridement Not yet FDA Approved

The osteochondral implants of TruFit CB® and MaioRegen®, both implanted without the addition of autologous cells, showed promise in early clinical trials, with post-operative patient scores superior to microfracture and slightly lower than mosaicplasty, but without the donor-site morbidity [99–102]. However, during extensive testing for FDA approval, TruFit CB® was pulled from the market due in part to a 70% revision rate for implant failure resulting from substandard subchondral bone regeneration, pain and swelling at 18-months [103].

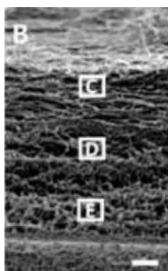
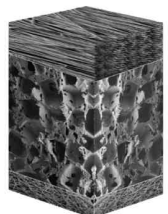
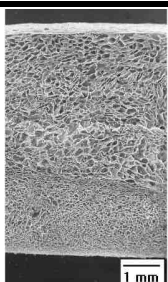
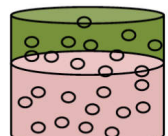
1.7.3 Multi-Layered Systems

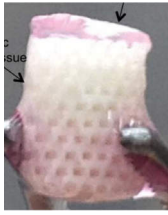
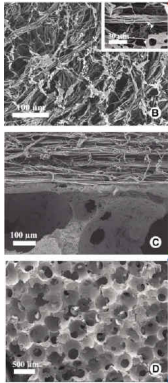
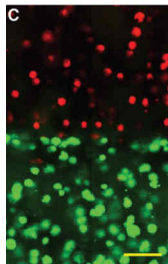
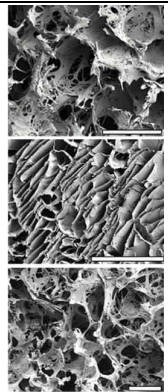
The majority of clinically implemented MACI techniques outlined use isotropic cartilage scaffolds, homogenous in mechanical, chemical, and biological properties, relying on the chondrocytes to

spontaneously form their own anisotropic structure. The generation of spontaneous anisotropy has been shown *in vitro* in scaffold-free cultures and hydrogels, but these systems lack the mechanical strength required for implantation in large defects [86,87]. Novocart 3D® and MaioRegen® are two exceptions, with superficial membrane attached to an open pore scaffold in in Novocart 3D® and a calcified cartilage interface between the cartilage and bone portions of MaioRegen®.

Investigations into the impact of anisotropic mechanical, morphological, chemical, and cellular gradients found in functional osteochondral tissue are a topic of growing interest in the field. Reviews of zonally organised, functionally graded, and interfacial scaffolds for articular cartilage regeneration highlight the wide array of approaches which have been investigated [104–107]. A selection of recent work, illustrating the diversity in the field has been summarised in Table 1-5.

Table 1-5. Selection of recent functionally graded and interfacial osteochondral scaffolds

Scaffold		Observations	Ref
3 Zones Sequentially electrospun PCL gradient of aligned 1 μm , random 1 μm , and random 5 μm fibre layers Scale = 100 μm		Seeded chondrocytes adopted zonen dependent morphologies and compressive moduli in the physiological range. Production of sGAG and type II collagen and upregulation of chondrogenic gene expression were positive but largely nonn zonal. Increasing fibre diameter from 1 to 5 μm increases pore size from $13 \pm 1 \mu\text{m}$ to $170 \pm 100 \mu\text{m}$.	McCullen <i>et al.</i> 2012 [90]
2 Zones Electrospun superficial zone fused to and 300 μm or 1000 μm porogen leached foam. 2 mm thick PCL.		Seeded with bovine chondrocytes and cultured in chondrogenic medium. Finer pores found to promote chondrogenic biochemical and gene expression profile. Large pores treated as 2D surfaces.	Steele <i>et al.</i> 2014 [108]
3 Zones Sequentially freeze-dried foam based on bovine type I collagen, supplemented with HAp, HAc, and type II collagen		This study produced a three-layer osteochondral foam with unique chemical environments but essentially isotropic mechanical properties with zones of 0.4 – 1.0 kPa. 14-day <i>in vitro</i> testing shows low cellular density and continued proliferation with no indication of zonal properties.	Levingstone <i>et al.</i> 2014 [109]
2 Zones Pre-differentiated MSC in multi-layered oligo(poly(ethylene glycol) fumarate) hydrogel		12-week <i>in vivo</i> data in a rabbit model shows promising hyaline cartilage from the addition of chondrogenically and osteogenically pre-differentiated MSC.	Lam <i>et al.</i> 2014 [110]

Scaffold		Observations	Ref
3 Zones Alginate/chondrocyte hydrogel partially embedded within an open-strut sintered resin-HAp- β TCP scaffold		12-week <i>in vivo</i> data in a rabbit model showed anchoring of the ceramic bone scaffold and hyaline-like cartilage in portions of the cartilage zone. Evidence of extensive healing in the empty defect and hydrogel-only controls.	Seol <i>et al.</i> 2014 [111]
2 Zones Oriented Poly(L-lactide-co-caprolactone)/ type I collagen electrospun fibres embedded in freeze-dried type I collagen/ HAc sponge on porous β TCP bone scaffold		Dynamic liquid electrospinning and freeze-drying used to produce a hybrid fibre-foam scaffold. Sponge and scaffold fused by Col1/HAc and freeze-drying. 21-days of chondrogenic culture resulted in full cellular penetration but minimal tissue formation. 12-week <i>in vivo</i> rabbit model showed improved healing relative to no treatment by ICRS scoring.	Liu <i>et al.</i> 2014 [112]
Scales = 100/100/500 μ m			
2 Zones Osteochondral construct of an agarose hydrogel and fused vertically-oriented starch-PCL fibres		Seeded with amniotic-fluid-derived stem cells and cultured in chondrogenic and osteogenic medium. Following differentiation, they were 'glued' together with agarose gel and cultured for 2 weeks in osteochondral medium. The osteogenic differentiation was robust and did not require additional BMP-2, the chondrogenic differentiation was not robust and required continued supplementation.	Rodrigues <i>et al.</i> 2012 [108]
Scale = 2 mm			
2 Zones Bioprinted UV-crosslinked PEG-MA with chondrocytes printed and simultaneously fixed in place, permitting 3D zonal organisation.		While not an interface paper, the capability for controlled cell deposition directly in a chondral defect with full native tissue integration was exhibited. The crosslinked PEG-dimethacrylate gel had an impressive 396 ± 80 kPa modulus	Cui <i>et al.</i> 2012 [113]
Scale = 100 μ m			
3 Zones Silk fibroin meniscus scaffold composed of two salt leached foams and a freeze dried foam, produced and seeded separately then fixed together with full-thickness plugs		When seeded with human fibroblasts and chondrocytes, and cultured in TGF- β 3, the cellularity, GAG, type I collagen and II contents increased, indicating a maintenance of fibrocartilage phenotype. Cell morphology was significantly different between the layers, showing aligned ECM deposition in the freeze dried foams. Stiffnesses, ranged from 165-294 kPa.	Mandal <i>et al.</i> 2011 [114]
Scales = 500 μ m			

The field of multi-zonal tissue engineering scaffolds remains predominantly one of osteochondral systems, with little attention paid to the anisotropic properties of cartilage beyond the calcified

interface. Additionally, many systems still investigate a gel-on-bone scaffold approach, which, while it produces structured hyaline cartilage in rabbit models, would suffer from implantation into a large defect in an adult. The prevalence of rabbit *in vivo* models for human diseases is discussed in more detail in Chapter 4.

1.8 Materials and Techniques for Cartilage Tissue Engineering

1.8.1 Materials

The selection of the material for tissue engineering scaffolds impacts surface chemistry, degradation mechanism and rate, stiffness, pore/fibre morphology, cellular adhesion, immunogenicity, and is therefore a very important consideration. Some commonly used polymers for cartilage tissue engineering, both natural and synthetic, are summarised in Table 1-6.

Table 1-6. Common polymers for articular cartilage scaffolds

Polymer	Effect	References
Agarose	Promotes ectopic chondrogenesis when placed between the cortical bone and periosteum. Mechanical properties improve with cellular density and matrix deposition, but remain weak at implantation (20-30 kPa). Issues with immunogenicity.	[88,89,108]
Alginate	Used as a chondrocyte scaffold, not as effective as agarose, with poorer mechanical properties, but improved immunogenicity.	[88,108,111,115]
Collagen	Culture in collagen gels and scaffolds maintains Col2 and GAG production in chondrocytes and induces chondrogenic differentiation <i>in vivo</i> in MSC. Collagen-mimetic peptides can be chemically modified to form the core of a biodegradable functionalised hydrogel.	[109,116–119]
Fibrin Glue	Chondrocytes and MSC in fibrin gels up-regulate Col2 and PG production without growth factors. Used to secure MSC into porous scaffolds for <i>in vivo</i> transplantation	[120–122]
Hyaluronic Acid	Chondrocytes on HAc scaffolds express Col2, aggrecan, and down-regulate Col1, catabolic factors, and apoptosis. Bone marrow MSC on HAc scaffolds express significantly higher Col2 than chondrocytes, and can differentiate <i>in vivo</i> in HAc gels under hypoxic conditions.	[89,119,123–125]
HYAFF	Benzyl-ester modified hyaluronic acid with maintained biological properties and degradability, but improved processability. Used commercially in Hyalofast and Hyalograft C cartilage implants.	[123,124]

Polymer	Effect	References
Poly(ethylene glycol) (PEG/PEO)	PEG provides an inert hydrogel which can be modified and copolymerised to degrade while supporting attachment, viability, proliferation and biosynthetic activity of chondrocytes. PEG modified with a collagen mimetic peptide promoted chondrogenesis and ECM secretion from BMSC.	[110,126–128]
Poly(lactide-co-glycolide) (PLGA/PLG)	PLGA exhibits improved sGAG and collagen production from chondrocytes and MSC, relative to poly(L-lactide). Used in a wide variety of FDA-approved polymeric implants.	[102,129]
Poly(ϵ -caprolactone) (PCL)	Degradable polymer which is easy to process into different geometries of greatly varying stiffness. Low cost, can be biofunctionalised in a controlled manner that does not interfere with processing. Hydrolyses slowly <i>in vivo</i> with no metabolite accumulation in tissue. Used in FDA-approved osteogenic scaffolds.	[130–133]
Poly(L-lactide-co- ϵ -caprolactone) (PLCL)	Biodegradable elastomer more resilient than PCL. Generally degrades faster than PCL but slower than PLA and PLGA, depending on composition.	[134–137]

The polymers in Table 1-6 can also be copolymerised, blended, and layered to further refine the desired surface chemistry, degradation rate and mechanical properties for a given architecture and application. Polymers can also be modified by the addition of bioceramics (hydroxyapatite (HAp), β -tricalcium phosphate (β TCP), or bioactive glasses) for the release of pro-osteogenic ions at the calcified cartilage interface and osteogenic zones of osteochondral scaffolds [86,111,112].

There is a divide in the literature and the clinic regarding the use of natural and synthetic polymers for tissue engineering, both of which are well represented in Table 1-5 and Table 1-6. The relative merits of each are open to interpretation and could be discussed at length. For example, the cell adhesion moieties and enzyme-mediated degradation of many natural materials is contrasted with the low adhesion and hydrolytic degradation of many synthetic polymers. For quick regeneration and integration, fibrin glue would be preferable to a PCL mesh; however, the PCL would heal slower and provide prolonged structural support in a more mechanically demanding application. New examples of synthetic functionalised polymers and peptide-polymer conjugates that mimic natural polymers in a controlled, reproducible manner, is revolutionising the field by integrating the merits of each [119,133].

PCL was selected as the optimal scaffold material for our application due to processability by multiple scaffold production techniques, high strength, biocompatibility, slow but complete biodegradation, ability for end-group modification, low cost, and extensive academic and clinical use

by leading researchers. As PCL can be processed using a wide variety of scaffold production methods it is ideal for the production of an anisotropic scaffold from a single polymer – preventing zone-specific material properties and surface chemistries from confounding the interpretation of the data. The history, applications, and chemistry of PCL, and its copolymers for biomedical applications were exhaustively reviewed by Woodruff and Hutmacher, providing an excellent resource for more in depth investigation [130]. Briefly, PCL is prepared by a ring-opening polymerisation of cyclic ϵ -caprolactone with a catalyst such as stannous octoate. The number average molecular weight can be varied from 3,000 to >80,000 g/mol by the addition of low molecular weight alcohols [130]. PCL degradation is enzyme-independent hydrolysis, with *in vivo* and *in vitro* rates showing no statistical differences, however, this point has been contested with high-surface-area-to-volume scaffolds such as electrospun fibres [130,138]. PCL degraded by hydrolysis to <3,000 g/mol, can be phagocytosed by macrophages and eliminated [131]. The only degradation product is 6-hydroxyl caproic acid, which enters the citric acid cycle and is not accumulated in the body [132]. While commonly cited as surface hydrolysis due to the hydrophobicity of PCL, on the scale of biomaterials, including electrospun fibres and foams, the degradation has a significant bulk hydrolysis aspect, as can be observed by the steady reduction in the molecular weight of constructs over time [138].

As porous PCL is less elastic than native cartilage and was perceived by some to degrade too slowly, the synthesis of poly(D,L-lactide-co-caprolactone) (PLCL) was also investigated (Chapter 5B) [134,135]. However, PCL is the predominant polymer used throughout this work as it possessed the uniformity and reliable chemistry of a bulk commodity polymer, eliminating batch-to-batch variability from our investigations into cellular responses to scaffold architecture [1,90].

1.8.2 Fabrication Methods

There are a number of different scaffold production techniques that produce a wide variety of microenvironments, replicating different aspects of the osteochondral system. Many of these methods were outlined in the previous discussion of commercially available and multi-zone scaffolds, and are outlined in more detail here.

Hydrogels

The use of hydrogels for cartilage and bone engineering has been widespread due to their ability to replicate the chemical microenvironment and diffusional properties of the natural tissue through tailorable chemistries. Stevens *et al.* discovered that a bolus of hyaluronic acid gel, supplemented with anti-angiogenic factors and TGF- β 1 placed beneath the periosteum of the femur will spontaneously recruit stem cells from the environment and generate hyaline-like cartilage, while

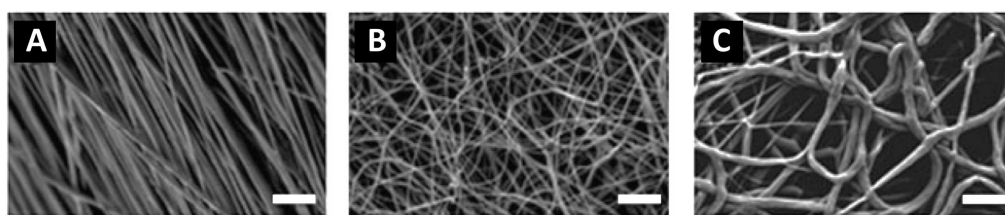
calcium alginate gels will produce bone [89]. Many studies have utilised the chondrogenic properties and simplicity of thermoset low-melting point agarose for chondral applications, most notably in studies on mechanical induction [139]. The versatility of hydrogels for regenerative medicine is highlighted in a review by Hunt *et al.* [140]. While the addition of chondrocytes increases the stiffnesses of a gels as ECM is produced, the mechanical properties remain too low, on the order of 1-20 kPa in compression, relative to the 150 kPa – 2 MPa values quoted for the different zones of cartilage [20]. Hydrogels for cartilage engineering are thoroughly reviewed by Kim *et al.* [141].

As stated in Table 1-4, there are a number of fibrin and type I collagen gel-based therapies currently in the market that benefit from the simplicity of injection, performed arthroscopically. Gels may lack the compressive mechanics of physiological cartilage, but are clinically used to stabilise and protect the clot formed by microfracture beneath a periosteum patch. A photoreactive PEG-diacrylate hydrogel was notably used by Sharma *et al.* in a 15-patient clinical trial, in combination with microfracture [128]. The hydrogel-microfracture condition showed significantly higher levels and density of tissue formation and increased tissue organisation, with less pain. Functional scoring at 6 months was equivalent for the two groups.

Electrospinning

Electrospinning is a simple scaffold production technique that produces a porous network of fibres on a 0.1-10 micron scale from polymer solutions. Fibres are produced by extruding polymer in a volatile solvent through a charged needle (up to 30 kV) to a grounded mandrel. As the solvent evaporates within the electric field, charge is localised to the surface of the fibre, causing the drying fibre to repel itself [142]. The process of repulsion-induced whipping thins the fibre even further from a macroscopic initial needle gauge (^{ex} 18G = 838 µm) to under 1 µm with the proper spinning conditions [1,90].

The non-woven fibre mats produced mimic the fibrous features in the native ECM and are therefore perceived by cells as a three-dimensional structure as opposed to a flat surface [91]. Electrospun mats have tuneable roughness, tensile strength, porosity and surface areas, as a function of polymer selection, fibre size, mat thickness, and fibre orientation, providing a useful platform technology for the modification of cellular behaviour [143–146].



Zone	Polymer Concentration (%w/w)	Working Distance (cm)	Linear Velocity (m/s)	Fibre Orientation	Fibre Size (μm)	Pore Size (μm)
A - Superficial	15	10	20	Aligned	1.1 ± 0.3	4.2 ± 1.0
B - Middle	15	10	0-1	Random	0.9 ± 0.2	12.9 ± 1.3
C - Deep	25	15	0-1	Random	4.6 ± 1.2	171 ± 102

Figure 1-9. Comparison of electrospinning parameters optimised to mimic superficial (A), middle (B), and deep zones (C) of cartilage, with quantified fibre and pore dimensions. Modified from McCullen *et al.* [90].

In a study by Coburn *et al.*, cultures of MSCs on poly(vinyl acetate-co-methacrylate) nanofibres were found to outperform pellet culture for cartilage-specific ECM production and gene expression. The improvement was attributed, in part, to the physical environment afforded by the nanofibre matrix [91].

PCL is the most commonly used polymer for electrospinning due to its optimal fibre forming abilities, biocompatibility and historical implementation in the tissue engineering industry [131]. Electrospun fibres have been used as a scaffold for most tissues, including cartilage and bone, and have proven to facilitate differentiation induction and maintenance, relative to 2D culture [147]. An article by McCullen *et al.* highlights the flexibility of the electrospun PCL system, producing a trilayered chondral scaffold of varying stiffness, fibre diameter, fibre orientation, and fibre spacing [90]. The fibre orientation was able to influence chondrocyte morphology, forming a smooth, aligned articular surface on oriented fibres and a more distributed cell population in the randomly aligned fibre zones. One prominent issue with electrospinning is poor cell infiltration, which can be partially overcome by sacrificial fibre spinning [148], or spinning into a liquid phase [91,112].

Direct Writing Melt Electrospinning

A variant of standard solution-based electrospinning is direct writing melt electrospinning, recently developed for tissue engineering applications by Prof. Dalton, Dr. Brown, Prof. Hutmacher and colleagues, which allows for control over electrospun pore geometries and interconnectivity in a manner akin to 3D printing. In order to achieve this high level of control, fine ($<10 \mu\text{m}$) fibre diameters are often conceded, but the fibres produced remain significantly smaller than the polymer struts in fused deposition modelling (FDM) [149]. The melt-electrospinning technique was recently used to great effect by Visser *et al.* to reinforce a hydrogel scaffold for cartilage tissue engineering,

illustrating the integration of multiple scaffold systems to develop an optimised composite system [25]. Melt electrospinning is a technology that will enable higher control and reproducibility in the field of tissue engineering.

Porogen Leaching

Porous polymer foams are able to provide a three-dimensional construct with variable stiffness, thicknesses, and cellular infiltration in a very simple, reproducible, and cost-effective manner. The formation of a porous sponge with controlled pore size is easily accomplished by porogen-leaching as it depends primarily on the geometry of the porogen. Foams are produced by suspending the porogen in a polymer solution or melt which is allowed to evaporate or cool, then the porogen is dissolved from the scaffold, leaving pores in the structure. The most commonly implemented porogen is sodium-chloride salt, others include ethanol-soluble Elvacite®, and thermoset gelatine [150,151]. Salt is readily soluble in water and is easily removed, however, the pores produced are jagged due to the irregular structure of the crystals. Microparticles of natural and synthetic polymers have the benefit of controlled spherical geometries, but limit solvent selection for the matrix phase and can raise questions regarding residual porogen polymer. In this way, thermoset gelatine, (developed for foams in chapter 3) is an ideal system as it is robust against many organic solvents but dissolves quickly in incubation conditions of 37°C in PBS. Any residual gelatine remaining as a film simply complements the requisite scaffold pre-incubation in bovine serum albumin (BSA) and/or foetal bovine serum (FBS), which is performed with all scaffolds to improve cellular adhesion.

A novel approach to porogen leaching published recently by Zhang *et al.* [152] used microspheres of ice to form pores in a collagen foam. The ice microspheres were produced using a very simple protocol where distilled water was simply sprayed into liquid nitrogen and the resulting particles were sieved at -15°C to obtain the correct particle size ranges. The resulting freeze-dried foams had spherical pores of defined size ranges with no contaminating porogen material (Figure 1-10).

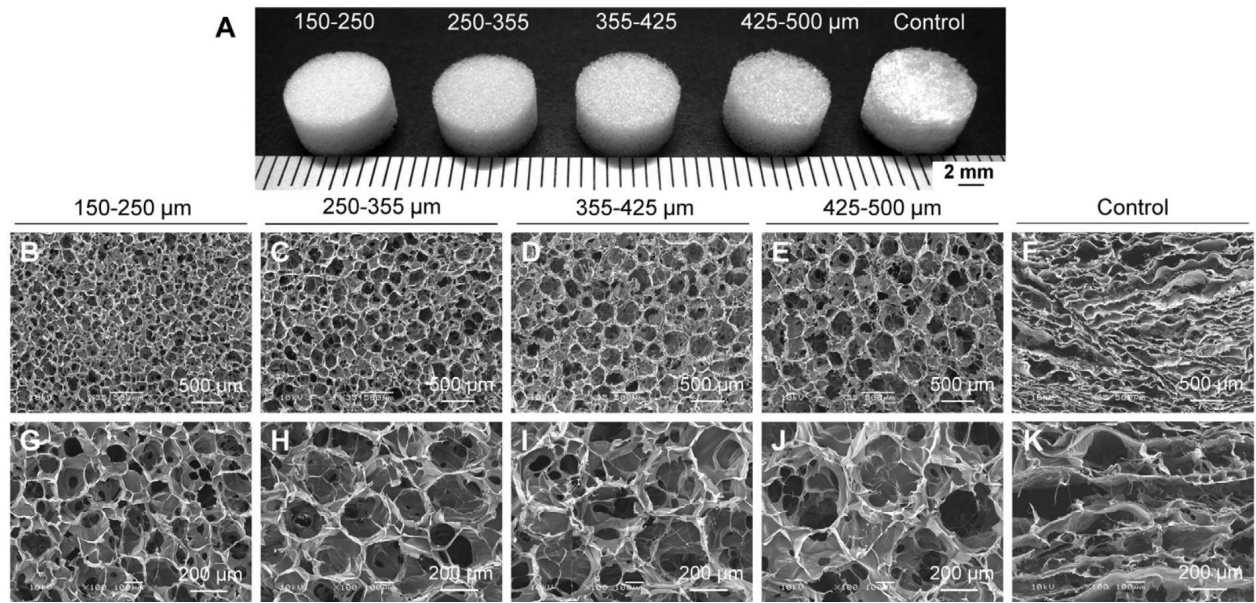


Figure 1-10. Macroscopic (A) and microscopic (B) morphology of ice microparticle porogen-leached collagen foams, from Zhang *et al.* [152].

One issue with porogen leaching addressed by Zhang *et al.* [92] in a comparison of porogen-leaching to directional freezing, is the potential issue of cellular ingress with small diameter pores. Pore interconnects are irregular and generally significantly smaller than the pore diameter, potentially limiting homogenous cellular distribution. With optimisation, this issue can be overcome and does not pose an impediment to further development of porogen-leached scaffolds [1].

Directional Freezing

In place of a physical porogen, the controlled formation and freeze-drying of solvent crystals can be used to produce interconnected pores in a process termed thermally-induced phase separation (TIPS). As a polymer solution is frozen, the solvent crystallises expelling polymer from the growing crystal, forming zones of crystallised solvent and zones of precipitated polymer. Removing the solvent by lyophilisation produces an open-cell foam. Foams produced by rapid cooling of polymer solutions have been used both academically and commercially for cartilage tissue engineering [109,153]. However, the isotropic structure of the foams produced with materials and porosities for tissue engineering applications result in poor mechanical properties [154].

Directional freezing, also called freeze casting, is a slight modification which freezes the solution in a controlled manner from a single plane at 0.1 to 10°C/min, producing vertically aligned solvent crystals perpendicular to the surface [155]. The structure of the crystals is dependent on the composition of the solvent phase and the freezing rate, with some producing more vertical columnar dendrites and others producing planar lamellae [155]. Following lyophilisation, a foam with vertical

channels is produced [92]. The freezing front takes on three distinct zones, a dense initial flash-frozen zone directly in contact with the plate, through a columnar phase into a lamellar phase as the solidified material creates an insulated zone between the freezing front and the plate [156].

Directional freezing has been used with ceramics, ceramic/metal, and ceramic/polymer hybrid systems to produce porous scaffolds for bone regeneration [157]. In a study by Deville *et al.* directionally frozen ceramics were infiltrated with epoxy to replicate the structure of nacre from mollusc shells, increasing the strength of porous ceramic bone scaffolds by over 4-fold relative to clinical standards, over 140 MPa at 40% porosity [158].

The use of directional freezing in polymer only systems is in clinical use for the preparation of ACI-Maix® from type I collagen solutions (Table 1-4). A new system for producing directionally frozen foams on a larger surface area was published recently by Pot *et al.*, which uses a wedge-based cooling system with a defined temperature gradient in the standard z-direction as well as the x-direction [159]. The simple system controls the crystallisation propagation throughout the width and height of the scaffold, increasing the regularity of the crystallisation across the x-y plane. The system was used to prepare collagen and poly(vinyl alcohol) (PVA) foams from aqueous solutions, to show the effect of polymer concentration and composition on pore size and architecture (Figure 1-11).

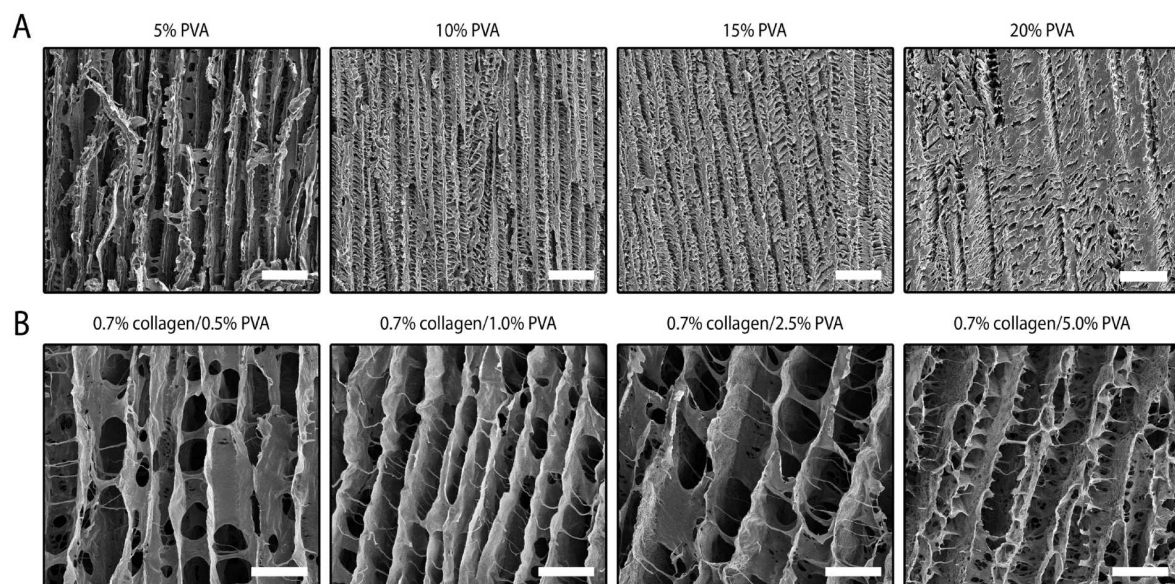


Figure 1-11. Morphologies of directionally frozen foams of different compositions and concentrations, from a study by Pot *et al.* [159]. Scale bars represent 100 μ m.

In a comparative study in a PLGA and dioxane system, Zhang *et al.* [92] discovered that the vertical nature of these channels promotes full cellular ingress and markedly increased stiffness, relative to salt-leached foams of the same porosity. The pore alignment also resulted in improved nutrient and waste transfer and GAG and collagen production, with significant effects after 4 and 8 weeks *in vitro*

and after 4 additional weeks *in vivo*. Additionally, the directionally frozen foams had volume and height shrinkage of less than 10% at 12 weeks, relative to the salt-leached foams which had a > 50% reduction in each [92]. The geometry of the vertically aligned stiff scaffolds is comparable to the deep zone of articular cartilage. In Chapter 3 and 5 this similarity is explored in the production of a multi-zone cartilage scaffold with significant mechanical and geometric anisotropy.

Rapid Prototyping

A number of techniques have been developed to print highly-defined scaffold geometries, including laser sintering [160] and fused deposition modelling (FDM)[161]. PCL is an ideal polymer for many of these techniques due to its low melting point, allowing for easy manipulation in the melt state [130]. The highly defined nature and reproducibility of prototyped scaffolds facilitates the study of different scaffold geometries. Woodfield *et al.* [18] investigated the use of FDM to produce polymer scaffolds with varying and highly defined pore-size gradients, the scaffolds promoted anisotropic cellular distribution and ECM production, but without a secondary support material such as a hydrogel [25], strut and pore sizes remain too macroscopic for three-dimensional culture *in vitro*.

The Hutmacher lab has had success with PCL- β tricalcium phosphate (PCL- β TCP) FDM scaffolds, forming a spinoff company Osteopore International Pte Ltd for the production of FDA-approved FDM scaffolds for craniofacial applications. Variants of these scaffolds produced by direct writing melt electrospinning, supplied by a collaboration with Prof. Hutmacher, were used to form the osteogenic portion of the scaffold structure for a preliminary surgical trial in a porcine model (Chapter 4).

Scaffold-Free Cartilage Engineering

Investigations into high-density chondrocyte and MSC culture to produce transplantable articular cartilage *in vitro* have been largely successful [162,163]. They have shown to produce tissues with chemical and structural similarities to native cartilage and with evidence of incorporation into the surrounding defect site [162,163]. However, these systems use very high concentrations of cells and require extensive *in vitro* expansion and culture (>8 weeks) before they are sufficiently robust for implantation [163]. Until a large-volume, phenotypically-stable chondrogenic stem cell population is developed, implementation of these scaffold-free cartilage tissue procedures in full-thickness load-bearing damage sites is currently infeasible and economically prohibitive.

1.8.3 Surface Modification

The surface modification of polymers allows for the efficient presentation of signalling molecules and ECM derivatives, while taking advantage of the superior mechanical properties and processability of commodity polymers like PCL. Covalent surface functionalisation of growth factors for tissue engineering, and the covalent binding chemistries were reviewed recently by Masters [164]. Factors which have been immobilised for cartilage engineering include glycosaminoglycans [91], glycosaminoglycan-binding peptides [133], and cell-binding epitopes [165,166].

The immobilisation of hyaluronic acid and chondroitin sulphate (CS) in freeze-dried type I collagen foams has been shown to increase cellular infiltration, ECM production, cartilage-specific gene expression, MSC differentiation and compressive modulus relative to type I collagen foams [153]. In a study by Coburn *et al.* [91], PVA-methacrylate nanofibres were bulk modified with CS, and showed a significant increase in type II collagen gene expression and synthesis relative to unmodified controls at three weeks *in vitro*, with comparable effects in a rat osteochondral defect model.

The production of peptide-polymer conjugates, in combination with scaffold forming processes that preferentially expose the conjugated functional group, such as electrospinning, have been investigated to simplify surface modification [133,166,167]. Gentsch *et al.* illustrated the use of electrospinning to concentrate a poly(lactic acid)-block-CGGRGDS peptide polymer conjugate, concentrated 11 times on the surface of electrospun fibres [166]. Chow *et al.* showed that chondroitin-sulphate and hyaluronic acid-binding peptide sequences can be displayed on electrospun PCL fibres in mirrored gradients throughout the thickness of the mat [133]. Additionally, the peptides retained the specificity to their intended ECM molecule throughout the electrospinning process forming a gradient of CS and HAc throughout the mat. Modification like these allow for the efficient coupling of high-value peptide on the surface of a fibre in a single, uniform process.

1.9 Cells Sources for Cartilage Tissue Engineering

In a 2015 analysis by Martin *et al.* of cellular and tissue therapies conducted in Europe in 2012, the field of chondral regeneration was divided on cell source, with 281 therapies using mesenchymal stem cells (MSC) (80% autologous) and 237 therapies using chondrocytes (70% autologous) [84]. The majority of cells were expanded *in vitro* (66%), untransduced (77%), unsorted (69%), and manually cultured (68%).

Interesting advances in the field in 2012 were the first use of transduced cells (120 patients) and the use of allogeneic cells (129 patients). The transduced cells were used for the treatment of patients

with early signs of osteoarthritis (OA) and rheumatoid arthritis (RA) through the upregulation of TGF β 1 in allogeneic chondrocytes [37,168]. Other chondrocyte transduction studies for OA and RA are summarised in a review by Evans *et al.* [168]. The allogeneic cells were transplanted in an encapsulated alginate system beneath a periosteal flap in the knee, with limited success, producing predominantly fibrocartilage [169–171].

Chondrocytes are extracted with a preliminary biopsy from the affected joint or the peripheral non-load-bearing area of articular cartilage. Chondrocytes are then extracted from their ECM by enzyme treatment and expanded for clinical applications. Clinical ACI preparations generally expand a few hundred thousand cells to 10-30 million, roughly 6-9 doublings [70]. The expansion process for chondrocytes typically requires 3-8 weeks of culture *ex vivo*. Autologous chondrocyte implantation suffers from the requirement for two procedures as well as the variable efficacy of chondrocytes from aging or osteoarthritic joints, which have reduced ECM production and proliferation rates [66]. One study, comparing human cartilage grown from pre-adolescent (7.5 ± 5 years) and adult chondrocytes (37.3 ± 14.5) showed that juvenile cells are able to regenerate 1.5 mm-thick articular cartilage *in vitro* in serum-free medium without exogenous growth factors, whereas adult cells were not able to regenerate tissue at all under the same conditions [172]. The authors hypothesized that this potentially indicates a loss of autocrine-signalling-induced cartilage homeostasis at puberty.

Dedifferentiation of chondrocytes by expansion can be largely reversed by culturing chondrocytes on 3D matrices, supplementation of the media with TGF- β 3 and dexamethasone, and physiological mechanical loading [173]. Redifferentiation protocols have been studied by gene expression as well as microRNA analysis, and in the case of microRNA expression, can be almost entirely restored with the proper physical and chemical cues [24].

The use of zonally-derived chondrocyte populations has been studied for the creation of zonally-seeded constructs for implantation. However, the lack of zonally-specific cell surface proteins required for efficient automated cell sorting techniques limits their clinical applicability [174]. Beyond zonal populations, the purification of progenitor cells isolated from cartilage by the stage-specific embryonic antigen-4 (SSEA-4) marker for undifferentiated stem cells has also proven of insufficient difference to warrant the additional complexity [175].

Stem cells are a promising avenue of investigation, especially adipose-derived stem cells, as they can be isolated with minimally invasive techniques and have low discomfort and donor site morbidity relative to bone marrow. However, the efficacy of chondrogenic differentiation of adipose-derived stem cells is significantly lower than bone-marrow derived stem cells [176]. Without chondrogenic stimulation MSC produce fibrocartilage *in vivo*, even when implanted in an otherwise promising

chondrogenic environment, such as within a collagen gel beneath a periosteum flap in an otherwise healthy 31-year old patient [177]. Bone marrow-derived stem cells grown in pellet culture have exhibited chondrogenic differentiation without induction [178], but are usually differentiated towards a chondrogenic lineage with approximately 10 ng/mL transforming growth factor β (TGF- β) [179]. In addition to TGF- β , differentiation protocols for MSC, embryonic stem cells (ESC) and induced pluripotent stem cells (IPS) have also included dexamethasone, FGF-2, BMP-2, or BMP-4 and encouraged chondrogenesis through culture in high-density pellets or suspension in agarose and alginate hydrogels [88,179–181].

Chondrogenically differentiated mesenchymal stem cells have proven to produce significantly more GAG than similarly-cultured chondrocytes, however, they fail to retain the GAG due to a lack of link protein [182,183]. Link protein binds proteoglycans to hyaluronic acid (Figure 1-2), retaining PG in the ECM [184], thus, MSC cultures with greatly reduced link protein production are unable to form a physiological ECM. Chondrogenically differentiated MSC cultures grown on Transwell cell culture inserts have been shown to produced spontaneous zonal organisation and tissue with properties approaching those of neonatal cartilage [163].

Therefore, the best cell type for cartilage regeneration is open to interpretation, with both chondrocytes and MSC used with roughly equal frequency in the literature and clinic, and both will continue to be used *in vitro* and *in vivo* for the foreseeable future.

1.10 Summary

The study of cartilage regeneration was initiated with the inception of tissue engineering a generation ago. A tissue with no innervation, no vascularisation, a single cell type, and a huge patient base – many assumed cartilage was the low-hanging fruit that would kick-start the tissue engineering economy. While commercially available products exist and are used in clinic, with significant clinical benefits for millions of patients world-wide, the wisdom of Hippocrates on the irreparable nature of cartilage remains essentially true for the average patient.

With the complex nature of even the simplest of tissues, no one factor will result in a perfect solution. Only through a combination of bioinspired material properties, microarchitectures, surface chemistries, cellular populations, mechanical environments, and environmental conditions can we hope to regenerate cartilage in a long-term functional capacity.

1.11 Project Aims

The primary objective of the following investigation was the development and validation of a zonally-organised porous polymer scaffold for the regeneration of articular cartilage. To achieve this objective, the project was broken into three distinct aims:

Aim 1: Design a Zonally-Organised Scaffold for Articular Cartilage Regeneration

Design a novel porous polymer scaffold for the regeneration of articular cartilage with superficial, intermediate and deep zone characteristics. The scaffold design had to be compatible with biodegradable polymers, mechanically integrated, interconnected, and amenable to fusion with an off-the-shelf scaffold for subchondral bone regeneration.

Chapter 2 – Combine electrospinning and salt-leaching polymer processing techniques to produce a scaffold with superficial zone characteristics and an intermediate/deep zone with an even cellular distribution.

Chapter 3 – Combine multiple scaffold production techniques to produce a scaffold with three distinct zones of biomimetic mechanical stiffness, with pore geometries able to induce zonally-distinct ECM deposition and cell phenotype. The proposed scaffold design is outlined in Figure 1-12.

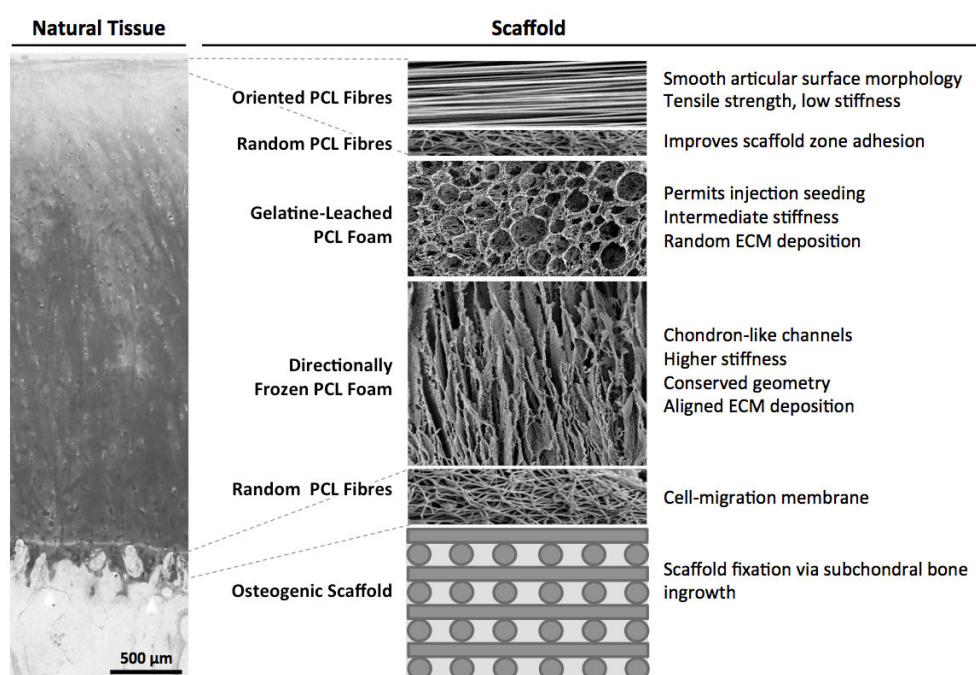


Figure 1-12. Aim 1 - Chapter 3: Proposed scaffold design and justification for the zonal regeneration of articular cartilage.

Aim 2: In Vivo Validation

Design a novel porous polymer scaffold for the regeneration of articular cartilage with superficial, intermediate and deep zone characteristics. The scaffold design had to be compatible with biodegradable polymers, mechanically integrated, interconnected, and amenable to fusion with an off-the-shelf scaffold for subchondral bone regeneration.

Chapter 4A – Combine optimised chondral scaffold from Aim 1 with an established bone scaffold to produce a fused osteochondral scaffold for implantation.

Chapter 4B – Preliminary large animal *in vivo* study of osteochondral scaffold to confirm animal model, surgical technique, and analytical protocols.

Chapter 4C – Full-scale large animal *in vivo* study with appropriate clinical and cellular controls, performed in a manner that promotes clinical translation.

Aim 3: Alternate Polymers

Investigate alternate biodegradable polymers for the production of a chondral scaffold with degradation and mechanical properties superior to 80 kDa poly(caprolactone) (PCL). If successful, use in place of PCL for Aim 1 and Aim 2.

Chapter 5A – Learn how to assess for biocompatibility of a new polymer for biomedical applications.

Chapter 5B – Produce poly(L-lactide-co-ε-caprolactone) of high molecular weight (>80 kDa) and random monomer distribution and test against 80kDa PCL for production of a tissue engineering scaffold for cartilage regeneration *in vitro*.

Chapter 5C – Test a commercially available biodegradable polyester against 80 kDa PCL for production of a tissue engineering scaffold for cartilage regeneration *in vitro*.

Following completion of these aims, the final objective is to advance the product towards clinical translation, as outlined in Chapter 6.

2 Combinatorial scaffold morphologies for zonal articular cartilage engineering

The content of this chapter, excluding section 2.4 and associated methods, was the basis for the publication “Combinatorial scaffold morphologies for zonal articular cartilage engineering” by Steele *et al.* published in 2014 [1], and included as Appendix A. The publication was co-authored by Dr. Seth McCullen, a post-doctoral research associate who developed the concept for the project and was instrumental in early supervision. However, the data collection and analysis contained in this document are my own, unless specifically noted.

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2.1 Introduction

2.1.1 Existing work and project aim

The development of a zonally-organised osteochondral scaffold system incorporating bio-inspired anisotropic mechanical and microenvironmental features that mimic the organisation of native articular cartilage was the cornerstone of a major project in the Molly Stevens group funded by the Wellcome Trust Medical Engineering Solutions in Osteoarthritis Centre of Excellence. Given the importance associated with clinical impact within the framework of the grant, care was taken to avoid materials and design complexities that could hinder translation potential. The investigation resulted in three generations of scaffold design characterised by incremental improvements based on the knowledge acquired with each iteration. The development of the second and third-generation scaffolds are the focus of the research elaborated in this thesis, with the second-generation scaffold covered in this chapter and the optimised design (third-generation) in chapters 3 and 5.

The first-generation design, developed by Dr. Seth McCullen and Dr. Anthony Callanan, was composed of three layers of electrospun poly(ϵ -caprolactone) (PCL) each with differing fibre alignment and diameter (Figure 2-1) [90,185].

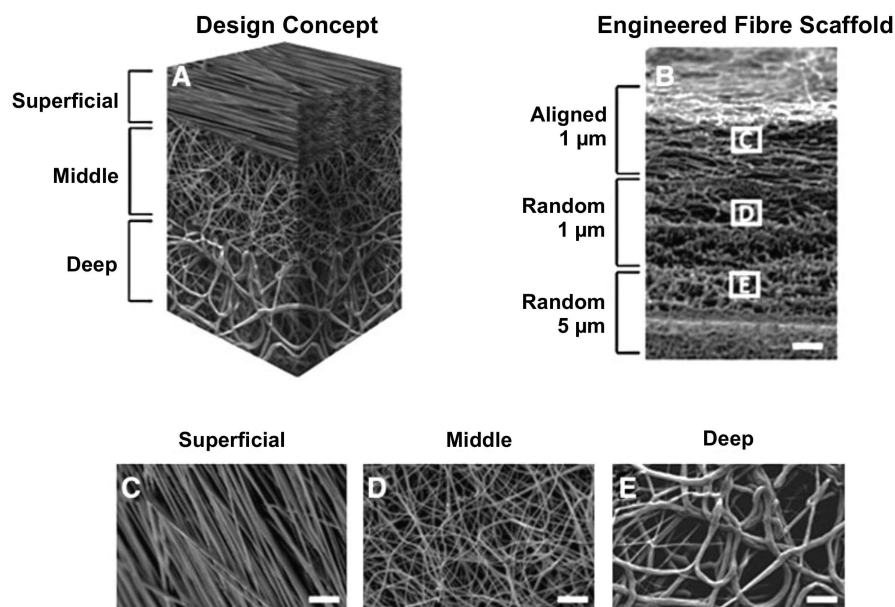


Figure 2-1. First-generation scaffold, composed of three electrospun PCL layers of differing alignment and fibre diameter. Reproduced with permission from McCullen *et al.* [90].

The three different zones were produced by altering key electrospinning parameters including the distance between the needle and the collection mandrel, the polymer solution concentration and the rotational velocity of the collection mandrel (Figure 2-1). High polymer concentrations, low

needle-mandrel separation and low velocities produced larger random fibres, while low concentrations, large distances and high rotational velocities organised the fibres into fine, aligned mats. The three zones of fibres produced a mechanical gradient of 'superficial', 'intermediate' and 'deep' zones. The tensile modulus increased with fibre alignment, transitioning from 35 MPa in the superficial zone to 7 MPa in the deep zone [90]. Conversely, in compression, the stiffness was dependent on fibre diameter, increasing from 137 kPa in the superficial zone to 512 kPa in the deep zone [90]. The gradient of compressive moduli was within the physiological range for human articular cartilage[20]. When seeded with primary bovine chondrocytes and cultured *in vitro* under chondrogenic conditions for five weeks the cells maintained high expression chondrogenic matrix genes (COL2A1, AGC) and produced sGAG-rich extracellular matrix. However, issues intrinsic to electrospinning – high fibre packing density and small pore size – hindered reproducible full-thickness cellularisation [143,186].

Key features of the first-generation scaffold investigation that were deemed advantageous to the design included the use of PCL as a base polymer, the tensile and topographical features of the electrospun superficial zone of aligned fibres, as well as the use of expanded primary bovine chondrocytes for cellularisation. PCL is a biodegradable polymer used in existing FDA-approved implants with proven ease of processing [130] and it was utilised in parallel within the group to develop a novel scaffold-biofunctionalisation approach that could be incorporated within the osteochondral scaffold designs presented in this thesis [133]. The superficial zone composed of aligned PCL fibres 1 μm in diameter possessed a tensile strength, compressive strength, and surface topography within the desired range for an articular cartilage scaffold and was therefore incorporated into the design of the second-generation scaffolds. The use of primary bovine chondrocytes at passage 2 allowed for the isolation and expansion of large quantities of primary cells from each donor. Passaged chondrocytes are a clinically relevant cell source with a strong chondrogenic response in the selected culture conditions.

Therefore, the aim of the second-generation scaffold investigation presented in this chapter was to develop and evaluate a methodology to produce a scaffold with improved cellularisation of the bulk portion of the scaffold in a manner that would interface with the electrospun superficial zone. The primary selection criteria for the new technique were high porosity, interconnectivity, and seeding efficiency.

2.1.3 Scaffold selection

A wide variety of scaffold fabrication techniques have been investigated for the production of *in vitro*-formed cartilage constructs. These have been outlined in detail in Section 1.8, but briefly include electrospinning, foaming, phase separation, porogen-leaching, and a wide variety of hydrogel formation techniques [18,92,145,187–190]. The scope of the project – a porous polymer scaffold composed of PCL with near-physiological mechanical stiffness – precluded the use of hydrogels as the primary structural component. While the different techniques all had their merits, porogen-leaching was selected for the second-generation scaffold as it produced an interconnected open-pore network that facilitated high seeding efficiency and reproducible pore size to modulate chondrogenesis [188]. A sodium-chloride porogen was used as it is insoluble in organic solvents used to dissolve the PCL, available in a range of particle sizes, leaves no cytotoxic residue, and is readily soluble in water - facilitating the leaching step that leaves pores within the polymeric structure.

Porogen-leaching was combined with electrospinning to produce a bilayered scaffold possessing the tensile strength and smooth surface morphology of the electrospun membrane and the high porosity of the underlying foam, which composed the bulk of the scaffold. At the time, multi-layered porous composite scaffolds had only been developed for vascular and urinary bladder applications [191–193]. Multi-layered cartilage tissue engineering strategies published at the time of investigation included layered hydrogels [113], the first-generation three-zone electrospun PCL fibre scaffold by McCullen *et al.* [90], and a multi-zone fused deposition modelling scaffold [18]. The investigation described in this chapter and published in Steele *et al.* (2014) was, to our knowledge, the first instance where electrospinning was combined with a traditional foaming technique to create multi-zone tissue engineering scaffolds for articular cartilage regeneration. It was believed that by replicating the anisotropic structure with distinct superficial and bulk characteristics, a tissue could be formed *in vitro* with zonally-differentiated phenotypes and gene-expression profiles from a single expanded primary chondrocyte population.

Two sodium chloride porogens of differing sizes were selected (0.03 mm^3 and 1.0 mm^3) in order to assess the effect of pore size, with the hypothesis that a smaller pore size would allow for the formation of cellular aggregates which have been shown to increase chondrogenic gene expression, while the larger pores would be perceived as two-dimensional surfaces by the cells [194].

2.3 Materials and Methods

2.3.1 Materials

All chemicals were acquired from Sigma Aldrich, UK and all cell-culture materials were acquired from Invitrogen, UK and used as received, unless otherwise specified.

2.3.2 Scaffold Production

Porogen Leaching

The bulk porous portion of the second-generation scaffold was composed of a single isotropic layer of PCL foam, produced by porogen-leaching. Fine 0.03 mm³ (S3014, 300 µm effective diameter) and coarse 1 mm³ (Coarse Sea Salt, Waitrose, 1000 µm effective diameter) varieties of sodium chloride salt were used for the production of porogen-leached scaffolds. A suspension of 30 g of salt in 15 mL of 15% (w/v) poly(ε-caprolactone) (70-90 kDa, 440744) in dichloromethane (DCM) was prepared in glass vials and mixed thoroughly. A 200 x 100 x 5 mm³ mould was custom machined from aluminium for the casting of the polymer-salt suspension. As the coarse salt packed less densely than the fine, four batches of salt-PCL suspension (120 g in 60 mL) were used to fill the mould for the coarse salt, while eight were used for the fine (240 g in 120 mL). The mould was topped with an additional layer of salt of the same variety, to avoid film formation on the surface, and stored in a fume hood overnight to allow the DCM to evaporate. When fully dry, the mould was immersed in distilled water for 48 hours to remove the salt, with regular water changes to accelerate dissolution. The PCL foam was then removed from the mould and dried under atmospheric conditions.

Electrospinning

The surface of the 200 x 100 x 2 mm³ porogen-leached foam was coated with PCL fibres electrospun directly onto the scaffold. Bilayer (BL) scaffolds (section 2.3) were coated with fibres on the upper surface, while trilayer (TL) scaffolds (section 2.4) were coated on both sides. In both cases, the upper surface was coated with oriented 1 µm diameter PCL fibres produced from a solution of 12% (w/v) PCL in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). To do so, the PCL foams were secured around an electrically grounded voltage-driven rotating mandrel 20 cm in diameter and 10 cm wide. The PCL-HFIP solution was loaded into a solvent-resistant syringe with a blunt 19 gauge needle, and extruded by a programmable syringe pump at 2 mL/h (Kd Scientific Model KDS 100 CE, Sandbach, Cheshire, UK). The rotating mandrel was placed at a working distance of 10 cm from the tip of the needle, charged to +16 kV by a high voltage power supply (Glassman, Bramley, Hampshire, UK). The electrically grounded mandrel was rotated at 1000 rpm (~10 m/s) to collect oriented fibres on the foam surface. To ensure a consistent thickness of fibres across the 10 cm width of the mandrel, the

needle was secured via silicone tubing to a custom-made device which translated $\pm$ 5 cm from the midpoint of the mandrel at a rate of 1 Hz. Upon completion, the scaffold sheet was cut from the mandrel and allowed to dry overnight. For trilayer (TL) scaffolds, the scaffold sheet was inverted and re-attached to the mandrel to coat the underside with fibres. The procedure was similar to that of the upper surface but with two differences. First, for the production of PCL-HAp fibres the 12% (w/v) PCL-HFIP polymer solution was supplemented with nanocrystalline hydroxyapatite (HAp) powder (677418) suspended at 10% (w/w PCL). Secondly, the speed of the mandrel was reduced to 300 rpm ($\sim$ 0.3 m/s) to produce a non-woven mat of randomly-aligned 1 μ m fibres.

2.3.3 Cytotoxicity

Cytotoxicity testing was performed on all components of the scaffolds as well as the final constructs, conforming to ISO 10993-5(2009)[195]. Briefly, acellular scaffolds were incubated in α -MEM for 24 hours at 37°C, 5% CO₂, at 6 cm²/mL for films and fibre mats and 3 cm²/mL for scaffolds to produce extract concentrates. MC3T3-E1 cells were cultured at 37°C, 5% CO₂, with 20,000 cells/cm² in a 96 well plate in α -MEM supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) non-essential amino acids, 1% (v/v) L-glutamine, 1% (v/v) antibiotic/antimycotic. Following 48 hours of culture, the medium was exchanged for a dilution series of the extract concentrates (1, 10, 25, 50 or 100% (v/v)) and the cells were cultured for an additional 24 hours. Viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay against a blank, a PVC 7500 positive control, and a high-density polyethylene negative control. Viability >70% relative to the positive control was deemed non-cytotoxic.

2.3.4 Chondrocyte Isolation

Bovine chondrocytes were isolated from young calves. The metacarpal-phalangeal joints were inspected for damage to the synovial capsule and if none was present, washed thoroughly with warm water and soap to remove dirt and massaged to remove residual blood. The skin was surgically resected and the leg was washed again with soap and 70% (v/v) ethanol. The hoof and exposed end were capped with nitrile gloves and the leg was sprayed with 70% (v/v) ethanol before dissection under a laminar hood. The joint was opened by cutting around the edge of the synovial capsule and dissecting through the ligaments to expose the articular surfaces. The full thickness cartilage was excised with a scalpel and incubated in Dulbecco's Modified Eagle's Medium (DMEM) + Glutamax (4.5 g/L glucose), 0.2% (w/v) pronase, 10 mM HEPES, 50 μ g/mL gentamycin, and 5% (v/v) foetal bovine serum (FBS) for 1 hour at 37°C. The minced cartilage was then digested for 24 hours at 37°C in the same base media with 0.04% (w/v) collagenase type I in place of the pronase. Following digestion, chondrocytes were isolated from the undigested matrix by 70 μ m filtration and

centrifugation (250 x g, 3 min). Chondrocytes were expanded in DMEM + Glutamax (4.5 g/L glucose), 10% (v/v) FBS, 50 µg/mL gentamycin, and 50 µg/mL ascorbic acid at 37°C and 5% CO₂. Primary chondrocytes were expanded to 90% confluency and cryopreserved in liquid nitrogen at 10M/mL in DMEM with 20% (v/v) FBS and 10% (v/v) dimethyl sulfoxide (DMSO). Chondrocytes at passage 2 (P2) were used for all experiments.

2.3.5 Scaffold Seeding and Culture

Scaffolds used to evaluate tissue formation were cut from the large sheets with a circular punch, producing scaffold discs 10 mm in diameter and approximately 2 mm in thickness. Prior to seeding, scaffolds were sterilised for 30 min in 70% ethanol, transferred to a laminar hood, washed three times with sterile PBS, and incubated in sterile 0.01 % (w/v) bovine serum albumin (BSA). Scaffolds were seeded with 10⁶ bovine chondrocytes (6.4x10⁶/cm³) at passage 2. A 100 µL bolus of expansion media containing 8x10⁵ chondrocytes was pipetted directly onto the porogen-leached foam surface of overturned sterile BL scaffolds and incubated for 2 hours at 5% CO₂, 37°C, and 100% RH. The scaffolds were then placed upright with sterile forceps to seed 2x10⁵ chondrocytes on the oriented-fibre surface in 50 µL expansion medium and incubated for an additional hour. Trilaminar scaffolds were seeded at similar concentrations and timelines, but required the 100 µL bolus of 8x10⁵ chondrocytes to be injected into the center of the salt-leached foam with a 21G needle and 1 mL syringe, as both upper and lower surfaces were coated in electrospun fibres. The seeded scaffolds were placed in 2% (w/v) agarose-coated 12-well plates to prevent cell adhesion to the tissue culture plastic and cultured in 1 mL of expansion medium for 24 hours. All subsequent culture used chondrogenic differentiation medium composed of DMEM + Glutamax (4.5 g/L glucose), 50 µg/mL L-proline, 50 µg/mL ascorbic acid, 0.1 mM sodium pyruvate, 1% (v/v) penicillin-streptomycin, 10 ng/mL TGF-β3, and 1% (v/v) ITS Premix (BD Biosciences, Oxford, UK) cultured at 37°C and 5% CO₂.

2.3.6 Biochemical Assessment

Papain Digestion

Scaffolds at weeks 0, 1, 2, and 4 were digested for 24 hours with 2.5 units papain/mL, 5 mM cysteine HCl, 5 mM EDTA, in sterile PBS at 60°C.

DNA Content

Double-stranded DNA content was quantified by the Quant-iT™ PicoGreen® assay, without alteration, using a calibration curve of calf-thymus DNA. Samples were analysed in technical triplicate with n ≥ 4 independent replicates per time point. Briefly, PicoGreen is a fluorescent

molecule with an excitation and emission of 480 nm and 520 nm, respectively. When bound to dsDNA, the fluorescence of PicoGreen increases >1000 fold due to constrained flexibility and polarisation, also serving to effectively eliminate the interference of signal from un-bound dye, which emits relatively low levels of fluorescence [196,197].

Sulphated Glycosaminoglycan Content

Aliquots of the papain digests were also used for the quantification of sulphated glycosaminoglycans (sGAG) by the Blyscan assay (Biocolor, Carrickfergus, UK) without alteration, against a calibration ladder of chondroitin sulphate. Samples were analysed in technical triplicate with $n \geq 4$ replicates per time point. Briefly, the mechanism of action in the Blyscan assay is similar to that of the dimethyl-methylene blue (DMMB) assay and uses the same dye. DMMB binds to sGAG in solution and forms a precipitate in the buffered system proprietary to the Blyscan kit. The residual unbound dye is removed with the supernatant and the DMMB-sGAG precipitate is re-solubilised with an anionic surfactant and absorbance is quantified at 656 nm [198]. The precipitation and re-solubilisation differentiates the Blyscan assay from the standard DMMB, as it reduces background interference to increase the precision of the assay.

Collagen Content

Scaffold samples at weeks 0, 1, 2, and 4 were digested overnight with 0.1 mg/mL pepsin in 0.5 M acetic acid at 4°C. Soluble collagen content was quantified by the Sircol assay (Biocolor, Carrickfergus, UK), without alteration against a calibration ladder of collagen reference standard. Samples were analysed in technical triplicate with $n \geq 4$ independent replicates per time point. Briefly, the Sircol dye is a sirius red dye in dilute picric acid that binds to fibrillar proteins, forming a precipitate [199]. The same mechanism is used in picrosirius red histological staining, discussed in section 2.3.11. The collagen-dye precipitate is dissociated by re-suspending in 0.5M sodium hydroxide and quantified by absorbance at 555 nm [199].

2.3.7 Gene Expression

RNA was extracted from samples at weeks 0, 1, 2 and 4 using the RNeasy kit (Qiagen, UK) according to the manufacturer's protocol and quality was assessed by the 260 nm/230 nm and 280 nm/230 nm absorbance ratios following concentration evaluation by a Nanodrop 2000c spectrophotometer (Thermo Scientific, UK). Purified RNA (200 ng) was transcribed to cDNA via the QuantiTect Reverse Transcription kit (Qiagen, UK) according to the manufacturer's protocol. Gene expression of collagen type I (COL1A1), collagen type II (COL2A1), and aggrecan (AGC) were analysed relative to the housekeeping gene B2M1 using the QuantiTect SYBR Green real time quantitative PCR kit on a Rotorgene 6000 (Qiagen, UK). The primers used and their efficiencies are listed in Table 2-1.

Table 2-1. qRT-PCR Primers

Gene	Forward	Reverse	Efficiency
COL1A1	CATTAGGGGTCAACAATGGTC	TGGAGTTCCATTTTCACCAG	60°C – 92%
COL2A1	CATCCACCCCTCTCACAGTT	GTCTCTGCCTTGACCCAAAG	60°C – 96%
AGC	GCTACCCTGACCCTTCATC	AAGCTTTCTGGGATGTCCAC	62°C – 93%
B2M	ACCTGCTGTCCACGCTGAGT	TGTTCAAATCTCGATGGTGCTGCT	60°C – 92% 62°C – 90%

Gene expression was normalised to the housekeeper gene B2M1 and expressed as a fold increase over week 0 (calibrator), calculated using the $\Delta\Delta C_t$ method. The COL2A1/COL1A1 ratio is calculated from the $2^{-\Delta C_t}$ of each gene as an estimation of the chondrogenic state of the construct.

2.3.8 Mechanical Characterisation

Mechanical testing of the scaffolds and their components was performed on an Instron 5540 testing machine (Norwood, USA) equipped with a 50N load cell in both compression and tension regimes. Unconfined uniaxial compression testing was performed on 10 mm diameter samples with a 0.05 N preload, a ramp of 0-10% strain at 0.05% strain per second, and a 5 minute relaxation at 10% strain. Sample thickness was measured with digital callipers before and after testing. 'Wet' samples were soaked in ethanol for 5 minutes, followed by PBS overnight. The modulus was determined from the slope of the linear portion of the stress-strain curve and the relaxation from the peak stress and equilibrium stress following the prescribed period at 10% strain. All measurements were performed with $n \geq 5$ independent samples.

Tensile testing was performed with the same apparatus, substituting the compression platens for tensile clamps. Scaffold and fibre samples were cut into standardised 50 mm x 10 mm strips, with a gauge length of 30 mm. Sample thicknesses were measured by digital callipers (Fisher Scientific, UK). The samples were tested at 10 mm/min. Ultimate tensile strength and Young's modulus were determined from the stress-strain curves, as the maximum stress and the slope of the linear region

of the stress–strain curve, respectively. The shear force required to delaminate the fibre and porogen-leached zones (and evaluate the interfacial properties) was measured in a lap test by carefully separating and removing one layer in the 1 cm² zone at one end of the 50 mm x 10 mm strips and the other layer at the opposite end, leaving a lapped gauge length of 30 mm in the center. Samples were extended at 10 mm/min to determine the peak load. Interfacial stiffness and interface peak load were tested in directions parallel and transverse to the upper surface fibre orientation. All measurements were performed with $n \geq 5$ independent replicates.

2.3.9 White Light Interferometry

The topography of acellular and cellularised electrospun mats, porogen leached foams, and bilayer scaffolds was assessed by white light interferometry (Wyko NT9100, Veeco). Representative 312 µm x 234 µm x 50 µm volumes were captured with a 20x objective. White light interferometry was performed in collaboration with Dr. McCullen and Dr. Dini, of Imperial College London.

2.3.10 Electron Microscopy

The microstructure of the scaffolds was examined using a JEOL 5610 (Herts, UK) environmental scanning electron microscope (SEM) with an accelerating voltage of 15 kV and a working distance of 15 cm. Samples were prepared for SEM by freeze-fracturing in liquid nitrogen or cutting liquid nitrogen-cooled samples with a clean histology blade and sputter-coating with 100 Å gold by an Emitech K550 sputter coater.

2.3.11 Histology

As PCL melts at 60°C and is soluble in warm xylene, traditional paraffin processing of acellular and cellularised scaffolds for histology was not possible. Additionally, as the scaffolds were micro-porous, embedding with traditional cryosectioning techniques also proved incompatible, due to hindered diffusion of the viscous cryoembedding media. Two custom histological techniques were developed for polymer scaffold applications with the assistance of Ms. Lorraine Lawrence of the Leukocyte Biology Histology Lab, Imperial College London, UK. These are portrayed in Figure 2-2 and described in more details below.

Method 1: Cryosectioning

Cell-laden scaffold constructs were washed in PBS, fixed in formalin for 15 minutes, and washed in PBS to remove residual fixative. Scaffolds were then partially dehydrated in 50% and 70% (v/v) ethanol before transferring to a 1:1 dilution of PBS and optimal cutting temperature medium (OCT, Cell Path, Newton, UK). The partial dehydration and subsequent rehydration aided in penetration of the OCT. The samples were stored in the OCT solution at 4°C for 48 hours, then vacuum cycled three times at 22 mmHg for 5 minutes to remove residual bubbles. After mounting in pure OCT the samples were flash frozen in isopentane at -160°C. OCT blocks were sectioned on a -20°C cryostat at 15 µm. Sections were maintained at room temperature to improve adhesion then rinsed in water to remove the OCT.

Method 2: Polyester Wax

Polyester wax embedding was optimised from a 1957 protocol by Steedman *et al.* to decrease section thickness and improve quality while maintaining the temperature low enough to prevent melting of the PCL scaffold [200]. The full SOP for embedding and sectioning, developed following expensive trial and error to apply the Steedman protocol to porous polymer scaffolds, can be found in Appendix B. Briefly, cell-laden samples were washed and fixed in the same manner as previously described for cryosectioning, then dehydrated in a 70/90/100/100% (v/v) ethanol series. Following 100% ethanol, samples were transferred to a 50:50 (v:v) solution of ethanol: polyester wax (VWR, UK) at 45°C and incubated overnight. Samples were then transferred to two lots of pure polyester wax at 45°C for 2 hours each, with the second step performed in a vacuum oven at 22 mmHg and 45°C. Sections were cut at 10 µm and adhered to Silane-Prep slides (Sigma Aldrich, UK), aided by 0.1% (w/v) gelatine or BSA solution where permissible and allowed to dry at room temperature. Slides were dewaxed in 100% ethanol and allowed to dry once again for ≥ 30 minutes to improve adhesion. Sections were then rehydrated with a 100/90/70/50% ethanol series followed by tap water in preparation for staining.

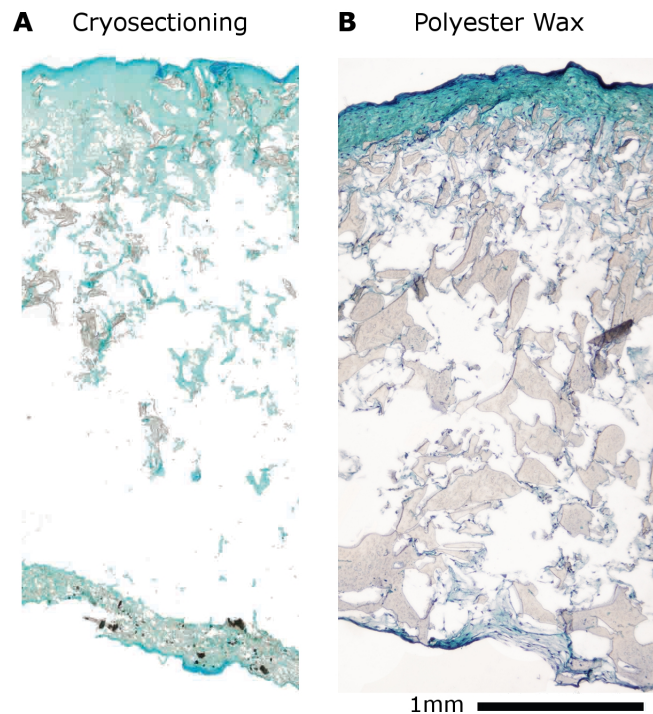


Figure 2-2. Comparison of cryosectioning and polyester wax sectioning. BL-0.03 scaffolds seeded with 1M chondrocytes and cultured *in vitro* for 4-weeks, stained with alcian blue to highlight sulphated glycosaminoglycans (blue). The two procedures are compared to highlight the greater retention of PCL foam structure with the polyester wax technique, despite the diffuse tissue.

Staining

Haematoxylin and Eosin

The most common histological stain is that of haematoxylin and eosin (H&E) for nuclear staining and general tissue counterstaining. Haematoxylin is a positively charged, basic dye molecule which forms an insoluble salt with DNA and RNA, as they are negatively charged acids, staining them red. The stain is then 'blued' in hard tap water, resulting in dark purple/blue nuclei [201,202]. Eosin is a bright pink fluorescent compound that binds electrostatically with positively charged amino acid residues, staining tissues intensely pink, as a counterstain to the dark blue/purple nuclei from Haematoxylin.

The staining process consists of 3 minutes in Harris' Haematoxylin, more than 3 min in running tap water, 3 dips in acid alcohol (3% HCl (v/v) in 70% (v/v) ethanol), more than 1 min in running water, 1 min in 1% (w/v) Eosin Y, and finally 1 min in running water. As with all aqueous stains, stained slides were dehydrated in a 70/90/100% ethanol series (1 min each) cleared in three 5 min changes of Histo-Clear II (National Diagnostics, UK) and mounted with Histomount. Slides were imaged on an IX51 epifluorescence microscope equipped with a DP70 digital camera under visible light (Olympus, UK) and all image processing was performed with GIMP 2.8.

Alcian Blue

Alcian blue was used at pH 2.5 to stain for sulphated polysaccharides and hyaluronic acid. These are main components of the cartilage extracellular matrix and thus intense alcian blue staining is indicative of a chondrogenic phenotype. Alcian blue staining at pH 2.5 results from the electrostatic attraction of positively charged dye to negatively charged ECM components (sGAG and HAc). The selectivity is due to the quick staining (5 min) and the large size of the molecule (2.5-3 nm) sterically hindering interaction with other negatively charged but compact intracellular components including chromatin [201]. As the charge is localised to the functional ends of the molecule and not dissipated throughout the aromatic structure, the electrostatic interaction results in the formation of an insoluble salt, Phthaloyanine Blue [201].

Hydrated samples were placed in 1% (w/v) Alcian blue in 3% (v/v) acetic acid, adjusted to pH 2.5 for 5 minutes and rinsed in running water for more than 5 minutes. Nuclei were counterstained with nuclear fast red for 1 minute, washed in running water, dehydrated and mounted as described previously. Nuclear fast red is used in place of haematoxylin as haematoxylin can result in a non-specific increase in background blue hue that can be erroneously associated with Alcian blue intensity.

Picrosirius Red

Collagen was stained using picrosirius red, which produced a birefringent green-red hue under cross-polarised microscopy, dependent on the diameter and alignment of the collagen fibrils [203]. Sections were stained for 30 seconds in celestin blue (Solmedia, UK) and 30 seconds in haematoxylin, with more than 1 min running water washes, followed by 1 hour in picrosirius red solution, 0.1 % (w/v) sirius red F3B (Solmedia, UK) in a saturated solution of picric acid in water ~1.3% (w/v). Slides were washed twice in acidified water (0.5% (v/v) acetic acid), dehydrated, and embedded as above. For crossed-polarised imaging of picrosirius red birefringence of collagen fibrils, the PCL in the scaffold sections was removed by soaking slides in xylene overnight followed by 30 min in chloroform, leaving only the cells and ECM. Without this step, the refraction of polarised light within the PCL sections produced a bright white glow that occluded the birefringence of the collagen fibrils.

Alizarin Red

Alizarin red was used to stain for calcium as an indication of calcification in the TL samples loaded with HAp. Samples were handled carefully to avoid dissolution of deposited calcium and placed in a 1% (w/v) solution of alizarin red adjusted to pH 4.2 (+/- 0.1) for 2 minutes. Sections were then

washed and dehydrated by 10 dips each in acetone, 50:50 acetone:xylene, and pure xylene and mounted immediately with Histomount.

Immunohistochemistry

Immunohistochemical staining (IHC) was used to visualise the distribution of type I collagen (Abcam ab34710), type II collagen (Abcam ab300) type X collagen (Abcam ab58632), and superficial zone protein (Abcam ab28484) with rabbit IgG (Vector Labs, UK) and PBS negative controls. Samples were pre-treated with hydrogen peroxide, an avidin and biotin blocking kit (Vector Labs, UK), and blocked with 5% (v/v) goat serum. Primary antibodies were incubated overnight at 1/200 in 5% (v/v) goat serum, followed by goat-anti-rabbit HRP at 1:100 for 1 hour. The slides were stained with a 3,3'-diaminobenzidine (DAB) kit (Vector Labs, UK) for 10 minutes and counterstained with haematoxylin. All sections were mounted with Histomount and viewed on an Olympus BX51 microscope equipped with an Olympus DP70 camera.

The horseradish-peroxidase (HRP)-DAB system is commonly used to localise the presence of a single antigen by IHC, as the slides produced can be observed through traditional microscopy and stored for extended periods of time without signal loss [204]. The HRP on the secondary antibody is able to oxidise DAB in the presence of hydrogen peroxide. The oxidation initiates a free-radical polymerisation (Figure 2-3) which produces a dark-brown insoluble precipitate, indirectly indicating the location of the antigen [204].

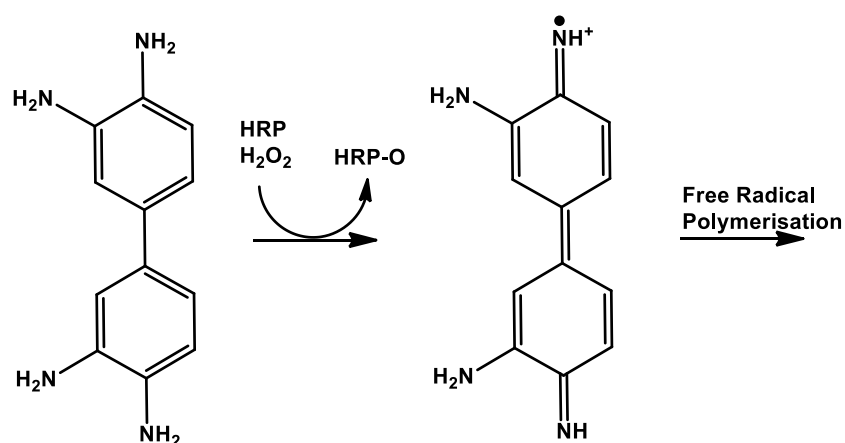


Figure 2-3. Polymerisation of DAB in the presence of horseradish peroxidase (HRP) [204,205].

2.3.12 Statistical Analysis

All data was collected with $n \geq 4$ for biochemical analysis and mechanical testing. Immunohistochemical and histological staining were performed on $n \geq 3$ samples. Data is presented as mean $\pm$ standard deviation and all significance (*) was determined by ANOVA with $p < 0.05$.

2.4 Results and Discussion

2.4.1 Scaffold Design

Two bilayer scaffold systems were developed and investigated to determine if unique zonally-appropriate biochemical signatures could be observed between the electrospun fibre zone (FZ) and the porogen-leached zone (PLZ). In addition to the zonal differences, the effect of pore size in the PLZ was also investigated with regards to scaffold seeding efficiency, tissue formation, and chondrogenic gene expression profiles. Figure 2-4 illustrates the bilayer scaffold design with SEM micrographs of the fibres, scaffold, and porogens for both the fine (0.03 mm^3) and coarse (1 mm^3) scaffolds.

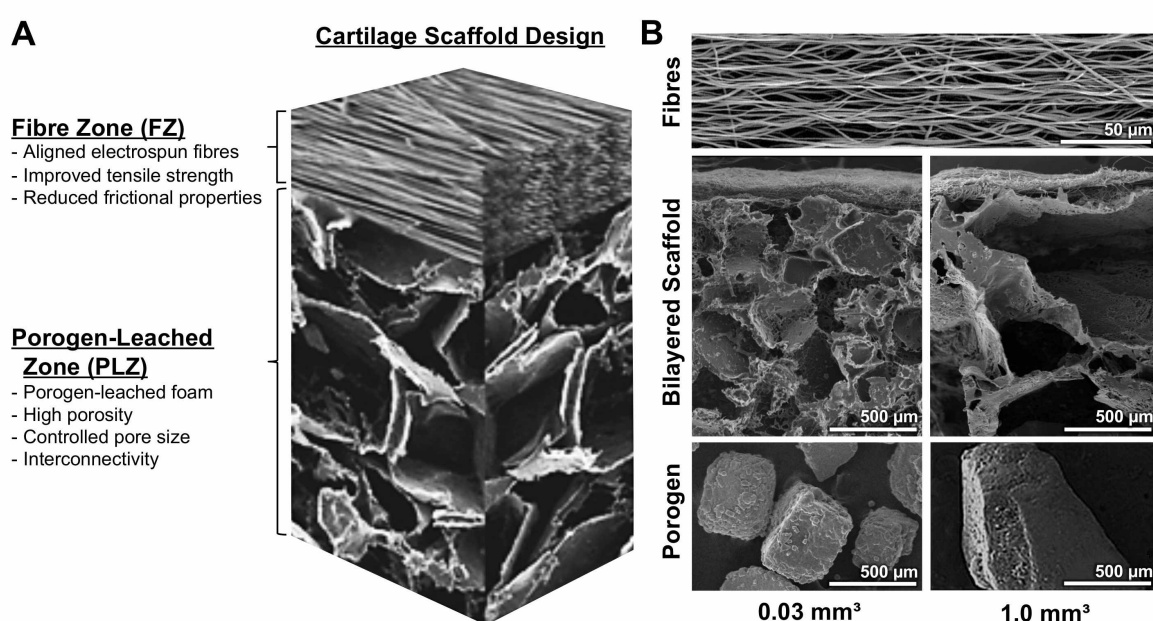


Figure 2-4. Bilayer scaffold schematic illustrating A) the properties of the two zones and B) SEM images of the fibres, 0.03 mm^3 and 1 mm^3 porogens used to form the PLZ and a cross-section of the two complete bilayered scaffold designs. Modified from Steele *et al.* [1]

2.4.2 Acellular Mechanical Characterisation

The mechanical properties of the separate zones (FZ/PLZ) and complete bilayer (BL) acellular scaffolds of both porosities were analysed under compression, tension, and tensile lap-tests for interfacial stiffness (Table 2-2). Surface roughness was calculated by white light interferometry.

Table 2-2. Mechanical properties of scaffold components, modified from Steele *et al.* [1][†].

Sample*	Tensile modulus (MPa)	Tensile stress at failure (MPa)	Compressive modulus (kPa)	Interfacial stiffness (aligned) (N mm ⁻¹)	Interface peak load (aligned) (N)	Surface Roughness (Ra; μm) [‡]
PLZ – fine	0.04 ± 0.02	0.05 ± 0.01	40 ± 5	N/A	N/A	16 ± 10
BL – fine	1.1 ± 0.3	0.4 ± 0.1	40 ± 21	0.24 ± 0.04	0.68 ± 0.03	3.0 ± 0.8
PLZ – coarse	0.10 ± 0.05	0.03 ± 0.01	20 ± 5	N/A	N/A	26 ± 20
BL – coarse	1.6 ± 0.5	0.4 ± 0.2	14 ± 4	0.23 ± 0.03	1.15 ± 0.03	2.1 ± 0.5
FZ	28 ± 2	12 ± 3	140 ± 20	N/A	N/A	3.0 ± 1.0

*PLZ = porogen-leached zone, BL = bilayered scaffold, FZ = fibre zone; fine = 0.03 mm³, coarse = 1.0 mm³ porogen,

[†]Data collected and analysed with Dr. Seth McCullen, Imperial College London

[‡]Data collected and analysed by Dr. Daniele Dini and Dr. Mario Accardi, Imperial College London

The tensile modulus and tensile stress at failure for the bilayer scaffolds were significantly greater than the PLZ alone. The tensile properties of the BL scaffolds are due to the presence of the aligned fibre zone, with a negligible contribution from the underlying PLZ foam.

The fibre zone has a significantly higher tensile modulus and stress at failure than the BL scaffold, despite owing their tensile strength to the same thickness of aligned electrospun fibres. This disparity is due to the statement of the properties in terms of stress (MPa) which is calculated based on the whole cross-sectional area of the BL scaffold (PLZ and FZ), despite the PLZ composing approximately 95% of the scaffold cross-sectional area, but contributing a negligible effect on the tensile properties. This finding is confirmed by the fact that the tensile forces at failure for the BL and FZ, which do not take into account cross-sectional area, were statistically comparable at 11 ± 5 N.

The stiffness of the fine-pore scaffold in compression was greater than the coarse-pore scaffold, with no statistical difference between the BL and PLZ, as the FZ did not greatly contribute to the compressive strength of the BL scaffold. The compressive properties of the FZ alone are greater than the BL and PLZ, as it is a densely packed aligned fibrous mat (Figure 2-4B), but the 100 μm thickness and softer underlying PLZ foam resulted in a negligible contribution to the bulk compressive stiffness of the 2 mm-thick scaffold.

A lap-test performed to analyse the strength of the interface indicates that the tensile stiffness of the interface was equivalent for the fine- and coarse-pores scaffolds but the samples with coarse pores had a higher peak load before failure. This was attributed to a greater contact area between the FZ and the PLZ in the coarse scaffold, as the coarse PLZ was known to produce a denser surface film, whereas the fine-pored foam exhibited a porous surface with reduced fibre-foam contact area.

The roughness of the surfaces was analysed by white light interferometry by collaborators Drs. Dini and Accardi. In the acellular analysis in Table 2-2, the electrospun surfaces of the BL foams had statistically similar roughnesses to those of the independent FZ sheets, while the underlying PLZ foams were far more variable, but coarser on average. This, along with the tensile measurements validated that the fibre deposition onto the surface of PLZ foams was of sufficient thickness and quality to confer the properties of an independent electrospun mat to the surface of the BL construct.

2.4.3 Biochemical Characterisation

Primary bovine chondrocytes were seeded at 10^6 /scaffold and cultured *in vitro* under serum-free chondrogenic conditions for up to 4 weeks. Samples were analysed mechanically and biochemically at 1, 2 and 4 weeks.

The primary objectives for adapting the scaffold design to include the porogen-leached foam were increased seeding efficiency, viability and proliferation within the scaffolds. These criteria were evaluated over a 7-day period in standard expansion medium, including 10% FBS. Analysis was performed on the whole scaffolds as well as the separated zones to determine if there were differential seeding efficiencies or early zone-specific divergences in proliferation (Figure 2-5).

PLZ scaffolds of both pore sizes led to a seeding efficiency greater than 75% after 2 hours of incubation. However, when the electrospun surface was added to the coarse-pored scaffold (BL-1.0) the seeding efficiency rose above 80% and was statistically greater than the fine-pored scaffold (Figure 2-5A). When the seeding efficiency is analysed by zones as shown in Figure 2-5B, it is clear that on day 1, both porogen-leached zones were seeded with roughly equal efficiency, yet there was a trend for greater seeding efficiency on the fibre zone of the large-pore scaffold (FZ-1.0), which contributed to the significant difference when complete scaffolds were compared.

The 7-day analysis of DNA accumulation (as an estimation of proliferation) within the scaffolds in Figure 2-5C,D indicated significant proliferation within both BL scaffolds and within each zone at days 4 and 7. There was a significantly greater quantity of DNA in the BL-1.0 scaffolds at all time points relative to BL-0.03. However, when analysed zonally neither FZ-1.0 nor PLZ-1.0 were significantly greater than their BL-0.03 counterparts (Figure 2-5D). This proliferation is likely indicative of the serum-supplemented expansion culture conditions in which the cells were cultured for 7 days and confirms the cytocompatibility of the scaffolds.

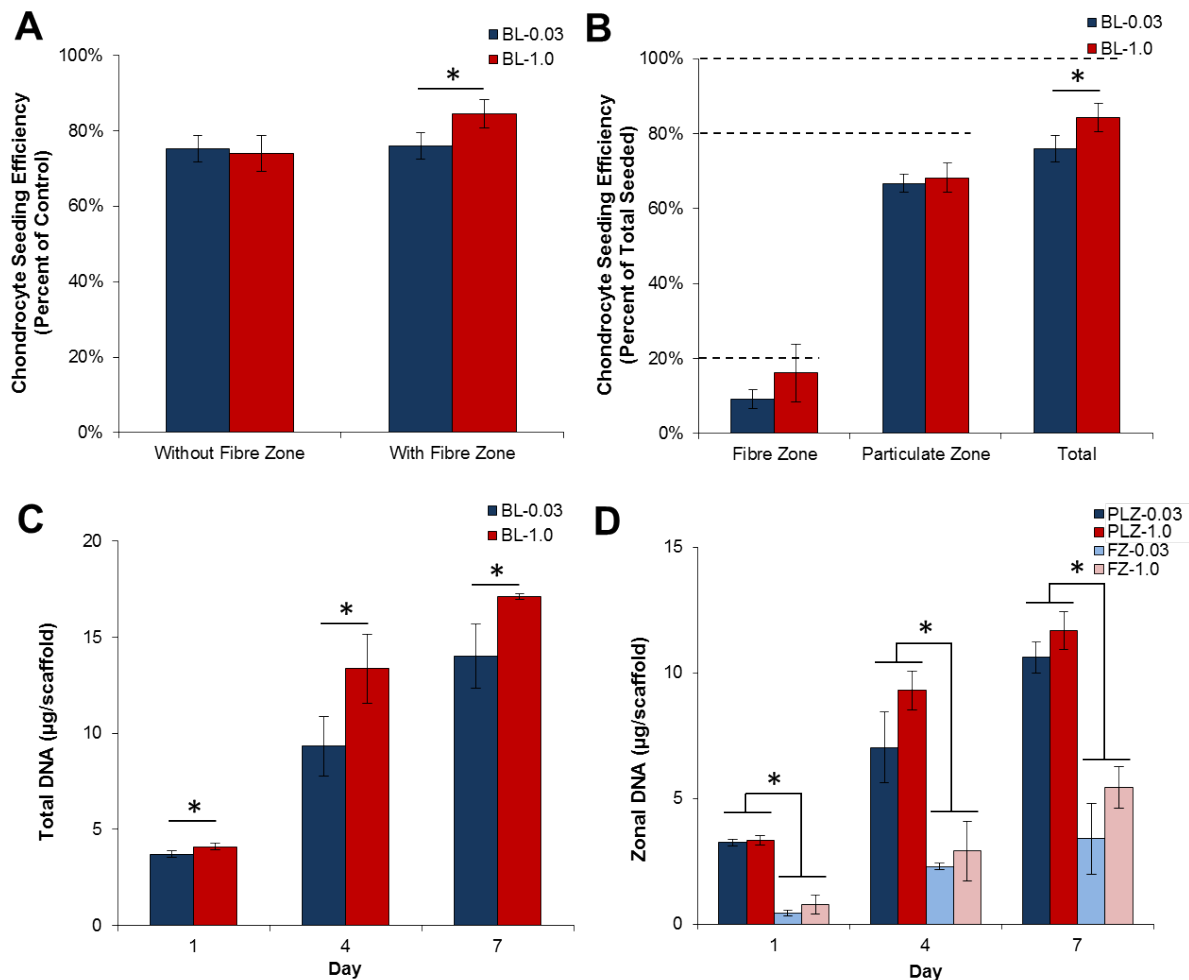


Figure 2-5. Initial seeding study. A) Seeding efficiency of scaffold with and without the superficial FZ at 24 hours. B) Bulk and zonal chondrocyte seeding efficiencies at 24 hours. C) DNA quantification in scaffolds over one week. D) DNA quantification over one week divided between the zones. *Significant difference ($p < 0.05$). Modified from Steele *et al.* [1].

A 4-week biochemical assessment of the *in vitro*-formed tissues was performed following incubation in serum-free chondrogenic media to assess the ability of chondrocytes to proliferate and produce a cartilage-like ECM (Figure 2-6). Total DNA within the scaffolds increased over time as in the previous study, but at a reduced rate, roughly doubling over 4-weeks, as opposed to the previous study which showed a 3-4 fold increase at 7-days. This pronounced difference is attributed primarily to the lack of serum in the chondrogenic medium and the presence of chondrogenic growth factors. Growth factors, like TGF- β 3 promote chondrogenesis and mature non-proliferating chondrocyte phenotypes as opposed to serum-supplemented expansion culture, which promotes proliferation at the expense of dedifferentiation. This difference was anticipated and desired for the formation of cartilaginous tissues, but is worth noting to avoid confusion.

DNA seeding efficiencies were not statistically different for both scaffold pore sizes at $86\% \pm 4\%$ and $77\% \pm 10\%$ for BL-1.0 and BL-0.03 respectively. At all non-zero time points there is significantly more DNA in the BL-1.0 system (Figure 2-6B). As in the previous short-term study, this can be attributed to increased proliferation in the larger pores.

The production of sulphated glycosaminoglycans (sGAG), indicative of chondrogenic phenotype, increased in both scaffolds over 4-weeks, with statistically more sGAG produced in the BL-0.03 scaffolds at all non-zero time points (Figure 2-6C). Additionally, in the zonal analysis (Figure 2-6D) the increased production in the complete BL-0.03 can be attributed to the PLZ-0.03, which had significantly more sGAG production than the PLZ-1.0, when normalised to DNA. The concentration of sGAG is expressed normalised to DNA as it dissociates matrix synthesis from proliferation and seeding density and is therefore more indicative of a cellular phenotype. Some publications also normalise to dry scaffold weight [162], wet scaffold weight [86], or unit volumes [18]. While equally applicable when discussing tissue sections [163], these normalisations can be confounded with the density and hydrophilicity of the polymer scaffold and seeding density in tissue engineering applications, producing data that is difficult to analyse and compare across scaffold varieties. When the scaffold sGAG values of $23\text{--}37\text{ }\mu\text{g}/\mu\text{g}$ DNA are compared to physiological values of $100\text{--}850\text{ }\mu\text{g}/\mu\text{g}$ DNA for adult human superficial and deep zone cartilage [2,6,22], the constructs appear to have low sGAG concentrations. However, this can be attributed to the early 4-week time point yielding a relatively immature tissue with low tissue density and a high cellular density. When compared to other 4-week *in vitro* scaffolds, which range from $5\text{--}58\text{ }\mu\text{g}/\mu\text{g}$ DNA (Appendix C), the values for sGAG are within the stated range, and on the high-end of the spectrum.

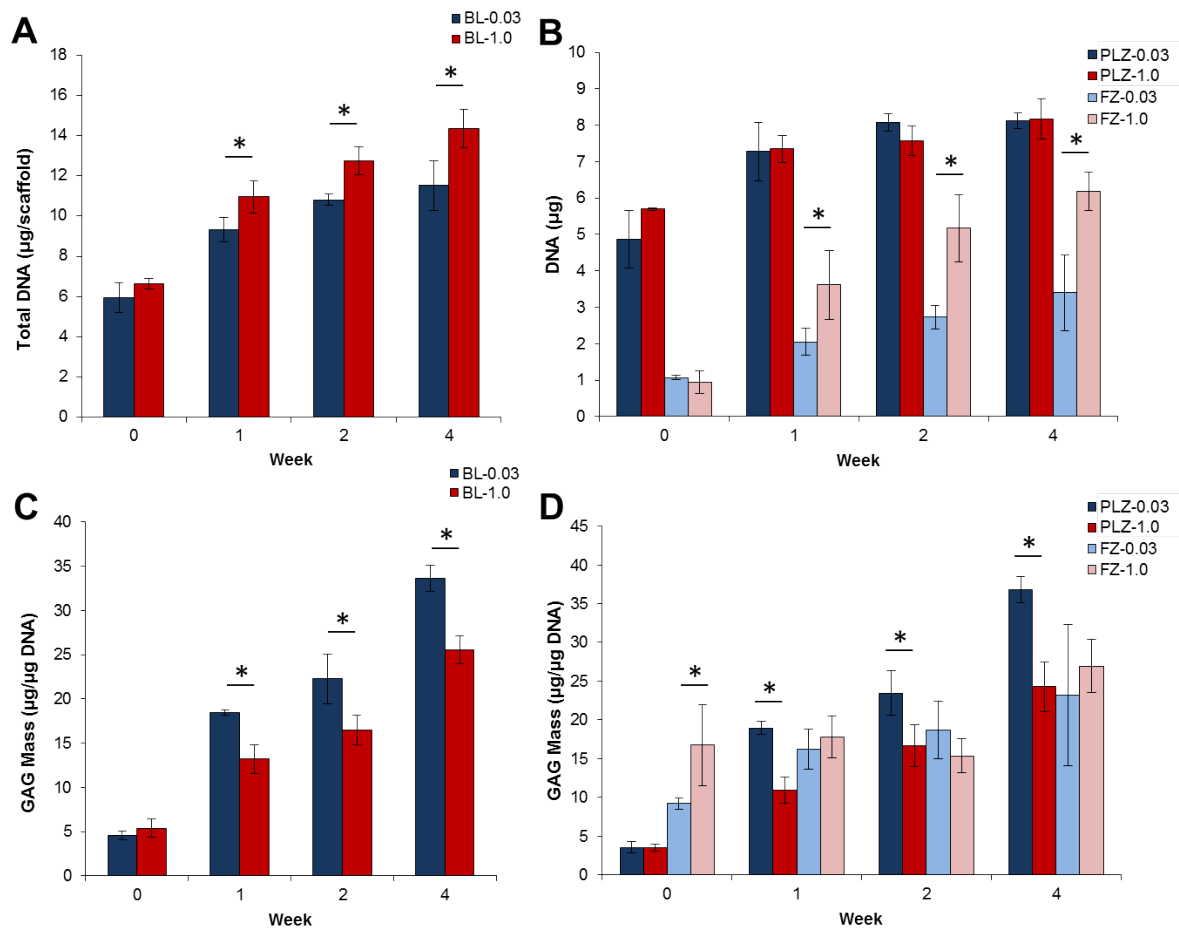


Figure 2-6. Biochemical assessment of scaffolds over 4-weeks *in vitro*. DNA (A,B) and DNA-normalised sGAG (C,D), on a bulk (A,C) and zonal (B,D) basis. *Significant difference ($p < 0.05$). Modified from Steele *et al.* [1].

It is common practice in many investigations to quantify collagen synthesis within cartilage scaffolds, as was performed in Figure 2-7. However, the utility of collagen quantification has been increasingly called into question as there are known issues with interference from serum, enzymatic digestion, and protein solubilisation [206]. Additionally, passaged chondrocytes produce a combination of collagens I and II, depending on the degree of dedifferentiation. Therefore, knowing the quantity of total collagen does not indicate the quality of the repair. Immunohistochemistry can be used to further enhance the qualitative assessment, while RT-qPCR provides a quantitative assessment of protein-specific gene regulation. Nevertheless, collagen quantification combined with immunohistochemistry and qPCR can provide a general appreciation of the quality of the cartilage-like tissue. The data in Figure 2-7 was omitted from the Steele *et al.* publication due to a lack of confidence with the Sircol collagen quantification assay, and the limited conclusions that can be drawn from it. The greatest trend observed in the data is the high level of collagen expression in the PLZ-1.0 at weeks 2 and 4. This was attributed to the increased proliferation and a potential

dedifferentiation effect of the large pore size on chondrocytes, generating a type I collagen-enriched fibrocartilage layer on the pore walls, as opposed to the more three-dimensional type II collagen-enriched chondrocyte aggregates observed in PLZ-0.03, as observed in the histological and immunohistochemical analysis in Figure 2-9.

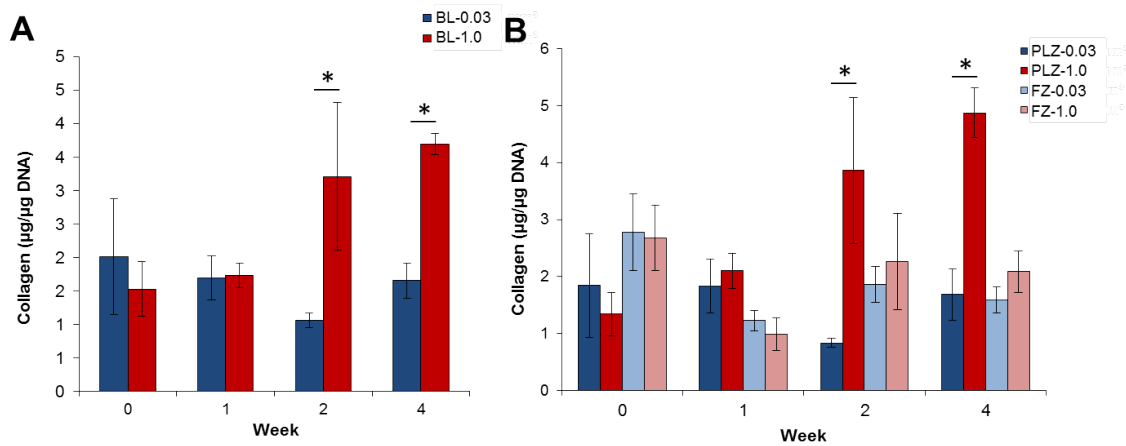


Figure 2-7. Quantification of DNA-normalised collagen synthesis in BL scaffolds seeded with 1M chondrocytes over 4-weeks *in vitro*. A) Total Scaffold, B) Zonal Assessment. *Significant difference ($p < 0.05$).

2.4.4 Gene Expression

Having assessed the biochemical and mechanical properties of the scaffold, the underlying gene expression resulting from the differential scaffold design was investigated by qRT-PCR at the same time points (Figure 2-8). Cellular scaffolds were separated into the two zones and RNA was extracted separately from each zone with gene expression was calculated relative to day zero. Up-regulation of AGC and COL2A1 at all time points and within both scaffold zones indicated a global increase in the production of aggrecan and type II collagen, both indicative of a chondrogenic gene expression profile (Figure 2-8 A,B). At weeks 1 and 2, the fine-pored PLZ-0.03 scaffolds had statistically greater increases in the expression of both chondrogenic genes AGC and COL2A1, with 2.6 and 2.9-fold up-regulations in gene expression at week 2 for aggrecan and type II collagen respectively, compared to PLZ-1.0. The significant difference between PLZ-0.03 and PLZ-1.0 can be attributed to the cellular microenvironment found within the two scaffolds. Within the fine pores of PLZ-0.03, chondrocytes are able to form cellular aggregates that bridge the pores and result in a three-dimensional tissue with a more differentiated phenotype as seen by histology and immunohistochemistry below. In the coarse pores of PLZ-1.0, the cells grow in flat fibroblastic layers, leading to dedifferentiation. As matrix accumulates by week 4, this significant difference is lost, presumably because the environmental differences between scaffolds are buffered by the deposition of appositional matrix

around the chondrocytes. An indicator of chondrogenic de-differentiation, type I collagen (COL1A1), was down-regulated at week 1 and only slightly up-regulated at the other time points, resulting in a significantly higher COL2A1/COL1A1 ratio for BL-0.03 over BL-1.0 at week 2, and a similar trend at weeks 1 and 4 (Figure 2-8 C,D). This gene expression profile indicates that the bilayer scaffolds with finer 0.03 mm³ pores are more chondrogenic than with the coarser 1.0 mm³ pores. The lack of statistical differences between FZ-0.03 and FZ-1.0 support the PLZ conclusions, as the fibre zones were identical in morphology and were therefore expected to have similar gene expression profiles.

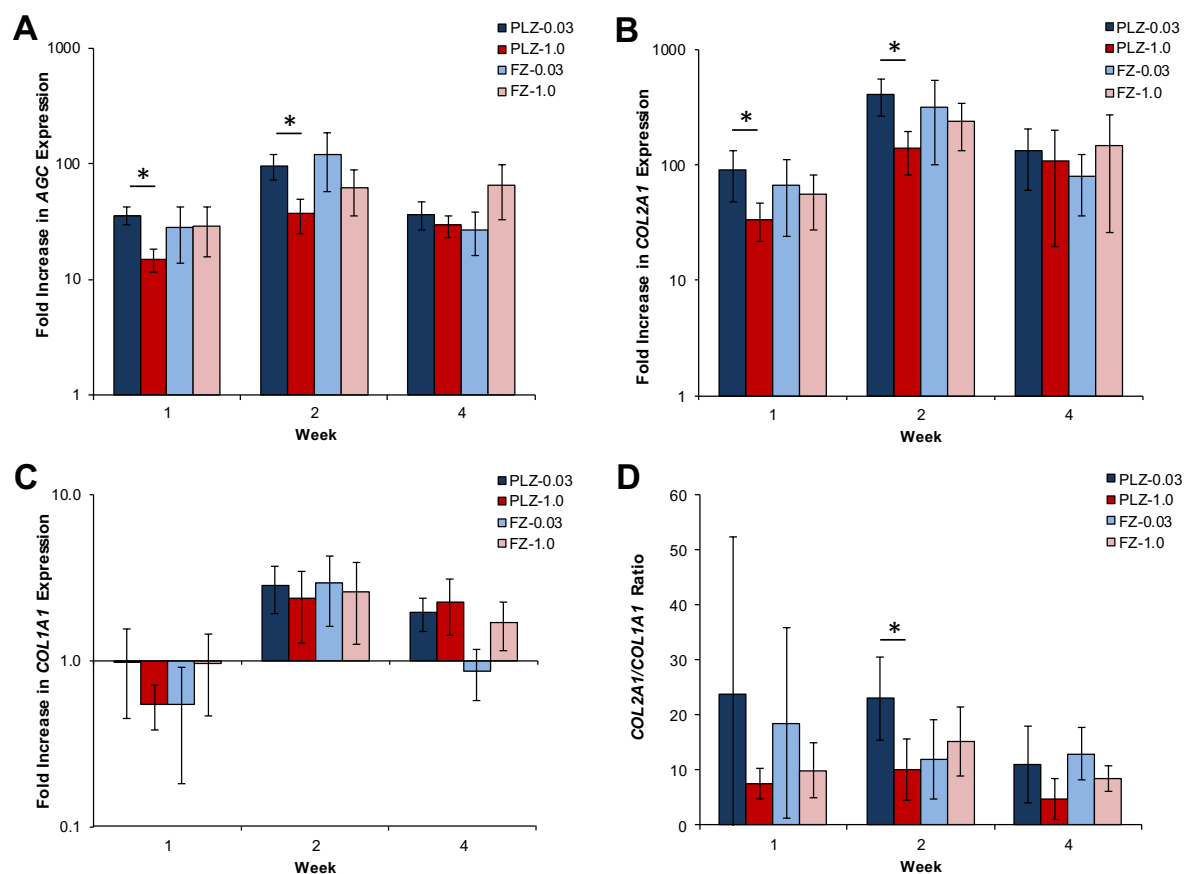


Figure 2-8. Gene expression profiles for both the porogen-leached zone (PLZ) and superficial fibre zone (FZ) following *in vitro* culture in chondrogenic medium. A) AGC, B) COL2A1 and C) COL1A1 are expressed as fold differences relative to seeded controls at day 0 and normalized to the B2M housekeeping gene. D) The COL2A1/COL1A1 ratio is an indicator of chondrogenic differentiation. *Significant difference ($p < 0.05$). Modified from Steele *et al.* [1].

2.4.6 Histology

Having determined that there was a biochemical difference observed between the two scaffold varieties and their zones, histological analysis and immunohistochemistry were required to confirm the quality and distribution of tissue formed at 4-weeks.

Polyester wax sections were stained with haematoxylin and eosin (H&E) to highlight tissue distribution throughout the scaffolds, alcian blue for glycosaminoglycan distribution, and picrosirius red for fibrillar collagen distribution and maturity (Figure 2-9). The BL-0.03 scaffold showed more intense alcian blue (sGAG) and picrosirius red (collagen) staining. Under cross-polarised microscopy of the picrosirius red-stained sections, the collagen fibres in the BL-0.03 superficial zone appeared more plentiful and mature due to their intense red/yellow colouration as opposed to the immature green fibres in BL-1.0. The tissue density in the electrospun fibre zones was the highest, showing intense staining throughout, while the tissue densities in both PLZ foams were lower than initially anticipated. The PLZ in BL-0.03 had more consistent tissue formation within the smaller pores, as chondrocytes at 10^6 /scaffold were able to bridge the pore and form tissue aggregates in some instances. Conversely, the PLZ of the BL-1.0 scaffold shows largely empty pores, with most cells present in a fibroblastic-like coating on the pore wall, as the spans were too great for the chondrocytes to form bridging aggregates within the cavities so that they essentially perceive their environment as a two-dimensional surface.

Immunohistochemical staining was performed to assess the distribution of type I collagen (Col I), type II collagen (Col II), type X collagen (Col X), and superficial zone protein (SZP) (Figure 2-9). In agreement with the gene expression which indicated that a combination of both type II collagen and type I collagen were produced, as would be expected of P2 chondrocytes despite the chondrogenic culture conditions, both collagens are found throughout the scaffold in a similar distribution to picrosirius red and H&E staining. Therefore, staining was indicative of general tissue distribution and not specialisation or zonal differentiation. The staining for type X collagen, an indicator of hypertrophy was minimal, while SZP, a superficial zone marker, was found in the pericellular domain of the chondrocytes throughout the scaffold [2,207].

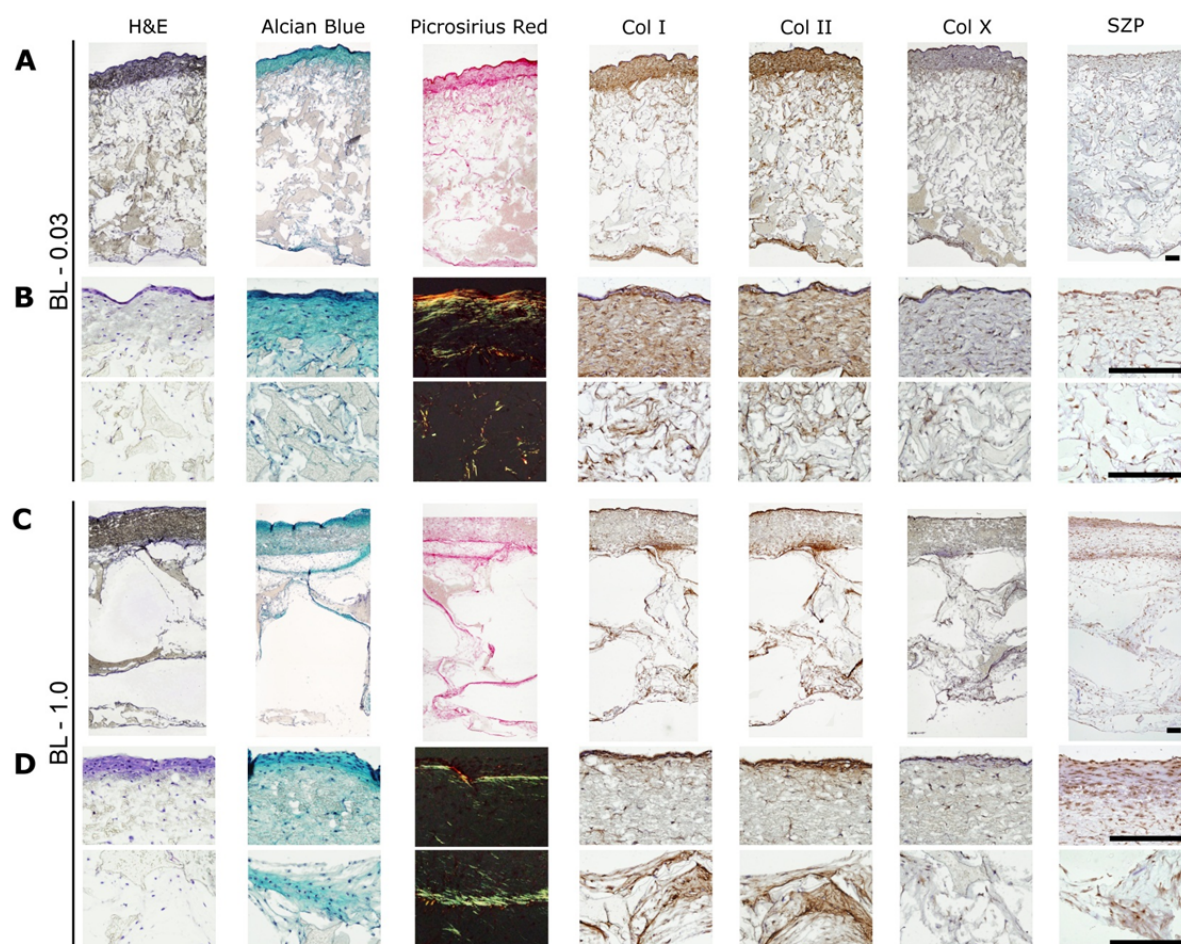


Figure 2-9 Histological analysis of bilayered scaffolds BL-0.03 (A, B) and BL-1.0 (C, D) following 4 weeks *in vitro*. All scale bars represent 250 µm. Full-thickness scaffold cross-sections (A, C) and high-magnification FZ and PZ zones (B, D) are shown. Scaffolds were stained with haematoxylin and eosin (H&E) for tissue distribution, alcian blue for sGAG, picrosirius red for collagens. Immunohistochemical analysis indicates the distribution of collagen type I, II and X, and superficial zone protein (SZP), respectively, from left to right. The visualisation of collagen birefringence under picrosirius red staining was visualized in sections from which the PCL has been removed, by cross-polarised microscopy. Modified from Steele *et al.* [1].

2.4.7 Cellularised Topography and Mechanical Characterisation

The surface roughness of scaffolds seeded with chondrocytes was assessed by white light interferometry to determine if the presence of cells and cell-deposited ECM significantly altered the acellular properties, as outlined in Table 2-2. The presence of cells slightly increased the surface roughness, as shown in Figure 2-10; however, high sample-to-sample variability resulted in no statistical differences being observed over time. Despite the variability, all scaffolds maintained roughness values well below the average of the acellular PLZ scaffolds alone (Figure 2-10 C). Therefore, the smooth surface for which the electrospun superficial zone was selected was maintained throughout 4 weeks of *in vitro* culture.

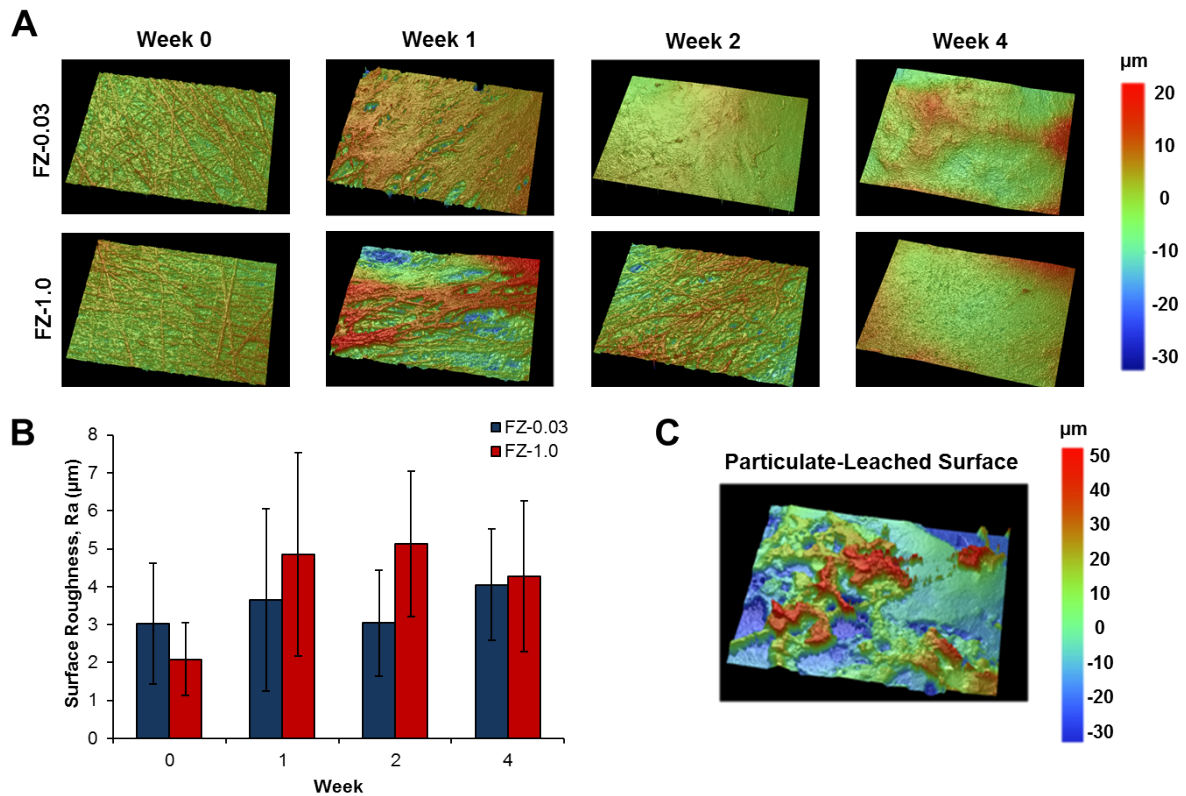


Figure 2-10. Surface roughness as determined by white light interferometry of (A,B) fibre zones of bilayer scaffolds over 4-weeks *in vitro*, seeded with chondrocytes. C) Roughness of the surface of an acellular PLZ-1.0. Figures in A and C are representative 312 x 234 μm areas. Modified from Steele *et al.* [1]. Data collected by Dr. Daniele Dini and Dr. Mario Accardi, Imperial College London.

Cultured scaffold samples were also tested mechanically at the same time points as the biochemical assessment to determine if the mechanical properties, either in compression or tension, were maintained or altered by the measured cellular proliferation and ECM deposition (Figure 2-11). Mechanical properties in both compression and tension were found to remain consistent throughout the culture period with no statistical differences relative to acellular controls. This presumed lack of contribution of the *in vitro*-formed tissue to the mechanical properties of the scaffold is consistent with the relatively low sGAG and collagen contents measured.

The BL-0.03 scaffold possessed a 2-3 fold higher stiffness compared to BL-1.0 in compression at all time points, with an initial stiffness of 44 ± 21 kPa versus 13.9 ± 4.3 kPa (Figure 2-11 A,B). Both compressive moduli were lower than the physiological value of 130 ± 40 kPa, obtained for full-thickness bovine articular cartilage explants with the identical testing regime, but they are well within the range of values published for articular cartilage scaffold stiffnesses and more than an order of magnitude stiffer than many hydrogel systems [33]. The stress relaxation, while statistically greater for the BL-1.0 in the acellular case, was roughly unchanged at all time points averaging $14.1 \pm 1.8\%$ over 5 minutes (Figure 2-11 D).

Similarly, there were no statistical differences in tension between scaffolds or culture time, with the exception of the superior ultimate tensile strength of BL-0.03 at week 1 (Figure 2-11 E,F). The similar tensile moduli and ultimate tensile strengths between samples were expected, as the tensile properties were attributed to the presence of the electrospun membrane, which was identical between scaffold varieties and was not expected to degrade over 4-weeks.

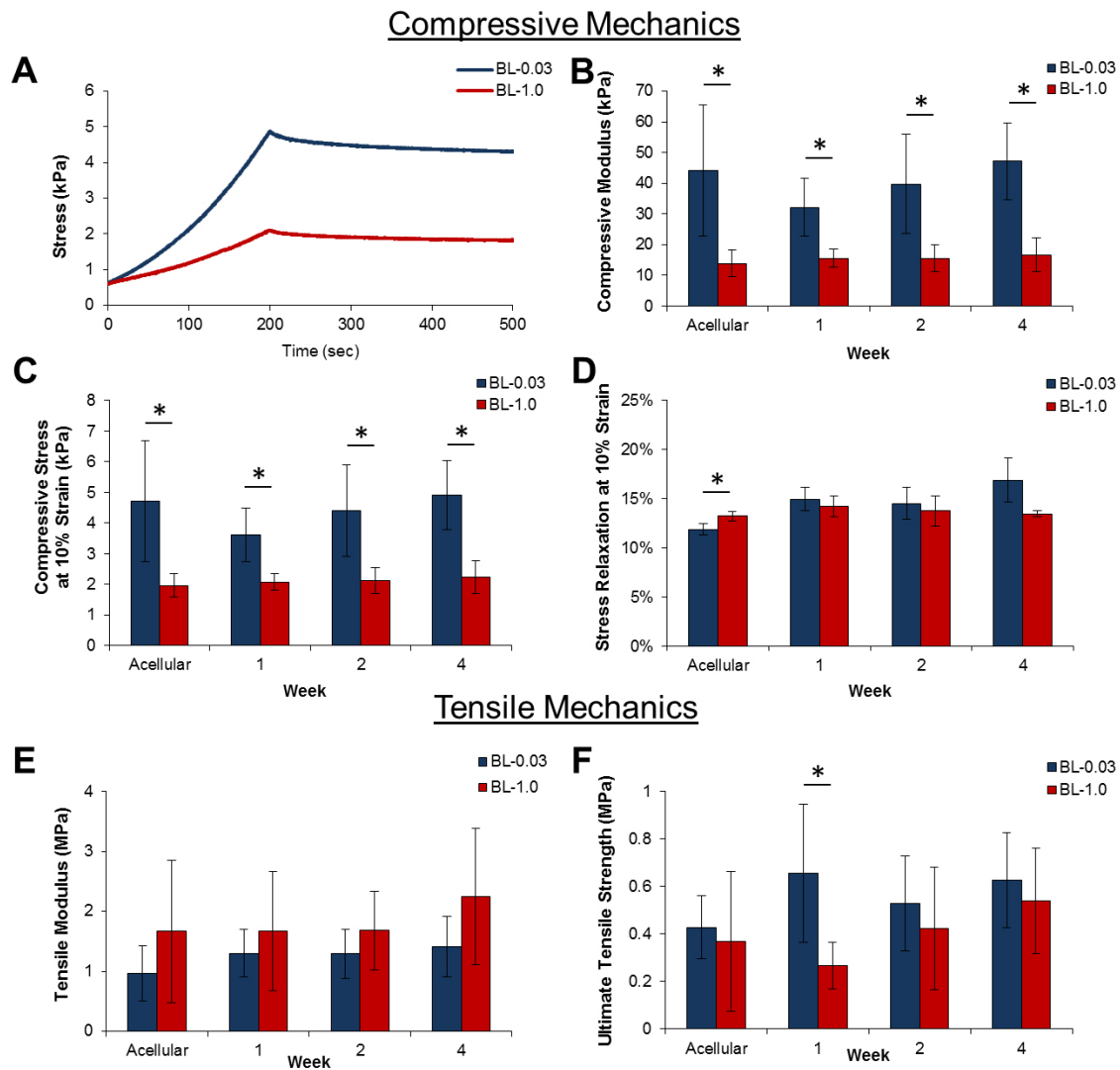


Figure 2-11 Mechanical properties of bilayered scaffolds, seeded with 1M chondrocytes, over 4 weeks culture *in vitro*. A) Representative stress-strain plots for acellular scaffolds under a ramp-and-hold test to 10% strain. B) Unconfined compressive modulus, C) Stress at 10% strain, D) Stress relaxation over 5 minutes, E) Tensile modulus, and F) Ultimate tensile strength. *Significant difference ($p < 0.05$). Modified from Steele *et al.* [1].

2.5 Second-Generation Scaffold Design Modifications

2.5.1 Addition of a Second Electrospun Zone and Increased Cellular Density

The next phase in the evolution of the second-generation scaffold design incorporated an electrospun layer with random-orientation on the underside of the scaffold, for the creation of a calcified-cartilage interface, as shown in Figure 2-12C and as specified in the original patent application [185]. For this scaffold modification, only the 0.03 mm³ fine pore size was selected, based on the chondrogenic findings in the bilayer system [1]. The PCL-HFIP solution used to electrospin fibres for the lower fibre layer was supplemented with 10% (w/w_{PCL}) hydroxyapatite (HAp) powder, hypothesized to nucleate calcification. HAp-free controls were also produced. The lower fibre layer was added to initiate hypertrophy and calcification of the chondrocytes on the underside of the scaffold that would lead to the production of a zone of calcified cartilage and to act as a barrier to cell-loss from the underside of the porous foam.

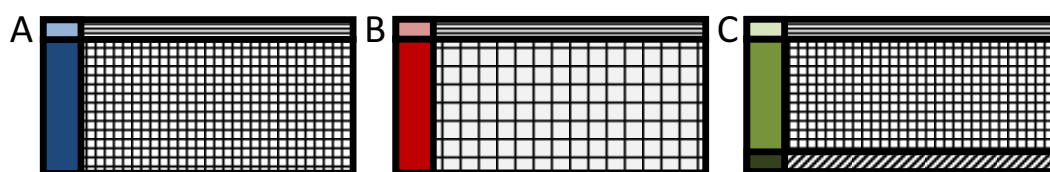


Figure 2-12. Trilayer scaffold schematics and zone colour-coding scheme for the bilayer (A,B) and trilayer (C) scaffold systems. The zonal colour coding is consistent throughout the results, with the PLZ colour representing the whole construct in complete scaffold data.

The new design necessitated one alteration to previous studies; the chondrocytes were injected into the center of the scaffold in a 100 µL bolus, as both surfaces were covered in electrospun-fibres. The number of chondrocytes seeded into the trilayer scaffolds was also doubled to 2 million, as the cell and tissue density of the previous study was deemed insufficient.

Histological analysis, performed using the cryosectioning approach, before the implementation of polyester wax protocols, Figure 2-13, showed improved ECM production in the TL scaffolds with 2M cells, relative to the bilayer system with 1M. An analysis of calcium distribution was performed with alizarin red staining (Figure 2-13B), highlighting the HAp particles in the lower fibre layer, visible as opaque black spots with red coroneae, and diffuse orange background staining in the remainder of the tissue. No additional calcification was observed in the tissue. H&E, alcian blue, and picrosirius red staining were performed to ascertain the distribution of ECM, sGAG, and collagen, respectively (Figure 2-13C, D, E).

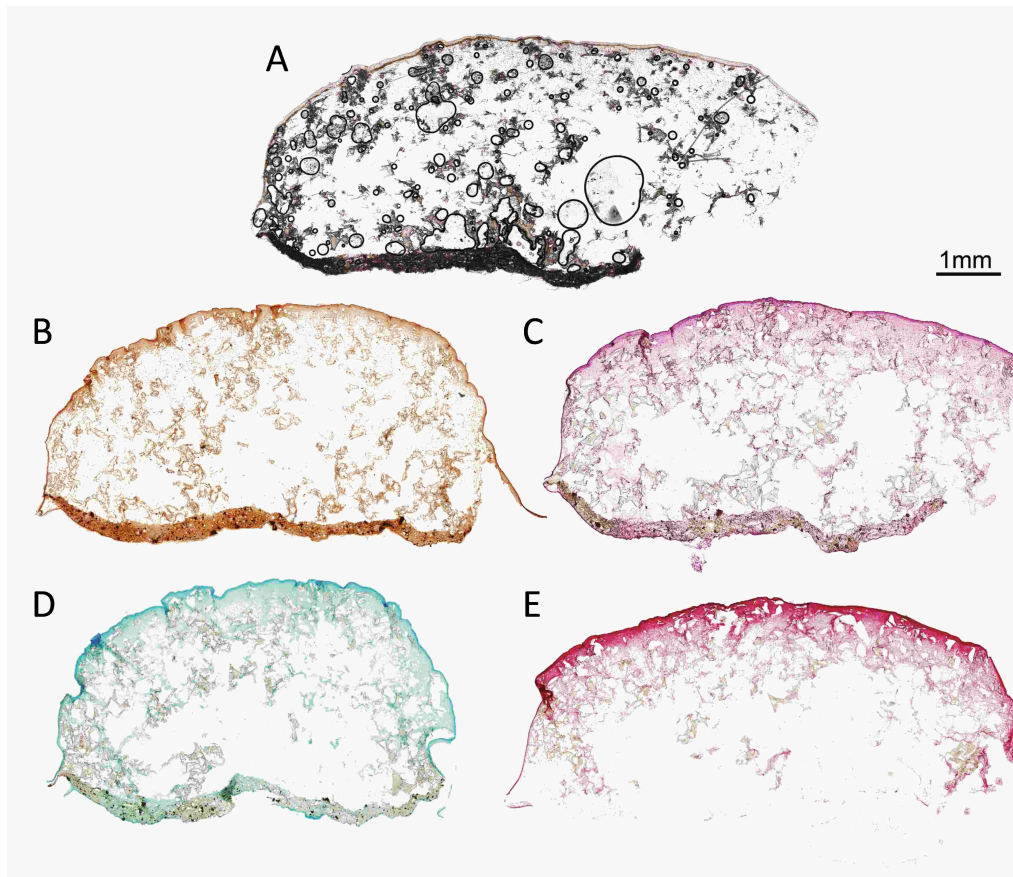


Figure 2-13. Histology of 2M chondrocyte-seeded trilaminar scaffolds. A – No washes or stain (air bubble artefacts are visible as dark-rimmed circles), B – Alizarin Red for calcium, C – H&E, D – Alcian Blue for glycosaminoglycans, E – Sirius Red for collagen (lower HA-fibre layer not present). Exaggerated Sample thickness and subsequent ECM voids are due to aforementioned issues with cryosectioning porous PCL.

The addition of HAP to the lower fibre layer failed to produce calcification within the scaffolds, however, the addition of 2M chondrocytes to the scaffold showed a visible increase in ECM production and density, which warranted further investigation.

2.5.2 Higher-Density Chondrocyte Culture

As the cellular and tissue density of the 1M-chondrocyte bilaminar and 2M-chondrocyte trilaminar scaffolds was deemed insufficient to form dense tissue *in vitro*, a 4-week study of BL-1.0, BL-0.03 and TL-0.03 scaffolds seeded with 10M chondrocytes was performed. The chondrocyte concentrations of 1, 2 and 10M bound the adult human chondrocyte concentration of 2.3 ± 0.2 million chondrocytes per 10 mm diameter plug, with higher-density culture more representative of foetal cartilage which has superior regenerative capacity [2,7,194].

The scaffolds were assessed for altered mechanical stiffness and relaxation at 4-weeks, and depicted relative to BL scaffolds with 1M cells for context in Figure 2-14. As with the full 1M-chondrocyte study, the BL-0.03 scaffold is significantly stiffer than BL-1.0 at all time points. There were no significant effect of chondrocyte seeding density on stiffness at 4 weeks. The stress relaxation (Figure 2-14B) in bilayer samples seeded with 10M cells showed significant increases over scaffolds with 1M cells at 4 weeks. This increase in stress relaxation was attributed to greater tissue density and ECM content. The 1M samples showed no increase over day zero, and therefore had insufficient ECM content to alter their mechanical properties. The addition of the second layer of electrospun fibres in the TL system, with or without HAp, had no significant effect relative to BL-0.03.

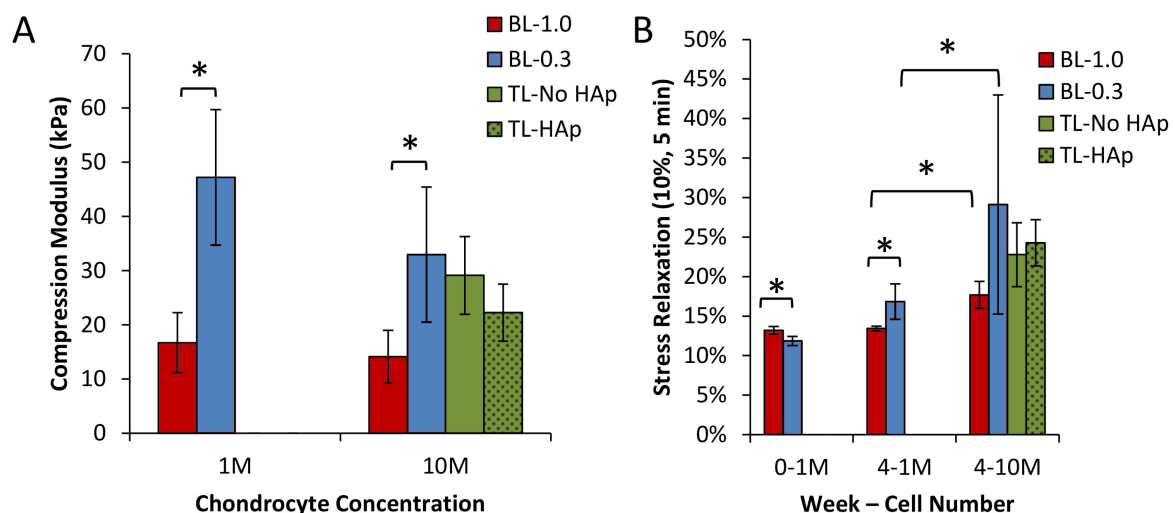


Figure 2-14. A) Compressive moduli at 10% strain for 10M-chondrocyte seeded scaffolds at 4-weeks, relative to 1M chondrocyte samples. B) Stress relaxation at 10% strain for 10M chondrocyte-seeded scaffolds at 4-weeks relative to 1M chondrocytes and week zero. BL = bilayer, TL = trilayer. *Significant difference ($p < 0.05$).

The observed increase in stress relaxation in BL scaffolds with 10M chondrocytes was attributed to the significantly greater biochemical content of the scaffolds at 4 weeks, as quantified in Figure 2-15.

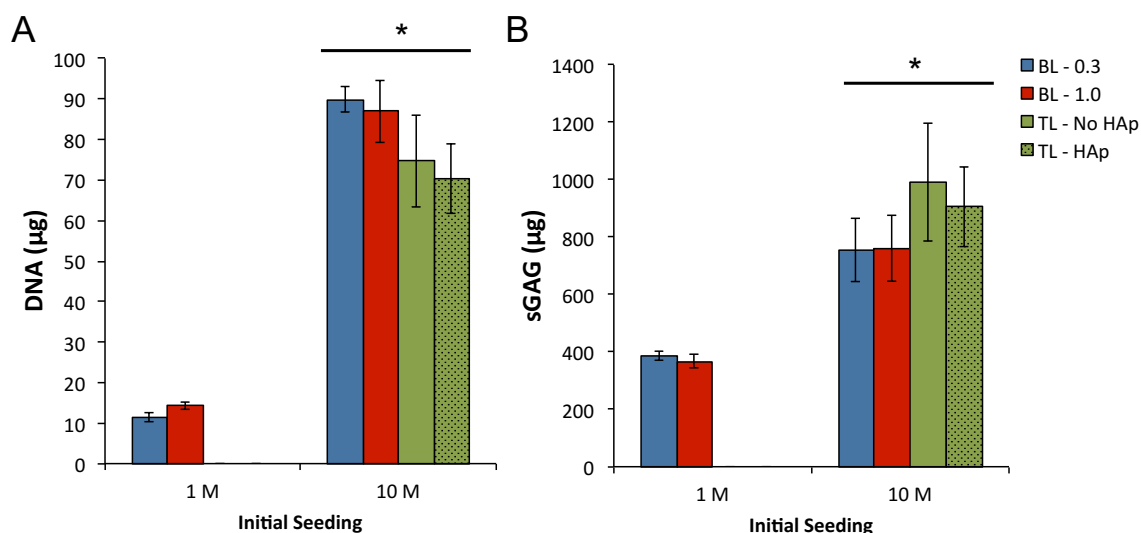


Figure 2-15. Biochemical analysis of A) DNA and B) sGAG in 10M chondrocyte-seeded scaffolds relative to 1M BL scaffolds after 4 weeks *in vitro* chondrogenic culture. *Significant difference ($p < 0.05$) vs 1M.

The cellular concentration within the scaffolds at 4 weeks, assuming 7.7 pg/cell [6], average 1.7M, 11.5M, and 9.4M for the BL-1M, BL-10M, and TL-10M systems, respectively. Increased chondrocyte seeding densities were associated with increased sGAG production and pore filling, but only by roughly 2-fold, resulting in a lower sGAG/DNA ratio at higher densities. Reduced or plateaued DNA-normalised sGAG production in tissue engineering scaffolds at higher seeding densities have been noted in the literature [208]. Higher density culture-induced chondrogenesis is supported by the use of high-density pellet culture with chondrocytes to preserve chondrogenic potential and pluripotent cells to differentiate towards chondrogenic phenotypes [194,209]. The use of high-density culture generated increased sGAG production and had no observed effect on chondrocyte viability. Therefore, this modification to the standard protocol was deemed worthy of further investigation in future scaffold investigations.

2.6 Conclusion

The design of the scaffolds, both BL and TL, were inspired by the success of the electrospun superficial zone in the first-generation scaffolds as well as the desire to increase cellular infiltration in a structure more representative of the intermediate/deep zone of cartilage.

The most significant outcome of this study was the conclusion that the fine 300 μm diameter pores in the BL-0.03 scaffold were superior to the large 1000 μm diameter pores in BL-1.0 both because of improved mechanical properties of the acellular scaffold and the increased cartilage-like matrix accumulation. The large pores of the PLZ-1.0 were treated as a flat surface by the chondrocytes, initiating increased dedifferentiation and proliferation. This was observed in the greater rate of DNA synthesis, reduced normalised sGAG accumulation, increased normalised collagen synthesis, and the reduced chondrogenic gene expression profile when compared to PLZ-0.03. The histological observations confirmed the dedifferentiated two-dimensional surface-coating morphology in the PLZ-1.0 scaffolds. The mechanical properties in compression were also superior in BL-0.03 relative to BL-1.0.

Modifications to the core design were of variable success. The introduction of an additional fibre zone to the underside of the scaffold, supplemented with hydroxyapatite to generate calcified cartilage failed to initiate calcification. The investigation of 2×10^6 and 10^7 chondrocytes per scaffold resulted in increased ECM production and increased stress relaxation attributed to the ECM synthesis.

2.7 Next Steps

One aspect of the second-generation scaffold investigation that left opportunities for further development was the use of a single zone of porogen-leached foam to act as both intermediate and deep zones. While the PLZ foam achieved the initial aim of improved seeding efficiency, the use of salt crystals produced an isotropic foam with irregular, angular pores and sub-physiological mechanical stiffness. The porogen-leached foam capped with electrospun fibres, while the first published instance of a fibre-foam system for cartilage engineering, left many opportunities for future design alterations to better mimic natural cartilage.

3 The development and *in vitro* characterisation of an anisotropic scaffold to mimic the structural organisation and biomechanics of articular cartilage

3.1 Introduction

Chapter 2 investigated the *in vitro* validation of bilayer and trilayer variants of the second-generation cartilage-engineering scaffold developed in the Stevens Group. The key findings from this study were instrumental in the further refinement of the design and culture conditions of an optimised scaffold. These include the use of smaller diameter (300 µm) pores, the ability to uniformly seed cells within a porogen-leached foam, as well as the tensile strength and surface smoothness provided by the electrospun superficial zone. Similarly, higher seeding densities (>1M per 10 mm Ø scaffold) were favoured to promote a chondrogenic phenotype.

The primary faults of the second-generation scaffolds were low compressive stiffness, low ECM production within the scaffold, and the absence of a deep-zone analogue within the design to direct vertical ECM and cellular alignment. As stated in the thesis introduction, directional freezing, a variant of thermally-induced phase separation (TIPS) or freeze-casting, can produce a porous scaffold with vertically aligned channels of relevant size and significant compressive strength, relative to scaffolds produced by typical fabrication methods with similar porosities and polymer compositions [92,155,210,211]. This vertical pore alignment and increased compressive strength are well-suited for the culture of chondrocytes as has been observed by others [212]. Jia *et al.* demonstrated that aligned pores in a directionally frozen scaffold composed of decellularised and solubilised cartilage ECM increased the stiffness of the resulting construct relative to non-aligned ECM foams of the same composition, while directing cells to maintain a chondrogenic phenotype [212]. However, the cartilage produced by Jia *et al.* was aligned throughout the entire thickness of the scaffold, thereby not incorporating the zonal complexity of articular cartilage in its design. The optimized scaffold design discussed in this chapter integrates this technique with porogen-leaching and electrospinning to reproduce the anisotropy of morphological and biomechanical features found in native cartilage, forming the third-generation scaffold, as outlined in Figure 3-1.

Oriented PCL Fibres
Smooth articular surface morphology
High tensile strength, low stiffness

**Gelatine-Leached
PCL Foam**
Permits injection seeding
Intermediate stiffness
Random ECM deposition

**Directionally
Frozen PCL Foam**
Chondron-like channels
Higher stiffness
Conserved geometry
Aligned ECM deposition

Random PCL Fibres
Cell-migration membrane

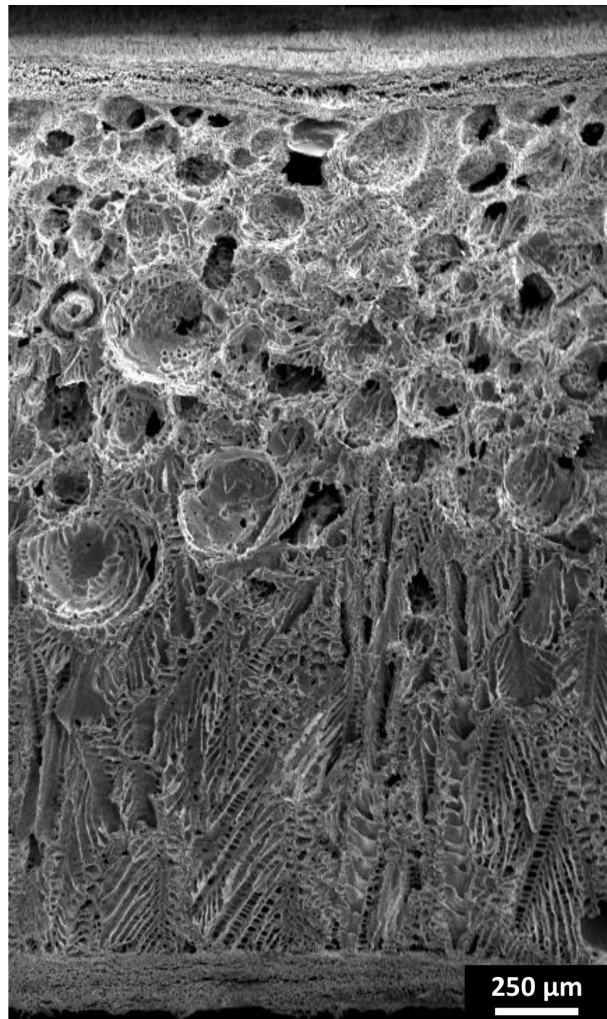


Figure 3-1. Third-generation scaffold describing scaffold zone fabrication methods and the functional attributes of each zone.

3.2 Materials and Methods

3.2.1 Materials

All chemicals were acquired from Sigma Aldrich, UK and all cell-culture materials were acquired from Invitrogen, UK and used as received, unless otherwise specified.

3.2.2 Scaffold Production

The third-generation composite scaffolds were produced in a step-wise manner, first generating a porogen-leached/directionally-frozen integrated foam then spinning the electrospun layers onto the top and bottom of the scaffold. Prior to scaffold fabrication, the processes of thermoset gelatine microsphere production and directional freezing had to first be optimised.

Gelatine Microsphere Production

The production of porogen-leached foams used uncrosslinked thermoset gelatine microspheres, as they can be prepared as populations of homogenous sizes and produce spherical pores, as opposed to crystalline rectilinear salt crystals. Gelatine microspheres were produced by an oil-water emulsion protocol developed and optimised for this scaffold and outlined in detail in the standard operating protocol (SOP) in Appendix D.

Briefly, a 20% (w/v) solution of porcine type A gelatine in distilled water at 60°C was added in a drop-wise manner to a bottle containing sunflower oil (Waitrose, UK) and 0.5% (v/v) Tween 20. Throughout, the system was mixed at 500 rpm with an upright laboratory mixer (Eurostar digital, IKA, UK). The oil/water emulsion was allowed to stabilise for 10 minutes, after which the mixture was cooled by jacketing with ice-cold water to thermoset the gelatine. Once the gelatine was set, an volume of cold acetone equal to the volume of oil was added and mixed overnight at 4°C to dehydrate the particles and cut the viscosity of the oil. The acetone/oil/water mixture was decanted and the particles washed with acetone at 4°C for 48 hours with 2 changes of acetone. The particles were sorted into 100-300 µm, 300-500 µm, and > 500µm fractions, dried under vacuum, and stored in sealed containers until required.

Directional Freezing

Directional freezing was performed on a specially customised PolarBear Plus (Cambridge Reactor Design Ltd., UK) automated cold-plate within polydimethylsiloxane (PDMS, SYLGARD® 184, VWR International, UK) barriers on a 450 µm-thick sheet of copper. The cold-plate mimicked the control and rates obtained by the traditional cold-finger approach to directional freezing [155], but

sacrificed some thermal homogeneity in exchange for the size of scaffolds produced (80x80 mm²). For directional freezing, 80 kDa PCL was dissolved in 1,4-dioxane at varying concentrations, with optimal performance at 8% (w/v). Samples were cooled from room temperature to -20°C on the Polar Bear, transferred to a -80°C freezer for > 20 minutes, and lyophilised within their PDMS/copper moulds overnight to remove the dioxane.

Scaffold Production Protocol

The detailed scaffold production SOP developed for this study is included in Appendix E and the scaffold sterilisation SOP for *in vitro* and *in vivo* experiments is included in Appendix F. The process is summarised in Figure 3-2.

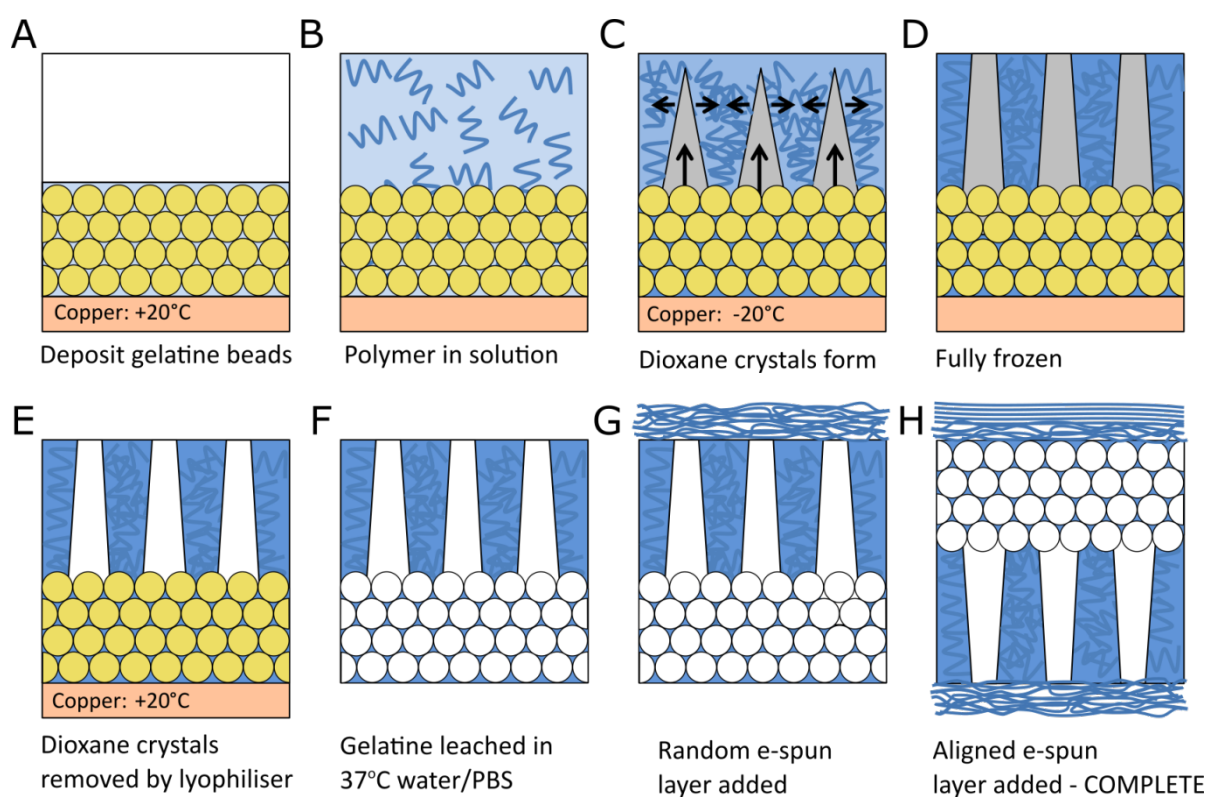


Figure 3-2. Third-generation scaffold production process. A) Gelatine microspheres in 8% (w/v) PCL-dioxane deposited on copper sheet, B) PCL-dioxane added above particles and frozen (C-D). Dioxane removed by lyophilisation (E) and gelatine removed by warm water (F). Surface electrospun with random (G) and random-to-aligned PCL fibres (H). Detailed in Appendix E.

Briefly, a slurry of 5 g of gelatine microspheres suspended in 7 mL of 8% (w/v) PCL-dioxane was deposited onto a clean copper plate to a thickness of 1 mm within an 80 x 80 x 2 mm³ PDMS edge mould and partially covered with glass to slow the evaporation of dioxane – reducing film formation – and allowed to dry overnight (Figure 3-2A). Additional 8% (w/v) PCL in dioxane was deposited onto the dried gelatine microspheres to a final depth of 2 mm total (Figure 3-2B). The copper was then

cooled by placing it directly onto a level -20°C surface, freezing the PCL/dioxane around the particles and nucleating dioxane crystals from the surfaces of the gelatine particles, concentrating the PCL at the crystal boundaries (Figure 3-2C). When fully frozen, the columnar dioxane crystals were removed by sublimation in a lyophiliser, leaving open columnar channels formed by the partitioned PCL (Figure 3-2D,E). The gelatine microspheres were dissolved in warm water followed by PBS and then dried (Figure 3-2F). A layer of randomly-oriented fibres was electrospun onto the directionally-frozen surface from a solution of 12% (w/v) PCL in HFIP, fusing to the underlying foam surface (Figure 3-2G). Finally, a second layer of fibres was electrospun onto the opposite particle-leached surface, transitioning from random to aligned fibre deposition by changing the rotation speed of the collector and fusing to the surface by starting the deposition at a shorter needle-to-collector distance (Figure 3-2H).

3.2.3 Chondrocyte Isolation

Primary bovine chondrocytes were isolated from 24-month old animals as previously outlined in section 2.2.4. Briefly, legs were obtained from an abattoir on the day of sacrifice and processed within 24 hours. The metacarpal-phalangeal joints were opened in a sterile manner and the cartilage was excised from the joint surfaces and minced. The cartilage was then digested at 37°C in 0.2% (w/v) pronase for 1h followed by 0.04% (w/v) collagenase overnight. The digest was filtered to remove ECM debris and the cells plated in expansion medium containing Dulbecco's Modified Eagles Medium (DMEM) with 4.5 g/L glucose and Glutamax, supplemented with 1% Anti-Anti and 10% (v/v) foetal bovine serum (FBS). Chondrocytes were expanded to 90% confluency and cryopreserved in liquid nitrogen at 10M/mL in DMEM with 20% (v/v) FBS and 10% (v/v) dimethyl sulphoxide (DMSO). Chondrocytes expanded from passage 1 to passage 2 (P2) were used directly without freezing for all experiments.

3.2.4 Scaffold Seeding and Culture

Initial seeding strategy tests were performed by injecting chondrocytes into scaffold variants at 0.5M, 1M, and 10M cells per 50 µL bolus of expansion media from a 21G needle on a 1 mL syringe. Chondrocytes were allowed to adhere for 2h at 37°C, 5% CO₂, 100% RH. All subsequent culture used chondrogenic differentiation medium composed of DMEM + Glutamax (4.5 g/L glucose), 50 µg/mL L-proline, 50 µg/mL ascorbic acid, 0.1 mM sodium pyruvate, 1% (v/v) anti-anti, 10 ng/mL TGF-β₃, and 1% (v/v) ITS Premix (BD Biosciences, Oxford, UK).

Scaffolds for histological and biochemical analyses were seeded at 10⁶ P2 chondrocytes/scaffold with 9x10⁵ injected into the intermediate zone and 1x10⁵ seeded on the superficial fibres. This

represented a significantly higher cell concentration than for the second-generation studies, as the scaffold size was reduced from 10 mm to 6 mm in diameter. Therefore, 10^6 chondrocytes in a third-generation scaffold was equivalent to 2.8×10^6 in a second-generation scaffold. The higher cell concentration was selected based on observations in the previous studies (Chapter 2) that suggested a higher cell density decreased chondrocyte dedifferentiation and therefore increased chondrogenic gene expression profiles and the quality of matrix produced. For gene expression analysis, samples were prepared at 1M and 10M chondrocytes per scaffold to examine the effect of increased seeding density.

To visualise the penetration and distribution of seeded cells, the scaffolds were fixed in 10% (v/v) paraformaldehyde (PFA), stained with 4',6-diamidino-2-phenylindole (DAPI) and the cross-section was imaged on an IX51 epifluorescence microscope equipped with a DP70 digital camera (Olympus, UK).

Zonal Seeding

To determine if the scaffolds could be seeded with three zonal sub-populations of cells, a zonal seeding protocol was developed. The validation of this protocol required three groups of P2 chondrocytes from a mixed zonal population, to be dyed red, green, and blue for discrimination. Live cell dyes were used (Table 3-1), to allow seeded chondrocytes to adhere to the scaffolds, rather than treating them as non-viable particulates.

Table 3-1. Live cell dyes used to stain passaged bovine chondrocytes for three-zone seeding

Dye	Dilution	Ex./Em.	Supplier
Green – Cell Tracker Green (CMFDA)	1/1000	492 nm 517 nm	C2925, Invitrogen, UK
Red – Wheat germ agglutinin Alexa Fluor 594	1/200	591 nm 618 nm	Image-iT LIVE Plasma Membrane and Nuclear Labelling Kit – I34406, Molecular Probes, UK
Blue – Hoechst 33342	1/1000	350 nm 461 nm	Image-iT LIVE Plasma Membrane and Nuclear Labelling Kit – I34406, Molecular Probes, UK

Briefly, T-175 flasks of near-confluent P2 primary bovine chondrocytes were washed with PBS and incubated with the dyes, diluted in DMEM cell culture medium without serum for 15 minutes at 37°C and 5% CO₂. The cells were then washed in PBS and cultured overnight to ensure no dye-associated cytotoxicity. The dyed cells were suspended by trypsinisation and concentrated to 1M/100 µL. The sequential zonal seeding protocol is summarised in Figure 3-3. The blue “deep zone” cells were injected first into the intermediate zone at 0.5M/50 µL with a 21G needle on a 1mL syringe, with the bevel facing downwards. Scaffolds were placed in a 24 well plate and transferred to a centrifuge with swinging baskets for cell-culture plates in which they were spun at 1300 x g for 5 minutes. The

scaffolds were then injected with 0.5M/50 μ L green “intermediate zone” cells in the same manner, without the centrifugation step. Approximately 0.2M red “superficial zone” cells were deposited on top of the scaffold as a single droplet of cells. The scaffolds were incubated for 2h at 37°C and 5% CO₂ to allow the cells to adhere before adding expansion media. The 6 mm $\varnothing$ scaffolds were fixed in PFA at 24 and 72h and the cross-section was imaged on an epifluorescence microscope.

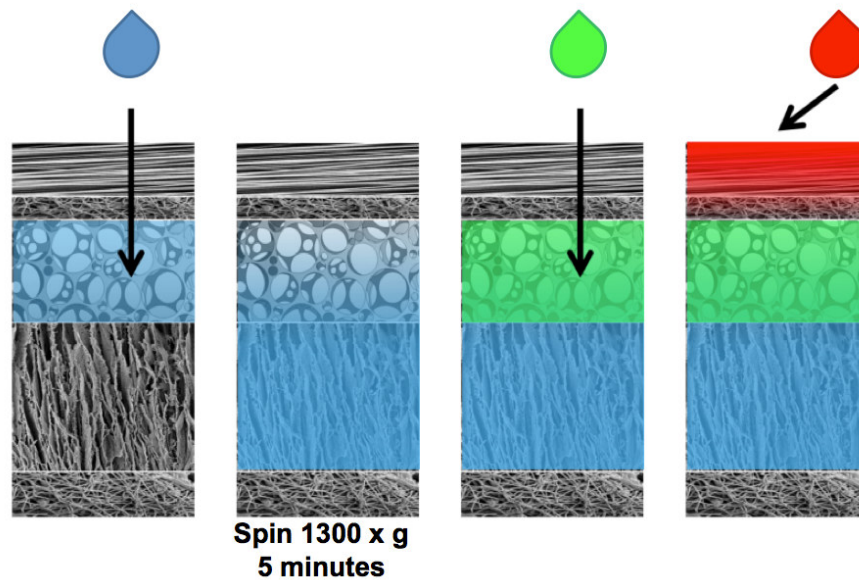


Figure 3-3. Zonal cell seeding protocol. Arrows signify injection into the intermediate zone with a 21G needle in a 50 μ L bolus of cell culture media. At the second step, scaffolds were spun in sterile 24 well plates at 1300 x g for 5 minutes.

3.2.5 Biochemical Assessment

Biochemical content evaluation of the tissues formed in the scaffolds proceeded in the same manner as in Chapter 2, with slight changes in the protocols. Only these alterations to previously described protocols will be discussed in detail, as full descriptions can be found in section 2.2.

The papain digestion was performed in the same manner as the previous study at week 0, 1, 2, and 4. As the scaffolds were more mechanically robust than the previous generation, the majority of the biochemical analysis was not performed zonally. In the instances where the scaffolds were separated, the electrospun layers were peeled from either side of the foam with forceps. To increase the accuracy of the PLZ-DF cut, the foams were cut in half radially with a sterile blade, then tipped onto the cut faces and bisected. These samples were then lyophilised, digested in papain and used for DNA and sGAG quantification, as before.

The assay used for sGAG quantification was changed from the Blyscan assay by Biocolor to the more traditional dimethylmethylene blue (DMMB) assay [213]. Recent studies into the interference of

DNA and other ECM molecules in the assay when the DMMB dye solution is used at pH 3.0 by Zheng and Levenston [214] prompted the switch to pH 1.5.

Briefly, a DMMB solution was prepared by dissolving 16 mg DMMB (341088) in 5mL ethanol and diluting to 1 L in distilled water with 40 mM NaCl and glycine. The solution was adjusted to pH 1.5 with 3M HCl and filtered to remove precipitate. Samples from papain digest were diluted 1/10 with papain digest buffer and combined with DMMB solution at 20:200 μ L respectively, in a 96-well plate. Plates were read immediately at 525 nm. sGAG was quantified against a 2-250 μ g/mL ladder of chondroitin-6-sulphate in papain digest buffer, run in duplicate in every plate. Samples were run in technical duplicate, with four replicates for each condition and time point.

3.2.6 Gene Expression

As before, RNA was extracted from samples using the RNeasy kit (QIAGEN, UK) and 200 ng RNA was transcribed to cDNA via the QuantiTect Reverse Transcription kit (QIAGEN, UK). Gene expression was analysed relative to the housekeeping gene 18S using the QuantiTect SYBR Green real time quantitative PCR kit on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, UK). The primers used and their efficiencies are listed in Table 3-2.

Table 3-2. qRT-PCR primers for the assessment of cartilage-specific gene expression in bovine chondrocytes

Gene	Forward	Reverse	Efficiency
COL2A1	CCACTGCAAGAACAGCATTG	CCAGTTCAGGTCTCTTAGAG	60°C – 98%
COL1A1	CATTAGGGGTCACAATGGTC	TGGAGTTCCATTTTCACCAG	60°C – 92%
AGC	CACTGTTACCGCCACTTCCC	GACATCGTTCCACTCGCCCT	60°C – 93%
COL10A1	ACAAGGACCTACAGGAGAAC	GCGGCAAGGAGTACAATG	60°C – 103%
SOX9	AACGCCGAGCTCAGCAAG	ACGAACGGCCGCTTCTC	62°C – 96%
PRG4	ATGCCTGAACCGACTCCTAC	TGCCGAAGCCTTGACTGG	61°C – 107%
18S	GTAACCCGTTGAACC	CCATCCAATCGGTAGTAGTAGCG	60°C – 104%

As before, genes are expressed as a fold increase over week 0, calculated using the $\Delta\Delta C_t$ method, and the COL2A1/COL1A1 ratio is calculated from the $2^{-\Delta C_t}$ of each gene.

In this study, the housekeeping gene 18S was used as a reference, in contrast to B2M in Chapter 2. The 18S gene codes for 18S ribosomal RNA, a basic component of all eukaryotic cells. The B2M gene codes for $\beta 2$ microglobulin protein, a basic component of the major histocompatibility complex. Both genes are commonly used as housekeeping genes for chondrocytes and their relative stability is open to interpretation [215–217].

With every *in vitro* assessment, four samples were prepared at every condition and time point for gene expression analysis by qRT-PCR. Issues with insufficient RNA quantity, quality, or reproducibility

resulted in incomplete or inconclusive data sets for all but one 7-day investigation. Multiple approaches were investigated to disrupt, dissolve, and macerate the scaffold samples in lysis buffer (RLT, QIAGEN) or phenol-chloroform (TRIzol®, Life Technologies) and extract the RNA with QIAGEN Cell Shredder and RNeasy Purification kits. Ultimately, in collaboration with Dr. Jean-Philippe St-Pierre it was determined that mincing the scaffolds with a scalpel and vortexing in RLT supplemented with β -mercaptoethanol (β ME) with RNeasy purification, while omitting the cell-shredder preparatory columns was the most reliable method for extraction of representative RNA samples.

3.2.7 Mechanical Characterisation

Mechanical testing of the scaffolds and individual scaffold zones was performed in an identical manner to that of Chapter 2, with compression tests carried out at a 0.05% strain per second ramp to 10% strain followed by a 600 s relaxation at constant strain. This testing was performed unconfined on an Instron 5540 testing machine (Norwood, USA) equipped with a 50 N load cell or a Bose ElectroForce 3200 Series III (TA Instruments, UK) equipped with a 5 lbf (22.2 N) load cell.

Confined compression was investigated early in the study, as it would provide a more accurate model for uniaxial compression *in vivo*. A 6 mm hollow cylinder and probe were produced to test scaffolds under confined conditions (Figure 3-4). Ultimately, there were no statistical differences in the moduli obtained from the two methods, but confined compression exhibited a 2.2 ± 0.2 fold greater standard deviation. Therefore, future mechanical testing was performed unconfined.

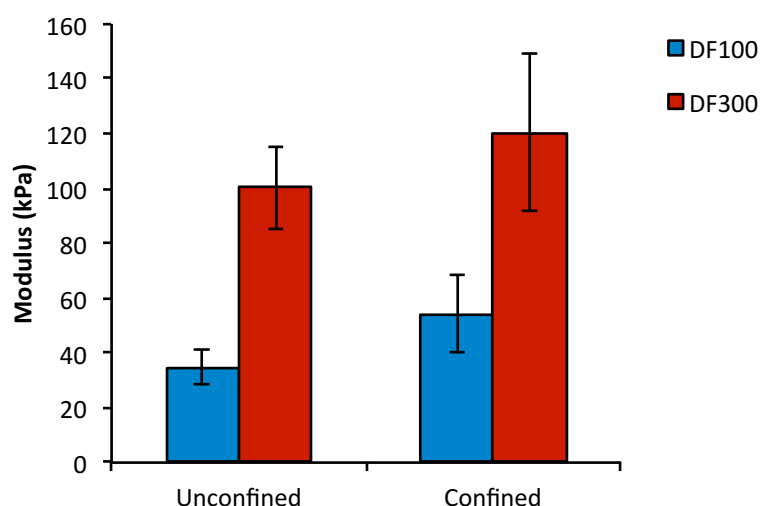


Figure 3-4. Comparison of confined and unconfined uniaxial compression of DF100 and DF300 scaffolds seeded with 10^6 chondrocytes after 4 weeks culture *in vitro*.

Dynamic mechanical analysis (DMA) was performed on a Bose ElectroForce 3200 Series III equipped with the WinTest DMA software package. Samples were tested dry and wet in unconfined uniaxial compression. Analysis was performed with a dynamic amplitude of 5% strain at 0.1, 1, and 10 Hz about a 5, 10, 15, 20% mean strain, under displacement control. The storage modulus (E') was calculated from the phase angle (Equations 1-1,2, and 3) and is stated for each iteration of mean strain and frequency. All scaffold varieties and conditions were tested with $n \geq 3$.

$$E' = K^* \cos(\delta) / \text{shape factor} \quad [\text{Eq. 1-1}]$$

$$K^* = F^* / X^* \quad [\text{Eq. 1-2}]$$

$$\text{shape factor} = (\pi(D/2)^2/h) \quad [\text{Eq. 1-3}]$$

Where K^* is the dynamic stiffness calculated as the ratio of the dynamic force and displacement amplitudes, F^* and X^* , respectively, as shown in Figure 3-5. The phase angle (δ) accounts for the viscous behaviour of the system and is a measure of the phase offset between the measured and reference channels – force and displacement, respectively. The shape factor (Eq. 1-3) is for a cylindrical sample under compression with a height (h) and diameter (D) [218,219].

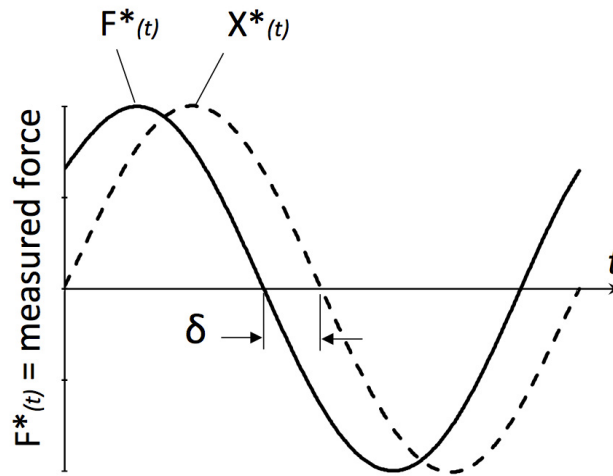


Figure 3-5. Elucidation of key terms for the calculation of storage modulus (E') by DMA analysis. F^* dynamic peak-to-peak force amplitude, X^* dynamic peak-to-peak displacement amplitude, δ phase angle. Modified from [218].

3.2.8 Electron Microscopy

Electron microscopy was performed in a similar manner to that outlined in section 2.2.10 with a JEOL 5610 (Herts, UK) environmental scanning electron microscope.

3.2.9 Histology

As the polyester wax protocol outlined in section 2.2.11 was developed alongside the initial aspects of this investigation, the protocol for histological preparation and sectioning from polyester wax was identical, defined within the Polyester Wax Histology SOP in Appendix B.

Staining

All H&E, alcian blue, and picrosirius red staining was performed on polyester wax sections as previously outlined in section 2.2.11.

Immunohistochemistry

All type I collagen and type II collagen immunohistochemistry using DAB was performed on polyester wax sections as previously outlined in section 2.2.11. The scaffolds and cartilage blocks used for multi-photon imaging by second harmonic generation were stained in a similar manner, but free-floating, with a 1/100 primary antibody dilution and 1/400 FITC-conjugated secondary. Samples were co-stained with phalloidin and Draq5 for actin cytoskeleton and nuclear localisation. Draq5 was used in place of DAPI as the multiphoton tuned to 860 nm excites at 430 nm, which is in the range of DAPI; Draq5 is a near-IR dye, which excites in the available spectral range above the FITC and phalloidin. Standard fluorophores were imaged on a Leica SP5 upright confocal microscope (FILM, Imperial College).

Multiphoton Microscopy

The collagen fibrils were imaged using label-free second harmonic generation. Fixed scaffold samples roughly 2 mm x 6 mm x 1 mm thick, stained in whole-mount for Col I or II by IHC and with Draq5 for nuclei, as described previously, were mounted in a petri dish filled with PBS and imaged on an upright multiphoton confocal microscope with a 25x dip objective (Leica, UK). The fluorescent dyes were imaged using standard confocal microscopy with the same objective for every frame to act as landmarks. The multiphoton apparatus used a tuneable two-photon laser (Spectra-Physics, USA) tuned to 860 nm excitation (2.5 W, 65% power) to penetrate the tissue and generate second harmonics at 430 nm, in the presence of organised collagen triple helices, detected on a 4-channel Non-Descanned Detector (NDD) [220,221]. Briefly, second harmonic generation (SHG) microscopy is a nonlinear coherent microscopy technique performed on a multiphoton microscope. SHG is able to detect a limited number of mesoscopic biomolecules, including collagen, myosin, and microtubules as they lack centrosymmetry and therefore have weak dipoles that are able to generate a SHG signal when excited by two coincident photons at a wavelength exactly twice the biomolecule excitation

wavelength [222,223]. As SHG is label-free technique and uses large wavelength infrared lasers, SHG is able to detect collagen with minimal processing artefacts, sample autofluorescence, and reference fluorophore bleaching, while effectively penetrating beyond the surface of tissues [222,224].

3.2.10 Statistical Analysis

All data was collected with $n=3$ independent experiments with 4 replicates for each condition in each experiments for biochemical analysis and $n \geq 4$ for mechanical testing. Data is presented as mean $\pm$ standard deviation and all significance (*) was determined by ANOVA with $p < 0.05$.

3.3 Results and Discussion

3.3.1 Scaffold Design

The primary aim of this investigation was the production of a third-generation porous PCL scaffold that mimicked the anisotropic structure and mechanical properties of articular cartilage in the knee. The proposed design consisted of an aligned fibre surface, intermediate isotropic zone and a distinct deep zone with higher stiffness and vertical pore morphology. The proposed design schematic includes spherical porogen-leached and directionally frozen foams with electrospun fibre layers on the upper and lower surfaces (Figure 3-6A). Through optimisations performed on the individual zones, a reproducible strategy for scaffold production was developed, as outlined in section 3.2.2 and in Appendix E, resulting in the complete integrated scaffold (Figure 3-6B).

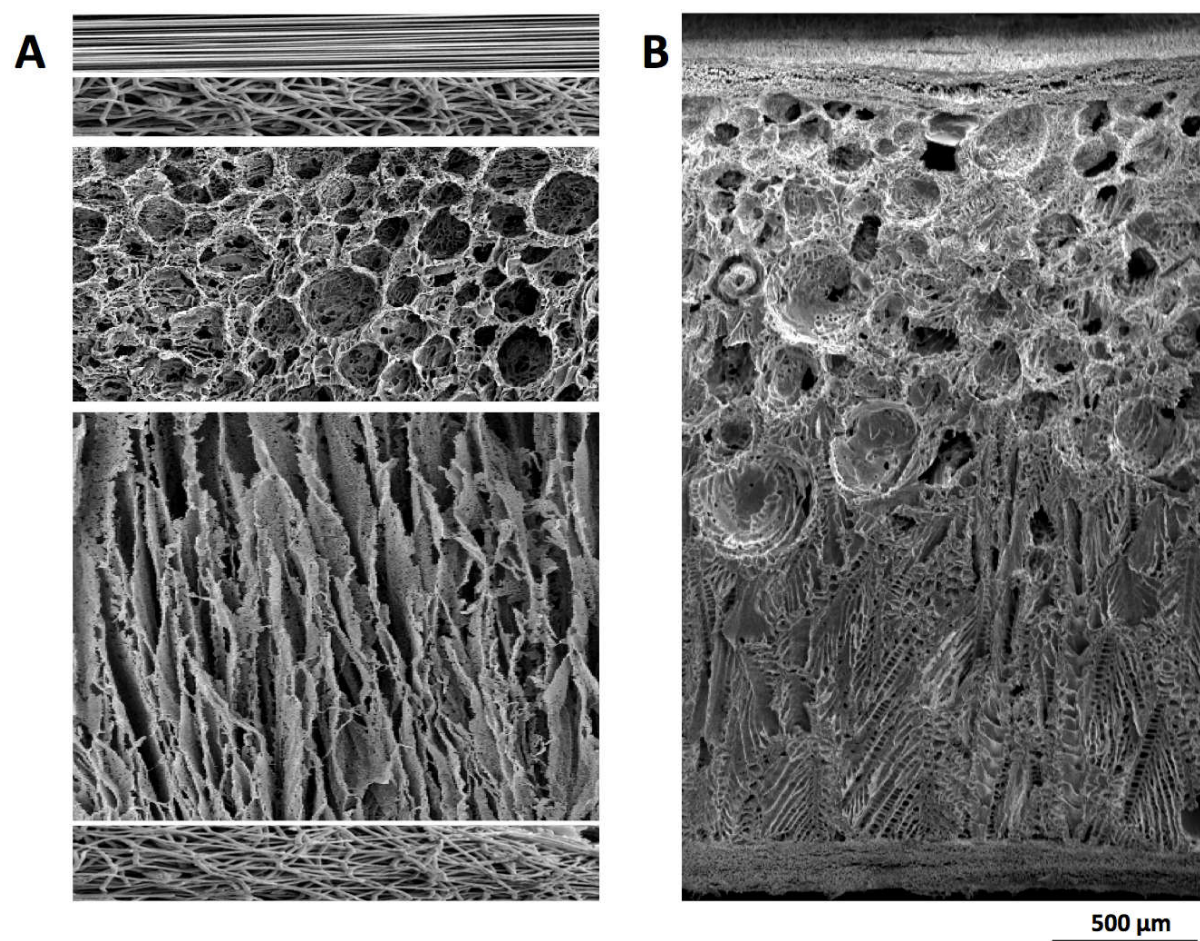


Figure 3-6. A) Proposed third-generation scaffold cross-section schematic and B) cross section of DF100 scaffold showing all layers successfully incorporated.

The study on second-generation scaffolds (Chapter 2) suggested that 300 μm diameter pores were superior to 1 mm pores for chondrogenic tissue formation. To further investigate this finding, two size ranges were selected for the pores in the intermediate zone of the third-generation scaffold: 100-300 μm and 300-500 μm . The complete scaffolds produced with these pore sizes within the PLZ are referred to throughout as DF100 (100-300 μm) and DF300 (300-500 μm).

During initial scaffold fabrication attempts, one consistent issue was apparent with the integrity of the design. While the PLZ and DF zone were incorporated into a single foam as per the design, the electrospun fibres were found to be weakly integrated and to delaminate easily from the highly porous foam portion (Figure 3-7A). The residual HFIP from the standard electrospinning process was insufficient to bind the two zones.

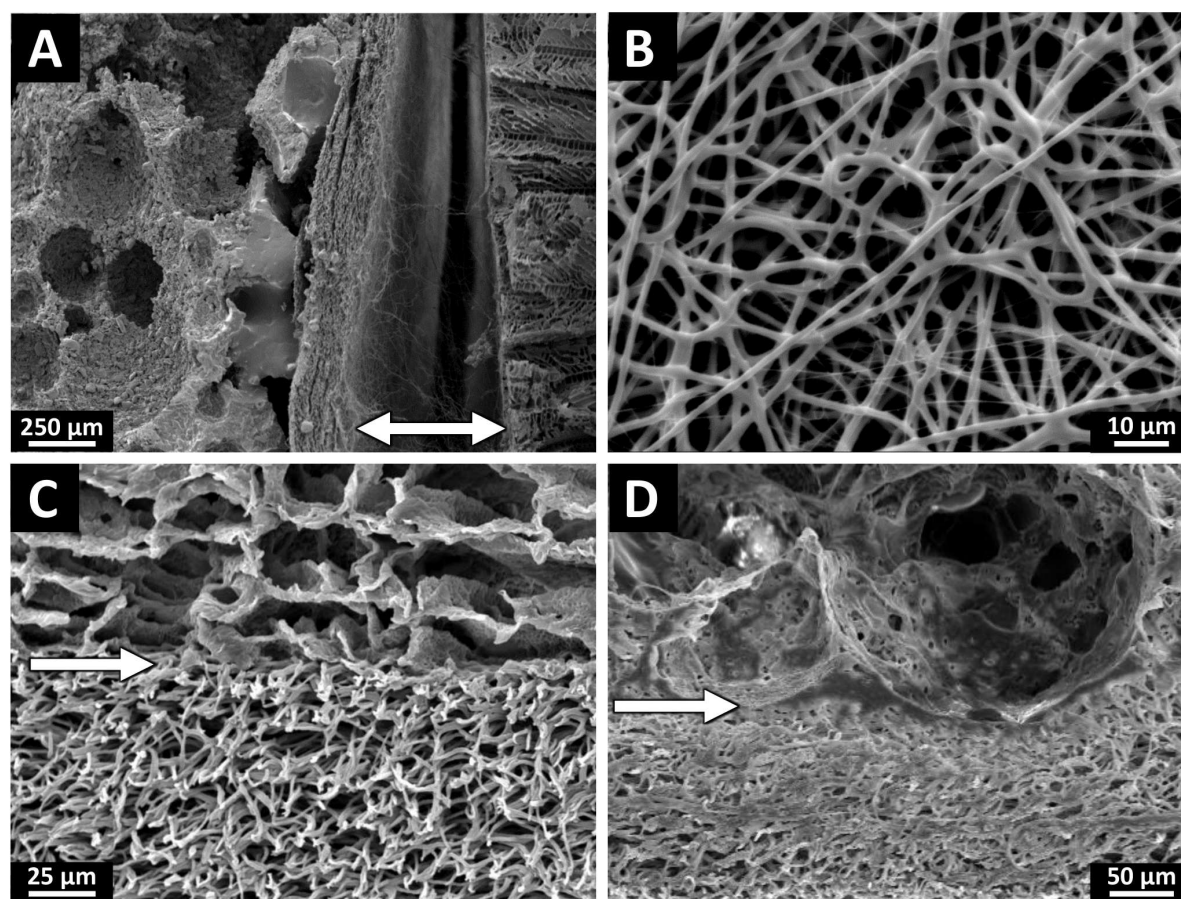


Figure 3-7. Fibre-foam adhesion issue and solution, illustrated by SEM. A) Issue: The separation of the DF foam and random fibre zone ($\leftrightarrow$) (fibre-zone melt-fused to porous StronBoneP). B) Solution: ‘Sticky’ PCL fibres used to adhere electrospun mat to underlying foam using residual solvent. C,D) Result: Fused DF-foam/fibre (C) and porogen-leached foam/fibre interfaces.

In the salt-leached foams used to develop the protocol for the second-generation scaffolds, the foam surfaces were composed of irregular low-density PCL fragments, which may have facilitated bonding. In the third-generation PLZ-DF foams, the surfaces – while porous – were smoother and denser. To overcome this, the first few layers of fibres were deposited with the needle 5 cm from the foam surface, 50% closer than the standard 10-11 cm. The resulting fibres had a higher residual HFIP content, resulting in increased fibre-fibre adhesion (Figure 3-7B) and fused DF-fibre and PLZ-fibre interfaces (Figure 3-7C,D). While adhesive, the fused fibre zone maintained a porous interface for diffusion and cell migration.

3.3.2 Mechanical Analysis

Initial mechanical analysis of the scaffolds was performed identically to that described in Chapter 2, wherein the 6 mm $\varnothing$ x 2 mm samples were compressed to 10% strain at 0.05% strain per second in unconfined conditions. This approach allowed for a direct comparison of the mechanical properties of the different scaffold types under compression. The porogen-leached foams of the second-generation scaffolds compression moduli were 20 ± 5 MPa and 40 ± 5 MPa for the coarse (1000 μm $\varnothing$) and fine-pored (300 μm $\varnothing$) foams, respectively. The PLZ of the third-generation scaffolds exhibited comparable compression moduli at 34 ± 5 MPa and 38 ± 7 MPa for the DF300 and DF100 foams.

Aside from the potential role of aligned pores in the matrix assembly, another rationale for the incorporation of a directionally frozen zone in the optimised scaffold design was to generate scaffolds with substantially increased compressive strength in the deep zone, thereby mimicking the mechanical gradients across the depth of articular cartilage. When the individual zones of the DF100 scaffold were analysed at 5% and 10% strain using the 0.05% strain/second ramp, the directionally frozen zone proved to be much stiffer, but highly variable under this testing regime, with compressive moduli of 145 ± 73 kPa and 2000 ± 700 kPa at 5% and 10% strain, respectively (Figure 3-8A). Representative stress-strain curves for the DFZ and PLZ components as well as a complete DF100 scaffold are illustrated in Figure 3-8B for the aforementioned testing regime. The stress-strain curves show the significantly greater stiffness of the DF foam, visualised on a semi-log plot, relative to the PLZ and the complete scaffold.

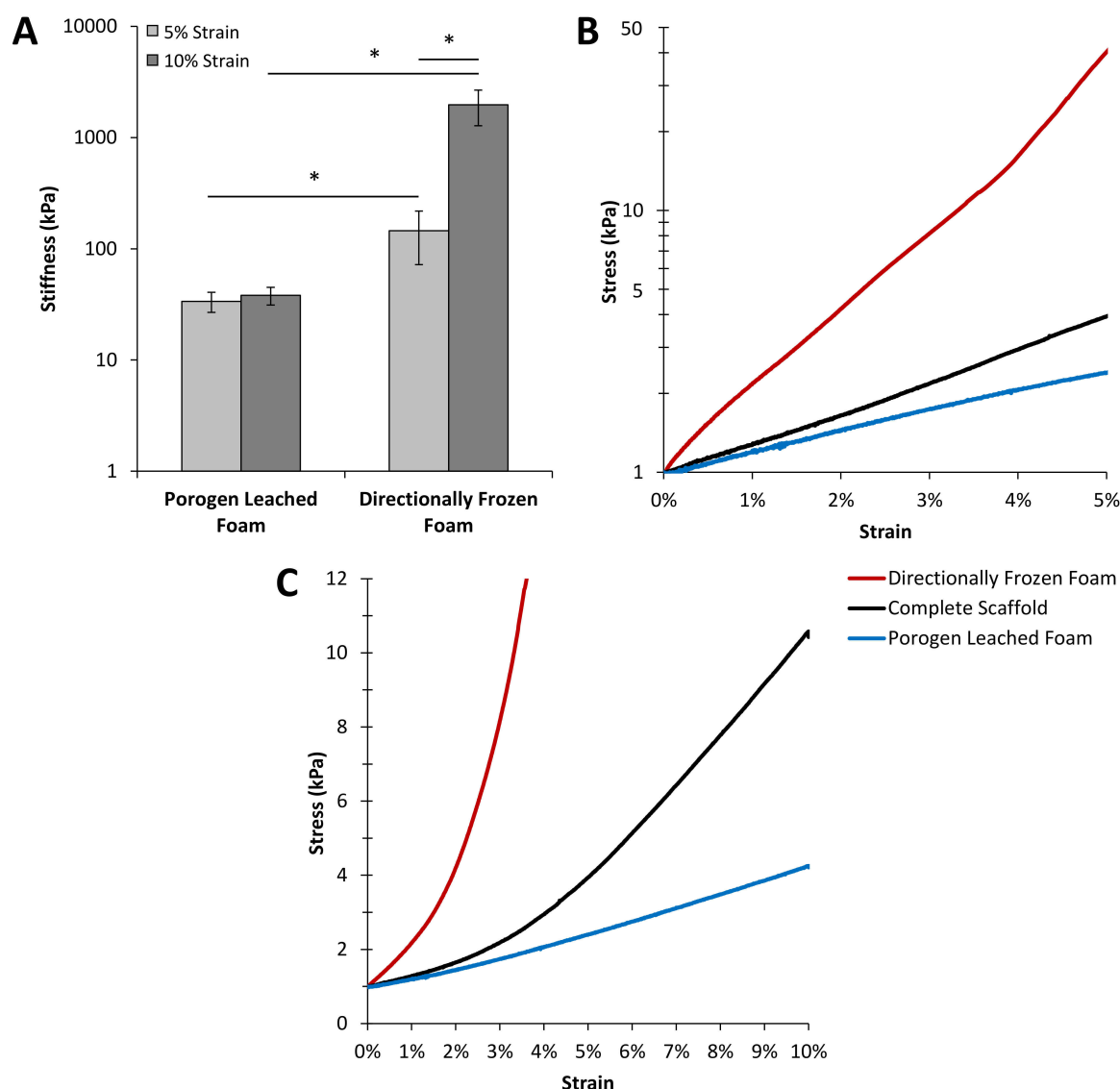


Figure 3-8. A) Stiffness of the porogen-leached 100-300 μm foam and directionally-frozen foam at 5% and 10% compressive stain, unconfined ($n = 4$, * $p < 0.05$). **B)** Representative stress-strain curve for the directionally frozen foam and porogen-leached foam components and the complete scaffold composed of equal layers of DF and PLZ. **C)** Same representative stress-strain curve, highlighting the divergence of PLZ and complete scaffold by plotting on a linear stress scale to 10% strain.

The divergence of the complete DF100 scaffold from the PLZ alone, more easily observed in Figure 3-8C, occurs in a non-linear manner, indicating that the initial strain is entirely within the PLZ zone, with the DF zone contributing more to the bulk stiffness as the forces increase. To visualise this strain regime scaffolds were loaded with fluorescent microbeads and measurements were performed on an epifluorescent microscope with a calibrated uniaxial actuator. A representative strain profile for an acellular DF100 scaffold from 0-25% bulk strain is illustrated in Figure 3-9. Here we see that the large majority of the strain occurs in the PLZ, with only minor strain in the DFZ, up to 25% bulk strain.

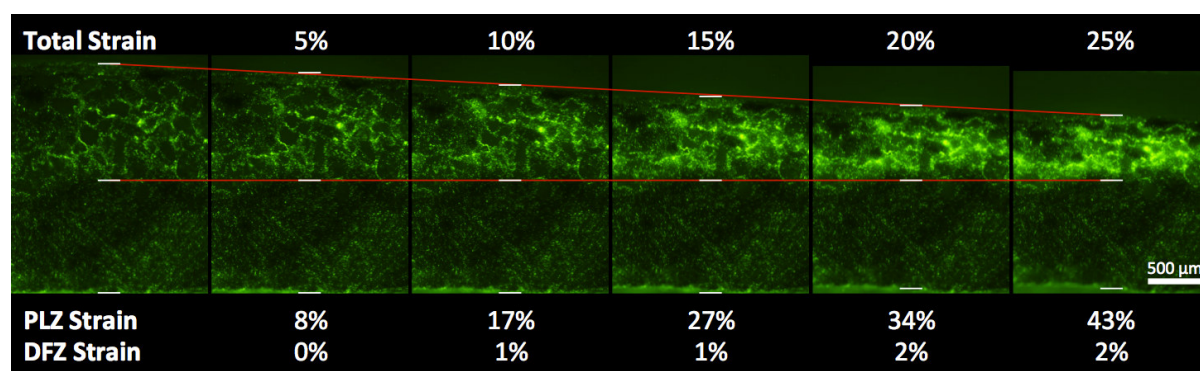


Figure 3-9. Mechanical compression of acellular DF100 scaffold decorated with fluorescent microbeads under 0-25% strain, with zone-specific strain for the PLZ and DF zone.

This strain profile is strikingly similar to the non-linear strain profile of native bovine cartilage, illustrated in Figure 1-6 and discussed in detail by Schinagl *et al.* [20]. The “mid-point” tracker in the Schinagl study deflects more than 2%, but begins in the upper third of the cartilage, not the true mid-point, as in Figure 3-9. A re-analysis of the true mid-point of the Schinagl study in the same manner as above gives 41% and 4% strain in the upper and lower halves respectively. Further investigation of the strain analysis using this method and cellularised scaffolds will provide further insight on the contribution of newly formed cartilage-like tissue in the mechanical properties of the construct.

The 0.05% strain/sec ramp test was performed to compare directly with previous studies. However, the simple protocol used for the second-generation study produced data with a single linear ramp at rates 400 times below physiological (10% strain at 1Hz). Additionally, the lack of a pre strain or cyclic testing resulted in high variability, as the data reflected incomplete initial contact area and initial plastic yielding as opposed to the more representative and clinically relevant average viscoelastic mechanical properties. To generate a more representative and robust data set, the scaffolds were assessed using dynamic mechanical analysis. DF100 and DF300 scaffolds were tested with a 5% dynamic strain amplitude about a mean of 5, 10, 15, 20% strain; at 0.1, 1, and 10 Hz. The most commonly reported condition for articular cartilage mechanotransduction and modelling is 10% strain at 1Hz, therefore the conditions selected bound this standard and test for strain and frequency-dependent variation.

In Figure 3-10A, the storage moduli of the scaffolds are plotted against mean strain by scaffold type and frequency. For increased clarity, the 1Hz measurements for each scaffold are highlighted separately in Figure 3-10B. Both the DF100 and DF300 scaffolds display an increasing stiffness with

strain for all data sets ($p > 0.05$), with DF100 scaffolds stiffer than DF300 at 15% and 20% strain ($p < 0.05$). While there is a consistent trend for increased stiffness with frequency, the difference is generally well within the distribution of the data and most 0.1-10 Hz pairs show no statistical difference. The exceptions are the DF300 scaffolds at 10% and 15% strain, which, due to strikingly low deviations, show statistical differences across the frequencies.

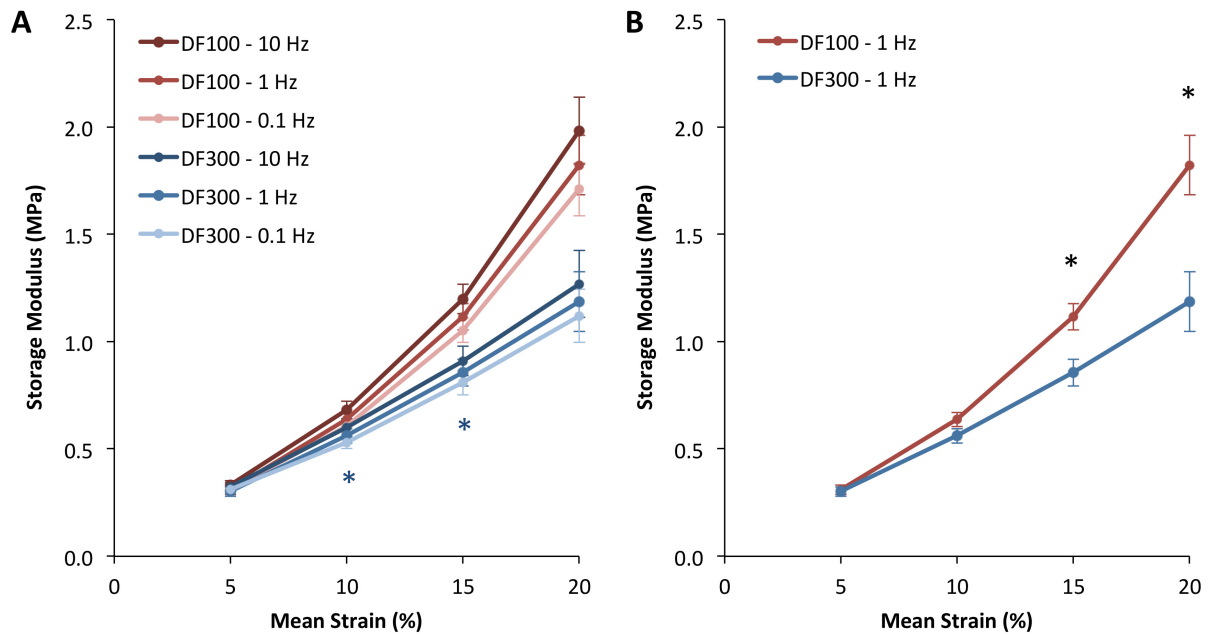


Figure 3-10. A) Mechanical characterisations of DF100 and DF300 scaffolds under strain and frequency sweeps by DMA. (* $p < 0.05$ for DF300 between all frequencies). B) A highlight of the 1 Hz data for direct comparison of DF100 and DF300 (* $p < 0.05$).

The greater stiffness of DF100, approaching 2 MPa at 20% strain, was a factor in the decision to select DF100 over DF300 for the later *in vitro* and *in vivo* studies. The DMA was performed under strain-control in a sinusoidal waveform and analysed from a 10-cycle dataset to determine truly representative moduli at each condition. However, the longer-term mechanical properties of the scaffold are of great interest. To test this, a force-controlled dynamic test was run at the standard condition of 1 Hz for 12h at a constant force, equivalent to a pre-test 10% strain. In Figure 3-11A the strain over 12h can be observed, showing deformation within the first 20-30 minutes, followed by a robust response with average strain beyond 3h increasing by only $0.044\% \pm 0.002\%$ strain per hour. To check this regularity and illustrate the testing regime, 5 second sets of stress and strain data from 3h intervals are included in Figure 3-11C and D, respectively. As the test was in force-control, the sinusoidal stress is consistent at every time point, whereas the strain shows the deviation between hours 0 and 3 followed by a standard waveform with minor deviation between hours 3-12, consistent with the trends observed in panel A. The stress-strain curves in Figure 3-11D show the

same trend, with the 0h time point showing a difference from the other time points which are clustered together, displaying only a slight drift towards increased strain.

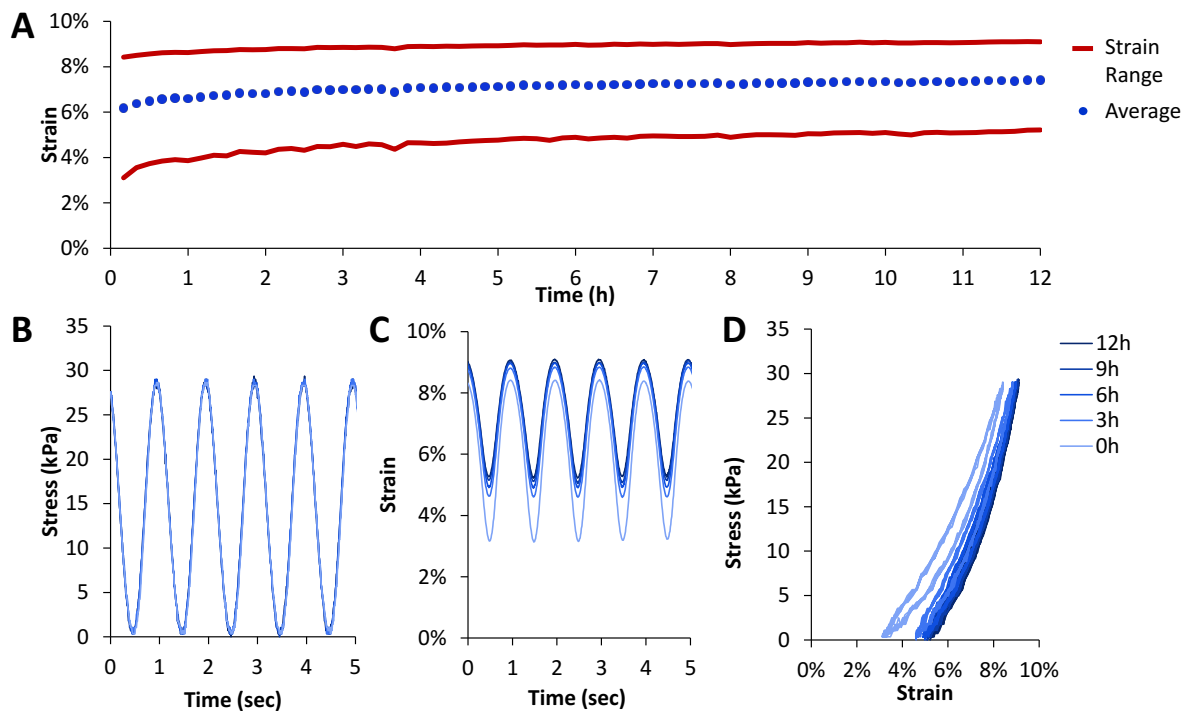


Figure 3-11. Extended cyclic mechanical testing of acellular DF100, at 1Hz over 12h, under force control. A) Time-dependent strain under constant force, showing average and max/min strains at 10-minute intervals. B,C) Stress and strain as a function of time at 3h intervals. D) Stress-strain curves of 10 consecutive cycles at 3h intervals.

The mechanical analysis of the scaffolds indicated that the mechanical properties of the scaffolds are much closer to those of articular cartilage than the previous generation of scaffolds. The low stiffness of the PLZ coupled with the high stiffness of the DFZ produce a non-linear strain profile, comparable to that of articular cartilage. The bulk storage modulus, as determined by dynamic mechanical analysis varies as a function of strain and falls within the physiological range for articular cartilage. The bulk modulus is dependent on the pore size in the PLZ, in agreement with the observed localisation of strain to the PLZ, with DF100 being stiffer than DF300 at strains above 10% at physiological strain rates. The bulk of the deformation of the scaffold under continuous physiological conditions occurs in the first 20 minutes, reaching a plateau to a slow steady rate beyond the first hour.

3.3.3 Interconnectivity and Cell Seeding

Interconnectivity between the zones was investigated to inform cell-seeding protocols. The second-generation scaffold study indicated that cells would not diffuse efficiently through the FZ-PLZ boundary, necessitating separate seeding steps. Therefore, initial interconnectivity study was focussed on the PLZ, DFZ, and PLZ-DFZ boundary, using foams without the addition of fibres. To determine if the pore interconnects were sufficiently large to seed P2 bovine chondrocytes across the PLZ-DFZ boundary an aliquot of 1M cells was seeded onto the exposed PLZ or DFZ surface of a PLZ-DFZ scaffold. The chondrocytes were observed to have effectively seeded the intermediate (Figure 3-12A,B) and deep (Figure 3-12C,D,E) zones of the scaffold and importantly the cells had passed the PLZ-DFZ interface. This result indicated sufficient pore and zone interconnectivity in the complete scaffold to commence more in depth cell experiments.

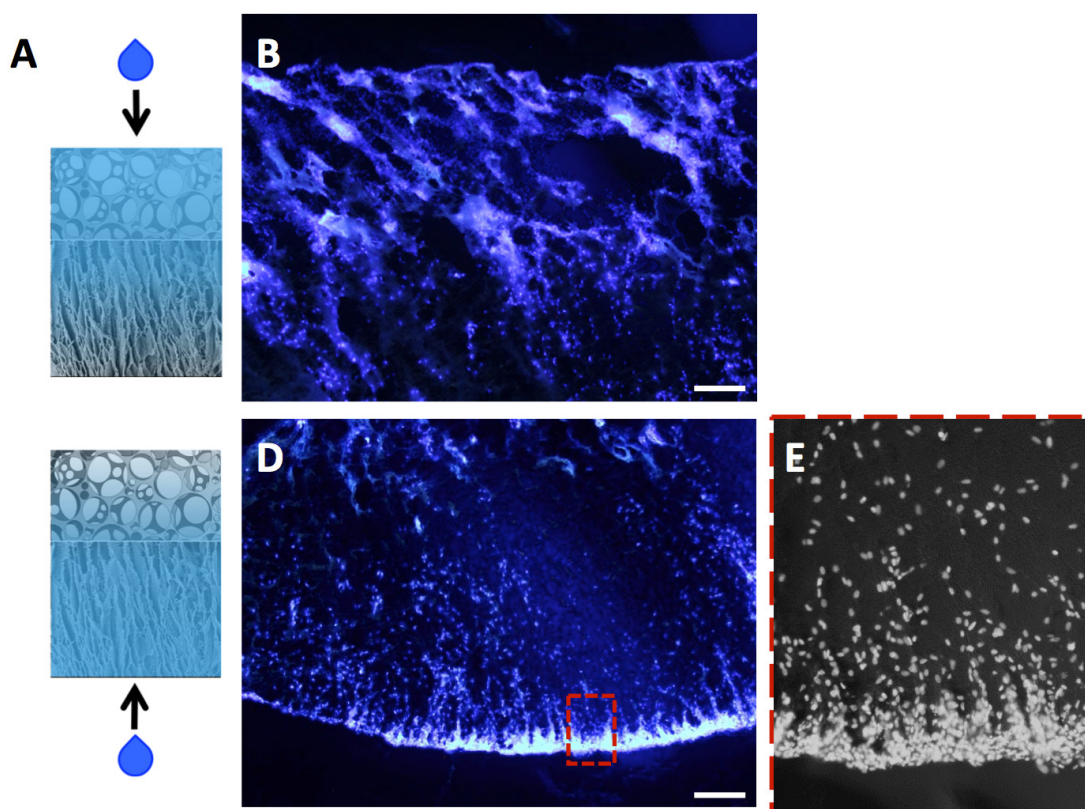


Figure 3-12. DF100 PLZ-DF foam interconnectivity/seeding study. Schematics (A,C) and epifluorescent cross-sections (B,D) of scaffolds seeded with an aliquot of Hoechst-stained P2 bovine chondrocytes via the upper (A,B) and lower (C,D) surfaces. E) Higher magnification inset from D) showing Hoechst-stained chondrocytes filling the aligned DF channels. Scale bars 250 μm .

Following the initial fibre-free seeding study, full-thickness scaffolds were seeded by direct injection of a 50-100 μL bolus of cell suspension containing 900K cells into the gelatine-leached portion of the

scaffold, with 100K cells subsequently deposited on the superficial electrospun surface. This injection protocol, developed for the second-generation tri-layer scaffolds, bypasses the electrospun membranes, ensuring full cellularisation of a scaffold with fibres on both the top and bottom.

The optimisation of the simple seeding protocol prompted a more complex proof-of-concept seeding trial. Articular cartilage is composed exclusively of chondrocytes; however, there are distinct populations of superficial, intermediate-zone and deep-zone chondrocytes, which are characterised by unique phenotypes and differential responses to chemical, structural and mechanical stimuli [23,24,66]. With this in mind, it was of interest to see if three distinct cell populations could be localised within the main zones of the scaffold.

Primary P2 bovine chondrocytes were labelled with live cell dyes to trace the location of each cell sub-population within the scaffolds following chondrocyte attachment. The stained chondrocytes were observed in a mixed population under epifluorescence microscopy to ensure that there was no cross-talk between the channels, which would result in false localisation within the scaffold (Figure 3-13).

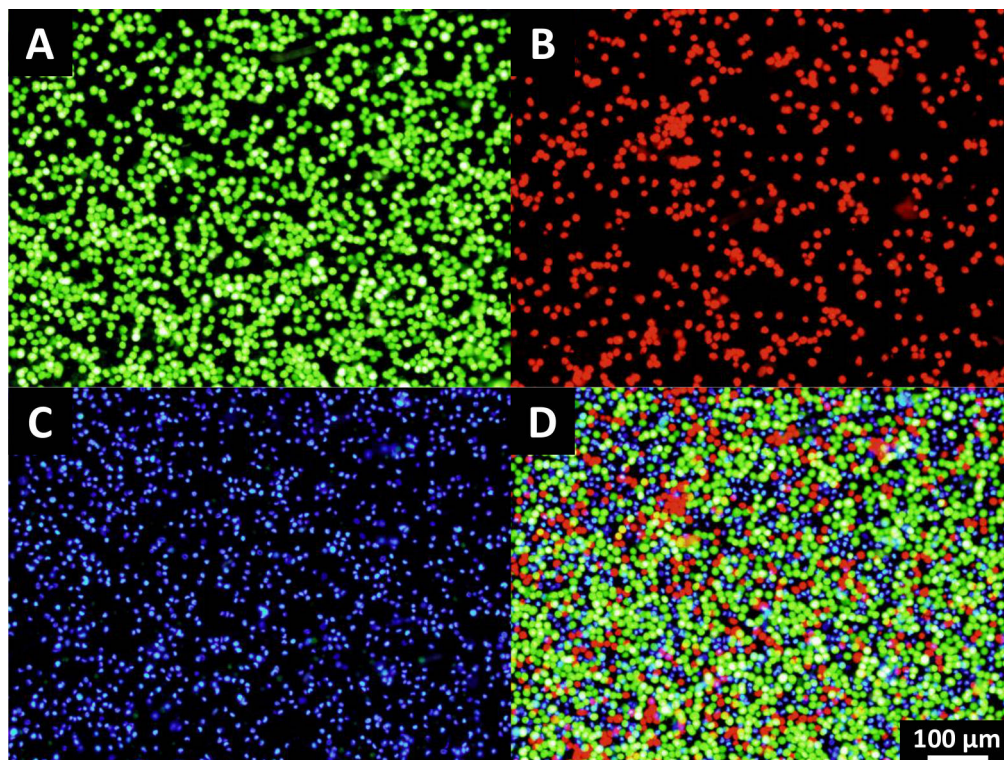


Figure 3-13. Independent channels (A,B,C) and composite image (D) of a mixed population of fluorescently stained live P2 bovine chondrocytes. Chondrocytes were separately stained with Cell Tracker Green (CMFDA) (A), Wheat germ agglutinin Alexa Fluor 594 (B) and Hoechst 33342 (C). A composite image (D) indicates no cross talk between the channels. Scale bar 100 μ m.

Complete scaffolds were seeded with a four-step seeding protocol that involved injecting the deep zone (blue) cells into the intermediate zone, spinning the scaffolds in a plate centrifuge, a second injection of intermediate-zone (green) cells, and the addition of superficial zone (red) cells to the fibre zone surface (Figure 3-14A,B). The cross-section of the resulting scaffold was observed under an epifluorescence microscope, and found to have three zones greatly enriched with their intended live cellular subpopulations at 24 hours (Figure 3-14C).

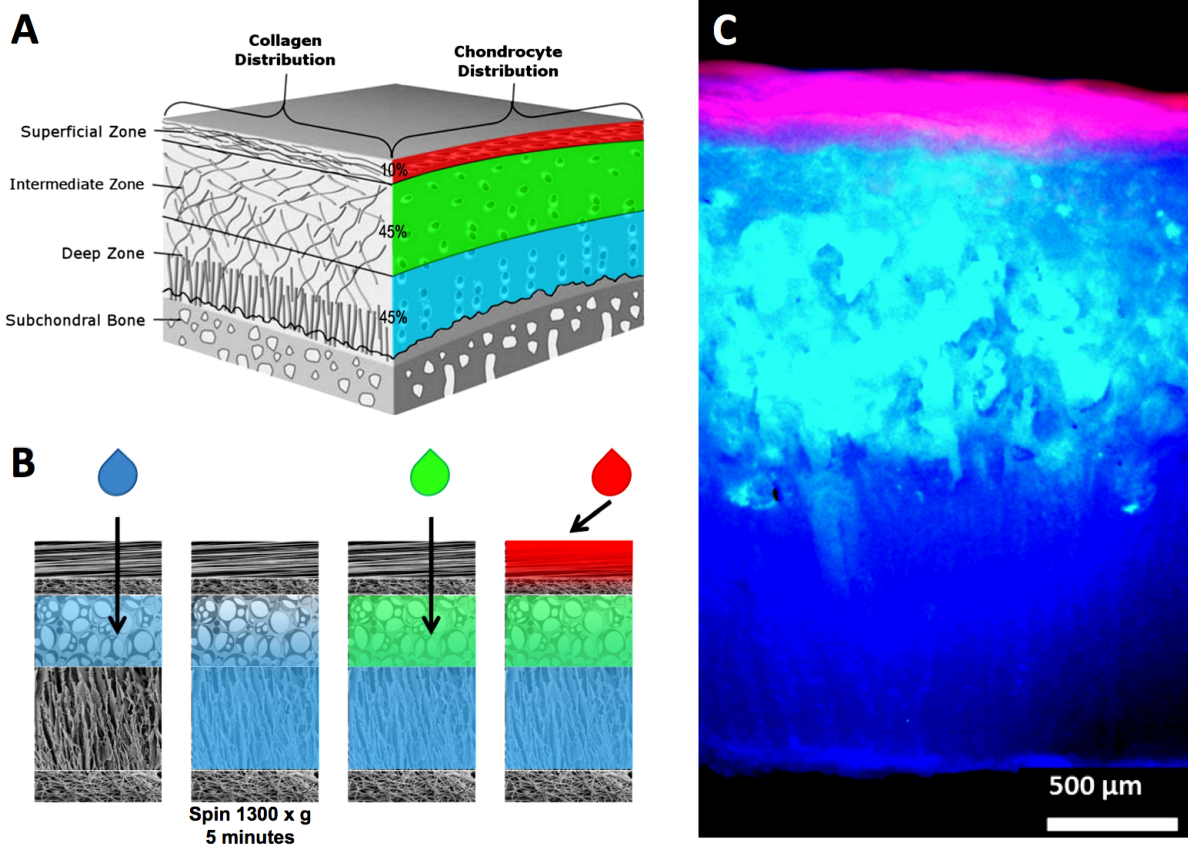


Figure 3-14. Proposal (A), optimised protocol (B) and epifluorescent scaffold cross-section (C) of three-zone scaffold seeding, visualised at 24 hours with RGB-stained live P2 bovine chondrocytes.

Subsequent thorough investigations into zonal scaffold seeding investigated seeding scaffolds with zonally-isolated (superficial, intermediate, deep) bovine chondrocytes. The studies seeded scaffolds with zone-matched cells and scrambled organisations, with the aim to track maintenance or induction of zonal differentiation as a function of scaffold zone by PCR. The studies were ultimately unsuccessful due to on-going issues with RNA isolation from the DF100 scaffolds. However, they remain technically possible due to the successful development of a zonal seeding protocol.

3.3.4 Histology of *in vitro*-formed cartilage-like tissue in the scaffold

Histological evaluation of scaffolds allowed for the visualisation of near-complete tissue infiltration at 8 weeks *in vitro* (Figure 3-15). The exceptional tissue density within the DF100 scaffolds, cultured under chondrogenic conditions indicates complete pore interconnectivity and cellular infiltration; observed by cellular and ECM distribution in the H&E stained sections and full-thickness sGAG production in the alcian blue staining (Figure 3-15A,B). Finally, in Figure 3-15C the interconnectivity between the spherical pores and the channels is visualised, with continuous tissue extending from the pore and flowing into the directionally frozen channels, one such example is highlighted (*).

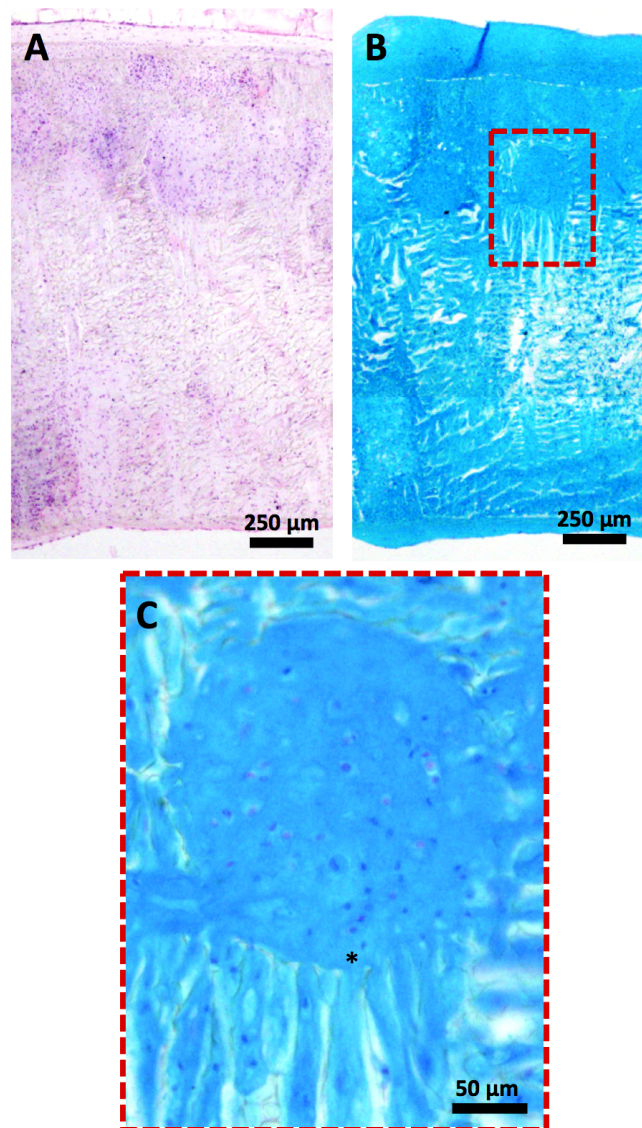


Figure 3-15. Full DF100 scaffold cross-section at 8-weeks showing full tissue infiltration by H&E (A) and Alcian Blue (B,C) staining. C) A higher-magnification visualisation of the PLZ-DF boundary, showing the interconnectivity of the two zones, with DF channels connected to pore faces (*) and the high tissue density of both zones.

When compared to the second-generation scaffold sections in Chapter 2 and Steele *et al.* [1], prepared and stained in the identical manner, the difference is striking. This difference can partly be attributed to the increased cellular seeding density (2.7 times greater), the smaller pore diameters throughout [225], the increased duration of culture, and donor variability. These parameters are known to influence matrix accumulation as the higher cellular density combined with the smaller pore size promote ECM infiltration and a three-dimensional morphology [225]. The scaffold depicted in Figure 3-15 present tissue formed for 8 weeks *in vitro*, as opposed to 4 weeks in the previous study (Chapter 2). However, week 4 sections with the optimised scaffold, also displayed tissue densities far in excess of the previous studies.

To assess the effect of higher chondrocyte density in the scaffolds, the morphology of the tissue formed within the spherical pores and the columnar DF channels, seeded with 10M cells and cultured for 4 weeks was observed at higher magnification, stained by H&E, alcian blue, and picrosirius red (Figure 3-16).

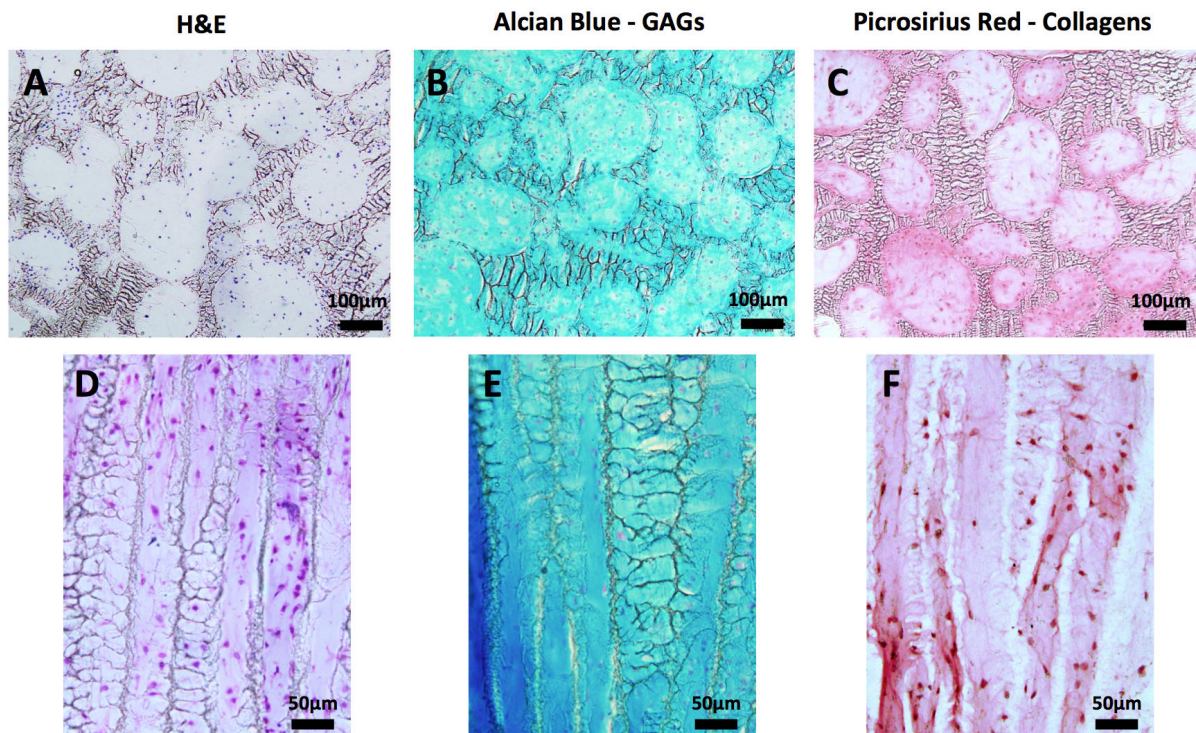


Figure 3-16. Histological analysis of cartilage formed within the DF100 scaffold, seeded with 10M bovine chondrocytes and cultured *in vitro* under chondrogenic conditions for 4-weeks. The porogen-leached zones (A,B,C) and directionally frozen zones (D,E,F) show complete tissue infiltration. Sections are stained with Haematoxylin and Eosin (A,D) for nuclear and non-specific tissue visualisation, Alcian Blue (B, E) for sGAG, and Picrosirius Red (C,F) for collagen distribution.

We can again see complete ECM filling of the scaffold porosity in both zones. Another interesting observation is that, while the chondrocytes were injected in a concentrated cell-media slurry without exogenous ECM or hydrogel carrier, most cells are not adhered to the walls of the pores in which they were initially seeded; instead, they are equally distributed throughout the de novo tissue. Therefore, the P2 chondrocytes filled the pores with ECM through appositional matrix deposition, evenly distributing cells and forming a three-dimensional tissue. In the spherical pores of both DF100 and DF300, few cells are observed in direct contact with the PCL pore surface. This was not true of the larger pores visualised in BL-1.0 scaffolds in Chapter 2, in which P2 chondrocytes instead formed dense sheets of collagen-rich ECM, treating the pores as a two-dimensional surface. The collagen deposition highlighted by the picosirius red staining in Figure 3-16C,F appeared to show random alignment in the spherical pores, with a higher density at the periphery of the pores where cells contact the PCL.

3.3.5 Multiphoton Microscopy and Immunohistochemistry of *in Vitro*-Formed Tissue in the Scaffold

To further investigate the alignment of the collagen fibrils, multiphoton microscopy was used to visualise collagen by label-free second harmonic generation using an 860 nm laser to produce SHG signal in collagen at 430 nm excitation. To validate the technique, a bovine osteochondral section was imaged with traditional staining for nuclei, actin, and IHC for type I collagen (Figure 3-17A). In this image, we can see the stark transition from subchondral bone to cartilage highlighted by the step-change in type I collagen density. In Figure 3-17B all fibrillar collagens are identified by second harmonic generation, with nuclear staining for chondrocyte localisation. The vertically aligned type II collagen fibrils in the deep zone of the articular cartilage as well as the mature type I collagen in the bone both have a strong SHG signal due to the alignment and maturity of the collagen fibres [222]. The intense SHG signal in the bone is correlated with the type I collagen IHC.

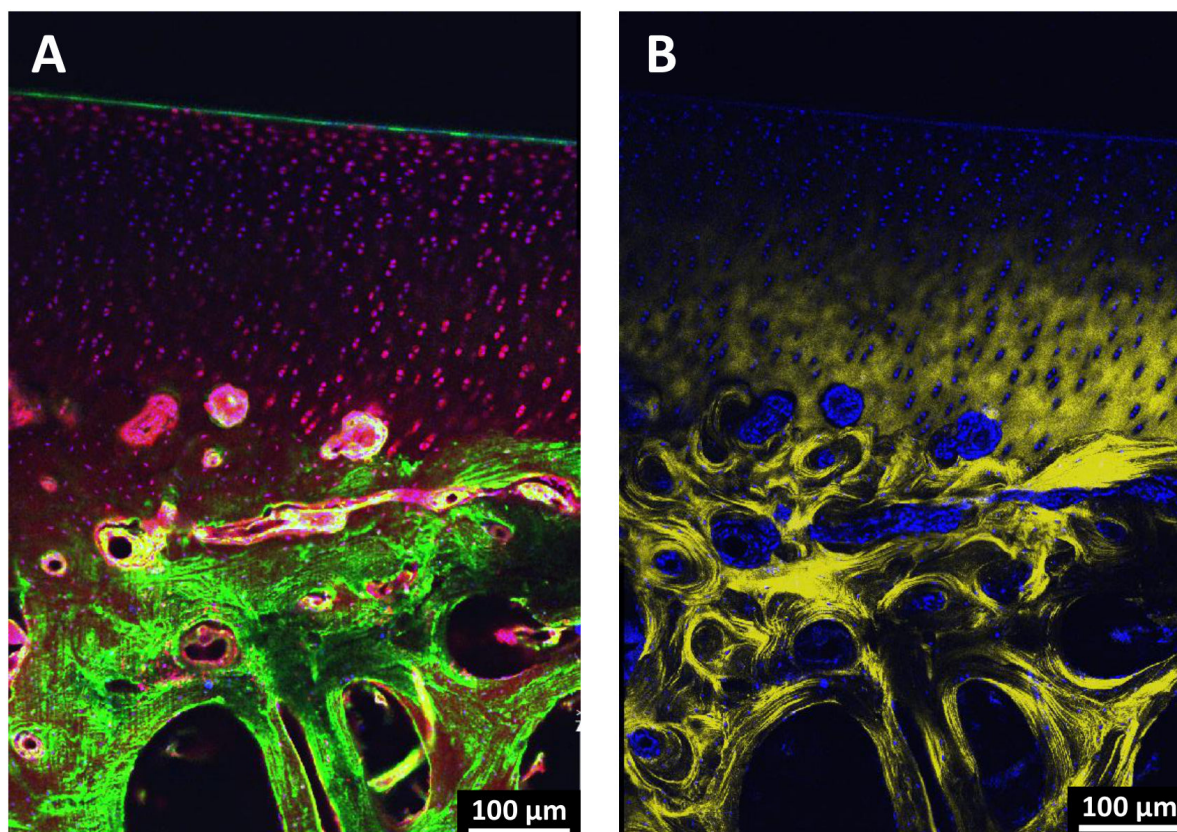


Figure 3-17. Validation of IHC staining and confocal/multiphoton imaging on a decalcified bovine osteochondral section. A) Traditional IHC staining of collagen type I (green), actin cytoskeleton (red) and nuclei (blue). B) Visualisation of collagen fibrils by second harmonic generation (430nm, yellow) with nuclear staining (blue) for reference. Scale bars are 200 µm.

DF100 scaffolds seeded with 1M cells from donor 2 and cultured for 12-weeks were analysed for collagen alignment in the same manner. For the evaluation of tissues inside the scaffolds type II collagen was used as the IHC counterstain to visualise the ECM and Draq5 to visualise the nuclei. The spherical pore from the PLZ in Figure 3-18A shows high cellular density by Draq 5 nuclear staining and full ECM infiltration within the pore by type II collagen IHC staining throughout the ECM, consistent with histological observations. The visualisation of collagen in the PLZ pore by SHG (Figure 3-18B) shows random collagen fibril alignment throughout the pore. The type II collagen IHC and SHG morphologies resembles the SHG imaging of chondrocytes in amorphous gelatine gels by Filova *et al.* [224]. The vertically-aligned channels from the DF zone in Figure 3-18E show a lower chondrocyte density by Draq 5 staining consistent type II collagen staining throughout the ECM, as was observed using traditional techniques in Figure 3-16D/E/F. Second harmonic generation shows a strong, highly aligned collagen signal within the vertically aligned pores, with a more conclusive intensity and alignment than that observed by picosirius red. The data generated by SHG visualisation of the collagen fibrils benefits from omitting sample sectioning, dehydration, and staining, reducing artefacts inherent in traditional histology.

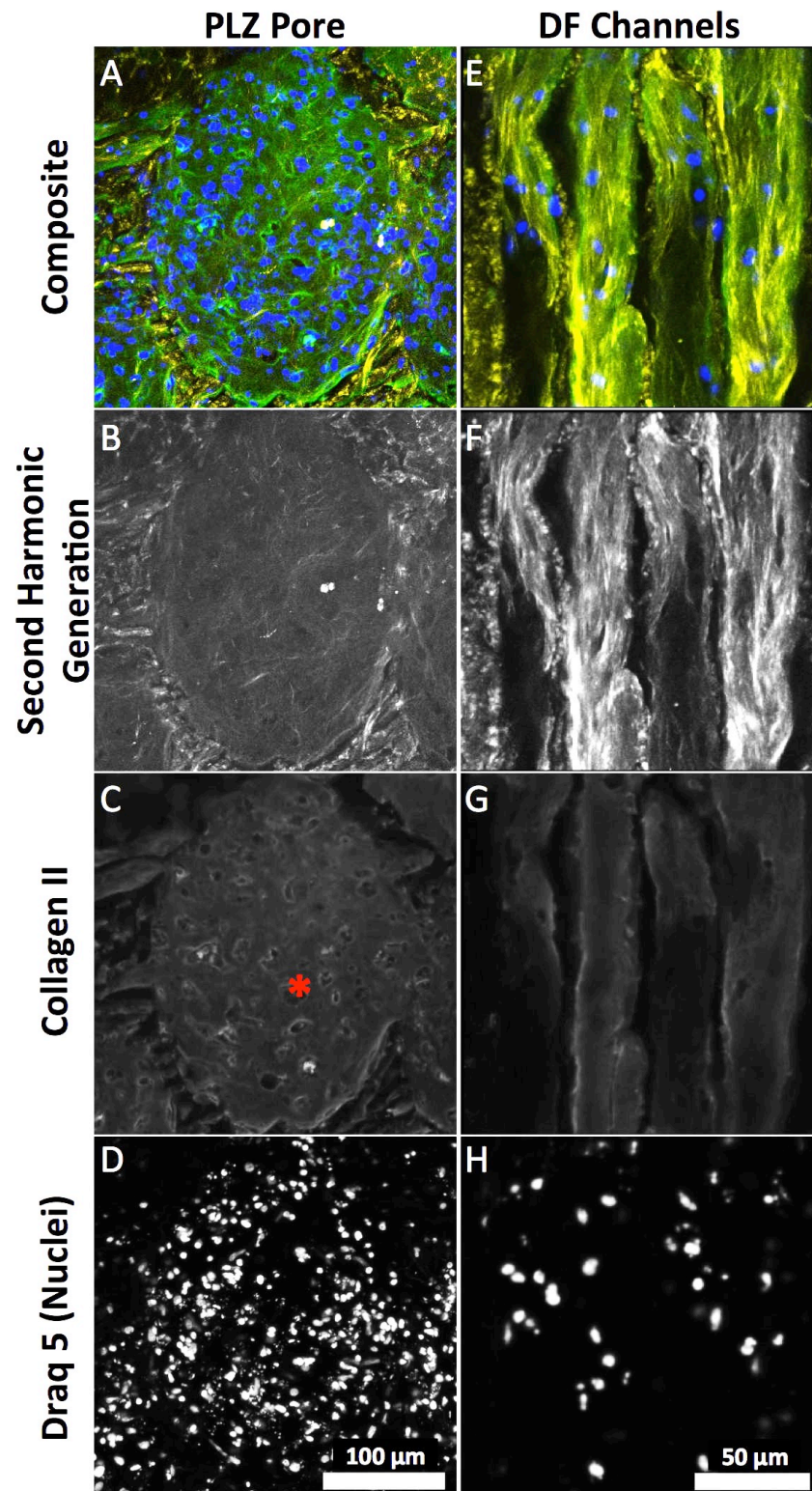


Figure 3-18. Differential tissue morphology of week 8 *in vitro* DF100 scaffolds (A,E), visualised by Second Harmonic Generation (SHG) (B,F), type II collagen IHC (C,G), and nuclear staining (Draq5)(D,H). The SHG shows a great contrast between the isotropic collagen fibril alignment in the intermediate zone (A) and the vertical collagen alignment within the directionally frozen pores in the deep zone (E). Type II collagen IHC distribution and the lacunae apparent in the image (C*) are indicative of hyaline articular cartilage, as opposed to fibrocartilage.

Therefore, the two primary zones of the DF100 scaffold, the PLZ and DFZ, generated two distinct collagen organisations from the same population of P2 bovine chondrocytes within two halves of the same complete scaffold under identical static *in vitro* culture conditions. The collagen orientation was reflective of physiological alignment within native articular cartilage and dependent on the tailored design of the microarchitecture within the zones. Potential confounding factors, such as differential nutrient diffusion within the scaffold zones would have resulted in collagen alignment varying along the radial and/or z-axis of the zones, which was not observed.

3.3.6 *In Vitro* Biochemical Characterisation of *In Vitro*-Formed Tissue in Response to Scaffold Architectures

Biochemical characterisation was performed to determine the effect of PLZ pore size and the impact of the different scaffold architectures. DF100 and DF300 scaffolds were seeded at 10^6 P2 chondrocytes/scaffold and cultured for 4 weeks *in vitro* under chondrogenic conditions. For analysis, the scaffolds were divided into the upper fibre zone (FZ), porogen-leached zone (PLZ), directionally frozen zone (DFZ), and lower fibre zone. PLZ-DF foams were separated with a scalpel along the visible midline. Therefore, the zonal biochemistry should be considered as PLZ or DFZ 'enriched' rather than absolute, taking into account the approximate nature of the division.

Scaffold zones were individually assessed for DNA, sGAG, and sGAG/DNA ratio (Figure 3-19A,C,E). The cumulative data was also displayed for complete scaffolds (Figure 3-19B,D,F) with indications of zonal contributions.

The zonal distribution of both sulphated glycosaminoglycans (sGAG) and DNA both reflected the relative volumes of the zones. The 900 μm thick PLZ and DFZ zones which each comprised $> 45\%$ of the scaffold thickness contained the majority of the sGAG and DNA, with no statistical differences observed between the two zones (Figure 3-19A,C). The total sGAG masses for DF100 and DF300 were statistically similar ($p = 0.80$) at $447 \pm 155 \mu\text{g}$ and $473 \pm 128 \mu\text{g}$ respectively (Figure 3-19B). Total DNA content trends lower in DF100 relative to DF300 with $33.0 \pm 12.9 \mu\text{g}$ and $41.8 \pm 5.9 \mu\text{g}$ for DF100 and DF300 respectively, but the high variability of DF100 precludes statistical significance ($p = 0.27$) (Figure 3-19D). The average sGAG and DNA density across both scaffold types are $8.1 \pm 2.3 \mu\text{g}/\text{mm}^3$ and $0.66 \pm 0.18 \mu\text{g}/\text{mm}^3$ ($86,000 \pm 23,000 \text{ cells}/\text{mm}^3$). These values indicate that the tissue formed at 4 weeks, despite being fully infiltrated, is immature, with a lower sGAG density than bovine cartilage ($25 \mu\text{g}/\text{mm}^3$) and an intermediate cellular density, between adult and foetal bovine cartilage ($30,000 - 150,000 \text{ cells}/\text{mm}^3$) (Table 1-1) [7,8].

The sGAG/DNA ratio was calculated for each zone (Figure 3-19E,F); however, the fibre zone data is depicted in grey to highlight the PLZ and DFZ, as they represent the majority of the scaffold and are calculated from the most robust data. While there is no difference between the identical DFZ zones ($p = 0.24$), the average sGAG/DNA ratio for DF100 PLZ trends roughly 40% higher than that of DF300 ($p = 0.062$). This trend is even stronger in the complete-scaffold ratios, where the average DF100 sGAG/DNA ratio is roughly 20% greater than that of DF300 ($p = 0.055$). The sGAG/DNA ratios for DF100 and DF300 are approximately half that of foetal bovine tissue (Table 1-1).

The biochemical data suggests a more differentiated chondrogenic phenotype in the smaller pores of DF100 with more sGAG bound per chondrocyte and reduced proliferation relative to the larger pores of DF300. Considering potential issues with scaffold subdivision and the similarity of the relatively fine pore distributions, the presence of such a strong trend in the PLZ sGAG/DNA ratio is notable.

The lack of statistical differences within the biochemical data was a reflection of a number of positive factors. All scaffold zones were successfully seeded, able to maintain chondrocyte viability, and permit the formation of extensive cartilaginous ECM. The pore sizes of the PLZ were selected to bound the 300 μm diameter shown in Chapter 2 and the literature to promote chondrogenesis [1,18,226,227]. The differences between the 100-300 μm and 300-500 μm pores are therefore less pronounced than those between 300 μm and 1000 μm pores in Chapter 2 as the both fall within the optimal range.

Therefore, the lack of statistical difference in DNA and sGAG content between the DF100 PLZ and DF300 PLZ indicate that both were well suited to support chondrocyte culture and cartilage-like ECM deposition, with DF100 displaying a slight advantage.

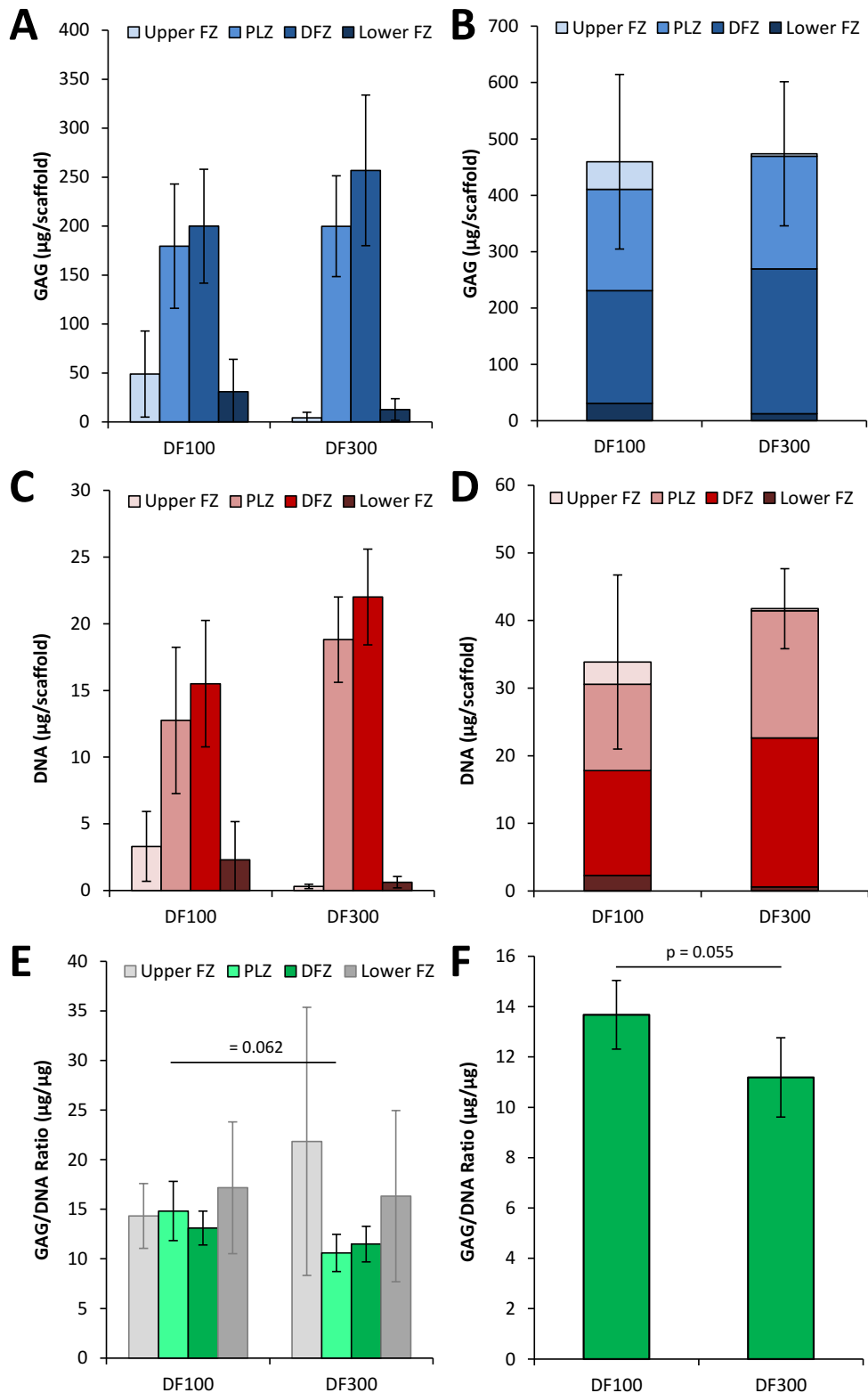


Figure 3-19. Biochemical zonal assessment of DF100 and DF300 scaffolds after 4-weeks of *in vitro* culture under chondrogenic conditions. Biochemical content is illustrated by zone for sGAG (A) DNA (C) and sGAG/DNA ratio (E). Complete-scaffold totals and standard deviations for DF100 and DF300 for sGAG(B), DNA (D), and sGAG/DNA ratio (F), with zonal contributions superimposed for sGAG (B) and DNA (D). Error bars = SD. n=4 with technical triplicates for all samples.

To investigate the biochemical changes over time, DF100 scaffolds were cultured *in vitro* for 4 weeks, with intermediate time points at day 0, week 1 and week 2. Due to improved mechanical performance and biochemical trends favouring DF100 over DF300, the latter was omitted from the investigation. Complete DF100 scaffolds were instead compared to scaffolds with a double-thickness porogen-leached zone in place of the directionally frozen zone, mimicking the BL-0.03 scaffold from Chapter 2. Direct comparison of DF100 and the 'No DFZ' control was intended to provide insight into the contribution of the directionally frozen zone while avoiding the variability associated with scaffold subdivision.

To investigate the variability from donor source, the full study was performed in triplicate with P2 chondrocytes from three 2-year old calf donors. All data was analysed in technical triplicate and $n = 4$ for each scaffold, time point and donor. Averaged sGAG, DNA, and sGAG/DNA ratio values are displayed in Figure 3-20A,C,E and the three donors are depicted individually in Figure 3-20B,D,F.

The average sGAG level increases significantly at all time points relative to week 0 (Figure 3-20A), with no statistical difference observed between DF100 and 'No DFZ' at any time point ($p > 0.05$). In the individual data (Figure 3-20B), it can be observed that there is considerable variability between donors at week 1, but all show a decrease in sGAG accumulation rate over 4 weeks and roughly converge by week 4. The sGAG production profiles, with an initial high rate of synthesis slowing in all cases between week 2 and 4 indicate rapid pore filling followed by maturation, in agreement with the histology, which displayed ECM-filled pores at week 4.

The average quantity of DNA in scaffolds increased over 4 weeks by roughly 2-fold, with no statistical differences between DF100 and 'No DFZ' at any time point (Figure 3-20C). DNA levels in DF100 are significantly greater than day zero at all time points. Variability between the donors at week 0 and 4 (Figure 3-20D) resulted in no statistical differences in the average 'No DFZ' scaffolds, relative to week 0 (Figure 3-20C). The increased donor 2 DNA levels at week 4 are notable, and potentially due to an uncontrolled variable stressing the system, resulting in dedifferentiation and proliferation.

Under the same donor, the 'No DFZ' and DF100 scaffolds show striking similarities, with similar trends throughout and no statistical differences between scaffold types at week 4. This agrees with the findings of the zonal analysis that, at week 4, chondrocytes produce a similar quantity of sGAG and DNA in the DFZ and PLZ. Therefore substituting one zone morphology for another did not impact the average ECM accumulation in the total scaffold. A greater variability is found between the donor cell populations than the scaffold architectures.

The average sGAG/DNA ratio for both scaffolds is statistically greater than week 0 at all time points and reaches the level of foetal bovine cartilage (22 $\mu\text{g}/\mu\text{g}$) in both scaffolds by week 2 (Table 1-1) (Figure 3-20E). In the only statistical difference exhibited between the two scaffold types, the ratio is statistically greater in the 'No DFZ' scaffold at week 2 ($p = 0.037$). There is also a weak trend for increased sGAG/DNA in 'No DFZ' at week 4, observed more clearly in the individual donors (Figure 3-20F). The decreased sGAG/DNA ratio in the DF100 scaffold at week 2, while remaining equivalent to foetal bovine cartilage, could be due to the greater surface area of cell-material interface in the DFZ resulting from the smaller pore diameter, increasing the probability of 2D culture conditions and subsequently impacting chondrogenic phenotype in a subset of cells. However, as the ratio remains within a physiological range, the scaffold is adequately suited for further investigation.

Through the zonal and temporal biochemical assessments, it was determined that the majority of the sGAG and DNA at week 4 is found within the porogen-leached and directionally frozen zones in roughly equal quantities, indicating that both are fully infiltrated with cartilaginous ECM. At week 4, the chondrogenic nature of the cells, as defined by the sGAG/DNA ratio, improved by roughly 40% within the smaller pore diameter of the DF-100 PLZ scaffolds, relative to DF300 PLZ, producing a more differentiated morphology ($p = 0.062$). Temporal studies saw levels of sGAG, DNA, and sGAG/DNA increase over 4-weeks, independent of donor. Temporal changes were also largely independent of the presence of the DFZ, with the only statistical difference between 'No DFZ' and DF100 occurring at week 2, when 'No DFZ' exhibited a higher sGAG/DNA ratio.

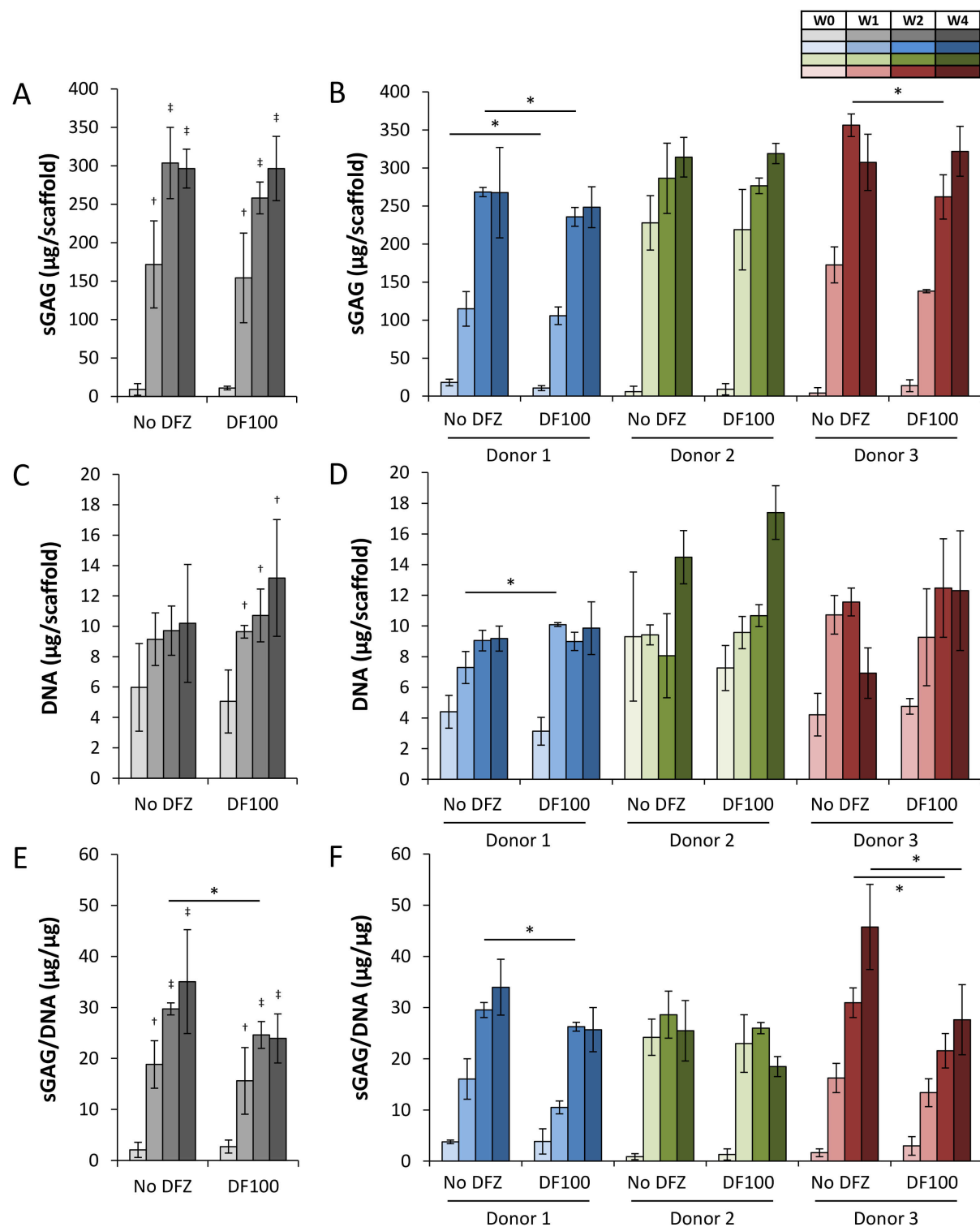


Figure 3-20. Four-week biochemical assessment of DF100 scaffolds with (DF100) and without (No DFZ) the directionally frozen zone. The data is shown as an averaged set (A,C,E) and separately for the three bovine donors (B,D,F). A,B) mass of sGAG per scaffold, C,D) mass of DNA per scaffold, and E,F) GAG/DNA ratio. Error bars = SD. (* $p < 0.05$, (A,C,E) relative to W0: † $p < 0.05$, ‡ $p < 0.005$).

3.3.7 Gene Expression

A 7 day PCR study was conducted on DF100 and DF300 scaffolds in a semi-zonal manner with two cellular densities: 1M and 10M P2 chondrocytes per scaffold. The analysis was semi-zonal as the scaffolds were divided at the PLZ-DF zone interface and fibre layers were not detached. The day 7 time point was selected to highlight the early cell-material interactions. Figure 3-21 outlines the expression of type II collagen (COL2A1), type I collagen (COL1A1), aggrecan (AGC), superficial zone protein (PRG4), Sox9, and type X collagen (COL10A1) relative to day zero and the housekeeping gene 18S.

The COL2A1/COL1A1 ratio, a metric of chondrogenic differentiation, is illustrated in Figure 3-21A. The higher-density culture exhibited increased ratios, significant in the DF300 scaffold zones, and trending in the DF100 scaffold zones. While increased variability eliminated significance in the COL2A1 and COL1A1 gene expression data, the trends remained evident, with greater COL2A1 values and reduced COL1A1 values for the higher density cultures across most scaffolds and zones (Figure 3-21B,C).

In the high-density culture condition aggrecan was significantly unregulated relative to low-density culture in the DFZ-half of DF100, with evidence of potential upregulation in the more variable PLZ half; no trend was observed in the DF300 scaffold (Figure 3-21D). Superficial zone protein (PRG4) was significantly upregulated in the high-density DF100 scaffold in both zones relative to low density; DF 300 showed a strong trend towards upregulation ($p = 0.074$) in the DFZ-half of the scaffold (Figure 3-21E). High-density culture conditions upregulated SOX9 relative to the low-density in the PLZ-half of DF100 (Figure 3-21F). There were no significant observations in type X collagen gene expression data; however, a trend towards increased expression in the high density DF100 scaffold was observed (Figure 3-21G).

While the gene expression data suffered from high variability, the significant differences indicated that the higher-density culture conditions were potentially preferable for chondrogenic culture within the scaffolds. Additionally, in agreement with biochemical observations, there were few significant differences in gene expression between the upper and lower zones for the genes investigated at day 7.

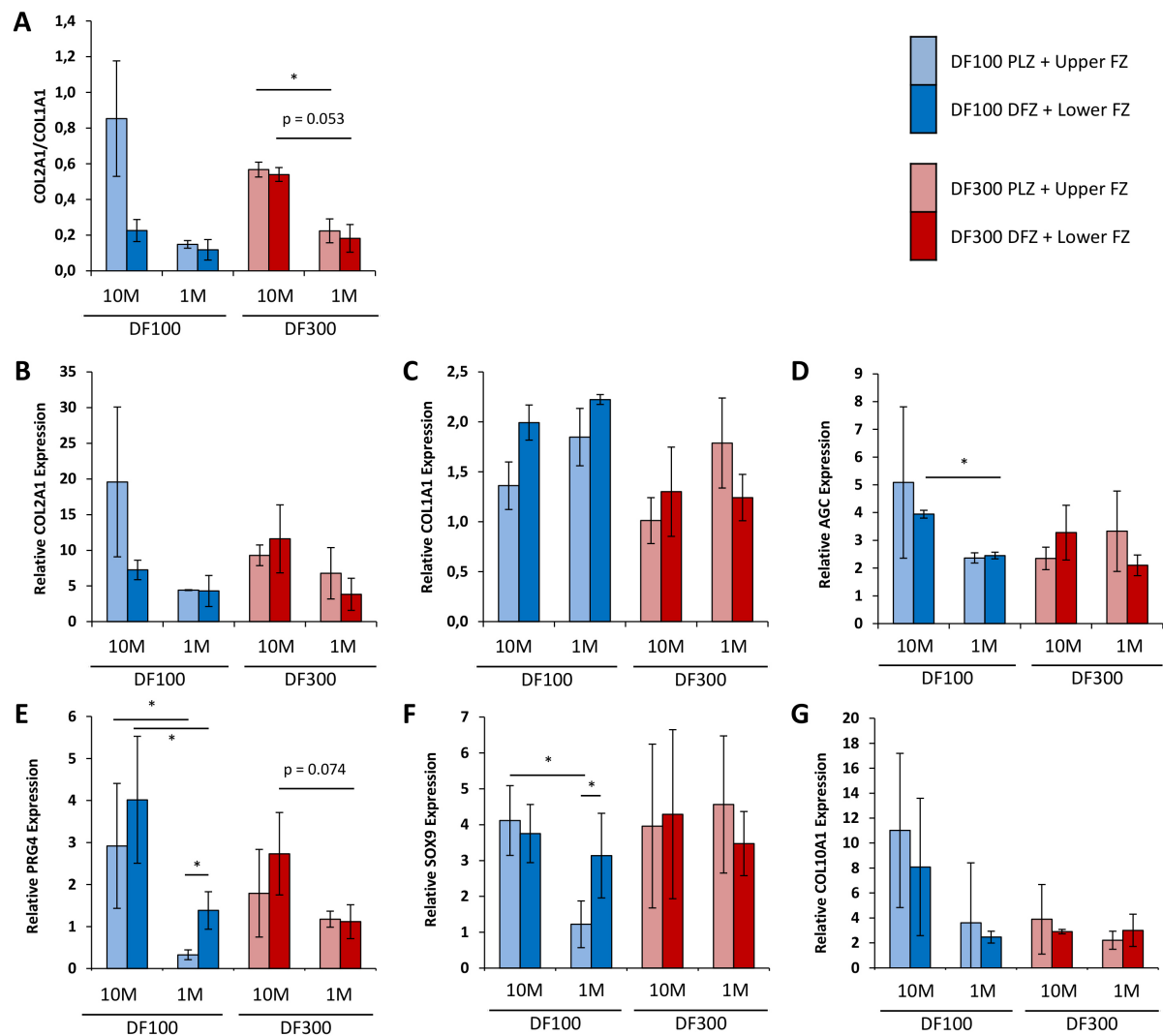


Figure 3-21. Gene expression profiles for DF100 and DF300 scaffolds with 10M or 1M P2 bovine chondrocytes following 7 days of *in vitro* culture in chondrogenic medium. For a semi-zonal analysis, the scaffolds were subdivided into two halves at the PLZ-DFZ boundary. A) The COL2A1/COL1A1 ratio is indicative of chondrogenic differentiation. B) type II collagen (COL2A1), C) type I collagen (COL1A1), D) aggrecan (AGC), E) superficial zone protein (PRG4), F) Sox 9, and G) type X collagen (COL10A1) are expressed as fold differences relative to day 0 controls and normalized to the 18S housekeeping gene. (* $p < 0.05$).

3.3.9 Critical Comparison

There are many approaches to the production of a cartilage scaffold, as outlined in Tables 1-4 and 1-5. Commercial products are starting to embrace an anisotropic design (ChondroGide®, MaioRegen®), however the majority of products on the market are composed of an isotropic foam, gel, or fibre-patch.

The drive towards innovative anisotropic cartilage scaffolds comes from the academic field exemplified by layered fibre and foam-fibre systems published within our group [1,90], layered foam systems by Levingstone *et al.* [154] under O'Brien and Gleeson at Trinity College Dublin, and 3D printing of cartilage gradient scaffolds supported by the independent work of Woodfield [18] and Hutmacher [104,228].

While the 3-layered foam scaffold produced by Levingstone has structural and chemical anisotropy [154], its mechanical gradient ranges from 300 Pa to 900 Pa, orders of magnitude below the second- or third-generation scaffolds developed as part of this thesis and native articular cartilage. Additionally, as only short 7 and 14-day cell incubation time points were investigated, the tissue produced within the scaffolds was very sparse with no indication of maturity and chondrogenic phenotype. Therefore, their approach of layered isotropic foams has great potential, but we are provided with limited ability for direct comparison to the third-generation scaffold design presented here.

The early 3D printing approaches by Woodfield using open-pore fused deposition modelling (FDM) provide physiologically-relevant stiffness in excess of those achieved in the third generation scaffold [229]. When printed with layers of different strut spacing, and therefore porosity, the tissue produced was statistically different, with a higher DNA concentration in the superficial zone [18]. However, the sGAG and collagen quantification, that also differs, is normalised to unit volume as opposed to DNA, and may therefore simply reflect differential cellular density as opposed to zonal morphology. While the FDM process is more reproducible than our third-generation scaffold process, the large struts of poly(ethylene glycol)-terephthalate–poly(butylene terephthalate) (PEGT/PBT) that provide the mechanical strength compose up to 40% (v/v) of the scaffold. Therefore, the local microenvironments may experience long-term stress shielding and effects from degradation by-products including inflammation that could lead to dedifferentiation and degeneration *in vivo*.

The 3D hydrogel printing, such as that by Klein and Hutmacher [228] provides the potential for chemical, stiffness, and cellular gradients that allows for precise control over the design. The use of

hydrogels provides a suspended-cell morphology that can improve the maintenance of chondrogenic phenotype; however, gels are generally much weaker than physiological tissue. Additionally, when considering clinical translation, the system would require personalised on-site sterile production, as the patient's own cells are incorporated into the bulk of the hydrogel structure. Scaffolds could not be produced and validated in large batches at a centralised facility and sold as an off-the-shelf product, as is the case for porous polymer scaffolds and simple gel systems which can be seeded at the time of implantation or as acellular matrices.

In general, while the third-generation design has a multi-step production protocol that relies on electrospinning and directionally freezing, two systems that still require optimisation for controlled reproducibility, it has merits that make it competitive in the field. It is mechanically robust, with anisotropic stiffness within the physiological range supported by the microarchitecture, distributing stress throughout the construct. Biologically, it has been shown to produce cartilage-like tissue with zonally-dependent collagen orientation *in vitro*. Commercially, it can be produced, sterilised, and validated off-site, making production and distribution economical. Clinically, it can be trimmed with surgical scissors if required and seeded with a single injection of cell suspension without the need for specialised skills or equipment, making it almost as simple as non-structured commercial products.

3.4 Conclusion

The third-generation scaffold has fulfilled the criteria as specified by the initial aim of the project. The series of zones result in a scaffold that is architecturally and mechanically anisotropic. The superficial zone of electrospun fibres imparts tensile strength and a smooth surface morphology while remaining porous and open to infiltration by chondrocytes and ECM. The fibre layer is fused to the underlying isotropic porogen-leached zone, with an intermediate stiffness and spherical pore morphology that has full interconnectivity throughout the PLZ and through the PLZ-DFZ interface into the channels of the directionally frozen deep zone. The DFZ has a stiffness that matches the deep zone of cartilage, exceeding 1 MPa at 10% strain, while maintaining high porosity conducive to full tissue infiltration. The tissue formed in both the PLZ and DFZ is able to completely fill pores at only 4 weeks, while maintaining a chondrogenic gene expression profile and staining for sGAG. The PLZ pores 100-300 μm in diameter in DF100 show trends that indicate superior chondrogenic potential over 300-500 μm pores in DF300. However, both pore varieties out-performed previous studies. The collagen generated within the pores by week 12 *in vitro* has a zone-specific alignment that matches the alignment of the native tissue, as detected by label-free second harmonic generation. The PLZ pores contain collagen fibrils of random alignment, while the DFZ contains vertically aligned organised collagen fibrils, matching those of the deep zone. Gene expression

analysis indicates chondrogenic profiles for DF100 and DF300, with improved upregulation of cartilage-specific genes at higher chondrocyte densities of 10^6 /scaffold.

3.5 Expansion of the Third-Generation Scaffold Study

The preceding study investigated the development of an unmodified PCL scaffold seeded with an expanded primary chondrocyte suspension. Modifications to these methods were investigated as proof-of-concepts for future expansion on the platform technology.

3.5.1 Human Mesenchymal Stem Cells

The use of mesenchymal stem cells in cartilage regeneration is an avenue of great interest both academically and clinically, as autogenic chondrocyte harvest generally requires a second surgical procedure with associated donor-site morbidity [230–232]. To expand the therapeutic potential, scaffolds were seeded with 10^6 human mesenchymal stromal cells (hMSC) in place of bovine chondrocytes, in a study carried out in collaboration with Dr. Hélène Autefage. The preliminary study showed seeding throughout the scaffold and viable cellular engraftment at 4 weeks with positive staining for sGAG by alcian blue (Figure 3-22).

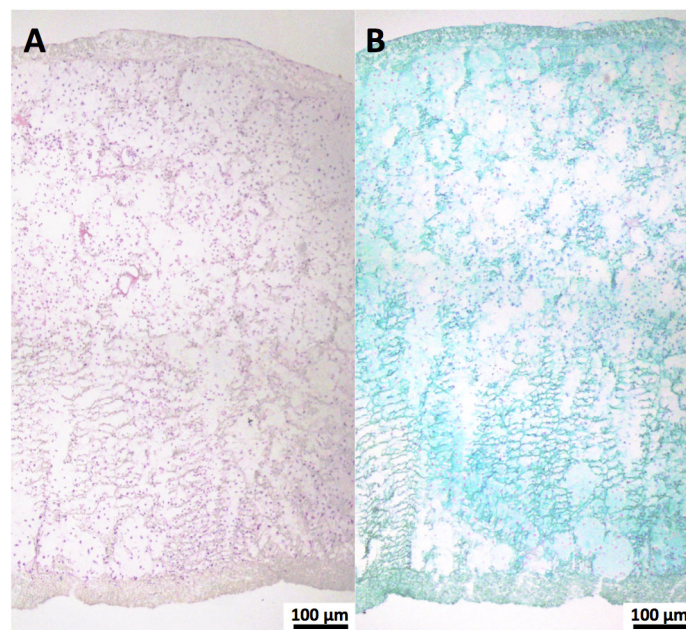


Figure 3-22. DF100 scaffolds seeded with hMSC and cultured under chondrogenic conditions for 4 weeks *in vitro*, stained with H&E (A) and alcian blue (B) to highlight general tissue and sGAG distribution, respectively.

A full zonal biochemical analysis was performed on the hMSC-seeded DF100 scaffolds at week 4; scaffolds were separated into their four constituent zones and analysed for DNA and sGAG (Figure 3-23). The production of DNA and sGAG followed the same distribution as the ECM produced by the

chondrocytes, with no statistical difference observed between the PLZ and DFZ (Figure 3-23A,B). For both DNA and sGAG the upper and lower fibre zones had values statistically different from the PLZ-DFZ zones and one-another. The quantity of DNA and sGAG in the hMSC-seeded scaffolds after 4 weeks of chondrogenic culture *in vitro* was comparable to that of P2 bovine chondrocytes. The sGAG/DNA ratios for all zones were not statistically different from the total sGAG/DNA ratio, indicating that hMSC in all zones behaved comparably, despite high variability in the lower FZ (Figure 3-23C).

Therefore, the preliminary hMSC investigation indicated that all zones of the DF100 scaffolds were able to support infiltration, viability, and ECM formation from a chondrogenically-differentiated hMSC population and generated sGAG-rich matrix comparable to that from expanded primary bovine chondrocytes.

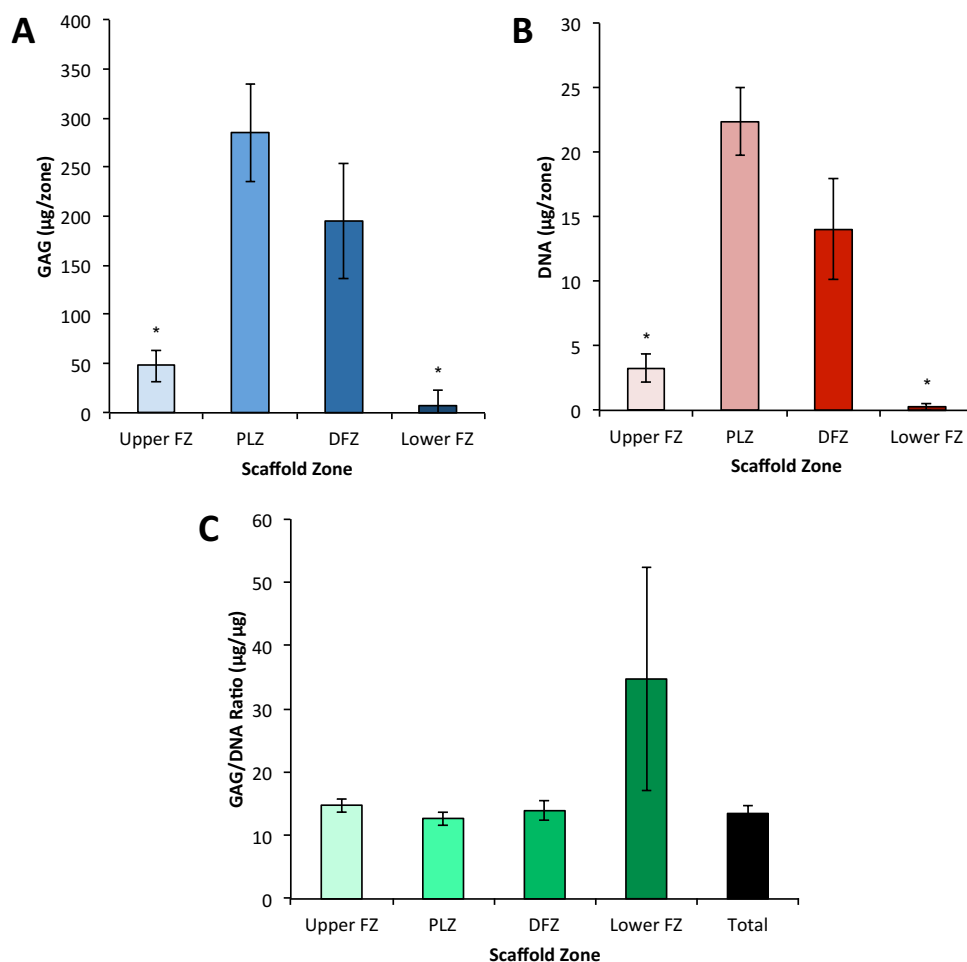


Figure 3-23. Zonal biochemical analysis of DF100 scaffolds seeded with 1M hMSC and cultured for 4 weeks *in vitro* under chondrogenic conditions. Biochemical content is illustrated by zone for sGAG (A) DNA (B) and sGAG/DNA ratio (C). n=4 with technical triplicates for all samples. (*significantly different from PLZ-DFZ, $p \leq 0.05$).

3.5.2 Injected Hydrogel Matrix

The seeding protocol was also modified with the incorporation of a hydrogel cell carrier developed in the group to encourage chondrogenesis. Hydrogels are used in conjunction with chondrocyte culture to maintain the cortical morphology of an adult chondrocyte, avoiding initial cell-material adhesion and resulting dedifferentiation [233]. A poly(ethylene glycol) (PEG) hydrogel with thiol and fluorescein bi-functionalised crosslinking agents prepared by collaborator Mr. Paresh Parmar was mixed with serum-free culture media and bovine chondrocytes at $10^6/\text{mL}$ and injected into a DF100 scaffold before setting. The hydrogel set quickly; however, as can be visualised in Figure 3-24, many of the pores were filled with both hydrogel and chondrocytes, but the cellular density in the DFZ appears to be significantly lower.

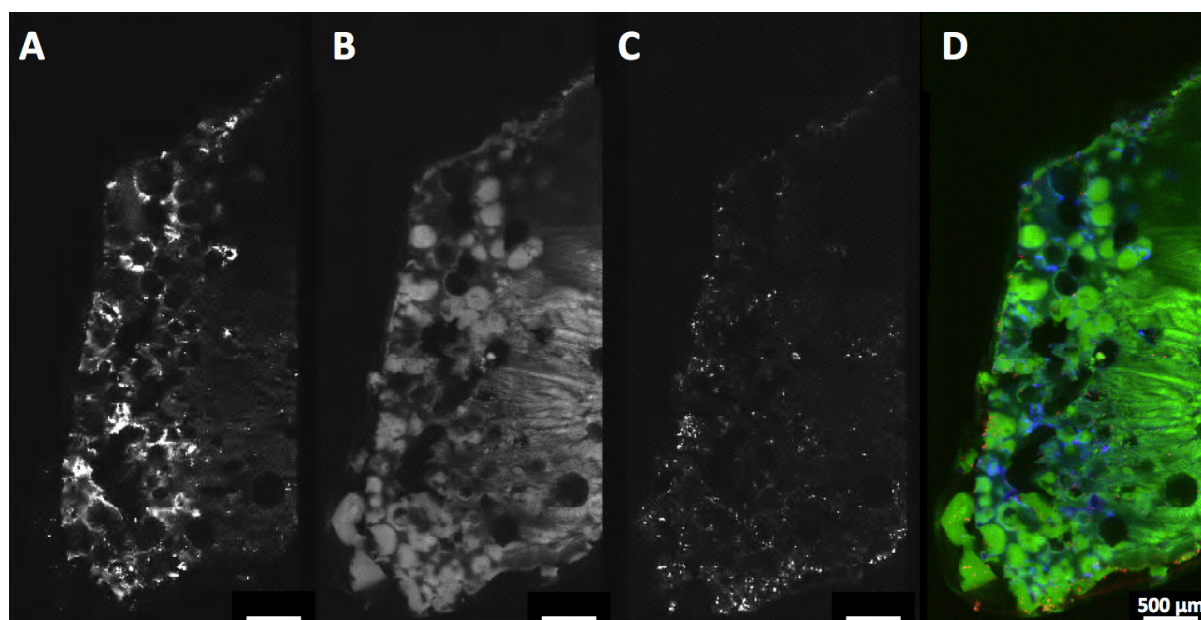


Figure 3-24. FITC-PEG hydrogel with bovine chondrocytes ($10^6/\text{mL}$) injected into DF100 scaffold and cultured overnight. A) DAPI, B) FITC, C) Alexa 648-phalloidin for actin, D) merged. Scale bars represent 500 μm .

A temporary hydrogel cell carrier could be optimised for the scaffold system, potentially further aiding in the retention of chondrogenic differentiation. Hydrogels tailored to direct chondrogenesis could be especially important clinically where adult chondrocytes would be slower to generate matrix and would be more likely to dedifferentiate.

3.5.3 Polymer Functionalisation

Throughout the previous two studies, PCL was used exclusively without further biofunctionalisation. On-going work by the Stevens lab has investigated the surface functionalisation of PCL with peptide sequences for the binding of endogenous chondroitin sulphate and hyaluronic acid [133]. The ability to functionalise the PCL surface with the addition of an additive to the manufacturing step would greatly increase the potential of the scaffold. To ascertain the applicability of such functionalisation schemes to the DF100 scaffold, a PCL-biotin polymer chain was produced by Dr. Lesley Chow of Imperial College London. This functionalised polymer was incorporated into PLZ and DFZ scaffolds at 1% (w/w), but otherwise the scaffold production methods were unchanged. Finished scaffolds were then labelled with fluorescein isothiocyanate (FITC) using streptavidin-FITC (Vector Labs, UK) (Figure 3-25). Both the PLZ and DFZ show full surface functionalisation, with the negative control showing no auto-fluorescence, indicating successful surface presentation of the biotin in the porogen-leaching and directional freezing protocols. One benefit of biotin functionalisation followed by streptavidin-FITC labelling is that only biotin present on the surface is able to bind with the streptavidin, indicating accessible surface functionalisation. As the purpose of testing functionalisation is for future surface modifications, the accessibility of the biotin or other ligand following scaffold production is key.



Figure 3-25. Porogen-leached (A) and directionally frozen (B) PCL scaffolds incorporating 1% (w/w) functionalized PCL-biotin and an unfunctionalised porogen-leached control for autofluorescence (C) stained with FITC-streptavidin, scale bars represent 200 μ m.

3.5.4 Alternate Geometries

Finally, the PLZ-DFZ foam was produced in a radial geometry for an angiogenesis application in collaboration with Dr. Adam Gormley. The central channel was designed to contain a hollow membrane of immunoisolated angiogenic cells, while the periphery would be seeded with endothelial cells. The radial channels would allow for the visualisation of cell migration through the scaffold. The production protocol was altered to use a central copper wire partially submerged in liquid nitrogen in place of a copper sheet, and the 300 μm NaCl porogen from Chapter 2 to line both the central pin and the outer wall. Otherwise, the protocol and resulting scaffold cross-section was remarkably similar (Figure 3-26B). Interconnectivity was confirmed by dye permeation and early *in vitro* work confirmed that the outer PLZ could be seeded with cells.

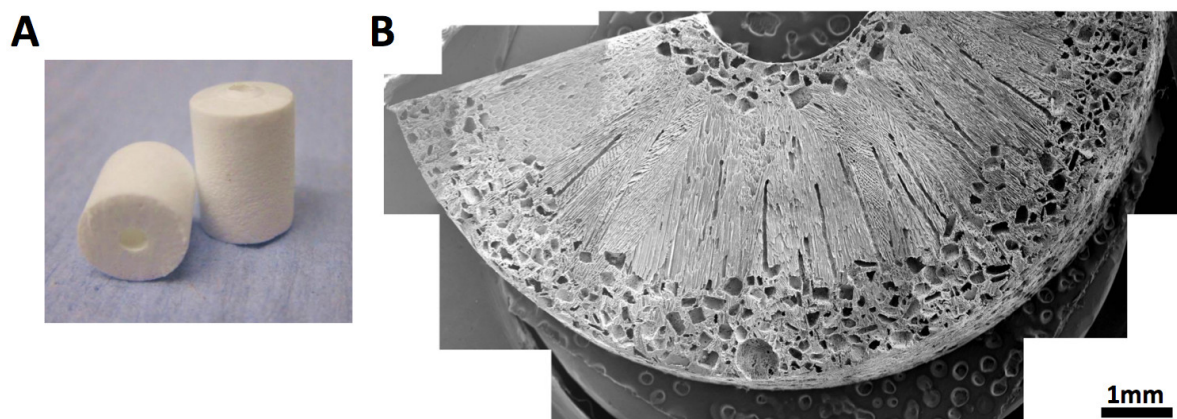


Figure 3-26. Radial variant of scaffold design. A) Cylindrical constructs produced and B) SEM micrograph displaying the radial PLZ-DFZ-PLZ zonal morphology.

4 The *in vivo* evaluation of anisotropic osteochondral scaffolds in a porcine model

4.1 Introduction

Three generations of anisotropic polymer scaffolds were developed to improve the state-of-the-art in matrix-based articular cartilage regeneration. The three designs and their advances are outlined in McCullen *et al.* [90], Steele *et al.* (Chapter 2) [1], and Chapter 3 of this thesis. The third generation scaffold design most closely mimics the zonal organisation of native articular cartilage both mechanically and in regards to alignment of the collagen deposited by passaged chondrocytes maintained under chondrogenic *in vitro* conditions. While *in vitro* culture is a powerful tool for scaffold optimisation, it does not adequately reflect the complex mechanical and biochemical environment of a native, functional joint that has sustained cartilage damage [234]. To determine if the optimised anisotropic polymer scaffold could aid in the formation of organised articular cartilage for the treatment of a focal osteochondral defect a large animal *in vivo* study was developed.

4.1.1 Large Animal Studies of Osteochondral Implants

The selection of an appropriate animal model is important in the study of osteochondral defects, as many commonly used and cost-effective animal models have limited translational value. The traditional animal models (typically mice, rats and rabbits) have small joints, thin articular cartilage and a superior intrinsic repair when compared to humans [234,235]. The zonal organisation of articular cartilage is not as pronounced in these small animal models and is therefore not suited to the evaluation of anisotropic scaffolds such as the one developed in this thesis. Large animal models have thicker articular cartilage and, when skeletally mature, have intrinsic repair rates and mechanisms that more closely resemble an adult human. However, for a pre-clinical test of an osteochondral scaffold, even canine, caprine, and mini-pig models suffer from articular cartilage layers significantly thinner than that of humans [235]. Hence, to truly mirror human dimensions, porcine and equine models are preferred [234]. Recent investigations of clinical studies and large animal models for osteochondral repair are summarised in Table 4-1. The table is limited to studies with full-thickness osteochondral defects in human (4), equine (1), porcine (8), ovine (3), and caprine (2) models. The clinical studies included are related to TruFit™ by Smith & Nephew and MaioRegen™ by FinCeramica, the two osteochondral scaffolds which have progressed to human

trials and (limited) commercial availability. As discussed in Chapter 1, TruFit CB™ for osteochondral applications has been withdrawn from the market due to cyst formation in clinical trials and is no longer available [103].

The primary insight gained from the review of the studies was the importance of a skeletally mature model. Additionally, in line with the difficulty in establishing a model with the correct cartilage thickness and zonal organisation, the majority of osteochondral studies in large animal models have been performed in a skeletally mature porcine model. Two studies used immature porcine models, including Sosio *et al.* [236] and Ho *et al.* [237], with detrimental consequences. Sosio *et al.* saw remarkable intrinsic repair in the 8 mm diameter defects due to the 3-month study being performed in a 4-month old porcine model. Many of the metrics ranked the empty defect as the most successful repair, with the histology indicating a near-complete intrinsic repair. The cell-free scaffold also performed significantly better than the chondrocyte-seeded scaffold, a result at odds with studies in skeletally mature porcine models [238,239]. The collagen scaffold used by Sosio *et al.* was remodelled quickly and was not visible in the histological study, potentially masking significant joint-growth. This issue was observed by Ho *et al.* who implanted osteochondral scaffolds within 6-month old animals that grew over 100% in mass with 22% and 32% increases in the width of the patellar groove and medial condyle respectively, by their 6-month time point. This joint growth resulted in the previously zonally aligned osteochondral scaffolds to be drawn entirely into the subchondral bone. The articulating surface either sealed-off the scaffold with a layer of bone and *de novo* cartilage, or formed a fissure of fibrocartilage that passed down into the joint to meet the scaffold [237].

Table 4-1. Recent clinical and large animal models of scaffold-based osteochondral regeneration

Author	Year	Model	Cartilage		Bone		Duration	Diam. or Area	Depth	Groups x N	Study Summary
			Material	Cells	Material	Cells					
Kon	2014 [240]	Human	Type I collagen	None	Type I collagen/HAp	None	5 yr	1.5-6.0 cm ²	6 mm	27	MaioRegen – In a 5 year follow-up, clinical scores significantly increased over the initial evaluation. At 5-years, complete defect filling was observed in 78% of the lesions, complete integration in 70%, and an intact repair tissue surface in 60.9%.
Hindle	2014 [102]	Human av 37.3 yr	PLG/calcium sulphate/PGA	None	PLG/calcium sulphate/PGA	None	1-5 yr	0.16-1.9 cm ²	8-12 mm	1 x 35 1 x 31	A study comparing the outcome of patients with mosaicplasty and TruFit. Concluded that while both had a statistically similar re-operation rate, mosaicplasty was superior, with significantly better outcomes in KOOS and EQ-5D scoring and faster return to sport.
Dhollander	2012 [100]	Human	PLG/calcium sulphate/PGA	None	PLG/calcium sulphate/PGA	None	1 yr	av 0.83 cm ²	8-12 mm	20	TruFit – At 1 year follow-up there were modest improvements in clinical scores. Following failure, 20% of implants were removed. Histology indicated the presence of fibrous vascularized tissue and foreign-body giant cells.
Kon	2010 [241]	Human	Type I collagen	None	Type I collagen/HAp	None	6 mo	1.5-5.9 cm ²	6 mm	13	MaioRegen - Scaffold attached and repair tissue present in 87% of patients and complete filling and smooth articular surface in 66%, as assessed by high-resolution MRI in all 13 patients and two second-look arthroscopies at 6 months. Oedema or sclerosis of the subchondral bone observed in 53% of patients.
Seo	2015 [242]	Horse 3.6 ± 2.3	gelatine/ 50% βTCP sponge	Auto. MSC	gelatine/ 50% βTCP sponge	Auto. MSC	4 mo	10 mm	5 mm	2 x 5	Sponges loaded with BMP-2 and autologous platelet-rich plasma and implanted below synovial membrane. Control was without synovial membrane. Treatment was histologically and macroscopically superior to the control group.
Steele	N/A	Pig 7 mo	Multilayer PCL	None	PCL βTCP strut	None	3 mo	5.5 mm	5 mm	2 x 2	One scaffold implanted in hind limb trochlear groove with empty defect control (n = 2). Implant fused with surrounding tissue, no sign of rejection. Scaffold migrated into subchondral bone due to joint growth. No evidence of bone ingrowth at 3 months.
Steele	N/A	Pig 24 mo (SM)	Multilayer PCL	None/Allo. Chond	Melt e-spun PCL	None	6 mo	6 mm	6 mm	4 x 6	Two scaffolds implanted in hind limb trochlear groove in skeletally mature pigs. Controls include MaioRegen and empty defect (n = 6). No complications following implantation. Study on-going.
Sosio	2014 [236]	Pig 4 mo	Type I collagen	Auto Chond	HAp	None	3 mo	8 mm	10 mm	3 x 12	Six defects per animal. The acellular control sample healed better than the cell-seeded scaffold by ICRS scoring. Untreated healed very well, defect hard to visualise at 3 months; biochemical analysis shows best GAG/DNA in untreated group. All treatments had low stiffness (20-100 kPa) at 3 months. Immature pigs were a poor model for osteochondral repair.
Zuo	2014 [252]	Pig (SM)	9:1 PLA-PGA 90% porous 100 µm pores	Auto. Chond.	N/A	N/A	6 mo	5 mm	2 mm	4 x 9	Full thickness defects created and left untreated for 8 weeks. Scaffolds implanted around mosaicplasty plugs with/without cells. Cells were either bone marrow mononuclear cells obtained on the day of implantation or 4-week <i>in vitro</i> pre-cultured chondrocytes. Repair with chondrocytes was better, but BMNC were superior to no-cells and empty defect.

Author	Year	Model	Cartilage		Bone		Duration	Diam. or Area	Depth	Groups x N	Study Summary
			Material	Cells	Material	Cells					
Jagodzinski	2013 [243]	Pig 14 mo (SM)	Type I/III collagen ChondroGide®	Auto. BMCC	Xenogenic bone TutoBone®	Auto. BMCC	3 mo	7 mm	10 mm	4 x 5	ChondroGide® type I/III collagen membrane was sutured in place on top of a TutoBone® xenogenic bone scaffold, with and without autogenic bone marrow cell concentrate (BMCC), with empty defect and inverted autogenic osteochondral cylinder controls. ICRS scores were higher for scaffolds with/without cells relative to controls which exhibited poor regeneration. The addition of BMCC had minimal impact on criteria other than slightly increased bone deposition.
Cui	2011 [239]	Mini-pig 7-8 mo (SM)	Porous PLGA	Auto. Chond.	Porous βTCP	Auto. OstBl./None	6 mo	8 mm	8 mm	3 x 8	Autologous chondrocytes and osteoblasts implanted into an osteochondral matrix in the weight bearing surface of the bilateral femoral medial condyles. Bone and cartilage zones sutured together before implantation. Groups included tissue engineered osteochondral, tissue engineered chondral, and empty defect. Osteochondral group produced statistically better repair.
Ho	2010 [237]	Pig 6 mo	Electrospun PCL	MSC in fibrin	βTCP-PCL	MSC in fibrin	6 mo	8 mm	5.5 mm	3 x 6	Implanted in the medial condyle and patellar groove. Repair achieved bone ingrowth, greater Young's modulus, and GAG production relative to controls. Cells and e-spun mesh improved outcome. Repair poorer in the patellar groove, joint grew around implants in both cases.
Im	2010 [244]	Mini-pig 23-25 mo (SM)	HAC- atelocollagen	Auto. Chond.	HAP-βTCP	None	5 mo	6 mm	7 mm	3 x 16 2 x 8	Four defects per animal in the medial and lateral condyles of the distal femur of both knees. Mechanically, treatments groups were similar to native cartilage. Col II staining throughout the subchondral bone, good morphology throughout.
Schleicher	2013 [245]	Sheep 2 yr	Type I/III collagen	Auto Chond.	Collagen/HAP	None	6 wk	9.4 mm	11 mm	1 x 6	One defect in the femoral condyle. Collapse of the graft, no cartilage produced, granulation tissue at surface, multinucleated macrophages throughout, potential cyst formation.
					Alogenic sterilised bone	None	6 wk	9.4 mm	11 mm	1 x 6	No cartilage formed on surface, bone interfaced well
Marquass	2010 [246]	Sheep 2-2.5 yr	Type I collagen hydrogel	Auto BMSC	βTCP, activated plasma	Auto. BMSC	6 mo 12 mo	4 mm	> 4mm	2 x 5	Chondrogenic pre-differentiation of MSC. Scaffold had superior cartilage bonding at 6 months. No significant macroscopic or mechanical difference from autograft at 12 months. Autograft showed superior cartilage matrix morphology at 12 months.
Kon	2010 [247]	Sheep 3-4 yr	Collagen	Auto Chond.	Collagen/HAP	None	6 mo	7 mm	9 mm	3 x 8	Maioregen – Integration of the chondral surface was observed in all groups except the control. Significantly improved bone regeneration was observed in the acellular scaffolds and autologous chondrocyte-seeded scaffolds. No difference in cartilage surface reconstruction and defect filling was observed between cell-seeded and cell-free groups.

Author	Year	Model	Cartilage		Bone		Duration	Diam. or Area	Depth	Groups x N	Study Summary
			Material	Cells	Material	Cells					
Kon	2014 [248]	Goat (SM)	HAc/ Aragonite (coral)	None	Aragonite (coral)	None	6 mo	6 mm	8 mm	4 x 7	Implants were replaced by hyaline cartilage and subchondral bone. The cartilaginous repair tissue showed a smooth contour and was well integrated into the adjacent native cartilage, with morphological evidence of hyaline cartilage confirmed by the presence of proteoglycans, collagen type II and the lack of collagen type I.
Miot	2012 [249]	Goat 1.5 yr	HAc (HYAFF®-11)	Auto. Chond.	65% HAp and HAc (HYAFF®-11)	None	8 wks. 8 mo	6 mm	400-500 µm	5 x 6-12	Scaffolds pre-cultured for 2 weeks performed best, only slightly better than 2-days, but better than 6-weeks, and acellular control. 6-week scaffolds had higher Col II/I ratio and GAG values, but didn't integrate as well, and had poor surface architecture.

Allo = allogeneic, Auto = autologous, BMCC = autogenic bone marrow cell concentrate, BMSC = bone marrow derived mesenchymal stem cells, Chond. = chondrocyte, Diam. = scaffold diameter, HAc = Hyaluronic acid, HAp = hydroxyapatite, OstBl. = osteoblast, SM = skeletally mature, βTCP = β tricalcium phosphate.

4.1.2 Cell Types Used in Osteochondral Models

If cells are seeded into the chondral portion of the scaffold, chondrocytes are the most commonly utilised cell population (Table 5-1). This observation is in agreement with the use of chondrocytes for full-thickness cartilage repair by autologous chondrocyte implantation (ACI) and matrix-induced ACI (MACI) [118,232,250]. The cell source for the subchondral bone portion of scaffolds is split between bone-marrow-derived cell sources and acellular approaches. Of note, the clinical trials in Table 5-1, including those of MaioRegenTM and TruFitTM, avoided the use of exogenous cells to facilitate translation of their products. Acellular approaches reduce the cost, risk, and logistical complexity of chondrocyte/MSC retrieval, amplification, validation, seeding, and potential need for cell expansion through *in vitro* culture prior to implantation. However, the logistical advantages of acellular scaffolds must be balanced with the reduced quality of repair [238,239,243].

Osteochondral reconstruction work performed in porcine models is focused primarily on autologous chondrocytes as a cell choice [239,244,251]. Minimal investigation has been performed into the immunogenicity of allogeneic cells in the porcine osteochondral model. Within the synovial joint, cartilage tissue formed from human juvenile allogeneic chondrocytes have been shown to be protected from the immune system in allogeneic and xenogeneic models by passive and active mechanisms [252]. Chondrocytes were shown to lack cell-surface markers required for T-cell induction (i.e. MHC Class II antigens) and to suppress the proliferation of activated T-cells through negative immune response regulators (i.e. chondromodulin-I and five members of the B7 family). Beyond surface markers, cartilage itself is immunoprivileged due to the lack of antigen presenting cells within the tissue and extensive ECM around the chondrocytes which sterically inhibits leukocyte mobility [252,253].

Miot *et al.*, employing a caprine model, suggested that 2-14 days of *in vitro* pre-culture of chondrocytes within the scaffold is beneficial to cartilage formation at 8-months *in vivo*. Most interestingly, the study also showed that excessive pre-culture (6 weeks) leads to a higher Col II/I gene expression ratio but can otherwise be detrimental, as the tissue integrates poorly, has lower GAG content, and develops different cellular architectures at the articulator surface [249]. This difference was attributed to the dense ECM in week-6 samples preventing the migration of precursor cells from the subchondral bone and neighbouring cartilage.

4.1.3 Control Groups

The correct selection of controls for a design greatly impacts the weight of conclusions that can be drawn from the subsequent analysis. Too often, studies use an empty defect as the only control external to their experimental groups [236,237,239,244,254]. While an empty defect is an important control for intrinsic repair, it only permits the claim that one or more experimental groups are better than no intervention. Some studies [243,246], compare a scaffold-based approach to autograft or mosaicplasty, allowing claims against a clinically proven intervention. While increasing animal numbers, the inclusion of an industry standard control greatly improves the value of the data generated, as the study can inform real world decisions and clinical practice. Without this control, the study can perform high-quality in-depth investigations on variations between experimental groups with/without cells, growth factors, pre-culture, etc., but does so in isolation from clinical context.

To validate the utility of the scaffold design and generate robust, translatable data, the decision was made to incorporate two experimental groups and two control groups into the study detailed in this chapter. The control groups were to be empty defect and a commercially available osteochondral scaffold industry control. The initial scaffold selected as a control was TruFit CBTM (Smith & Nephew, UK), as it was a PLGA-based osteochondral scaffold with an isotropic chondral zone [99,255]. However, over the course of scaffold development and *in vitro* validation, TruFit CBTM was pulled from the market. The control selected to replace TruFit CBTM was MaioRegenTM by FinCeramica (Italy), a scaffold with three isotropic zones of equine collagen and 0, 40, 70% (w/w) magnesium-enriched hydroxyapatite [241,254,256].

4.2 Scope of Contribution to Study

The following *in vivo* scaffold validation studies were performed in collaboration with A/Prof Mia Woodruff and the Medical Engineering Research Facility at the Queensland University of Technology, Brisbane, Australia. This team was selected due to their experience with polymer scaffolds in large animal bone defect models, their existing contributions to FDM scaffold design and successful previous collaborations. Additionally, their commitment to the highest quality animal husbandry, GLP-grade implantation, explantation, and histology ensured that the data collection would be robust and translatable.

The following is an outline of work that I performed in contribution to the collaborative project:

- The design, production, and sterilisation of osteochondral scaffolds for the preliminary and full-scale study.
- The selection of the animal model and writing of the study design, animal ethics, and surgical protocol, with subsequent assistance from MERF to align with their best practises.
- The histological sectioning (thick section, thin section, and paraffin), staining, and digitisation of preliminary study explants at Queensland University of Technology, Brisbane, Australia.
- The isolation and *in vitro* expansion of allogeneic porcine chondrocytes from osteochondral cylinders and scaffold at MERF, Brisbane, Australia.
- The seeding of pre-cultured scaffolds and *in vitro* culture at MERF, Brisbane, Australia.
- Assistance with full-scale study implantations, including sample preparation, documentation, and authorised assistance with pre- and post-operative care.

Chapter 4A – Osteochondral Implant Design

In the introductory aims of this thesis, the production of an osteochondral implant for *in vivo* investigation was outlined, prompting the design of chondral scaffold able to fuse with an off-the-shelf osteogenic scaffold, producing an osteochondral implant. The addition of the bone scaffold was intended to anchor the scaffold in the defect, improving integration at the osteochondral interface and the quality of defect repair. The third generation optimized scaffold design, comprehensively covered in Chapter 3, was modified for the *in vivo* study by the addition of an osteogenic scaffold beneath the lower fibre zone. The PCL scaffold design was designed such that it could be combined to a number of appropriate bone substitutes by heat bonding, as previously used in PCL implants by the group of Prof. Hutmacher [257].

The bone scaffold selected for the preliminary surgical study was composed of poly(ϵ -caprolactone) supplemented with osteogenic β -tricalcium phosphate (β TCP). Bone scaffolds from PCL- β TCP manufactured by fused deposition modelling (FDM) are currently used clinically, with commercial products from Osteopore International Ltd [258]. PCL is also widely used in the field of bone scaffold engineering, and has been investigated heavily by Prof. Dietmar Hutmacher and A/Prof. Mia Woodruff at QUT due to its appropriate mechanical properties, relatively slow biodegradation, and easy processing by additive manufacturing methods [130,258–260].

Strontium-substituted porous bioactive glasses (StronBone P) developed at Imperial College London were also considered for the lower portion of the scaffold. StronBone P had proven to osseointegrate more completely than any known bone substitute, producing higher-quality organised bone with low organic content, relative to controls. Despite their non-polymeric composition, fusion with the chondral scaffold was achieved (Figure 3-7A). However, it was decided to use the PCL fused-deposition modelling constructs as they were established in the field, were composed of the same polymer as the chondral scaffold, and would allow the scaffold to be assessed without the confounding effect of bioactive strontium ion release.

4.3 Materials and Methods

DF100 chondral scaffolds were produced according to the established SOP as outlined in Chapter 3 (Appendix E). Fused-deposition modelling scaffolds produced from poly(ϵ -caprolactone) in a 0°-60°-120° deposition pattern (Figure 4-1C) were kindly manufactured in 3 mm thick sheets by Prof. Dietmar Hutmacher (QUT, Australia) to form the subchondral bone portion of the scaffold. DF100 and FDM scaffolds were punched into 6 mm discs with sterile biopsy punches (Stiefel, Schuco, UK) and cleaned in 70% ethanol for 30 minutes. Scaffold components were then dried in a laminar flow hood under UV irradiation for 3 hours. Once dry, FDM scaffolds were melt-fused to the DF100 scaffolds according to established protocols for electrospun-fibre-FDM scaffold fusion [257]. To do so, the FDM scaffolds were suspended 10 mm above a 300°C hotplate for 10 seconds and immediately pressed onto an overturned DF100 scaffold. Scaffolds were allowed to cool, and were manually tested for mechanical integrity. Successful scaffolds were then sterilised in 70% (v/v) ethanol and dilute peracetic acid (PAA) according to the established SOP (Appendix F), and sealed in separate sterile vials for transport to the Queensland University of Technology Medical Engineering Research Facility (QUT MERF), Brisbane, Australia.

4.4 Results and Discussion

The final osteochondral scaffold, shown in Figure 4-1 was a cylindrical implant 6 mm in diameter and 5 mm in height with a 2 mm DF100 chondral portion and 3 mm FDM subchondral bone portion. The cylindrical osteochondral design was similar to that of TruFit CB, with the intent to act in a similar manner to that of mosaicplasty. Viewing the scaffold from the side (Figure 4-1A), the PLZ and DFZ zones of the chondral scaffold are visible, each composing 1 mm of the scaffold thickness. The electrospun superficial zone ensures a smooth articular surface (Figure 4-1B), while the FDM scaffold composed of alternating layers of parallel struts arranged in a 0°-60°-120° deposition pattern provides open pores for bone ingrowth (Figure 4-1C).

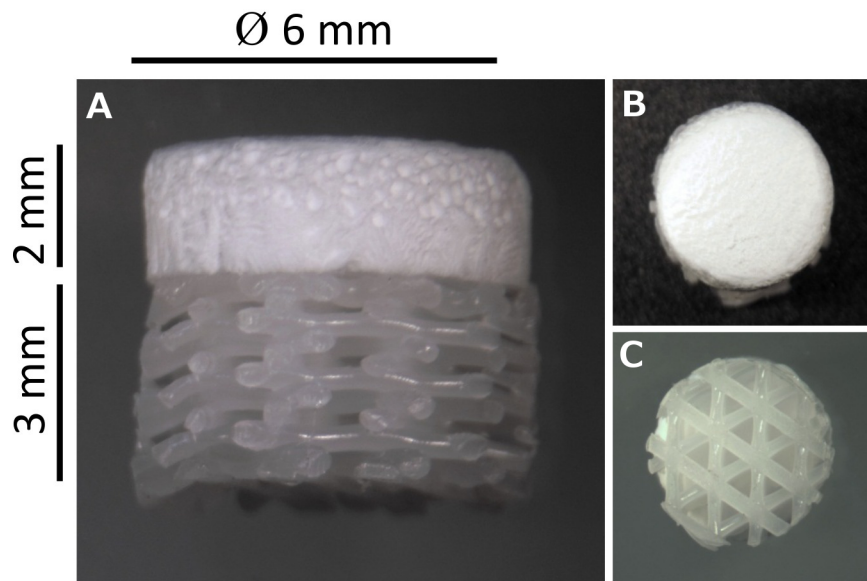


Figure 4-1. Preliminary surgical study osteochondral implant. The 2 mm-thick DF100 PCL cartilage scaffold is fused to the 3 mm-thick β TCP-PCL FDM bone scaffold. A) Side, B) top and C) bottom perspectives are shown.

The mechanical integrity of the scaffold fusion was confirmed by manual testing. Quantitative uniaxial tensile interfacial strength testing using a Bose Electroforce 3200 was attempted. The subchondral bone portion of the scaffold was held in place with a tensile clamp while the chondral scaffold was gripped with two perpendicular sutures run through the scaffold at the PLZ-DFZ interface. Testing was unsuccessful due to the sutures cutting into the chondral scaffold.

4.5 Conclusion

The integrated osteochondral scaffold was deemed appropriate for implantation and approved for implantation at the Medical Engineering Research Facility, Queensland University of Technology.

Chapter 4B – Preliminary *in vivo* Study

A skeletally mature 24-month old porcine model was selected for the full *in vivo* study with two 6 mm Ø defects in the trochlear groove of the right stifle joint, implanted to align the cartilage-bone interface to the scaffold zones (Figure 4-2).

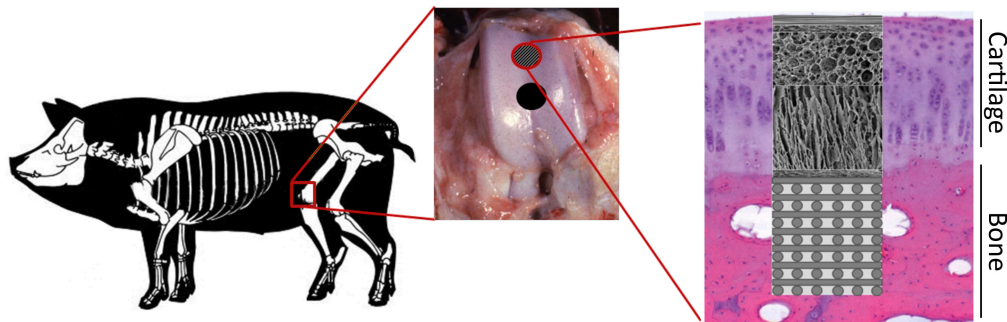


Figure 4-2. Placement of two scaffolds within the trochlear groove of the porcine stifle joint.

Refinement of the study design included placing two samples in the trochlear groove, reducing animal numbers by half as well as the use of only one joint per animal, improving mobility and reducing postoperative discomfort.

Additionally, the study was divided between a 3-month preliminary surgical pilot study and the full 12-animal 6-month investigation. This division provided an opportunity to investigate the surgical approach, scaffold implantation technique, recovery and animal husbandry before committing to the full study with larger animal numbers.

The preliminary study, which is complete and therefore the focus of this chapter, had only two conditions in duplicate: acellular scaffolds and empty defects. The full study, detailed in section 4C, is on-going, with twenty-four defects in twelve animals divided between four conditions (n=6):

- 1 – PCL osteochondral scaffold - acellular
- 2 – PCL osteochondral scaffold - pre-seeded with allogeneic chondrocytes and cultured *in vitro*
- 3 – Empty defect (intrinsic repair control)
- 4 – MaioRegenTM acellular scaffold (industry control)

Pooled allogeneic chondrocytes were selected for the cellularised condition to eliminate the need for a second procedure and instead use the tissue removed from defect drilling in the other 3 conditions. With the findings of Adkisson *et al.* [252] regarding the immunogenicity of allogeneic chondrocytes we were confident in the validity of this cell source. Additionally, there is a benefit in using a pooled allogeneic cell population, as it reduces donor-related variability between the replicates. Following the observations of Miot *et al.*, scaffolds were pre-cultured for 1 week *in vitro* under chondrogenic conditions.

Through analysis of existing large animal osteochondral studies and consultation with the Queensland University of Technology Animal Ethics Committee, a study was designed to ensure high quality data with clinical and translational relevance, while remaining mindful of animal numbers and wellbeing.

The overall aims of the project were:

1. To validate the small-breed porcine model at the Queensland University of Technology (QUT) Medical Engineering Research Facility (MERF) including the surgical approach, scaffold implantation technique, recovery and animal husbandry.
2. To validate the scaffold extraction and analysis protocols to produce sufficient evidence of superior healing and cartilage formation at 6-months relative to an industry control.
3. To implant the scaffolds with and without porcine chondrocytes and assess performance relative to an appropriate commercially available industry standard and an empty defect control.
4. To analyse the samples with validated techniques to allow for clear quantitative conclusions and produce a strong pre-clinical data package to raise interest in the biomedical industry, ultimately benefitting young patients with focal cartilage damage.

4.6 Materials and Methods

The following methods cover the complete preliminary surgical trial. Modifications and additions to these protocols for the full study are included separately in Chapter 4C. All cell culture materials were acquired from Invitrogen Australia and used as received, unless otherwise specified.

4.6.1 Ethics

All procedures, including the surgical pilot and the full-scale 6-month animal trial currently *in vivo* were approved by the Queensland University of Technology Animal Ethics Committee in July 2014 (Animal Research Ethics Approval Certificate: 1400000378). All work was performed at the QUT Medical Engineering Research Facility under strict QUT/MERF cGLP protocols.

4.6.2 Pre-Operative Care

Two female 70 kg large white cross saddleback pigs were obtained by MERF for the surgical implantation trial. Skeletally immature animals (7 mo) were used for the surgical trial as they were suited to the aims of the preliminary surgical trial and more easily sourced. Animals were examined by a veterinary surgeon prior to selection and quarantined for 2 weeks to ensure biosecurity.

Animals were starved of food for 12 hours, and water from 2 hours prior to anaesthetic induction. Each animal received an opiate/midazolam combination sedative prior to surgical anaesthesia. Vascular access was obtained through the placement of an 18G intravenous catheter placed in the lateral ear vein. Anaesthesia was induced with intravenous propofol, and maintained with inhalational isoflurane at 2-4% following the placement of a cuffed endo-tracheal tube.

Animals were placed on a surgical table in lateral recumbancy, with the desired limb on a table surface. The limb was shaved and aseptically prepared for surgery with chlorhexidine surgical scrub (5%), with a final wash in 70% (v/v) ethanol. The animals were draped with sterile re-usable drapes with surgical field exposed and an lobanTM dressing applied.

4.6.3 Surgical Implantation

A 15-20 cm parapatellar incision was made and the patella and patellar ligament exposed with blunt tissue dissection and haemostasis. The patella was then dislocated laterally exposing the trochlear groove. Two defects, 6 mm in diameter and 5 mm in depth, were created in the central trochlear groove using sequentially larger diameter drill bits with dimensions confirmed by calliper measurement. Care was taken not to damage the cartilage surrounding the defect. Thorough irrigation was used to remove any particulate matter from the defect and surrounding tissues. Sterile samples were placed into the defect site according to group designation, either in the proximal or distal defects. Samples were manually press-fit into the defect, flush with the surface of the joint.

The patella was then relocated and all internal tissue layers sequentially closed with absorbable sutures, with particular attention given to any divided muscle tissue to ensure satisfactory tissue apposition prior to wound closure. The skin was closed with 0-Vicryl interrupted sutures following adequate haemostasis. The wound was cleaned and dressed with an Ioban™ dressing.

Animals were recovered from surgery with extubation on return of laryngeal reflexes. Pulse-oximetry, electrocardiography, and capnography were monitored throughout with animals only returned to a recovery area once conscious and stable. Animals were given access to food and water once weight bearing and fully conscious.

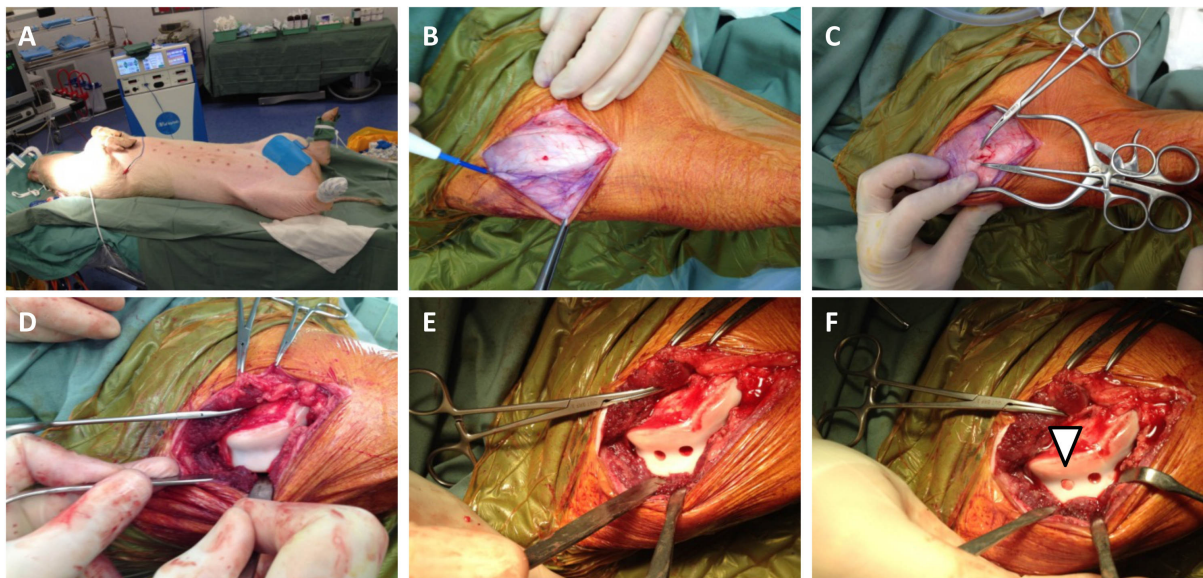


Figure 4-3. Surgical implantation at MERF. A) Animal is positioned for surgery, B) Parapatellar skin incision, C) Joint capsule opened, D) patella dislocated laterally to expose the trochlear groove, E) Two Ø6mm x 5mm deep cylindrical defects made in the femoral trochlear ridge, F) Acellular scaffold placed in one site (▽), the other site treated as empty defect control.

4.6.4 Post-Operative Care

Post-operatively, animals were maintained in recovery slings for 2 days with limited weight bearing. Animals were closely monitored for 72 hours post-surgery, with wound inspection, appetite and lameness assessment as priorities. During this period a daily analgesia protocol of low-dose intramuscular buprenorphine and oral NSAIDs were administered, and adjusted according to individual requirements.

Acute monitoring in the first seven days consisted of observing the animals' demeanour, being vigilant for signs of pain or distress. Particular note was made of appetite, thirst, urination,

defecation, interest in surroundings, and mobility. The wound dressing was removed several days following surgery, the wound inspected, and the incision sprayed with antiseptic.

After the first week, the animals were observed three times per week in the paddocks. Notes were made regarding their demeanour, appetite, drinking, weight change, mobility and any signs of pain or distress. The surgical site was observed for any signs of infection or inflammation, none of which were detected.

4.6.5 Sample Recovery and Preparation

Animals were euthanized at 3-months, the defect areas harvested and gross pathology of the defect site was recorded. Samples were fixed in a 4% (w/v) paraformaldehyde solution at room temperature for 24 hours. Fixed samples were cut in half, bisecting the scaffold perpendicular to the articulating surface to expose the full osteochondral gradient and integration with surrounding bone and cartilage. Samples were then transferred to 70% (v/v) ethanol and stored at 4°C. Samples are designated QM00#X where # is the animal number (1 or 2) and X is the defect (A – empty defect, B – acellular scaffold).

4.6.6 Micro-computed tomography

Bisected samples were scanned in 70% (v/v) ethanol in a micro-computed tomography (μ CT) scanner (μ CT 40, Scanco Medical, Brüttisellen, Switzerland) by collaborators at QUT. The scans were reconstructed with an isotropic voxel size of 30 μ m.

4.6.7 Ground Resin Histology

One bisected sample from each condition for QM002 were embedded in poly(methylmethacrylate) (PMMA) resin (Technovit 9100 NEU, Heraeus Kulzer, Wehrheim, Germany) without decalcification. Thick sections (~200 μ m) were cut using a low-speed diamond saw (Buehler Isomet, Buehler GmbH, Düsseldorf, Germany) and ground to 50 μ m for histological analysis with a series of silicon carbide grit papers on a grinder followed by handheld polishing with 1 μ m alumina oxide paste. Sections were stained with Goldner's trichrome stain to visualise tissue morphology and mineralisation.

4.7 Results and Discussion

Macroscopic evaluation of the joint surfaces at the 3 month time point reveal well-integrated scaffolds with no evidence of rejection (Figure 4-4). Qualitatively, the acellular scaffold group exhibits more complete restoration of joint contour and cartilage colouration relative to the empty defect control.

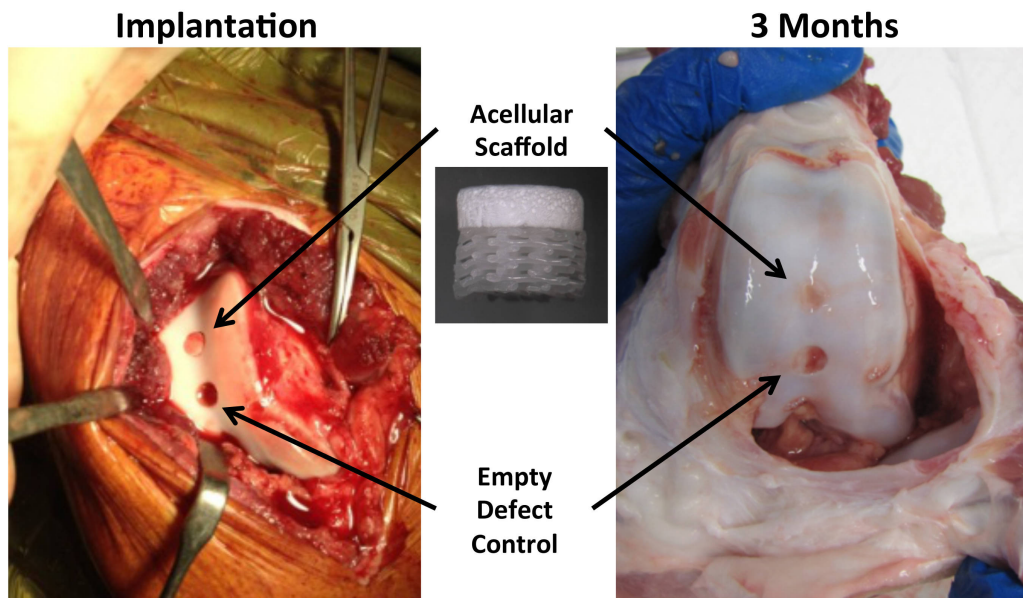


Figure 4-4. Joint surface at implantation and 3-month termination of the preliminary surgical trial, showing acellular and empty defect sites in the trochlear groove for animal QM002.

Subchondral bone morphology at 3 months was assessed by μ CT (Figure 4-5). All defects and animals exhibited a degree of subchondral bone repair at 3 months. However, the two animals healed differently, with more intrinsic repair in the empty defect of QM002 but more bone ingrowth into the bone scaffold in QM001. The empty defect of QM001 (Figure 4-5A) shows a cylindrical void in the subchondral bone, smaller than the original 6 mm x 5 mm defect, indicating limited bone repair. The empty defect in QM002A (Figure 4-5B, C) shows a bony projection into the empty defect and a greater degree of repair. The cross-sectional bone morphology of QM002 is highlighted in greater detail, indicating the approximate location of the drill site (Figure 4-5C,D) and clarified in the ground resin sections of the cross-section stained with Goldner's trichrome (Figure 4-6A).

Whereas QM002 showed greater intrinsic repair in the empty defect, QM001 showed a greater degree of repair in the acellular scaffold system. In QM001B, aside from a cylindrical void 1-2 mm deep within the bounds of the original defect, the remainder of the bone appears to have healed (Figure 4-5A). In comparison, in QM002B (Figure 4-5D) there are indications of poor bone ingrowth

within the FDM scaffold, scaffold migration into the subchondral bone, and bone remodelling in the periphery of the chondral scaffold, this is more easily visualised histologically (Figure 4-6B).

The shifting of the scaffold into the subchondral bone is attributed to the growth of the animals increasing from 60 kg and 61 kg to 100 kg and 105 kg respectively, an average increase of 69% over 3 months. The resultant joint growth has caused the scaffold to be drawn into the subchondral bone as was also observed by Ho *et al.* [237]. This observation was not unexpected, as younger animals were used for the pilot study due to the unavailability of skeletally mature animals for the preliminary trial. As the primary aim of the initial investigation was the validation of the surgical protocol, recovery, animal husbandry, and the technical aspects of sample retrieval and processing, the risk of scaffold subduction was a secondary concern. Of note, from the μ CT visualisation of the subchondral bone, the shifting of the scaffold in QM001B appears to be less prominent, with no bony projections formed over the DF100 cartilage scaffold, as is observed in QM002B. However, the presence of a cylindrical void in the subchondral bone suggests the final location of the DF100 scaffold and therefore zonal misalignment and scaffold overgrowth with soft tissue. Without a skeletally mature model or an early time point for comparison, the possibility that the misalignment occurred during implantation cannot be excluded.

The remodelling of the subchondral bone adjacent to the DF100 scaffold, while not observed in the other scaffold condition (QM001B), is of interest. The remodelling, only observed in the condition with the bone overgrowth may be due to an immune-response from the surgical procedure. Alternatively, the irritation could be due to the accumulation of 6-hydroxyl caproic acid, the degradation by-product of PCL; however, as PCL degrades slowly and 6-hydroxyl caproic acid is readily metabolised in the citric acid cycle, this is less probable [132].

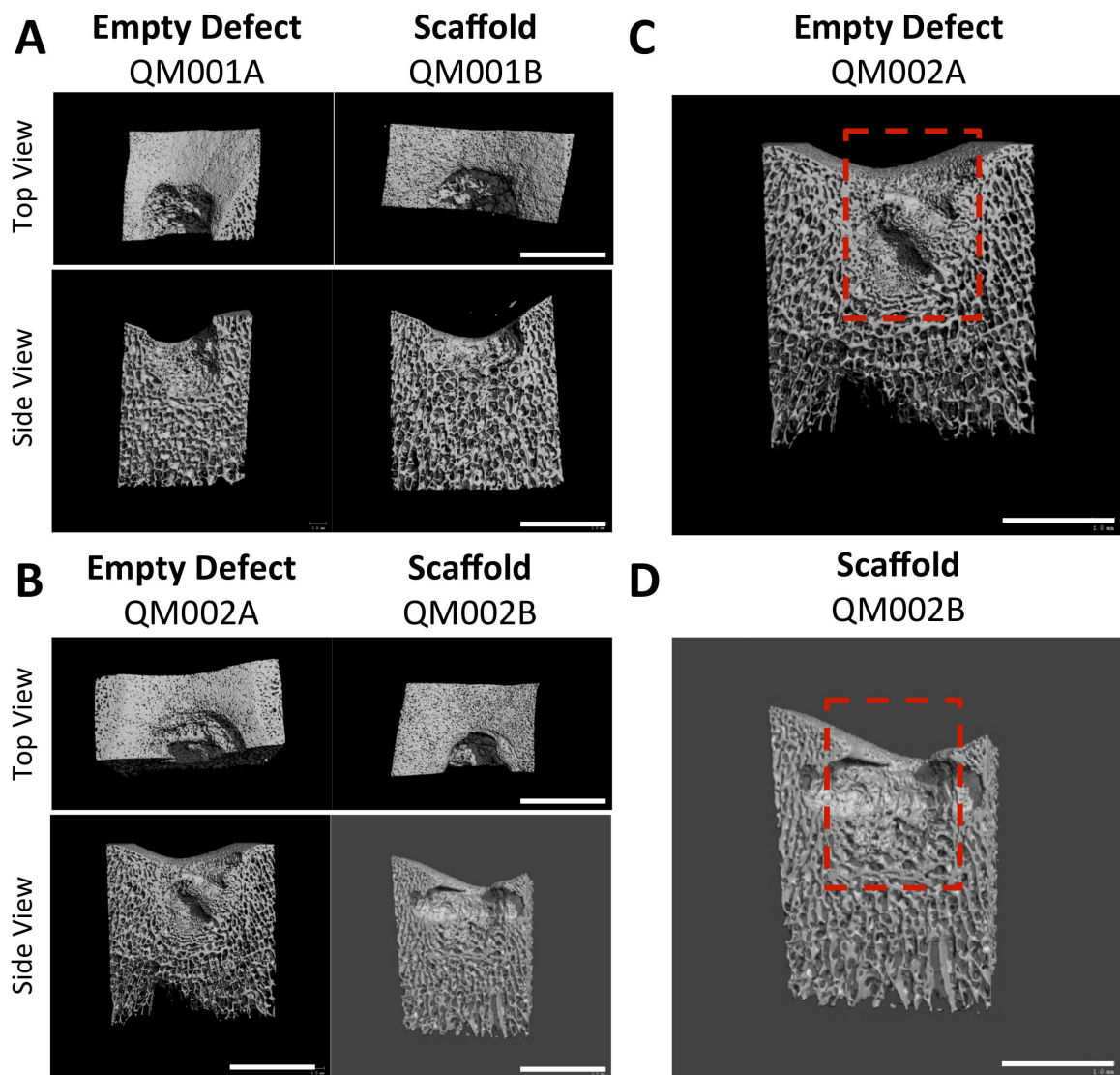


Figure 4-5. Micro-CT of bisected trochlear samples at 3 months for the two surgical trial animals (A, B). Enlarged cross-sections for QM002 indicating the approximate implant site. Scale bar represents 5 mm.

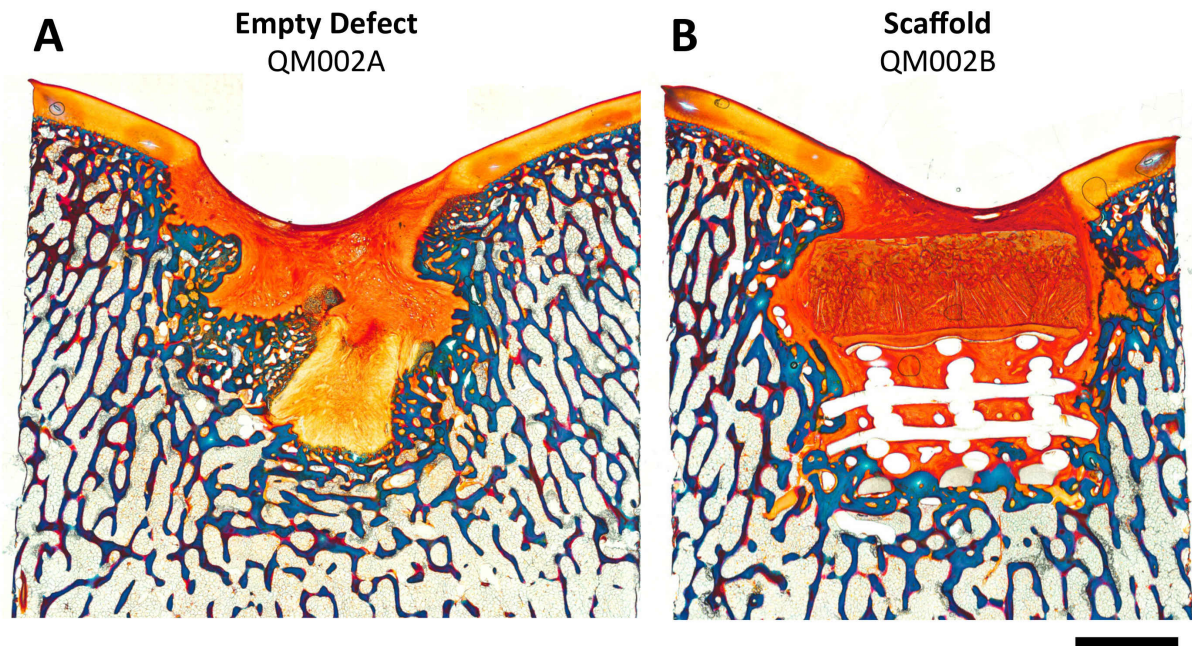


Figure 4-6. Ground resin sections of an (A) empty defect (QM002A) and (B) acellular scaffold (QM002B) from the same animal at 3-months. Sections stained with Goldner's trichrome. Scale bar represents 2mm.

The purpose of this preliminary trial was to validate the surgical procedure, animal care protocols, sample retrieval and processing for CT and histology. As these aims were all successfully achieved, it was tempting to prematurely analyse the data and draw early conclusions. However, with only two disparate replicates in an immature model no conclusions can be drawn, and must await the completion of the full-scale study.

The scaffold subduction and subchondral bone overgrowth observed in the preliminary trial are anticipated to be significantly less prominent in the full-scale study, as they will be conducted in skeletally mature animals.

4C – Full-Scale Study Design

Following the successful surgical outcome of the preliminary pilot trial, the full-scale study was organised, with adjustments to the animal model and scaffold design.

Twelve skeletally mature (24 mo) female small breed pigs (Large white cross-bred, 70-100 kg) were sourced for the study, to avoid joint growth and scaffold misalignment. To improve bone ingrowth, the FDM polymer scaffold was replaced with a melt-electrospun scaffold, as detailed in Section 4.7.1. The study is on-going, with the revised protocols (where applicable), and initial post-surgical recovery outlined in the following subsections.

4.7.1 Scaffold Production

The same DF100 cartilage scaffold design was used for the full study, however, an alternate bone scaffold was used for the lower portion. The new bone scaffold, kindly provided by A/Prof. Mia Woodruff (Queensland University of Technology, Australia) was produced by the technique of 3D direct writing melt electrospinning, currently being developed at QUT. The new scaffold was selected as it is currently under investigation by our collaborating partners A/Prof Woodruff and Prof. Dietmar Hutmacher for the regeneration of bone and allows for previously unavailable control over three-dimensional structure and porosity. The 4 mm thick scaffold was in the form of an open-pore lattice composed of 120 layers of 20 µm diameter fibres spaced 200 µm apart in a 90° square grid. The scaffold was printed from PCL (Perstop CAPA 6400) using custom-built equipment. The PCL was heated to 73°C and extruded at 40 µL/h from an 18G needle charged to +3.2 kV. The polymer was extruded onto an x-y-z computer-controlled collector plate charged to -3.2 kV. The scaffolds were treated in the same manner as the FDM scaffolds: united with DF100 by heat-bonding, producing 6 mm Ø x 6 mm osteochondral scaffold plugs. Scaffolds were sterilised by PAA-treatment in the same manner as previously outlined (Appendix F).

4.7.2 Chondrocyte Isolation and Expansion

Osteochondral plugs were obtained from defects produced for acellular experimental groups and controls. Tissue plugs were stored for up to 24 hours in expansion media containing Dulbecco's Modified Eagles Medium (DMEM) with 1.0 g/L glucose, 1% (v/v) Penicillin-Streptomycin (P/S), 1% (v/v) MEM Non-Essential Amino Acids (100x), 1% (v/v) HEPES (100x), 0.4 mM L-Proline, 0.1 mM ascorbic acid, and 10% (v/v) foetal bovine serum (FBS). Cartilage was excised from the plug surface, pooled, and digested at 37°C in 0.15% (w/v) collagenase type 2 (Worthington, Australia) in DMEM

overnight in a sterile spinner flask at 60 rpm. The digest was filtered to remove ECM debris, washed in expansion media, and plated in tissue culture flasks. Chondrocytes were expanded to passage 2 (P2) and used directly without cryopreservation. This degree of expansion matches the *in vitro* validation and is within the range used clinically for ACI and MACI procedures.

4.7.3 Scaffold Seeding and Culture

Scaffolds were seeded in a similar manner to that of the high-density DF100 scaffolds in Chapter 2. Briefly, P2 chondrocytes were suspended with 0.5% trypsin and rinsed in serum-free chondrogenic media composed of DMEM (4.5 g/L glucose), 1% (v/v) ITS (100x), 1% (v/v) P/S, 1% (v/v) MEM Non-Essential Amino Acids (100x), 1% (v/v) HEPES (100x), 1% (v/v) GlutaMAX (100x), 0.4 mM L-Proline, 0.1 M dexamethasone, 0.1 mM ascorbic acid, and 10 ng/mL TGF- β 3. Live cells were quantified by haemocytometer and trypan-blue staining and concentrated to 10M/50 μ L. Sterile scaffolds were rinsed three times in sterile PBS prior to cell seeding. Cells were injected into the scaffolds in a 45 μ L bolus (9×10^6 cells) through a 25G needle into the PLZ, bevel facing towards the DFZ. A further 5 μ L drop (1×10^6 cells) of the cell suspension was deposited onto the top of the scaffold. The bone scaffold was rinsed with sterile PBS and the scaffolds were transferred upright to a 50mL Falcon tube with a filter cap and incubated at 37°C, 5% CO₂, 100% RH for 2 hours. The scaffolds were then suspended in chondrogenic media within the falcon tubes and cultured for 1 week, with media changes every 2 days.

On the day of implantation, scaffolds were rinsed and transported in sterile PBS, with no more than 15 minutes between removal from the incubator and implantation.

4.7.4 MaioRegen™ Preparation

MaioRegen™ (JRI Orthopaedics, UK) was purchased in the form of a sterile 35 mm x 35 mm x 6 mm sheet. Control scaffolds were prepared in a sterile laminar hood at 6 mm $\varnothing$, and stored with the acellular osteochondral scaffolds under sterile conditions until implantation.

4.7.5 Implantation

The pre-operative surgical, and post-operative protocols used in the full study are identical to those of the preliminary surgical trial, with the exception of the creation of a deeper 6 mm defect depth to accommodate the larger scaffolds, and the additional conditions. Six replicates for each group were implanted into the 24 defects. The empty defects, acellular scaffolds, and MaioRegen™ controls were paired randomly, while the cellularised scaffolds were implanted together in pairs in three animals. The segregation of the cellularised scaffolds from the other conditions was performed to

isolate the effect of any immune response to the allogeneic cell population and to mitigate the effect of chondrocyte migration through the synovial fluid to the neighbouring defect site through joint movement.

4.8 Preliminary Observations

All animals were weight bearing immediately following anaesthetic recovery. The immediate post-operative period was uneventful for all animals, with none of the potential complications described for the para-patellar surgical approach (e.g. patellar subluxation or wound dehiscence).

Since April 2015 the animals have been housed at the MERF rural farm facility, with regular access to exercise and daily monitoring. No complications have been reported to date. Euthanasia is scheduled for autumn 2015 with comprehensive analysis continuing into 2016, which is beyond the scope of this thesis.

4.9 Conclusion

A large animal surgical model was developed at Queensland University of Technology Medical Engineering Research Facility (QUT MERF) through collaboration with A/Prof. Mia Woodruff. A small-breed porcine model was selected to model the geometry of the adult human joint.

The surgical protocol, animal husbandry, sample retrieval, and analysis were validated in a two-animal preliminary surgical trial in 7 month old female pigs. The preliminary trials indicated full recovery without adverse immunological reaction, improved joint resurfacing with the scaffold relative to empty defect controls, and no sign of scaffold rejection. Sample processing for μ CT and ground-resin histology were also successful.

Preliminary data collected from the study show the artefacts of 69% animal growth and resulting joint growth. Significant intrinsic repair was observed in one of the empty defects, and in another, the scaffold was drawn into the subchondral bone, resulting in bony projections and bone remodelling in the periphery of the DF100 scaffold in line with expectations

The full-scale study, with four conditions and six replicates per condition used a commercially available control and allogeneic P2 chondrocyte-seeded additional conditions. No adverse effects were reported during immediate recovery and throughout the 5 month experimental period that has elapsed to date. The inclusion of a commercially available osteochondral scaffold control will permit higher-impact conclusions and facilitate human translation.

5 Investigation of alternate biodegradable polymer compositions for cartilage tissue engineering

5.1 Introduction

A wide array of natural and synthetic polymers are used in the production of tissue engineering scaffolds, each with unique cellular responses, mechanical properties, ease of functionalisation, ease of processing, degradation rate, and degradation by-products. Polymers for biomedical applications are summarised briefly in section 1.8.1, and in comprehensive reviews [261,262].

Poly(ϵ -caprolactone), an aliphatic polyester, was the polymer used in the investigations detailed in Chapters 2-4. The history and breadth of research into PCL as a biomaterial was comprehensively reviewed by Woodruff and Hutmacher [130]. Briefly, benefits of PCL include the ease of processing via different scaffold production modalities and solvents, inexpensive production, and a controlled degradation rate, largely independent of cellular processes.

The following brief studies outline investigation into co-polymer formulations, all of which include the ϵ -caprolactone monomer but have been supplemented with one or more additional monomers to improve supercritical foaming of scaffolds (Chapter 5A) or scaffold elasticity and degradation rate (5B and 5C). The polymers were produced in collaboration with Prof. Charlotte Williams and Dr. Min Tang (5A), synthesized independently with the assistance of Dr. Robert Chapman (5B), and kindly donated by Dr. Seth McCullen of Poly-Med Inc. (5C).

The scope of the following three investigations are all unique, but focus on how the alteration of the polymer chemistry modulates their utility as a material for cartilage tissue engineering, relative to PCL.

Study 5A – Cytocompatibility of acetyl-modified poly(ϵ -caprolactone) copolymer for supercritical foaming

The work contained in Chapter 5A is the cytocompatibility validation study and materials characterisation performed as part of an investigation by Dr. Min Tang and Prof Charlotte Williams, Department of Chemistry, Imperial College London. The data was included in Tang *et al.* 2013 [263], however, all data, figures, and analysis within the results and discussion are my own. Figures and tables are reproduced and adapted with permission from Tang *et al.*, copyright 2013 American Chemical Society (Appendix G).

5.2 Introduction

Tissue engineering scaffolds are often designed as open-pored structures that provide a mechanical support and architecture for cellular seeding and guided tissue production. In many cases, the scaffolds are designed to breakdown over time, either through active degradation by cells or through simple hydrolysis, independent of the cellular population. A commonly used polymer in tissue engineering scaffold design is poly(ϵ -caprolactone) an aliphatic polyester that degrades by hydrolysis; however, PCL is hydrophobic and lacks functional domains, reducing its biocompatibility and slowing degradation [130]. To increase hydrophilicity, a modified caprolactone monomer (Monomer 1) was developed by Dr. Min Tang from naturally occurring D-gluconolactone. Monomer 1 contains two acetyl functional groups, relative to caprolactone that are responsible for the increased hydrophilicity (Figure 5-1) [264]. Monomer 1 was copolymerised with caprolactone to produce random copolymers of varying compositions, from 0 to 25 mol% monomer 1.

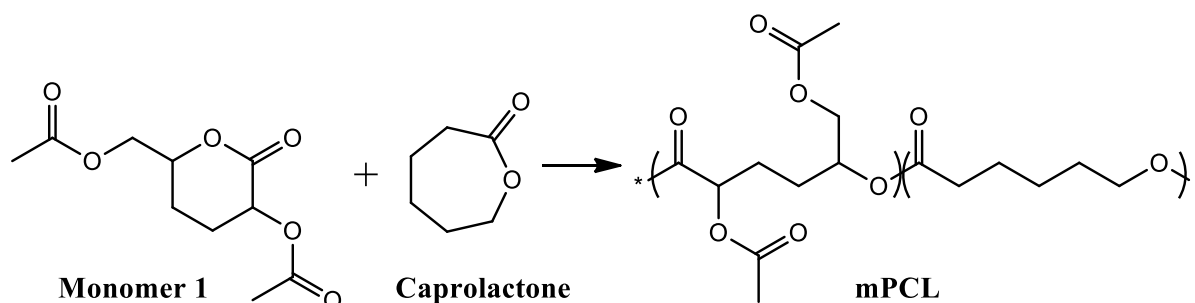


Figure 5-1. Synthesis of mPCL by ring opening polymerisation of monomer 1 and caprolactone
Modified from Tang *et al.* [264].

The ability to load polymer scaffolds with growth factors and bioactive proteins has therapeutic applications for drug delivery and cell homing. However, these approaches are traditionally limited to hydrophilic polymers and hydrogels as the techniques used to form scaffolds from many polymers involve harsh organic solvents and/or elevated temperatures that can cause denaturation of proteins [265]. Supercritical carbon dioxide (scCO₂) is able to dissolve many polymers at low temperatures (74 bar, 31°C), facilitating blending with proteins and other biomolecules. Additionally, the dissolved CO₂ can serve a second purpose, nucleating bubbles within the polymer as the pressure is reduced, forming a foam [189,266]. However, the application of this scCO₂ foaming technique to PCL has necessitated polymer blending or temperatures above 60°C; otherwise, there are issues with pore interconnectivity, which is paramount to applications as a tissue scaffold [130,189,264,266,267] .

The acetyl groups that improve the hydrophilicity of the caprolactone monomer in monomer 1 also aid in the solubility of the polymer with scCO₂, reducing the processing temperatures required and allowing for the incorporation of selected bioactive proteins.

Ten copolymers were produced by Tang *et al.* with varying monomer 1 compositions. Following physical characterisation and foaming studies (Figure 5-2), pure PCL and four copolymers with 1, 2, 3, and 4 mol% monomer 1 (P1, P2, P3, and P4, respectively) were selected. The addition of 4 mol% monomer 1 to PCL decreased the molecular weight of the polymer from 178 kDa to 65.9 kDa [264]. With the reduction of molecular weight and altered chemistry, the melting point of P4 also decreased to 50.5°C from 54.3°C for pure PCL.

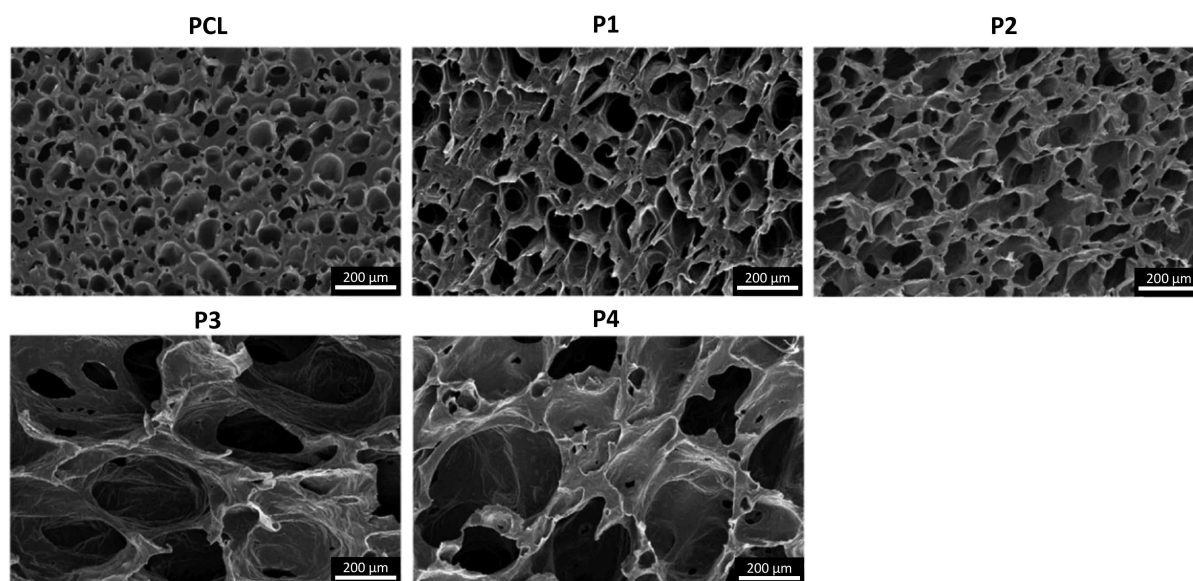


Figure 5-2. Supercritical CO₂ foaming study of monomer1:CL copolymers. PCL) 0:100, P1) 1:99, P2) 2:98, P3) 3:97, P4) 4:96. Modified from Tang *et al.* [264]. Scale bars 200 μm.

The morphology and stiffness of the resulting foams were also highly dependent on the composition, with pore sizes increasing from $71 \pm 17 \mu\text{m}$ to $319 \pm 91 \mu\text{m}$ and compression moduli decreasing from 22.8 MPa to 6.8 MPa between 0% and 4 mol% monomer 1 [264]. It was difficult to interpret the direct effect of polymer chemistry on these properties as the stiffness, pore size, and melting point were confounded with the decreased molecular weight. For this reason, the study of biocompatibility was performed predominantly on polymer films as they had a consistent surface area and topographical microenvironment for all polymer compositions.

PCL, P1, P2, P3, P4, and P5 were initially selected for polymer cytocompatibility investigation and protein release studies, while only PCL and P4 were selected for cytocompatibility investigations on scCO₂ foams.

5.3 Materials and Methods

5.3.1 Materials

All chemicals were acquired from Sigma Aldrich, UK and all cell-culture materials were acquired from Invitrogen, UK and used as received, unless otherwise specified.

Scaffolds in the form of pre-foamed cylinders and hot-rolled films were obtained from Dr. Min Tang, Imperial College London. All polymers, mPCL and PCL, were synthesized by Dr. Min Tang for this experiment through ring-opening polymerisations of purchased caprolactone and synthesized monomer 1, detailed in Tang *et al.* [264].

5.3.2 Cytocompatibility - ISO10993-5

MC3T3-E1 pre-osteoblasts (ATCC, Germany) were selected to evaluate the cytocompatibility of the polymers in accordance with ISO 10993-5. MC3T3-E1 were seeded at $2 \times 10^4/\text{cm}^2$ in 96-well tissue culture-treated plates and cultured in Minimum Essential Medium α (α -MEM) with 10% (v/v) foetal bovine serum (FBS) and 50 $\mu\text{g}/\text{mL}$ gentamycin at 37 °C, 5% CO₂, 100% RH. Cells were expanded for 48 hours to form a subconfluent monolayer. Hot-rolled polymer films (PCL, P1-P5), scCO₂ foams (PCL and P4), nontoxic PVC tubing (Med7539 noDop) (negative cytotoxicity control), and organo-tin stabilized PVC sheet (Raumedic, Germany) (positive cytotoxicity control) were sterilized with 70% (v/v) ethanol for 30 minutes, washed three times with sterile PBS, and pre-treated in complete medium. Following 24 hours pre-treatment, media was exchanged for fresh complete medium at 5

mL/g polymer and conditioned for 24 hours at 37 °C, 5% CO₂. The conditioned media was collected and used at 100% concentration to culture the MC3T3-E1 monolayers. Viability was assessed at 72 hours with the 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The insoluble MTT product was quantified in DMSO by the difference in absorbance between 570 and 650 nm on a Spectra Max M5 plate reader (Molecular Devices, UK), with values normalized to the negative control.

5.3.3 2D Film MC3T3-E1 Culture and Metabolic Activity

PCL and P1-P5 hot-rolled films were punched into 8 mm diameter disks and sterilized in ethanol, washed, and pre-treated in complete medium as previously stated. MC3T3-E1 cells were seeded onto the disks at $2 \times 10^4/\text{cm}^2$ and cultured in non-adherent agarose-coated wells for 14 days.

The metabolic activity of the samples at 14 days was assessed by the Alamar Blue assay, using a 1:10 dilution in complete medium incubated with the MC3T3-E1 for 4 hours. The medium was removed and analysed at 540 nm excitation and 580 nm emission. Six replicates were analysed in duplicate for each polymer formulation.

5.3.4 3D Foam Primary Chondrocyte Culture

Primary bovine chondrocytes were isolated from the metacarpal-phalangeal joints of 24-month old animals as previously outlined in section 2.2.4. Chondrocytes were expanded to passage 2 in Dulbecco's Modified Eagles Medium supplemented with 10% (v/v) FBS and 50 µg/mL gentamycin. PCL and P4 foam scaffold cylinders 8 mm in diameter were cut into 2 mm thick discs, sterilized with 70% (v/v) ethanol for 30 minutes, washed three times with sterile PBS, and pre-treated with complete medium for 24 hours. Scaffolds were placed in 24-well plates lined with non-adherent agarose, seeded with 2×10^6 chondrocytes in 100 µL aliquots of growth medium, and allowed to adhere for 2 hours at 37°C, 5% CO₂ and 100% RH. Scaffolds were cultured in complete medium for 7 days.

5.3.5 Microscopy

Cells on polymer films and within scaffolds were washed with PBS and fixed in 3.7% w/v formalin for 15 minutes. Cells were stained with phalloidin 568 for 15 minutes to highlight the actin cytoskeleton of the MC3T3-E1. Both the films and the foams were stained with the nuclear dye 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 15 minutes. Samples were washed with PBS and visualized on an upright Olympus BX51 epifluorescent microscope equipped with an Olympus DP70 camera.

5.4 Results and Discussion

5.4.1 Cytotoxicity

The cytotoxicity of PCL and modified PCL copolymers P1-P5 prepared in the form of hot-rolled films as well as scCO₂ foams (PCL and P4 only) were determined according to ISO 10993-5 [195]. The ISO protocol probed for the presence of toxic leachates in media pre-conditioned with the polymer for 24 hours, not through direct cell-material contact. Cytotoxicity was defined as <70% metabolic activity relative to the negative control. The P1-P5 copolymers films and both scCO₂ foams showed no significant toxicity (Figure 5-3). However, the pure PCL film did show toxicity, with a viability of only $64 \pm 6\%$, below the cytotoxicity threshold.

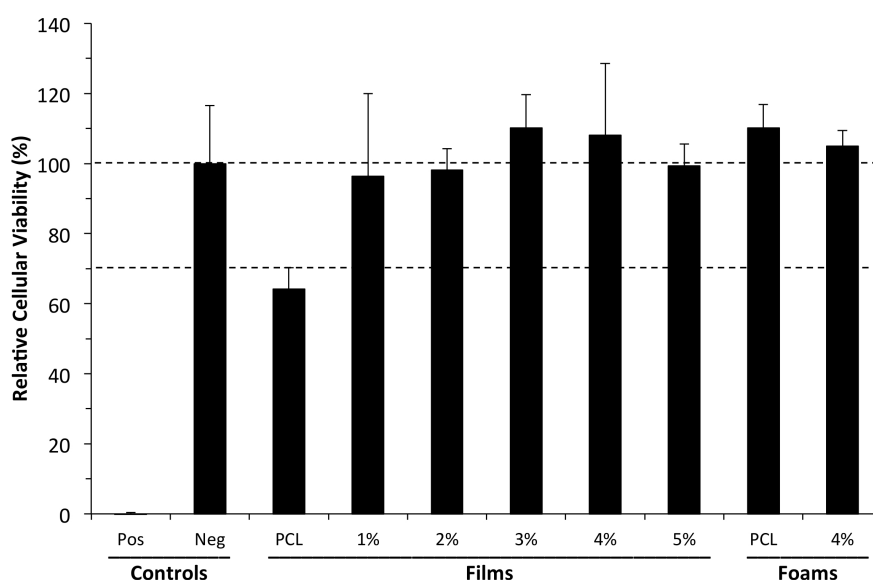


Figure 5-3. ISO 10993-5 Biocompatibility Test. All foams and modified PCL films pass the cytotoxicity test, the PCL film failed the test, as determined by MTT of MC3T3-E1 cultured for 72 hours in MEM pre-conditioned for 24 hours at 5 mL/g polymer. Cytotoxicity defined as <70% viability [195]. Modified from Tang *et al.* [264].

The toxicity of PCL was attributed to residual tin octoate catalyst from the ring-opening polymerisation. The cytotoxic tin may have been retained in greater concentrations in the PCL due to the significantly higher molecular weight of the PCL (178 kDa) relative to the modified copolymers P1-P5 (88-63 kDa). This hypothesis is corroborated by the lack of toxicity in the scCO₂ PCL foams that underwent additional processing and possessed a greater surface area/volume than the hot rolled film, allowing additional opportunity for the toxic catalyst to diffuse out during scCO₂ foaming, scaffold sterilisation, and surface preconditioning. The PCL batches were further purified by multiple precipitations in diethyl ether to avoid issues with tin toxicity in subsequent 2D *in vitro* tests.

5.4.2 2-Dimensional *in Vitro* Culture

The effect of polymer formulation on cell growth was assessed by 2D *in vitro* culture. Two-dimensional films were selected for initial assessment as they eliminated the confounding parameters of scaffold porosity, interconnectivity, and stiffness. MC3T3-E1 cells cultured on PCL and P1-P5 films for 14 days and analysed for metabolic activity by Alamar Blue (Figure 5-4). The cells on the PCL had the lowest average metabolic activity; however, high variability resulted in reduced significance. Relative to PCL, there was statistically more metabolic activity on P2 ($p = 0.04$) and a strong trend on P4 ($p = 0.072$).

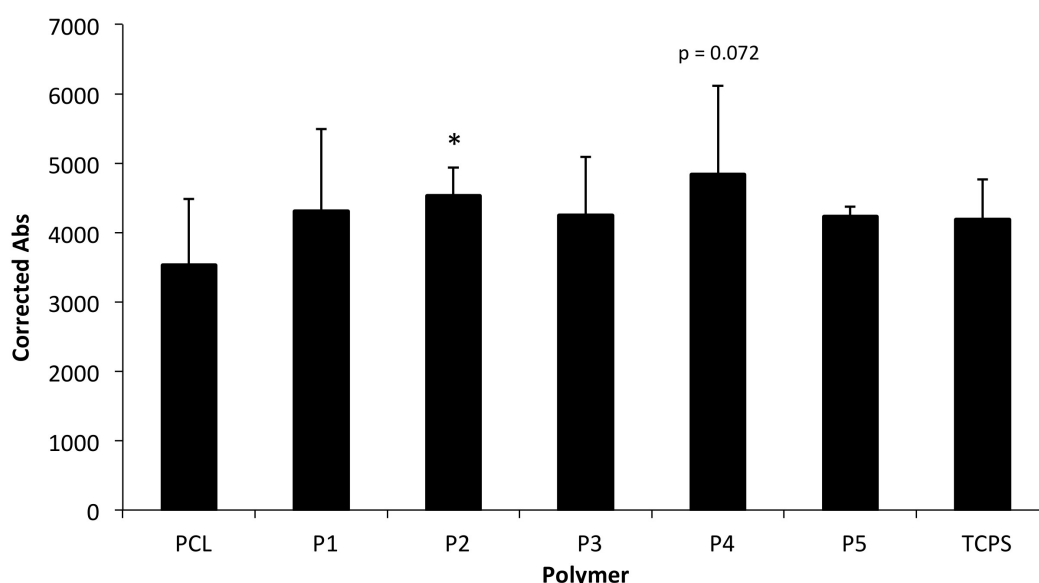


Figure 5-4. Metabolic activity at 14 days on polymer films of different compositions. PCL – poly(ϵ -caprolactone), P1-P5 – monomer 1-PCL copolymer of 1-5 mol% monomer 1, TCPS – tissue culture polystyrene control. (* $p < 0.05$ relative to PCL). Modified from Tang *et al.* [264].

To validate the observation of metabolic activity, cells were visualized on PCL and P4 surfaces, and showed markedly different morphologies (Figure 5-5). The increased adhesion in P4 was attributed to the increased density of surface charge in the form of negatively charged acetyl groups and the subsequent increased hydrophilicity of the copolymer, validated by the moderate reduction in contact angle from 98 ± 4 to 94 ± 3 degrees, as determined by Tang *et al.* [263].

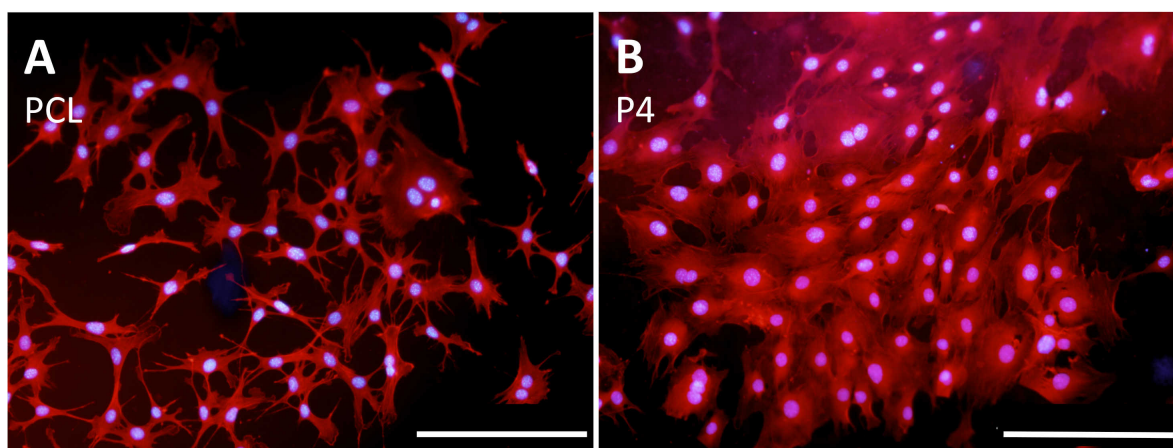


Figure 5-5. Differential adhesion of MC3T3-E1 cells to A) PCL and B) P4 hot-rolled films at 14 days *in vitro*. Scale bar represents 500 μm . Modified from Tang *et al.* [264].

5.4.3 Three-Dimensional *in Vitro* Culture

To assess the interconnectivity of the scCO_2 foams for tissue engineering applications, primary bovine chondrocytes were seeded onto foam scaffolds and cultured for 7 days. The foams were stained with DAPI to visualise cellular distribution in the scaffolds. Chondrocytes penetrated the more porous and hydrophilic P4 foam, while the distribution was limited to the exposed surfaces and first tier of pores in the more dense PCL foam (Figure 5-6). As can be observed in Figure 5-6 C,D, and SEM images of scaffold cross-sections (Figure 5-2), the PCL and P4 foams had different architectures, porosities, and interconnect sizes, all of which were the direct result of the differential polymer chemistry and molecular weight of PCL and P4 [263].

The proof-of-concept 3D culture illustrated that P4 formed a scaffold that was more conducive to chondrocyte penetration *in vitro* when P4 and PCL scCO_2 foams, as prepared in Tang *et al.*, were scCO_2 -foamed at the same conditions.

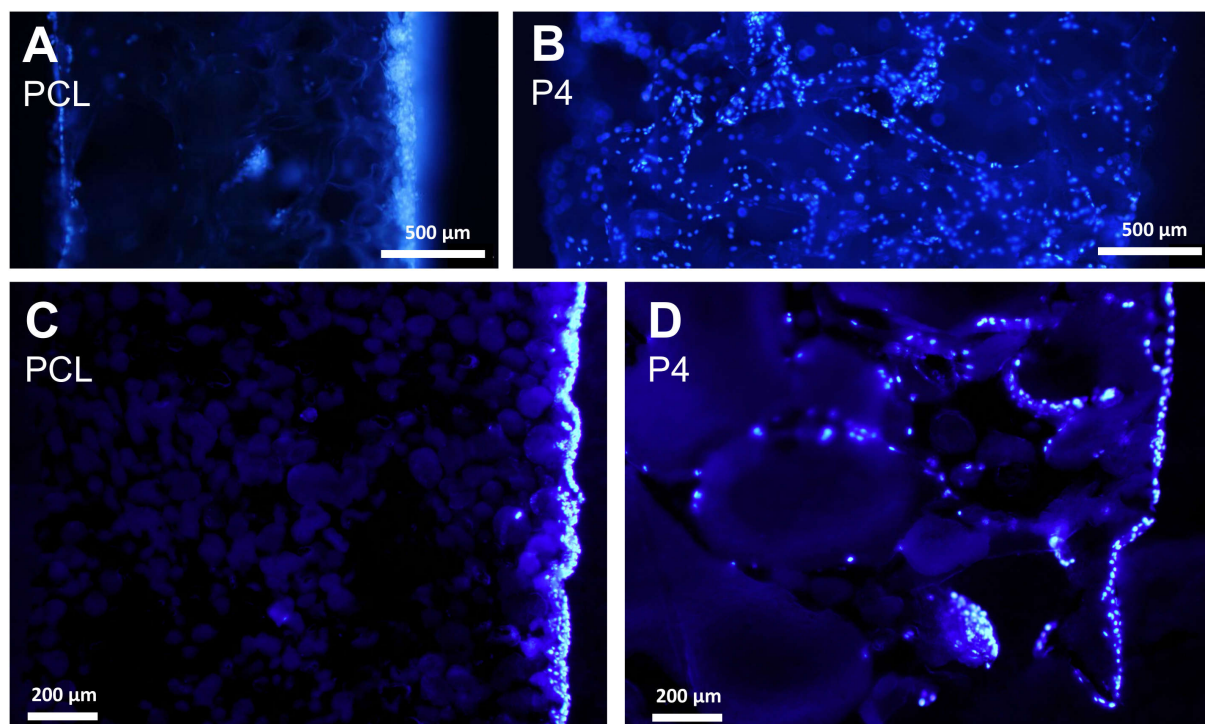


Figure 5-6. Chondrocyte seeding on PCL (A,C) and P4 (B,D) scaffolds, indicated by DAPI staining of the nuclei shows a significantly different cellular distribution in cross-section (A,B) and thin section (C,D). Modified from Tang *et al.* [264].

As the co-polymer composition is confounded with a significant change in molecular weight, steps were taken to ensure that the findings, while a preliminary proof-of-biocompatibility, were valid. The two-dimensional static film testing was incorporated to ensure that the culture surfaces were roughly equivalent with the exception of surface chemistry. The films also permitted greater utility from a limited supply of synthesized polymer.

5.5 Conclusion

Copolymers formulated with 1-5 mol% monomer 1, an acetylated monomer intended to increase the hydrophilicity and CO₂ solubility were analysed as polymer films and scCO₂ foams. The copolymers and scaffold foams were found to be non-cytotoxic when sufficiently purified. MC3T3-E1 cultured on polymer films for 14 days showed slightly increased adhesion on the modified surfaces, with a more adherent cellular morphology. In three-dimensions, the P4 scCO₂ foams were significantly more amenable to chondrocyte infiltration than PCL controls.

Study 5B – Production of poly(L-lactide-co-caprolactone) for cartilage tissue engineering

5.6 Introduction

Poly(L-lactide-co- ϵ -caprolactone) (PLCL) is a biodegradable thermoplastic elastomer first used for biomedical applications in 1991 [134,137]. It is produced by the ring-opening polymerisation of ϵ -caprolactone and L-lactide [137].

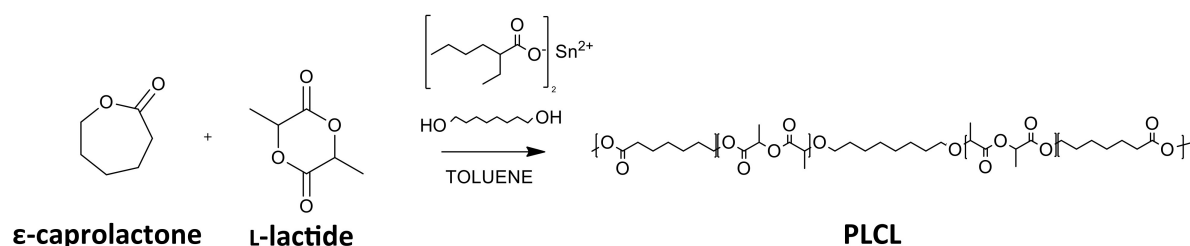


Figure 5-7. Ring opening polymerisation of PLCL from ϵ -caprolactone and L-lactide using a stannous octoate catalyst and 1,8-octanediol initiator.

PLCL was introduced as a non-toxic alternative to diphenylmethane diisocyanate (MDI) for the production of nerve guides, as MDI was found to have toxic degradation by-products [134]. The degradation of PLCL, produces 6-hydroxyhexanoic acid and L-lactic acid, which are quickly metabolised.

The mechanical properties of PLCL are elastomeric, but vary greatly depending on the monomer ratio, distribution, molecular weight, and chirality of lactide [135–137,268]. Pure L-lactide forms a brittle crystalline polymer, while amorphous poly(lactide) is soft, with a glass transition temperature (T_g) above body temperature. Copolymers with caprolactone:lactide (C:L) monomer ratios of 50:50 and 60:40 form elastomers fit for tissue engineering applications, by improving backbone flexibility with the additional ester-bond in the lactide monomeric unit, while hindering L-lactide crystallisation with the random incorporation of caprolactone. The addition of blocks of L-lactide results in phase separation over time as crystalline domains form and enlarge, resulting in altered properties during polymer storage. A study of 30:70 C:L PLCL observed an increase in crystallinity from 0-23% with an associated increase in elastic modulus from 12-128 MPa after only 5 weeks at 21°C [137].

In addition to the C:L ratio of the PLCL produced, the mechanical and degradation properties of the polymer are highly dependent on the distribution of lactide within the copolymer. During PLCL polymerisation, the growing end of the polymer chain is more reactive towards lactide; therefore,

reactions are often run for extended periods of time to increase caprolactone conversion, in effect producing a lactide-caprolactone gradient copolymer [269]. L-lactide domains will crystallize under physiological conditions resulting in significant reductions in degradation rate by hydrolysis [137]. The highly crystalline domains can remain in the body for years after the caprolactone has degraded, triggering a foreign body response [270]. Amorphous lactide domains hydrolyse significantly faster than poly(caprolactone) and crystalline lactide, a trait desired in many tissue engineering applications. The issue of controlled lactide incorporation has been resolved by a number of methods, including the use of racemic lactide to prevent crystallisation but excessive racemic lactide will result in a sticky polymer without structural tissue engineering applications [136,137].

5.7 Materials + Methods

5.7.1 Materials

All chemicals were acquired from Sigma Aldrich, UK and used as received, unless otherwise specified. ϵ -caprolactone was purchased from Alfa Aesar (UK, A10299-36) and L-lactide was kindly provided by Purac Biomaterials (UK, Purasorb L) and stored under argon at -20°C.

5.7.2 Polymerisation and Purification

Three polymerisation conditions were studied: bulk, solution, and continuous lactide addition, each with the objective of increasing control over the molecular weight and C:L ratio and distribution of the polymer produced.

Bulk Polymerisation

Bulk polymerisation under melt conditions was performed following a protocol from Fernández *et al.* [137]. Monomers were combined at equimolar amounts for a 10 mmol polymerisation. The catalyst, tin(II) 2-ethylhexanoate, was dissolved in dry toluene and degassed under dry nitrogen. Monomers were degassed by the freeze-pump-thaw method and heated to 150°C with continuous mixing, under dry nitrogen. Catalyst was injected at 1:2000 molar ratio (5 μ mol), and the reaction progressed for 75 minutes at 150°C. PLCL polymer was precipitated in cold methanol, dissolved in chloroform and precipitated a second time in cold diethyl ether.

Solution Polymerisation

The protocol for bulk polymerisation was modified by diluting monomers in dry toluene to 2 mol/L total monomer, necessitating a temperature reduction to 110°C, the boiling point of toluene. The reaction proceeded for 24 hours under dry argon, at which point it was quenched by the addition of 10 µL water and precipitated in cold diethyl ether.

Continuous Lactide Addition

To improve the distribution of lactide throughout the copolymer, controlled addition of lactide into the reaction was investigated. Reagents were prepared in the standard manner for a solution polymerisation, with the omission of lactide. Lactide was dissolved in anhydrous chloroform at 0.4 g/mL and extruded at 0.190 mL/h for 6 hours into the polymerisation performed at 110°C under inert argon. A positive pressure was maintained within the Schlenk flask by the addition of a balloon of dry argon. The reaction was sampled for $^1\text{H-NMR}$ at 0, 1, 2, 4, 6, and 24 hours. At 24 hours, the polymerisation was quenched with 10 µL water, and precipitated into cold diethyl ether.

Monomer Distillation

To increase the purity of the caprolactone monomer in the final set of polymerisation it was distilled over calcium hydride (CaH_2). Caprolactone was dried over powdered CaH_2 under dry nitrogen for 2 hours with continuous mixing. The caprolactone- CaH_2 was then distilled under high vacuum at 100°C. Fractions were collected, sealed under nitrogen, and used within 24 hours.

$^1\text{H-NMR}$

Polymerisations were analysed by preparing samples for $^1\text{H-NMR}$ (400 MHz) in deuterated chloroform (CDCl_3) at various time points. The α and ϵ methylenes of caprolactone and methine of lactide were used to monitor conversion and purified polymer composition (Figure 5-8).

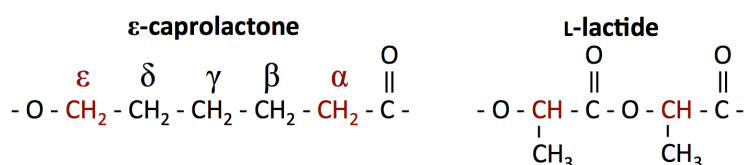


Figure 5-8. Proton assignment within the caprolactone and lactide monomeric units, adapted from Fernández *et al.* [137].

In the monomeric form, the lactide methines have a quartet centred at 4.9 ppm that shifts to 5.16 ppm upon polymerisation. The ϵ and α methylenes of ϵ -caprolactone have monomeric signal at 4.2

ppm and 2.65 ppm that shifts to 4.1 ppm and 2.35 ppm, respectively [137]. The C:L ratio in the purified polymer was calculated as the ratio of integrated peaks indicative of polymerisation for each monomer: 4.1 ppm and 5.16 ppm. The conversion of caprolactone and lactide were calculated from integrations of the monomer (M, 4.9 ppm or 4.2 ppm) and polymer (P, 5.16 ppm or 4.1 ppm) peaks according to Eq.5-1.

$$\text{Conversion} = P/(M+P) \quad [\text{Eq 5-1}]$$

As the polymer is composed of a random mix of L-L, C-C, and C-L dyads, the triplets representative of the ϵ and α methylenes of caprolactone and quartet from lactide methines each present twice in ^1H -NMR and can be used to determine the distribution of the monomers [271]. The peaks at chemical shifts 4.06 ppm and 2.30 ppm correspond to C-C dyads, while those at 4.11 and 2.37 ppm correspond to C-L. The overlapped signal for the lactide methines hinders analysis in this manner, but the shoulder at $\sim$ 5.07 ppm on the quartet centred at 5.16 ppm corresponds to C-L dyads, with the remainder of the signal attributed to L-L.

Size Exclusion Chromatography

The molecular weight of the polymers was characterised by size exclusion chromatography on a GPCMax VE 2001 (Viscotek, UK) size exclusion chromatography (SEC) apparatus. Samples were prepared at 1 mg/mL in the eluent N,N-Dimethylformamide (DMF), and run at 0.7 mL/min. Two PSS Gram DMF columns were used at 35°C and calibrated against poly-methyl methacrylate molecular weight calibration standards (Agilent Technologies, UK) without correction.

5.7.3 Scaffold Production

Directionally frozen scaffolds were produced from various polymer compositions following an identical protocol to that of PCL, outlined in Chapter 3.2.2. Briefly, a solution of 8% (w/v) PLCL (60:40 C:L, 69 kDa) in dioxane was cast in a PDMS edge mould on top of a 450 μm thick copper sheet to a depth of 2 mm. The copper sheet was placed on the PolarBear Plus at -20°C for 20 minutes, transferred to the -80°C freezer for 20 minutes and lyophilised overnight.

5.7.4 Mechanical Testing

Directionally frozen foams prepared from PLCL and PCL were tested in compression at 0.05% strain per second to 10% strain followed by a 600 s relaxation at constant strain. Testing was performed unconfined and hydrated on a Bose ElectroForce 3200 Series III (TA Instruments, UK) equipped with

a 5 lbf (22.2 N) load cell. Sample thickness was measured with digital callipers. The modulus was determined from the slope of the linear portion of the stress-strain curve.

5.7.5 Cytotoxicity

PLCL was assessed for cytotoxicity by ISO 10993-5, as outlined in detail in Section 5.3.2. Growth media was preconditioned with 1 g/mL PLCL powder at 37°C for 24 hours and tested against sub-confluent MC3T3-E1 pre-osteoblasts for 72 hours. Cytotoxicity was determined by MTT assay and stated relative to a polymer-free media control. MC3T3-E1 culture and MTT were kindly performed by Ms. Anu Solanki, Department of Materials, Imperial College London.

5.8 Results and Discussion

5.8.1 Polymerisation and Purification of PLCL

The primary aim of the investigation was the optimisation of the PLCL polymerisation, with the aim of producing a PLCL with a 50:50 ϵ -caprolactone:L-lactide composition and a molecular weight in excess of 50 kDa. Different protocols were investigated to optimise control and molecular weight, including bulk, solution, and controlled addition of lactide to solution polymerisation.

Bulk and Solution Polymerisation

Bulk polymerisation was performed with a stoichiometric ratio of 1:1 for the production of a 50:50 C:L polymer. The reaction proceeded quickly, forming a thick polymer within 75 minutes. Analysis by size exclusion chromatography (SEC) (Figure 5-9A,B) indicated a retention time comparable to the 70-90 kDa PCL, used in previous investigations, but highlighted the high polydispersities of both. Although bulk polymerisation is a standard protocol for tissue engineering applications, solution polymerisation, was investigated to improve reproducibility and control [136,137,272]. Improved control was determined by a decrease in polydispersity index ($PDI = M_w/M_n$), visible as a decrease in the SEC peak width. An initial investigation performed in toluene and monitored over 4 hours, produced a PLCL product with comparable retention time in the SEC and a narrower peak, indicating increased control over molecular weight (Figure 5-9C). The shifting and slightly broadening peaks in (Figure 5-9C) indicate that the polymer is increasing in molecular weight over the course of the 4-hour reaction while also increasing in polydispersity.

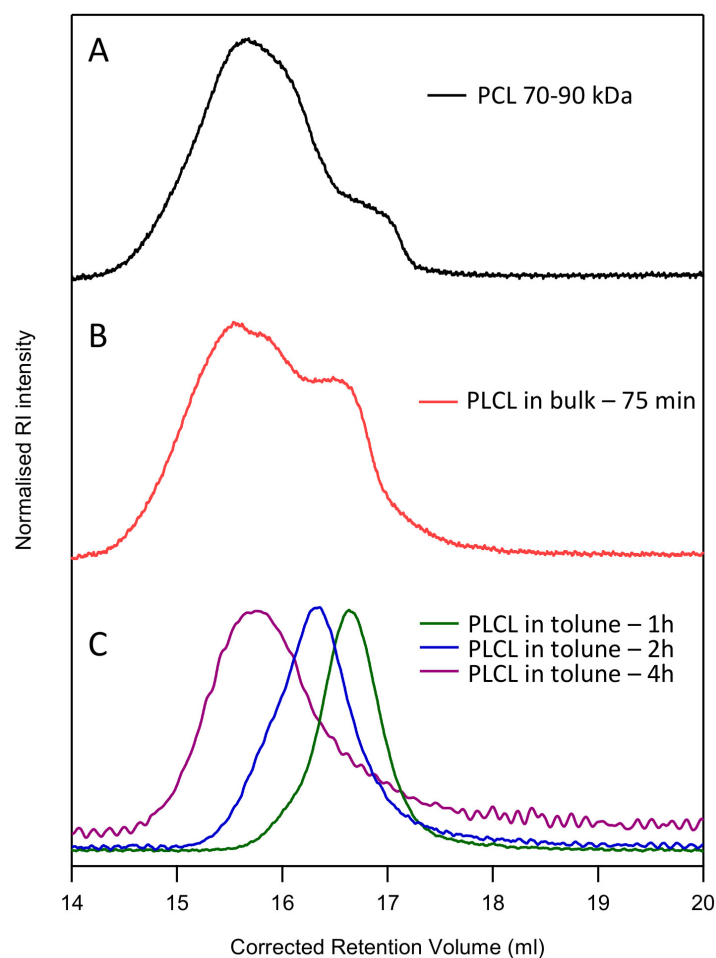


Figure 5-9. Size exclusion chromatogram of A) Commercial 70-90kDa PCL, B) PLCL from bulk polymerisation, C) PLCL from 2M solution polymerisation in toluene at 1, 2, and 4 hours.

The initial polymerisations were performed in toluene with a monofunctional t-butanol initiator. An optimisation of initiator to monomer molar ratio indicated that 1:667 and 1:1000 (mol:mol) improve molecular weight of the polymer product while maintaining high conversion of lactide and sufficient conversion of caprolactone (Table 5-1).

Table 5-1. Optimisation of initiator:monomer molar ratio in solution PLCL polymerisation with t-butanol initiator.

Initiator : Monomer	Conversion (¹ H-NMR)		Mn (kDa)
	Lactide	Caprolactone	
1 : 1000	99%	55%	79.9
1 : 667	99%	60%	69.0
1 : 500	99%	58%	39.0
1 : 400	99%	57%	43.0

As caprolactone was known to be less reactive, the polymerisations were performed with 65:35 C:L monomer molar ratios, resulting in a final composition of 47-49% lactide.

With the desired molecular weight and C:L monomer ratio achieved, the distribution of caprolactone throughout the polymer was determined by ^{13}C -NMR with assistance from Dr. Robert Chapman, Department of Materials, Imperial College London. While the signal was low, the data suggested that $69\% \pm 7\%$ of triads in the polymers were lactide (LLL) and caprolactone (CCC) (Figure 5-10), indicating that the polymers produced had the characteristics of block copolymers [273]. Therefore, while the polymer possessed a 50:50 molar ratio and a high molecular weight, the distribution of caprolactone and lactide could be improved to eliminate crystalline lactide domains.

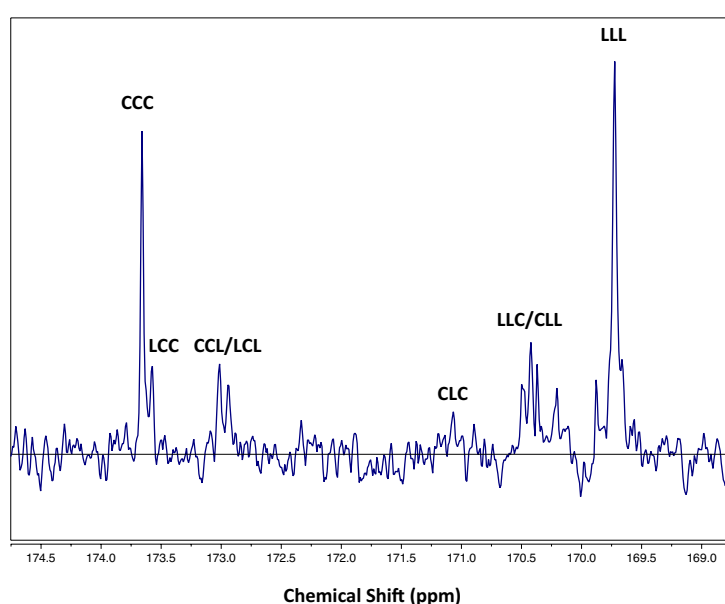


Figure 5-10. ^{13}C -NMR of 80 kDa 50:50 PLCL produced from a 65:35 C:L monomer stock. CCC and LLL indicate peaks representing pure caprolactone and lactide triads, while the others indicate triads including a combination of both C and L [273].

Continuous Lactide Addition

The growing end of the aliphatic polyester polymer chain thermodynamically favours lactide addition over caprolactone, and regularly achieves 99% lactide conversion. To promote a more random monomer distribution throughout the polymer, the concentration of lactide was maintained at low levels by continuous addition over six hours. The metered addition of lactide ensured an excess of caprolactone in the system, promoting incorporation into the polymer. The time course of lactide and caprolactone addition into polymer was assessed by ^1H -NMR, and displayed in Figure 5-11.

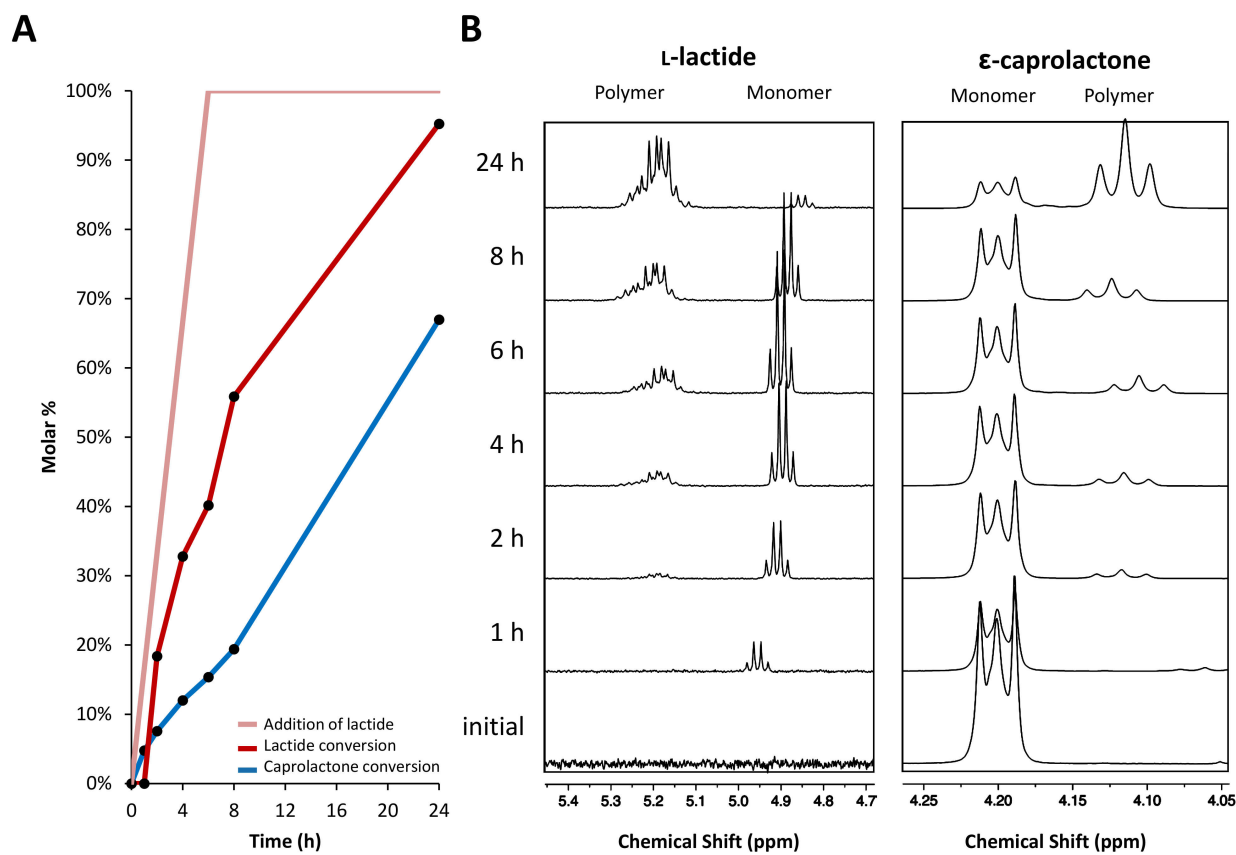


Figure 5-11. A) Delivery of L-lactide and conversion of L-lactide and ε-caprolactone in the continuous lactide addition polymerisation. B) Excerpts of ^1H -NMR of a PLCL time series used to generate panel A, focusing on the area of interest for L-lactide and ε-caprolactone.

In **Figure 5-11A**, the addition of lactide and conversion of the monomers are visualised, with data extracted from ^1H -NMR included in **Figure 5-11B**. The lactide is added in a linear manner over six hours, with the conversion of lactide increasing over that period to a final conversion of 95%. The conversion of caprolactone, while slower, as previously discussed, increased linearly over time, and reached a conversion of 67%.

With the success of the continuous addition protocol, an attempt was made to scale up the polymerisation from 500 mg to 5 g batches of PLCL. The polymer PLCL6 was selected for analysis as it exemplifies the successes and unresolved issues with the scale-up of controlled lactide addition. The scale-up was successful in achieving a consistent blend of C and L. The time-course of the controlled lactide addition polymerisation of PLCL6 is shown in **Figure 5-12**. By depicting the plot in moles of monomer incorporated into the polymer, the initial high level of caprolactone incorporation into the polymer is evident, with a 49:51 C:L molar composition at 6 hours and 55:45 at 24 hours.

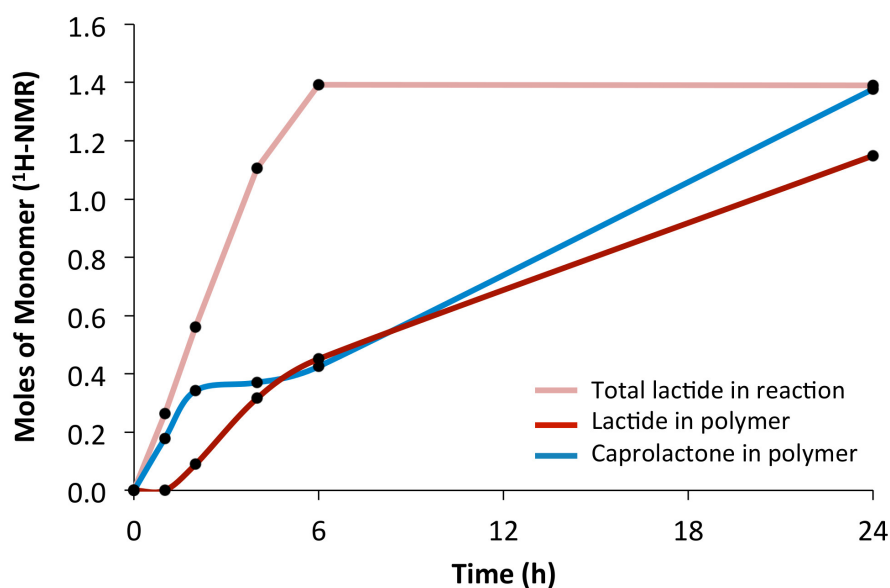


Figure 5-12. Time-course of PLCL6 polymerisation by controlled-addition of lactide indicating moles of lactide in the reaction and moles of caprolactone and lactide in the PLCL polymer. Data extracted from ^1H -NMR.

The distribution of caprolactone and lactide within PLCL6 was assessed by comparing diads in the ^1H -NMR spectra, as the signal was more robust than the ^{13}C -NMR spectra used previously. The relative integrations of caprolactone peaks at 4.05 ppm (CC diad) and 4.15 ppm (CL diad) were compared and it was determined that 38% of caprolactones were bound to a lactide in PLCL6. However, the molecular weight of PLCL6 suffered from the scale-up and was only 14.5 kDa with a PDI of 1.54, well below the desired molecular weight. Further optimisations of the system were unsuccessful, producing either a well-blended polymer or a large molecular weight.

In order to produce a PLCL of improved molecular weight and control, a final attempt was made to optimise traditional solution polymerisation using distilled caprolactone, believed to be the source of contamination. A polymer, PLCL11 was produced at 112 kDa, with a 42:58 C:L molar ratio and a ^1H -NMR diad analysis revealing 25% of caprolactones bound to a lactide.

The two samples were analysed by differential scanning calorimetry (DSC) by Celia, Department of Materials, Imperial College London. The T_m of each was found to be 49.7°C for PLCL11 and 46.7°C for PLCL6, within the range of published melting points for PLCLs [273].

5.8.2 Scaffold Production

An early batch of 80kDa PLCL produced by solution polymerisation was used to produce directionally frozen scaffolds for mechanical and cytocompatibility assessment. The PLCL polymer produced a directionally frozen scaffold with discernible vertical pores without need for modification to the protocol for PCL (Figure 5-13).

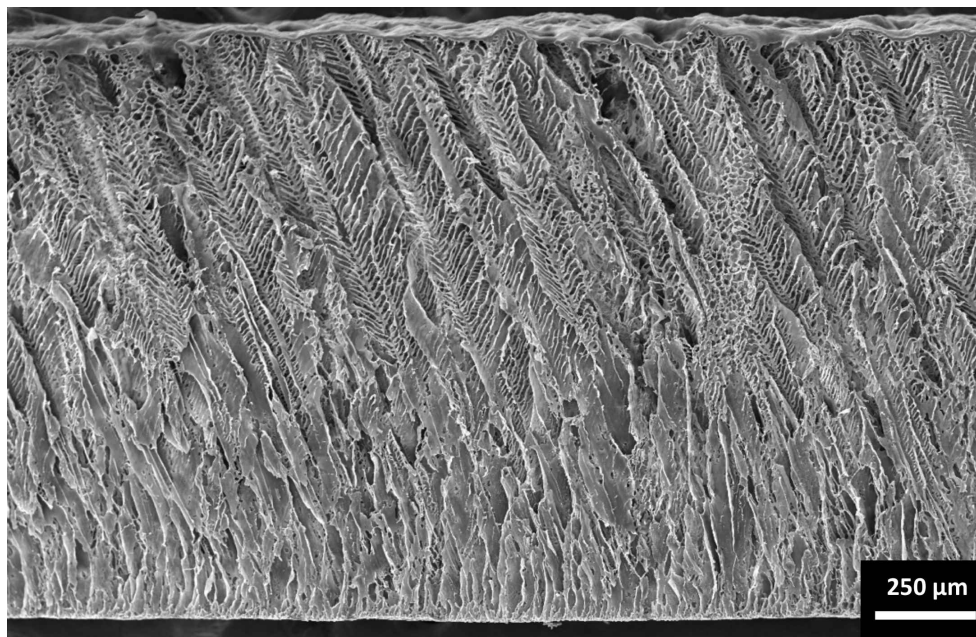


Figure 5-13. Directionally frozen scaffold produced from 80kDa PLCL.

5.8.3 Mechanical Testing

Directionally frozen foams prepared from 80 kDa PLCL were tested against 80 kDa PCL foams prepared in the same manner at 0.05% strain/sec followed by a 5 minute relaxation at 10% strain. Representative stress-strain curves for the two scaffolds show similar responses for the two polymers with the exception that there is a larger toe region for PLCL, shifting the stress-strain curve by 2.5% strain (Figure 5-14A). The average compressive moduli of the two scaffolds over 10% strain are 2.29 ± 0.19 MPa and 1.76 ± 0.25 MPa for PCL and PLCL respectively.

The stress relaxation in the PLCL scaffold is greatly increased ($p = 3 \times 10^{-5}$) relative to PCL, at $42.0\% \pm 0.4\%$ and $17.6\% \pm 0.3\%$ respectively (Figure 5-14B). This significant increase in stress relaxation was expected for the more elastomeric PLCL.

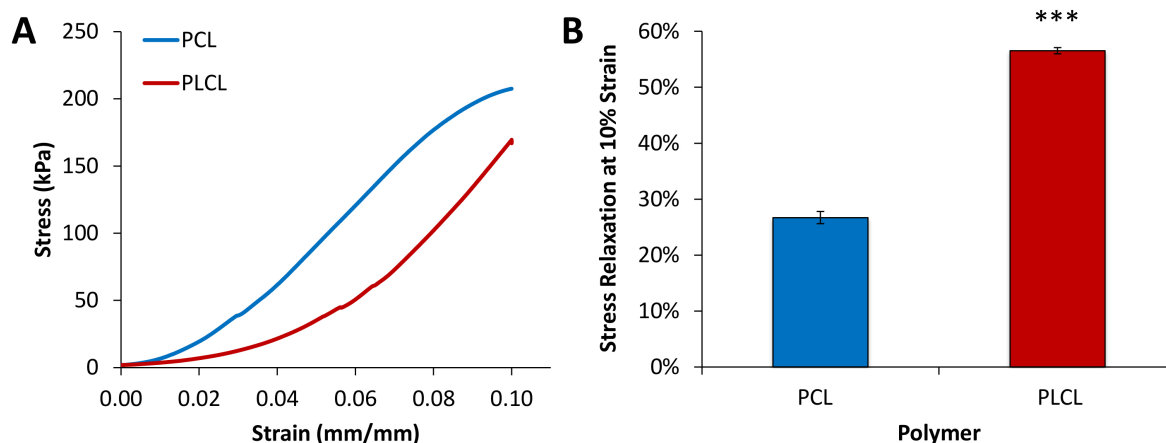


Figure 5-14. Mechanical characterisation of DF scaffolds composed of PCL and PLCL. A) Representative stress-strain curve for scaffold compression at 0.05% strain/sec to 10% strain. B) Stress relaxation in PCL and PLCL scaffolds over 5 minutes at 10% strain ($p < 0.001$ relative to PCL).**

5.8.4 Cytotoxicity

The same batch of PLCL used for mechanical testing was assessed for cytotoxicity by ISO 10993-5, as previous polymerisations utilising a tin-catalyst required additional purification to overcome toxicity from residual catalyst (Chapter 5A). Preliminary testing by Ms. Anu Solanki, Department of Materials, Imperial College London indicate that growth media preconditioned with PLCL is not statistically different from a blank control, suggesting no issue of PLCL toxicity (Figure 5-15).

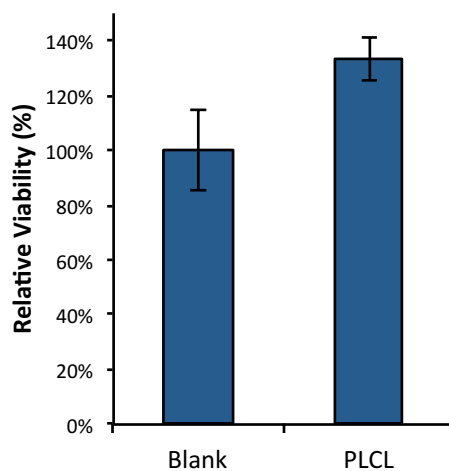


Figure 5-15. ISO 10993-5 Biocompatibility Test. PLCL is biocompatible, as determined by MTT of MC3T3-E1 cultured for 72 hours in MEM pre-conditioned for 24 hours at 1 g/mL polymer, relative to a blank control. Cytotoxicity is defined as <70% viability [195].

5.9 Conclusion

Through optimisation of solution polymerisation of distilled ϵ -caprolactone and L-lactide with a 1,8-octanediol initiator and stannous octoate catalyst, a 112 kDa poly(L-lactide-co- ϵ -caprolactone) with a 42:58 molar C:L ratio was produced. The polymer was not a block co-polymer, with 25% CL diads (caprolactone monomeric units bound to lactide).

Investigation into improved caprolactone incorporation produced polymers with up to 38% CL diads, a 40% increase over standard solution techniques. However, the modified technique resulted in greatly reduced molecular weights, as low as 14.5 kDa. The reduced molecular weight was attributed to contamination from atmospheric water penetrating the reaction over the 24 hours of polymerisation. In the absence of a controlled atmosphere environment, the sealed multi-component system was too prone to contamination to be considered for further investigation.

An early batch of 80kDa PLCL was determined to be cytocompatible by ISO10993-5, and was used to produce directionally frozen scaffolds using the protocol for PCL without modification. The average stiffness of the PLCL scaffold was 1.76 ± 0.25 MPa, not statistically different from PCL. However, the stress relaxation of the directionally frozen PLCL scaffold ($42.0\% \pm 0.4\%$) was significantly greater than PCL.

The variability of ring-opening polymerisation of aliphatic polyesters, evidenced by the exceptionally large PDI for commercial poly(ϵ -caprolactone) requires large controlled batch synthesis to ensure reproducibility. New implantation-grade biodegradable elastomeric polymers are now entering the market at competitive prices. In the interest of efficiency, clinical translation, and reproducibility, these commercial polymers are superior to the PLCL synthesised in this study.

Study 5C – Preliminary assessment of Strataprene™ for cartilage tissue engineering

5.10 Introduction

While optimising the synthesis of PLCL, a commercially available elastomeric aliphatic polyester was investigated as an alternative. Strataprene® 3534 (Poly-Med Inc.) is a proprietary linear block copolymer comprised of 35% caprolactone, 34% lactide, 17% glycolide, and 14% trimethylene carbonate (Figure 5-16)[274].

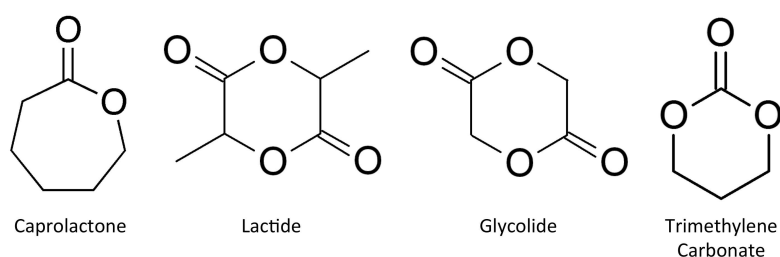


Figure 5-16. Monomers within Strataprene 3534.

Strataprene® 3534 is a polymer of interest, as it is similar to the PLCL compositions being synthesized, containing 70 mol% caprolactone and lactide, while also possessing high elasticity and hydrophilic properties that suggested it would be suitable for cartilage engineering [274]. Additionally, as it was commercially available at implant-grade, the batch-to-batch variability would be greatly reduced and the path to clinical translation would be facilitated. As the series of polymers were new, there are currently no publications using Strataprene® to reference.

5.11 Materials + Methods

5.11.1 Materials

All chemicals were acquired from Sigma Aldrich, UK and all cell-culture materials were acquired from Invitrogen, UK and used as received, unless otherwise specified. Strataprene® 3534 was kindly provided by Poly-MED Inc. (Anderson, SC, USA).

5.11.2 Scaffold Production

Complete DF100 scaffolds, DF100 porogen-leached foams and directionally frozen foams were produced from Strataprene® 3534 in a manner identical to Chapter 3.2.2 (Appendix E). Scaffolds were sterilised by peracetic acid (Appendix F) and rinsed thoroughly in sterile PBS.

5.11.3 Materials Testing

Mechanical characterisation, was performed on complete DF100 scaffolds composed of poly(ϵ -caprolactone) and Strataprene® 3534 by dynamic mechanical analysis in a manner identical to Chapter 3.2.7 using the Bose ElectroForce 3200 Series III equipped with the WinTest DMA software package. Samples were tested dry and wet in unconfined uniaxial compression. Analysis was performed with a dynamic amplitude of 5% strain at 0.1, 1, and 10 Hz about a 5, 10, 15, and 20% mean strain, under displacement control. The storage modulus was calculated from the phase angle and is stated for each iteration of mean strain and frequency. All scaffold varieties and conditions were tested with $n \geq 3$. PCL DF100 scaffold data is reproduced from Figure 3-10 as a reference and was collected using identical protocols.

5.11.4 Cell Seeding and Culture

Expanded (P2) primary bovine chondrocytes were seeded at 10^7 /scaffold, in a manner identical to Chapter 3.2.4. Briefly, a bolus of 10^7 cells per 50 μ L DMEM was injected into the porogen-leached zone of the sterile 6 mm $\varnothing$ DF100 complete scaffold. Chondrocytes were adhered for 2 hours at 37°C, 5% CO₂, 100% RH. All subsequent culture used chondrogenic differentiation medium composed of DMEM + Glutamax (4.5 g/L glucose), 50 μ g/mL L-proline, 50 μ g/mL ascorbic acid, 0.1 mM sodium pyruvate, 1% (v/v) anti-anti, 10 ng/mL TGF- β 3, and 1% (v/v) ITS Premix (BD Biosciences, Oxford, UK).

5.11.5 Gene Expression

Gene expression in Strataprene scaffolds was determined by qRT-PCR in an identical manner to Chapter 3.2.6 and is displayed alongside PCL with equivalent seeding density and PLZ pore diameter as a reference, reproduced from Figure 3-21 (DF100 - 10M). Scaffolds were divided with a sterile scalpel at the interface between the particulate-leached zone and the directionally frozen zone and analysed separately. Genes investigated at 7 days were COL2A1, COL1A1, AGC, COL10A1, SOX9, PRG4, and the housekeeping gene 18S (Table 3-2). Genes are expressed as a fold increase over week 0, calculated using the $\Delta\Delta C_t$ method, and the COL2A1/COL1A1 ratio is calculated from the $2^{-\Delta C_t}$ of each gene.

5.12 Results and Discussion

DF100 complete scaffolds were produced from Strataprene® 3534 and tested against PCL scaffolds of the same PLZ pore size (100-300 μm) and thickness by dynamic mechanical analysis. Scaffolds were tested under wet and dry conditions by unconfined compression of a 6 mm diameter scaffold. Both conditions were tested as Strataprene was known to be more hydrophilic than PCL, and therefore more likely to be affected by scaffold hydration.

The dynamic mechanical analysis was performed with a strain sweep (Figure 5-17 A) and a frequency sweep (Figure 5-17 B). Under every condition tested, the PCL scaffold had a greater storage modulus (E') than the Strataprene ($p < 0.023$ for dry and $p < 0.0028$ for wet), as clearly shown by the dry, 1Hz data in (Figure 5-17 C). The storage moduli at 10% mean strain 1 Hz are summarised in Table 5-2.

Table 5-2. Storage moduli of scaffolds tested by DMA at 1Hz, 10% mean strain, 5% dynamic amplitude.

Material	Storage Modulus (kPa) $\pm$ SD	
	Dry	Wet
Strataprene	295 $\pm$ 33	102 $\pm$ 28
PCL	638 $\pm$ 68	753 $\pm$ 26

The storage modulus increased with mean strain, with statistically greater storage moduli at 20% over 5% for every condition ($p < 0.033$) (Figure 5-17A). There was no significant change in storage modulus from 0.1 Hz to 10 Hz in the Strataprene or PCL samples (Figure 5-17B), with the exception of wet PCL tested at 5% mean strain ($p < 0.01$).

The effect of hydration was prominent in the Strataprene scaffolds, with all samples at all testing conditions softening when wet with an average decrease in storage modulus of $63\% \pm 2\%$. In contrast, the storage modulus of the PCL scaffolds was unchanged, with a decrease of $11\% \pm 12\%$. The considerable decrease in compressive stiffness between PCL and hydrated Strataprene reduces the utility of the polymer from a mechanical perspective. However, the modulus of 102 kPa is still greater than that of the second generation scaffold and most clinically approved hydrogels and scaffolds composed of collagen, other biopolymers, and poly(ethylene glycol) [1,97,109,110,116–119,140].

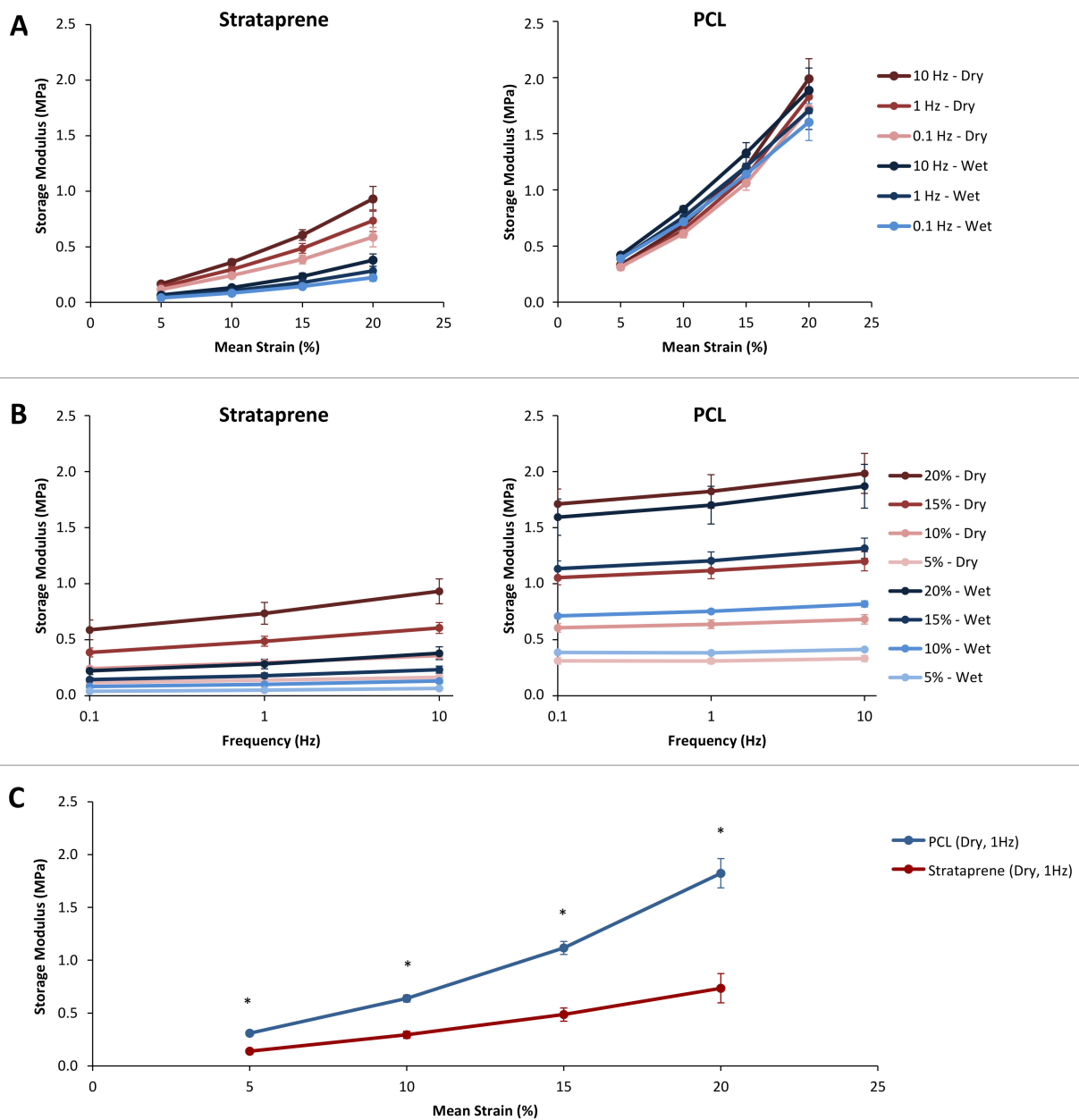


Figure 5-17. Mechanical characterisation of DF100 scaffolds composed of Strataprene and PCL under wet and dry conditions determined by DMA with 5% strain amplitude. A) Mean strain sweep for each polymer with data sets denoting frequency and hydration. B) Frequency sweep for each polymer with data sets denoting mean strain and hydration. C) Mean strain sweep of Strataprene and PCL samples at 1Hz, 10% mean strain, dry conditions (* $p < 0.05$ PCL v Strataprene). $n = 3$, error = SE.

Strataprene and PCL DF100 complete scaffolds seeded with 10^7 primary bovine chondrocytes were assessed for chondrogenic gene expression by qRT-PCR at day 7. Scaffolds were divided into the upper and lower halves to determine if the effect of the polymer was independent of geometry.

Generally, the Strataprene scaffolds had a reduced chondrogenic gene expression profile at day 7 relative to PCL (Figure 5-18). Collagen type II expression, a key marker of chondrogenic differentiation, trended lower in the Strataprene scaffolds, relative to PCL. There were also no statistical differences in collagen type I expression between the polymers, or within the Strataprene zones. The resulting collagen II/I ratios in the upper half of the scaffolds indicated that Strataprene was likely leading to a dedifferentiated phenotype ($p = 0.080$) relative to PCL, but due to the variability of the collagen type I and II expression, none of the conclusions based on collagen gene expression are statistically significant.

The expression of aggrecan is statistically lower in the deep zone of the Strataprene scaffold, relative to PCL (Figure 5-18D). PRG4, superficial zone protein, is higher in the lower half of the Strataprene scaffold, but both halves trend lower than their PCL counterparts (Figure 5-18E). The same reduction relative to PCL is observed statistically in both halves of the Strataprene scaffold for Sox9 expression (Figure 5-18F). Collagen type X expression is reduced relative to PCL in the upper half of the scaffold, with a strong trend in the lower half ($p = 0.086$), due again to high variability in the PCL gene expression (Figure 5-18G).

Taken together, the reduction in collagen type II, aggrecan, PRG4, and Sox9, either statistically or as a strong trend, all point to a dedifferentiated phenotype relative to PCL scaffolds with the same architecture. Without more extensive investigation, with histological observations, this data set is merely an interesting observation. PCL was selected for its ease of processing, slow degradation rate, uniformity, and versatility as a substrate for future surface modification, not as a chondrogenic substrate [130]. The fact that PCL had superior performance to a more elastic and hydrophilic polymer was entirely unexpected.

One interesting observation is the reduction in collagen type X expression, an indicator of hypertrophy, within the Strataprene scaffolds. This reduction could potentially be due to the reduced stiffness of the scaffold, relative to PCL.

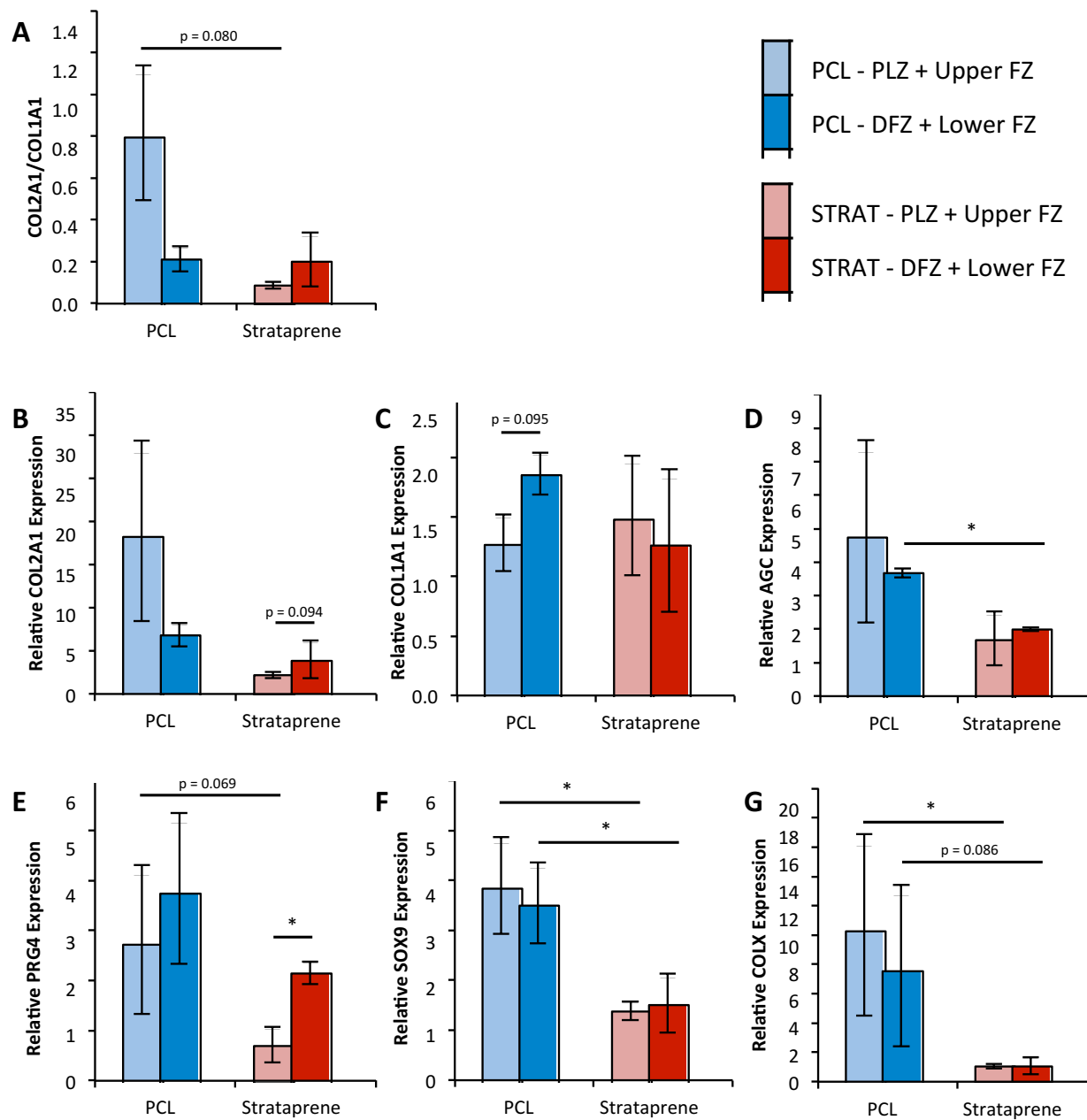


Figure 5-18. Gene expression analysis at 7 days of 10M bovine chondrocytes in DF100 scaffolds composed of PCL or Strataprene (Strat). For a semi-zonal analysis, the scaffolds were subdivided into two halves at the PLZ-DFZ boundary. A) The COL2A1/COL1A1 ratio is indicative of chondrogenic differentiation. B) type II collagen (COL2A1), C) type I collagen (COL1A1), D) aggrecan (AGC), E) superficial zone protein (PRG4), F) Sox 9, and G) type X collagen (COL10A1) are expressed as fold differences relative to day 0 controls and normalized to the 18S housekeeping gene. (*p < 0.05).

5.13 Conclusion

Strataprene® 3534 is commercially available implant-grade block copolymer of caprolactone, lactide, glycolide, and trimethylene carbonate with improved elasticity and increased degradation relative to poly(ϵ -caprolactone). While it was easily processed into DF100 complete scaffolds, the resulting structures had a greatly reduced stiffness of only 295 ± 33 kPa, relative to PCL (638 ± 68 kPa). The storage modulus decreased an additional $63\% \pm 2\%$ when hydrated, leaving the complete scaffold at 102 ± 28 kPa; a scaffold far softer than native tissue. Gene expression at seven days in high-density chondrocyte-seeded scaffolds indicated reduced expression of genes indicative of a differentiated phenotype, relative to PCL. Collagen type X, an indicator of chondrocyte hypertrophy, was down-regulated, potentially indicating a stiffness effect or complete dedifferentiation into a fibroblastic phenotype. While Strataprene® 3534 was easy to process and had unique mechanical properties, it showed no improvements over 80 kDa poly(ϵ -caprolactone) and is therefore not suited for use in the DF100 scaffold system for articular cartilage regeneration.

5.14 Polymer Summary

The three sections of Chapter 5 investigated copolymer variations of poly(ϵ -caprolactone): 0-5 mol% acetyl-modified PCL synthesized by Dr. Min Tang (Chapter 5A), poly(L-lysine-co- ϵ -caprolactone) polymerised by an assortment of techniques (Chapter 5B), and commercial Strataprene® 3534 (Chapter 5C). While all three polymers were degradable aliphatic polyesters, the effect of monomer composition, monomer distribution, and polymer molecular weight had pronounced effects on their ability to form scaffolds and impact cellular behaviour.

The beneficial effects of copolymer modification were investigated relative to PCL, the commodity polymer that was used for all scaffolds in Chapters 2 and 3. Through these investigations the value and complexity of multi-parametric optimisations became evident. The incorporation of acetylated monomer in Chapter 5A did greatly improve the porosity and interconnectivity of scaffolds produced by supercritical CO₂ foaming; however, the variations in polymer molecular weight that were confounded with degree of modification also contributed to this effect. The influence of co-polymer composition on molecular weight was also observed in the PLCL syntheses, where different compositions, techniques, and production scales produced polymers of varying molecular weights. With optimisation, PLCL was produced within the molecular weight range of commercial 80 kDa PCL, allowing for a more accurate comparison. Efforts to improve monomer incorporation, distribution, and synthesis scale resulted in deviation from this comparable 80 kDa molecular weight. In recognition of the complexities of controlled, reproducible ring-opening polymerisation a new

commercial product was investigated. Strataprene® 3534 had the benefit of industrial standardisation, large batch sizes, and implantable-grade designation. Unfortunately, as was observed with the acetylated PCL, the change in polymer chemistry had a significant undesired effect on cellular behaviour in addition to the mechanical properties. This effect was significantly detrimental to the maintenance of a differentiated state in primary bovine chondrocytes.

With the complexities encountered in tissue engineering applications, the choice of polymer is not one that should be taken without considerable investigation. The data generated within each of the three studies was valuable in that it informed decisions and developed skills that influenced the subsequent polymer sections within of this chapter. The data also informed the final decision to use poly(ϵ -caprolactone) without modification in the third-generation scaffold study in Chapter 3 and the on-going *in vivo* investigation that it prompted, summarised in Chapter 4.

6 Conclusion and Future Directions

6.1 Conclusion

Articular cartilage is an anisotropic tissue organised in compositional and functional layers. The primary conclusion of this work is that different scaffold production techniques can be combined to better replicate this intricate structure. The biomimetic zonal organisation of mechanical and microenvironmental properties impacts cellular distribution and key aspects of extracellular matrix deposition including collagen fibre orientation. These properties are manifested *in vitro* without requirements for chemical modification or culture under mechanical loading.

Three generations of anisotropic cartilage scaffolds have been developed. The first-generation scaffold by McCullen *et al.* [90] was built upon in Chapters 2 and 3 with second- and third-generation design concepts. Each scaffold generation had a distinct general design and involved a variety of zonal design optimisations.

Second-Generation Scaffold

The second-generation scaffold was the first example of a dual-zone cartilage scaffold to incorporate an electrospun fibre articular surface onto a porous particulate-leached foam [1]. The combination of fibre and foam integrated a smooth articular surface ($R_a = 3.0 \pm 1.0 \mu\text{m}$) with high tensile strength and a foam that permitting full-thickness seeding. Particulate-leached foams of two pore diameters were investigated, 300 μm and 1000 μm . The scaffolds with finer 300 μm pores (BL-0.03) proved to have statistically superior mechanical performance with a compressive modulus of $40 \pm 5 \text{ kPa}$, relative to $20 \pm 5 \text{ kPa}$ for the coarser foam. The BL-0.03 scaffold also had superior biological performance, attributed to the BL-1.0 scaffold being perceived by cells as a series of flat surfaces, while BL-0.03 had sufficiently small pores to initiate three-dimensional pore bridging and tissue infiltration by the passaged chondrocytes. The three-dimensional growth resulted in decreased proliferation and re-differentiation on the foam portion of the BL-0.03 scaffolds, quantified by decreased DNA synthesis, greater chondrogenic ECM accumulation (sGAG/DNA), and upregulated gene expression for indicators of chondrogenesis (aggrecan, collagen type II, collagen II/I ratio), relative to BL-1.0. Investigation into 2-fold (2×10^6) and 10-fold (10^7) increases in seeding density resulted in increased ECM deposition and stress relaxation within the scaffolds. The addition of a hydroxyapatite-loaded PCL fibre layer on the underside of the scaffold with the aim of initiating hypertrophy and calcified cartilage mineralisation was not sufficient to elicit a significant response.

Third-Generation Scaffold

The knowledge gained from the second-generation scaffold informed decisions for the next iteration of design, which incorporated an additional layer of anisotropy to the scaffold for increased biomimicry. The third-generation design consisted of the aligned electrospun PCL articular surface identical to the previous generation, a particulate-leached foam intermediate zone, a directionally frozen deep zone, and a lower fibre zone composed of randomly arranged electrospun fibres. The spherical pores of the intermediate zone had diameters bounding the optimal 300 μm from the second-generation design, 100-300 μm in DF100 and 300-500 μm in DF300. The columnar pores in the deep zone were perpendicular to the articular surface and found to be interconnected with the spherical pores of the PLZ. The scaffold possessed an anisotropic mechanical profile with more than an order of magnitude difference in compressive stiffness, while containing only one material and retaining full interconnectivity, porosity, and mechanical integration. The stiffness of the deep zone exceeded 1MPa at 10% strain, matching the stiffness of the native tissue.

When seeded with a single injection of passaged chondrocytes, the scaffolds filled with uniformly distributed ECM by 4 weeks while maintaining a chondrogenic gene expression profile and staining for sGAG. The collagen alignment was determined to be zone-specific, as visualised by label-free second harmonic generation. Collagen fibrils were aligned perpendicular to the surface in the deep zone and with an isotropic distribution in the intermediate zone, mirroring the natural tissue. Assessment of chondrogenic gene expression found significant improvements at high seeding densities ($10^7/\text{scaffold}$) in the DF100 scaffolds. This density and scaffold type were selected for further investigation in a large animal model.

Large Animal Investigation

Following *in vitro* validation, a skeletally mature porcine model of full-thickness osteochondral defects was developed with the Queensland University of Technology Medical Engineering Research Facility (QUT MERF) in collaboration with A/Prof. Mia Woodruff. The protocol and associated husbandry and analytical techniques were validated in a successful preliminary surgical trial in two skeletally immature animals. Acellular scaffolds 6 mm in diameter were implanted in the trochlear groove of the hind limb with no adverse immunological reactions and improved joint resurfacing relative to empty defect controls. Issues observed with intrinsic repair and subchondral bone remodelling were attributed to the 69% growth of the immature animals over the course of the 3-month study and are not anticipated in the skeletally-mature animals.

The 6-month full-scale study has commenced with four scaffold conditions and six replicates per condition in a total of twelve skeletally mature pigs. Scaffold conditions include acellular DF100, allogeneic-chondrocyte seeded DF100, a MaioRegen® commercially available control scaffold, and an empty defect. All animals recuperated and completed the 6-month experimental period without adverse events. The subsequent data analysis is beyond the scope of this thesis. The design of the large animal study will permit clinically-relevant conclusions regarding the implantation of DF100 as it incorporates an approved scaffold currently used in humans. Therefore, conclusions relevant to the standard of care, in addition to the empty defect control, can be drawn.

Alternate Polymers

In addition to scaffold design, variations on poly(ϵ -caprolactone) were investigated for osteochondral tissue engineering. The first polymer investigated was a copolymer of caprolactone and 1-5 mol% acetylated CO₂-philic monomer. The polymers were designed and synthesised by collaborator Dr. Min Tang for improved super-critical CO₂ foaming of PCL at low temperatures [264]. The study was limited to an early investigation of cytocompatibility, cellular adhesion, and scaffold infiltration. The polymers and the resultant foams were determined to be non-cytotoxic, once sufficiently purified by re-precipitation. Polymer films were assessed for MC3T3-E1 pre-osteoblast adhesion over 14 days, with improved cell spreading and adhesion on the modified surfaces. Polymer scaffolds produced by super-critical foaming with 4 mol% modified monomer had pore diameters and interconnectivities sufficient to enable chondrocyte infiltration, visualised by histological section. The modified polymer was superior to PCL for scCO₂ foaming and supported viable, adherent cell populations.

The CO₂-philic nature of the polymer developed by Dr. Tang was developed for scCO₂ foaming and was therefore not intended for the second- or third-generation scaffold design. Instead, the synthesis of elastomeric poly(L-lactide-co- ϵ -caprolactone) was investigated directly. Through a series of optimisations, 112 kDa PLCL with a C:L molar ratio of 42:58 was produced by solution polymerisation of distilled ϵ -caprolactone and L-lactide at 2 mol/L in dry toluene with a 1,8-octanediol initiator and stannous octoate catalyst. The polymer had an intermediate distribution of monomers with 25% of caprolactones directly bound to a lactide, avoiding the formation of a block co-polymer with large poly(L-lactide domains). PLCL produced by this method was determined to be non-cytotoxic, and was incorporated into the directional freezing protocol, producing a scaffold with a stiffness of 1.76 ± 0.25 MPa and a greatly increased stress relaxation of $42.0\% \pm 0.4\%$, relative to PCL. Investigation into controlled monomer addition improved caprolactone incorporation by up to 40%, producing polymers with 38% CL diads. However, the modified

technique resulted in greatly reduced molecular weights due to atmospheric contamination when scaled to multi-gram batches.

Finally, Strataprene® 3534, a commercially available implant-grade copolymer of caprolactone, lactide, glycolide, and trimethylene carbonate was investigated to replace PCL in the DF100 scaffold. The DF100 scaffolds produced from Strataprene® 3534 were significantly less stiff than PCL in compression, at only 295 ± 33 kPa, decreasing an additional $63\% \pm 2\%$ when hydrated. The gene expression profiles of high-density bovine chondrocytes at 7 days indicated a less differentiated phenotype, relative to PCL controls. While Strataprene® 3534 was easy to process and had unique mechanical properties, it showed no improvements over 80 kDa poly(ϵ -caprolactone) and was therefore not suited as an alternative for use in the DF100 scaffold system.

Summary

The work contained within this thesis has expanded the field of anisotropic scaffold design, with implications for articular cartilage engineering. The combination of electrospun fibres and anisotropic foams for scaffold engineering was the first in the field when published in Steele *et al.* [1]. The design of the third-generation scaffold is new to the field, as is the order-of-magnitude increase in stiffness in a porous polymer scaffold while maintaining interconnectivity and polymer composition. The observation of differentially aligned ECM within a single multi-layer scaffold without zonally distinct materials or surface functionalisation is also believed to be the first in the field. The design, validation, and implementation of the porcine *in vivo* model have produced an effective GLP test of an osteochondral scaffold. The study of alternate polymer compositions concluded that 80 kDa poly(caprolactone) remained the best polymer for the production of a mechanically robust porous scaffold for cartilage regeneration. The objectives of the project were met, with a zonally organised design possessing biomimetic stiffnesses and capable of inducing zone-specific ECM deposition. The next steps are finalising the on-going *in vivo* validation and continuing efforts towards clinical translation.

6.2 Future Directions – Clinical Translation

The clinical translation of the scaffold investigated *in vitro* in Chapter 3 and as part of an osteochondral scaffold *in vivo* in Chapter 4 involves multiple steps, summarised as validation, protection, communication, and acquisition.

In Vivo Validation

The first step in translation is the completion of the full-scale large animal study, in compliance with GLP protocols for histological preparation and analysis according to existing frameworks including the International Cartilage Repair Society (ICRS) Osteochondral Histology Score [275]. If scoring shows significant advancement in cartilage repair, considerable work will be undertaken to move the scaffold towards clinical translation. If the repair does not warrant translation, the scaffold will undergo further development within Imperial College London to fix identified issues and further improve the quality or repair for later translation.

Defining Unmet Need

In order to bring a product to market, it must meet an unmet need. The unmet need identified in the cartilage regeneration field is the generation of organized, clinically functional articular cartilage. We aim to show evidence of meeting this need, relative to a current clinically approved control with the analysis of the full-scale *in vivo* validation. Additionally, few interventions are mechanically similar to articular cartilage immediately upon implantation in load bearing applications. This secondary unmet need was considered a key aspect of our scaffold development, as it would encourage earlier joint loading, mobility, and impose important mechanical stimuli on the regenerating tissues.

Market Analysis

A total of 1.2 million surgical interventions are performed each year for cartilage repair, with a world-wide market of £1.5 billion a year. An initial market analysis on scaffold-based cartilage repair has been performed for this thesis, and can be found in Table 1-4. Notable academic publications *in vitro* and *in vivo*, which will inform future markets and competition were also summarized in Table 1-5 and Table 4-1. A more thorough analysis will need to be performed, and will be conducted in collaboration with Imperial Innovations.

In line with the market analysis, further surgical consultation will be performed to flag any issues raised with the scaffold or the implantation procedure. The scaffold was designed to interface with existing tools for mosaicplasty, in a manner identical to that developed for TruFit CB by Smith &

Nephew to facilitate translation and promote surgical confidence in the procedure. While all testing was performed with 6 mm diameter scaffolds, the scaffolds are produced as 80 x 80 mm sheets and could be cut to any standard or custom dimensions, to be determined by consultation with the end users. The use of standardised mosaicplasty cylinder dimensions was the approach taken by TruFit, while the tailorable surgeon-trimmed custom-shape approach was implemented by FinCeramica for MaioRegen. Therefore both options have existing clinical markets, potentially marketed in parallel depending on defect size and shape.

GMP-Compliant Scaffold Production

The existing scaffolds were produced in an academic lab, and therefore do not meet Good Manufacturing Practices (GMP) production standards. The production process will have to be moved to a GMP facility and implement GMP-approved materials and existing protocols. This can be arranged through partnership with established osteochondral medical implant manufacturers, including, but not limited to Smith & Nephew, Stryker, Zimmer, and DePuy or smaller contract-based Original Equipment Manufacturer (OEM) GMP-compliant facilities.

First Target Clinical Population for Testing

The scaffold has been designed to promote the repair of healthy articular cartilage, however, it requires a healthy cell population, either from expanded exogenous cells or active endogenous cells to form the tissue. Therefore, along with other MACI and AMIC techniques, it is not applicable to the formation of hyaline cartilage in older patients or those with advanced osteoarthritis. Therefore, in a first clinical study, young (18-40 year old) patients with unipolar femoral or patellar cartilage defects 1-3 cm² would be selected. Patients would have to present significant pain, but those with osteoarthritis, significant ligament damage, or a BMI >30 would be rejected to ensure joints are loaded correctly.

Regulatory Approval

To open an early revenue stream, the first regulatory step which will be investigated is the CE mark allowing for sales within the European Economic Area. To gain a CE mark the device has to prove to be safe in a limited clinical population as outlined in EU Directive 90/385/EEC.

The larger and more profitable market is the United States, which requires FDA approval. Premarket Approval (PMA) defined under section 515 of the FD&C Act is required, as the scaffold is designated as a Class III device. PMA is the most stringent approval process for a medical device, and must confirm that the device is both safe and effective for its intended application.

Acquisition and Licensing

Once the in vivo analysis and market analysis are completed, discussions will begin between Imperial College, Imperial Innovations, and commercial partners. The intellectual property is currently owned in a joint venture between Imperial College London and Imperial Innovations [185]. Common successful approaches towards translation of medical devices from academic labs are the licensing or acquisition of intellectual property to existing medical device companies. The cost and complexity of clinical trials, regulatory approval, and marketing of a medical device are too great for all but the most successful university spin-out companies. Occasionally a spinout company is initially formed with early private or institutional investment for the early clinical trials, at which point the company is acquired, as the IP would then have more value and less risk.

As the scaffold is patented, with the compilation of the in vivo data package, in collaboration with Imperial Innovations, Smith & Nephew, Stryker, Zimmer, and DePuy will be contacted to gauge interest in IP licensing.

6.3 Future Directions – Advanced Analysis

In addition to the preliminary work summarised in the Chapter 3 extension (3.4 Expansion of the Third-Generation Scaffold Study) there are many potential directions for future work including non-traditional analysis of the existing scaffolds and modification of the design by zonal surface functionalisation. Finally, improvements in the emerging technology of 3D direct writing melt electrospinning could be utilised to generate complex but reproducible scaffold geometries on a similar scale.

In-depth analysis of in vitro scaffolds

There are a wide variety of analytical methods not traditionally applied to tissue engineering scaffolds that would refine the analysis and improve the resolution of scaffold-dependent cellular responses. Three of these approaches which were investigated in a preliminary manner but found to require extensive optimisation beyond the scope of this thesis were Raman spectroscopy-based histological assessment, x-ray diffraction tracking of collagen dynamics, and laser-capture microdissection for localised qRT-PCR.

Raman Spectroscopy

Work within the Stevens group has shown the power of Raman spectroscopy for the assessment of cells and tissues, revealing chemical changes within these systems on a sub-micron scale. Examples include the observation that hMSC and ESC differentiated towards an osteogenic line are chemically distinct [276], that radio-frequency fusion of intestinal mucosa and arterial wall alter collagen organisation and crosslinking [277], and that hMSC exposed to ions from strontium-substituted bioactive glasses have significantly increased levels of cytoplasmic cholesterol and lipids [278]. This technology could be applied to *in vitro* or *in vivo* grown cartilage to assess chemical composition and zonal organisation. Existing work on the zonal segregation of developmental cartilage shows promise for further refinement of the technique [279]. The benefit of Raman spectroscopy is the wealth of data that is collected in a label-free, assumption-free manner from a single sample of hydrated tissue, foregoing histological artefacts. Additionally, as the procedure is non-destructive, this tissue can then be embedded for correlated traditional histology. The adoption of Raman-based imaging for tissue engineering will advance the understanding of cell-scale changes in ECM composition within different scaffold architectures, zones, and proximities to the cell-material interface.

Laser Capture Microdissection

As Raman spectroscopy provides insights into the chemical composition with cell-scale resolution, laser-capture microdissection can be used to improve the resolution of qRT-PCR. Within this work, scaffolds were physically dissected to provide zonal gene expression analysis, a refinement of the traditional whole-scaffold approach to PCR. However, the differentiation of gene expression within the zones suffered from the variability attributed to the range of microenvironments within each zone. For example, within the PLZ, RNA from cells located at the center of spherical pores, at the cell-material interface and within the interstitial space between the larger pores. The same can be said for the deep zone channels.

Laser-capture microdissection (LCM) is a tool used to cut specific tissue structures from thin sections for further analysis. Briefly, a section of tissue prepared by cryosectioning is mounted on a slide and loaded within a LCM-equipped microscope. The tissue is visualised, the area of interest is traced with an ultraviolet laser, propelled from the slide surface with an infrared laser, and collected in a microcentrifuge tube. The small sample of tissue can then be used for localised analysis, in this case, gene expression by qRT-PCR. Preliminary work on LCM with the DF100 scaffold system encountered issues with cryosectioning samples sufficiently thin such that the PCL scaffold could be cut with the laser without burning the adjacent tissue. This issue could be overcome with sufficient time to optimise the protocol.

Dynamic Collagen Alignment

Multi-photon microscopy was used to visualise the alignment of collagen within the zones of the DF100 scaffold. An advancement on this analysis would be the assessment of collagen alignment under dynamic loading conditions by x-ray diffraction within a synchrotron. An attempt was made to utilise this technique to assess alignment in 8-week *in vitro* cultured DF100 samples in collaboration with Dr. Angelo Karunaratne, Department of Bioengineering, Imperial College London at the Diamond light source. However, due to issues with the x-ray opacity of the dynamic loading rig and the low signal from the immature collagen fibrils within the scaffolds, no data was generated by the preliminary study. With optimisation, the strain-dependent zonal alignment of collagen within a macroscopic scaffold could be visualised and quantified.

Zonal Surface Functionalisation

All three generations of scaffold were produced from un-modified PCL. The development of the scaffold was performed in parallel with the development of PCL surface functionalisation by peptide and polymer brushes. While in the Stevens Group Dr. Lesley Chow developed a method to

functionalise the surfaces of electrospun polymer fibres with functional peptide sequences incorporated into peptide-polymer conjugates before processing. By eliminating the need for post-functionalisation, the localisation of the peptides was controlled by the production technique, allowing the formation of functional gradients [133]. An early proof of concept for the peptide functionalisation of DF100 was included in Chapter 3.4, and would permit surface functionalisation of each zone with different peptide sequences. Additionally, work performed in collaboration with Ms. Rachael Harrison, Stevens Group, Imperial College London, has produced surface modification of PCL fibres with functional polymer brushes, spatially localised by PCL-initiator incorporation into the electrospinning solution [280]. These two surface functionalisation techniques could be adapted to the zonal modification of the DF100 scaffold, potentially improving the cell-material interface and ultimately the zonal differentiation of cells seeded within the scaffold. However, the extensive matrix accumulation observed inside the scaffold pores suggests that such functionalization approaches might not be necessary for improved cartilage repair.

3D Melt Electrospinning

The primary aim of the thesis was to control pore shape, alignment, and location to permit control over cellular microenvironment and mechanical properties. The early additive manufacturing work for cartilage by Woodfield and Klein investigated the effect of porosity, architecture, and alignment to produce zonally-organised structures; however, the limitations of additive manufacturing at the time resulted in features with millimetre-scale resolution [18,228,229]. The current resolution of traditional additive manufacturing for tissue engineering scaffolds is limited to 50-100 μm , still beyond the cellular scale. The first-generation scaffold design which this project originated from approached scaffold anisotropy with layers of traditional electrospinning of 1-10 μm fibres [90]. However, an issue intrinsic to traditional electrospinning is poor control of porosity, fibre alignment, and three-dimensional scaffold shape, all of which impacted the utility of the final product.

Concurrent with my PhD, a new technique has been developed and improved by Prof. Paul Dalton, Dr. Toby Brown and collaborators, including Prof. Dietmar Hutmacher and A/Prof. Mia Woodruff [149]. The technique of 3D direct writing melt electrospinning combines the cell-scale topographical features of traditional electrospinning with the spatial control and geometrical complexity of fused-deposition modelling (FDM) [149,281]. This technique brings cell-scale geometries to additive manufacturing with a resolution as precise as 800 nm [282]. While currently used with bulk polymers, the development of composite structures with highly controlled and reproducible geometries, compositional gradients, surface functionalities, and anisotropic mechanical properties is an exciting new frontier in the field of tissue engineering.

7 References

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Appendices

- A** Steele *et al.* 2014. Combinatorial scaffold morphologies for zonal articular cartilage engineering
- B** SOP - Polyester Wax Histology
- C** Comparison of Normalised ECM Concentrations
- D** SOP - Gelatine Microbead Emulsion
- E** SOP - Multilayer Scaffold Production
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Combinatorial scaffold morphologies for zonal articular cartilage engineering[☆]



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ABSTRACT

Articular cartilage lesions are a particular challenge for regenerative medicine strategies as cartilage function stems from a complex depth-dependent organization. Tissue engineering scaffolds that vary in morphology and function offer a template for zone-specific cartilage extracellular matrix (ECM) production and mechanical properties. We fabricated multi-zone cartilage scaffolds by the electrostatic deposition of polymer microfibres onto particulate-templated scaffolds produced with 0.03 or 1.0 mm³ porogens. The scaffolds allowed ample space for chondrocyte ECM production within the bulk while also mimicking the structural organization and functional interface of cartilage's superficial zone. Addition of aligned fibre membranes enhanced the mechanical and surface properties of particulate-templated scaffolds. Zonal analysis of scaffolds demonstrated region-specific variations in chondrocyte number, sulfated GAG-rich ECM, and chondrocytic gene expression. Specifically, smaller porogens (0.03 mm³) yielded significantly higher sGAG accumulation and aggrecan gene expression. Our results demonstrate that bilayered scaffolds mimic some key structural characteristics of native cartilage, support in vitro cartilage formation, and have superior features to homogeneous particulate-templated scaffolds. We propose that these scaffolds offer promise for regenerative medicine strategies to repair articular cartilage lesions.

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1. Introduction

Osteoarthritis is the predominant form of arthritis and remains a leading cause of disability [1]. Arthritic joints are characterized by lesions in hyaline cartilage that result in severe pain, loss of motion and eventually require surgical intervention [2]. Cartilage tissue engineering has emerged as a treatment method for articular cartilage lesions and this approach has employed a variety of scaffold materials which act as a carrier of and delivery vehicle for autologous chondrocytes and/or progenitor cells capable of cartilage formation [3–5]. Such strategies incorporate scaffold materials that range from autologous periosteal membranes to

highly fibrillar materials derived from both biopolymers and degradable polymers including collagen, hyaluronic acid and aliphatic polyesters [6,7]. The materials used are predominantly isotropic in regards to functional characteristics including mechanical performance, cellular distribution, and cellular interaction with the matrix. Articular cartilage is a highly organized tissue that provides a low-friction and wear-resistant bearing surface and exhibits anisotropic mechanical properties as a result of depth-dependent differences in the density and structural arrangement of its extracellular matrix (ECM). Articular cartilage is comprised of four main zones: the superficial, middle, deep and calcified cartilage zone [8,9]. Each zone varies in regards to biochemical content, morphology and biomechanical function, with increased proteoglycan concentration and stiffness with depth, while conversely cellular density decreases; the total collagen concentration is unchanged with depth [8,10]. From a functional perspective, the cartilage portion of the osteochondral gradient can be simplified into two main regions: the superficial zone which exhibits a high tensile strength and low coefficient of friction to maintain smooth articulation; and a dense ECM region rich in proteoglycan molecules which contribute to the compressive mechanical

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properties by producing a high osmotic pressure within the tissue [5,11].

The superficial, middle and deep zones possess distinct gene and microRNA expression profiles, as thoroughly summarized by Grogan et al. [12]. When chondrocytes are expanded in vitro to obtain sufficient cell quantities for implantation, they dedifferentiate, losing their characteristic gene expression profile of collagen II and aggrecan by over an order of magnitude at the first passage, and begin expressing high levels of collagen I [13]. Dedifferentiation can be reversed by culturing chondrocytes on 3-D matrices, supplementation of the media with transforming growth factor (TGF)- β 3 and dexamethasone, and physiological mechanical loading [14]. The redifferentiation protocols have been studied by gene expression as well as microRNA analysis, and in the case of microRNA expression can be almost entirely restored with the proper physical and chemical cues [13]. The use of zonally derived chondrocyte populations has merits in creating zonal constructs for implantation; however, the lack of efficient automated cell-sorting techniques limits their clinical applicability, leaving some to question whether zonal properties can be derived from an expanded redifferentiated chondrocyte population by simpler methods [15]. Beyond zonal populations, the use of purified progenitors isolated from cartilage using the classic stage-specific embryonic antigen-4 (SSEA-4) marker for undifferentiated stem cells has also proven of insufficient difference to warrant the additional complexity [16]. For this reason, a mixed population of passage-2 chondrocytes, cultured in a redifferentiation medium, remains the most widely investigated cell population for clinically applicable cartilage tissue engineering.

Current strategies for scaffold fabrication which have been shown to support cartilage formation include electrospinning of fibrous materials, particulate-leaching, gas-foaming and phase separation [9,17–21]. Each of these techniques offer specific advantages, yet none can fully encompass all requirements for optimal scaffold performance including zonal organization, adequate mechanical properties, full cellular ingress and physiological levels of ECM formation. Electrospinning is a facile technique which produces flexible, densely packed fibre networks with tuneable mechanical, physical and biological properties based on polymer selection, fibre size, network orientation and overall thickness [22,23]. Previous research has shown that aligned electrospun fibres are able to mimic the highly oriented morphological and tensile properties of the superficial zone of articular cartilage, and have been shown to support in vitro cartilage formation and chondrogenesis [18]. Nevertheless, electrospun scaffolds are generally limited in regards to dimensional thickness and lack of cellular infiltration due to the dense packing of fibres [22,24]. One exception is melt electrospinning, which can completely eliminate these issues, but concedes fine ($<10\ \mu\text{m}$) fibre sizes for control, forming scaffolds intermediate between solution electrospinning and fused deposition modelling [25]. Particulate-templated scaffolds offer their own unique advantages including greater control of pore size, interconnectivity, geometry and overall scaffold porosity based on the size, shape and loading of the particulate [20].

Electrospinning directly onto a foam substrate produces a bilayered scaffold that possesses the tensile strength and fibrous morphology of the electrospun membrane and the high porosity of the underlying foam. The two-zone approach has been previously applied to vascular and bladder scaffolds which utilize the layered design to mimic the structural variations within their respective tissues [26–28]. As previously stated, articular cartilage also possesses a depth-dependent multi-zone morphology, with varied mechanical properties and function. A scaffold that mimics the structure of the native cartilage may offer a superior template for in vitro cartilage formation and ECM organization. Some approaches to multi-layer articular cartilage scaffolds include mul-

ti-layer hydrogels [29], multilayer electrospun fibres [18] and anisotropic fused deposition modelling [9]. This investigation is the first to study a zonally organized articular cartilage scaffold with an electrospun fibre articulating surface deposited onto a particulate-leached foam.

Herein, we generate bilayered poly(ϵ -caprolactone) (PCL) scaffolds comprised of an aligned fibre zone, which mimics the morphology and mechanics of the superficial zone of articular cartilage, laminated to a bulk porous particulate-templated scaffold which allows for full cellular infiltration and extensive ECM deposition. We hypothesized that the addition of aligned electrospun fibres would increase tensile properties, reduce surface roughness, and provide morphological similarities to native articular cartilage. Additionally, we varied the particulate size (0.03 vs. $1\ \text{mm}^3$) in the particulate-templated scaffolds and hypothesized that smaller pores would support in vitro cartilage formation by bovine chondrocytes. Our results demonstrate that the electrostatic deposition of fibres with aligned orientation onto particulate-templated foams produces scaffolds which mimic key mechanical and functional characteristics of native cartilage and are able to support in vitro cartilage formation, indicating their promise in regenerative medicine strategies to repair articular cartilage lesions.

2. Materials and methods

2.1. Scaffold fabrication

2.1.1. Particulate-templated scaffolds

PCL with a number average molecular weight, M_n , of 80,000 Da, 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) and dichloromethane (DCM) were procured from Sigma–Aldrich (Dorset, UK). Particulate-templated scaffolds were fabricated by mixing 30 g NaCl of varying sizes (0.03 or $1\ \text{mm}^3$) with 8% w/v PCL in DCM to form a homogeneous paste that was cast into a custom aluminium mould ($2\ \text{mm} \times 140\ \text{mm} \times 240\ \text{mm}$). The solvent was allowed to evaporate overnight, salt was extracted for 3 days in distilled water with water changes every 12 h, and scaffolds were air-dried for 1 day. Macroscopic sheets were formed with a final thickness of $\sim 2\ \text{mm}$.

2.1.2. Bilayered fibre zone (FZ)/particulate-templated zone (PZ) scaffold construction

Particulate-templated scaffolds (PZ) were mounted onto a voltage-driven rotating mandrel (diameter 6.4 cm, length 10 cm) and aligned fibres (FZ) were deposited by electrospinning 5 ml of 12% w/v PCL in HFIP using a custom-made electrospinning setup that included a programmable syringe pump (Kd Scientific Model KDS 100 CE, Sandbach, Cheshire, UK) operating at $2\ \text{ml h}^{-1}$, a Glassman high-voltage power supply series WR (Glassman, Bramley, Hampshire, UK) operating at 16 kV with an air gap distance of 11 cm, and a variable speed mandrel rotating at a linear velocity of $10\ \text{m s}^{-1}$. Bilayered fibre scaffolds had a fibre membrane thickness of $\sim 200\ \mu\text{m}$ with a fibre diameter of $1\ \mu\text{m}$.

2.2. Scaffold characterization using electron microscopy

Scaffolds were imaged by scanning electron microscopy (SEM) using a JEOL 5610 (Herts, UK). Specimens were coated with 100 Å Au using an Emitech K550 sputter coater and imaged at an accelerating voltage of 15 kV and a working distance of 15 cm. Cross-sectional examination of scaffolds was performed on freeze-fractured scaffolds prepared in liquid nitrogen.

2.3. Mechanical characterization of scaffolds

Particulate-templated and bilayered scaffolds were mechanically tested in tension to failure using an Instron Model 5540 testing machine (Norwood, MA, USA), equipped with a 50 N load cell operated at a crosshead speed of 10 mm min⁻¹. Specimens had a gauge length of 30 mm and width of 10 mm; thickness was measured by digital callipers. Ultimate tensile strength and Young's modulus were determined from stress–strain curves where the ultimate tensile strength was taken as the maximum stress and the Young's modulus was calculated from the linear region of the stress–strain curve. All cellular and acellular mechanical measurements were performed with $n \geq 5$ independent replicates.

Shear strength at the interface between the fibre and particulate-templated zones of the bilayered scaffolds was measured by carefully separating the individual zones of 50 mm length specimens to expose 10 mm of the particulate-templated zone or the fibre zone for fixation at either end, with a gauge length of 30 mm in between. Specimens were tested in both aligned and transverse fibre orientations; peak load (N) and stiffness (N mm⁻¹) were determined.

Compression mechanics of bilayered scaffolds was assessed from 10 mm diameter samples that were pre-loaded to 0.05 N and compressed to 10% strain at a crosshead speed of 0.5% strain min⁻¹. Following compression, samples underwent stress relaxation, which was quantified as the percentage decrease in force after 300 s at 10% strain. Tangent modulus and peak stress were calculated from the linear portion of the stress–strain curve and the maximum stress level achieved, respectively.

2.4. Bovine chondrocyte isolation

Bovine cartilage was harvested from the lower leg joints of a young calf. Briefly, the ankle joint was cut open along the joint line and cartilage tissue was cut with a scalpel into thin sections parallel to the subchondral bone. Chondrocytes were isolated by digesting in Dulbecco's modified Eagle's medium (DMEM) + Glutamax (4.5 g l⁻¹ glucose) with 0.2% w/v pronase, 10 mM HEPES and 50 µg ml⁻¹ gentamycin (all reagents from Invitrogen, Paisley, UK) for 1 h at 37 °C with agitation. This digest was removed and replaced with DMEM + Glutamax (4.5 g l⁻¹ glucose) supplemented with 10 mM HEPES, 50 µg ml⁻¹ gentamycin, 5% v/v fetal bovine serum (FBS) and 0.04% w/v collagenase (Sigma) overnight at 37 °C with agitation. After digestion, isolated chondrocytes were filtered through a 70 µm pore size filter, centrifuged at 250 × g for 3 min, and plated in DMEM (4.5 g l⁻¹ glucose) with 10% v/v FBS, 50 µg ml⁻¹ ascorbic acid (Sigma) and 50 µg ml⁻¹ gentamycin (expansion medium).

2.5. Assessment of chondrocyte/bilayered scaffold interaction and in vitro cartilage formation

Particulate-templated and bilayered scaffolds were assessed for cell-seeding efficiency, cell infiltration and for cellular proliferation. Scaffolds 2 mm thick × 10 mm diameter were punched from macroscopic sheets. Scaffolds were sterilized in 70% ethanol for 30 min followed by three washes with sterile phosphate-buffered saline (PBS) and then soaked in 0.01% w/v bovine serum albumin (BSA) (Sigma–Aldrich, UK) in PBS overnight to promote chondrocyte adhesion. Bilayered scaffolds and control scaffolds (particulate-templated with no fibre membrane) were seeded with 1×10^6 chondrocytes. A 20 µl aliquot of expansion medium containing 2×10^5 chondrocytes (passage 2) was placed on the fibre side of each bilayered scaffold and cells were allowed to adhere for 2 h. Constructs were then inverted and the particulate-templated side was seeded with an additional 8×10^5 chondrocytes

in 80 µl of expansion medium, which were allowed to adhere for an additional 2 h before returning the constructs to the upright position and adding 2 ml expansion medium. Seeded scaffolds were cultured for up to 7 days and assessed for seeding efficiency, proliferation and chondrocyte distribution. Chondrocyte-seeding efficiency was assessed by comparing the DNA content of the seeded fibre zones (FZ-0.03/FZ-1.0) and particulate-templated zones (PZ-0.03/PZ-1.0) to the DNA content of 2×10^5 and 8×10^5 chondrocytes, respectively, as determined using a Quant-iTTM PicoGreen[®] kit (Invitrogen, UK), following the manufacturer's instructions.

In vitro cartilage formation was assessed by culturing bilayered scaffolds seeded with 1×10^6 chondrocytes, following the same seeding protocol, for up to 4 weeks in chondrogenic differentiation medium consisting of DMEM (4.5 g l⁻¹ glucose), supplemented with 50 µg ml⁻¹ L-proline (Sigma), 50 µg ml⁻¹ ascorbic acid (Sigma), 0.1 mM sodium pyruvate (Sigma), 10 ng ml⁻¹ TGF-β3 (Lonza, Slough, UK) and 1% v/v ITS Premix (BD Biosciences, Oxford, UK) at 37 °C and 5% CO₂. Medium was changed twice weekly. Bilayered scaffolds were assessed at 1, 2 and 4 weeks after seeding. All biochemical assays were performed with $n \geq 4$ independent replicates.

2.6. Chondrocyte biochemical assessment

After 0, 1, 2 and 4 weeks of culture, chondrocyte-seeded bilayered scaffolds were carefully separated into fibre and particulate-templated zones, freeze dried, and digested individually in papain digest solution (2.5 units papain ml⁻¹, 5 mM cysteine HCl, 5 mM EDTA, in PBS (all reagents from Sigma Aldrich, UK)) at 60 °C overnight. Digested samples were assayed for total DNA content using a Quant-iTTM PicoGreen[®] kit and for sulfated glycosaminoglycan (sGAG) using the Blyscan Kit (Biocolor, Carrickfergus, UK) as per the manufacturers' instructions. All biochemical assays were performed with $n \geq 4$ independent replicates.

2.7. Mechanical testing and surface characterization of chondrocyte-seeded bilayered scaffolds

Tensile and compressive mechanics were assessed for acellular and for chondrocyte-seeded bilayered scaffolds at time points of 1, 2 and 4 weeks as described in Section 2.3. Surface roughness of particulate-templated and bilayered fibre scaffolds was assessed with white light interferometry (Wyko NT9100, Veeco) with a 20 × objective and digital images were captured over a 312 µm × 234 µm area with a z-axis resolution of 50 µm.

2.8. Histological examination of chondrocyte-seeded scaffolds

After 4 weeks in culture, bilayered scaffolds were fixed in formalin, dehydrated in an ethanol series and embedded in polyester wax (VWR, UK) at 45 °C. Sections were cut at 10 µm, adhered to Si-lane-Prep slides (Sigma Aldrich, UK) and dewaxed in Histo-Clear II (Fisher Scientific, UK). Dewaxed sections were stained for deposited sulfated proteoglycans (sGAG) using alcian blue (pH 2.5), collagen using picrosirius red, and with haematoxylin and eosin (H&E) for cell nuclei and extraneous matrix, respectively. For crossed polarized imaging of picrosirius red birefringence, the PCL was removed from sections with xylene overnight followed by an additional 30 min in chloroform to prevent the birefringence of PCL from hindering collagen fibril visualization. Immunohistochemical staining (IHC) was performed for collagen I (Abcam ab34710), collagen II (Abcam ab300) and collagen X (Abcam 58632) with rabbit IgG and PBS negative controls. Samples were pretreated with hydrogen peroxide, an avidin and biotin blocking kit (Vector Labs, UK), and blocked with 5% v/v goat serum. Primary

Table 1
qPCR primers.

Gene	Forward	Reverse
<i>B2M</i>	ACCTGCTGTCCACGCTGAGT	TGTTCAAATCTCGATGGTGCTGCT
<i>ACAN</i>	GCTACCCTGACCCCTTCATC	AAGCTTTCTGGGATGTCCAC
<i>COL1A1</i>	CATTAGGGGTCAATGGTC	TGGAGTTCATTTCACCAG
<i>COL2A1</i>	CATCCACCTCTCACAGTT	GTCTCTGCCTTGACCCAAAG

antibodies were incubated overnight at 1/200 in 5% v/v goat serum, followed by goat-anti-rabbit HRP at 1:100 for 1 h, stained with a 3,3'-diaminobenzidine (DAB) kit (Vector Labs, UK) for 10 min, and counter-stained with haematoxylin. All stained sections were mounted with Histomount (Fisher Scientific, UK), and viewed on an Olympus BX51 microscope equipped with an Olympus DP70 camera.

2.9. Gene expression analysis

After 0, 1, 2 and 4 weeks, total RNA was isolated from the bilayered scaffolds (scaffold zones were separated prior to isolation using sterile forceps) by using an RNeasy kit and cDNA was obtained from reverse transcription using the QuantiTect Reverse Transcription kit (all from Qiagen, UK). Real-time quantitative polymerase chain reaction (qPCR) was carried out on a Rotorgene Corbett PCR, using previously published primer sequences. All qPCR measurements were performed with $n \geq 4$ independent replicates. qPCR were performed on β -2 microglobulin (*B2M*), aggrecan (*ACAN*, U76615), collagen I (*COL1A1*, NM-174520) and

collagen II (*COL2A1*, X02420) using the QuantiTect SYBR Green PCR kit in accordance with the manufacturer's recommendations (Qiagen, UK). Primers, summarized in Table 1, were validated and the efficiencies of the primers were found to be between 0.90 and 1.07. The gene expression levels were normalized to the expression of the housekeeping gene *B2M* and were expressed as fold changes relative to week 0 passaged chondrocyte control samples (2 h post-seeding). The relative mRNA levels of *ACAN*, *COL1A1* and *COL2A1* were calculated using the $\Delta\Delta C_t$ method and the *COL2A1/COL1A1* ratio corresponds to the $2^{-\Delta C_t}$ of each gene.

2.10. Statistical analysis

All experimental groups had a sample size of at least $n = 4$ for biochemical and qPCR and $n \geq 5$ for mechanical property analyses. Data are presented as average $\pm$ standard deviation. Statistical significance was determined by performing one-way ANOVA with a significance accepted at p -value < 0.05 .

3. Results

3.1. Fabrication of bilayered scaffolds

Macroporous scaffolds were fabricated with two zones, the fibre zone (FZ) and porous zone (PZ) (Fig. 1A). The PZ was produced by particulate leaching of either 0.03 or 1.0 mm³ salt crystals embedded in a matrix of PCL followed by the electrostatic deposition of aligned PCL microfibres on their surface. Fig. 1B is composed of SEM micrographs of the aligned fibre layer, both bilayered

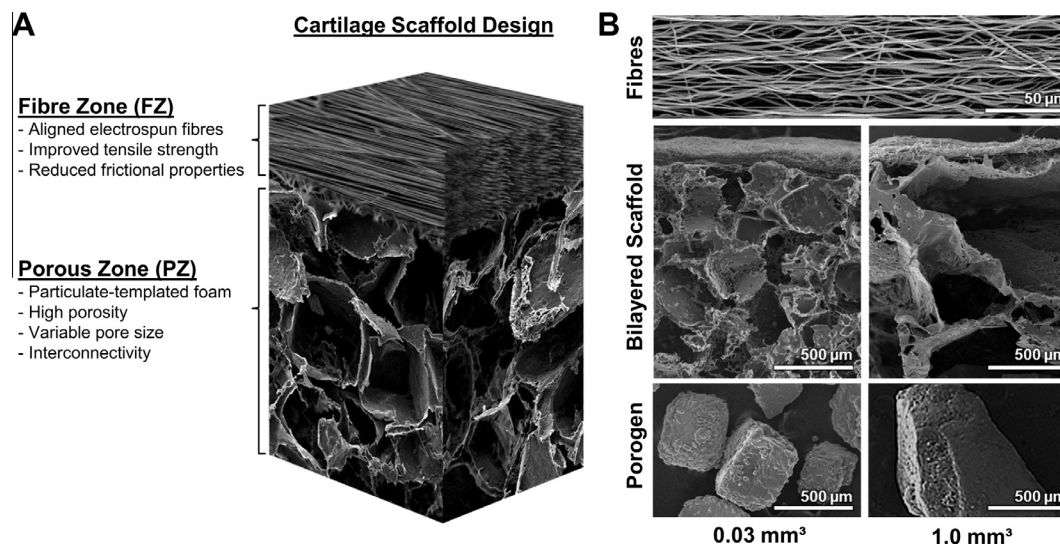


Fig. 1. Bilayered cartilage scaffold schematic. (A) A diagram illustrating the electrospun fibre zone (FZ) deposited on a particulate-templated foam (PZ). The combination of the two distinct zones is designed to yield an anisotropic scaffold with a smooth articulating surface and a more porous region for ECM deposition. (B) Electron microscopy images of (top) the aligned fibre zone that is shared between both scaffold varieties, (middle) the complete bilayered scaffolds with 0.03 mm³ (left) and 1.0 mm³ (right) pores, and (bottom) the sodium chloride porogens used to produce their respective scaffolds.

Table 2
Properties of particulate-templated (PZ) and bilayered (BL) scaffolds produced with 0.03 or 1.0 mm³ porogens. Addition of the aligned fibre membrane in the BL scaffolds significantly enhanced tensile mechanics and lowered surface roughness when compared to single-component particulate-templated scaffolds.

Scaffold type	Tensile modulus (MPa)	Tensile stress at failure (MPa)	Compressive modulus (kPa)	Compressive stress (kPa) at 10% strain	Interfacial stiffness (aligned) (N mm ⁻¹)	Interface peak load (aligned) (N)	Surface roughness (R _a ; μ m)
PZ – 0.03	0.04 $\pm$ 0.02	0.05 $\pm$ 0.01	40 $\pm$ 5	5 $\pm$ 1	N/A	N/A	16 $\pm$ 10
BL – 0.03	1.1 $\pm$ 0.3	0.4 $\pm$ 0.1	44 $\pm$ 21	4.7 $\pm$ 2.0	0.24 $\pm$ 0.04	0.68 $\pm$ 0.03	3.0 $\pm$ 0.8
PZ – 1.0	0.10 $\pm$ 0.05	0.03 $\pm$ 0.01	20 $\pm$ 5	2 $\pm$ 1	N/A	N/A	26 $\pm$ 20
BL – 1.0	1.6 $\pm$ 0.5	0.4 $\pm$ 0.2	14 $\pm$ 4	2.0 $\pm$ 0.4	0.23 $\pm$ 0.02	1.15 $\pm$ 0.03	2.1 $\pm$ 0.5
Fibre zone	28 $\pm$ 2	12 $\pm$ 3	140 $\pm$ 20	70 $\pm$ 4	N/A	N/A	3 $\pm$ 1

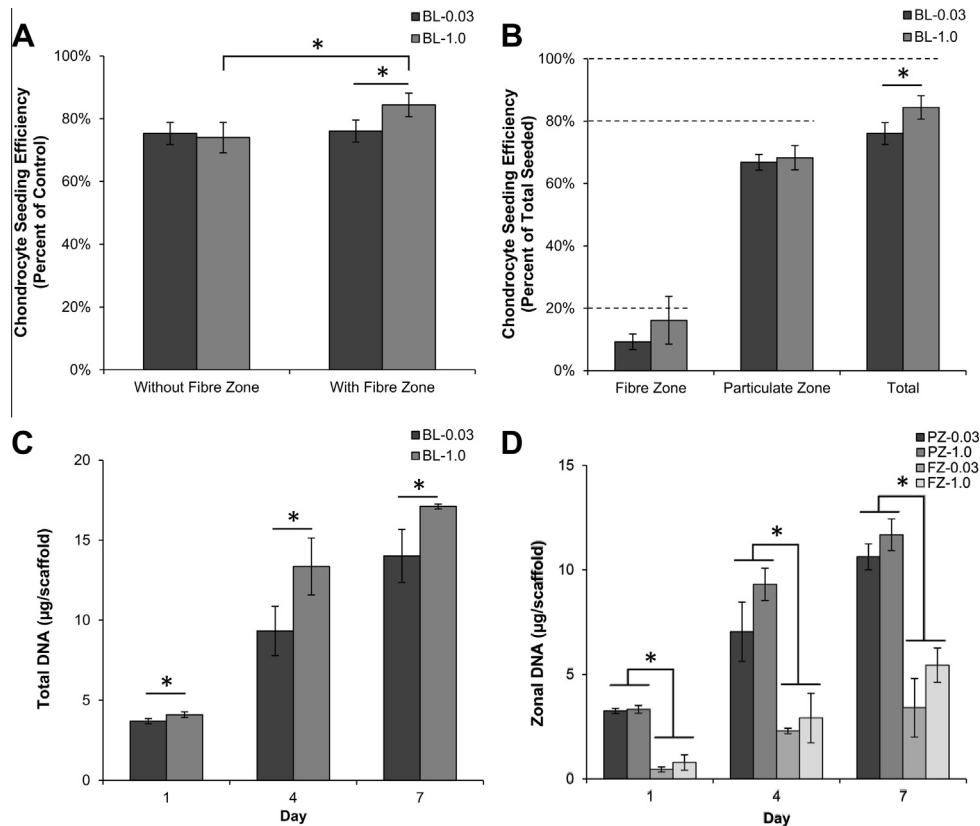


Fig. 2. Chondrocyte-seeding efficiency and proliferation analyzed by DNA quantification. (A) Scaffold-seeding efficiencies with and without the fibre zone. Increased seeding efficiency was observed in BL-1.0 relative to BL-0.3 and both of the fibre-free scaffolds. (B) Seeding efficiency by scaffold zone showing the maintenance of the 20:80 FZ:PZ ratio at which they were initially seeded. (C,D) Total chondrocyte proliferation in the bilayered scaffolds (C) and subdivided into the FZ/PZ zones (D) over 7 days. *Significant difference ($p < 0.05$).

scaffolds with fibre and porous zones (0.03 and 1.0 mm³), and the particulates used to create the differing pore architectures. Compressive properties of the particulate-templated scaffolds were influenced by particulate size with the smaller particulate (0.03 mm³) yielding a significantly stiffer scaffold ($p < 0.005$, Table 2), whilst both particulate-templated scaffolds had similar tensile properties (Table 2). Electrostatic deposition of aligned fibres on the particulate-templated scaffolds resulted in the adhesion of the fibre layer which significantly enhanced the tensile properties compared to the singular particulate-templated scaffolds (Table 2). Additionally, the surface roughness of the fibre layer was significantly lower compared to both particulate-templated scaffolds (Table 2). To assess the integration of the fibre layer with the particulate-templated scaffolds, interfacial mechanics were performed in both the aligned and transverse direction of fibre alignment. Interfacial stiffness was significantly higher in the aligned fibre direction (0.24 ± 0.04 N mm⁻¹), relative to the transverse direction (0.08 ± 0.05 N mm⁻¹) ($p < 0.001$). Peak loads of attachment for the 30 mm $\times$ 10 mm FZ–PZ interface were also significantly higher in the aligned direction than the transverse, with average values of 1.15 ± 0.3 and 0.47 ± 0.03 N respectively for BL-1.0 ($p < 0.001$) and 0.68 ± 0.03 and 0.430 ± 0.05 N respectively for BL-0.03 ($p < 0.05$).

3.2. Chondrocyte-bilayered scaffold interaction

We first evaluated the ability of chondrocytes to adhere and proliferate on bilayered scaffolds for 7 days in vitro. Chondrocyte-seeding efficiency was assessed with and without the addition of the fibre zone to the particulate-leached scaffolds (Fig. 2). There was a significant increase in seeding efficiency from

$74 \pm 5\%$ to $84 \pm 4\%$ in the BL-1.0 scaffolds with the addition of the fibre membrane ($p = 0.045$), but no significant difference in the BL-0.03 scaffolds, which averaged $76 \pm 3\%$. Chondrocytes were well distributed throughout the scaffolds with and without the fibre membrane as assessed by DAPI staining (data not shown). Zonal seeding efficiency also demonstrated that chondrocytes were preferentially attached based on the seeding protocol with 9–16% of chondrocytes seeded on the fibre membrane, 66–68% seeded in the particulate-templated scaffold, yielding a total seeding efficiency range of 75–84%. Chondrocyte proliferation maintained this trend for both particulate scaffold types and demonstrated temporal increases in chondrocyte number as measured via DNA content (Fig. 2C, D).

To evaluate in vitro cartilage formation, bilayered scaffolds were cultured for up to 4 weeks in chondrogenic medium. Chondrocytes were seeded with similar efficiencies on bilayered scaffolds of both porosities at week 0 and proliferated throughout the culture period. Total DNA was significantly higher for the bilayer-1.0 scaffolds vs. bilayer-0.03 at weeks 1, 2, and 4 ($p < 0.05$) (Fig. 3A). Zonal analysis demonstrated no significant differences between either PZ-0.03 or PZ-1.0, yet a significant increase in DNA content for the FZ-1.0 compared to FZ-0.03 at weeks 1, 2 and 4 ($p < 0.5$) (Fig. 3B). Sulfated GAG normalized to DNA content was also quantified as a marker of chondrocyte matrix accumulation, and increased in both a zonal and temporal fashion for both bilayered scaffolds with greater normalized accumulation in the BL-0.03 scaffolds at weeks 1, 2 and 4 (Fig. 3C). Total sGAG content was significantly higher for the bilayer-0.03 scaffold at weeks 1, 2 and 4 ($p < 0.05$). For the particulate-templated zones, there was significantly higher normalized sGAG accumulation on weeks 1, 2 and 4 for the PZ-0.03 yet no statistical differences for either fibre zone (Fig. 3D).

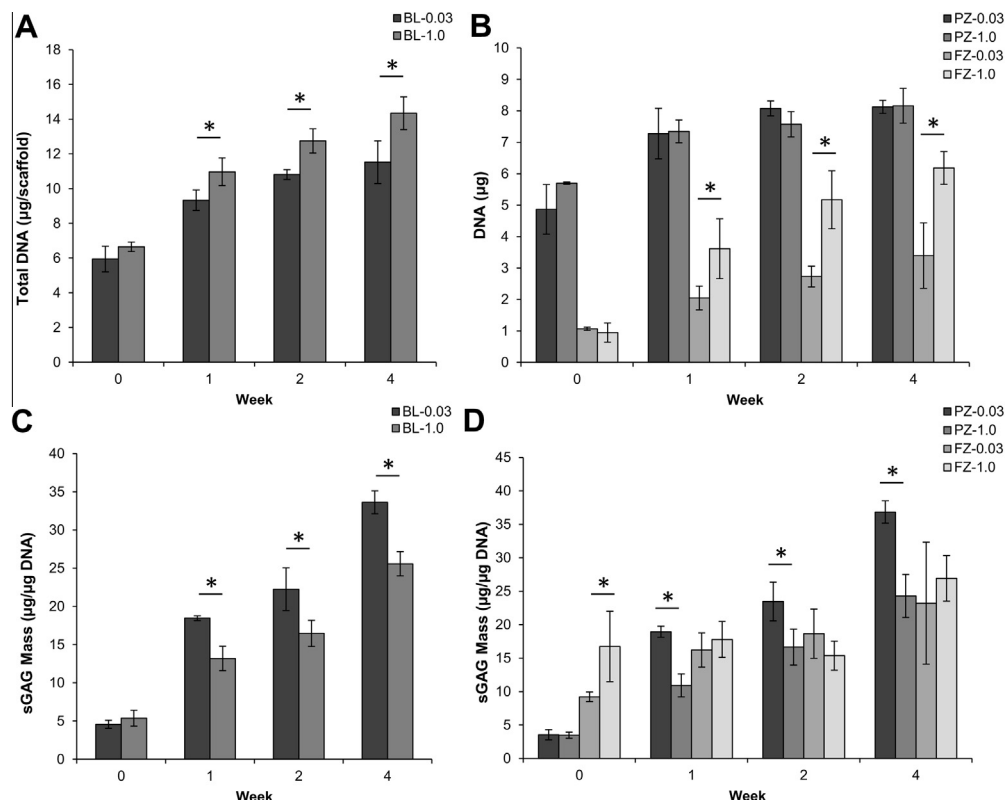


Fig. 3. DNA and sGAG content in chondrocyte-seeded bilayered scaffolds over the 4 week culture period. (A) Total DNA content quantification per scaffold. (B) Zonal analysis of total DNA content. (C) Quantification of total sGAG per scaffold normalized to DNA content. (D) Zonal analysis of sGAG production normalized to DNA content. At all non-zero time points, the BL-1.0 had statistically higher proliferation due to differences observed in the fibre zone, while the BL-0.03 had statistically higher normalized sGAG production due to differences observed in the porous zone. *Significant difference ($p < 0.05$).

Gene expression profiles over the 4 weeks of culture in chondrogenic differentiation medium were quantified by scaffold zone (Fig. 4). mRNA levels of *ACAN* and *COL2A1* were significantly elevated at all time points compared to week 0 values and a significant peak in expression was observed at week 2 for both genes in the PZ-0.03 scaffold, while the other zones showed strong trends towards increased expression (Fig. 4A, B). The PZ-0.03 scaffold exhibited higher *ACAN* and *COL2A1* fold increase expression than the PZ-1.0 zone at both weeks 1 and 2 ($P < 0.01$ for *ACAN* and $P < 0.05$ for *COL2A1*). Expression of *COL1A1* was not significantly modified during the 4 week culture period and remained low (Fig. 4C). The differentiation index for chondrocytes (i.e. ratio of expression *COL2A1*/*COL1A1*) remained high throughout the culture period, with a significantly higher ratio for the PZ-0.03 at week 2 when compared to PZ-1.0, indicating maintenance of chondrogenic gene expression profile during the experimental time course (Fig. 4D).

To evaluate temporal changes in surface roughness, white light interferometry was performed on the fibre zone and particulate-templated portions of the bilayered scaffolds on acellular samples and over 4 weeks of in vitro culture with chondrocytes (Fig. 5). The PZ-0.03 and PZ-1.0 mm scaffold surfaces were difficult to image as both exhibited high surface roughness with R_a values ~ 15 – 35 μm (Table 2). The deposition of fibre zones onto both scaffolds resulted in significantly smoother surfaces compared to their respective particulate-templated counterparts with average R_a values of 3.0 ± 0.8 and 2.1 ± 0.4 μm for the FZ-0.03 and FZ-1.0, respectively. ECM accumulation was evident on the surface of the fibre zones throughout the 4 week culture period. Due to cellular infiltration and ECM deposition, the surface morphology was altered and the resulting R_a values slightly increased to 4.1 ± 0.7 and 4.3 ± 0.9 μm for the FZ-0.03 and FZ-1.0, respectively.

Bilayered scaffolds were assessed for both compressive and tensile mechanics (Fig. 6). Compressive mechanics did not vary during the 4 week culture period and were similar to acellular controls, and the bilayer-0.03 scaffold was consistently significantly stiffer when compared to bilayer-1.0. Representative stress-relaxation curves demonstrated that the scaffolds were significantly different in regards to compressive modulus and compressive stress, yet both displayed similar relaxation profiles. Tensile mechanics of chondrocyte-seeded bilayered scaffolds displayed maintenance of tensile modulus and ultimate tensile strength throughout the culture period with no significant changes based on ECM deposition.

Histological evaluation of the scaffolds after 4 weeks of in vitro culture indicated a dense cellular infiltration and ECM production in the fibre zone, intermediate cellular density and ECM production in the PZ-0.03, and low cellular density and ECM production in the PZ-1.0 (Fig. 7). Immunohistochemical staining showed a correlation between collagen I, collagen II and picrosirius red intensity, as would be expected from expanded P2 chondrocytes. Collagen X staining, an indicator of chondrocyte hypertrophy [30], is minimal. Superficial zone protein (SZP), a marker for the superficial zone [8], is found throughout the scaffolds within the pericellular matrix.

4. Discussion

Zonal organization of scaffolds that mimic the in vivo architecture and the structural design of cartilage are of crucial importance in regenerating the morphological as well as functional aspects of this challenging tissue. In this study, we have successfully fabricated bilayered cartilage scaffolds from PCL that possess zonal organization by utilizing a combinatorial strategy of electrostatic

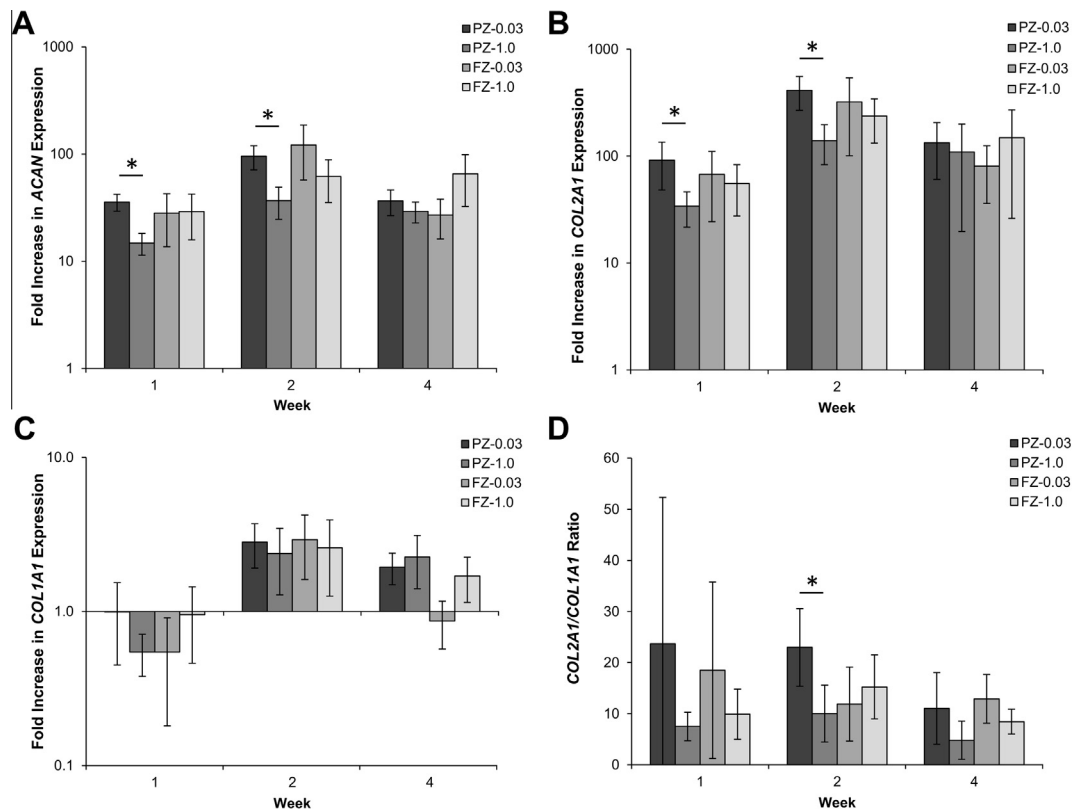


Fig. 4. Zonal gene expression profiles of bovine chondrocytes cultured on the bilayered scaffolds in chondrogenic differentiation medium. mRNA levels of (A) *ACAN*, (B) *COL2A1* and (C) *COL1A1* are represented as fold difference relative to week 0 controls (2 h post-seeding) after normalization with the housekeeping *B2M* gene. (D) The *COL2A1*/*COL1A1* ratio was high throughout the culture period. *Significant difference ($p < 0.05$).

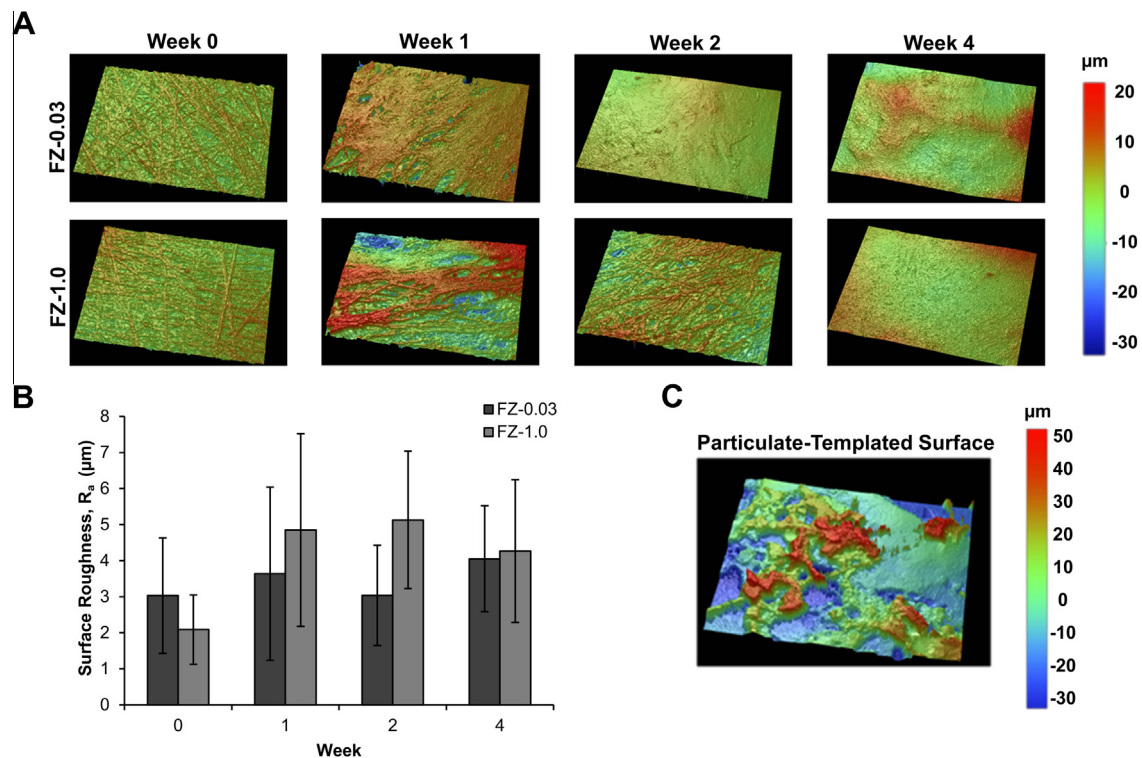


Fig. 5. White light interferometry surface analysis. (A, B) Fibre zone surface roughness in bilayered scaffolds, analyzed over 4 weeks of culture. Representative areas measure $312 \mu\text{m} \times 234 \mu\text{m}$. (B) Surface roughness quantification of scaffolds with a fibre-zone. (C) Particulate-templated zone without a fibre-zone coating. The omission of the smooth fibre zone led to much greater surface roughness (R_a) values ranging from 15 to $35 \mu\text{m}$.

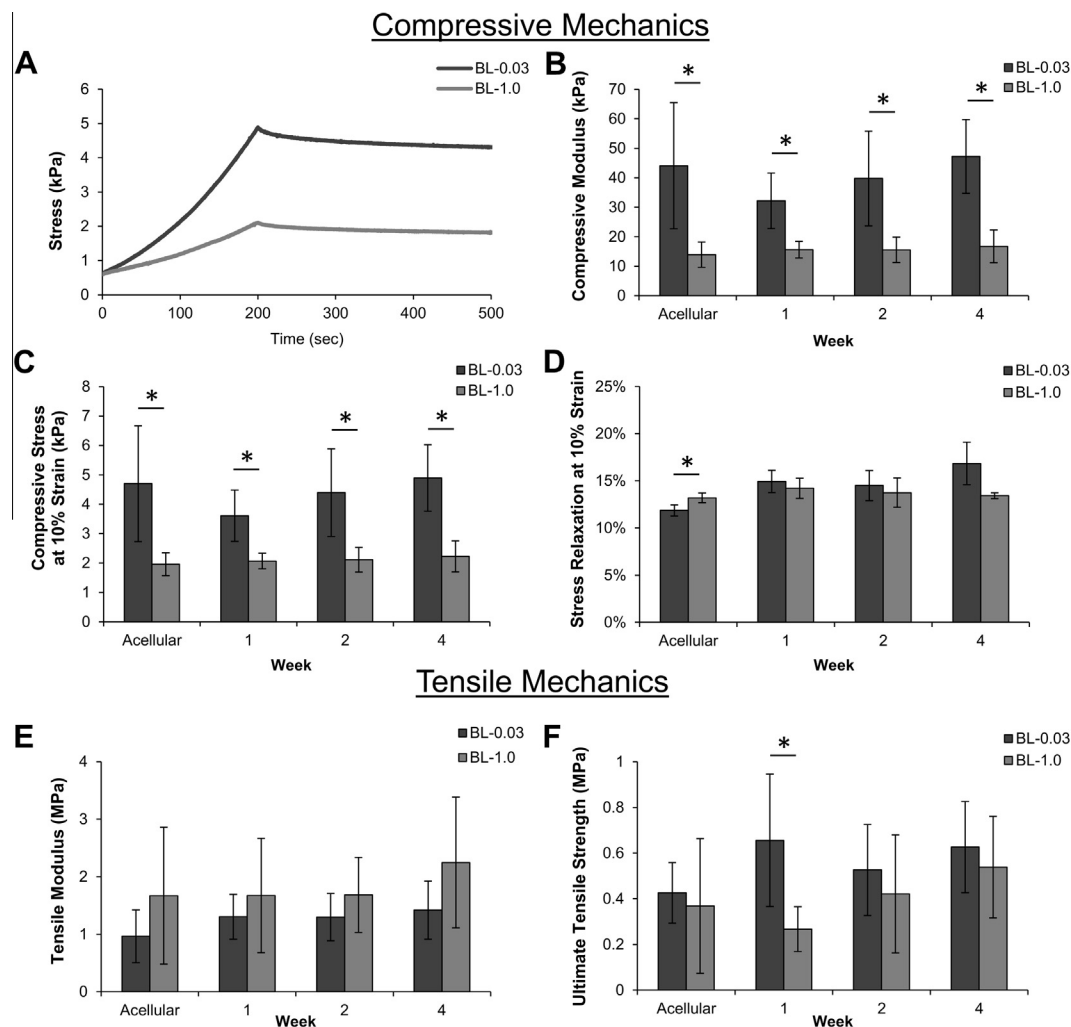


Fig. 6. Compressive and tensile mechanics of bilayered scaffolds over 4 weeks of culture with chondrocytes. (A) Representative acellular responses for each scaffold under compression loading to 10% strain, followed by relaxation. Compressive modulus (B), stress at 10% strain (C) and stress relaxation (D) determined from the stress-relaxation test. Tensile modulus (E) and ultimate tensile strength (F) were not significantly different between the two scaffold groups. *Significant difference ($p < 0.05$).

deposition of fibres on a particulate-templated scaffold. We selected PCL as it is used frequently in the field of musculoskeletal tissue engineering due to its biodegradable nature, facile processing capacity, elasticity and current use in FDA-approved medical devices [31]. To assess the performance of our scaffolds we investigated the in vitro cartilage formation of bovine chondrocytes. We performed zonal analysis of bovine chondrocyte attachment, proliferation and matrix production over 4 weeks in vitro as well as assessing the effect of particulate size (0.03 vs. 1.0 mm³) on chondrocyte gene expression, matrix accumulation and global scaffold mechanics. Our results demonstrate that the addition of aligned microfibrils did not alter chondrocyte-seeding efficiencies (Fig. 2) and resulted in zonal differences in chondrocyte density and ECM formation (Fig. 3). Chondrocytes proliferated significantly on the FZ-1.0 zone, based on the large pores in the particulate-leached zone allowing complete and rapid cellular ingress and attachment to the fibre membrane. Despite the significant increase in chondrocyte number on the FZ-1.0 zone, sGAG content was not significantly different from the FZ-0.03 zone when normalized to DNA content (Fig. 3), signifying the importance of pore size on ECM production.

The addition of aligned microfibrils significantly reduced the surface roughness of particulate-templated scaffolds and enhanced the tensile mechanics, regardless of particulate size (Table 2 and

Fig. 5). Surface roughness (a key characteristic of the articulating surface for hyaline cartilage [32]) was evaluated using white light interferometry: the measurements of the topographical changes based on temporal evolution in ECM deposition showed that the scaffolds maintain a relatively smooth and increasingly homogeneous surface (maximum $R_a = 4 \mu\text{m}$). Particulate-templated scaffolds demonstrated high heterogeneity in surface roughness based on creation of large pores via particulate removal (Fig. 5). Tensile mechanics of the bilayered scaffolds were significantly enhanced based on the contribution of the tensile properties of the aligned fibres (Table 2). The enhanced porosity of the particulate-leached zone (PZ) and large pore size caused a significant decrease in the compressive modulus, when compared to the aligned electrospun membrane comprised of densely packed fibres. The benefit of our scaffold design is that both cellular ingress and ECM deposition in the PZ zone are possible, while the aligned fibre membrane provides a means to mimic the topographical, tensile, and frictional characteristics of articular cartilage. Moreover, it is our understanding that having the PZ zone with a slightly lower modulus should be favourable as this will not provide stress-shielding to cells as they experience compressive forces.

As indicated in Fig. 3B, we quantitatively determined that the chondrocytes were seeded in an 80:20 ratio at day 0. Over the next 4 weeks of culture, while there was proliferation within both

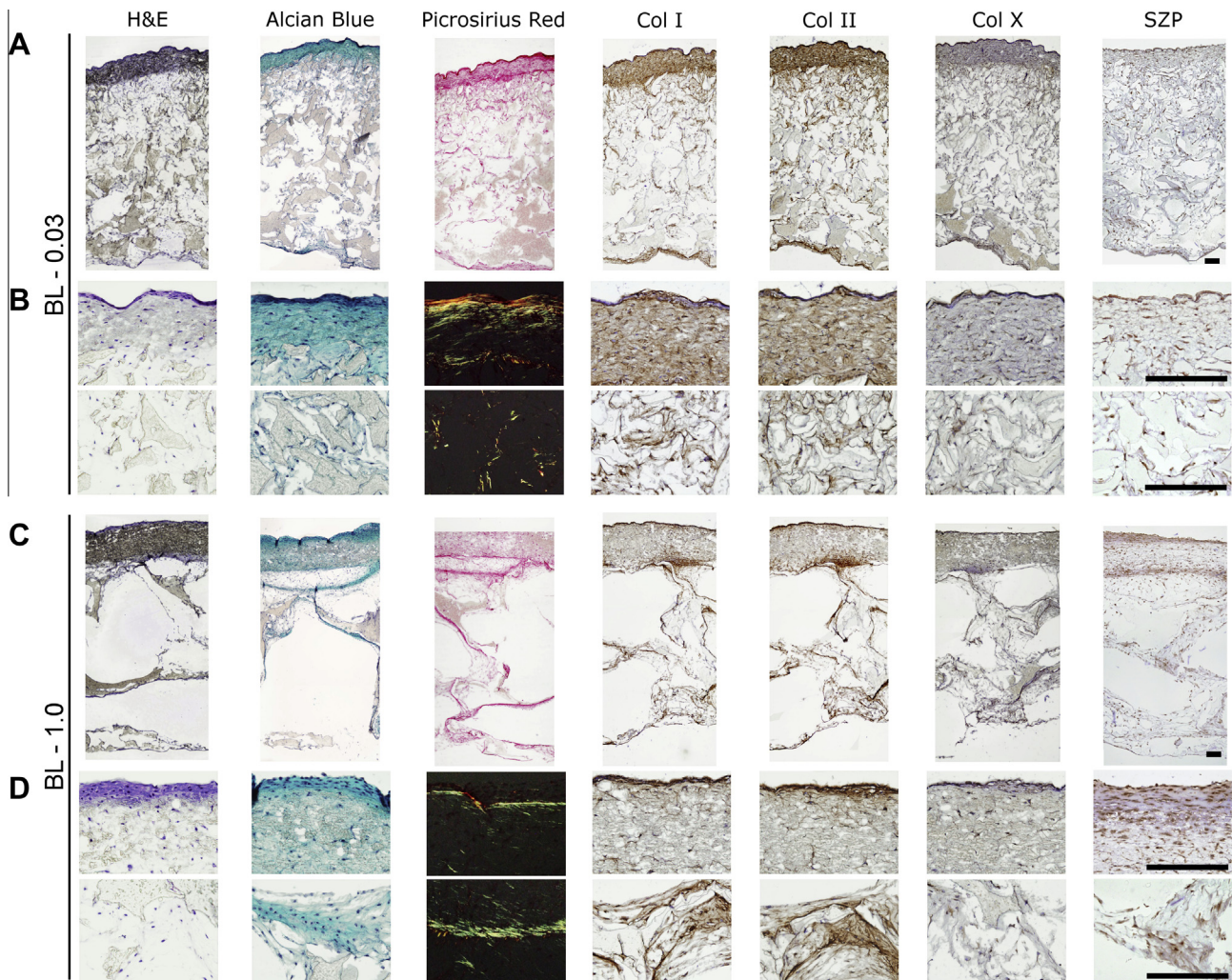


Fig. 7. Histological examination of bilayered scaffolds BL-0.03 (A, B) and BL-1.0 (C, D) after 4 weeks in vitro. All scale bars represent 250 μ m. Samples are shown at full thickness (A, C) and high-magnification FZ and PZ zones (B, D). Scaffolds are stained with haematoxylin and eosin, alcian blue for sGAG, picrosirius red for collagens, and by IHC for collagen I, II and X, and superficial zone protein (SZP), respectively, from left to right. The high-magnification images of the picrosirius red staining use cross-polarized microscopy, on sections where the PCL has been removed, to visualize collagen fibril birefringence.

zones, the DNA concentration within the superficial zone increased at a greater rate. As the fibre layer only comprises 10% of the total scaffold thickness, the initial seeding as well as the greater proliferation concentrated chondrocytes in the upper fibre layer, as is found in the native tissue, leading to increased ECM production in the superficial zone, as observed in the histological sections (Fig. 7).

Zonal analysis also revealed that a particulate size of 0.03 mm³ significantly upregulated expression of both *Aggrecan* and *Col2 α 1*, in PZ-0.03, and yielded the highest amount of proteoglycans as measured via sulfated GAG content. The benefit of the smaller pore size was localized to the particulate-leached zones, as the fibre zones from BL-1.0 and BL-0.03, which had identical morphologies, had similar gene expression profiles (Fig. 4). The observed increase in normalized sGAG production in the less porous PZ-0.03 is consistent with native tissue, wherein proteoglycan density has been correlated with decreased scaffold permeability and the resulting hypoxic gradients [33]. In both scaffold systems sGAG density was highest in the fibre zone, as observed by alcian blue staining intensity (Fig. 7). However, while the cellular density of the porous zone was lower than the fibre zone at 4 weeks in vitro, and proliferation had slowed (Fig. 3B), sGAG/DNA continued to increase (Fig. 3D). As the tissue had not yet reached a mature state, with

time, the scaffold would be expected to fill with ECM and adopt a more deep-zone-like phenotype, characterized by low DNA and high sGAG concentrations [8,10]. The observed expression of SZP throughout the scaffold, in a non-zonal manner (Fig. 7), may be due to the use of a mixed population of chondrocytes cultured over 4 weeks in vitro in chondrogenic medium. Extended culture conditions have been shown to significantly increase expression of SZP in all zones of cartilage explants as well as in different chondrocyte morphologies including monolayer culture and inert hydrogel encapsulation [34]. Additionally, the lack of mechanical stimulation during culture would both have contributed to the lack of zonal SZP organization, leaving only the background chondrogenic-culture induced expression [35].

Lu et al. have shown that varying pore sizes in directionally frozen collagen scaffolds also affects cartilage formation—specifically, larger pores of 0.7 mm were ineffective in producing cartilage when seeded with bovine chondrocytes; however, in support of this work, pore sizes of 0.03 mm exhibited higher cell-seeding efficiencies and also more sulfated GAG per cell [36]. Tanaka et al. were able to vary both pore size and porosity of particulate-templated scaffolds using sugar crystals. Scaffolds of particulate size 0.03 mm³ and porosities of 90 or 95% yielded the best cartilage in terms of sulfated GAG content when compared to scaffolds formed from 1 mm³ size

particulates, agreeing with our results presented here [37]. Mandal et al. demonstrated that layered scaffolds of varying pore size and morphology were able to support chondrocyte and fibroblast proliferation and matrix production, with smaller pores in the range of 60–80 µm producing more sGAG on a per cell basis after 4 weeks in culture [38]. A contrary observation has also been made by Woodfield et al. in melt electrospun scaffolds which show greater tissue production in large pores, attributed to nutrient availability, reduced cell–cell contact, and available voids to fill with ECM [9]. As tissue engineering investigations are highly multifactorial, the true cause of the differential ECM production is open to interpretation and warrants further investigation.

Our results demonstrate that morphological differences in bilayered scaffolds are able to contribute to gradations in chondrocytic gene expression and ECM deposition. By designing a scaffold organized into zones of differing mechanical and geometrical microenvironments, cellular-level changes have been shown to be imparted in a continuous construct. The addition of the aligned fibre membrane is able to enhance functionality of the salt-leached foam by reducing surface roughness and enhancing tensile mechanics while mimicking the orientation of the superficial zone of cartilage.

Bilayered scaffold structures have been used previously for blood vessel regeneration through the combination of different polymer systems. Here, we demonstrate that scaffold zones can be produced with different morphologies by utilizing the same polymer, and that these morphological changes are able to provide desired functionality while impacting both chondrocytic gene expression and ECM accumulation.

Previous studies have shown that a variety of scaffold morphologies can support in vitro cartilage formation including particulate-templated foams, electrospun fibrous scaffolds and rapid-prototyped scaffolds [9,17–21]. Electrospun fibres are able to act as functional membranes that can be engineered to have varying morphologies, mechanics and cellular responses, but standard solution electrospinning techniques are generally limited due to the fibres' overall thickness as well as lack in porosity inhibiting full cellular colonization [18]. Particulate-templated scaffolds enable a high level of cellular ingress but can lack suitable mechanical properties based on their isotropic and highly porous nature. Combining these materials results in a zonally organized scaffold with benefits of both electrospun fibres (tensile/surface properties) and particulate-templated scaffolds (variation in pore size, porosity, pore interconnectivity and permeability). By taking a combinatorial approach to the use of different scaffold production techniques and morphologies we were able to yield engineered materials that provide zonal organization within a single scaffold system.

5. Conclusions

We fabricated bilayered scaffolds that mimic the functional organization of articular cartilage by the electrostatic deposition of fibres onto particulate-templated scaffolds. Our scaffolds overcome common limitations associated with homogeneous scaffolds by mimicking the functional interfaces of this tissue as well as supporting in vitro cartilage formation. Our analysis demonstrated zonal differences in cellular proliferation, biochemical composition and gene expression. Taken together, our results suggest that scaffolds inspired by the structural and functional organization of the native tissue may find use in developing regenerative medicine strategies to treat articular cartilage lesions.

Acknowledgements

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Appendix A. Figures with essential colour discrimination

Certain Figures in this article, particularly Figs. 5 and 7 are difficult to interpret in black and white. The full colour images can be found in the on-line version, at <http://dx.doi.org/10.1016/j.actbio.2013.12.030>

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Appendix B

SOP - Polyester Wax Histology

STANDARD OPERATING PROCEDURE

File Reference:			
Document Title:	Polyester Wax Histology		
SOP Number:		Version #:	v1.0
Author:	Joe Steele	Prepared Date:	12/05/2015
Reviewed By:		Review Date:	
Approved By:		Approval Date:	

1. PURPOSE

To take thin, paraffin-like sections of heat or xylene-sensitive materials which cryosection poorly, most notably poly(caprolactone) tissue engineering scaffolds.

Benefits over paraffin include:

- can be used with heat-sensitive materials (PCL) as it melts at ~40°C
- can be used with xylene-sensitive materials (like PCL) as it's soluble in ethanol
- no/minimal antigen retrieval is required for IHC due to low temp and no xylene

Protocol modified by Joe Steele and Lorraine Lawrence of Imperial College London from Steedman et al [1] to improve embedding of porous PCL foams. Outlined briefly in Steele et al [2].

2. DEFINITIONS

PCL = poly(caprolactone)

BSA = Bovine Serum Albumin

3. MATERIALS

EQUIPMENT

Balance
General Laboratory Glassware (graduated cylinders, beakers)
Vacuum oven, preheated to 45°C
24-well plates (2 for every 6 samples)
50 mL tubes or 200-500 mL sealable plastic specimen jars (work best)
Microscope Slides, Standard – [Fisher #12-550B & 12-550-15]
Microscope Slides, Silane Prep – [Sigma-Aldrich# S4651-72EA]
Plastic Transfer Pipettes – [VWR#612-1681]
Disposable Tissue Moulds – [Solmedia#CAS821]
Staining Dishes & Racks

REAGENTS

Polyester wax – [VWR #100496-094]
Ethyl (ETOH) Alcohol 190 & 200 proof – [AAPER Alcohol]
Gelatine – [Sigma-Aldrich#G1890]
Bovine Serum Albumin – [Sigma-Aldrich# A2153]
DI Water
Tap Water

SOLUTIONS

A. 70% Ethly Alcohol

Distilled water – 263 mL
190 Proof Ethanol – 737 mL

B. 90% Ethyl Alcohol

Distilled water – 53 mL
190 Proof Ethanol – 947 mL

C. 50:50 EtOH:Wax

Polyester Wax – 23g (roughly 25 mL) – can re-used old/solidified poly.wax
200 Proof Ethanol – 25 mL
Dissolve in a 50 mL falcon tube at room temperature on a roller or in a sealed container on a stir plate to ensure the wax is evenly dissolved and distributed

D. Melted Wax

Polyester Wax – 45g (roughly 50 mL)
Melt in a sealed plastic specimen jar at 45°C overnight, prepare as-needed and don't let solidify, stir before use, avoid bubbles.

E. Mounting Solution (0.1% w/v)

Gelatine – 100 mg
Distilled water – 100 mL mL
or
Bovine Serum Albumin – 100 mg
Distilled water – 100 mL mL

4. PROCEDURE

*** Samples should be fixed in PFA for 15 minutes, rinsed in PBS and stored at 4°C until embedded.**

A. DEHYDRATION AND EMBEDDING

1. Fill the wells of a 24-well plate with 70%, 90%, 100% and 100% ethanol, 2 mL each, with one set for each sample.

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
70%	70%	70%	70%	70%	70%
90%	90%	90%	90%	90%	90%
100 %	100 %	100 %	100 %	100 %	100 %
100 %	100 %	100 %	100 %	100 %	100 %

2. Move samples from PBS directly to 70% ethanol, allow to dehydrate for 30 min

3. Move samples down one row from 70-90% ethanol, leave 30 min.

4. Repeat for both 100% ethanol dehydrations, 30 min each

5. Fill the top row of a 24-well plate with 1 mL 50:50 EtOH:Wax, move samples directly from 100% to 50:50, and store overnight at 45°C, plate sealed with parafilm

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
50:50	50:50	50:50	50:50	50:50	50:50
Wax 1	Wax 1	Wax 1	Wax 1	Wax 1	Wax 1
Wax 2	Wax 2	Wax 2	Wax 2	Wax 2	Wax 2

6. The next day, fill remaining wells with fresh pure wax, transfer samples to wax 1, incubate for 3 hours at 45°C.

7. Transfer samples to Wax 2, incubate for >1h at 45°C, vacuum cycling at least once to 22"Hg to remove any remaining air bubbles.

8. Block out samples as with paraffin into the disposable plastic moulds using fresh wax, ensuring to cool the wax slowly. A cold block can be used, but keep it ~4°C.

9. Allow to cool at room temperature overnight, then transfer to the 4°C fridge

10. Allow ~3 days to fully harden

B. SECTIONING

1. Cut blocks directly from fridge, or, place on ice with aluminium foil between the blocks and the ice. Avoid getting blocks wet.
2. Section as with paraffin. Trim the block slowly with $<20\ \mu\text{m}$ bites, as the wax will flake and make a mess.
 - * If it squeaks, it will cut nicely. Cool on ice/fridge between trimming and sectioning.
 - * If scaffolds are sectioning poorly, cut at $10\ \mu\text{m}$.
3. Place 2-3 drops of mounting solution onto each slide directly before mounting.

Mounting Solution	Slide	Application
0.1% Gelatine	Standard	Most histological stains, use for all acellular samples, best adhesion
0.1% BSA	Standard	Better for Collagen I/II/X IHC, less background
DI Water	Silane-Prep	For tissue-filled sections where gelatine and BSA both interfere with staining.
0.1% BSA	Silane-Prep	For IHC of samples with minimal tissue and previous issues with detachment
DI Water	CaF ₂	For FTIR imaging
DI Water	MgF ₂	For Raman imaging

4. As they are cut, lay the sections onto the droplets and allow them to flatten. Wick away solution before the wax starts to break up and dry upright at room temperature overnight. Don't dry in an oven or the sections will melt!

C. DEWAXING

1. Dewax in pure ethanol for 10 min
2. Allow sample to dry at room temperature >30 min. If not sufficiently de-waxed, repeat, allowing the sample to dry in between, to prevent detachment
3. Rehydrate with a standard ethanol series and stain following existing protocols.
 - * As adhesion is poorer than paraffin, minimise the agitation and duration of washes, if possible.

5 NOTES

Optimised for 3 x 6 x 2 mm, >80% porous PCL with 0-100% tissue infiltration

You can use biopsy cassettes and standard automated ethanol dehydration if processing many samples at once, however, the pure wax steps should be performed in a 24-well plate to minimise the volume of wax needed.

6 LIMITATIONS

There are known issues with citrate buffer and protease K antigen retrieval. The citrate is too hot and melts PCL samples. Standard protease treatments can degrade the gelatine/BSA mounting and cause the samples to detach. Check with a positive control to determine if antigen retrieval is even necessary, if so, use minimal protease.

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Appendix C

Comparison of Normalised ECM Concentrations

Table C1. Compilation of approximately 4r week in vitro DNAr normalised ECM data from tissue cartilage tissue engineering scaffolds and comparison to 4r week bilayer data

Scaffold	Time Point	Approx. GAG ($\mu\text{g}/\mu\text{g DNA}$)	Approx. Collagen ($\mu\text{g}/\mu\text{g DNA}$)	Ref
PCL Bilayer 1.0 mm ³ fibre	4 weeks	24.3 $\pm$ 3.2	2.1 $\pm$ 0.4	Chapter 2 [1]
Zonal Study 1.0 mm ³ foam	4 weeks	36.8 $\pm$ 1.7	4.9 $\pm$ 0.4	
0.3 mm ³ fibre	4 weeks	26.9 $\pm$ 3.4	1.6 $\pm$ 0.2	
0.3 mm ³ foam	4 weeks	23.2 $\pm$ 9.1	1.7 $\pm$ 0.5	
Macroporous PEG cryogels	4 weeks	30	4.3	[2]
4% (w/v) agarose hydrogels	3 weeks	11-19	25	[3]
	6 weeks	9-30	27-43	
PEG hydrogel	4 weeks	5-27	0.07-0.13 (Col2)	[4]
Freezedried HAp-Col1 foam	4 weeks	27-58	-	[5]
Col1 gels with HAc and CS	4 weeks	37-40	-	[6]
PCL scaffold	4 weeks	3-4.5	-	[7]
Salt-leached silk foam	4 weeks	200-270*	3300-3600*	[8]
*DNA data is questionable				
Adult human cartilage (superficial)	-	108	1080	[9]–[11]
Adult human cartilage (deep)	-	843	3370	[9]–[11]

* Un-validated DNA data can drastically alter normalised ECM production values. For example, in this study of chondrocytes in a salt-leached silk foam published in *Biomaterials* by Mandal *et al.* [8], the DNA quantification corresponds to a 2.6-2.9% seeding efficiency, based on their intended seeding concentration and 7.7 pg DNA/chondrocyte [11]. Therefore, their DNA quantification is likely significantly underestimated, and their extraordinary physiological-levels for DNA-normalised data after 4-weeks *in vitro* are therefore called into question.

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Appendix D

SOP - Gelatine Microbead Emulsion

STANDARD OPERATING PROCEDURE

File Reference:

Document Title: Gelatine Microbead Emulsion

SOP Number: Version #: v1.0

Author: Joe Steele Prepared Date: 26/04/2015

Reviewed By: Review Date:

Approved By: Approval Date:

1. PURPOSE

To prepare gelatine beads for multi-layer cartilage scaffolds.

2. SCOPE

The production of gelatine beads is required for the porogen-leached zones of DF100 and DF300 scaffolds, which is covered in a separate SOP. The primary protocol outlined is the oil emulsion, a chloroform variant is outlined in a separate SOP.

3. DEFINITIONS

-

4. ABBREVIATIONS

-

5. BACKGROUND

The particles are used to replace the salt crystals used previously in Steele et al [1]. The technique was developed by Joe Steele (Lab books: JS-2/3).

6. MATERIALS

Supplies:

- >10 g porcine type A gelatine (G1890, Sigma Aldrich)
- 250 mL sunflower oil (Waitrose, UK)
- 1.25 mL Tween 20 (P1379, Sigma Aldrich)
- 2L ice water slurry
- thermometer
- Acetone
- 200 mL glass bottle for gelatine
- 500 mL glass bottle for emulsion
- 60 mL syringe fitted with 10 cm silicone tubing

Equipment:

- Insulated Styrofoam container larger than 30 cm x 30 cm x 20 cm
- upright laboratory mixer with two-bladed impeller (Eurostar digital, IKA, UK)
- hotplate with temperature probe
- stirplate and 6 cm stirbar at 4°C
- large jackstand
- Stainless steel sieves (710, 500, 300, 100 µm) (VJ Tech, UK)

7. PROCEDURE

All work with acetone should be performed in a chemical safety cabinet

7.1 Prepare equipment and materials:

- Prepare 20% (w/v) gelatine in distilled water, 50 mL per emulsion, with excess, generally 180 mL for three emulsions. Prepare at 60°C on a hotplate with magnetic mixing, avoid bubbles. Store excess at 4°C for a few days if necessary, and quickly re-heat in the microwave, with caution and frequent stirring.
- Place stirplate in 4°C fridge
- Prepare icewater

7.2 Setup stand mixer with the impeller within the 500 mL glass bottle inside the insulated container on the jackstand, elevated to ~15 cm. This setup will allow the bottle to be jacketed with icewater, and for the bottle/icewater/insulated container to be removed from the mixer easily.**7.3 Add 250 mL oil and 1.25 mL Tween 20 to the 500 mL bottle, adjust height of jackstand to immerse impeller blades.****7.4 Turn on mixer to 500 rpm****7.5 Fill 60 mL syringe with 50 mL gelatine by removing the plunger and pouring into the tube. Insert plunger and remove air. Place 10 cm of tubing over the end of the syringe to make the next step easier.**

* Don't use a luer-lock hosebarb, just get tubing the correct diameter to fit over the cylindrical 'nub' at the tip of the syringe.

7.6 Add the gelatine dropwise (30 sec total) into the mixing oil, avoiding dripping on the impeller and bottle edges. Allow to equilibrate for 10 minutes.**7.7 Add the icewater into the insulated container around the glass bottle to the same level as the oil/gelatine. Allow to cool for 30 minutes to thermoset the gelatine. Do not interrupt/stop the mixing.**

- 7.8 Stop mixing and remove the impeller.
- 7.9 Quickly add 250 mL -80°C acetone and the 6 cm magnetic stirbar to the bottle, seal the cap, shake gently, and place on 4°C mixer at 200 rpm overnight.
- 7.10 Stop the stirplate and allow the particles to settle for 15 minutes at 4°C.
- 7.11 Decant as much oil/acetone supernatant as possible, through the 100 µm sieve to catch floating particles.
- 7.12 Return any captured particles to the bottle and add 250 mL fresh acetone to remove residual oil and water.
 - * At this point, multiple batches can be pooled into one bottle
- 7.13 Mix at 4°C overnight at 200 rpm.
- 7.14 Decant off acetone over the 100 µm sieve and rinse with acetone until oil is no longer visible.
- 7.15 Prepare a particle/acetone slurry and pour over the stack of sieves 710-500-300-100 µm place on top of a beaker to collect acetone. Recycle acetone until the particles are fully sorted by size.
 - * This process is faster if performed in multiple small batches
 - * Much easier to perform while wet with acetone, as the dry particles have too much static charge
 - * Can substitute 100% ethanol for acetone and perform on bench top
- 7.16 Allow particle fractions to dry in hood and then overnight under high vacuum
- 7.17 Weigh fractions to determine portion of particle within acceptable range, keep for future reference/adjustment.
- 7.18 Store in sealed containers to avoid excess humidity

8 NOTES

- All work with acetone must be performed in a chemical safety cabinet.
- If particles are non-spherical, adjust mixing speed and/or gelatine/oil ratio.
- If dry particles are greasy and don't flow, mix overnight in acetone at room temperature and re-dry. Residual oil cannot be easily removed from the PCL scaffolds, and should be avoided (frequent changes of ethanol)

9 LIMITATIONS

-

10 REFERENCES

- [1] J. A. M. Steele, S. D. McCullen, A. Callanan, H. Autefage, M. A. Accardi, D. Dini, M. M. Stevens, *Acta Biomater.* **2014**, *10*, 2065–75.

Appendix E

SOP - Multilayer Scaffold Production

STANDARD OPERATING PROCEDURE

File Reference:			
Document Title:	Multilayer Scaffold Production		
SOP Number:		Version #:	v1.0
Author:	Joe Steele	Prepared Date:	24/04/2015
Reviewed By:		Review Date:	
Approved By:		Approval Date:	

1. PURPOSE

To prepare multi-layer cartilage scaffolds for in vitro or in vivo investigation.

2. SCOPE

The production of DF100 and DF300 scaffolds is covered from production through to pre-sterilisation. The production of gelatine microbeads and sterilisation are covered in a separate SOP.

3. DEFINITIONS

DF100 – 2 mm thick multi-zone cartilage scaffold with 1 mm of porogen-leached foam with **100-300 μm pores**, 1 mm of directionally frozen foam, and electrospun surfaces.

DF300 – 2 mm thick multi-zone cartilage scaffold with 1 mm of porogen-leached foam with **300-500 μm pores**, 1 mm of directionally frozen foam, and electrospun surfaces.

4. ABBREVIATIONS

PCL – poly(ϵ -caprolactone)

PBS – phosphate buffered saline

PDMS – poly(dimethylsiloxane)

HFIP – 1,1,1,3,3,3-hexafluoroisopropanol

5. BACKGROUND

The scaffold was developed by Joe Steele (Lab books: JS-2/3). Based on principles of electrospinning, porogen-leaching, and directional freezing from McCullen et al [1], Steele et al [2], and Wegst et al [3].

6. MATERIALS

Supplies for 'N' 80 mm x 80 mm x 2 mm DF100 scaffolds:

- N x 5 g – Gelatine Microbeads – see SOP
- N x 17 mL – 8% (w/v) PCL in 1,4-dioxane (Sigma Aldrich 440744 and 360481)
- N x 10 mL – 12% (w/v) PCL in HFIP (Sigma Aldrich 440744 and 105228)
- N - Clean copper plates 100 mm x 100 mm x 450 μm
- N - PDMS wells 80 mm x 80 mm x 2 mm (SYLGARD 60, VWR, 1/15 mix)
- N - 90 mm x 90 mm glass plate (histology staining tray lid)
- PBS from tablets
- 70% Ethanol
- Spatula

- N - 50 mL falcon tube
- Fine-grit sandpaper
- Acetone
- 2N - Solvent-proof 10 mL syringes
- Dry ice
- New razor blades
- Sealable plastic container large enough to hold scaffolds
- Clean glass slide
- Tape to affix scaffold to mandrel
- Aluminium foil
- 18G blunt needle

Equipment:

- Drum lyophiliser, with insulated/pre-cooled blocks (RSM Lab)
- -80°C freezer (or dry ice)
- PolarBear Plus, levelled (Cabridge Reactor Design Ltd, RSM Lab)
- Electrospinner (Optimised for 2011-2015 setup, Bessemer lab)
- 37°C waterbath
- Centrifuge for 50 mL tubes

7. PROCEDURE

All work with dioxane and HFIP should be performed in a chemical safety cabinet

7.1 Prepare materials:

- Ensure gelatine particles are dry and sorted by size
- Ensure PCL is fully dissolved in dioxane (make a large batch, mix with stirbar overnight, protected from light, store with solvents) and HFIP (make up small batches in 28 mL glass vials, roll overnight to dissolve, store with e-spinning solutions)
- clean copper sheet with fine-grit sandpaper and water, rinse well with water, and clean with acetone to remove oxidation and residual PCL
- clean PDMS wells in acetone to remove residual PCL
- turn on PolarBear Plus, level, clean, and set to -20°C with lid on
- clean and turn on lyophiliser

7.2 Weigh out gelatine particles in 50 mL falcon tubes, masses are approximate and vary with particle size and composition, when combined with PCL-dioxane and spun at 2000 x g, the particles should settle to 7.5 mL with 1.5 mL of supernatant.

DF 100 – 5.0 g 100-300 µm gelatine particles, 7 mL 8% PCL-dioxane

DF 300 – 4.5 g 300-500 µm gelatine particles, 9 mL 8% PCL-dioxane

- * dispense PCL-dioxane with solvent-proof syringe to ensure accuracy
- * never use 15 mL tubes as it is too hard to remove particle-PCL-dioxane slurry
- 7.3 Spin particle-dioxane suspension at 2000 x g for 5 min to distribute evenly and remove air bubbles.
- 7.4 Place PDMS well onto copper sheet and press firmly to seal the edge. Add the particle suspension to the centre of the well and distribute with a spatula, suspension should flow and evenly cover copper sheet.
- 7.5 Cover with glass, exposing one corner to allow for particles to settle while slowly evaporating dioxane, leave for >2 h, overnight.
- 7.6 Add **12 mL 8% PCL-dioxane** onto the dried particles with a solvent-proof syringe, tilting to distribute evenly. Cover completely with glass and allow 90 min to settle evenly. Perform this step as close to the PolarBear as possible and on a surface that allows you to easily lift the copper sheet up and onto the PolarBear without tilting or jarring, which would re-suspend or re-distribute particles.
- 7.7 Place on PolarBear at -20°C with cooling power at 100%. Place evenly and quickly to avoid shifting when partially frozen. Allow to freeze for 10 minutes.
- 7.8 Transfer on dry ice to -80°C freezer, or store directly on dry ice until all replicates are frozen.
- 7.9 Place in lyophiliser quickly, stacked at 45° rotations to prevent sealing. Place on metal blocks pre-cooled to -80°C or Styrofoam to avoid any thawing before high-vac is achieved. Leave overnight to allow for complete sublimation of dioxane.
- 7.10 Turn off lyophiliser, remove samples. Collect dioxane from cooling coil with warm water, dispose of as aqueous waste.
- 7.11 Trim 1-2 mm from the edges of the samples with a clean razor blade to remove cupping from capillary action at the side of the wells. Check for correct thickness, 2 mm, and complete gelatine particle-directionally frozen foam partitioning at 1 mm each. If unsatisfactory, dissolve in acetone, spin 1000 x g 5 min, collect gelatine, repeat wash with acetone twice and dry under vacuum.
- 7.12 Place scaffolds in a sealable plastic container, immerse in 70% (v/v) ethanol for 5 minutes to increase hydrophilicity of the scaffolds.
- 7.13 Rinse with distilled water, fill container with distilled water, seal, and place in 37°C waterbath. Leave for 1h to begin dissolving gelatine particles. Repeat until scaffold is visibly white and the used water does not leave a smudge on a clean glass slide when a drop is dried. (Generally 48h)

* Immerse in 70% ethanol for 15 minutes at least twice per day to sterilise the scaffold and treat the newly-exposed surfaces.

** Can be accelerated at 45°C, but be cautious to avoid temperatures above 50°C, as PCL begins to soften and collapse, melting at 60°C.

*** Once cleared, exchanging PBS for water in the last few washes is useful for removing gelatine, but salts will show up on the droplet-glass test.

7.14 Dry flat between absorbent towels with a slight weight on top (glass from previous step) to keep as flat as possible.

7.15 Re-measure scaffold and layer thicknesses to ensure correct.

7.16 Select an electrospinning mandrel (Small – 64 mm Ø or Large – 200 mm Ø, both 100 mm wide) and cover with clean aluminium foil. Mount scaffold with tape at the corners, with the directionally-frozen foam side facing upwards.

7.17 Load 12% (w/v) PCL-HFIP into a solvent-proof syringe, attach an 18G blunt needle, and place into the electrospinning syringe pump with a 50 mm gap between the needle tip and the scaffold surface, attach electrodes.

7.18 Electrospin at +16 kV, 2 mL/h at the following speeds and distances for the appropriate mandrel, following all safety precautions as outlined in the electrospinning risk assessment:

Table 1. Electrospinning schedule for bottom fibre zone				
Mandrel	Step	Duration	Speed	Distance
Small	1	30 min	100 rpm	50 mm
	2	120 min	100 rpm	100 mm
Large	1	1 h	100 rpm	50 mm
	2	7 h	100 rpm	100 mm

7.19 Cut the scaffold from the mandrel with a clean razor blade, being cautious to cut through the overhanging fibres and tape, but not the foam. Flip the scaffold, placing the porogen-leached foam side facing upwards and re-affix with tape around the edges.

7.20 Electrospin at +16 kV, 2 mL/h at the following speeds and distances for the appropriate mandrel, following all safety precautions as outlined in the electrospinning risk assessment:

Table 2. Electrospinning schedule for upper fibre zone

Mandrel	Step	Duration	Speed	Distance
Small	1	30 min	100 rpm	50 mm
	2	30 min	100 rpm	100 mm
	3	30 min	1000 rpm	100 mm
	4	60 min	3000 rpm	100 mm
Large	1	1 h	100 rpm	50 mm
	2	1 h	100 rpm	100 mm
	3	1 h	1000 rpm	100 mm
	4	5 h	2000 rpm	100 mm

* The velocities and timings of the large v small mandrel are different. All 2014-2015 scaffolds were spun on the small mandrel.

7.21 Cut the scaffold from the mandrel with a clean razor blade, being cautious to cut through the overhanging fibres and tape, but not the foam.

7.22 Trim the scaffolds to remove tape and edge-effects.

7.23 When cutting or punching, use a sharp blade and spray with 70% ethanol to aid in a clean cut without binding, especially when using biopsy punches.

7.24 Sterilise with 70% ethanol and peracetic acid (see sterilisation SOP) and rinse with sterile PBS before *in vitro* or *in vivo* applications.

8 NOTES

- Training must be undertaken before using any of the PolarBear, lyophiliser, and electrospinner.
- All work with dioxane and HFIP must be performed in a chemical safety cabinet.
- All syringe-pump repositioning should be performed with a non-conductive wooden stick.
- When measuring scaffolds, it is often helpful to cut the 80 mm x 80 mm scaffolds into two 40 mm x 80 mm strips and measure at the center. If a whole scaffold is preferable to save material and avoid excessive edge effect, a biopsy-punch from the center can also be used to measure.
- If a 'fanner' is not available to translate the electrospinning needle +/- 50 mm from the center-point of the mandrel, cut the scaffolds into 40 mm x 80 mm strips and position length-wise in a 40 mm x 160 mm strip around the mandrel to avoid large variations in fibre zone thickness.

9 LIMITATIONS

-

10 REFERENCES

- [1] S. D. McCullen, H. Autefage, A. Callanan, E. Gentleman, M. M. Stevens, *Tissue Eng. Part A* **2012**, 18, 2073–83.
- [2] J. A. M. Steele, S. D. McCullen, A. Callanan, H. Autefage, M. A. Accardi, D. Dini, M. M. Stevens, *Acta Biomater.* **2014**, 10, 2065–75.
- [3] U. G. K. Wegst, M. Schecter, A. E. Donius, P. M. Hunger, *Philos. Trans. A. Math. Phys. Eng. Sci.* **2010**, 368, 2099–2121.

Appendix F

SOP - PCL Scaffold Sterilisation

STANDARD OPERATING PROCEDURE

File Reference:			
Document Title:	PCL Scaffold Sterilisation		
SOP Number:		Version #:	v1.0
Author:	Joe Steele	Prepared Date:	26/04/2015
Reviewed By:		Review Date:	
Approved By:		Approval Date:	

1. PURPOSE

To sterilise PCL scaffolds for *in vitro* and *in vivo* studies.

2. SCOPE

The sterilisation of scaffolds composed of PCL by peracetic acid and ethanol for the eradication of microbial and fungal contamination, including spores.

3. DEFINITIONS

-

4. ABBREVIATIONS

PAA – peracetic acid

PCL – poly(ε-caprolactone)

5. BACKGROUND

In the past, PCL scaffolds have been sterilised solely with 70% ethanol [1]. While it does a good job, ethanol does not kill spores. As PCL cannot be autoclaved, is damaged by irradiation, and ethylene oxide is not available, a new protocol was suggested which uses peracetic acid (PAA). The method below is modified from “*Peracetic Acid: A Practical Agent for Sterilizing Heat-Labile Polymeric Tissue-Engineering Scaffolds*” by Yoganarasimha et al [2]. The modifications include the re-insertion of 15-min in ethanol to remove residual PAA and ensure that, in the event of PAA expiry, this sterilisation procedure is equivalent to the old ethanol-only protocol.

6. MATERIALS

1000 ppm peracetic acid (PAA) in 20% ethanol:

- 50 mL:
- 10 mL absolute ethanol (Sigma Aldrich)
 - 40 mL de-ionised water
 - 128 µL 39% PAA in acetic acid (390,000 ppm, 77240, Sigma Aldrich)
- Stable for 3 weeks at room temperature

0.1% BSA:

- 50 mL:
- 50 mg BSA (A2153, Sigma Aldrich, store at 4°C)
 - 50 mL PBS (tablets, non-sterile)
- Fully dissolve in 50 mL falcon tube at room temperature on the lab rollers
- Run through 0.2 µm filter to sterilise.
- Store at 4°C.

7. PROCEDURE

All work with PAA must be performed in a chemical safety cabinet

- 7.1 In chemical hood: Place dry scaffolds in 50mL flacon tube containing PAA solution.
- 7.2 Roll for 15 minutes at RT on the lab roller
- 7.3 In chemical hood: Decant PAA solution into waste container
- 7.4 In chemical hood: Add 70% ethanol
- 7.5 Roll for 15 minutes at RT on the lab roller
- 7.6 Transfer to sterile laminar hood
- 7.7 Rinse three times in sterile PBS (3 x 5 min on roller/shaker)

In vitro:

- 7.8 Place in sterile 0.1% BSA in PBS overnight at 4°C
- 7.9 Rinse in sterile PBS

In vivo:

- 7.8 Dry under UV for 1 h per side
- 7.9 Seal in autoclaved containers

8 NOTES

- All work with PAA must be performed in a chemical safety cabinet.
- For 6 mm Ø scaffolds for *in vivo* work, seal in autoclaved 1.5 mL Eppendorf tubes

9 LIMITATIONS

-

10 REFERENCES

- [1] J. A. M. Steele, S. D. McCullen, A. Callanan, H. Autefage, M. A. Accardi, D. Dini, M. M. Stevens, *Acta Biomater.* **2014**, *10*, 2065–75.
- [2] S. Yoganarasimha, W. R. Trahan, A. M. Best, G. L. Bowlin, T. O. Kitten, P. C. Moon, P. a Madurantakam, *Tissue Eng. Part C. Methods* **2014**, *20*, 714–23.

11 RISK MANAGEMENT

* Peracetic Acid



- Sigma 77240
- Store 4-8°C, well-sealed with parafilm and upright
- Always use and dilute in chemical safety cabinet

Hazard statement(s)

H226 Flammable liquid and vapour.
H314 Causes severe skin burns and eye damage.
H335 May cause respiratory irritation.
H400 Very toxic to aquatic life.

Precautionary statement(s)

P261 Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
P273 Avoid release to the environment.
P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.
P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P310 Immediately call a POISON CENTER or doctor/ physician.

Appendix G

Tang *et al.* Reproduction Permission



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**Title:** Porous Copolymers of ϵ -Caprolactone as Scaffolds for Tissue Engineering
Author: Min Tang, Matthew Purcell, Joseph A. M. Steele, et al
Publication: Macromolecules
Publisher: American Chemical Society
Date: Oct 1, 2013
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Author: Michiel W. Pot, Kaeuis A. Faraj, Alaa Adawy, et al

Publication: Applied Materials

Publisher: American Chemical Society

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Woodfield TBF, Van Blitterswijk C a, De Wijn J, Sims TJ, Hollander a P, Riesle J. Polymer scaffolds fabricated with pore-size gradients as a model for studying the zonal organization within tissue-engineered cartilage constructs. Tissue Eng 2005;11:1297–311. doi:10.1089/ten.2005.11.1297.

Figure 1

McCullen SD, Autefage H, Callanan A, Gentleman E, Stevens MM. Anisotropic fibrous scaffolds for articular cartilage regeneration. Tissue Eng Part A 2012;18:2073–83. doi:10.1089/ten.TEA.2011.0606.

Figure 1

Table 1

Joseph Steele

Appendix I

List of Publications

- Steele JAM**, McCullen SD, Callanan A, Autefage H, Accardi A, Dini D, Stevens MM. Combinatorial scaffold morphologies for zonal articular cartilage engineering. *Acta Biomaterialia*. 2014, 10: 2065–2075.
- Steele JAM**, Halle JP, Poncelet D, Neufeld R. Therapeutic Cell Encapsulation Techniques and Applications in Diabetes. *Advanced Drug Delivery Reviews*. 2014, 67–68: 74–83.
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- Campagnolo P, Gormley AJ, Chow LW, **Steele JAM**, Madeddu P, Stevens MM. Pericyte-coated dual peptide vascular grafts for improved endothelialisation. *Bioamaterials*. (in review).
- He M, Callanan A, Lagaras K, **Steele JAM**, Stevens MM. Optimisation of perfusion decellularisation in whole kidney bioscaffolds for whole kidney tissue engineering. *J Biomed. Materials Res. Part A*. (in review).
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- de la Rica RR, Mendoza E, Cloyd KL, Bertazzo S, Chow LW, Watkins HC, St. Pierre JP, **Steele JAM**, Stevens MM. Self-Assembly of Collagen Building Blocks Guided by Electric Fields. *Small*. 2014, 10(9):3876–3879.
- Su L, Cloyd K, Arya S, Hedegaard MAB, **Steele JAM**, Elson DS, Stevens MM, Hanna GB. Raman Spectroscopic Evidence of Tissue Restructuring in Heat-Induced Tissue Fusion. *Journal of Biophotonics*. 2014, 7: 713–723.
- Tang M, Purcell M, **Steele JAM**, Lee KY, McCullen S, Shakesheff KM, Bismarck A, Stevens MM, Howdle SM, Williams CK. Porous Copolymers of ϵ -Caprolactone as Scaffolds for Tissue Engineering. *Macromolecules*. 2013, 46 (20): 8136–8143.

