Engineering of Natural Cartilage Substitution Biomaterials

Hazel Louise Fermor

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The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapter 3 is based on a joint authorship publication, Fermor, H.L., McLure, S.W.D., Taylor, S.D., Russell, S.L., Williams, S., Fisher, J., Ingham, E. Biological, biochemical and biomechanical characterisation of articular cartilage from the porcine, bovine and ovine hip and knee. Bio-Medical Materials and Engineering. In press. As the lead author the candidate was responsible for the laboratory work, analysis and writing of the paper. McLure, S.W.D. and Taylor, S.D. were responsible for laboratory work relating to the biomechanical testing of specific animal osteochondral tissues as indicated in Table 3.1. The contributions of the co-authors included discussions on experimental design of the study, critique and proof reading of the manuscript and obtaining funding for the study.

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Abstract

Cartilage lesions cause pain and a loss of joint motion, and if not repaired will further degenerate into an osteoarthritic state. Currently the only treatment options for end stage osteoarthritis are total joint replacements, which are not preferable for younger or more active patients. Early intervention therapies to repair initial cartilage damage and stem the progression of osteoarthritis are thought to be a more favourable treatment option in appropriate cases. It is hypothesised that an acellular xenogeneic osteochondral scaffold could be used in mosaicplasty-like operations to provide an immunocompatible, off-the-shelf biomaterial for osteochondral lesion repair, retaining the same natural composition, structure and function as native cartilage.

Initial characterisation of cartilages from different species (pig, cow and sheep) and joint regions of the hip and knee revealed differences in cartilage biology, biochemistry and biomechanics. Osteochondral tissues from skeletally immature porcine medial condyles were selected along with mature bovine femoral groove tissues as source materials for decellularisation, primarily based on cartilage thickness and glycosaminoglycan (GAG) content.

Bovine osteochondral pins were subject to a number of decellularisation protocols and were shown to be successfully decellularised following use of a water pik to remove bone marrow, four cycles of freeze-thaw (two of which were in a hypotonic buffer with protease inhibitors), two cycles of hypotonic buffer followed by incubation in 0.1 % (w/v) SDS with protease inhibitors and treatment with nucleases and sterilisation with 0.1 % (v/v) peracetic acid. The process removed all whole cell nuclei, as visualised by histology and reduced cartilage DNA content per cartilage dry weight to 39 ng.mg⁻¹. However, the process removed 99% of GAGs and resulted in reduced biomechanical properties.

Porcine osteochondral pins were fully decellularised following use of the above process with one cycle of incubation in hypotonic buffer and 0.1 % (w/v) SDS in hypotonic buffer. Cartilage DNA content was reduced by 98% and the osteochondral tissues contained no visible cell nuclei. Cartilage GAG content was reduced by 60 %. Further alterations to the protocol to improve GAG retention revealed cartilage damage; histologically appearing highly porous and having greatly increased water content and almost
complete GAG loss. A comprehensive investigation was conducted to identify the cause of the damage, however no protocol could be developed which completely eradicated cartilage damage.

Further characterisation of acellular bovine osteochondral scaffolds showed that a low concentration of SDS remained in the bone, and that this had cytotoxic effects when incubated in contact with BHK cells. Further washes in PBS were added to the protocol to remove excess SDS, however this increased washing led to damage of the bovine cartilage, as seen previously in porcine osteochondral decellularisation.

Finally, the decellularisation process was applied to whole porcine condyles in which the ratio of cut edge to cartilage area was minimised and minimal damage to the decellularised cartilage was seen. In summary, the complex microstructure of cartilage has been shown to be surprisingly fragile \textit{ex vivo} and a novel approach to the development of clinically relevant acellular osteochondral grafts is required; however significant advances have been made in the current study.
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<tr>
<td>ACI</td>
<td>Autologous chondrocyte implantation</td>
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<tr>
<td>ACL</td>
<td>Anterior cruciate ligament</td>
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<tr>
<td>ADAMTS</td>
<td>A disintegrin and a metalloprotease with thrombospondin motifs</td>
</tr>
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<td>AMCI</td>
<td>Autologous matrix induced chondrogenesis</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumen</td>
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<tr>
<td>BSE</td>
<td>Bovine spongiform encephalopathy</td>
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<tr>
<td>CDMP</td>
<td>Cartilage-derived morphogenic protein</td>
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<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
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<tr>
<td>CILP</td>
<td>Cartilage intermediate layer protein</td>
</tr>
<tr>
<td>CL</td>
<td>Confidence level</td>
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<td>CMP</td>
<td>Cartilage matrix protein</td>
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<tr>
<td>COMP</td>
<td>Cartilage oligomeric matrix protein</td>
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<tr>
<td>CPM</td>
<td>Counts per minute</td>
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<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
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<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
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<tr>
<td>DABCO</td>
<td>1,4-diazobicyclo-(2, 2, 2)-octane</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
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<tr>
<td>DMB</td>
<td>1,9- dimethylmethylene blue</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
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<td>Deoxyribonuclease</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>FE</td>
<td>Finite element</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>Gal</td>
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<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
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<tr>
<td>G1cUA</td>
<td>Glucuronate</td>
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<tr>
<td>GMEM</td>
<td>Glasgow’s minimal essential media</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan (hyaluronic acid)</td>
</tr>
<tr>
<td>HAS</td>
<td>Hyaluronan synthase</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte-associated</td>
</tr>
<tr>
<td>ICRS</td>
<td>International cartilage repair society</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGD</td>
<td>Interglobular domain</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KS</td>
<td>Keratan sulphate</td>
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<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
</tr>
<tr>
<td>LP</td>
<td>Link protein</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LVDT</td>
<td>Linear variable differential transducer</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MACI</td>
<td>Matrix assisted chondrocyte Implantation</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MSD</td>
<td>Minimum significant difference</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin M</td>
</tr>
<tr>
<td>PAA</td>
<td>Peracetic acid</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease-activated receptor</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid and Schiff’s reagent</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Posterior cruciate ligament</td>
</tr>
<tr>
<td>PCL</td>
<td>poly (ε-caprolactone)</td>
</tr>
<tr>
<td>PEGDA</td>
<td>Poly(ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>PERV</td>
<td>Porcine endogenous retrovirus</td>
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<tr>
<td>PGA</td>
<td>Polyglycolic acid</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
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<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNase</td>
<td>Ribonuclease</td>
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<tr>
<td>SAL</td>
<td>Surface amorphous layer</td>
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<tr>
<td>SDC</td>
<td>Sodium deoxycholate</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SIS</td>
<td>Small intestinal submucosa</td>
</tr>
<tr>
<td>SLRP</td>
<td>Small leucine-rich repeat protein</td>
</tr>
<tr>
<td>SZP</td>
<td>Superficial zone protein</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TPB</td>
<td>Tryptone phosphate broth</td>
</tr>
<tr>
<td>TSE</td>
<td>Transmissible spongiform encephalopathys</td>
</tr>
<tr>
<td>VAS</td>
<td>Visual analogue scale</td>
</tr>
<tr>
<td>α-gal</td>
<td>Galactose α1-3 galactose</td>
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</table>
Chapter 1

Introduction

1.1 General introduction

Osteoarthritis (OA) is a major public health concern, causing high levels of morbidity within the population and posing a large socioeconomic burden (Brooks, 2006). It is the most common disease affecting joints, and is characterised by irreversible progressive articular cartilage damage leading to joint failure (Herrero-Beaumont et al., 2009). Increasingly, OA is being recognised as a disease of the entire joint. Initial injury to the cartilage itself or other joint tissues such as ligaments can affect load distribution and friction during joint articulation, this abnormal activity leads to progressive erosion of the cartilage and malalignment of the joint resulting in further damage, including damage to the muscles and tissue inflammation (Felson, 2009).

It is clear from the progressive nature of the disease that initially minor cartilage surface damage can lead to a much greater physiological disorder. Therefore early intervention treatments are crucial to prevent or delay the development of debilitating OA, and avoid end stage treatments such as total joint replacement. There are currently a number of therapies available to treat initial cartilage lesions, however these are often ineffective, only having short-term therapeutic benefit or causing difficulties such as donor site morbidity. Considering these therapies, it is proposed that a new tissue engineered approach may provide a better treatment option.
1.2 Anatomy

1.2.1 The hip joint (acetabulofemoral)

The hip joint (Figure 1.1) is commonly affected by OA, as a load bearing joint, the surface cartilage is exposed to high mechanical stress leading to wear. Here the acetabulum of the pelvis articulates with the head of the femur through a synovial ‘ball and socket’ joint allowing for flexion, extension and rotation. The articulating surfaces of the acetabulum and femoral head are lined with hyaline cartilage which reduces friction allowing the joint to function. The hemi-spherical femoral head is held in the acetabulum by a fibrocartilaginous labrum lip which extends past the bony element of the acetabulum.

![Figure 1.1 The hip joint. Diagram entailing the main features of the joint. Figure adapted from http://www.noc.nhs.uk/hipandknee/information/hip/joint-anatomy.aspx.](image-url)
The synovial membrane and fibrous capsule are attached to the femoral neck (zona orbicularis) and the labrum encasing the femoral head and acetabulum, they contain synovial fluid which provides lubrication and cushioning for the joint. The capsule along with one intracapsular (ligamentum teres) and three extracapsular ligaments (iliofemoral, ischiofemoral and pubofemoral) provide mechanical stability for the joint (Gray, 2000; Sinnatamby, 2006).

1.2.2 The knee joint

The knee joint (Figure 1.2) is the largest synovial joint in the body. An effective ‘hinge’ joint, it allows for flexion and extension, as well as slight rotation in some positions. It is a complex joint which articulates between a) the femur and the patella, via a sellar joint whereby the patella slides through the patellofemoral groove, and b) the tibio-femoral joint, which articulates the medial and lateral condyles of the femur with the tibial plateau; these interactions are partially divided by the two menisci. The condyles of the femur and the tibial plateau are, like the articular surfaces of the hip joint, lined by hyaline cartilage, allowing reduced friction. The medial and lateral menisci are composed of fibrocartilage, which has different biological and mechanical properties from hyaline cartilage, allowing it to cushion the joint effectively.

The joint is surrounded by an articular capsule which is composed of a strong fibrous membrane lined by the synovial membrane. The capsule is attached to the cartilage margins on both the femur and the tibia and to the head of the fibula. The presence of a suprapatellar bursa anteriorly on the femur results in the joint capsule being attached more proximally than the cartilage. As with the hip joint, the capsule provides stability to the joint, and encloses the synovial fluid which provides lubrication for the joint. Ligaments act alongside the capsule to provide stability to the joint by limiting movement. Intracapsular ligaments include the anterior (ACL) and posterior cruciate ligaments (PCL), injury to which may often result in damage to cartilage, leading to OA (Felson & Zhang, 1998).
transverse ligament, anterior and posterior meniscofemoral and the meniscotibial ligaments are also intracapsular. The patellar tendon is associated with the quadriceps tendon to secure the patellar. The sides of the patellar are attached to the capsule, allowing its articulating surface to be intracapsular but the non-articulating face of the patellar to be extracapsular. The patellar tendon is attached to the extracapsular non-articulating face of the patella. Extracapsular ligaments include the medial and lateral collateral ligaments and the oblique and arcuate popliteal ligaments. There are a number of bursae associated with the knee joint, these fluid sacks and synovial pockets surround and communicate with the joint cavity, cushioning impact between bones and tendons and/or muscles, and they also act to further reduce friction (Gray, 2000; Sinnatamby, 2006).

**Figure 1.2 The knee joint.** Articulation between the femur, patella and tibia. Figure adapted from http://www.digital-doc.com/images/knee.jpg.
1.3 Cartilage

There are three forms of cartilage; elastic cartilage (for example the ear), fibrocartilage (menisci) and hyaline cartilage (synovial joints; Roughley, 2006). Hyaline cartilage covers the articulating surfaces of bones at joints, the function of which is to reduce friction between articulating surfaces during load bearing and movement. Hyaline cartilage is a tough tissue formed predominantly of extracellular matrix (ECM) composed of interwoven collagen fibres, proteoglycans and interstitial water. These components are secreted by chondrocytes which reside dispersed within the ECM. Cartilage is avascular and aneural, qualities which result in limited repair capacity of the tissue.

1.3.1 Cartilage composition

Cartilage is a highly specialised tissue, able to effectively lubricate articular joints throughout a lifetime of movements without experiencing any significant wear. This is possibly due to its ideal composition and structure. The key components of cartilage are collagen, proteoglycans, interstitial water, chondrocytes and lubrication by synovial fluid.

1.3.1.1 Collagen

Collagen comprises ~15% of cartilage ECM and it is present as a network of interwoven fibres. Each procollagen molecule is composed of three α-chains which contain a repeated Gly-X-Y sequence where X is most commonly a proline residue and Y a hydroxyproline residue. This repeat region is flanked by short linear telometric regions and globular N- and C-terminal pro-peptides. The stoichiometry of these amino acids results in the chain being a left-handed α-helix. The three chains interact with one another to form a superhelix. These chains can form hetero- or homotrimerers, the composition of which, determine the type of collagen fibre produced (Cremer et al., 1998). Cleavage of the procollagen terminal peptides by specific proteases (metalloproteases and disintegrin) allows the spontaneous formation of collagen fibrils (Steplewski et al., 2007).
The most dominant form of collagen in cartilage ECM is type II collagen, a homotrimer of $\alpha_1$(II) chains, it constitutes ~80-85% of the total collagen content. Collagen IX and XI are the second most common comprising ~3-10% each. Both are heterotrimeric, collagen type IX has a large N-terminal globular domain from an $\alpha_1$(IX) chain, it also has a ‘kink’ mid chain from which a chondroitin sulphate rich glycosaminoglycan (GAG) chain extends. It runs antiparallel to the fibre and is situated at points of regular D-periodicity (alternating gap and overlap regions along the fibre exterior (Steplewski et al., 2007)). Type XI collagen resembles type II in that it is also a straight chain, however some molecules have an $\alpha_3$(XI) chain propeptide at the N-terminus which projects from the fibril, this molecule is covalently cross-linked to collagen type II molecules in the fibre backbone at hydroxylysine residues. Collagen X is a straight chained cartilage specific homotrimer, although shorter than collagens II or XI, these form a multimeric collagen fibril (Figure 1.3). Type VI collagen is also found sparsely in cartilage (Cremer et al., 1998).

**Figure 1.3 Collagen fibril composition.** A diagrammatical representation of cartilage fibril composition and the collagen molecules which are specific to articular cartilage. Fibril composition is mainly type II collagen (solid line) with internal type XI collagen (dotted line) molecules and surface type IX molecules (bent line with globular domain). Type X is not intrinsic to the fibril formation and is found elsewhere in the collagenous matrix of cartilage. Figure cited from Cremer et al. (1998).
The backbone of heteropolymeric cartilaginous fibrils is mainly formed from type II collagen, type IX is thought to play a role in facilitating the interaction of fibrils with proteoglycans along with acting as a ‘spacer’ between fibrils and a ‘glue’ to bind the type II lattice. Collagen type XI regulates fibril length (Mendler et al., 1989; Cremer et al., 1998). Type VI collagen forms independent microfibrils in the ECM around lacunae (Cremer et al., 1998) and plays a role in chondrocyte attachment to the ECM (Poole, 1997) Type X is most abundant in areas where hypertrophic chondrocytes are found (Cremer et al., 1998) and is thought to play a role in the organisation and distribution of cartilage matrix components (Shen, 2005).

Collagen fibres are organised with a specific orientation in the cartilage matrix, known as the arcade concept (Figure 1.4). In the deep calcified zone of cartilage, collagen fibres are anchored to the bony component and run vertically toward the surface in the radial zone, these then turn obliquely in the transitional zone and run parallel to the surface in the tangential zone (Jeffery et al., 1991). The anchorage of collagen fibres into the subchondral bone ensures the soft cartilage tissue and solid bone are firmly fixed together. The specific organisation of collagen fibres in the cartilage structure has a major impact on the mechanical properties of the tissue, as will be discussed later. This inhomogeneity of collagen fibre distribution within cartilage results in differing values of permeability throughout the layers of the tissue with the middle zone being relatively permeable compared to the superficial and deep zones. There are dense collagen fibres at the superficial zone, but a high charge density in the deep zone (Mow et al., 1984).
Figure 1.4 Arcade concept of collagen organisation. Collagen fibre orientation is altered though the different layers of the cartilage tissue.

1.3.1.2 Proteoglycans

Proteoglycans are highly glycosylated proteins comprised of a core protein covalently linked to one or more glycosaminoglycan (GAG) chains. A GAG is a disaccharide unit which is repeated to form a long unbranched chain. Aggrecan (Figure 1.5) is a highly abundant, key proteoglycan in cartilage; it exists in the form of large aggregates with hyaluronan (HA) and link protein (LP). Aggrecan is a modular protein with three globular domains (G1, G2 and G3) which contain cystine residues for the formation of disulphide bonds. There is a short interglobular domain between G1 and G2, and between G2 and G3 there is a long GAG attachment region rich in keratan sulphate (KS) and chondroitin sulphate (CS). The N-terminal G1 domain is responsible for attachment to HA, the function of G2 is currently unknown, G3 is key for normal post-translational modification of aggrecan, ensuring correct secretion from the cell (Roughley, 2006). The GAG attachment region is divided into 3 sections, the keratan sulphate attachment region, which is closest to G2, and the adjacent chondroitin sulphate attachment region is comprised of two sections.
Figure 1.5 Aggrecan. Aggrecan molecule showing all three globular regions (G1, G2 and G3), the interglobular domain (IGD) and the GAG attachment region comprised of one keratan sulphate binding region (KS) and two chondroitin sulphate binding regions (CS1 and CS2). Figure adapted from Dudhia (2005).

Aggrecan exists as large aggregates in cartilage (Figure 1.6) entrapped within the collagenous network. These aggregates are composed of one long HA molecule attached to up to 100 aggrecan molecules stabilised by a link protein (Roughley, 2006). The link protein is similar in structure to the aggrecan G1 region. Through its ability to bind both HA and aggrecan G1 regions, the presence of link protein stabilises the attachment of aggrecan to HA, retaining association of the aggregate under physiological conditions. It also facilitates association of newly secreted aggrecan molecules with HA and finally along with the G1 region of aggrecan forms a protein coat surrounding the HA molecule to protect it from degradation by enzymes or free radicals (Roughley, 2006).
Figure 1.6 Proteoglycan aggregate. Proteoglycan aggregate of aggrecan molecules attached to a single hyaluronan chain stabilised by link protein (LP). Three aggrecan molecules are shown here, but up to 100 can be attached to a single hyaluronan molecule. Figure cited from Dudhia (2005).

HA is a unique GAG of notably large molecular weight, it is unsulphated. The HA polysaccharide chain is composed of a repeated disaccharide unit of glucuronate (GlcUA) and N-acetylgalactosamine (GlcNAc; Figure 1.7), the size of the chain decreases as HA abundance increases with age, which may be relevant to osteoarthritis onset later in life. The decrease in size is thought to be related to degradation of the GAG post synthesis (Roughley, 2006). HA molecules have carboxyl groups along their length which are fully ionised at physiological pH, resulting in the molecule having high osmotic activity allowing it to affect the distribution and movement of water (Fraser et al., 1997). Formation of secondary hydrogen bonds along the chain produces twists resulting in some stiffness of the molecule and hydrophobic patches which assist the molecule in interacting with other HA chains to form a network and allow association with lipid structures such as cell membranes (Scott, 1992). HA is synthesised at the chondrocyte plasma membrane by hyaluronan synthase (HAS) of which there are three types. The HAS’s produce the same composition, however vary in the length of HA chain they synthesise, which may have implications of the overall matrix composition (Roughley, 2006).
The GAG side chains are the key to aggrecan function; they have a fixed negative charge which allows hydrophilic interactions between the proteoglycan molecules of the cartilage ECM and interstitial water, controlling the movement of water through the collagen network. Chondroitin sulphate and keratan sulphate (Figure 1.7) are the GAGs associated with aggrecan (Roughley, 2006), they attach at serine residues located along the core protein chain within the GAG attachment region of aggrecan.

![Glycosaminoglycan molecular structures](image)

**Figure 1.7 Glycosaminoglycan molecular structures.** A - Chondroitin sulphate disaccharide unit, showing specific sites where the sugar can be sulphated, unit is repeated many times to form a GAG chain. B – keratan sulphate disaccharide unit, also showing possible sulphated regions, unit is repeated many times. C – Hyaluronan disaccharide unit, characteristically unsulphated, repeated to form a long polysaccharide chain. Figure adapted from Dudhia (2005).

GAGs are polysaccharides composed of repeating disaccharide units of one of two modified sugars: GalNAc or N-acetylglucosamine, and a uronic acid: GlcUA, iduronate or galactose (Gal). The sulphate and acidic substitutions on the chains of KS and CS result in the molecule being polyanionic. CS is post translationally modified with sulphate ester
substitutions at carbon-4-O-hydroxyl or carbon-6-O-hydroxyl positions of many of the GalNAc residues, which can be unsulphated, monosulphated or disulphated. These negatively charged groups within the GAG are fixed to the aggrecan protein and provide the proteoglycan with its hydrophilic properties (Dudhia, 2005).

KS GAG chains associate with the aggrecan protein in the KS region, where there is a repeated residue motif of Glu-Glu/Lys-Pro-Phe-Pro-Ser (Antonsson, 1989), KS molecules attach at the serine residue. There are also KS chains in the G1 domain attached by N- and O-linked substitutions. A few KS chains are also found in the CS rich regions of aggrecan attached via O-linked substitutions on threonine residues (Barry et al., 1992). The proline-rich repeats of the KS attachment region have a moderate affinity for collagen, allowing interaction with the collagenous network of the cartilage matrix (Hedlund et al., 1999). The CS rich region of aggrecan also contains a repeated motif of either Ser-Gly-X-Gly in the CS1 region or (Asp/Glu)-X-Ser-Gly in the CS2 region (Doege et al., 1997).

1.3.1.3 Non-collagenous proteins

There are other proteins in the cartilage matrix besides aggrecan aggregates and collagens, these include other proteoglycans, such as members of the small leucine-rich repeat protein (SLRP) family. There are also a number of structural proteins which are of importance in cartilage function. Other proteins found in articular cartilage play a regulatory role in controlling cellular activities; their involvement is not directly relevant to the mechanical function of the cartilage matrix (Roughley, 2001). These proteins play various roles in the cartilage matrix, some of which are listed in Tables 1.1 and 1.2.
<table>
<thead>
<tr>
<th>Proteoglycan</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decorin</td>
<td>SLRP associated with collagen fibrils as a decorating proteoglycan, carries a dermatan or chondroitin sulphate chain, thought to play a role in fibril diameter regulation. Decorin knockout mice have shown reduced tensile strength of skin and tendons.</td>
<td>Knudson &amp; Knudson, 2001, Danielson et al., 1997</td>
</tr>
<tr>
<td>Biglycan</td>
<td>SLRP with two chondroitin or dermatan sulphate chains is found primarily in the pericellular matrix of cartilage. Biglycan interacts with collagen type IX and II regulating fibril formation and stability.</td>
<td>Knudson &amp; Knudson, 2001 Chen et al., 2006</td>
</tr>
<tr>
<td>Lumican</td>
<td>SLRP with keratan sulphate GAG chains is able to bind collagen fibres to modulate fibril formation and the GAG chain acts to regulate interfibrillar spacing.</td>
<td>Nikitovic et al., 2008</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>Another keratan sulphate SLRP acts along with decorin, biglycan and lumican to regulate collagen fibril diameter and stability. It is probable that there is a level of redundancy between these small glycoproteins.</td>
<td>Hedlund et al., 1994 Roughley, 2001</td>
</tr>
<tr>
<td>Perlecan</td>
<td>Perlecan is commonly substituted with heparin or chondroitin sulphate GAG chains. Located pericellularly, perlecan is able to stimulate fibrillogenesis when glycosylated with 4-6-disulphated chondroitin sulphate residues.</td>
<td>Melrose et al., 2008 Kvist et al.; 2006</td>
</tr>
<tr>
<td>SZP/Lubricin</td>
<td>A secreted glycoprotein, lubricin is present at the superficial zone of articular cartilage. It functions to lubricate the articular surface, reducing wear, and also to inhibit invasion of the cartilage surface by synoviocytes and prevent protein deposition onto the surface by synovial fluid.</td>
<td>Rhee et al., 2005 Roughley, 2001</td>
</tr>
</tbody>
</table>

Table 1.1 Non-collagenous Proteins – Proteoglycans. A list of the less common cartilage structural proteins, detailing their function within the tissue. SZP = Superficial zone protein.
<table>
<thead>
<tr>
<th>Structural protein</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COMP</td>
<td>A disulphide-bonded pentamer, the carboxy-terminal regions of which interact with collagen fibrils, the correct folding of the molecule is determined by calcium binding. The protein has also been shown to interact with cell surface integrins supporting cellular attachment. COMP is thought to play an important role in matrix assembly.</td>
<td>Chen et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hecht et al., 2005</td>
</tr>
<tr>
<td>Matrilin-1/CMP &amp; Matrilin-3</td>
<td>Matrilins are a family of proteins, matrilin-3 is normally associated with matrilin-1, however matrilin-1 is only expressed at low/negligible levels in articular cartilage whereas matrilin-3 is more abundant. It is thought that matrilins are able to interact with aggrecan, COMP and decorin molecules as well as collagen fibrils, acting in a structural capacity.</td>
<td>Wagener et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Segat et al., 2001</td>
</tr>
<tr>
<td>CILP</td>
<td>Found in the intermediate zone of articular cartilage, this protein is thought to play a role in modulating the architecture of the cartilage matrix, but has also shown to be an IGF-1 antagonist.</td>
<td>Johnson et al., 2003</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>There is a unique splice variant of fibronectin produced by chondrocytes. This protein is involved in cell-matrix interactions.</td>
<td>Roughley, 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Burton-Wurster et al., 1998</td>
</tr>
</tbody>
</table>

**Table 1.2 Non-collagenous Proteins – Structural Proteins.** A list of the less common cartilage structural proteins, detailing their function within the tissue. COMP= Cartilage oligomeric matrix protein (thrombospondin-5), CMP= Cartilage matrix protein (matrilin-1), CILP= Cartilage intermediate layer protein.
1.3.1.4 Interstitial water

The interstitial water of the ECM plays an integral role in cartilage function through interactions with GAG molecules, it forms 60-85% wet weight of cartilage. Water is composed of two hydrogen atoms covalently bound to an oxygen atom. The molecule is not linear, and oxygen has a higher electronegativity than hydrogen, resulting in a dipole. The slight positive charge on the hydrogen atoms allows for interaction with charged molecules.

Water within the cartilage tissue contains small proteins, metabolites and a high concentration of cations (Buckwalter & Mankin, 1998). The immobilised sulphate and carboxyl groups on CS and KS of aggrecan become charged in solution and produce a net negative charge in the tissue relative to the surrounding fluid. These negative fixed charges are situated close together in the collagen network and repel one another, this unbalanced charge density is neutralised by increased positive sodium ion concentration, and repulsion of negative chloride ions in the fluid. The overall increase in total inorganic ion concentration results in increased osmolarity of the tissue – creates a Donnan effect, causing water to enter the tissue and swell the cartilage. However, this Donnan osmotic pressure is resisted by the tensile strength of the collagen network, allowing the tissue to resist deformation (Mow et al., 1984; Buckwalter & Mankin, 1998).

1.3.1.5 Chondrocytes

Articular cartilage is exclusively populated by one cell type, chondrocytes. The cells are sparsely dispersed and exist in the cartilage matrix as chondrons, a unit comprising one cell linked at its surface to a pericellular glycocalyx which is encased in a fibrillar pericellular capsule. The morphology of chondrons differs throughout the different layers of the cartilage tissue (Figure 1.8), in the superficial zone the chondrocytes are flattened disc-shape, these become rounded and spherical in the middle layers. In the deep layer, chondrocytes are rounded and arranged in linear columns. In the calcified layer, chondrocytes are also rounded and surrounded by uncalcified lacunae (Poole, 1997).
Figure 1.8 Chondrocyte distribution and morphology. The distribution and morphology of chondrocyte chondrons within the cartilage layers.

There is a degree of polarity, with the chondron pole facing the articular surface being more dense than the basal pole (Figure 1.9). A loosely woven ‘tail’ can be seen at the basal pole and in columns of multiple chondrons these ‘tails’ form interconnecting segments between chondrons, ensuring that adjacent chondrons are aligned linearly (Poole, 1997).

The composition of cartilage matrix has already been defined in terms of horizontal matrix subdivisions, however many studies have highlighted a circumferential differentiation of matrix composition, based on pericellular, territorial and interterritorial matrices surrounding each chondrocyte. The pericellular matrix is in direct contact with the chondrocyte, and contains high concentrations of HA, sulphated proteoglycans, biglycan and a number of glycoproteins including link protein, fibronectin and laminin. Collagen composition also differs in these concentric subdivisions, with thinner fibrils being tightly interwoven to encase the cell in the pericellular region. In the territorial region, the collagen fibrils are larger and form radial bundles; this region is dense in chondroitin sulphate, whereas keratan sulphate is most concentrated in the interterritorial regions where the collagen fibres are largest and can form the classical ‘collagen arcade’ structural organisation. As described previously, there are different types of collagen, these are not uniformly distributed in the matrix, with type VI
being exclusively pericellular. Type IX is also more concentrated in the pericellular region (Poole, 1997).

The pericellular matrix of the chondron is thought to be highly specialised, providing hydrodynamic protection for the chondrocyte during physiological loading due to high pericellular concentrations of HA and aggrecan in combination with differential organisation of specific collagen molecules. The pericellular matrix also plays a metabolic role in retention, temporal assembly and spatial migration of synthesised macromolecules in the different chondron territories (Poole, 1997).

Figure 1.9 Chondron composition and morphology. Three-dimensional cutaway representation of a single chondron showing the central chondrocyte, dense collagen organisation at the articular pole (Ap) and more loosely packed collagen tail at the basal pole (Bp). Figure from Poole (1997).

The role of chondrocytes is to secrete and maintain components of the ECM, these processes are tightly controlled by a number of regulatory factors (Goldring, 1999). Chondrocytes are cells of the mesenchymal lineage (Pettenger et al., 1999) and differentiate from mesenchymal stem
cells to initially produce an osteochondral precursor cell, which further differentiates down a chondrogenic route to produce chondrocytes, thought to be the default differentiation state of the precursors (Hartmann, 2006). Sox9 is the key transcription factor responsible for ensuring differentiation of an osteochondral progenitor cell towards a chondrogenic rather than osteogenic lineage, it recruits further chondrocyte specific transcription factors Sox5 and Sox6 (Lefebvre et al., 2001; Hartmann, 2006).

### 1.3.1.6 Synovial fluid

The synovial intima is the thin cellular layer which lines the luminal surface of the synovial membrane in the joint capsule. This layer is populated by synoviocytes, which can be two to three cells deep. There are two different types of synoviocytes, type A are resident macrophages which phagocytose debris and waste from the joint cavity, and the more abundant type B are fibroblast-like cells which are involved in secretion of matrix constituents such as HA. Type B cells have a characteristically high endoplasmic reticulum content, suggesting that they are active in protein synthesis. These cells secrete HA, collagens, fibronectin and other glycosaminoglycans into the intimal interstitium and joint cavity. Type B cells have long membranous processes that extend into the joint cavity allowing a larger surface area for delivery of secreted products (Iwanaga et al., 2000).

The composition of synovial fluid is almost identical to that of blood plasma, with the exception of larger globulins which are filtered out as plasma passes through synovial capillary walls. There are additional molecules secreted into the synovial fluid by synoviocytes. The level of glucose in synovial fluid is lower than that of plasma, as it is metabolised by chondrocytes (Gerwin et al., 2006).

HA and lubricin have been reported to determine the viscosity of synovial fluid, allowing its primary function as a lubricant, they cause the fluid to be a thixotropic non-newtonian fluid which decreases in viscosity as the shear strain rate increases. The fluid forms a thin film over the synovial membranes and joint surfaces to provide effective hydrodynamic
lubrication at low shear rates. The viscoelasticity of HA allows it to behave as a viscous lubricant at low impact movements, and as an elastic shock absorber under high impact movement, maintaining joint homeostasis. Under high load, lubricin is thought to play a role by interacting with surface-active phospholipids (Gerwin et al., 2006).

### 1.3.2 Cartilage function

The role of cartilage in the synovial joint is to effectively transmit loads and articulate with low levels of friction. During normal walking conditions, articular cartilage in the lower joints often has to withstand very high loads of up to 4 times body weight, the mechanical properties of hyaline cartilage allow it to perform this function throughout a person’s life. Cartilage distributes the load across the joint, over a wide area to minimise stress and allow movement with minimal friction. A number of different lubrication regimes operate in cartilage to allow minimal friction under a vast range of different loading conditions. These many different lubrication mechanisms can be categorised under three main types occurring in synovial joints: fluid film, boundary and biphasic lubrication.

#### 1.3.2.1 Fluid film lubrication

Fluid film lubrication (Figure 1.10 A) works well under many physiological conditions, however cannot alone completely account for low friction in joints, as it is unsuccessful in certain conditions, such as start-up after a period of standing. Fluid film lubrication is based on two solid bearing surfaces being separated by a thin film of lubricant, so that the load bearing and friction capacity is governed by the laws of hydrodynamics, rather than the behaviour of rubbing solid surfaces (Dowson, 1995). This film must be thick enough to prevent any isolated asperity on one surface from touching a similar peak on the opposing surface. Fluid film lubrication encompasses hydrodynamic, elastohydrodynamic and squeeze film lubrication (Walker et al., 1968). Entraining action is the mechanism by which lubricant is dragged back into the contact area, this is due to the motion of two opposing surfaces sliding across one another.
Hydrodynamic lubrication (Figure 1.10 B) occurs when two rigid surfaces are separated by a thin film of viscous lubricant, but they oppose each other at an angle, so the fluid is in a ‘wedge’ shape. When the surfaces move tangentially, fluid is moved into the diminishing gap by viscous forces. The pressure in the fluid is sufficient to support the transverse load (Walker et al., 1968).

If the pressure in the fluid becomes too great, it may result in deformation of the solid surfaces and if the geometric alteration is great enough then elastohydrodynamic lubrication (Figure 1.10 C) operates. Here, the softer, more elastic surfaces allow a larger volume of fluid to be drawn into the converging gap. With increased relative radius of curvature of the solid, viscosity of the lubricant, sliding speed and increased softness of the material, the subsequent lubricant layer is thicker (Dowson et al., 1995).

Squeeze film lubrication (Figure 1.10 D) occurs when the surfaces produce force on one another in a normal direction not necessarily with transverse movement. Increased load results in increased pressure and lubricant viscosity. The approaching surfaces attempt to squeeze out the intervening fluid; this is, however, resisted by viscous forces and the film thickness is maintained, preventing solid-to-solid contact (Walker et al., 1968).

Unsworth (1991) believed fluid film lubrication to be of great importance in diarthroidial joints. However, the activity of such lubrication mechanisms within synovial joints has been disputed by Ateshian (2009).
Figure 1.10 Fluid film lubrication. Schematic representations of fluid film lubrication mechanisms. A: Fluid film lubrication showing two surfaces or articular cartilage separated by a thin film of viscous synovial fluid lubricant. B: Hydrodynamic lubrication, fluid is forced into wedge-shaped gap by viscous forces during motion. C: Elastohydrodynamic lubrication, where deformation of the surface occurs and film is maintained due to viscous forces. D: Squeeze film lubrication force in the normal direction causes opposing surfaces to come together and pressurise fluid which is maintained by viscous forces.

1.3.2.2 Boundary lubrication

Boundary lubrication occurs when the fluid film thickness becomes very reduced resulting in increased asperity contact between two solid surfaces (Walker *et al.*, 1968). This encompasses boosted lubrication, mixed lubrication, and boundary lubrication.
Boosted lubrication was first proposed by Walker et al. (1968) whereby pressure from load causes water and other small molecules from synovial fluid to enter the porous cartilage tissue. High molecular weight glycoproteins such as HA, for instance, are too large to be forced into the tissue so become highly concentrated at the surface, thus the resulting lubricating film is highly viscous (Walker et al., 1968).

Prior to the full use of boundary lubrication there is a stage of mixed lubrication (Figure 1.11 A), during which the majority of the surface is lubricated by fluid film mechanisms, however some points of contact between asperities on the surface use boundary lubrication.

During boundary lubrication (Figure 1.11 B) the fluid film is negligible so the surfaces touch; however, they are protected by boundary lubricants, which in the synovial joint are GAGs such as HA and, to a lesser extent, phospholipids. These molecules bind to the surface and prevent direct contact between the surfaces, instead sliding occurs between GAG molecules within the boundary lubricant layers. These GAG layers are replenished by the synovial fluid, ensuring they remain (Walker et al., 1968).

**Figure 1.11 Boundary lubrication.** Schematic representation of boundary lubrication regimes. Articular cartilage lined with a layer of boundary lubricants (blue line). A: Mixed lubrication where the fluid film is discontinuous, and boundary lubrication occurs at asperities which touch. B: Boundary lubrication where all the load is carried on the solid surface which is protected by a layer of boundary lubricants.
1.3.2.3 Biphasic lubrication

The biphasic mechanism of lubrication is possibly the most important in articular cartilage, it describes how initial load is supported by the interstitial water or fluid phase of the cartilage tissue, then load is gradually passed to the solid phase as water content of the tissue depletes. This lubricating mechanism can maintain low friction under extreme loading conditions in the absence of surface lubricant (Forster & Fisher, 1996).

The solid phase of cartilage denotes the collagen matrix and associated proteoglycans. The collagenous network has great tensile stiffness, however, poor compressive stiffness due to its fibrillar morphology, it has negligible charge. The proteoglycan component of the ECM, conversely, has many fixed negatively charged groups, which allow repulsion between molecules and therefore, considerable expansion of the volume which they occupy. However, proteoglycans are entrapped within the collagen network, which prevents them expanding to their full potential, also, the high concentration of free counterions within the interstitial fluid phase results in high Donnan osmotic pressure, slight external mechanic or ionic environment alterations lead to the loss or gain of water (Mow et al., 1984).

The architecture of the collagen matrix and the hydrophilic nature of the proteoglycans results in a micro-porous tissue. With increased compression, the tissue becomes less permeable based on its physiochemical composition. The permeability of the tissue to fluid movement and the equilibrium of swelling force from ionic interactions with collagen network tensile strength are directly affected by the volume of interstitial water (and the hyaluronic acid content). This pressurised interstitial water is capable of supporting the load (Katta et al., 2008; Mow et al., 1984).

Under compression, the forced flow of water from the tissue results in higher organic material concentration, and increased charge density. When charge density, collagen tension and applied load are balanced, a new equilibrium compressive stiffness is reached. Proteoglycan molecules interact with the collagen network to provide the tissue with shear strength.
So, in the solid phase, flow independent behaviours arise, where cross-linking between collagen fibres in the network and also interactions with this network and proteoglycan aggregates provide strength to resist deformation (Mow et al., 1984).

With significant fluid flow (under load) the frictional drag causes flow-dependant viscoelastic effects such as creep and stress relaxation. The collagen network provides resistance to tensile stress while the proteoglycans provide compressive stress resistance; there is also diffuse resistance as fluid flows through the ECM (Mow et al., 1984).

Under compression, fixed charge density increases which leads to reduced permeability. Compaction of the solid matrix under compression results in increased drag forces against the fluid flowing through, therefore a further reduction in permeability. Permeability is also dependent on the viscosity of the fluid (Mow et al., 1984).

The coefficient of friction is lower when load is carried by the fluid phase as compared to the solid phase. The biphasic lubrication mechanism is successful over long periods of load bearing. When movement occurs, the water content of the previously loaded tissue is replenished as the load is transferred to a different contact area of the cartilage surface. Continued movement of the load allows for the fluid phase to continue bearing the load at a lower coefficient of friction than if the solid phase was allowed to bear the load (Forster & Fisher, 1999; Forster & Fisher, 1996).

It has been noted that alterations of the counterion concentrations within the interstitial fluid can have major effects on the interaction of proteoglycans, which would have severe effects on the compressive resistance of the tissue, it has therefore been suggested that this ionic phase may be highly crucial in cartilage mechanics, so a triphasic model has been proposed by Lai et al. (1991).

Above the superficial tangential zone of articular cartilage there is a surface amorphous layer (SAL) comprised of sulphated sugars, glycoproteins and lipids. This layer is suggested to also be biphasic in nature and play a role
in friction reduction during shock loads, to prevent damage to the underlying bulk of cartilage (Katta et al., 2008).

1.4 Osteoarthritis

Osteoarthritis is the most common disorder affecting joints (Herrero-Beaumont et al., 2009). The progressive, degenerative disease causes degradation of articular cartilage which becomes more severe over time, as cartilage is unable to repair itself. More recently OA has been highlighted as a disease of the whole joint, not merely the cartilage and underlying bone (Felson, 2009). OA can result in great pain and morbidity for sufferers and can be debilitating at later stages, when the whole joint fails (Felson, 2009).

1.4.1 Epidemiology

OA has a major impact globally, with an estimated 26.9 million adults aged 25 or over in the USA presenting with the disease in some joint (Lawrence et al., 2008). In the United Kingdom 8.5 million people are estimated to be living with osteoarthritis, and this number is predicted to double by 2030 (OANation2012). There is also a gender difference in OA prevalence, with more cases reported in women than men (Gabriel & Michaud, 2009). Arthritis Research UK report statistics that 1.8 % of males and 3.0 % of females consulting with their general practitioner per year are presenting with OA of any body part. Extrapolated, this would total 435,500 males and 769,000 females presenting with OA each year (www.arthritisresearchuk.org). Although it is possible to detect OA in x-rays by observing joint space narrowing, it is unethical to x-ray patients who do not present with symptoms, so estimates are restricted to those with symptomatic painful OA, and the percentage of the population with unsymptomatic OA is unknown. Along with increasing age and female sex, obesity is a major risk factor associated with OA (Gabriel & Michaud, 2009) so it would appear that with the increasing life expectancy of the
population, and increased incidence of obesity, OA can only become more prevalent unless effective treatments can be found, Felson & Zhang (1998) have proposed a doubling in prevalence from 2000 to 2020.

OA is a major economic burden, the most recent figures report around 58,952 primary hip replacements and 62,150 primary knee replacements were performed in the UK in 2006/2007, at a cost of around £430 million and £460 million respectively (www.arthritisresearchuk.org). In the USA, musculoskeletal procedures (predominantly knee arthroplasty and hip replacements) cost $31.5 billion in 2005. More recently it has been suggested that OA in the USA has a direct cost of $81 billion per year (Lützner et al., 2009). However, it is not just costs from hospital procedures and care, but loss of wages through inability of sufferers to work; estimated as indirect costs of $47 billion in the USA (Lützner et al., 2009). Clearly OA poses a major economic burden to the population as well as causing great morbidity in the many sufferers, therefore reduction in the incidence of OA is imperative (Gabriel & Michaud, 2009).

1.4.2 Pathogenesis

There are classically thought to be two forms of OA, primary and secondary. Secondary OA is caused by well-recognised predisposing conditions such as anatomic abnormalities, trauma, metabolic and inflammatory disorders. Conversely, primary OA has no apparent triggering event and occurs in previously undamaged joints (Herrero-Beaumont et al., 2009). There are many different factors which can lead to the onset of OA, these are outlined in Figure 1.12. Any combination of factors can lead to OA onset, it is likely that systemic factors predispose a person to OA, and the application of local biomechanical factors triggers the process of cartilage disruption (Felson & Zhang, 1998).
Figure 1.12 Osteoarthritis pathogenesis. A schematic representation of the pathogenesis of osteoarthritis detailing the different types of risk factors involved. HRT = hormone replacement therapy. Figure adapted from Felson & Zhang (1998).

Osteoarthritic cartilage is altered from a normally silvery/white, firm tissue to become yellow, pitted, soft, uneven and fissured. This condition progressively spreads across an articular surface. Degradation of articular cartilage can occur through mechanical wear processes; however there can also be a molecular basis to cartilage degeneration (Martel-Pelletier et al., 2008).

The subchondral bone undergoes areas of necrosis and resorption, which appear as cysts. The tissue undergoes sclerosis where new bone is laid down in areas under stress. Marginal osteophytes arise at the joint periphery, composed of a centre of new bone with a surface layer of cartilage containing a mixture of hyaline and fibrocartilage (Altman, 1987). At the clinical stage of OA there is also inflammation of the synovial membrane. Failure results from an inability of chondrocytes to balance this progressive degradation with the synthesis of new matrix. When synovial inflammation occurs the production of inflammatory mediators regulates chondrocyte homeostasis, favouring catabolic activities resulting in matrix loss (Martel-Pelletier et al., 2008).
Cartilage matrix is turned over throughout life and many cellular factors regulate cartilage homeostasis, balancing anabolic and catabolic activities, however disruption of this balance can lead to increased catabolism and therefore degradation of the tissue (Martel-Pelletier et al., 2008).

One of the key anabolic factors in cartilage homeostasis is transforming growth factor β (TGF-β) which is thought to play a proactive role in regulating cell proliferation and ECM synthesis. The growth factor functions to increase proteoglycan synthesis and to inhibit interleukin 1β (IL-1β) mediated proteoglycan synthesis suppression. It also acts to inhibit production of matrix metalloproteinase 9 (MMP-9) and MMP-1, molecules which are involved in ECM destruction. TGF-β is also able to induce MMP-13 and the aggrecanase ADAMTS-4 (a disintegrin and a metalloprotease with thrombospondin motifs-4) allowing macromolecular turnover in normal cartilage. (Martel-Pelletier et al., 2008). TGF-β is usually present at low levels in healthy joints, but levels are increased in OA cartilage. Other anabolic factors include bone morphogenic proteins (BMPs), cartilage-derived morphogenic proteins (CDMPs), insulin-like growth factors (IGFs) connective tissue growth factor (CTGF), hepatocyte growth factor (HGF) and fibroblast growth factor (FGF). These factors, combined, work to stimulate matrix deposition, cell proliferation and inhibit catabolic activity (Martel-Pelletier et al., 2008).

Of greater interest are the catabolic factors, many of which appear to have increased production or function in OA cartilage, which may act as catalysts or amplifiers of OA development. These include proteases, in particular matrix metalloproteinases (MMPs). MMPs are classified into groups: collagenases (MMP-1, -8 and -13 which degrade specific collagen molecules), stromelysins (MMP-3 and -11 which degrade proteoglycans, fibronectin, elastin and laminins), gelatinases (MMP-9 and -2 which degrade collagen types IV and V along with denatured collagen and gelatine), and adamalysin (ADAMTS-5 which has aggrecanase activity). These MMPs have all been shown to be uniquely expressed or upregulated in OA cartilage. Other proteases involved in OA development include certain serine and thiol proteases (Martel-Pelletier et al., 2008).
Inflammatory factors also play a key role in the catabolism of cartilage, the proinflammatory cytokine IL-1β is thought to be the most important factor responsible for the catabolic processes in OA. It forms a positive amplification loop in chondrocytes leading to increased production of itself, proteases, cytokines and other inflammatory factors. It is also able to inhibit antagonists of such enzymes and inhibit synthesis of ECM constituents (Daheshia & Yao, 2008). Other proinflammatory factors involved in OA development include tumour necrosis factor alpha (TNF-α) (Goldring, 1999), oncostatin M (OSM) (Hui et al., 2003), leukaemia inhibitory factor (LIF) (Lotz et al., 1992), IL-17, IL-18 (Martel-Pelletier et al., 2008), nitric oxide (NO) (Vuolteenaho et al., 2007), eicostanoids (prostaglandins and leukotrienes) and also protease-activated receptors (PARs) (Martel-Pelletier et al., 2008).

There are a number of factors other than proteases and inflammatory molecules which have catabolic activity in cartilage which are upregulated in OA including fibronectin fragments (Homandberg et al., 1998), leptin (Teichtahl et al., 2005) and neuropeptides (Martel-Pelletier et al., 2008).

OA was historically thought to be a disease of the cartilage, but more recent evidence indicates that it is a disease of the whole joint. The initiating lesion resulting in osteoarthritis may arise in many different tissues of the joint. McGonagle et al. (2010) discuss a site specific pathogenic classification of OA in which the disease can arise from cartilage, ligaments, meniscus, synovium, bone or a combination of many of these tissues (Halstead et al., 2010). They suggest that injury or disregulation of these tissues could progress to whole joint effects resulting in OA, and with improved magnetic resonance imaging (MRI) technology, these initial tissue lesions can be detected earlier, before clinical onset of OA.

### 1.4.3 Articular cartilage defects

Many cases of OA stem from an initially minor cartilage defect; these can be caused by acute injury to the joint, or from osteochondral pathology, such as osteonecrosis or osteochondritis dissecans. Due to the poor ability
of cartilage to heal, these small defects progressively deteriorate under regular joint loading and articulation, and advance to clinical OA (Chaing & Jiang, 2009). Not only do these lesions pose a risk of deterioration to OA, but are troublesome in themselves, often causing pain, swelling and mechanical symptoms such as locking of the joint, reducing the quality of life for patients (Minas & Nehrer, 1997). An estimated 10,000 patients in the UK each year suffer knee cartilage damage warranting repair (NICE TA89, 2008).

To prevent onset of OA and reduce symptoms, it is possible to repair these defects. There are various approaches, although these depend on the severity and position of the defect (Schindler, 2011). Cartilage defects can be graded by severity, highlighting cartilage properties, as well as the size, shape and depth of lesions. There are many systems for categorising cartilage defects; two different approaches were developed by Outerbridge (1961) and Bauer & Jackson (1988). The latter is useful in a more descriptive sense, allowing for association with recent trauma (grades I-IV) or lesions undergoing a degenerative process (grades V & VI), as it defines fracture pattern. However, the Outerbridge system is most useful to delineate a symptomatic focal area suitable for repair as it is concerned with depth of the lesion (Minas & Nehrer, 1997). More recently, the ICRS grading system has been widely used (Figure 1.13).
Figure 1.13 ICRS cartilage grading system. Normal (grade 0): healthy cartilage, grade 1: the cartilage has a soft spot or blisters, grade 2: minor tears visible in the cartilage, grade 3: lesions have deep crevices (more than 50% of cartilage layer), grade 4: the cartilage tear exposes the underlying (subchronal) bone.

It has been suggested that cartilage defects can result in increased cartilage breakdown, leading to a decrease in cartilage volume resulting in decreased joint space, and breakdown of collagen type II fibres (Ding et al., 2005). Due to the link between cartilage defects and development of early OA, it is proposed that repair of these defects can prevent further cartilage degradation and progression to OA.
1.5 Current approaches to defect repair

Current treatments aim to restore the integrity of the joint surface and allow a full range of motion with the absence of pain. These treatments include; lavage and debridement, marrow stimulation techniques, perichondral/periosteal grafts, mosaicplasty and autologous chondrocyte implantation (Minas & Nehrer, 1997). When determining which of these procedures to use, it is important to take into account the location and severity of cartilage damage, the patient’s characteristics and risk factors (Figure 1.14; Lützner et al., 2009; Schindler, 2011). Many of these techniques can be performed in the knee, but are inappropriate in the hip due to difficulties in accessing the joint, although arthroscopy is becoming more advanced, usually, end stage total joint replacement is the only option for hip arthritis.

**Figure 1.14 Treatments for focal cartilage defects in the knee.** The selection of a cartilage repair procedure based on location, lesion size and whether it is a first or second line treatment. * = treatment for young/high demand patient, ** = treatment with no medium-long term clinical results available to date. Figure from Schindler (2011).
1.5.1 Lavage & debridement

The aim of lavage is to remove loose articular debris and inflammatory mediators from the joint space through arthroscopy, therefore reducing inflammatory synovitis and pain and improving function (Minas & Nehrer, 1997, Lützner et al., 2009). Debridement is often performed alongside lavage to provide advanced benefit, with the intention of improving mechanical conditions by removing loose bodies, hypertrophied synovium and, in the knee, torn meniscal fragments, and often removing flaps and shaving fibrillated cartilage (Lützner et al., 2009).

Following a randomised six month controlled trial, lavage and tidal irrigation of the knee were found have an equal effect on pain relief to corticosteroid injection into the joint space. The effect was more prolonged with lavage and tidal irrigation, lasting up to 6 months in 64 % patients compared to only 29 % of patients receiving corticosteroid injection (Arden et al., 2008). Hubbard (1996) compared the effects of lavage alone to the use of combined debridement and lavage, and found more effective pain relief and sustained results when both techniques were used (Hubbard, 1996). Beneficially, lavage and debridement are cheap procedures which are relatively simple to perform.

However, many studies have suggested that the pain relief from debridement is no greater than that from lavage alone (Chang et al., 1993) and no greater than can be achieved with a placebo (Moseley et al., 2002). Kirkley et al. (2008) found no significant benefit of arthroscopic surgery over optimised physical and medical therapy alone. This view however is still controversial, and the treatment may be beneficial in a small number of patients with specific lesion types. Furthermore, the application of this treatment does not replace lost tissue and has not been shown to defer progression into OA (Lützner et al., 2009).

1.5.2 Marrow stimulation techniques

The aim of bone marrow stimulation is to cause bleeding into the joint space from the subchondral bone resulting in fibrin clot formation in the
defect. This is followed by migration of undifferentiated mesenchymal stem cells to the lesion where they form fibrocartilagenous tissue to fill the full thickness cartilage defect (Minas & Nehrer, 1997; Lützner et al., 2009, Schindler, 2011). Techniques used to penetrate the subchondral bone are drilling, microfracturing and abrasion arthroplasty. Of these microfracturing is most commonly used as it is easiest to perform (Figure 1.15). Firstly debridement is used to remove unstable cartilage from the rim of the lesion, and then holes are made 2-4 mm deep and 3-4 mm apart in the centre of the defect. Rehabilitation involves passive motion and partial weight bearing for 6-8 weeks (Minas & Nehrer, 1997; Lützner et al., 2009).

Figure 1.15 Microfracture procedure. Firstly debridement is used to define the edge of the defect, holes are made in the bone to stimulate bleeding, a fibrin clot forms in the defect which overtime forms repair fibrocartilage. Figure from Schindler (2011).

Steadman et al. (2003) found that microfracture marrow stimulation greatly reduced pain and improved knee function for a minimum of 7 years in patients under the age of 45 (Steadman et al., 2003). Bae et al. (2013)
reported that 67.9 % of patients who underwent microfracture surgery did not require further treatment for cartilage pathologies at 10 year follow up. However, this repair technique produces fibrocartilage which has inferior biomechanical properties and durability compared to the original hyaline cartilage (Steinwachs et al., 2008; Lützner et al., 2009). It is unlikely, therefore, to be a curative measure or prevent disease progression (Lützner et al., 2009).

Autologous matrix induced chondrogenesis (AMCI® Geistlich Pharma) has been developed to produce more consistent results from microfracture procedures. A cell free collagen matrix is secured into the defect to provide an environment for mesenchymal stem cells and aid chondrogenesis. The success of AMIC® has been reported for the repair of osteochondral lesions of the knee in a two year follow up study (Gille et al., 2013). Furthermore, the technique (alongside the use of autologous bone grafting) has been reported to be successful at 24 month follow up for the repair of osteochondral lesions of the ankle joint (Valderrabano et al., 2013). The long term clinical benefits of this procedure are currently unknown.

1.5.3 Perichondrial/periosteal grafting

The key problem with marrow stimulation techniques is that they produce an inferior fibrocartilaginous repair tissue. It has been shown that autogenous rib perichondrium, when grafted onto a cartilage defect which had undergone debridement, produced a repair tissue which resembled hyaline cartilage. Autogenous periosteal grafts have also been performed and produce hyaline-like repair cartilage (O’Driscoll et al., 1988). Therefore perichondrial/periosteal grafting is possibly a superior alternative to microfracture (Minas & Nehrer, 1997). A study conducted by Homminga et al. (1990) in 25 patients under the age of 45 with 30 chondral lesions used periostium grafts from the rib and anchored them to cartilage lesions of the knee using fibrin glue. They found a mean increase in knee function when questioning patients on the Ranawat knee scoring system, and 14 people
maintained that score after two years. In 28 cases, the defect was completely filled with hyaline cartilage (Homminga et al., 1990).

Bouwmeester et al. (2002) performed a retrospective analysis of two studies comparing patients receiving perichondrial grafting and those receiving subchondral drilling. The success rate of perichondrial grafting was 78% (three grafts failed) compared to a surprisingly high 100% success rate of subchondral drilling. No long term advantage of having a hyaline-like repair tissue compared to fibrocartilage was found within the 10 year scope of the study. Grafting has to be performed via an ‘open’ procedure, whereas microfracture techniques are performed arthroscopically, which is preferable. Furthermore there was a degree of calcification of the grafts (Bouwmeester et al., 2002). Perichondral grafting is now more commonly performed as part of autologous chondrocyte implantation procedures (Section 1.5.5; Chaing & Jiang, 2009).

1.5.4 Osteochondral autografts/allografts (mosaicplasty)

During autologous osteochondral transplantation (Figure 1.16), pins of cartilage attached to subchondral bone, which contain metabolically active chondrocytes, are taken from non-load-bearing areas of the affected joint and like periosteal/chondrial grafting are fixed to the site of chondral defects. Osteochondral grafts are thought to have greater stability along with the benefit of producing hyaline cartilage repair tissue (Minas & Nehrer, 1997). Koulalis et al. (2004) performed osteochondral autografts in knee joints of 18 patients with a follow-up time of 27 months. All showed defect coverage 12 months postoperatively and all cases had osseous integration, although there was some indication of donor site morbidity in 11 patients which decreased over time. This is a good approach to defect repair; however the factor of donor site morbidity is a disadvantage (Koulalis et al., 2004).

Solheim et al. (2010 and 2013) performed mosaicplasty surgeries on 69 patients (mean age 33) and recorded Lysholm score and visual analogue scale (VAS; 0=no pain; 100=worst possible pain) of pain. At 12 month
follow up the mean Lysholm score and VAS of pain improved from 48 and 62, respectively, at the time of surgery to 81 and 24, respectively. However, at mid-term (5-9 years) follow up pain levels were observed to have increased and mean scores of 68 and 32 were reported, respectively (Solheim et al., 2010). At long-term (10-14 years) follow up, mean pain scores were still improved from baseline, at 72 and 27 respectively. However an unsatisfactory Lysholm score of < 64 was reported in 40 % of patients. Although, the outcome was found to be improved in a specific demographic of patients (males under 40 years old) with small (< 3 cm²) initial defects (Solnheim et al., 2013). It is therefore necessary to accurately select recipients for mosaicplasty to improve long term clinical success.

Figure 1.16 Mosaicplasty procedure. The use of autologous osteochondral pins taken from non-load-bearing joint surfaces to repair lesions in load bearing regions. Figure from www.kneejointurgery.com.

To overcome the issues with donor site morbidity, osteochondral allografts, which still contain metabolically active chondrocytes, have been used. As they are allogeneic, rather than taken from donor sites, grafts of a much larger size are able to be produced. The grafts are anueral and avascular and so were thought to be immuneprivileged, making them ideal for
allogenic transplantation, however, immune reactions can be evoked from the immersing synovial fluid (Chaing & Jiang, 2009). There are concerns about possible transmission of infection for allograft recipients, so further steps are required to sterilise grafts (LaPrade et al., 2009). Storage and preparation of allografts, if not used fresh, is troublesome, as chondrocytes die during the process (Chaing & Jiang, 2009).

1.5.5 Autologous chondrocyte implantation (ACI)

ACI is a technique developed in the late 1900s whereby articular cartilage is harvested from a patient and the chondrocytes from which are expanded in vitro. These expanded, autologous chondrocytes are then reimplanted into chondral defects of the same patient, beneath a perichondrial patch (Chaing & Jiang, 2009). Grande (1989) initially performed this procedure on rabbits and found improved defect healing and decreased synovitis in joints in which chondrocytes had been implanted compared to controls where just the perichondrial flap had been attached. The repair cartilage was hyaline-like, and cell labelling revealed that the implanted cells were present in and responsible for production of the repair cartilage. A follow-up study by Minas (2001) looked at the capacity of ACI to repair cartilage defects in the human knee. In a sample of 169 patients a 87% success rate was found. Moradi et al. (2012) found a substantial improvement in all clinical outcome parameters at up to 14 years post-operatively, with complete defect filling in 52 % of patients as observed by MRI.

Despite having a relatively high success rate and producing a mechanically and biologically superior hyaline-like repair tissue with a non-immunogenic graft, there are disadvantages to this procedure. The treatment involves an open technique, as opposed to arthroscopic which results in a more prolonged recovery period for patients (Minas, 2001). Also, the procedure is technically demanding, with multiple stages, so differences in surgical skill may well correlate to altered outcomes of the treatment (Grande et al., 1989).
A variation of ACI, matrix assisted chondrocyte Implantation (MACI®; Genzyme, Cambridge, MA), has been in clinical use for a number of years. Similarly to AMCI®, this procedure uses a collagen matrix onto which the autologous chondrocytes are cultured prior to implantation into the defect. Beneficially, use of MACI enables the procedure to be performed arthroscopically, as harvest of periosteum is not required (Getgood et al., 2009). Good clinical mid-term results have been reported with this approach (Kon et al., 2009).

1.6 Current tissue engineering approaches

As current surgical interventions for osteochondral defect repair have shown variable outcomes, there is need for the development of a biologically and biomechanically superior repair biomaterial which will avoid the use of autologous tissue and circumvent donor site morbidity. Tissue engineered approaches are currently undergoing research and development to fulfil this clinical niche.

There are three key components in tissue engineering, cells which characterise the tissue type, biomaterial scaffolds which provide a habitat for the cells and chemical, mechanical or biological environmental factors which determine cell behaviour (Chaing & Jiang, 2009).

Cells can be used in regenerative medicine associated with a scaffold, or injected directly, without a scaffold. Often these cells are extracted from donor tissue and either expanded or implanted directly into the host. These cells can be allogeneic (from another human) but most preferably autologous, from the individual themselves, as these will be immunocompatible. Enough cells may not be available from a tissue biopsy, or cells extracted may not be easily expanded, in these circumstances, stem cells are an alternative source. Stem cells can be embryonic, foetal-related tissue (amniotic fluid or placenta) or adult stem cells derived from bone marrow, skin etc. (Atala, 2007). The growth of research in the field of stem cell biology has opened up new possible cell
sources including pluripotent stem cell lines created by somatic cell nuclear transfer (Wakayama et al., 2001), and induced pluripotent stem cells (Takahashi et al., 2007), which may be of use in the future. Often scaffolds can be implanted without cells, to allow native cell in growth in vivo (Atala, 2007).

Biomaterials used to form a scaffold need to be tailored to the tissue being engineered, to mimic the biological and mechanical characteristics of the natural ECM. The scaffold should ideally be biodegradable and bioresorbable. These materials can be natural decellularised ECM, naturally derived materials formed in vitro or synthetic polymers. Beneficially, natural materials can be recognised biologically and thus avoid rejection, however polymers have the ability to be reproduced on a large scale, and the properties of strength, degradation and the microstructure can be easily controlled (Atala, 2007).

Cartilage is a complex tissue and as such there have been a number of different approaches taken to engineer the tissue for use in articular cartilage defect repair. These approaches include synthetic polymer and natural biomaterial scaffolds.

1.6.1 Synthetic scaffolds

Synthetic polymers are used in research to engineer many different tissue types as they are easy to manufacture and their specific properties can be accurately controlled to mimic the natural ECM. However, the biomechanical properties of such materials are inferior to natural cartilage (Atala, 2007). Some examples of polymers which have been used as cartilage scaffolds are shown in Table 1.3. A single material can be used, but more commonly they are used in combination to provide better physical characteristics.

Problems encountered with synthetic polymers include low GAG content of repair tissue and inconsistent subchondral bone reformation (Chu et al., 1996). van Susante et al. (1998) found that the repair cartilage formed with hydroxyapatite scaffolds was inferior fibrocartilage. Scaffolds are designed
to biodegrade, so if cell seeding, retention or metabolism is not sufficient, the ECM will not be replicated by resident cells before the scaffold degrades and the implant will fail. The use of copolymers allows control of degradation rate (Frenkel & DiCesare, 2004). Other problems have been found with integration of successful polymer hydrogels with the existing cartilage, composites of polyvinyl alcohol (PVA) gels with nanohydroxyapatite allowed for better integration with host tissues (Pan & Xiong, 2009).

Recently, nanofabrication technology has been used to produce an anisotropic cartilage repair biomaterial. The electrospinning of poly (ε-caprolactone) (PCL) fibres enabled tangential surface alignment and random orientation in the rest of the material, increased fibre diameter was included in the base of the material to mimic natural cartilage zonal microstructure with promising in vitro results (McCullen et al., 2012).

Some synthetic scaffolds have begun to undergo testing in clinical trials such as Trufit® plug (Smith&Nephew, Andover, USA) and BioMatrix® Cartilage Repair Device (Arthrex, Karlsfeld Germany).

<table>
<thead>
<tr>
<th>Synthetic biomaterial</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polylactic acid (PLA)</td>
<td>Chu et al., 1996 Giurea et al., 2003</td>
</tr>
<tr>
<td>Polyglycolic acid (PGA)</td>
<td>Liu et al., 2002 Schaefer et al., 2002</td>
</tr>
<tr>
<td>PLA-PGA copolymer</td>
<td>Cohen et al., 2003</td>
</tr>
<tr>
<td>Poly(ethylene glycol) diacrylate (PEGDA)</td>
<td>Kim et al., 2003</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>van Susante et al., 1998 Pan &amp; Xiong, 2009</td>
</tr>
<tr>
<td>Polycaprolactone (PCL)</td>
<td>McCullen et al., 2013</td>
</tr>
<tr>
<td>Polyvinyl alcohol (PVA)</td>
<td>Pan &amp; Xiong, 2009</td>
</tr>
</tbody>
</table>

**Table 1.3 Synthetic biomaterials.** Synthetic biomaterials used as scaffolds in cartilage tissue engineering for repair of osteochondral defects.
1.6.2 Natural scaffolds

Natural biomaterials have the advantage of biological recognition, however, they are more difficult to use than synthetic polymers, resulting in scaffolds which are less reproducible (Atala, 2007). Collagen for example, has great advantages as a biomaterial for cartilage production, as it is the main ECM component of the natural tissue, and has specific RGD sequences which allow cell adhesion and phenotype retention (Atala, 2007). Fibrin is also a logical candidate, as the clots which form during the natural cartilage repair process are fibrin based (Frenkel & DiCesare, 2004). Some of the natural biomaterials which have been used in the production of cartilage scaffolds are shown in Table 1.4. As with synthetic polymers, these materials can be used in isolation, or in combination with a number of other materials to produce the optimum properties to function as a scaffold.

<table>
<thead>
<tr>
<th>Natural biomaterial</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>Wakitani et al., 1994</td>
</tr>
<tr>
<td></td>
<td>Frenkel et al., 1997</td>
</tr>
<tr>
<td>Collagen-GAG-calcium phosphate</td>
<td>Getgood et al., 2012</td>
</tr>
<tr>
<td>Fibrin</td>
<td>Hendrickson et al., 1994</td>
</tr>
<tr>
<td></td>
<td>Kreuz et al., 2009</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Chang et al., 2004</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>Butnariu-Ephrat et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Grigolo et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Dausse et al., 2003</td>
</tr>
<tr>
<td>Agarose and alginate</td>
<td>Marijnissen et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Dausse et al., 2003</td>
</tr>
<tr>
<td>Bacterial cellulose</td>
<td>Müller et al., 2006</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Hoemann et al., 2005</td>
</tr>
<tr>
<td>Gellan Gum</td>
<td>Oliveira et al., 2010</td>
</tr>
</tbody>
</table>

**Table 1.4 Natural biomaterials.** Natural biomaterials which have been used as scaffolds for cartilage tissue engineering.

Good results have been seen in terms of hyaline-like cartilage repair tissue formation in animal studies, however as with synthetic materials, there were problems with scaffold integration into the existing tissue, and also
with the thickness of the resulting tissue, being thinner than the surrounding cartilage. In many cases repair tissues were biologically and mechanically inferior to natural cartilage. It is however, suggested that despite the promising functionality of some biological scaffold materials, there are still concerns with batch variation, and the potential for pathogen transfer, especially bovine spongiform encephalopathy from prions within bovine collagen sources (Frenkel & DiCesare, 2004).

1.6.3 Natural acellular scaffolds

As discussed previously, the architecture of the cartilage ECM is essential for its load bearing and lubrication functions. Therefore, using a simplistic homogenous scaffold may not be sufficient to recreate the biological and mechanical properties of natural cartilage (Chaing & Jiang, 2009). An approach taken in the engineering of other tissues has been to use natural acellular ECM scaffolds. These collagen rich matrices are produced by chemical, enzymatic and mechanical manipulation of natural tissues resulting in the removal of resident cells. These scaffolds slowly degrade once implanted and ingrowing or transplanted cells secrete ECM proteins to slowly replace and remodel the scaffold (Atala, 2007). Acellular scaffolds also have the benefit of being biocompatible, biodegradable and bioresorbable as they are natural materials. Additionally, acellular tissue matrices can be bioactive, retaining cell attachment motifs, growth factors and other biological cues (Hofmann & Garcia-Fuentes, 2011).

Work is currently underway in developing acellular porcine bladder for use in tissue engineering (Bolland et al., 2007), along with meniscus (Stapleton et al., 2008), other acellular matrices have been developed for tendon (Lee, 2008), heart valves (Korossis et al., 2002), blood vessels (Cronklin et al., 2002), skin (Chen et al., 2004), nerves (Kim et al., 2004) and small intestinal submucosa (Badylak et al., 1999) amongst others.

Decellularised small intestinal submucosa (SIS) has been used clinically for over a decade to repair tissues such as skin, muscle and tendon (eg. Restore™, DePuy Orthopaedics; Surgisis®, Cook Biotech Inc). Although
the application of decellularised SIS for osteochondral repair has not been reported.

1.6.3.1 Immunogenicity of tissue transplants

If allogeneic or xenogeneic tissue engineered biomaterials are to be of use clinically, it is necessary to understand the immunological response to such materials upon implantation, to avoid the rejection of tissue engineered implants.

1.6.3.1.1 Immunological response to allografts

The extracellular matrix components of biological tissues such as collagens and proteoglycans are conserved between individuals and across species, so are not recognised as foreign upon transplantation. However, human leukocyte-associated (HLA) proteins are present on the cell membrane of every nucleated cell and are encoded for by highly polymorphic set of major histocompatibility complex (MHC) genes. Each individual has six sets of MHC I and six sets of MHC II genes, three from each parent, which are expressed co-dominantly. The histocompatibility proteins expressed are therefore unique to each individual (excluding monozygotic twins, who share the same genetic material). It is the presence of these histocompatibility proteins on the surface of cells which enables identification and distinction between “self” and “non-self”, and therefore poses a barrier in transplantation of allogeneic and xenogeneic tissues.

The role of the MHC molecules is to present foreign antigens, such as epitopes from cytoplasmic or internalised pathogens. The presentation of a foreign antigen elicits a cell mediated immune response, however, as the MHC molecules are specific to each individual, allogeneic “non-self” MHC molecules will also be recognised as foreign, without presenting an antigen. Initial acute rejection of transplants can occur through direct recognition of allogeneic MHC Class II proteins presented on the surface of antigen presenting cells (APC’s) by CD4+ Helper T- cells (Figure 1.17). The binding of T-cell receptor to allogeneic MHC Class II, followed by binding of co-stimulatory cell surface molecules results in the formation of an immunological synapse which activates the CD4+ T-cell. Once
activated, the CD4+ T-cell produces interleukin-2 (IL-2) which is able to stimulate CD8+ T-cells. CD8+ T-cells have a receptor which binds MHC Class I on all nucleated cells. Once CD4+ and CD8+ T-cells are activated they undergo clonal expansion, to rapidly produce many more T-cells reactive against the specific MHC molecule shape recognised as “non-self”. Clonal expansion produces both memory and effector cells. Effector CD4+ T-cells release cytokines which stimulate APC’s to kill foreign cells, for example Interferon-γ (IFN-γ) release stimulates macrophages to destroy foreign cells via the production of oxygen radicals and enzymes. Effector CD8+ T-cells are themselves able to kill the cell presenting allogeneic MHC Class I which they have bound. Destruction of transplanted cells results in the tissue becoming necrotic and dying. Memory T-cells are long lived cells which reside in the body to rapidly expand if the same foreign MHC molecules are presented again in the future, resulting in a much more rapid response (Janeway, et al., 2005, Knight & Ingham, 2006).

**Figure 1.17 Direct antigen recognition.** Helper T-cells recognise allogeneic MHC Class II on allogeneic antigen APC and release IL-2 to stimulate cytotoxic T-cells which then recognise allogeneic MHC Class I. Th cell = CD4+ Helper T-cell, Tc = CD8+ Cytotoxic T-cell. Figure adapted from Knight and Ingham (2006).
In addition to the mechanism of recognising allogeneic MHC molecules described above, MHC molecules shed by cells of the transplant tissue can be picked up by antigen presenting cells, and epitopes derived from the MHC molecule presented, leading to a cell mediated immune response. Cytotoxic CD8+ T-cells can then respond to MHC Class I molecules presented on all allogeneic cells (Figure 1.18; Janeway, et al., 2005, Knight & Ingham, 2006).

![Diagram of indirect antigen recognition](image)

**Figure 1.18 Indirect antigen recognition.** MHC fragments are shed by allogeneic cells, these are then taken up and epitopes presented on MHC Class II by APCs. Helper T-cells recognise allogeneic MHC epitopes release IL-2 to stimulate cytotoxic T-cells which then recognise allogeneic MHC Class I. Th cell = CD4+ Helper T-cell, Tc = CD8+ Cytotoxic T-cell. Figure adapted from Knight and Ingham (2006).

1.6.3.1.2 Immunological response to xenografts

Although xenogeneic cells will elicit immune rejection via the response described for allogeneic cells, they primarily induce hyper-acute rejection. Cells of xenogenic tissues possess a cell surface sugar, galactose α1-3
galactose (α-gal), which is present in all mammals except for humans and old world monkeys (Galili et al., 1987). Humans have a high titre of antibodies against a sugar epitope present on bacteria in the human gut which is similar in shape to α-gal. Upon transplantation of xenogeneic cells expressing α-gal, antibodies rapidly bind to the epitope on endothelial cells of blood vessels, activating the complement cascade leading to inflammation and blood clotting, the vessels become blocked which results in hypoxia and death of the transplant tissue (Janeway, et al., 2005, Knight & Ingham, 2006).

By removing whole cells, membranes and cytoplasmic proteins from allogeneic or xenogeneic tissues, only the nonimmunogenic ECM proteins will remain. The tissue will retain its biological/biochemical structure and function yet will not elicit an immune response upon implantation.

1.6.3.1.3 Macrophage response to decellularised tissues

Macrophages are large mononuclear cells of the immune system which have been reported to be “classically activated” or “alternatively activated”. More recently macrophage phenotype has been shown to be a continuum between the two forms (Mills et al., 2000). Classically activated or M1 macrophages are aligned with the Th1 CD4+ T-cell response. The secretion of IFN-γ by T-cells or the presence of TNFα or bacterial lipopolysaccharide (LPS) activate macrophages with enhanced microbicidal capacity and increased proinflammatory cytokine secretion. Alternatively activated, M2 macrophages are aligned with the Th2 CD4+ T-cell response. The secretion of IL-4 and IL-13 by T-cells activate macrophages with a role in the immune response to parasites, but also in allergy, wound healing, and tissue remodelling (Cassetta et al., 2011).

Brown et al. (2009) identified a change in macrophage polarisation in response to transplanted tissues with or without a cellular component. In vivo, cellular xenografts were infiltrated by M1 type macrophages which resulted in a proinflammatory response and scar tissue formation, whereas acellular xenografts and allografts were infiltrated with M2 polarised macrophages which resulted in constructive tissue remodelling.
Furthermore, Kean et al. (2012) have reported an association between decellularisation efficacy and macrophage polarisation, with ineffective removal of cell debris (despite histological confirmation of whole cell removal) resulting in a higher M1:M2 ratio.

It is therefore, not only important to ensure the full removal of cells to avoid eliciting an immune response and graft rejection, but it is necessary to ensure effective removal of cellular debris, to tailor the tissue remodelling response from macrophages.

1.6.3.2 Natural acellular (osteo)chondral scaffolds

With the success of natural acellular scaffold materials for the repair of other tissues, it is possible that the most optimal treatment for articular cartilage repair is to use decellularised xenogeneic or allogeneic (osteo)chondral tissues. These constructs would ideally be structurally, biologically and mechanically relevant to natural articular cartilage. Acellular natural materials would also be biocompatible, biodegradable and bioresorbable. Removal of the existing allogeneic/xenogeneic cells would allow the construct to be immunocompatible. A limited number of approaches for natural cartilage tissue decellularisation are currently undergoing research and development (Benders et al., 2013).

1.6.3.2.1 Decellularisation of homogenised cartilage matrix

Yang et al. (2008) developed a method whereby human cadaveric cartilage was physically shattered in PBS containing protease inhibitors and then centrifuged to produce microfilaments. Treatment with triton X-100 (1 % v/v in hypotonic tris-HCl) and hypotonic solution, nucleases and extensive phosphate buffered saline (PBS) washes resulted in decellularised cartilage microfilaments.

Yang et al. (2010) developed a method whereby bovine cartilage was lyophilised and ground to a powder prior to decellularisation with trypsin, nucleases, hypotonic buffer and triton X-100 (1 % v/v in PBS) in the presence of protease inhibitors and antimicrobial agents, again followed by extensive PBS washes.
In both instances, the acellular cartilage matrix was then freeze dried into moulds and cross linked to produce a biomaterial. Scaffolds produced from decellularised homogenised cartilage have the benefit of retaining bioactive cues for cell growth and are also more customisable; however, they lack the 3D architecture and structure of native cartilage and therefore have reduced biomechanical function.

1.6.3.2.2 Decellularisation of cell derived matrix

Elder et al. (2009) cultured week old calf chondrocytes in agarose to produce a 3D disc shaped cell-derived cartilage ECM. They then investigated the efficacy of different detergents to remove the cells from the construct. Discs were treated with a PBS decellularisation solution containing nucleases, protease inhibitors and antibiotics and either sodium dodecyl sulphate (SDS; 1% and 2% w/v), tributyl phosphate (TnBP; 2% w/v) or triton X-100 2% (v/v) then washed in PBS. Although decellularised cell-derived-ECM scaffolds have no issues regarding biocompatibility, the need to culture cells renders the process time consuming and this approach requires cell harvesting from the patient, increasing the number of invasive procedures performed and potentially causing donor site morbidity. The 3D structure and composition of the cell derived matrix is currently not comparable to natural cartilage tissues.

1.6.3.2.3 Decellularisation of native cartilage

Elder et al. (2010) developed a decellularisation protocol (based on earlier work; Elder et al., 2009) for 4 week old calf distal femur articular cartilage with the intention of producing a facet joint cartilage repair biomaterial. The short protocol included one freeze/thaw cycle followed by the use of 2% SDS (w/v) in PBS with deoxyribonuclease (DNase), ribonuclease (RNase), ethylenediaminetetraacetic acid (EDTA) and penicillin/ streptomycin/ fungizone, then a final wash step in PBS. Different durations of SDS incubation were investigated. Incubation for 2 hours had no detrimental effects on cartilage GAG content or biomechanics, however no significant reduction in DNA content was seen and whole cell nuclei were still present, although the number was reduced. Incubation of cartilage for 8 hours in
SDS enabled the removal of all whole cell nuclei, however the DNA content was still 3861 ng.mg\(^{-1}\), only a 40 % reduction from fresh. In addition, increased duration of SDS wash incubations led to loss of GAGs and a reduction in biomechanical properties. With > 50 ng.mg\(^{-1}\) DNA remaining, the treated cartilage did not meet the criteria set by Crapo et al. (2011) to be defined as a decellularised matrix. The poor DNA reduction with this protocol was likely due to the initial high cellularity seen in the immature source tissue and also potential inhibition of nucleases through inappropriate application conditions. Nucleases require the presence of magnesium to perform nucleotide cleavage and would be denatured by SDS within the buffer.

Schwarz et al. (2012) aimed to develop a porous collagen matrix from natural cartilage tissues, including meniscus and nasal septum cartilage from porcine and human source tissue. As such they did not make an attempt to retain the natural proteoglycan structure and biomechanical properties of the tissues. Cartilages were successfully decellularised using a protocol involving osmotic cell lysis with distilled water, following which sodium hydroxide (> pH 12) was used to inactivate pathogens, denature DNA/RNA and remove cellular debris. Samples were defatted in 70 % (v/v) ethanol and GAGs and other non-collagenous proteins were denatured and removed using a guanidine hydrochloride (1 M) solution containing sodium acetate (0.05N). Cartilages were finally sterilised using hydrogen peroxide (5% v/v). This produced a highly porous collagen scaffold, devoid of GAGs, which would encourage rapid cell infiltration. This protocol was not intended for articular cartilage decellularisation, and the produced matrix would not provide an acceptable repair material.

The decellularisation of intact native cartilage tissues has the potential to produce an ECM with bioactive cues, the natural biological, biochemical and biomechanical composition and structure. However, the clinical relevance/success of such products may be limited, as such approaches do not provide a subchondral bone component for integration and stability of a cartilage repair biomaterial upon implantation.
1.6.3.2.4 Decellularisation of osteochondral tissues

Here it is proposed that the decellularisation of osteochondral xenogeneic tissues may provide the best osteochondral lesion repair graft material. The addition of a bony component to the overlying cartilage layer may improve integration of the graft to the natural joint to avoid problems associated with graft loosening. Using a xenogeneic donor allows for a larger range of graft sizes, as many treatments such as mosaicplasty are limited by available graft sizes and donor site morbidity. Another benefit of the proposed biomaterial would be the ‘off the shelf’ availability, with no reliance on donor human tissue, allowing greater ease of use and convenience during surgery. Acellular osteochondral pins could easily be adopted into current surgical procedure for mosaicplasty.

Preliminary work has been performed to determine the capacity for decellularising natural xenogeneic tissues to use as an osteochondral graft. Kheir et al. (2011) investigated the use of a decellularisation protocol developed by Booth et al (2002) to develop an acellular porcine scaffold. Osteochondral tissues were subject to four cycles of freeze thaw (the final two in hypotonic solution) and then incubated through 6 cycles of 24 h in hypotonic solution followed by 24 h in 0.1 % (w/v) SDS in hypotonic solution, both containing aprotinin and EDTA at 45 °C with agitation. Blocks were than treated with nuclease and sterilised using 0.1 % Peracetic acid (PAA; v/v). The process was completed by 2 x 30 min PBS washes and a short 24 h final PBS wash. This technique enabled complete decellularisation with no detectable remnants of cellular debris or the α-gal epitope, but still with retention of original collagen content. However there were severely depleted levels of GAGs within the tissue and the collagen fibres seemed to be disrupted, appearing more loosely packed. The decellularisation process had the effect of massively decreasing the compressive stiffness of the tissue in mechanical studies, with the resulting matrix being inappropriate as a graft material.

This previous work has outlined the possibility of using acellular xenogenic scaffolds, however it highlighted various issues which need to be addressed. The loss of GAGs and collagen disruption seen when full
decellularisation was achieved, led to the loss of the biomechanical properties of cartilage. The initial starting material for decellularisation may not have been optimal, Kheir et al. (2011) used porcine osteochondral tissues extracted from 4 month old pigs. Characterisation of osteochondral pins from various species of different ages may determine which material has properties most similar to that of human cartilage and may indicate more robust materials which will be least affected by harsh decellularisation protocols. Materials with higher levels of GAGs and more defined structure initially may show less disruption.

The decellularisation protocol used previously employed EDTA as a proteinase inhibitor, however, this also acts to decalcify the bony component of osteochondral pins. Removal of EDTA from the process may retain the structural stability of decellularised pins. Previous studies have highlighted problems with detergents removing GAGs from the tissue. Altering the concentration of detergents may reduce the effect of decellularisation protocols on GAG content. Fewer wash cycles or reduced cycle times may also act to retain GAGs, but adversely may not adequately remove cellular components. Taking into account these possible alterations, a more effective protocol for osteochondral decellularisation may be developed.
1.7 Aim & Objectives

Hypothesis

It is hypothesised that a biocompatible xenogeneic osteochondral matrix can be developed with the structure and function of native tissue ECM for use in osteochondral lesion repair.

Aim

The aim of this project is to develop a natural decellularised xenogeneic osteochondral biomaterial with similar biological, biochemical and biomechanical properties to natural articular cartilage for use in articular cartilage defect repair.

Objectives

- To determine the optimal starting material for decellularisation by assessing the biological, biochemical and biomechanical characteristics of osteochondral tissues taken from hips and knees of bovine, porcine and ovine animals.
- To optimise the protocol for decellularisation xenogeneic osteochondral tissues.
- To determine the biological and biochemical and biomechanical characteristics of the acellular osteochondral tissues by histological, immunohistochemical and quantitative methods
- To investigate the regenerative capacity of the biomaterial in terms of cytotoxicity and biocompatibility.
Chapter 2
Materials and Methods

2.1 Materials

2.1.1 Equipment

The equipment used and equipment suppliers are listed in appendix A Table 1.

2.1.2 Chemicals

The chemicals/reagents used and chemical suppliers are listed in appendix A Table 2.

2.1.3 Consumables

The consumables and plastic ware used and suppliers are listed in appendix A Table 3 and appendix A Table 4 respectively.

2.1.4 Cells

The cells used in this study and suppliers are listed in Table 2.1.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Type</th>
<th>Species</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3 Cells</td>
<td>Fibroblasts</td>
<td>Murine</td>
<td>European collection of cell cultures</td>
</tr>
<tr>
<td>BHK</td>
<td>Fibroblasts</td>
<td>Hamster</td>
<td>Health Protection Agency</td>
</tr>
</tbody>
</table>

Table 2.1 Cells used throughout the study.

2.1.5 Glassware

Glassware (beakers and bottles [100 ml, 1,000 ml and 2,000 ml]) were cleaned by immersion in a 1% (v/v) solution of phosphate-free detergent (Neutracon®, Decon Laboratories Ltd) overnight. Following this, glassware
was rinsed in tap water, and a final rinse in distilled water to remove any residual detergent. Glassware was then dried and sterilised (180 °C for 4 h, as required) by dry heat.

2.1.6 General stock solutions

2.1.6.1 Phosphate buffered saline (PBS)

One PBS tablet was dissolved in 100 ml distilled water. The pH was adjusted to pH 7.2 -7.4.

2.1.6.2 Baby hamster kidney (BHK) cell culture medium

Glasgow's minimal essential media (GMEM) was used with 5 % (v/v) foetal bovine serum (FBS), 2.5 % (v/v) tryptone phosphate broth (TPB), 1 mM L-glutamine, 100 U.ml⁻¹ penicillin and 100 U.ml⁻¹ streptomycin.

2.1.6.3 3T3 cell line culture medium

Dulbecco's modified Eagle's medium (DMEM) was used with 10 % (v/v) FBS, 2 mM L-glutamine, 100 U.ml⁻¹ penicillin and 100 U.ml⁻¹ streptomycin.
2.2 Methods

2.2.1 General methods

2.2.1.1 Measurement of pH

The pH of solutions was measured using a Jenway 3020 pH meter. The pH meter was firstly calibrated using solutions of pH 4, 7 and 10 made from buffer tablets dissolved in deionised water. The pH of solutions was measured using temperature compensation. For pH adjustment of solutions, 1M hydrochloric acid or 1M sodium hydroxide was added drop-wise with stirring.

2.2.1.2 Microscopy

Bright-field microscopy was carried out using an Olympus BX40 microscope. In order to use polarized-light microscopy, a polarizer and analyser were incorporated in the usual bright-field set up. Fluorescence microscopy was carried out with the fluorescent vertical illuminator (BX51-RFA) and appropriate filters using the same microscope. Cells in tissue culture plates were viewed using an inverted microscope (Olympus IX71) set up for phase contrast microscopy. Images were captured using an attached Olympus digital camera controlled through Cell^B software (image capture and digitalisation).

2.2.1.3 Sterilisation

Solutions and equipment were sterilised using one of three procedures

- Dry heat sterilisation – Items suitable for dry heat sterilisation were placed in a hot air oven for 4 h at 180 °C.
- Moist heat sterilisation – Items which were not suitable for dry heat sterilisation were sterilised in an autoclave for 20 min at 121 °C at a pressure of 103 kPa.
- Filter sterilisation – Solutions which were not suitable for heat sterilisation were filtered using 0.2 µm pore sized filters using a disposable syringe in a class II safety cabinet.
2.2.2 Tissue acquisition

2.2.2.1 Dissection equipment

The dissection equipment used and suppliers are listed in Table 2.2 and shown in Figure 2.1.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Size/model</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bench top Clamp</td>
<td>-</td>
<td>Irwin</td>
</tr>
<tr>
<td>Corer (serrated drill attachment)</td>
<td>9 mm diameter</td>
<td>University of Leeds</td>
</tr>
<tr>
<td>Corer (serrated)</td>
<td>9 mm diameter</td>
<td>University of Leeds</td>
</tr>
<tr>
<td>Corer (smooth)</td>
<td>9 mm diameter</td>
<td>University of Leeds</td>
</tr>
<tr>
<td>Hand drill</td>
<td>-</td>
<td>Bosch</td>
</tr>
<tr>
<td>Hand saw</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rat toothed forceps</td>
<td>125 mm length</td>
<td>Fisher Scientific Ltd</td>
</tr>
<tr>
<td>Scalpel handle</td>
<td>Size 4</td>
<td>Scientific Laboratory Supplies Ltd</td>
</tr>
<tr>
<td>Straight fine point forceps</td>
<td>115 mm length</td>
<td>Fisher Scientific Ltd</td>
</tr>
<tr>
<td>Straight scissors with fine point</td>
<td>125 mm length</td>
<td>Fisher Scientific Ltd</td>
</tr>
</tbody>
</table>

Table 2.2 Dissection equipment used throughout the study.


2.2.2.2 Dissection

Porcine, ovine and bovine legs were supplied by local abattoirs, usually within 24 h of slaughter (bovine legs within 96 h). Pigs were aged ~ 6 months, sheep ~ 8-12 months and > 4 yr and cows ~ 18 months. Joints were used fresh, or stored at 4 °C overnight for dissection the following day.
Porcine and ovine legs were dissected as shown in Figure 2.2. Excess flesh was removed from the skeletal structures to allow easier access and maneuverability. Joints were exposed by cutting the surrounding tissue and extracapsular ligaments, then the joint capsule was excised. All ligaments were severed and the menisci removed from the knee so that all joint surfaces were exposed. Any exposed cartilage surfaces were kept hydrated throughout by covering in PBS soaked tissue. Bovine legs were supplied without the acetabulum and with the meat already removed, so only excision of the patella and menisci was required.

2.2.2.3 Pin extraction

Osteochondral pins 9 mm in diameter and ~12 mm deep were extracted from cartilage surfaces of the acetabulum, femoral head, femoral groove, medial and lateral condyles and the medial and lateral tibial plateau. The side of each joint was removed with a hacksaw to ease pin extraction. The pins were initially marked out then a power drill with a specialist corer drill bit was used to cut into the subchondral bone. A handheld corer was then used to loosen the pin and extract it from the joint. Images showing the procedure for the bovine femoral groove are shown in Figure 2.3.

Figure 2.3 Extraction of osteochondral pins. A – Intact bovine femoral groove. B – Edge of groove removed for ease of extraction. C – Position of extraction marked out with smooth corer. D – Indentations made with serrated corer to allow drill attachment to grip. E – Cores drilled out. F – Pins fully extracted.

2.2.2.4 Storage

Pins were rinsed in PBS before being placed in plastic storage pots on PBS moistened filter paper and kept at -20 °C for up to 3 months.

2.2.3 Basic histological techniques

2.2.3.1 Fixation

Osteochondral pins were placed in a universal with 15 ml 10 % (v/v) neutral buffered formalin (NBF) for 48 hours to achieve complete fixation prior to decalcification.
2.2.3.2 Decalcification

2.2.3.2.1 Decalcification solution (12.5 % (w/v) EDTA)

Ethylenediaminetetraacetic acid (EDTA; 250 g) and NaOH (25 g) were dissolved in 1.75 L distilled water. The pH was adjusted to 7.0 using 6 M HCl or 6 M NaOH if necessary.

2.2.3.2.2 Decalcification procedure

Bone was decalcified using 25 ml 12.5 % (w/v) EDTA for 2 weeks (or until bone was soft enough to be sectioned) at 45 °C with agitation. Decalcification solution was changed every 2-3 days.

2.2.3.3 Paraffin wax embedding

Osteochondral pins were bisected as described in Figure 2.4 before being placed in plastic cassettes (Histocette®) and cycled in the Leica TP 120 automated tissue processor. Tissues were processed by immersion in 70 % (v/v) ethanol for 1 h followed by 1 h in 90 % (v/v) ethanol. Tissues were then immersed in absolute ethanol for 2 h 20 min, 3 h 20 min then 4 h 20 min. Immersion in xylene followed for cycles of 1 h, 1 h 30 min and 2 h. Tissues were then embedded with molten paraffin wax for 1 h 30 min then 2 h. Tissue cassettes were taken from the automated processor, tissues removed and orientated cut-surface-down in moulds. Samples were covered in molten wax, left to set overnight at room temperature, after which, excess wax was trimmed away.
Figure 2.4 Orientation of osteochondral pins for histology. Pins were bisected before being placed in histology cassettes, then the cut surface was laid face down when moulded in wax, so that sections could be cut from the cartilage surface through the osteochondral face.

2.2.3.4 Sectioning and slide preparation

Wax embedded tissues were sectioned on a microtome (Leica RM2125RTF) to a thickness of 6 μm, sections were taken from the cartilage surface through the bone as shown in Figure 2.4, to avoid dragging of bone fragments through the cartilage. Using forceps and a brush, sections were carefully placed in a water bath at 40 °C. The wax sections were then transferred onto Superfrost Plus slides and placed directly onto a hotplate at 45 °C to bake on and dry.

2.2.3.5 Dewaxing and rehydration

Sections were dewaxed in xylene for 10 min, then in fresh xylene for another 10 min. Sections were then dehydrated in 3 successive immersions in 100% (v/v) ethanol for 3 min, 2 min then 2 min followed by immersion in 70% (v/v) ethanol for 2 min. Slides were then placed under running tap water for 3 min to rehydrate the sections.

2.2.3.6 Dehydration and mounting

Stained sections were dehydrated by immersion in 70% (v/v) ethanol for 5 sec, followed by successive washes in 100% ethanol for 1 min, 2 min and 3 min. Sections were then immersed twice in xylene for 10 min each time. Cover slips were mounted to slides using a drop of DPX mountant, avoiding
bubbles. Slides were left to dry in a fume hood for a minimum of 4 h before visualizing using microscopy.

2.2.4 Histological staining methods

2.2.4.1 Haematoxylin and eosin staining

Haematoxylin and eosin (H&E) was used to stain cell nuclei and general tissue architecture. Once paraffin embedded tissue sections had been dewaxed and rehydrated (Section 2.2.3.5) slides were immersed in pre-made haematoxylin (Mayer's) for 1 min, rinsed under running tap water until the water ran clear then immersed in eosin for 3 min. Slides were then dehydrated and mounted (Section 2.2.3.6).

2.2.4.2 Sirius red/ Miller's staining

Reagents:

- 5 % (w/v) potassium permanganate
  - 15 g potassium permanganate
  - 300 ml distilled water
- 1 % (w/v) oxalic acid
  - 1 g oxalic acid
  - 100 ml distilled water
- Weigert’s haematoxylin
  - 1 part solution A
  - 1 part solution B
- 0.1 % (w/v) Sirius Red
  - 0.1 g Sirius red
  - 100 ml saturated piric acid solution

Sirius red stains collagen fibers, while Miller's stains for elastin fibers. Following dewaxing and rehydration sections were immersed in potassium permanganate for 5 min then rinsed using distilled water. Sections were immersed in oxalic acid for 2 min then distilled water for 1 min then 4 min. Sections were then dehydrated in 70 % (v/v) ethanol for one min then 95 % (v/v) ethanol for one minute before being immersed in Millers stain for 1 h. Sections were rinsed in 70 % (v/v) ethanol until clear, immersed in 95 % (v/v) ethanol for 1 minute then rinsed in running tap water for 2 min. Sections were immersed in Weigert’s haematoxylin for 10 min then rinsed in
running tap water for 1 min then distilled water for 30 sec. Sections were immersed in Sirius red for 1 h, rinsed in distilled water and blotted dry before being dehydrated and mounted (Section 2.2.3.6). Sections were viewed using polarized light microscopy.

### 2.2.4.3 Alcian blue staining

Alcian blue was used to stain cartilage proteoglycans, a counter stain of periodic acid and Schiff’s reagent (PAS) was used to stain other structures and nuclei were stained with haematoxylin (Gills III). Following dewaxing and rehydration sections were immersed in pre-made 1 % (w/v) alcian blue for 15 min. Sections were rinsed in distilled water then immersed in pre-made periodic acid for 5 min. Sections were again rinsed (three times) in distilled water before immersion in Schiff’s reagent for 15 min. Sections were cleared in running tap water for 5 min before being immersed in haematoxylin for 90 sec. Sections were returned to running tap water to clear before being dehydrated and mounted (Section 2.2.3.6).

### 2.2.4.4 Van Gieson staining

**Reagents:**

- **1.0 % (v/v) Acid alcohol**: 2 ml hydrochloric acid (37 %, v/v)  
  200 ml 70 % (v/v) ethanol

Van Gieson stains for collagen fibres, nuclei are stained with haematoxylin. Following dewaxing and rehydration sections were immersed in Weigert’s haematoxylin for 15 min, then running tap water for 15 min. Sections were then differentiated in acid alcohol for 2 min and cleared in running tap water for 3 min before being immersed in van Gieson stain for 3 min. Each section was blotted dry before being dehydrated and mounted.

### 2.2.4.5 Safranin O/ fast green staining

**Reagents:**

- **0.1 % (w/v) Safranin O**: 0.1 g safranin O  
  100 ml distilled water

- **0.02 % (w/v) Fast green**: 0.02 g fast green
100 ml distilled water

- **1.0 % (v/v) acetic acid**
  - 3 ml glacial acetic acid
  - 300 ml distilled water

Safranin O stains proteoglycans while fast green stains collagen, nuclei are stained with Weigert’s haematoxylin. Following dewaxing and rehydration, sections were immersed in Weigert’s haematoxylin for 3 min then running tap water for 10 min. Sections were differentiated in 1 % (v/v) acid alcohol for 1 min then rinsed for 3 min in running tap water. Sections were immersed in fast green for 5 min before being rinsed in acetic acid for 10-15 sec. sections are immersed in safranin O for 4 min before being dehydrated and mounted.

### 2.2.4.6 DAPI staining

**Reagents:**

- **Sodium hydrogen carbonate buffer (1 M, pH 9.0)**
  - 4.2 g sodium hydrogen carbonate
  - 500 ml distilled water

- **2.5 % (w/v) DABCO (pH 9.0)**
  - 2.5 g 1,4-diazobicyclo-(2, 2, 2)-octane (DABCO)
  - 100 ml 1 M sodium hydrogen carbonate buffer

- **DABCO:glycerol mountant**
  - 10 ml 2.5 % (w/v) DABCO
  - 90 ml glycerol

- **Dye Buffer (pH 7.4)**
  - 1.211 g Trizma base
  - 0.3724 g EDTA
  - 0.0058 g sodium chloride
  - 1 L distilled water

- **DAPI dye solution**
  - 10 mg DAPI
  - 10 ml nuclease free water

- **DAPI working solution (pH 7.4)**
  - 20 µl DAPI dye solution
200 ml dye buffer
4′,6-diamidino-2-phenylindole (DAPI) binds DNA which enables cell nuclei and DNA remnants to be visualised. Following dewaxing and rehydration, sections were immersed in DAPI working solution for 10 min in the dark. Sections were then washed 3 times for 10 min each in PBS in the dark before being mounted in DABCO:glycerol. Sections were kept in the dark until visualised using an upright fluorescent microscope and a DAPI filter.

2.2.5 Tissue culture

Cells were cultured in cell culture medium (Section 2.1.6). All cell culture work was performed aseptically in a class II safety cabinet. Cultures were incubated at 37 °C in 5 % CO₂ (v/v) in air. Before use, all culture medias and additives were equilibrated to 37 °C.

2.2.5.1 Resurrection and maintenance of cells

Following removal from liquid nitrogen storage, cells were thawed in a 37 °C water bath. Thawed cell stock (2 ml) was added to a T75 cell culture flask containing 13 ml of the appropriate, pre-warmed cell culture medium. Flasks were incubated for at least 18 h at 37 °C in 5 % CO₂ (v/v) in air to allow cells to attach to the culture plastic. Culture medium was changed every 2-3 days until cells were confluent and could be passaged.

2.2.5.2 Cell passaging

Cell culture medium was aspirated and the monolayer gently washed two times with 10 ml PBS without calcium or magnesium. Trypsin/EDTA (1.5 ml) was added to the flask and incubated for 5 min at 37 °C in 5 % CO₂ (v/v) in air. Flasks were gently tapped to detach cells and 10 ml culture medium was added to suspend cells and inhibit trypsin activity. The cell suspension was then centrifuged for 10 min at 150 g. The supernatant was carefully aspirated from the cell pellet, which was then resuspended in 5 ml culture medium. Following a count of viable cells (Section 2.2.5.3), the appropriate cell density was seeded into a fresh flask with culture medium and the medium replaced every 2-3 days until confluent and ready for passage again.
2.2.5.3 Cell viability

In order to test the viability of cells, trypan blue was added to cell suspensions before counting. Trypan blue is able to enter dead cells due to a loss of membrane potential, so live cells microscopically appeared transparent while dead cells appeared blue and could therefore be excluded from cell counts.

To perform a cell count, 20 µl cell suspension was added to 20 µl trypan blue and added to an Improved Neubauer counting chamber. Viable cells were counted in \( n \) number of grids which resulted in a cell count of between 100-300 cells. The total number of cells per ml suspension was calculated as follows:

\[
Number of cells.m l^{-1} = \frac{Number of viable cells}{n} \times 10^4 \times Dilution \text{ factor}
\]

Where \( n \) = number of grids used in count and

And \textit{Dilution factor} = correction required due to dilution of cell suspension in trypan blue (in this case \textit{Dilution factor} = 2)

2.2.5.4 Cell cryopreservation

Cells were harvested from flasks and a cell count performed as detailed previously (Section 2.2.5.2 and 2.2.5.3). Cells were resuspended at \( 10^6.\text{ml}^{-1} \) in cryopreservation medium (DMEM containing 20 % (v/v) FBS and 10 % (v/v) filter sterilised dimethyl sulphoxide (DMSO)). Cell aliquots (1 ml) were put in cryovials and placed into cryofreezing pots containing isopropanol. Pots were frozen at -80 °C for 12 – 24 h following which vials were transferred to liquid nitrogen for long term storage.

2.2.6 Statistical analysis

2.2.6.1 Confidence limits

Numerical data was analysed using Microsoft Excel (version 2007, Microsoft) and presented as the mean (n\( \geq 3 \)) ± 95 % confidence level (CL). The 95% confidence intervals (\( \alpha = 0.05 \)) were calculated using the descriptive statistics part of the data analysis package in Microsoft Excel.
\[ x = \text{Individual value} \]
\[ n = \text{Sample number} \]
\[ \Sigma = \text{Sum of} \]

\[ \text{mean} = \frac{\Sigma x}{n} \]
\[ \text{StDev} = \sqrt{\frac{\Sigma x^2}{n-1}} \]
\[ SE \text{ mean} = \frac{\text{StDev}}{\sqrt{n}} \]

\[ 95 \% CL = \text{mean} \pm t \times SE \]

### 2.2.6.2 Statistical analysis

The student’s t-test was used to compare groups of two means or a one-way analysis of variance (ANOVA) was used when comparing the means of more than two groups. Individual differences between group means were identified by calculating the minimum significant difference (MSD) at \( p = 0.05 \) using the T-method, or T'-method when comparing groups of unequal sample size (Sokal & Rohlf, 1995).

\[ Q = \text{Critical value} \]
\[ \alpha = p = 0.05 \]
\[ k = \text{number of groups} \]
\[ v = \text{degrees of freedom (n-1)} \]

\[ MSD = Q(\alpha[k,v]) \times SE \]

### 2.2.6.3 Arcsin transformation

Where data is presented as a percentage or proportion, values were transformed to arcsin to allow accurate calculation of 95 % CL and statistical analysis. Following analysis, values were transformed back for presentation.
2.2.6.4 Linear regression analysis

When interpolation of results was required, a linear regression analysis was performed on standard curves.

\[
\text{unknown } x = \frac{\text{mean measured } y \text{ variables}}{y \text{ value of linear regression line equation}}
\]
Chapter 3
Characterisation of porcine, bovine and ovine osteochondral tissues

3.1 Introduction

To provide successful long-term cartilage lesion repair, a biomaterial should closely mimic the function of natural articular cartilage. As discussed in Chapter 1, the composition and structure of cartilage is essential for the load bearing, low friction function of the tissue. The glycosaminoglycans (GAGs) within the cartilage matrix create a Donnan osmotic pressure, reducing fluid flow within the tissue under loading, providing the compressive strength of cartilage, and reducing friction between solid phase components of the matrix. The collagen fibres provide tensile strength for the tissue (Mow et al., 2005, Roughly, 2006). Variations in the biochemical composition and biological structure alter the function of articular cartilage.

Here, it is proposed that the use of an acellular osteochondral biomaterial will enable successful repair of osteochondral lesions. Procedures to decellularise tissues are performed with the aim of producing an immunocompatible scaffold which retains the original tissue function, so the properties of any source material should be retained in the final scaffold. Cartilage biology, biochemical composition and biomechanical function (Athanasiou et al., 1991) varies between species and joint regions (Rogers et al., 2006). Before developing a decellularisation protocol it was sensible to assess the potential tissue sources currently available and to identify which species/joint region has properties most similar to that of human cartilage. Furthermore, assessment of composition will allow any cartilages with high GAG content to be identified, as sulphated proteoglycan concentration is known to be diminished following decellularisation using SDS (Stapleton et al., 2008; Elder et al., 2010; Kier et al., 2011; Crapo et al., 2011; Benders et al., 2013).
To achieve good clinical outcomes in osteochondral repair with an acellular xenogeneic scaffold it was therefore necessary to characterise and understand the biochemical composition, biological structure and biomechanical function of any potential source materials and chose a tissue with properties desirable in the decellularised scaffold.
3.2 Aims and objectives

Aims:
The aim of the study presented in this chapter was to characterise osteochondral tissues from various joint regions and species. The intention was to determine the most suitable material for use in the development of a decellularisation process.

Objectives:

- To qualitatively assess the osteochondral tissues using histology, looking at the general histoarchitecture, GAG distribution and to measure cellularity and cartilage thickness.
- To quantify biochemical components of the cartilage, including sulphated proteoglycan, collagen and water content.
- To characterise biomechanical properties of cartilage, measuring the percentage deformation and cartilage thickness, then to determine cartilage permeability and equilibrium elastic modulus using a finite element method.
3.3 Biological methods

Osteochondral pins were collected from the acetabulum, femoral head, femoral groove, medial and lateral condyles and medial and lateral tibial plateau as described in Section 2.2.2. Porcine (~6 month old), bovine (~18 month), ovine (~1 yr and >4 yr) and human cadaveric tissues were characterised. Acetabular tissues were not available from cows, so were not characterised in this study.

3.3.1 Histological evaluation of osteochondral tissues

The general histological methods used in this study can be found in Section 2.2.3.

3.3.2 Cell count

Sections were stained with H&E and the resulting images were analysed using Image-Pro Plus software (Media Cybernetics). For each image n=6, 500 μm² regions were manually selected to sample cells from all three cartilage zones (two regions of superficial cartilage, two of middle zone cartilage and two of deep zone cartilage). All cells which fell in that defined region were counted manually. The mean number of cells was calculated for each image. Images from n=5 animals were counted for each joint region. The mean number of cells for each species and joint region and the 95 % CL were calculated.

3.3.3 Thickness measurement

Image-Pro Plus software (Media Cybernetics) was used to measure the thickness of cartilage from all animals, using images of sections stained with alcian blue. Measurements (n=6) were made through the depth of the cartilage at even intervals across the image, the cartilage-bone interface was defined as when the blue stain colour finished and became red/pink staining. Again, the mean thickness of each image was calculated, then the mean of n=5 animals determined plus 95 % CL for that species/joint region.
3.4 Biochemical methods

3.4.1 Sample preparation

3.4.1.1 Lyophilisation

Cartilage tissue was cut away from the subchondral bone and macerated. Samples were weighed three times and a mean wet weight calculated. Samples were placed in a freeze dryer (Thermo, Savant ModulyoD) at -50 °C, 0.15 – 0.2 mbar, and the weight measured every 24 h until constant (48 - 72 h).

3.4.1.2 Papain digestion

For sulphated proteoglycan quantification, macerated, freeze-dried tissues were digested using papain.

- **Digestion buffer (pH 6.0)**
  - 0.788 g L-cystine hydrochloride
  - 1.8612 g EDTA
  - 1 L PBS

- **Digestion solution**
  - 1250 U papain
  - 25 ml digestion buffer

Papain digestion solution (5 ml) was added to 10 – 20 mg (dry weight) lyophilised cartilage in a bijou and incubated in a water bath at 60 °C for 36 – 48 h, until fully digested.

3.4.1.3 Acid hydrolysis

For quantification of hydroxyproline residues, cartilage was macerated, lyophilised and hydrolysed in hydrochloric acid. Tissues (15 mg) were placed in polypropylene universals and 5 ml 6 M hydrochloric was added. Samples were incubated in a block heater at 120 °C for 8 - 16 h as verified in Figure 3.1, before being neutralised with 6 M sodium hydroxide.
Figure 3.1 Hydroxyproline concentration after varying durations of acid hydrolysis. Data is displayed as the mean (n=3) ± 95 % CL. * Represents a significant difference from 16 h, one-way ANOVA (p< 0.05).

3.4.2 Water content

The percentage water content of tissue was determined by weighing before and after lyophilisation (Section 3.4.1.1). The water weight was calculated from the dry and wet weights and was then presented as percentage of tissue wet weight.

3.4.3 GAG quantification

Quantification of GAGs (sulphated/carboxylated proteoglycans) in tissues is possible by colorimetric analysis, using a method first developed by Farndale et al. (1982). Under acidic conditions the cationic dye 1,9-dimethylemethylene blue (DMB) undergoes metachromatic changes upon binding with carboxyl or sulphate groups of GAG chains, this can then be measured spectrophotometrically.

Reagents:

- Phosphate buffer (pH 6.8)
  - 137 ml sodium di-hydrogen orthophosphate (0.1M)
  - 63 ml di-sodium hydrogen orthophosphate (0.1M)
- 75 -

- **DMB dye (pH 3.0)**
  - 16 mg DMB
  - 5 ml ethanol
  - 2 ml formic acid
  - 2 g sodium formate
  - Make up to 1 L with distilled water

**Method:**

Tissues were lyophilised and papain digested (Sections 3.4.11 & 3.4.1.2). Standard calibration solutions of dermatan sulphate were made up in phosphate buffer at 0, 3.125, 6.25, 12.5, 25, 50, 100, 150 and 200 µg.ml\(^{-1}\). Test samples were neat, or diluted 1:10, 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000 in phosphate buffer, a dilution of 1:50 was found to be optimal. Each standard and sample (40 µl) were added to a clear, 96 well flat bottomed plate in triplicate, to which 250 µl DMB dye was added. Plates were gently shaken for 2 min on a plate shaker before the optical density of each well was read at 525 nm using a micro plate spectrophotometer. A standard curve was plotted of the absorbance of the dermatan sulphate standards vs concentration, linear regression analysis was performed (Section 2.2.6.4) to interpolate the GAG concentration of test samples based on their absorbance. Dilution factors were then accounted for, and the concentration of GAG was determined for tissue dry weight.

3.4.4 Hydroxyproline quantification

Hydroxyproline residues are a major constituent of collagen, and are unique to this protein in articular cartilage. Hydroxyproline residues can be oxidised to pyrroles which produce a red chromophore with p-dimethylaminobenzaldehyde, this can then be measured colorimetrically. This method is based on that of Edwards & O'Brien (1980). The hydroxyproline levels can then be used to calculate collagen content.

**Reagents:**

- **Hydroxyproline buffer (pH 6.2)**
  - 13.3 g citric acid
  - 3.2 ml glacial acetic acid
Method:

Samples were lyophilised and acid hydrolysed (Section 3.4.1.1 & 3.4.1.3). Standard concentrations of trans-4-hydroxy-L-proline were made up in hydroxyproline buffer at 0, 2, 4, 6, 8, 10, 15, 20, 25 & 30 µg.ml⁻¹. Test solutions were neat and diluted 1:5, 1:10, 1:25, 1:50, 1:100 in assay buffer, a dilution of 1:10 was found to be optimal. Standard and test solutions (50 µl) were added to a clear, flat bottomed 96 well plate in triplicate. Chloramine T (100 µl) was added to each well and the plate shaken gently for 5 min to oxidise the hydroxyproline residues. Ehrlich’s reagent (100 µl) was then added to each well and the plate incubated in a water bath at 60 °C for 45 min. The optical density of each well was then measured at 570 nm using a micro plate spectrophotometer. A standard curve was produced of absorbance vs. concentration, from which the hydroxyproline concentration of test samples could be interpolated. Neutralisation and dilution factors were accounted for and the hydroxyproline concentration was determined for cartilage dry weight.
3.5 Biomechanical methods

3.5.1 Creep indentation

In order to assess the time dependent biomechanical behaviour of articular cartilage, osteochondral pins underwent creep indentation. The equipment used is shown in Figure 3.2. Pins were secured in a collet, mounted in a sample holder and submerged in PBS to maintain cartilage hydration (Figure 3.3). An impermeable, stainless steel, hemispherical indenter (3 mm diameter) was used to indent the cartilage with a load of 0.82 N over 1 h. The indenter was positioned ~ 1 mm above the cartilage surface and the lowering of the indenter shaft was controlled via a silicone oil filled dashpot, to reduce the speed of impact, the full load was applied within 0.2 sec. The displacement of the indenter was measured using a linear variable differential transducer (LVDT; RDP D5-200H, Electroence, PA, USA) and the resistance force measured using a piezo-electric force transformer (Part No. 060-1896-02, Electroence, PA, USA). LabView 8 software (National Instruments, TX, USA) was used to collect and store data produced by the force transducer and LVDT.
Figure 3.2 Indentation apparatus. The equipment used to perform creep indentation testing to determine deformation of osteochondral pins.

Figure 3.3 Sample holder and indenters. A – collet, collet holder and sample bath, B – Pin held in place, C – hemispherical and needle indenters used during testing.
3.5.1.1 Calibration†

Calibration was required to convert the voltage outputs of the LVDT and force transducer into millimetres and Newtons respectively. Standard steel slip gauges were used to calibrate the LVDT, the voltage was recorded for each height increase following the addition of slip gauges. Voltage was plotted against slip gauge height and the linear trend line calculated, the equation of which was used as a calibration factor (Figure 3.4).

\[
y = -0.5221x + 1.2077 \\
R^2 = 1
\]

![Figure 3.4 LVDT calibration. Calibration of LVDT from the indentation apparatus using standard height slip gauges to determine factor to convert output voltage to displacement.](image)

To calibrate the force transducer, the voltage was recorded over incremental increases of known mass. This again, was plotted as a graph and the equation of the trend line used to convert voltage to Newtons (Figure 3.5).

† Work performed by Abdellatif Abdelgaied
Calibration of the load cell from the indentation apparatus using standard known masses to determine the factor to convert output voltage to load.

### 3.5.2 Needle indentation

The thickness of cartilage was measured in order to normalise deformation data for each osteochondral pin so that the percentage deformation could be presented and the material properties could be derived. Cartilage thickness was assessed using needle indentation. An Instron material testing machine (Instron 3365, Bucks, UK) was used for this purpose (Figure 3.6). Osteochondral pins were secured in a PBS bath as described in Section 3.5.1, a needle attached to the instron arm was manually positioned ~1 mm above the cartilage surface. During testing the arm was controlled via a PC graphic user interface and lowered at a rate of 4.5 mm.min\(^{-1}\). The resistance to motion was measured using a 500 N load cell.

Cartilage thickness was defined as the distance between the increase in resistance from initial needle contact with the surface and the steep increase in resistance from needle contacting the much stiffer bone (Figure 3.7). Each pin was indented 6 times and the mean thickness calculated.

![Graph showing load cell calibration](image-url)

**Figure 3.5 Load cell calibration.** Calibration of the load cell from the indentation apparatus using standard known masses to determine the factor to convert output voltage to load.
Figure 3.6 Instron material testing machine. Equipment used to indent osteochondral pins with a needle indenter to determine cartilage thickness.

Figure 3.7 Needle indentation graph interpretation. This schematic shows how cartilage thickness was determined from needle indentation.
3.5.3 Finite element method for derivation of material properties

Cartilage deformation curves generated in Section 3.5.1 along with cartilage thicknesses measured in Section 3.5.2 were used to derive the permeability and equilibrium elastic modulus of cartilage. An axisymmetric biphasic poroelastic finite element (FE) model (ABAQUS, version 6.9-1 Dassault Systemes, Suresnes Cedex, France) created and published by Pawaskar et al. (2010, 2011) was used for the derivation of these properties (Figure 3.8).

**Figure 3.8 Finite element model of an osteochondral pin.** This schematic shows the axis of symmetry, and the imposed fluid flow restrictions.

Cartilage was modelled as a poroelastic material and meshed using four-node bilinear displacement and pore pressure axisymmetric elements (CAX4P), cartilage was assigned the water content value calculated in section 3.4.2 and a Poisson’s ratio of zero was assumed. Bone was meshed using four-node bilinear elastic axisymmetric elements (CAX4), A Poisson’s ratio of zero and was assigned and elastic modulus of 17 GPa (Pawaskar, 2010).
The FE model mimicked the method for collecting experimental data (Section 3.5.1), the load was increased from 0 – 0.82 N over 2 sec through the indenter and was applied for 3600 sec in total. A deformation/time graph was produced by the FE model based on the material properties (equilibrium elastic modulus and permeability) set. The closeness of fit between the final 30 % of the FE generated and experimental curves was assessed using MATLAB (version 7.4, MathWorks Inc, Boston, MA, USA; Figure 3.9). The input material properties were incrementally changed to minimise squared error, an $R^2$ value greater than 0.6 was accepted as significant for biological tissues. The material properties input into the FE model which gave the highest $R^2$ were taken as the equilibrium elastic modulus and permeability for that cartilage.

![Figure 3.9 Experimental and modelled cartilage displacement curves.](image)

The FE modelled curve was fitted to the deformation curve produced by experimental data to reach the highest possible agreement by altering the input material properties.
3.6 Results

3.6.1 Macro scale observations

Upon dissection, physical differences were observed between species. The length of the femurs from femoral head to the front of the condyles was ~ 20 cm in both pigs and sheep; however this was double in cows, ~ 40 cm (Figure 3.7). This size difference was reflected in the size of the joints; the bovine femoral head measured ~ 8 cm from the terres ligament to femoral neck, while the porcine was ~ 4 cm and ovine only ~ 2 cm. This pattern in joint size was seen for both the hip and knee. Cartilage generally appeared white, glossy and healthy with exception of ovine (> 4 yr) cartilage which had a yellowed appearance in most cases. Upon drilling, the bone of porcine and young ovine was notably softer than that of bovine and older ovine samples. Old ovine bone, despite being hard, was also found to be quite brittle when cores were extracted from the joints.

![Figure 3.10 Dissected femurs. a) porcine, b) bovine, c) ovine ~ 1 yr and d) ovine > 4 yr.](image)
3.6.2 Histological evaluation of cartilage

3.6.2.1 H&E

H&E staining of osteochondral sections from each species showed characteristic cell orientation (Figure 3.11), with smaller flattened cells at the superficial zone becoming rounder and more randomly distributed in the middle zone and then forming linear columns perpendicular to the cartilage surface in the deep zone. This characteristic cell organisation was less pronounced in porcine (a) and young ovine (c) cartilage and cells in the deep zone showed a more hypertrophic appearance.

Figure 3.11 H&E staining of cartilage from the medial condyle. a) porcine, b) bovine, c) ovine ~1 yr, d) ovine > 4 years. Size bars are 500 µm.

3.6.2.2 Alcian blue

Alcian blue staining of osteochondral sections from each species showed characteristic proteoglycan distribution (Figure 3.12). All cartilage tissues showed strong alcian blue staining in the middle and deep zones, while the superficial zones and deep calcified cartilage and bone showed reduced
alcian blue staining, highlighted by the presence of pink periodic acid-Schiff counterstaining.

Figure 3.12 Alcian blue and periodic acid-Schiff staining of cartilage from the medial condyle. a) porcine, b) bovine, c) ovine ~1 yr, d) ovine > 4 years. Size bars are 500 µm.

3.6.3 Cell count

From the images of H&E stained sections a cell count was performed to identify cell density of cartilage from the different species and joint areas. Cartilage from the younger animals (pig and young sheep) tended to have the highest concentration of cells and cartilage from old sheep was least cellular, this trend was significant in a number of joint regions (Figure 3.13). Within species, cartilage from the porcine tibial plateau had a significantly higher cell concentration that cartilage from the femoral groove, unlike bovine tibial plateau cartilage, which was significantly less cellular than all other joint regions except for the femoral head. Old ovine cartilage from the acetabulum and medial tibia had significantly fewer cells than other joint regions. No significant difference was seen in young ovine cartilage cellularity across joint regions (p<0.05 ANOVA).
3.6.4 Cartilage thickness measurement

Thickness of cartilage for each species was measured from digital images of the stained sections (Figure 3.14). Ovine cartilage tended to be thinner than porcine and bovine cartilage, significantly so in a number of joint regions. Porcine tibial cartilage was significantly thinner than that of the groove and medial condyle, young ovine femoral head cartilage was significantly thinner than that of the medial tibia, no significant difference between joint areas was seen in bovine or old ovine (p<0.05 ANOVA). There was a trend toward cartilage of the condyles and femoral groove being thicker than other joint regions.
Figure 3.14 Thickness of cartilage from various joint regions of the pig, cow and sheep. Data is expressed as the mean (n=5) ± 95 % confidence limits. Data for a given tissue site was analysed by one-way analysis of variance and individual means compared by the T-method. * indicates p<0.05.

3.6.5 Water content

The water content of cartilage did not vary significantly between any species except for bovine lateral tibial cartilage which had a significantly higher water content than cartilage from the lateral tibia of older sheep (Figure 3.15). Significant intra-species water content variation was only seen in bovine cartilage, with that from both sides of the tibia having a significantly higher water content than bovine femoral head and medial groove cartilage, cartilage from the lateral tibia also had significantly higher water content than from the bovine femoral head (p<0.05 ANOVA). All cartilages had a water content between 70 - 82 %.
Figure 3.15 Water content of cartilage from various joint regions of the pig cow and sheep. Data was subject to arcsin transformation prior to calculation of the 95% confidence limits and analysis of variance. Data is expressed as the back transformed mean (n=5) ± 95% confidence limits. Data for a given tissue site was arcsine transformed then analysed by one-way analysis of variance and individual means compared by the T-method. * indicates p<0.05.

3.6.6 GAG quantification

The GAG content of porcine cartilage was significantly higher than that of other species in a number of joint regions (Figure 3.16), cartilage from old sheep tended to have the lowest GAG concentration. There was a trend seen across the joint regions, similar to that seen for cartilage thickness, with femoral knee (groove & condylar) cartilage having higher concentrations of GAGs compared to other joint regions. In the pig, cartilage of the groove and both condyles had a significantly higher GAG content than that of the medial side of the tibial plateau, only the medial condyle and groove showed higher concentration than the lateral side. Cartilage from the medial condyle of the older sheep had a significantly higher GAG content than that of the acetabulum, groove and tibia. Cartilage of the bovine lateral condyle had significantly fewer GAGs than the medial tibia (p<0.05 ANOVA).
Figure 3.16 GAG content of cartilage from various joint regions of the pig cow and sheep. Data is expressed as the mean (n=5) ± 95 % confidence limits. Data for a given tissue site was analysed by one-way analysis of variance and individual means compared by the T-method. * indicates p<0.05.

3.6.7 Hydroxyproline quantification

Quantification of hydroxyproline content (relative to collagen content) showed there was an inverse relationship to GAG content (Figure 3.17). Porcine cartilage had the highest GAG content in most regions, and had the lowest hydroxyproline content, whereas old ovine cartilage had low GAG content, but had highest hydroxyproline content. Cartilage of the porcine lateral condyle had significantly lower hydroxyproline content than that of the medial condyle, there were no other significant differences within species (p<0.05 ANOVA).
Figure 3.17 Hydroxyproline content of cartilage from various joint regions of the pig cow and sheep. Data is expressed as the mean (n=5) ± 95% confidence limits. Data for a given tissue site was analysed by one-way analysis of variance and individual means compared by the T-method. * indicates p<0.05.

3.6.8 Percentage deformation

The mechanical properties of cartilage from the different species and joint regions are shown in Figure 3.18 and Table 3.1, detailing the percentage deformation of cartilage after one hour, the permeability, elastic equilibrium modulus and thickness of each tissue. Properties could not be determined for ovine groove cartilage as the joints were too small to collect suitable pins.

Cartilage of the hip (femoral head and acetabulum) tended to have a lower percentage deformation than that of the tibial plateau and lateral condyles. Porcine tibial cartilage showed significantly greater percentage deformation than all other joint areas. Bovine lateral condyle and lateral tibial cartilage had significantly higher percentage deformation than that of the femoral head and medial condyle and tibia. Cartilage from the young ovine acetabulum deformed significantly less than that of the femoral head. Acetabular cartilage from the older sheep had a significantly lower percentage deformation than cartilage from the lateral condyle and tibial plateau, and acetabular cartilage from young sheep and pigs.
Figure 3.18 Percentage deformation of cartilage from various joint regions of the pig cow and sheep. Data was subject to arcsin transformation prior to calculation of the 95% confidence limits and analysis of variance. Data is expressed as the back transformed mean (ovine 8-12 months, acetabulum and groove n=5; all other n=6) ± 95 % confidence limits. Data for a given tissue site was arcsine transformed then analysed by one-way analysis of variance and individual means compared by the T-method. * indicates p<0.05. a) From Taylor et al. (2011), b) from McLure et al. (2012), all other data from the present study.

3.6.9 Equilibrium elastic modulus, permeability and thickness

The equilibrium elastic modulus was generally lowest for porcine cartilage, increasing in bovine then young ovine cartilage, with old ovine having a much higher equilibrium elastic modulus than the other species. A trend was seen across the joint regions in all species, with cartilage of the tibial plateau having a lower elastic modulus than the femoral knee and hip.

Permeability showed an inverse relationship with equilibrium elastic modulus. Old ovine cartilage was the least permeable tissue and porcine tended to be most permeable. Permeability was highest in the tibial plateau and cartilage of the hip had the lowest permeability.
<table>
<thead>
<tr>
<th>Material property</th>
<th>Porcine</th>
<th>Bovine</th>
<th>Ovine (8-12 month)</th>
<th>Ovine (&gt; 4 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Equilibrium elastic modulus (MPa)</strong></td>
<td>Mean</td>
<td>R²</td>
<td>Mean</td>
<td>R²</td>
</tr>
<tr>
<td>Acetabulum</td>
<td>1.30</td>
<td>0.99</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Femoral head</td>
<td>a1.13</td>
<td>b0.74</td>
<td>a1.59</td>
<td>b0.81</td>
</tr>
<tr>
<td>Groove</td>
<td>1.04</td>
<td>0.93</td>
<td>0.86</td>
<td>0.94</td>
</tr>
<tr>
<td>Medial condyle</td>
<td>b0.74</td>
<td>0.63</td>
<td>b1.17</td>
<td>0.80</td>
</tr>
<tr>
<td>Lateral condyle</td>
<td>b0.66</td>
<td>0.67</td>
<td>b0.81</td>
<td>0.81</td>
</tr>
<tr>
<td>Medial tibia</td>
<td>b0.88</td>
<td>0.86</td>
<td>b0.85</td>
<td>0.73</td>
</tr>
<tr>
<td>Lateral tibia</td>
<td>b0.70</td>
<td>0.86</td>
<td>b0.81</td>
<td>0.85</td>
</tr>
<tr>
<td><strong>Permeability, k x10⁻¹⁶ (m⁴/Ns)</strong></td>
<td>Mean</td>
<td>R²</td>
<td>Mean</td>
<td>R²</td>
</tr>
<tr>
<td>Acetabulum</td>
<td>3.04</td>
<td>0.99</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Femoral head</td>
<td>5.70</td>
<td>0.97</td>
<td>1.78</td>
<td>0.91</td>
</tr>
<tr>
<td>Groove</td>
<td>4.49</td>
<td>0.93</td>
<td>6.33</td>
<td>0.94</td>
</tr>
<tr>
<td>Medial condyle</td>
<td>7.18</td>
<td>0.63</td>
<td>4.49</td>
<td>0.80</td>
</tr>
<tr>
<td>Lateral condyle</td>
<td>6.77</td>
<td>0.67</td>
<td>5.45</td>
<td>0.81</td>
</tr>
<tr>
<td>Medial tibia</td>
<td>8.09</td>
<td>0.86</td>
<td>4.52</td>
<td>0.73</td>
</tr>
<tr>
<td>Lateral tibia</td>
<td>10.29</td>
<td>0.86</td>
<td>5.75</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Table 3.1a Material properties of cartilage from various joint regions of the pig, cow and sheep.
Table 3.1b Material properties of cartilage from various joint regions of the pig, cow and sheep.

Thicknes data is expressed as the mean (ovine 8-12 months, acetabulum and groove n=5; all other n=6) ± 95% confidence limits, Elastic equilibrium modulus and permeability data was modelled on mean deformation data and R² values for modelled and experimental deformation curve agreement are presented. a) from Taylor et al. (2011), b) from McLure et al. (2012), all other data from the present study.

<table>
<thead>
<tr>
<th>Thickness</th>
<th>Porcine Mean</th>
<th>95% CL</th>
<th>Bovine Mean</th>
<th>95% CL</th>
<th>Ovine (8-12 month) Mean</th>
<th>95% CL</th>
<th>Ovine (&gt; 4 years) Mean</th>
<th>95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetabulum</td>
<td>1.12</td>
<td>0.15</td>
<td>-</td>
<td>-</td>
<td>0.85</td>
<td>0.12</td>
<td>1.06</td>
<td>0.45</td>
</tr>
<tr>
<td>Femoral head</td>
<td>1.22</td>
<td>0.05</td>
<td>1.32</td>
<td>0.13</td>
<td>0.48</td>
<td>0.07</td>
<td>0.52</td>
<td>0.10</td>
</tr>
<tr>
<td>Groove</td>
<td>1.70</td>
<td>0.53</td>
<td>1.36</td>
<td>0.10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Medial condyle</td>
<td>2.23</td>
<td>0.20</td>
<td>1.28</td>
<td>0.17</td>
<td>0.87</td>
<td>0.12</td>
<td>0.71</td>
<td>0.24</td>
</tr>
<tr>
<td>Lateral condyle</td>
<td>2.08</td>
<td>0.23</td>
<td>0.93</td>
<td>0.15</td>
<td>0.80</td>
<td>0.22</td>
<td>0.55</td>
<td>0.18</td>
</tr>
<tr>
<td>Medial tibia</td>
<td>0.87</td>
<td>0.15</td>
<td>1.70</td>
<td>0.36</td>
<td>0.76</td>
<td>0.23</td>
<td>0.61</td>
<td>0.24</td>
</tr>
<tr>
<td>Lateral tibia</td>
<td>0.82</td>
<td>0.08</td>
<td>1.02</td>
<td>0.21</td>
<td>0.70</td>
<td>0.28</td>
<td>0.59</td>
<td>0.27</td>
</tr>
</tbody>
</table>
3.7 Discussion

A xenogeneic source material for decellularisation should have properties as similar as possible to those of human osteochondral tissues to improve the chances of clinical success. Additionally a higher GAG content in source materials compared to that of human cartilage may be beneficial, as GAGs are depleted following decellularisation with SDS which can result in diminished mechanical properties (Stapleton et al., 2008; Elder et al., 2010; Kier et al., 2011; Crapo et al., 2011; Benders et al., 2013). The work in this chapter looked at the biological, biochemical and biomechanical properties of different osteochondral tissues. Porcine, bovine and ovine osteochondral tissues from various regions of the hip and knee were assessed to determine the most suitable source material for the development of a decellularisation protocol.

Initial macroscopic observations of cartilage from the different species showed that old ovine cartilage (>4 years) had a yellowed appearance seen in older cartilage tissues (Altman, 1987), usually attributed to the presence of advanced glycation end products (Bank et al., 1998). These non-enzymatic crosslinks affect the mechanical properties of tissues, increasing the stiffness (Bank et al., 1998; Verzijl et al., 2002). Yellowed, aged ovine cartilage would make an inferior substitute for healthy young cartilage in vivo. Joint size may impact on the usefulness of species as donors of osteochondral tissues, as a greater number of osteochondral samples can be harvested from a bovine joint compared to a porcine joint, and even more so than a small ovine joint. When drilling, the apparent softness of porcine and to some extent younger ovine bone highlighted the skeletal immaturity of these tissues.

H&E staining of cartilage tissues showed varying extents of cell organisation, the highly ordered cell orientation seen in bovine and old ovine cartilage was indicative of mature tissues, while the lack of organisation and the increased presence of hypertrophic chondrocytes showed the relative immaturity of porcine and young ovine cartilages. As in humans, the cellularity of animal cartilage decreased with age (Stockwell, 1976), with older sheep (>4 years)
having fewest cells and pigs (6 months) having the most. Porcine cartilage cellularity varied according to the thickness of the cartilage from the different joint regions, so cartilage from the groove, which was significantly thicker than that from the tibia, also had significantly fewer cells. Thicker cartilage has been reported to have lower cell density than thin cartilage (Stockwell 1971). A source material with fewer cells would be preferable for decellularisation.

Cartilage thickness has been reported to increase as body weight increases (Stockwell, 1971). This was not necessarily supported by the work in the current chapter. Sheep, with a lower body mass than pigs or cows did have thinner cartilage, however, bovine cartilage was thinner than porcine, despite cows having greater body mass. Julkunen et al. (2009) showed that cartilage thickness varies with skeletal maturity, that immature animals have thick cartilage and that cartilage thickness decreases with maturity. This was supported here by the comparison of two age groups of sheep, with the mature ovine cartilage tending to be thinner than that of sheep around 1 year of age. In all species, cartilage of the groove and condyles tended to be thicker than that of the hip and tibia, this trend was also seen when comparing the thickness of cartilage from the same joint regions in humans (Athanasiou et al., 1991; Shepherd & Seedhom, 1999a; Thambya et al., 2006). Simon et al. (1973) identified a relationship between cartilage thickness and joint congruity, with more congruent joints such as the hip having thinner cartilage than that of less congruent joints such as the knee.

Human cartilage, on average, tended to be thicker than that of any of the species studied for a given joint region. Shepherd & Seedhom (1999a) report the thickness of human femoral head cartilage to range between 1.08-2.40 mm whereas ovine cartilage was only ~ 0.68 mm in this area, porcine 1.14 mm and bovine 1.53 mm. Cartilage from the human femoral groove ranged between 1.76-2.59 mm, ovine cartilage was again much thinner; 0.86 mm in young sheep and 0.63 mm in old sheep. Cartilage from the porcine groove was 2.04 mm and bovine 1.52 mm, within or approaching the range of human cartilage thickness. The relative thickness of human cartilage probably reflects the increased loads placed on human knee cartilage due to the bipedal posture, as discussed by Stockwell (1971). For an acellular
xenogenic osteochondral graft to integrate and function well when implanted in a human joint, it is likely that a graft with similar thickness to the surrounding host cartilage would be best, therefore porcine and bovine tissues have preferred cartilage thickness for this purpose.

The GAG content of cartilage from each species was assessed qualitatively with alcian blue staining of tissue sections and quantitatively using a colourimetric assay with DMB dye. Qualitatively, all species showed characteristic zonal distribution of sulphated proteoglycans. Only staining of old ovine cartilage showed a distinct tidemark below which acidic mucins were not found, suggesting that this tissue was fully mature. Quantitatively, porcine cartilage had the highest GAG concentration, likely due to the young age of the tissue, as the concentration of proteoglycans is reportedly higher in immature cartilage, and decreases with age (Roughley & White, 1980). It has been suggested that age related reductions in cartilage GAG concentration are due to changes in chondrocyte activity; aggregcan and link protein synthesis decreases with maturity (Bolton et al., 1999). Except for cartilage of the medial condyle, there was no significant difference in the GAG content of bovine and ovine cartilage. A continuing trend showed cartilage of the groove and condyles having higher GAG content than other joint regions. The relationship in these joint areas of increased GAG content alongside increased thickness has been observed when tissues undergo increased loading or activity (Kiviranta et al., 1987). However, forces acting on the healthy knee are reportedly lower than those of the hip (Paul, 1976). This trend across the joint regions may again be due to the congruence of the joints, affecting the contact area and therefore stresses. Increased load on a very local scale may cause specific remodelling at that site, to increase thickness and GAG concentration so to decrease contact pressure.

Information on human cartilage GAG content is limited in the literature. Various figures have been quoted for the GAG concentration of cartilage from the femoral head, Vilim & Krajickova (1991) quote 54.7 and 38.6 mg.g\(^{-1}\) GAG per wet weight cartilage from two donors, while Hollander et al. (1991) reported values ranging between 25 – 40 μg.mg\(^{-1}\) wet weight. In the current study, porcine femoral head cartilage was 78 ± 30 μg.mg\(^{-1}\), bovine was 68 ± 8 μg.mg\(^{-1}\), young ovine was 45 ± 18 μg.mg\(^{-1}\) and old ovine was 62 ± 16
μg.mg⁻¹, all higher than values reported for human cartilage. GAG content was reported to be between 5-10 % total cartilage mass, as described for human cartilage by Mow et al. (2005). All species have a relatively high GAG content compared to human cartilage, however, as loss of GAGs is a limitation in decellularisation processes with SDS (Stapleton et al., 2008; Elder et al., 2010; Kier et al., 2011; Crapo et al., 2011; Benders et al., 2013), porcine cartilage, with the highest GAG content would be preferred.

The hydroxyproline content of cartilage from different species showed an inverse relationship with GAG content. Porcine cartilage had highest GAG content, but also had lowest hydroxyproline content. Muir et al. (1970) found great variation in human cartilage collagen content between individuals and joint regions, no correlation between collagen content and age was seen. The present study only quantitatively assessed collagen content of articular cartilage and did not investigate collagen fibre alignment or the ratios of different collagens present within the cartilage. Although collagen content appeared constant across species and joint regions there may be qualitative differences which have not been identified here.

Water content of the cartilages from different species and joint areas did not vary significantly on the whole, only significant differences were seen with bovine tibial cartilage. The water content of human cartilage varies between 68-85 % (Mow et al., 2005), the water content of cartilage from all joint regions and species lies within this range. Any species would therefore make an acceptable starting material, in terms of cartilage water content, however this parameter may differ once the tissue has undergone decellularisation.

Percentage deformation of cartilage appeared to vary with joint region, cartilage of the tibia tended to have higher deformation than the hip, this was reflected in the equilibrium elastic modulus derived for each type of tissue, with tibial cartilage showing a lower modulus. Tibial cartilage, with low GAG content, had higher permeability. As proteoglycans are responsible for restriction of fluid flow through the tissue, increased fluid flow results in reduced load supported by the fluid phase so tissues showed greater deformation. The relationship between material properties and
biological/biochemical composition was most pronounced in the bovine knee; cartilage of the medial condyle and medial tibia was thicker and had higher GAG content than its lateral counterpart, cartilage of the medial side of each joint also showed lower permeability, and stiffer equilibrium elastic modulus (lower percentage deformation).

Porcine cartilage was least stiff, and cartilage from old sheep was most stiff. Despite young ovine cartilage having higher GAG content, old ovine cartilage was stiffer, bovine cartilage was also stiffer than porcine cartilage, again despite the GAG content of porcine cartilage being higher. This is possibly due to the quality of the GAGs within these younger tissues. Cartilages from these more skeletally mature animals may have more effective proteoglycan structures which are more adequately immobilized within the cartilage matrix resulting in lower permeability and stiffer cartilage. As mentioned previously, old ovine cartilage had a yellowed appearance indicative of the presence of advanced glycation end products, the increased crosslinking of the collagen matrix results in a stiffer cartilage, which may explain the reduced deformation and increased equilibrium elastic modulus of old ovine cartilage (Bank et al., 1998; Verzijl et al., 2002). Old ovine cartilage being stiffer than bovine may be an effect of yet further maturity in GAG structures or an effect of ovine cartilage being much thinner than bovine. In this study, thinner cartilage tended to have a stiffer equilibrium elastic modulus. The equilibrium elastic modulus of bovine lateral condyle cartilage was similar to the equilibrium aggregate modulus quoted for human (0.70 MPa) and bovine (0.89 MPa) previously (Athanasiou et al., 1991; Mow et al., 2005). Porcine cartilage also had a similar elastic modulus, but was lower; this was possibly as a result of having softer subchondral bone. Old ovine cartilage had a much higher elastic modulus, so may be too stiff to replace human cartilage. Cartilage from all species was less permeable than values reported for human cartilage (Mow et al., 2005; Athanasiou et al., 1991).

Many of the differences seen in cartilage properties in the current chapter appear to be as a result of animal age and therefore skeletal maturity. The age of the species considered in this study was restricted by the availability of animals bred from the food chain for human consumption. Not only should
the specific parameters of each cartilage be considered here, but the
potential pros and cons of using a skeletally mature versus a more
biologically active immature tissue as a osteochondral scaffold as the
regenerative capacity may differ.

The characterisation of cartilage from different species and joint areas was
carried out with the aim of identifying a source material for decellularisation
with properties as similar as possible to those of human cartilage. Porcine
cartilage from 6 month old animals was the thickest of all cartilages studied,
however was not as thick as human. The GAG content of porcine cartilage
was high, however cartilage was highly cellular and did not have a fully
defined structure due to the immaturity of the tissue. Bovine cartilage from
18 month old animals, conversely, was mature. The GAG content of bovine
cartilage was lower than that found for porcine cartilage, but still within the
range of human tissues. Bovine cartilage was also relatively thick. Both
porcine and bovine cartilages had an equilibrium elastic moduli similar to the
equilibrium aggregate modulus reported for human cartilage. Ovine cartilage
from both age ranges was very thin, young ovine cartilage was not fully
mature with high cell density and less well defined structural organisation.
Ovine cartilages had lower GAG content than porcine.

Based on the parameters characterised in this chapter, osteochondral
tissues from the porcine medial condyle and bovine medial groove were
identified as the most suitable source materials for development of an
osteochondral decellularisation protocol.
Chapter 4
Development of a protocol for the decellularisation of bovine osteochondral tissues

4.1 Introduction

Surgical repair techniques for osteochondral lesions have shown varying degrees of efficacy, producing fibrocartilaginous repair tissue in the case of microfracture (Steinwachs et al., 2008; Lützner et al., 2009), or in the case of procedures such as autologous chondrocyte implantation (ACI) or mosaicplasty producing more hyaline-like repair materials but with varying degrees of donor site morbidity (Chaing & Xiong, 2009; Minas et al., 2001; Koulalis et al., 2004). Tissue engineering approaches have focused upon production of synthetic or natural scaffold materials, which avoid issues with donor site morbidity, but are unable to produce a hyaline-like repair tissue with the same composition, structure and function of natural articular cartilage (Frenkel & di Cesare, 2004).

Decellularisation of xenogeneic tissues has the potential to produce a natural extracellular matrix (ECM) scaffold, with the same biological, biochemical and biomechanical characteristics of natural tissue, and does not require tissue harvesting from a host donor site. Ideally, the decellularisation process will remove all immunogenic proteins and avoid issues of immunological rejection upon implantation (Crapo et al., 2011).

As discussed in Chapter 1, the ECM plays a key role in the function of articular cartilage as a low friction, load distributing tissue. In addition, it is understood that the molecularly diverse natural ECM is biologically active, having involvement in chemotaxis, cell mitogenesis (Bornstein & Sage, 2002) and differentiation and promoting tissue homeostasis. These attributes are thought to stem from the specific microarchitecture, surface
topology and composition of the ECM (Crapo et al., 2011). This interactivity with cells may facilitate the recellularisation and regeneration of acellular ECM more effectively than synthetic or artificially arranged natural material scaffolds, potentially resulting in improved clinical outcomes.
4.2 Decellularisation of natural tissues

There are many different methods which can be employed to decellularise tissues including chemical, enzymatic and physical techniques which can be used to varying extents depending on the type of tissue being treated. An acellular tissue, as defined by Crapo et al. (2011), should meet the following criteria:

- <50 dsDNA per ECM dry weight
- <200 bp DNA fragment length
- Lack of visible nuclear material in tissue sections stained with DAPI or H&E

Most decellularisation processes use a combination of chemical, enzymatic and physical methods to achieve adequate decellularisation of natural tissues. Each of these approaches is discussed below.

4.2.1 Chemical methods

4.2.1.1 Acids and bases

Acids and bases can hydrolyse biomolecules, this enables solubilisation of cellular membranes and removal of nucleic acids. Peracetic acid (PAA) has been successfully used at low concentrations (0.1 % v/v in 4% v/v ethanol) as a decellularisation agent for a number of thin tissues, including porcine small intestinal submucosa (SIS) and layers of the urinary bladder with minimal effect on the ECM structure (Freytes et al., 2004; Gilbert et al., 2008). Higher concentrations of PAA (5 % v/v) have, however, been shown to greatly alter the ECM structure (Wu et al., 2011). Concomitantly with decellularisation, PAA is a sterilant since it can penetrate microorganisms and generate oxygen free radicals which oxidise microbial proteins (Pruss et al., 1999). Other acids such as hydrochloric acid and sulphuric acid and bases such as NaOH (Schwartz et al., 2012) are also able to disrupt cellular membranes and decellularise matrices, but have been shown to dissociate GAGs from the tissue. Applying lime (a mixture of calcium hydroxide, soda
ash and sodium sulphate) to porcine dermis leading to severely reduced biomechanical and structural properties (Reing et al., 2010).

4.2.1.2 Hypotonic and hypertonic solutions

Hypertonic saline solutions have been shown to dissociate DNA from proteins (Cox & Emili, 2006) while hypotonic solutions induce cell swelling and bursting by osmosis. These solutions are also used to aid washout of cellular debris, however further chemical or enzymatic treatments are likely required to fully remove DNA and cellular debris.

4.2.1.3 Detergents

Detergents are amphipathic molecules comprising of a hydrophilic head group and a hydrophobic tail. In aqueous solution, these molecules spontaneously form micelles (Seddon et al., 2004). Detergents disrupt cellular membranes by inserting into lipid bilayers. At concentrations above the critical micelle concentration detergents form micelles containing native lipids and encapsulating membrane proteins which can then be washed from the tissue.

4.2.1.3.1 Ionic detergents

Ionic detergents have a hydrophilic head with either a negative or positive net charge. Ionic detergents are extremely effective in the solubilisation of membrane proteins but are almost always denaturing to some extent through the disruption of protein-protein interactions (Seddon et al., 2004). SDS (Figure 4.1) is an anionic detergent commonly used in decellularisation processes (Booth et al., 2002, Stapleton et al., 2008, Kheir et al., 2011)

![Sodium dodecyl sulfate (SDS)](image)

Figure 4.1 Molecular structure of sodium dodecyl sulphate (SDS). Adapted from Seddon (2004).
The use of ionic detergents such as SDS and sodium deoxycholate (SDC) has been shown to be more effective than the use of non-ionic or zwitterionic detergents for tissue decellularisation (Booth et al., 2002). However, ionic detergents have been shown to disrupt matrix proteins, with tissues showing a loss of GAGs and collagen damage when ionic detergents, such as SDS have been used at high concentrations (1.0 – 2.0 % w/v; Samouillan et al., 2000; Elder et al., 2010). These effects may, in part, have been the result of matrix metalloproteinase activity following release of cellular proteases during cell membrane disruption and cell lysis. These deleterious effects have been minimised by using low detergent concentrations (SDS, 0.1 % w/v; SDC 0.5 % w/v) and protease inhibitors (Booth et al., 2002).

4.2.1.3.2 Non-ionic detergents

Non-ionic detergents have uncharged hydrophilic head groups and have minimal effects on protein–protein interactions, so do not have a deleterious effect on the protein components of the ECM (Seddon et al., 2004). Triton X-100 is one of the most widely used non-ionic detergents for decellularisation treatments (Courtman et al., 1995; Vavken et al., 2009).

\[
\begin{align*}
  x &= 9, \text{Triton}^\text{®} \text{ X-100} \\
  x &= 7-8, \text{Triton}^\text{®} \text{ X-114}
\end{align*}
\]

**Figure 4.2 Molecular structure of triton X-100.** Adapted from Seddon (2004).

The effects of Triton X-100 as a decellularisation agent have been shown to be variable and dependent on exposure time and tissue type. Decellularisation of porcine heart valves with Triton X-100 (1 % v/v) with 0.2 mg.ml\(^{-1}\) DNase and 20 μg.ml\(^{-1}\) RNase for 24 h at 37 °C was successful, however this lead to a near complete loss of GAG as well as reduction of laminin and fibronetin ( Grauss et al., 2005). Conversely, Woods & Gratzer (2005) did not see deleterious effects upon GAG or collagen content of ACL-bone tissues following decellularisation with 1% (v/v) Triton X-100 in
combination with hypotonic and nuclease treatment, however full cell removal was not achieved.

4.2.1.3.3 Zwitterionic detergents
Zwitterionic detergents possess both a negative and positive charge at different regions of the molecule. As such, they display properties of both ionic and non-ionic detergents, having greater effect on disrupting protein-protein interactions than non-ionic detergents.

\[ x = H, \text{CHAPS} \]
\[ x = OH, \text{CHAPSO} \]

**Figure 4.3 Molecular structure of CHAPS.** Figure adapted from Seddon (2004).

One of the most commonly used zwitterionic detergents for the decellularisation of tissues is 3-[[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS). Du *et al.* (2010) found that decellularisation of porcine corneas with 0.5 % and 1.0 % (w/v) CHAPS for 36 h histologically had little effect in removing cells and cellular debris, and destroyed tissue histoarchitecture. Booth *et al.* (2002) found no histological differences in the biochemical components of porcine aortic tissue following 72 h incubation with CHAPS (0.5 % w/v), however decellularisation was not achieved with this detergent.

### 4.2.2 Enzymatic methods

Enzymes such as nucleases, collagenase, lipase and α-galactosidase have been used as part of decellularisation procedures alongside other agents to provide higher specificity for removal of undesirable constituents (Gilbert *et al*., 2006; Crapo *et al*., 2011). Only trypsin has been used on its own as a
successful decellularisation technique. Cebotari et al. (2002) treated human aortic and pulmonary heart valves with a solution of trypsin/EDTA (containing 0.5 % w/v trypsin and 0.2 % w/v EDTA) in PBS (1:10 ratio) for 48 h at 37 °C. Complete decellularisation was reported with preservation of ECM. Conversely, Grauss et al. (2005) used 0.5 % (w/v) trypsin and 0.05 % (w/v) EDTA in the presence of DNase, RNase and gentamycin for 17 h at 37 °C to decellularise porcine aortic valves and found that pyknotic cell nuclei remained and that significant damage to the elastin and collagen fibres had been sustained, along with a moderate reduction in GAG content seen histologically.

Nucleases are commonly used to cleave nucleic acids and enable the more effective wash out of nuclear material from tissues. The successful use of nucleases to reduce tissue DNA content following detergent treatment has been reported for a number of tissues, including heart valves, tendon, meniscus and osteochondral tissues without adverse effect on tissue ECM (Booth et al., 2002; Ingram et al., 2007; Stapleton et al., 2008; Kheir et al., 2011).

Treatment of xenogeneic tissues with α-galactosidase can be required as part of a decellularisation bioprocess to specifically remove galactose α1-3 galactose (α-gal), a xenogeneic cell membrane protein which leads to hyperacute rejection of xenografts (Stapleton et al., 2011).

### 4.2.3 Physical methods

A number of physical methods can be employed in combination with chemical and enzymatic methods to decellularise tissues. These include freezing, the application of direct pressure, agitation and sonication (Gilbert et al., 2006, Crapo et al., 2011). Freezing is commonly used and has been included in processes for cartilage (Kheir et al., 2011) and meniscus (Stapleton et al., 2008) decellularisation, amongst other tissues. Freezing produces ice crystals within the cells, which disrupt membranes, resulting in cell lysis. The formation of crystals within the ECM also acts to increase tissue permeability, improving perfusion of subsequent solutions through tissues and enhancing the wash out of cellular debris. Freeze-thawing has,
however, been linked to the denaturation of proteins (Cao et al., 2003), which may result in adverse effects on the biomechanical performance of the tissues. As ice crystal size is affected by freezing rate, it is important to select the correct temperature at which to freeze tissues so as to balance damage to the matrix against cell lysis (Gilbert et al., 2006).

Funamoto et al. (2010) have reported successful decellularisation of porcine aortic blood vessels using high-hydrostatic pressure. Vessels were sealed in a bag containing PBS and placed in a cold isostatic pressurization machine. An atmospheric pressure of 980 MPa was gradually applied (at a rate at which the starting temperature was maintained) then sustained for 10 min, following which vessels were washed in PBS for 14 days. At high pressures, the temperature at which water freezes is increased, so temperature and magnitude of pressure needs to be carefully optimised. The rate of pressure increase/decrease should also be monitored, as drastic changes in pressure alter the temperature. The temperature of the pressurisation chamber was maintained above 30 °C at 980 MPa, to prevent ice crystal formation and avoid deleterious effects on the tissue ECM. No histological damage was seen in tissues pressurised at 30 °C, however those decellularised using a starting temperature of 10 °C showed damage to the collagen fibre network following formation of ice crystals as a result of high pressure. Both conditions resulted in reduction of the mechanical properties of the tissue compared to native vessels.

In combination with chemical or enzymatic treatment, mechanical scraping has been employed to remove epithelial layers from basement membranes such as the amniotic membrane. However, results have been inconsistent and often lead to damage of the basement membrane (Hopkinson et al., 2008).

Agitation and sonication can be employed to increase the penetration of enzymatic and chemical decellularisation solutions and therefore improve cell lysis and wash out of cell debris. Orbital shakers, magnetic stirrers and low profile rollers have been used to agitate solutions and the tissues within them (Gilbert et al., 2006). The rate of agitation can affect the wash out of DNA from a tissue, and can vary depending on the size and density of the
tissue to be decellularised. Increasing agitation rate has been reported to increase DNA wash out, but adversely can also increase the wash out of ECM components, so needs to be optimised for each tissue type (Crapo et al., 2011). Sonication has been employed in decellularisation bioprocesses to increase the permeability of dense tissues and improve recellularisation (Ingram et al., 2007). However, optimisation of sonication frequency and duration is necessary to avoid excess structural damage to the tissue.
4.3 Aims and objectives

Aims:

The aim of the research described in this chapter was to develop a process using low concentration SDS plus proteinase inhibitors to decellularise bovine osteochondral pins obtained from the medial femoral groove.

Objectives:

- To develop a protocol to remove cells and cellular debris from bovine osteochondral pins while retaining the natural histoarchitecture, biochemical composition and biomechanical function of the native osteochondral tissues.

- To characterise the resultant decellularised ECM and compare to native tissue using:
  - Histological characterisation to identify the presence/lack of cells and to observe any changes in the histoarchitecture of the tissue.
  - DNA quantification to determine the reduction in DNA compared to native tissue following decellularisation.
  - Determination of the GAG and collagen content of decellularised scaffolds.
  - Determination of the mechanical properties of cartilage following decellularisation.

- To characterise immunohistochemically the distribution of collagen type VI and COMP in the native and decellularised bovine osteochondral matrix.
4.4 Methods

4.4.1 Decellularisation solutions

4.4.1.1 PBS

A solution of PBS was produced as described in Section 2.1.7.1. When required, PBS was solution was sterilised by moist heat, as described in Section 2.2.1.3. Solution was stored at room temperature for up to 1 month.

4.4.1.2 PBS with aprotinin (10 KIU.ml⁻¹ aprotinin)

A solution of PBS was produced as described in Section 2.1.7.1. When required, the solution was sterilised by moist heat, as described in Section 2.2.1.3. Solution was stored at room temperature and used within 1 month. Just prior to use, 1 ml aprotinin (10,000 KIU.ml⁻¹) was added to 1 L PBS.

4.4.1.3 Hypotonic buffer (10 mM Tris, 10 KIU.ml⁻¹ aprotinin)

Trisma base (1.21 g) was dissolved in 990 ml distilled water. The pH was adjusted to 8.0 – 8.2 using 6 M HCl and the volume made up to 1 L. When necessary, the solution was sterilised by moist heat, as described in Section 2.2.1.3. Solution was stored at room temperature for up to 1 month. Just prior to use, 1 ml aprotinin (10,000 KIU.ml⁻¹) was added.

4.4.1.4 SDS in hypotonic buffer (0.1 % w/v SDS, 10 mM Tris, 10 KIU.ml⁻¹ aprotinin)

SDS (10 g) was dissolved in 100 ml distilled water to produce an SDS solution. SDS solution (10 ml) was added to 990 ml hypotonic solution. When necessary, the solution was sterilised by moist heat, as described in section 2.2.1.3. Solution was stored at 4 ºC and used within 1 week. Just prior to use, 1 ml aprotinin (10,000 KIU.ml⁻¹) was added.

4.4.1.5 Nuclease solution (50 mM Tris, 10 mM MgCl₂, 50 U.ml⁻¹ DNAase, 1U.ml⁻¹ RNAase)

Trisma base (6.1 g) and magnesium chloride (MgCl₂; 2 g) were dissolved in 80 ml distilled water. The pH was adjusted to 7.5 – 7.7 using 6 M HCl. The volume was then made up to 990 ml using distilled water. At this stage, when required, the solution was sterilised by moist heat, as described in
Section 2.2.1.3. Just prior to use, 5 ml DNase (10,000 U.ml\(^{-1}\)) and 10 ml RNase (100 U.ml\(^{-1}\)) were added. Solution was used within 10 min of preparation.

4.4.1.6 Peracetic acid solution (0.1 % v/v)

Peracetic acid (PAA; 1.57 ml) was added to 500 ml distilled water and adjusted to pH 7.2 – 7.5 using 6 M NaOH. Solution was used within 1 h of preparation.

4.4.1.7 EDTA solution (12.5 % w/v)

EDTA solution was produced as described in Section 2.2.3.2.1, and if necessary was sterilised by moist heat as described in Section 2.2.1.3. Solution was stored at room temperature and used within 6 months of preparation. Just prior to use 1 ml aprotinin (10,000 KIU.ml\(^{-1}\)) was added to 1 L EDTA solution.

4.4.2 Decellularisation processes

Decellularisation methods using low concentration 0.1 % (w/v) SDS plus proteinase inhibitors were developed based on the initial process described by Booth et al. (2002) and adapted following methods developed by Stapleton et al. (2008) and Kheir et al. (2011). Osteochondral pins (9 mm diameter, 10 – 12 mm deep) were placed 4 – 5 per 150 ml pot into which 125 ml of each solution was added. All incubations were carried out at 42 ± 2 °C except the nuclease treatment which was performed at 37 ± 1 °C and the PAA treatment which was performed at 27 ± 1 °C. All solutions were pre-warmed to the appropriate temperature. Agitation on an orbital shaker at 240 rpm was carried out during each incubation excluding the nuclease step, which was agitated at 80 rpm, pots were laid horizontally on the shaker.

4.4.2.1 dCELL 1 (basic decellularisation)

Osteochondral pins were harvested from the bovine medial femoral groove, as described in Section 2.2.2 and stored at – 20 °C on PBS moistened filter paper. Pins (3 – 5 pins per pot from n=3 animals) were thawed at room temperature (RT) for 1 - 2 h or until fully defrosted and then re-frozen on
PBS moistened filter paper at -20 °C for 3 h. Pins were again thawed at RT for 1 – 2 h or until fully defrosted before hypotonic buffer was added and pins were frozen at -80 °C. Pins were thawed at 45 °C in a water bath for 1 - 2 h or until fully defrosted before 1 further cycle of freeze/thaw in hypotonic solution was completed. Pins were washed for 3 x 10 min in PBS with aprotinin before being incubated in hypotonic buffer for 16 h. An incubation in SDS in hypotonic solution then followed for 24 h. Pins were washed for 3 x 10 min in PBS with aprotinin and incubated in nuclease solution for 3 h. Pins were then washed for 3 x 10 min in PBS without aprotinin and incubated in PAA solution for 3 h. A final set of 3 x 10 min PBS without aprotinin washes were performed and the pins were stored frozen at -20 °C or used immediately for analysis.

4.4.2.2 dCELL 2 (extended PBS wash)

This process was exactly the same as that described in Section 4.4.2.1 with the addition of a 48 - 60 h extended final PBS wash.

4.4.2.3 dCELL 2 (hypotonic)

This process differed from dCELL 2 described in Section 4.4.2.2 only in that all four freeze/thaw cycles were conducted in hypotonic buffer containing 10 KUI.ml⁻¹ aprotinin.

4.4.2.4 dCELL 2 (distilled water)

This process differed from dCELL 2 described in Section 4.4.2.2 only in that all freeze/thaw cycles were conducted in distilled water containing 10 KUI.ml⁻¹ aprotinin.

4.4.2.5 dCELL 2 (+ ultrasonication)

This processed followed the same method outlined in Section 4.4.2.2 however, prior to the initial freezing, pins were ultrasonicated. The technique was based on work by Ingram et al. (2007) and Stapleton (2008b). Each osteochondral pin was placed in a custom wire mesh holder and positioned under an ultrasonic probe in a bath of ice cold PBS. Each sample was sonicated at 70 watts for 15 min with 6 second pulses.
4.4.2.6 dCELL 3 (water pik)

Water pik treatment of the subchondral bone was added to the dCELL 2 method described in Section 4.4.2.2. Following the first thaw, blood clots and marrow from the subchondral bone were removed using a water pik (Figure 4.4). A thin jet of PBS (200 - 400 ml per pin) was applied directly to the bone to force out marrow components.

Figure 4.4 Water pik. PBS (stored in the reservoir) was pumped through the nozzle and forced into a thin jet when directed through the tip. The intensity of the jet can be controlled using the dial.

4.4.2.7 dCELL 3 (hirudin)

A 16 h incubation in the anticoagulant enzyme hirudin (1 U.ml⁻¹ in PBS with 10 KUI.ml⁻¹ aprotinin) at RT was added to the process described in Section 4.4.2.6 before treatment with the water pik.

4.4.2.8 dCELL 3 (hirudin applied to the bone only)

Rather than incubating the entire osteochondral pins in hirudin solution (1 U.ml⁻¹ in PBS with 10 KUI.ml⁻¹ aprotinin), as described in Section 4.4.2.7, pins were placed in 6 well plates and 8 ml hirudin solution was added to each well (Figure 4.5). The lid of each plate was lined with PBS moistened
tissue paper to maintain a moist environment for the exposed cartilage. Plates were incubated at 37 °C on an orbital shaker at 80 rpm for 18 h.

Figure 4.5 Anticoagulant application to bony region of osteochondral pins. Pins were placed in a 6 well plate with the bony component submerged in an anticoagulant solution.

4.4.2.9 dCELL 3 (monosodium citrate applied to the bone only)

As an alternative to the hirudin used in Section 4.4.2.8, the bone component was incubated in a 0.4 % (w/v) solution of monosodium citrate. Sodium hydrogen carbonate (NaHCO₃; 1.98 g) and citric acid (C₆H₈O₇; 3.92 g) were dissolved in 1 L distilled water and pH adjusted to 7.0. Plates were incubated at 37 °C on an orbital shaker at 80 rpm for 18 h.

4.4.2.10 dCELL 3 (PBS)

The same method was followed as in Section 4.4.2.7, however the hirudin solution was substituted for PBS and the incubation was completed at RT.

4.4.2.11 dCELL 4 (PBS at 45 °C)

This followed the method outlined in Section 4.4.2.10 however the incubation in PBS prior to the water pik treatment was carried out at 45 °C rather than RT.
4.4.2.12 dCELL 4 (+ extra nuclease)

In this process, an additional nuclease treatment was added to the protocol outlined in Section 4.4.2.11. Following the first 3 h incubation in nuclease solution, pins were washed for 3 x 10 min in PBS without aprotinin before being subject to a second nuclease treatment for a further 3 h incubation.

4.4.2.13 dCELL 4 (+ decalcification)

The process described in Section 4.4.2.11 was adjusted to include a decalcification step. Following water pik treatment, pins were decalcified in 12.5 % (w/v) EDTA for 1, 2, 4, 8 or 12 days with incubation at 45 °C and agitation at 240 rpm. Incubation for 4 days was selected for further process development.

4.4.2.14 dCELL 5 (+ hypotonic/SDS cycle)

The dCELL 4 process described in Section 4.4.2.11 was adapted to include a further cycle of washing with hypotonic buffer and SDS in hypotonic buffer. Following the first 24 h incubation in SDS solution, pins were incubated in hypotonic buffer for 24 h and again in SDS in hypotonic buffer for 24 h before continuing with PBS washes, nuclease and PAA treatments.

4.4.3 Histological characterisation

Histological characterisation of fresh and decellularised osteochondral tissues was carried out as described in Section 2.2.3. H&E, DAPI and alcian blue stains were performed.

4.4.4 DNA quantification

4.4.4.1 Tissue lyophilisation

Tissues were freeze dried prior to digestion as described in Section 3.4.1.1. Cartilage was removed from the bone using a scalpel and macerated prior to freeze drying.
4.4.4.2 Cartilage digestion using proteinase K

Tissues were digested using solutions provided in the QIAGEN DNeasy blood and tissue kit using a method based on the manufacturers’ instructions. Briefly, lyophilised native cartilage tissue (25 g wet weight) was placed in a sterile 2 ml eppendorf tube. Digestion buffer ATL (180 µl) and proteinase K (20 µl) were pre-mixed then added to the tissue. Digests were vortexed briefly then incubated at 56 °C for 16 h with agitation at 80 rpm.

Lyophilised decellularised cartilage tissue (~250 g wet weight) was digested as above, but using 360 µl buffer ATL with 40 µl proteinase K. If digestion was not complete after 18 h, a further 180 µl ATL and 20 µl proteinase K were added and incubation continued until digestion was complete.

4.4.4.3 Cartilage DNA purification

Once digestions were complete, DNeasy spin columns were used to extract and purify DNA from the digests. The manufacturers’ instructions were followed for native tissue. For decellularised tissue digests, the volumes of ethanol and buffer AL were doubled when precipitating DNA, as the digest volume was doubled. Columns were rinsed and then the isolated DNA was eluted from the membrane using 200 µl buffer AE. Elution was repeated a second time into the same eppendorf to ensure that all of the DNA was recovered.

4.4.4.4 DNA quantification

Purified DNA was quantified using a nanodrop nanospectrophotometer. Samples and standards (2 µl) were pipetted onto the pedestal and their absorbance measured at 260 nm. Absorbance was normalised to a blank of AE buffer. A standard curve was produced using known concentrations of calf thymus DNA to calibrate the nanodrop spectrophotometer.

4.4.5 Biochemical characterisation

The GAG, hydroxyproline and water content of fresh and decellularised tissues was determined using methods described in Section 3.4.
4.4.6 Biomechanical characterisation

The percentage deformation of decellularised cartilage was determined using the indentation methods described in Sections 3.5.1 and 3.5.2.

4.4.7 Immunohistochemical labelling

4.4.7.1 Reagents

- **Hydrogen peroxide solution** (3 % v/v) 20 ml hydrogen peroxide (30 % v/v) 180 ml PBS
- **Tris buffer (2 M, pH 7.6)** 242.26 g Trizma base 758 ml distilled water
- **Sodium chloride solution (3M)** 175.32 g sodium chloride 1 L distilled water
- **Tris buffered saline (TBS)** 25 ml Tris buffer (2 M) 50 ml sodium chloride solution (3 M) 925 ml distilled water
- **TBS containing 0.05 % (w/v) Tween 20** (TBS-T) 500 µl Tween 20 1 L TBS
- **Bovine serum albumin** (BSA; 5 % w/v) 2.5 g BSA 500 ml PBS
- **Antibody diluent** 6 ml Sodium azide (1 % w/v) 300 µl BSA (5 % w/v) 54 ml TBS 1 L distilled water

4.4.7.2 Sample preparation

Fresh and decellularised tissues (n=3) were fixed in 10 % (v/v) NBF, decalcified, wax embedded, sectioned and transferred onto slides as
described in Sections 2.2.3.1 – 2.2.3.4, sections were then dewaxed and rehydrated as described in Section 2.2.3.5.

4.4.7.3 Antigen retrieval
As tissues had been NBF fixed, antigen retrieval was performed to re-expose tissue epitopes for antigen binding. Following dewaxing and rehydration, each section was circled using a hydrophobic marker and proteinase K solution applied drop wise until sections were fully covered. Sections were incubated at room temperature for 20 min.

4.4.7.4 Antibodies
All antibodies were diluted to optimised working concentration in antibody diluent.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Supplier</th>
<th>Product code</th>
<th>Isotype</th>
<th>Stock concentration</th>
<th>Dilution/ Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen Type VI</td>
<td>AbD Serotech</td>
<td>AHP2049</td>
<td>IgG</td>
<td>1.0 mg.ml(^{-1})</td>
<td>1 in 500 2.0 mg.L(^{-1})</td>
</tr>
<tr>
<td>COMP</td>
<td>AbCam</td>
<td>ab74524</td>
<td>IgG</td>
<td>0.4 mg.ml(^{-1})</td>
<td>1 in 80 5.5 mg.L(^{-1})</td>
</tr>
<tr>
<td>IgG</td>
<td>AbCam</td>
<td>ab27478</td>
<td>-</td>
<td>0.2 mg.ml(^{-1})</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.1 Antibodies. Antibodies and isotype controls used during immunohistochemical analysis of native and decellularised bovine osteochondral tissues

4.4.7.5 Labelling of tissue sections using monoclonal antibodies
Following antigen retrieval (Section 6.3.1.3) sections were immersed in hydrogen peroxide (3 %, v/v) in PBS for 10 min at room temperature to block endogenous peroxidase activity, and then washed for 3 min in running tap water. Sections were briefly washed with TBS on a plate rocker. To block non-specific background staining, dual endogenous enzyme block (25 µl) from the Ultra vision kit was added and sections were incubated for 10 minutes, followed by two ten min washes in TBS on a plate rocker. The primary antibody (Table 6.1) was then applied at working concentration and incubated at room temperature for 1 h. Sections were then washed twice for
10 min in TBS-T, then twice for 10 min in TBS on a plate rocker. Labelled polymer-HRP (20 µl) from the Ultra vision kit was then added to each section and incubated for 30 min in the dark at room temperature. Sections were again washed twice for 10 min in TBS-T, then twice for 10 min in TBS on a plate rocker. Excess buffer was tapped off before substrate chromogen (20 µl liquid 3,3-diaminobenzidine (DAB) chromagen plus 1 ml substrate buffer) was added to each section and incubated at room temperature for 10 min. Sections were rinsed four times in distilled water before being immersed in haematoxylin (Mayer’s) for 10 sec. Sections were washed for 3 min in running tap water before being dehydrated and mounted in DPX mountant, as described in Section 2.2.3.6. To verify specific antibody binding, an isotype control was performed on native and decellularised tissue sections using IgG in place of the primary antibody at the same concentration as the primary antibody had been used. An antibody-free negative control was also included, in which sections were incubated in antibody diluent alone in place of the primary and secondary antibody. Once dry, sections were viewed using normal light microscopy.
4.5 Results

Initial attempts to decellularise bovine osteochondral tissues were not successful and this led to numerous alterations to the process which are documented below.

Images of sections of native bovine osteochondral tissue and tissue subject to various decellularisation protocols stained with H&E, DAPI or alcian blue/PAS are presented in Figures 4.6, 4.7 and 4.8 respectively. DNA, GAG and hydroxyproline content of native bovine cartilage and tissues subject to various decellularisation protocols are presented in Figures 4.9, 4.10 and 4.11 respectively. The percentage deformation of native osteochondral tissues and tissues subject to various decellularisation protocols is presented in Figure 4.12.

4.5.1 Native tissue

Native bovine medial groove osteochondral sections stained with H&E (Figure 4.6 a) showed characteristic cartilage architecture and cell organisation, cells were present throughout the depth of the cartilage tissue and were situated in chondrons. At the superficial layer, cells were smaller and more randomly orientated, in the mid and deep zones linear columns of cells aligned perpendicularly to the cartilage-bone interface were seen. There was a darker stained layer at the cartilage surface indicating a healthy, smooth articulating surface. Pink eosin staining become more intense in the bone and the intratrabecular spaces of the bone could be seen, small, round osteocytes were nestled in lacunae within the bone tissue. Bone marrow was also present within the subchondral bone with a high cell density. This organisation of cells and extent of cellularisation could also be seen in DAPI stained sections (Figure 4.7 a). Staining with alcian blue showed more intense blue GAG staining in the middle zone and slightly less staining in the deep and superficial zones. There was an absence of GAGs in the bone (Figure 4.8 a). Native cartilage contained 261.1 ± 29.3 μg.mg⁻¹ sulphated proteoglycans per cartilage dry weight (Figure 4.10).
Native cartilage had a DNA content of 119 ± 36 ng.mg$^{-1}$ wet weight (Figure 4.9).

4.5.2 dCELL 1

After application of the dCELL 1 basic protocol developed by Stapleton et al. (2008), staining of treated tissue sections with H&E revealed that there were still a number of cells present in the cartilage and bone (Figure 4.6 b). In the superficial zone, a number of empty lacunae could be seen, which were slightly enlarged. The general structure of the tissue was maintained, marrow was present in the bone, but did not contain whole cell nuclei. DAPI staining confirmed a reduction in cell nuclei, but showed that a large number of cells remained (Figure 4.7 b). There was no significant reduction in cartilage DNA content compared to native tissue (Figure 4.9). There was a slight loss of intensity of alcian blue staining throughout the cartilage, and PAS staining was slightly present at the very superficial layer (Figure 4.8 b), indicating that loss of GAGs has occurred. The GAG content of cartilage following treatment with the dCELL 1 protocol was not significantly different from native tissue, however a reduction was noted (Figure 4.10).

4.5.3 dCELL 2

Following the introduction of an extended PBS wash to the end of the basic protocol, sections of osteochondral pins decellularised using the dCELL 2 protocol as outlined in Section 4.4.2.2 showed a much greater reduction in cell content (Figure 4.6 c). Empty lacunae, again, appeared slightly enlarged and within the empty lacunae, there was light eosin staining, which may have indicated the collapsed collagen type VI shell, as previously reported by Kheir et al. (2011). These stained regions within lacunae did not stain with haematoxylin or DAPI (Figure 4.7 c) so do not contain nuclear material. DNA quantification showed a significant reduction from native tissues (Figure 4.9). However, nucleated cells were still visible in the deep/calcified cartilage regions and in the subchondral bone. There appeared to be an
overall reduction in GAGs in cartilage tissues (Figure 4.8 c), which when quantified, was not significantly different from native tissues (Figure 4.10).

To enhance osmotically induced cell lysis in the deep/calcified regions and improve decellularisation, alterations were made to dCELL 2 protocol to freeze/thaw entirely in hypotonic buffer or distilled water. As seen in Figure 4.6 d & e and Figure 4.7 d & e cells were still present in the dense deep cartilage and bone in similar numbers to those seen following use of the dCELL 2 protocol. The DNA content of cartilage treated with hypotonic freeze/thaw did not differ significantly from native cartilage, however distilled water freeze/thawed tissue did show a significant reduction (Figure 4.9). Both protocols resulted in similar GAG reduction to that of dCELL 2 protocol (Figure 4.8 d & e), which when quantified, was not significantly different from native tissues (Figure 4.10).

Ultrasonication was employed in an attempt to increase the permeability of the matrix and aid perfusion of decellularisation solutions through the deeper cartilage and bone. No histological changes could be seen in cartilage or bone structure following treatment with the sonicating probe (Figure 4.6 f). Similar to the freeze/thaw alterations to the dCELL 2 protocol, ultrasonication did not improve the removal of cells (Figure 4.7 f) nor did it have any additional effects on GAG retention (Figure 4.8 f and Figure 4.10) although DNA content was significantly reduced from that of native tissue (Figure 4.9).

### 4.5.4 dCELL 3

To increase decellularisation solution access to the bone and deep cartilage layers a water pik was used to remove bone marrow from within the trabecular spaces (dCELL 3 protocol). Visibly, not much marrow was removed following application of the water jet. Histologically, no damage to the cartilage or bone was seen (Figure 4.6 g), there was no additional adverse effects on cartilage GAGs (Figure 4.8 g and Figure 4.10), although cells were still visible in the tissue and within denser areas of bone (Figure 4.7 g). The DNA content of cartilage was significantly reduced compared to native cartilage (Figure 4.9).
As bovine tissues were received 3 – 4 days post-slaughter, blood within the marrow had clotted, potentially making removal using the water pik difficult. To overcome this issue, an anticoagulant, hirudin, was applied to osteochondral pins to increase the efficacy of the water pik treatment. Marrow was more easily removed, and histologically, there appeared to be a greater extent of cell removal (Figure 4.6 h and Figure 4.7 h) compared to use of the water pik alone. The DNA content of cartilage was significantly reduced compared to native cartilage (Figure 4.9). However, blue staining was less intense, indicating a greater loss of GAGs with use of the enzyme (Figure 4.8 h), however the reduced GAG content was not significantly different to native cartilage (Figure 4.10).

Since application of hirudin to the cartilage had an adverse effect on GAGs, a different method was developed to submerge only the bony component of osteochondral pins into the enzyme (section 4.4.2.8), a non-enzymatic anticoagulant, monosodium citrate, was also used in this novel set up to avoid enzymatic removal/degradation of GAGs. The incubation temperature was increased to 37 °C to increase the rate of diffusion. This method was unsuccessful as there was significant loss of GAGs with hirudin treatment (Figure 4.8 i and Figure 4.10). Monosodium citrate did not cause as great a reduction in cartilage GAGs as hirudin (Figure 4.8 j & Figure 4.10). Cell removal was not as effective following either of these treatments as was seen when pins were completely submerged in hirudin (Figure 4.6 i & j and Figure 4.7 i & j). However, the reduction in DNA was still significant (Figure 4.9).

As a negative control for the anticoagulant activity of hirudin, pins were also incubated in PBS prior to use of the water pik. Water pik treatment was equally effective at removing marrow following incubation of pins in PBS as it was with hirudin incubation (Figure 4.6 k and Figure 4.7 k). The reduction in DNA content was equally acceptable (Figure 4.9). The adverse effects on GAGs were minimised using PBS rather than hirudin (Figure 4.8 k and Figure 4.10).
4.5.5 dCELL 4

As bone marrow has a high lipid content, it was thought that marrow wash out would be more effective at a higher temperature, when marrow fats would be liquid rather than solid. To this end, the temperature of the pre-water pik treatment PBS incubation was increased to 45 °C. This greatly improved ease and extent of marrow removal with the water pik (dCELL 4 protocol). However no improvement in cell presence or DNA reduction in cartilage tissue was seen compared to incubation in PBS at room temperature (Figure 4.6 l, Figure 4.7 l and Figure 4.9), and a significant reduction in GAG content compared to native tissue was observed (Figure 4.8 l, Figure 4.10).

To improve DNA removal, an additional nuclease step was included in the dCELL 4 protocol. No improved cell removal was seen compared to the use of a single nuclease treatment (Figure 4.6 m and Figure 4.7 m) and again, GAG content was significantly lower than that of native cartilage (Figure 4.8 m and 4.10).

As cell retention appeared to occur in dense bony regions and calcified cartilage, it was thought that decalcification of the osteochondral pins might improve access to these regions. Cells were still visible in the deep cartilage regions (Figure 4.5 n and Figure 4.7 n) and cartilage DNA content did not differ from dCELL 4 (Figure 4.9). GAG content of EDTA treated cartilage was significantly lower than native tissues (Figure 4.8 n and Figure 4.10).

4.5.6 dCELL 5

To achieve full decellularisation, other processes have required repeat cycles of SDS and hypotonic washes (Stapleton et al., 2008; Kheir et al., 2011), however these have resulted in greater GAG loss. Two cycles of SDS were incorporated into the dCELL 5 protocol, so cells were exposed to additional osmotic stress and membranes would be more effectively removed. Following decellularisation with dCELL 5 protocol, cartilage only contained 7 ± 6 ng.mg⁻¹ DNA per wet weight (39 ng.mg⁻¹ DNA per dry weight), a reduction of 94 % (Figure 4.9). This protocol also led to full
removal of whole cells from both cartilage and bone (Figure 4.6 o and Figure 4.7 o). Empty lacunae were enlarged throughout the cartilage ECM (Figure 4.6 o). However, following the application of the protocol, the presence of GAGs within the cartilage tissue was greatly reduced (Figure 4.8 o). The GAG content of the acellular cartilage was significantly reduced to 2.5 ± 2.8 µg.mg⁻¹; a 99 % (w/w) reduction from native tissue (Figure 4.10). Previous protocols, despite resulting in reduced cartilage GAG content, did not have adverse effects on the tissue biomechanics. Following application of this protocol however, the severe reduction in GAGs was found to result in a significant increase in the percentage deformation from 30.4 (+ 7.6, - 7.1) % deformation of native cartilage to 48.4 (+ 20.1, - 21.2) % (Figure 4.12). The reduction in GAG content resulted in a significant increase in the cartilage collagen content as a proportion of tissue dry weight from 72.3 ± 26.5 µg.mg⁻¹ (native) to 146.3 ± 65.1 µg.mg⁻¹ (dCELL 5; Figure 4.11).

To further characterise the acellular bovine osteochondral matrix produced following the application of the dCELL 5 protocol, immunohistochemical analysis was performed to localise specific matrix proteins in native and decellularised tissues. Tissues were labelled with antibodies for collagen type VI (Figure 4.13). In native tissues, brown staining was seen throughout the superficial and middle zones of the cartilage, with greater intensity in the superficial region, little to no staining was seen in the deep zone. Intense staining was found localised to the cartilage chondrons around chondrocytes. Decellularised tissues showed very little staining, and no increased intensity could be seen at chondrons. Isotype and antibody free controls did not show any brown staining for native tissue, however slight brown staining was seen in the decellularised tissue isotype control.

Images of tissue sections labelled with antibodies against COMP care presented in Figure 4.14. Staining was evenly dispersed across the native cartilage, but was absent in the deep calcified region and was slightly depleted at the cartilage surface. Decellularised tissues also showed similar staining, however there was a more distinct loss of staining at the surface layer and at higher magnification (100 x) a graininess could be seen in the stain, which was not present in the native tissue stains. Again, isotype and antibody-free controls were negative for native tissue, however the isotype
control for decellularised tissue was positive (Figure 4.14 g), although the graininess seen when labelling the decellularised cartilage with antibody against COMP was not present in the isotype.
Figure 4.6 H&E stained sections of native and decellularised bovine osteochondral pins. a) fresh, b) dCELL 1, c) dCELL2, d) dCELL 2 with hypotonic freeze/thaw, e) dCELL 2 with distilled water freeze/thaw, f) dCELL 2 with ultrasonication, g) dCELL 3, h) dCELL 3 with hirudin, i) dCELL 3 with hirudin applied to bone only, j) dCELL 3 with monosodium citrate, k) dCELL 3 with PBS, l) dCELL 4, m) dCELL 4 with two nuclease washes, n) dCELL 4 with EDTA pre-treatment, o) dCELL 5. Scale bar = 500 µm.
Figure 4.7 DAPI stained sections of native and decellularised bovine osteochondral pins. a) fresh, b) dCELL 1, c) dCELL2, d) dCELL 2 with hypotonic freeze/thaw, e) dCELL 2 with distilled water freeze/thaw, f) dCELL 2 with ultrasonication, g) dCELL 3, h) dCELL 3 with hirudin, i) dCELL 3 with hirudin applied to bone only, j) dCELL 3 with monosodium citrate, k) dCELL 3 with PBS, l) dCELL 4, m) dCELL 4 with two nuclease washes, n) dCELL 4 with EDTA pre-treatment, o) dCELL 5. Scale bar = 500 µm.
Figure 4.8 Alcian blue stained sections of native and decellularised bovine osteochondral pins. a) fresh, b) dCELL 1, c) dCELL2, d) dCELL 2 with hypotonic freeze/thaw, e) dCELL 2 with distilled water freeze/thaw, f) dCELL 2 with ultrasonication, g) dCELL 3, h) dCELL 3 with hirudin, i) dCELL 3 with hirudin applied to bone only, j) dCELL 3 with monosodium citrate, k) dCELL 3 with PBS, l) dCELL 4, m) dCELL 4 with two nuclease washes, n) dCELL 4 with EDTA pre-treatment, o) dCELL 5. Scale bar = 500 µm.
Figure 4.9 Cartilage DNA content. Data is shown as the mean (fresh and dCELL 1 n=5, others n=3) ± 95 % confidence limits. * significant difference compared to native tissue p<0.05 (ANOVA).

Figure 4.10 Cartilage GAG content. Data is shown as the mean (fresh and dCELL 1 n=5, others n=3) ± 95 % confidence limits. * significant difference compared to native tissue p<0.05 (ANOVA).
**Figure 4.11** Cartilage hydroxyproline content. Data is shown as the mean (fresh and dCELL 1 n=5, others n=3) ± 95% confidence limits. * significant difference compared to native tissue p<0.05 (ANOVA).

**Figure 4.12** Percent deformation of cartilage. Data was subject to arcsin transformation prior to calculation of the 95% confidence limits and analysis of variance. Data is shown as the back transformed mean (fresh and dCELL 1 n=5, others n=3) ± 95% confidence limits. * significant p<0.05 (ANOVA).
Figure 4.13 Localisation of collagen type VI in native and decellularised bovine osteochondral tissues. a) native tissue (20 x magnification) scale bar = 1000 µm, b) decellularised tissue (20 x mag.) scale bar = 1000 µm, c) native tissue (40 x mag.) scale bar = 500 µm, d) decellularised tissue (40 x mag.) scale bar = 500 µm, e) native tissue (100 x mag.) scale bar = 200 µm, f) decellularised tissue (100 x mag.) scale bar = 200 µm.
Figure 4.14 Labelling of COMP in native and decellularised bovine osteochondral samples. a) native tissue (20 x magnification) scale bar = 1000 µm, b) decellularised tissue (20 x mag.) scale bar = 1000 µm, c) native tissue (40 x mag.) scale bar = 500 µm, d) decellularised tissue (40 x mag.) scale bar = 500 µm, e) native tissue (100 x mag.) scale bar = 200 µm, f) decellularised tissue (100 x mag.) scale bar = 200 µm, g) decellularised tissue isotype control (100 x mag.) scale bar = 200 µm.
4.6 Discussion

This study aimed to produce an acellular bovine osteochondral pin for use in osteochondral lesion repair. The aim was to achieve complete removal of whole cells and cellular debris from the ECM scaffold to eliminate the immunogenic effects of xenogeneic cellular antigens. As the fundamental macromolecules of the ECM, such as collagens, are conserved throughout mammalian species, these matrix proteins should not evoke an immune response.

A protocol for decellularisation was developed, based on previous work by Booth et al. (2002). Increased incubation temperatures (Stapleton et al., 2008 and Khier et al., 2011) were required to overcome issues with dense matrix and high GAG concentrations of osteochondral tissues which can inhibit the diffusion of decellularisation solutions. Additionally, EDTA, incorporated as a protease inhibitor in existing protocols was initially removed from the process to avoid decalcification of the bony component of the osteochondral pins.

Histological analysis of osteochondral tissues decellularised using the dCELL 5 protocol showed the full removal of whole cells and large cellular debris, and the DNA content of cartilage was significantly reduced to below 50 ng.mg⁻¹. To achieve complete decellularisation, bone marrow first needed to be loosened and removed. This was achieved by incubation in PBS at 45 °C with agitation for 18 h and then use of a water pik. Use of anticoagulants and lower temperatures did not prove as effective at loosening marrow. Removal of bone marrow enabled better diffusion of decellularisation solutions through the bone and up to the subchondral plate. Four cycles of freeze/thaw were required (two of which were in hypotonic solution) to open up the ECM through formation of ice crystals to improve diffusion of solutions through the tissue. The use of hypotonic buffer or distilled water at all stages did not improve the efficacy of freeze/thaw cycles and ultrasonication of tissues prior to decellularisation to increase diffusion was not advantageous. Two applications of hypotonic buffer and low concentration SDS in series were required to fully remove cells from calcified cartilage and the subchondral bone plate, as these areas were most dense.
This number of SDS cycles was fewer than required in the protocol developed by Khier et al. (2011) for porcine tissue, likely due to the smaller volume of tissue being decellularised. Extensive washing in PBS following nuclease treatment and PAA sterilisation was required to fully remove cell remnants from the tissue.

Although decellularisation was achieved, there were detrimental effects on the ECM. Histological analysis of the acellular matrix showed an increase in cartilage porosity. A reduction in GAGs was seen with alcian blue staining which was confirmed by sulphated proteoglycan quantification. This loss of GAG had an adverse effect on the mechanical properties of the tissue resulting in a significant increase in cartilage deformation.

The DNA content of the bony section of the osteochondral tissue was not quantified, so the true extent of osteochondral decellularisation is currently unknown. Satisfactory digestion of the mineralised tissue could not be achieved following preliminary alterations to the protocol provided with the Qiagen kit, further work is required to quantify bone DNA. Retention of DNA within the bone is not thought to be problematic, as whole cell nuclei have been shown to be removed following histological analysis. The presence of DNA in the bone will not elicit an immune response, only the presence of cellular membranes and proteins (Knight & Ingham, 2006). Residual DNA is known to result in calcification of decellularised tissues following implantation (Schoen & Levy, 2005), however this would not pose and issue in an already mineralised tissue such as bone.

Immunohistochemical analysis of decellularised tissues showed that most collagen type VI was lost from the tissue. Collagen type VI is a key component of the cartilage ECM and is usually localised to the chondrons (Cremer et al., 1998) where it plays a role in chondrocyte attachment to the ECM (Poole et al., 1997). As cells were removed from the tissue during the process, it was expected that some damage to the immediate pericellular matrix would occur. Damage to collagen type VI following decellularisation was also seen by Kheir et al. (2011), where the collagen shell of the chondron had collapsed in.
COMP is a non-collagenous matrix protein which plays a role in matrix assembly. The protein is uniformly distributed in the interterritorial matrix, with slightly increased density in the deep zone and decreased density around the tidemark and calcified cartilage (DiCesare et al., 1995). Depletion of COMP has been seen in osteoarthritic cartilage (DiCesare et al., 1996), and is thought to be a good soluble biomarker for the disease (Saxne & Heinegård, 1992). Immunolabelling of tissue sections showed COMP to be uniformly distributed throughout native bovine cartilage, with some reduction in staining at the superficial zone and an absence of staining in the calcified cartilage. Decellularised tissues showed similar stain distribution, however there was a distinct boundary of stain loss at the superficial zone, and throughout the cartilage staining appeared grainy, suggesting some clumping of COMP proteins.

Isotype controls were negative for native tissues but positive for decellularised tissue sections, showing non-specific binding of the secondary antibody. It is likely that residual SDS from the decellularisation process is interacting ionically with the secondary antibody, resulting in positive staining.

Other studies have been conducted to decellularise bovine articular cartilage without the subchondral bone. Elder et al. (2010) used 2 % (w/v) SDS over a range of incubation times to decellularise immature calf cartilage. They found that minimal detrimental effects to GAG content and therefore mechanical function occurred at short (2 h) incubation times, however, there were also minimal reductions in cell and DNA content. To achieve decellularisation, longer incubation (8 h) with SDS was required, however this lead to detrimental effects on GAG content and mechanical properties, as also seen in this study.

Yang et al. (2012) achieved successful decellularisation of mature bovine cartilage and retained GAGs, however their protocol involved freeze drying and homogenisation of cartilage to a powder before decellularising and re-forming, thus completely removing the structure of the natural ECM.

The regenerative capacity of the acellular bovine osteochondral scaffold developed in this study will need to be determined. The biological and
structural changes which have occurred following decellularisation and the biocompatibility of the resultant matrix need to be investigated further. As a number of alterations to the protocol for the decellularisation of bovine tissues have been explored in the current study, with varying degrees of success, the efficacy of some of these initial protocols will be investigated using porcine tissues. It may be possible to produce a porcine ECM scaffold which retains more of the characteristics of native tissues than was seen for bovine tissues.
Chapter 5
Investigation of methods for the
decellularisation of porcine osteochondral
tissues

5.1 Introduction

As introduced previously (Chapter 1 and Chapter 4), an acellular xenogeneic osteochondral scaffold may provide a more effective early intervention treatment for osteochondral lesions. An analysis of osteochondral tissues from different species and joint areas in Chapter 3 identified bovine medial groove and porcine medial condyle tissues as appropriate source materials for decellularisation. A protocol was developed for the successful decellularisation of bovine osteochondral tissues using a water pik to remove bone marrow followed by four cycles of freeze-thaw (two of which were in hypotonic buffer solution), two cycles of hypotonic buffer solution followed by incubation in 0.1 % (w/v) SDS, treatment with nucleases and sterilisation with 0.1 % (v/v) peracetic acid. In this chapter, the process required to decellularise porcine osteochondral tissues was investigated.
5.2 Aims and objectives

Aims:

The aim of the research in this chapter was to investigate methods using low concentration SDS plus proteinase inhibitors to decellularise porcine osteochondral pins obtained from the medial condyle.

Objectives:

- To develop a protocol to remove cells and cellular debris from porcine osteochondral pins while retaining the same natural histoarchitecture, biochemical composition and biomechanical function as the native osteochondral tissues.

- To characterise the resultant decellularised ECM and compare to native tissues
  - To characterise the tissue histologically in order to identify the presence/lack of cells and to observe any changes in the histoarchitecture of the tissue.
  - To quantify the total DNA, to identify the reduction in DNA compared to native tissue following decellularisation
  - To characterise the tissue biochemically to assess GAG and collagen content of decellularised scaffolds
  - To determine the biomechanical properties in order to determine any changes in the mechanical properties of cartilage following decellularisation.
5.3 Methods

5.3.1 Decellularisation solutions

Solutions for decellularisation were produced as described in Section 4.4.1. Additional solutions used in this part of the study are described below.

5.3.1.1 0.05 % (w/v) SDS in hypotonic buffer

SDS (10g) was dissolved in 100 ml distilled water to produce a 10 % (w/v) SDS solution. SDS solution (5 ml) was added to 995 ml hypotonic buffer. When required, the solution was sterilised by autoclaving and could be stored at room temperature for up to 3 months. Just prior to use, 1 ml aprotinin (10,000 KIU.ml\(^{-1}\)) was added to give a final concentration of 10 KIU.ml\(^{-1}\).

5.3.1.2 PBS with aprotinin and EDTA (0.1% w/v)

A solution of PBS was produced as described in Section 2.1.7.1. EDTA (1 g) was dissolved in 1 L PBS and the pH adjusted to 7.2 – 7.4. When required, the solution was sterilised by autoclaving and could be stored at room temperature for up to 3 months. Just prior to use 1 ml aprotinin (10,000 KIU.ml\(^{-1}\)) was added to give a final concentration of 10 KIU.ml\(^{-1}\).

5.3.1.3 PBS with mannitol (1 % w/v)

A solution of PBS was produced as described in Section 2.1.7.1. Mannitol (10 g) was dissolved in 1 L PBS and the pH adjusted to 7.2 – 7.4. When required, the solution was sterilised by autoclaving and could be stored at room temperature for up to 3 months. Just prior to use 1 ml aprotinin (10,000 KIU.ml\(^{-1}\)) was added to give a final concentration of 10 KIU.ml\(^{-1}\).

5.3.1.4 PBS with mannitol (2 % w/v)

A solution of PBS was produced as described in Section 2.1.7.1. Mannitol (20 g) was dissolved in 1 L PBS and the pH adjusted to 7.2 – 7.4. When required, the solution was sterilised by autoclaving and could be stored at room temperature for up to 3 months. Just prior to use 1 ml aprotinin (10,000 KIU.ml\(^{-1}\)) was added to give a final concentration of 10 KIU.ml\(^{-1}\).
5.3.2 Development of the decellularisation process

Decellularisation processes developed for bovine cartilage in the previous chapter were used to determine the response of porcine osteochondral tissues to decellularisation. Porcine osteochondral pins (9 mm diameter, 10 – 12 mm deep) were harvested from the porcine medial condyle and stored frozen as described in Section 2.2.2.

5.3.2.1 dCELL 1 (basic decellularisation)

Porcine osteochondral pins were decellularised as described in Section 4.4.2.1.

5.3.2.2 dCELL 3 (water pik)

Porcine osteochondral pins were decellularised as described in Section 4.4.2.6.

5.3.2.3 dCELL 3 (0.05 % w/v SDS)

Porcine osteochondral pins were decellularised as described in Section 4.4.2.6, however a reduced concentration (0.05 % w/v) of SDS in hypotonic buffer was used.

5.3.2.4 dCELL 3 (6 h 0.1 % w/v SDS)

Porcine osteochondral pins were decellularised as described in Section 4.4.2.6, however the 24 h 0.1 % (w/v) SDS in hypotonic buffer wash was replaced with 6 h in 0.1 % (w/v) SDS followed by 18 h in PBS.

5.3.2.5 dCELL 3 (0 % w/v SDS)

Porcine osteochondral pins were decellularised as described in Section 4.4.2.6, however the 24 h 0.1 % (w/v) SDS wash was replaced with 24 h in hypotonic buffer without SDS.

5.3.3 Cartilage characterisation

Following application of the decellularisation process detailed in Section 5.3.2.5, the cartilage was assessed histologically using the methods detailed in Section 2.2.3.
5.3.3.1 Denatured collagen quantification

5.3.3.1.1 Lyophilisation
Cartilage (50 mg) was lyophilised to a constant weight as described in Section 3.4.1.1.

5.3.3.1.2 α-chymotrypsin digestion
To determine the extent of collagen denaturation, macerated, freeze-dried tissues were digested using α-chymotrypsin.

- **Digestion Buffer (pH 7.8)**
  - 1.21 g trizma base
  - 0.15 g calcium chloride
  - 100 ml distilled water

- **Digestion solution**
  - 100 mg α-chymotrypsin
  - 20 ml digestion buffer

α-chymotrypsin digestion solution (5 ml) was added to each sample and incubated at 30 °C for 24 h.

5.3.3.1.3 Acid hydrolysis of α-chymotrypsin digest supernatant
Following digestion, each sample was centrifuged at 600 g for 10 min. The supernatant (4 ml) was collected in a polypropylene universal and hydrolysed with hydrochloric acid (6 M; 4ml) and then neutralised as described in Section 3.4.1.3.

5.3.3.1.4 Hydroxyproline quantification
The hydroxyproline residues from denatured collagen in the hydrolysed supernatant were quantified using the method detailed in Section 3.4.4. The supernatant was not diluted before assaying.

5.3.4 Investigation of the cause of cartilage damage
Osteochondral pins were harvested from the medial condyle of n=3 pigs. Unless otherwise stated, two pins from each individual were placed in one pot and three pots (n=3) were used for each condition. One pin from each pot was analysed.
5.3.4.1 Analysis of incubation temperature

Osteochondral pins (2 pins per pot, n=3) were decellularised as described in Section 5.3.2.4. One group was decellularised at 40 °C and the other group at 45 °C. The solution temperatures were measured with a thermometer at the beginning and end of each wash step and the temperature inside the incubator measured at the end of each wash step.

5.3.4.2 Systematic removal of major steps of the decellularisation process

Osteochondral pins (2 pins per pot, n=3) were decellularised using the dCELL 3 protocol as described in Section 5.3.2.2 at 42 °C. The complete protocol was used as a positive control for cartilage damage. Each major step of the protocol was systematically removed as detailed in Table 5.1 to identify the effect on cartilage. Incubation in PBS with aprotinin for the same duration was performed as a negative control.

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Table 5.1 Protocols with systematic removal of major steps of the process. The wash steps of the decellularisation protocol are listed in order. Variations are labelled a-f and positive (+ve) and negative (-ve) control protocols are also listed. The presence of each individual step is denoted with (+) and if the step was omitted this is indicated with (-).
5.3.4.3 Matrix metalloproteinase Inhibition

Osteochondral pins (2 pins per pot, n=3) were incubated in PBS with aprotinin and EDTA (0.1 % w/v) for 5 days at 42 °C with agitation. Another set of pins (2 pins per pot, n=3) were incubated in PBS without aprotinin or EDTA as a positive control.

5.3.4.4 Analysis of solution tonicity

5.3.4.4.1 Osmolality measurements

The osmolality of synovial fluid (collected from the porcine knee cavity using a 1 ml syringe, n=3) and of PBS containing 1 % and 2 % (w/v) mannitol was measured. Each sample (20 µl, n=3) was measured using a micro-osmometer (Advanced Instruments, 3MO Plus Freezing Point Osmometer).

5.3.4.4.2 Incubation of pins in PBS with mannitol

Osteochondral pins (2 pins per pot, n=3) were incubated in solutions of 1 % and 2 % (w/v) mannitol in PBS for 5 days at 42 °C with agitation.

5.3.4.4.3 Decellularisation of pins using PBS with mannitol

Osteochondral pins (2 pins per pot, n=3) were decellularised following the dCELL 3 process detailed in Section 5.3.2.2 however PBS washes were carried out in 1 % or 2 % (w/v) mannitol in PBS.

5.3.4.5 Analysis of pig skeletal maturity

Osteochondral pins were harvested from the medial condyle of 3 month old (n=3) and ~ 3 year old (n=3) pigs as described in Section 2.2.2. Pins were decellularised (1 pin per pot) following the dCELL 3 process detailed in Section 5.3.2.2.

5.3.4.6 Decellularisation with 1 vs 5 pins per pot

Osteochondral pins were decellularised by dCELL 3 as described in Section 5.3.2.2 with either 1 or 5 pins per pot (n=3).

5.3.5 Histological characterisation

Histological characterisation of native and decellularised osteochondral tissues was carried out as described in Section 2.2.3.
5.3.6 DNA quantification

The DNA content of native and decellularised cartilage and bone was quantified as detailed in Section 4.4.4.

5.3.7 Biochemical characterisation

The GAG, hydroxyproline and water content of native and decellularised tissues was assessed using methods described in Section 3.4.

5.3.8 Biomechanical characterisation

The percentage deformation of decellularised cartilage was determined using the indentation methods described in Sections 3.5.1 and 3.5.2.
5.4 Results

5.4.1 Decellularisation optimisation

5.4.1.1 dCELL 1

Following application of the dCELL 1 basic protocol the general tissue structure was maintained, however, there were still a number of cells present in the cartilage and bone (Figure 5.2 b and 5.3 b). Only a 77.5 % reduction in cartilage DNA was found (Figure 5.5). Native cartilage contained 262.1 ± 72.9 ng.mg\(^{-1}\) DNA per cartilage wet weight while cartilage which had been subject to the dCELL 1 protocol had 58.9 ± 9.9 72.9 ng.mg\(^{-1}\). There was some loss of intensity of alcian blue staining (Figure 5.4 b) and PAS staining was stronger in the middle zone, suggesting loss of GAGs in this zone. Native cartilage contained 377.4 ± 54.2 µg.mg\(^{-1}\) GAG per cartilage dry weight while cartilage which had been subject to the dCELL 1 protocol had 151.7 ± 105.1 µg.mg\(^{-1}\), a reduction of 59.8 % (Figure 5.6). This reduction in GAGs had a detrimental effect on the biomechanics of the tissue (Figure 5.7), with the percentage deformation of the cartilage increasing from 22.8 (+1.1, -1.0) % (native) to 34.7 (+7.3, -6.9) % (dCELL 1). The hydroxyproline content of cartilage following dCELL 1 was significantly increased from 43.6 ± 14.6 µg.mg\(^{-1}\) per dry weight (native) to 115.2 ± 16.4 µg.mg\(^{-1}\) (Figure 5.8). The water content of cartilage was not significantly different following the application of dCELL 1 (Figure 5.9).

5.4.1.2 dCELL 3

As application of the dCELL 1 protocol did not result in complete decellularisation of porcine osteochondral tissues, dCELL 3 as developed for bovine tissues in the previous chapter was employed. This again resulted in the general ECM structure being maintained (Figure 5.2 c) and following this protocol no whole cell nuclei could be seen (Figure 5.2 c and 5.3 c). The DNA content of cartilage was reduced by 98 % to 5.3 ± 0.4 ng.mg\(^{-1}\) cartilage wet weight (Figure 5.5). Images of alcian blue stained section showed a loss of blue intensity and increased PAS staining in the superficial and middle zones (Figure 5.4). The GAG content of cartilage had reduced by 60.3 % to 149.8 ± 284.6 µg.mg\(^{-1}\) cartilage dry weight (Figure 5.6), however variation
between samples was high, with one sample only having 19.2 µg.mg\(^{-1}\). This again resulted in significantly increased percentage deformation (Figure 5.7) to 41.9 (+21.5, -20.0) %. The hydroxyproline content was not significantly different to native cartilage (Figure 5.8), however the water content had increased to 83.5 (+3.6, -4.0) % (Figure 5.9).

5.4.1.3 dCELL 3 (0.05 % SDS)

Upon completion of the dCELL 3 protocol using 0.05 % (w/v) SDS in place of 0.1 % (w/v) SDS, following the extended PBS end wash the solutions containing the osteochondral pins (n=3) were cloudy, and in one of the three pots, cartilage had dissolved away from the bone. Another pot of pins were subject to the process to achieve n=3. The cartilage in the remaining pots appeared gelatinous and had shrivelled away from the edge of the bone (Figure 5.1).

Figure 5.1 macroscopic observation of damaged pins. a) side view and b) top view of dCELL 3 pin (left) and dCELL 3 with 0.05% SDS pin (right).

No cell nuclei were observed in the decellularised tissue (Figure 5.2 d and 5.3 d) however the cartilage structure was highly porous and had reduced alcian blue staining for GAGs (Figure 5.2 d and Figure 5.4 d). The cartilage DNA content of these samples was significantly reduced by 98.2 % (Figure 5.5). The GAG content of cartilage was reduced by 82.1 % to 67.7 ± 161.7 µg.mg\(^{-1}\) (Figure 5.6). The hydroxyproline content of the tissue following this protocol was significantly increased compared to native tissue and was 115.2 ± 48.9 µg.mg\(^{-1}\) (Figure 5.8). The water content of the tissue was significantly increased to 84.2 ± 0.8 % compared to native tissue (Figure
5.9. Following measurement of deformation using a hemispherical indenter, this damaged cartilage did not recover, so needle indentation could not subsequently be performed to measure cartilage thickness. No percentage deformation data is therefore available for presentation.

5.4.1.4 dCELL 3 (6 h)

Similarly to the previous protocol using 0.05 % SDS, when samples were incubated in 0.1% SDS for only 6 h, cartilage damage was seen again. Cartilage was porous, the surface was wrinkled (Figure 5.2 e) and cartilage GAG content was depleted (Figure 5.4 e). No cell nuclei were present in the tissue (Figure 5.2 e and 5.3 e). The DNA content of this cartilage was significantly reduced by 90.6 % compared to native tissue. No sulphated proteoglycans could be detected using the DMB dye assay, there was no significant difference in the absorbance of the sample to that of the blank (ANOVA). The hydroxyproline content of the tissue was significantly increased compared to native tissue to 137.3 ± 59.1 µg.mg⁻¹. The cartilage water content was also significantly increased to 93.8 ± 0.3 %. Again, tissue did not recover following indentation, so the percentage deformation of the cartilage could not be determined.

5.4.1.5 dCELL 3 (0.0% SDS)

As seen following the two previous decellularisation protocols, cartilage appeared shrivelled and gelatinous, the tissue volume was greatly reduced. The histological appearance was also as previously reported (Figure 5.2 f, 5.3 f and 5.4 f). Since limited tissue was available, this group of damaged pins were used to investigate the nature of the cartilage damage by performing histological analysis and quantitative analysis of collagen damage (Section 5.4.2).
Figure 5.2 H&E stained sections of native and decellularised osteochondral pins. a) native, b) dCELL 1, c) dCELL 3, d) dCELL 3 (0.05 % SDS), e) dCELL3 (6 h SDS) and f) dCELL 3 (0.0 % SDS). Scale bar = 500 µm.
Figure 5.3 DAPI stained sections of native and decellularised osteochondral pins. a) native, b) dCELL 1, c) dCELL 3, d) dCELL 3 (0.05 % SDS), e) dCELL3 (6 h SDS) and f) dCELL 3 (0.0 % SDS). Scale bar = 500 µm.
Figure 5.4 Alcian blue/PAS stained sections of native and decellularised osteochondral pins. a) native, b) dCELL 1, c) dCELL 3, d) dCELL 3 (0.05 % SDS), e) dCELL3 (6 h SDS) and f) dCELL 3 (0.0 % SDS). a, b, e and f scale bar = 500 µm, c and d scale bar = 1000 µm.
**Figure 5.5 Cartilage DNA content.** Data is shown as the mean (native and dCELL1 n=5, others n=3) ± 95% confidence limits. * significantly different to native (p<0.05 ANOVA).

**Figure 5.6 Cartilage GAG content.** Data is shown as the mean (native and dCELL 1 n=5, others n=3) ± 95% confidence limits. * significantly different to native (p<0.05 ANOVA).
**Figure 5.7 Percent deformation of cartilage.** Data was subject to arcsin transformation prior to calculation of the 95% confidence limits and analysis of variance. Data is shown as the back transformed mean (native n=6, dCELL 1 and 3 n=3) ± 95% confidence limits. * significantly different to native (p<0.05 ANOVA).

**Figure 5.8 Cartilage hydroxyproline content.** Data is shown as the mean (native and dCELL 1 n=5, others n=3) ± 95% confidence limits. * significantly different to native (p<0.05 ANOVA).
Figure 5.9 Cartilage water content. Data was subject to arcsin transformation prior to calculation of the 95% confidence limits and analysis of variance. Data is shown as the back transformed mean (native and dCELL 1 n=5, others n=3) ± 95% confidence limits. * significantly different to native (p<0.05 ANOVA).

5.4.2 Cartilage damage characterisation

5.4.2.1 Histological analysis

Cartilage which was damaged following application of the dCELL 3 (0.0% SDS) protocol was analysed histologically to identify changes to the general structure. H&E stained sections (Figure 5.2 f) showed that the cartilage surface was much rougher than native cartilage, no cell nuclei were visible, and lacunae appeared enlarged. The cartilage also appeared to be generally thinner. The bone appeared histologically normal. Use of alcian blue (Figure 5.4 f) and safranin O staining of cartilage sections (Figure 5.10 a & b) showed a severe loss of GAGs. There was no safranin O staining visible in the damaged cartilage and PAS staining was present throughout all layers of the cartilage in the damaged samples. Staining of sections with van Geison (Figure 5.10 c & d) and Sirius red (Figure 5.10 e & f) was performed to visualise collagen fibres within the cartilage ECM. Both stains showed a clumping of the collagen fibres into a more mesh-like network in the damaged samples as opposed to the evenly dispersed collagen seen in the native tissues. The attachment of cartilage to the underlying bone in the
damaged samples also appeared to be less secure, with long threads of collagen sparsely attaching to the underlying bone.

![Figure 5.10 Histological characterisation of cartilage damage following application of the dCELL 3 (0.0 % SDS) protocol. Safranin O/Fast green stained sections of a) native and b) damaged cartilage, van Gieson stained sections of c) native and d) damaged cartilage, Sirus red/Millers elastin stained sections of e) native and f) damaged cartilage. Scale bar = 500 µm.]

**5.4.2.2 Denatured collagen quantification**

To determine whether collagen fibres were being denatured during the decellularisation processes, samples of native and treated cartilage (dCELL 3, 0.0 % SDS) were digested with α-chymotrypsin which can only cleave denatured collagen. The digest was hydrolysed and the hydroxyproline
content quantified to determine the amount of denatured collagen in the tissues. There was no significant difference between the levels of denatured collagen in cartilage subject to the dCELL 3 (0.0 % SDS) protocol compared to native tissue (Figure 5.11).

Figure 5.11 Denatured collagen content of native and damaged cartilage (dCELL 3, 0.0% SDS). Data is shown as the mean (n=3) ± 95% confidence limits. * significantly different to native (p<0.05 ANOVA).

5.4.3 Investigation into cartilage damage

To determine the cause of the cartilage damage which was occurring during decellularisation, key steps in the process were changed or omitted systematically to determine their effect on cartilage stability.

5.4.3.1 Analysis of incubation temperature

To better understand the temperature changes during the decellularisation process and how temperature could affect cartilage stability, the temperature of all solutions and incubators was measured when set to 45 °C which was used as standard and at a lower temperature of 40 °C. The incubator and solution temperatures recorded during dCELL 3 (6 h 0.1 % SDS) process are shown in Figure 5.12. The highest solution temperature reached during incubation at 40 °C was 43 °C, whereas solutions incubated at 45 °C
reached temperatures of 47.5 °C during the extended PBS end wash. At the end of the decellularisation process the cartilage was still attached to all pins decellularised at 40 °C, however the cartilage on the pins decellularised at 45 °C had all become detached and had begun to completely dissolve in solution. All subsequent decellularisation processes were performed at 42 °C to minimise any detrimental effects of high temperature on the cartilage structure.

Figure 5.12 Incubator and solution temperatures during decellularisation. The solution temperature before and after incubation (n=1) and the incubator temperature at the end of each incubation are shown for incubators set at 40 °C and 45°C.
5.4.3.2 Systematic removal of key steps of the decellularisation process

Key stages of the decellularisation process were removed to identify how the level of cartilage damage was affected by the presence/absence of that step, as described in Section 5.3.4.2. Histological assessment of tissues following these varied decellularisation procedures are shown in Figure 5.13, 5.14 and 5.15.
Figure 5.13 H&E stained sections (n=3) of cartilage following decellularisation following protocols with steps systematically removed. Panel a) complete decellularisation dCELL 3 process (positive control), b) dCELL 3 process with the dry freeze/thaw cycle (-20 °C) omitted, c) dCELL 3 process with the all four freeze/thaw cycles omitted, d) dCELL 3 process with the hypotonic wash omitted, e) dCELL 3 process with the SDS wash omitted, f) dCELL 3 process with the PAA sterilisation step omitted, g) dCELL 3 process with the extended PBS end wash omitted, h) PBS only (negative control). Scale bar = 200 µm.
Figure 5.14 van Gieson stained sections (n=3) of cartilage following decellularisation following protocols with steps systematically removed. Panel a) complete decellularisation dCELL 3 process (positive control), b) dCELL 3 process with the dry freeze/thaw cycle (-20 °C) omitted, c) dCELL 3 process with the all four freeze/thaw cycles omitted, d) dCELL 3 process with the hypotonic wash omitted, e) dCELL 3 process with the SDS wash omitted, f) dCELL 3 process with the PAA sterilisation step omitted, g) dCELL 3 process with the extended PBS end wash omitted, h) PBS only (negative control). Scale bar = 200 µm.
Figure 5.15 Alcian blue stained sections (n=3) of cartilage following decellularisation following protocols with steps systematically removed. Panel a) complete decellularisation dCELL 3 process (positive control), b) dCELL 3 process with the dry freeze/thaw cycle (-20 °C) omitted, c) dCELL 3 process with the all four freeze/thaw cycles omitted, d) dCELL 3 process with the hypotonic wash omitted, e) dCELL 3 process with the SDS wash omitted, f) dCELL 3 process with the PAA sterilisation step omitted, g) dCELL 3 process with the extended PBS end wash omitted, h) PBS only (negative control). Scale bar = 200 µm.

All tissues showed signs of damage, having much larger lacunae than native tissue and showing a rougher cartilage surface. Clumping of the collagen network could be seen at the cartilage surface on most pins. Surprisingly, however, even the PBS negative control also showed cartilage damage.

5.4.3.3 Matrix metalloproteinase inhibition

Since incubation in PBS alone for 5 days at 42 °C resulted in damage to the cartilage, EDTA was added to the PBS plus aprotinin in order to inhibit the activity of matrix metalloproteinases which may have been actively degrading the cartilage ECM. H&E, van Gieson and alcian blue stained sections of pins incubated in PBS with aprotinin and EDTA are shown in Figure 5.16, and pins incubated in just PBS plus aprotinin (as a positive control) can be seen in Figure 5.17.

Damage to cartilage was minimal in both sets, enlarged lacunae and roughened cartilage surface seen previously were less evident in these images, even in the PBS only positive control.
Figure 5.16 Osteochondral pins incubated in PBS with EDTA plus aprotinin for 5 days at 42 °C. Panel a) H&E stained tissue sections, scale bar = 500 µm, b) van Gieson stained tissue sections, scale bar = 500 µm, and panel c) alcian blue stained tissue sections, scale bar = 1000 µm.
Figure 5.17 Osteochondral pins incubated in PBS plus aprotinin only for 5 days at 42 °C. Panel a) H&E stained tissue sections, scale bar = 500 µm, b) van Gieson stained tissue sections, scale bar = 500 µm, and panel c) alcian blue stained tissue sections, scale bar = 1000 µm.

5.4.3.4 Analysis of solution tonicity

5.4.3.4.1 Osmolality measurements

The osmolality of synovial fluid obtained from porcine knee joints was measured to determine whether PBS was at the correct tonicity for cartilage, as although it is isotonic to the cells, it may not be isotonic to cartilage ECM.

The osmolality of porcine synovial fluid was found to be 329 ± 59 mOsmol.Kg⁻¹ and ranged between 302 - 344 mOsmol.Kg⁻¹ (Figure 5.18). PBS had a lower osmolality than synovial fluid, but not significantly so. Mannitol was added to PBS at to different concentrations, 1 % (w/v) mannitol in PBS resulted in a solution with an osmolality of 352 ± 6 mOsmol.Kg⁻¹, and 2 % (w/v) mannitol in PBS had significantly higher osmolality than that of synovial fluid at 401 ± 5 mOsmol.Kg⁻¹.
Figure 5.18 Osmolality of porcine synovial fluid, PBS and PBS with mannitol. Data is shown as the mean (n=3) ± 95% confidence limits. * significantly different to synovial fluid (p<0.05 ANOVA).

5.4.3.4.2 Incubation in PBS with mannitol

After incubating osteochondral pins in PBS with 1 % (w/v) and 2 % (w/v) mannitol for 5 days at 42 °C, pins were assessed histologically (Figure 5.19 & 5.20). As previously observed (Section 5.4.3.3), there was no major damage visible. It was hypothesised that in the absence of agents such as SDS to remove the cells, the process was less harsh and occurrence of damage more variable. It was therefore decided to test the hypertonic wash solutions in the complete decellularisation process.
Figure 5.19 Osteochondral pins incubated in 1% (w/v) mannitol in PBS for 5 days at 42 °C. Panel a) H&E stained tissue sections, scale bar = 500 µm, b) van Gieson stained tissue sections, scale bar = 500 µm, and panel c) alcian blue stained tissue sections, scale bar = 1000 µm.
Figure 5.20 Osteochondral pins incubated in 2 % (w/v)mannitol in PBS for 5 days at 42 °C. Panel a) H&E stained tissue sections, scale bar = 500 µm, b) van Gieson stained tissue sections, scale bar = 500 µm, and panel c) alcian blue stained tissue sections, scale bar = 1000 µm.

5.4.3.4.3 Decellularisation of pins using PBS with mannitol

By not incorporating decellularisation steps, the cells remained in the cartilage when washed with PBS alone for five days at 42 °C and the cells may have acted to stabilise the tissue and not allow cartilage damage as seen in Section 5.4.3.4.2. The hypertonic mannitol in PBS solutions were therefore tested in place of standard PBS washes in a decellularisation protocol so that the effect of solution osmolality could be determined.

Histological analysis of pins decellularised using dCELL 3 are shown in Figure 5.21, pins decellularised using dCELL 3 with PBS washes containing 1 % (w/v) and 2 % (w/v) mannitol are shown in Figures 5.22 and 5.23 respectively. All pins showed a severe reduction in GAGs and some signs of damage, including enlarged lacunae, however damage was not as severe as observed previously, and was not improved by the addition of mannitol.
Figure 5.21 Osteochondral pins decellularised with the dCELL 3 protocol at 42 °C. Panel a) H&E stained tissue sections, scale bar = 500 µm, b) van Gieson stained tissue sections, scale bar = 500 µm, and panel c) alcian blue stained tissue sections, scale bar = 1000 µm.
Figure 5.22 Osteochondral pins decellularised with the dCELL 3 protocol using 1 % (w/v) mannitol in PBS at 42 °C. Panel a) H&E stained tissue sections, scale bar = 500 µm, b) van Gieson stained tissue sections, scale bar = 500 µm, and panel c) alcian blue stained tissue sections, scale bar = 1000 µm.
Osteochondral pins were taken from skeletally mature pigs (2.4 - 3.6 yr) and from immature pigs (3 months). These tissues were analysed by histology in their native form and following decellularisation using the dCELL 3 protocol at 42 °C. H&E stained sections and alcian blue stained sections of native and decellularised mature porcine osteochondral tissues are shown in Figure 5.24 and 5.25 respectively, H&E stained native and decellularised immature porcine osteochondral sections are shown in Figures 5.26 and 5.27, and alcian blue stained sections are shown in Figure 5.28 and 5.29.

Mature cartilage was notably thinner (~ 1.2 mm) than immature cartilage (~ 5.0 mm) and had a lower cell density. The cells of mature cartilage had a much more defined orientation, with linear columns of cells aligning perpendicular to the cartilage-bone interface. The chondrons of immature
cartilage often contained more than one cell, especially in the deep and hypertrophic regions. The subchondral bone plate of mature porcine tissues was well formed, dense and thick, whereas the boundary between cartilage and bone in immature cartilage was much less defined. Trabecular struts of mature bone were much thicker than those of immature bone. Cartilage from immature pigs also appeared to be highly vascularised, vascular tissues could be seen upon dissection and cross sections of vasculature were clearly visible in histological sections, for example Figure 5.28 a and b, where pink PAS stained blood vessels were observed to cross over from bone to cartilage. Vascular tissues have been observed in 6 month old pigs (Figure 5.19 b), however, no vascularisation was present in mature porcine cartilage.

During decellularisation, all osteochondral pins from mature pigs remained intact, cartilage detached from two of the three pins from immature pigs. Upon histological analysis, no damage to the cartilage surface was observed on any of the pins, even those from which the cartilage had detached. Cartilage and bone from immature pigs appeared mostly decellularised, however cells remained in the tissues from mature pigs.
Figure 5.24 H&E stained sections of native and decellularised (dCELL 3 process at 42 °C) mature porcine osteochondral tissues. a) Native (2.4 yr)  b) decellularised (2.4 yr), c) native (3.6 yr), d) decellularised (3.6 yr), e) native (3.6 yr), f) decellularised (3.6 yr). Scale bar = 500 µm.
Figure 5.25 Alcian blue stained sections of native and decellularised (dCELL 3 process at 42 °C) mature porcine osteochondral tissues. a) Native (2.4 yr) b) decellularised (2.4 yr), c) native (3.6 yr), d) decellularised (3.6 yr), e) native (3.6 yr), f) decellularised (3.6 yr). Scale bar = 500 µm.
Figure 5.26 H&E stained sections of native immature porcine osteochondral tissues. a) 73 days old, b) 74 days old and c) 77 days old. Scale bars = 500 µm.
Figure 5.27 H&E stained sections of immature porcine osteochondral tissues following decellularisation with dCELL 3 process at 42 °C. a) 73 days old, b) 74 days old and c) 77 days old. Scale bars = 500 μm.
Figure 5.28 Alcian blue stained sections of native immature porcine osteochondral tissues. a) 73 days old, b) 74 days old and c) 77 days old. Scale bars = 500 µm.
Figure 5.29 Alcian blue stained sections of immature porcine osteochondral tissues following decellularisation with dCELL 3 process at 42 °C. a) 73 days old, b) 74 days old and c) 77 days old. Scale bars = 500 µm.
5.4.3.6 Decellularisation with 1 vs 5 pins per pot

Pins from mature and immature animals showed no sign of cartilage damage, however, they were decellularised 1 pin per pot, as opposed to the usual 2 - 5, so that the pins from each individual pig could be identified. To determine whether the lack of cartilage damage seen in the previous study was due to having a single pin per pot and therefore altering the flow of solutions in each pot during agitation, two further sets of porcine (6 month old) osteochondral pins were decellularised with either one or five pins per pot.

Figure 5.30 Osteochondral pins decellularised using the dCELL 3 protocol at 42 °C with 1 pin per pot. Panel a) H&E stained tissue sections, scale bar = 500 µm, b) van Gieson stained tissue sections, scale bar = 1000 µm, and panel c) alcian blue stained tissue sections, scale bar = 1000 µm.
Figure 5.31 Osteochondral pins decellularised using the dCELL 3 protocol 42 °C with 5 pins per pot. Panel a) H&E stained tissue sections, scale bar = 500 µm, b) van Gieson stained tissue sections, scale bar = 1000 µm, and panel c) alcian blue stained tissue sections, scale bar = 1000 µm.

Stained sections of osteochondral pins decellularised with one pin per pot are shown in Figure 5.30. Minimal damage was seen, with the structure appearing normal, but with some loss of GAGs. When 5 pins per pot were decellularised, damage was seen in pins in two of the three pots (Figure 5.31), the cartilage was thinner and the surface had become more rough, complete loss of cartilage GAGs was seen in one of the pins.
5.5 Discussion

As discussed in the previous chapter, the aim of this study was to produce an acellular osteochondral scaffold for use in lesion repair. A protocol was developed to successfully decellularise bovine osteochondral tissues. The aim of the work in this chapter was to optimise the previously developed protocol for decellularisation of porcine osteochondral tissues.

5.5.1 Decellularisation optimisation

Following completion of the basic dCELL 1 protocol it was evident that cells were more easily removed from porcine tissues than bovine. Histologically, far fewer cell nuclei were present in porcine tissues than bovine tissues following decellularisation. The porcine cartilage DNA content was reduced by 77.5 % whereas a non-significant reduction of only 20.9 % was seen for bovine cartilage. The inclusion of two cycles of 0.1 % (w/v) SDS and hypotonic washes in dCELL5 were necessary to fully decellularise bovine tissues, however with the apparent ease of decellularisation of porcine tissue, a shorter protocol was preferred to minimise tissue damage. As use of the water pik and extended PBS end wash were shown to significantly increase cell and DNA removal in bovine tissues compared to the basic protocol, the dCELL 3 protocol was performed on porcine osteochondral pins, and was successful. This was in contrast to a study by Kheir et al. (2011) in which 3 cycles of low concentration SDS and hypotonic washes were required to fully decellularise porcine tissues, however this work was performed on large osteochondral cuts, as opposed to 9 mm diameter pins used in the current study.

Loss of cartilage proteoglycans was observed following decellularisation of porcine tissues using dCELL 3, and this adversely affected the mechanical properties of the tissue. Further optimisation with reduced SDS concentration and incubation times were employed to minimise GAG loss, however, following these protocols, cartilage damage was seen, with cartilage detaching from the bone and becoming gelatinous. Further analysis
was required to characterise the cartilage damage and to elucidate the cause.

**5.5.2 Cartilage damage**

Damaged tissue had a distinct histological appearance as evidenced with multiple staining techniques. The tissue became highly porous, with lacunae of chondrons becoming greatly enlarged and the collagen network between chondrons becoming clumped and net-like as opposed to evenly distributed. The cartilage surface became rough, likely due to a loss of tension in the collagen fibres which run parallel to the surface. Cartilage became very loosely attached to the bone, as lacunae which previously housed hypertrophic chondrons became stretched and enlarged, leaving only thin collagen fibres tying cartilage to the subchondral bone. No GAGs could be quantified in severely damaged tissues (6 h SDS) and there was a significant increase in water content, up to 95 %.

The distinct histological appearance of damaged cartilage was also witnessed by Schwartz *et al.* (2012) when developing acellular nasal septum cartilage. Schwartz *et al.* employed a decellularisation process involving osmotic cell lysis with distilled water, following which sodium hydroxide (> pH 12) was used to inactivate pathogens, denature DNA/RNA and remove cellular debris. Samples were defatted in 70 % (v/v) ethanol and GAGs and other non-collagenous proteins were denatured and removed using a guanidine hydrochloride (1 M) solution containing sodium acetate (0.05 N). Cartilages were finally sterilised using hydrogen peroxide (5% v/v).

The histological appearance of the treated nasal septum cartilage was more pronounced in porcine (7 - 8 months old) compared to human (18 - 74 years old) cartilage. The process resulted in a significant GAG reduction from 4.82 ± 0.94 % to 1.50 ± 0.43 % as a percentage of dry weight, and an almost complete loss of alcian blue staining. They saw an increase in tissue porosity from 19 ± 12 % to 29 ± 12 %. They concluded that loss of GAG and increased porosity were advantageous for cell infiltration during recellularisation.
A study to produce acellular osteochondral tissues by Kheir et al. (2011), also used immature porcine tissue (4 months old), and employed a similar protocol to that described in this study, based on that of Stapleton et al. (2008). They used 50 mm x 10 mm x 10 mm osteochondral blocks from the porcine femoral groove and found that 6 cycles of hypertonic buffer and SDS in hypertonic buffer (0.1 %, w/v) were required to fully remove all cell nuclei and achieve a 98 % reduction in cartilage DNA, however, no histological damage to cartilage was apparent following this more harsh protocol.

To further assess the cartilage damage seen in the current study, digestion of cartilage with α-chymotrypsin was performed to cleave denatured collagen which could then be quantified using the hydroxyproline assay. This was performed with the aim of identifying whether collagen damage was occurring during the decellularisation process and contributing to the histological appearance and tissue degeneration. No significant increase in denatured collagen was found compared to native tissue, suggesting that collagen fibres had remained intact.

5.5.3 Investigation into cartilage damage

To identify the cause of cartilage damage, many different factors associated with the decellularisation protocol were investigated. Although no collagen denaturation was observed, damage to other non-collagenous proteins may have occurred at high temperatures of incubation. Osteochondral pins decellularised at 45 °C (reaching temperatures of 48 °C) disintegrated more readily than those incubated at 40 °C, however, cartilage damage was still present at lower temperatures. The increased damage at higher temperature may have been as a result of increased diffusion. As temperature had had an effect on the extent of damage, all subsequent protocols were performed at the lower temperature of 42 °C, as this would not range above 45 °C, and would remain at a higher temperature than 37°C, avoiding the optimal temperature for bacterial growth.

As reduction in incubation temperature had not eliminated cartilage damage, each aspect of the decellularisation process was considered and removed from the process to determine the individual effect on cartilage stability.
Many phases of the decellularisation protocol had the potential to cause damage; freeze-thaw cycles create interstitial and intracellular ice crystals, which increase matrix permeability, hypotonic buffer has an osmotic effect on the cells and tissue, SDS denatures proteins and solubilises membranes (Seddon et al., 2004) and PAA causes protein damage by oxidative stress (Pruss et al., 1999). Although PBS washes alone were not thought to be damaging to the tissue, cartilage disintegration tended to occur during the final end wash, so this was also isolated as a potential damage causing factor. As a negative control, pins were washed in PBS alone. Surprisingly, all protocols, including the PBS alone control resulted in some degree of tissue damage. This indicated that damage was not as a direct result of any particular stage of the decellularisation process, and other protocols based on the same sequence of decellularisation treatments had not resulted in tissue damage (Booth et al., 2002; Stapleton et al., 2008; Kheir et al., 2011).

Cartilage damage had occurred when osteochondral pins were incubated in PBS with aprotinin (10 KUI.ml⁻¹) for 5 days. Advancing on protocols developed by Stapleton et al. (2008) and Kheir et al. (2011), EDTA, a matrix metalloproteinase inhibitor had been omitted from the PBS wash buffer in this study, as it also functions to decalcify bone and has been linked to cytotoxicity (Koulaouzidou et al., 1999). Pins were incubated in a PBS wash buffer containing EDTA (0.1 %, w/v) and aprotinin (10 KUI.ml⁻¹) for 5 days, to see if addition of the MMP inhibitor reduced or prevented the cartilage damage. No damage was seen, however, neither was it seen in the PBS only positive control. Although addition of EDTA did not clarify the role of MMPs in cartilage damage, due to the lack of denatured collagen in damaged samples, it is unlikely that collagenase activity of MMPs was responsible for cartilage degradation.

Another factor associated with the PBS wash buffer to consider was the tonicity. PBS is isotonic for cells, but may not necessarily be isotonic to the cartilage ECM. According to Baumgarten et al. (1985), the osmolality of normal synovial fluid is 404 ± 57 mOsmol.Kg⁻¹, however following exercise this reduced to isotonic levels, 301 ± 19 mOsmol.Kg⁻¹. Synovial fluid from knee joints of individuals suffering from OA and rheumatoid arthritis was found to have reduced osmolality, being 297.3 ± 16.9 mOsmol.Kg⁻¹ and
279.6 ± 7.5 mOsmol.Kg⁻¹ respectively (Shanfield et al., 1988). Having linked reduced osmolality of synovial fluid to arthritic joints, Bloebaum & Magee (1989) developed and patented a modified ringers solution containing mannitol for use as a hypertonic irrigating solution during arthroscopic lavage. In this study the osmolality of porcine synovial fluid was found to be 329 ± 59 mOsmol.Kg⁻¹. To increase the osmolality of the PBS wash buffers used for decellularisation, mannitol was added. A 1 % (w/v, 350 mOsmol. Kg⁻¹) solution was used to match porcine synovial fluid and a 2 % (w/v, 400 mOsmol.kg⁻¹) solution of mannitol in PBS was used to match values stated for human synovial fluid. After incubation at 42 °C with agitation in PBS with mannitol for 5 days, all osteochondral pins appeared histologically undamaged, however as stated before, the PBS only control also did not show signs of damage. Potentially the retention of cells in the tissue was stabilising the structure, so mannitol enhanced PBS wash buffers were used as part of the dCELL 3 protocol. Following decellularisation with the mannitol solutions, both showed slight histological signs of damage, as did the decellularisation using PBS without mannitol. Use of hypertonic PBS did not prevent cartilage damage during decellularisation.

It was noted that although porcine cartilage showed signs of damage following application of the dCELL 3 protocol, bovine tissues did not; the quality of the porcine source material was therefore considered a potential factor in cartilage degeneration. Characterisation of osteochondral tissues from different species in Chapter 3 highlighted differences in the maturity of porcine and bovine tissues. To assess the effect of skeletal maturity on cartilage degeneration, two further age ranges of pigs were selected. As pigs are skeletally mature from 2.5 years (Reinwald & Burr., 2008), a skeletally mature group aged between 2.4 and 3.6 years and a very immature group aged 3 months old were selected for decellularisation. Histologically the native osteochondral tissues from immature pigs showed characteristically thick cartilage with high cell density and indistinct cell orientation and intense alcian blue staining for GAGs (Julkunen et al., 2009), vascularisation was also seen throughout the young cartilage, indicative that the tissue was not fully formed and still growing, and the cartilage-bone interface was not clear and appeared full of hypertrophoc cells. Conversely, tissue from the older
pigs was fully mature, with much thinner cartilage, which had a lower cell density and much more defined cell orientation. The tissues from mature pigs showed a defined, thick subchondral bone plate.

Upon decellularisation, neither the immature group nor the mature group showed any signs of cartilage damage. Although cartilage had become detached from the bone of 2/3 pins from immature pigs during washing, this was likely due to the poor attachment originally and shear stress applied to the attachment site during coring of osteochondral pins from the joint, and not due to the decellularisation process. Of interest was the fact that immature tissues appeared to be fully decellularised following application of dCELL 3, however mature porcine tissues (similarly to bovine osteochondral tissues) were not fully decellularised, likely due to inadequate solution infiltration through the dense subchondral bone plate.

To identify which osteochondral pin came from which individual pig when assessing the effect of skeletal maturity on cartilage degradation, each pin was decellularised in a single pot. Usually 3-5 pins are decellularised in one pot at a time. A single pin agitated in a pot of solution will likely have different effect on how the fluid flows, and may affect the mechanical shear forces experienced by that single pin compared to a larger number of pins, explaining why neither mature nor immature porcine osteochondral pins showed cartilage damage. To this end one pin and five pins per pot were decellularised using the dCELL 3 protocol. Damage to varying degrees was seen in both conditions, so the number of pins per pot is not thought to influence cartilage damage.

Of note throughout this study was the variability with which cartilage responded to any of the processes detailed. The presence or absence of damage and extent to which cartilage damage occurred was inconsistent within treatment groups and between processes. This unpredictability made it difficult to conclusively determine the effect of any treatment on porcine cartilage. The lack of consistency may be due to the stage of development of the porcine tissue, as minor variations in pig age could have had pronounced effects on the cartilage stability.
5.5.4 Conclusion

Although initially a protocol was developed to successfully decellularise porcine tissues, further investigation revealed that the cartilage was damaged, which in some cases resulted in the cartilage dissolving completely in the decellularisation solutions, and in other cases resulted in the cartilage becoming devoid of GAGs, having a greatly increased porosity and water content and having drastically reduced biomechanical properties. High temperatures were found to increase the severity of cartilage damage, likely through increased diffusion.
Chapter 6
Biocompatibility of acellular bovine osteochondral scaffolds

6.1 Introduction

A protocol was developed to successfully decellularise bovine osteochondral tissues in Chapter 4. Histological analysis of the acellular scaffold was performed and the DNA, GAG and hydroxyproline content quantified as well as an evaluation of the biomechanical properties. The tissue was found to have severely reduced GAG content, which resulted in reduced mechanical properties. To further characterise the effect of decellularisation on the osteochondral extracellular matrix, immunohistochemistry was used to locate specific matrix constituents.

Furthermore, SDS used in the decellularisation has been reported to have cytotoxic effects when not effectively removed from decellularised tissue matrices (Rieder et al., 2004). Residual SDS within the scaffold may prevent ingrowth of cells in vivo and result in graft failure. Residual SDS within the tissue was quantified and the contact cytotoxicity of the acellular scaffold was determined.
6.2 Aims and objectives

Aims:
The aim of the research in this chapter was to investigate changes to specific matrix proteins in bovine osteochondral tissues following decellularisation and to assess the biocompatibility of the acellular scaffold.

Objectives:

- To determine the biocompatibility of the acellular osteochondral matrix.
  
  - To determine the concentration at which SDS has cytotoxic effects on BHK and 3T3 cell lines.
  
  - To quantify the SDS removed from the tissues during wash cycles and determine the concentration of residual SDS left in bovine cartilage and bone following decellularisation.
  
  - To determine biocompatibility of the acellular scaffold in vitro using the contact cytotoxicity technique with BHK and 3T3 cell lines.

Additional objectives were not pursued due to unforeseen issues with the initial biocompatibility testing.
6.3 Methods

6.3.1 SDS quantification

Reagents

In addition to reagents listed in Section 4.4.1, the following reagents were used.

- $^{14}$C radiolabelled SDS in 750 ml SDS in hypotonic solution hypotonic buffer (0.1 % w/v, 75 µl $^{14}$C SDS 0.01 µCi.ml$^{-1}$)
- MicroScint™-20

Method

To quantify the SDS washed out of the decellularised tissues in each wash solution (n=3) and also to quantify the residual SDS in the decellularised tissues (n=3), the decellularisation protocol described in Section 4.4.2.14 was performed using SDS in hypotonic solution which had been spiked with $^{14}$C radiolabelled SDS, produced as described in Section 6.3.2.1. Solutions and tissues from the decellularisation were collected.

A set of standards were produced from the spiked SDS solution to give SDS concentrations of 1000 µg.ml$^{-1}$, 750 µg.ml$^{-1}$, 500 µg.ml$^{-1}$, 250 µg.ml$^{-1}$, 125 µg.ml$^{-1}$, 50 µg.ml$^{-1}$, 25 µg.ml$^{-1}$, 10 µg.ml$^{-1}$ and 0 µg.ml$^{-1}$. Standard (40 µl), test solution (40 µl) or macerated decellularised cartilage or bone (40 mg) were added in triplicate to a flat bottomed 96 well optiplate™ and MicroScint™-20 (160 µl) was added to each well. Counts per minute were measured using Top count™, with each well being counted for 20 min. A standard curve of counts per minute was produced from the known concentrations of SDS, from which the concentration of SDS in the test solutions and tissue could be interpolated.

6.3.2 Determining the toxic concentration of SDS

To determine the concentration at which SDS had cytotoxic effects on two cell lines, 3T3 and BHK cell lines were incubated with varying concentrations
of SDS solution. Cell culture was performed as described in Section 2.2.6. Cells were seeded at subconfluent numbers onto flat bottomed 96 well culture plates and incubated overnight at 37 °C in 5 % CO₂ (v/v) in air. Once cells had attached, culture medium was aspirated and replaced with 200 µl of medium containing a known concentration of SDS or DMSO positive control (40 %, v/v) in cell culture medium. SDS was dissolved in appropriate cell culture medium for each cell type at concentrations of 750 µg.ml⁻¹, 500 µg.ml⁻¹, 250 µg.ml⁻¹, 100 µg.ml⁻¹, 50 µg.ml⁻¹, 25 µg.ml⁻¹, 10 µg.ml⁻¹, 5 µg.ml⁻¹ and 0 µg.ml⁻¹ and sterilised by passing through a 2 µm filter. Cells were incubated in these test media for 24 h at 37 °C in 5 % CO₂ (v/v) in air. Following incubation cell viability was determined using the ATPLite-M® assay.

6.3.2.1 ATPLite-M® cell viability assay

Cell viability was determined using the ATP-lite M® assay kit, where cellular adenosine triphosphate (ATP; present in all metabolically active mammalian cells) reacts with d-luciferin in the presence of luciferase to produce light which can be quantified using a scintillation counter. In cells which have undergone apoptosis, ATP will rapidly deplete, so little to no light will be detected.

Cell culture medium was aspirated from cells cultured in a 96 well plate and 50 µl of the appropriate cell culture medium was added to each well. Mammalian cell lysis solution (50 µl) from the ATPLite-M® assay kit was added to each well and the plate was shaken for 5 min on a microshaker. The contents from each well was transferred to a 96 well optiplate™ and 50 µl substrate solution was added to each well. The plate was covered in foil and agitated for a further 5 minutes on a microshaker. Any air bubbles were removed by carefully flaming the plate before determining the luminescence of each well using Top count™ scintillation counter.
6.3.3 Contact cytotoxicity

Bovine osteochondral pins (2 pins per pot from n=3 animals) were decellularised using dCELL 5 (Section 4.4.2.14). Each pin was dissected into small pieces as described in Figure 6.1 and 3 pieces from one pin were each placed in a well of a 6 well culture plate and secured using a steri-strip. A drop of cyanoacrylate contact adhesive was applied to the centre of one well on each plate to act as a positive control for cell growth inhibition and a steri-strip adhered to another as a negative control, a final well was left empty to observe normal cell growth.

![Figure 6.1 Dissection of osteochondral pins for contact cytotoxicity assay. Pins were cut along the dotted lines using a sterile scalpel blade to produce four rectangular osteochondral pieces exposing the innermost part of the pin.](image)

Plates were washed using PBS without calcium or magnesium three times for ten minutes each before being seeded with 3T3 (n=3) or BHK cells (n=3) at numbers appropriate to become confluent after 48 h. Plates were incubated for 48 h at 37 °C in 5 % CO₂ (v/v) in air. Each well was examined microscopically to detect changes in cell morphology, vacuolisation, detachment and cell lysis. The medium was aspirated and each well washed in PBS before fixation of the monolayer with 10 % (v/v) NBF for 10 min. NBF was aspirated and Giemsa stain was applied to each well for 5 min. Excess Giemsa stain was then removed using tap water. Again, changes to the cells were visualised by light microscopy.
6.3.4 Modified protocol for the production of biocompatible bovine osteochondral scaffolds

The protocol developed to decellularise bovine osteochondral pins (Section 4.4.2.14) was augmented to include further PBS end washes, with the intention of improving SDS wash out to produce a biocompatible scaffold. Two extended protocols (7 day and 14 day extended PBS washes) were developed to determine the optimum end wash duration, where sufficient SDS wash out was achieved without unnecessarily extending the protocol. The basic 48 - 60 h weekend end wash protocol (Section 4.4.2.12) was augmented with 4 x 24 h PBS washes after the final weekend wash (7 day PBS end wash) or 4 x 24 h PBS washes, followed by a second 48 – 60 h PBS wash and a further 4 x 24 h PBS washes (14 day PBS end wash). All PBS washes were performed at 42 °C with agitation. The three protocols (2 day, 7 day and 14 day PBS end wash) were performed alongside each other, 2 pins per pot from n=3 animals were used for each protocol.
6.4 Results

6.4.1 SDS quantification

To quantify SDS washed out from tissues during decellularisation and to determine the concentration of residual SDS in the decellularised osteochondral matrix, tissues were decellularised using solutions spiked with radiolabelled SDS. Quantification of radiolabelled SDS was performed by adding scintilant to spiked solutions/ tissues and measuring the activity on a scintillation counter. A standard curve of SDS concentration against counts per minute was produced (Figure 6.2), from which unknown SDS concentrations could be interpolated.

![Figure 6.2 Linear relationship between SDS concentration and counts per minute. Data is shown as the mean (n=3) ± 95% confidence limits.](image)

The concentration of SDS in each wash solution of the decellularisation process is shown in Figure 6.3. High concentrations of SDS were measured in the SDS wash solutions (750 ± 210 µg.ml⁻¹), however this was lower than the 1000 µg.ml⁻¹ expected from a 0.1 % (w/v) SDS solution. Error was high in the SDS solutions and some SDS from the solution would have entered the tissues, explaining the lower value. The hypotonic wash (24 h) after the first SDS incubation showed a relatively high concentration of SDS (59 ± 87
µg.ml⁻¹) compared to the PBS wash (10 min) following the second SDS incubation, suggesting a more thorough wash out of SDS during that first 24 h wash period. All solutions showed low concentrations of SDS, suggesting that SDS was still being washed out of the tissue during the last PBS wash.

**Figure 6.3 Concentration of SDS in decellularisation solutions following quantification of ¹⁴C SDS.** Data is shown as the mean (n=3) ± 95 % confidence limits.

Quantification of SDS in the tissue showed that decellularised bovine cartilage contained 364 ± 43 ng.mg⁻¹ SDS and decellularised bone contained 374 ± 187 ng.mg⁻¹ SDS (Figure 6.4), approximately 0.037 % (w/w) SDS per tissue wet weight.
Figure 6.4 Concentration of residual SDS in decellularised bovine cartilage and bone. Data is shown as the mean (n=3) ± 95 % confidence limits.

6.4.2 SDS toxicity

To determine the concentration at which SDS had cytotoxic effects, cell lines were incubated with varying concentrations of SDS in cell culture medium and incubated overnight. Cell viability was determined using the ATP-lite M® assay.

The cytotoxic effect of varying concentrations of SDS on BHK and 3T3 cell lines can be seen in Figure 6.5. At SDS concentrations of 25 µg.ml\(^{-1}\) and below, no cytotoxic effect was seen with either cell line. At ≥ 50 µg.ml\(^{-1}\) SDS, BHK cells showed a significant reduction in counts per minute (CPM), indicating that reduced cell viability had occurred at these concentrations. 3T3 cells only showed a significant reduction in CPM at SDS concentrations ≥ 250 µg.ml\(^{-1}\) suggesting this cell type had lower sensitivity to SDS cytotoxicity.
Figure 6.5 Cytotoxicity of varying concentrations of SDS to BHK and 3T3 cell lines. Data is shown as the mean (n=3) ± 95% confidence limits. * Significant, p<0.05 (ANOVA).

6.4.3 Contact cytotoxicity

Decellularised tissues were incubated with either 3T3 cells (Figure 6.6) or BHK cells (Figure 6.7) for 48 h to determine contact cytotoxicity. Growth of both cell types appeared normal when seeded into a blank well of the culture plate. Those cultured with a steri-strip negative control also had normal morphology and grew up to and against the strip. Cells cultured with cyanoacrylate positive control did not adhere next to the glue and had a rounded morphology characteristic of dead cells. 3T3 cells cultured with decellularised cartilage and bone had normal morphology and grew up to the tissue. However when BHK cells were cultured with decellularised tissue one of the 3 tissues (Figure 6.7 d & e) showed cytotoxic effects, with cells not adhering next to the tissue and having a rounded ball morphology.
Figure 6.6 Contact cytotoxicity of decellularised bovine osteochondral tissues when cultured for 48 h with 3T3 cells. Panel a) cells only, panel b) cyanoacrylate positive control, panel c) steri-strip negative control, panel d) decellularised bovine bone, panel e) decellularised bovine cartilage. 100 x magnification, scale bars = 200 µm.
Figure 6.7 Contact cytotoxicity of decellularised bovine osteochondral tissues when cultured for 48 h with BHK cells. Panel a) cells only, panel b) cyanoacrylate positive control, panel c) steri-strip negative control, panel d) decellularised bovine bone, panel e) decellularised bovine cartilage. 100 x magnification, scale bars = 200 µm.
6.4.4 Modified protocol

Following application of the 7 and 14 day PBS wash modified protocols (Section 6.3.5), cartilage damage occurred in all samples between the 4 and 5 day point. The cartilage detached from a number of samples as observed with porcine tissues (Chapter 5). Further analysis could not be performed.
6.5 Discussion

The aim of this study was to further characterise the decellularised bovine osteochondral matrix to identify whether the resultant scaffold was biocompatible.

For acellular scaffolds to be used in patients, it is essential that they are not toxic or harmful. ISO 10993 is in place to ensure thorough and appropriate testing of all medical devices is completed during product development. Initial basic in vitro biocompatibility testing should be performed prior to in vivo testing and use in animal models to ensure the scaffold has no immediate toxic effects before more advanced immune responses can be investigated.

SDS, a detergent included in the decellularisation process to solubilise cellular membranes has been shown to have cytotoxic effects when not thoroughly removed from dcellularised tissues (Rieder et al., 2004). SDS is able to impart a negative charge on proteins, which may inhibit cell attachment and proliferation, it is also able to denature proteins, which may affect cell survival (Seddon, 2004).

Quantification of the residual SDS in the decellularised tissues, found that the majority of SDS was removed during wash steps, and that tissues only contained ~ 370 ng.mg⁻¹ SDS per wet weight following decellularisation. However, incubation of cells with varying concentrations of SDS showed concentrations of ≥ 250 µg.ml⁻¹ to be cytotoxic to 3T3 cells and even lower concentrations of ≥ 50 µg.ml⁻¹ to be cytotoxic to BHK cells. Assuming 370 ng.mg⁻¹ SDS present in tissues is equivalent to 370 µg.ml⁻¹, the residual SDS in decellularised tissues may have resulted in them being cytotoxic.

Contact cytotoxicity assays with 3T3 cells did not show any cytotoxic effects of the tissues, however, BHK cells, which showed higher sensitivity to SDS did show cytotoxicity with one sample. It may be that, although the concentration of SDS in the tissues was high, it was not immediately accessible from the dense 3D solid tissues to the cell monolayer, as it would be expected that all samples would be cytotoxic with both cell types at this concentration.
As some cytotoxicity was seen, modifications to the protocol were included to increase the number and duration of PBS washes at the end of the process to improve SDS wash out. When additional PBS washes were included, tissue samples began to show signs of damage, as seen in porcine tissue. The cartilage shrivelled in from the bone and in cases became detached. It appeared that the same problem was present with bovine and porcine osteochondral decellularisation.

The potential causes of cartilage damage were thoroughly investigated in Chapter 5, however the size of osteochondral pin decellularised is a common factor in porcine and bovine decellularisation processes which had not been investigated. To conclusively eradicate cartilage damage during the decellularisation process, further methods using larger osteochondral constructs were investigated in the next chapter.
Chapter 7
Decellularisation of large osteochondral constructs

7.1 Introduction

Previously in this study, a protocol was developed for the decellularisation of osteochondral pins from the knee condyles of 6 month old pigs (Chapter 5). A limitation to this process was that the cartilage often became irreversibly damaged during decellularisation. The degree to which cartilage damage occurred varied greatly, resulting in a range of tissue morphologies from slight increase of porosity at the cartilage surface, to shrinkage in from the cut edges, complete loss of GAGs and increase in water content to 95% (w/w), to the cartilage completely dissolving away in the decellularisation wash solutions. The cause of the damage was thoroughly investigated, however, no protocol could be developed which fully eradicated cartilage damage. Prior to studies with porcine cartilage, it had been shown that bovine cartilage pins from the knee condyles of 18 month old animals could be decellularised without sustaining any overt damage (Chapter 4), however, with increased wash cycles to reduce residual SDS levels in the tissue, the bovine cartilage pins also exhibited similar damage (Chapter 6).

During the course of this study, Schwarz et al., (2012) reported upon the decellularisation of porcine and human nasal cartilage. It was of interest to note, that although not recognised by the authors as damaged, the histology images of the decellularised cartilage showed similar morphology to the damaged cartilage found in the present study. A similarity was that Schwarz et al. performed decellularisation on cylindrical cuts of cartilage (5 mm diameter discs, 1 mm depth). Collagen fibres in hyaline cartilage are under tension, this tension plays an important role in cartilage structure and function. The collagen network interacts with GAGs to resist repulsion between the negatively charged proteoglycans, holding the ECM together
(Mow et al., 2005). It was hypothesised that when extracting cartilage pins and producing a cut edge, a loss of tension in the collagen fibres occurred and this facilitated the decellularisation solutions to cause damage to the tissue. In order to test this hypothesis it was logical to determine whether damage would occur when larger cuts or uncut cartilage (large bovine plates and whole porcine condyles) were subject to the decellularisation process and observing the effect on cartilage structure.
7.2 Aims and objectives

Aims:
The aim of the study presented in this chapter was to apply the decellularisation protocols developed in Chapter 5 for porcine cartilage and Chapters 4 and 6 for bovine cartilage to whole porcine condyles and large bovine osteochondral cuts and to identify any structural damage caused to the cartilage.

Objectives:

- To apply the decellularisation protocol for porcine cartilage pins to whole porcine medial condyles (with the cancellous bone removed).
- To apply the decellularisation protocol for bovine pins to bovine osteochondral plates (40 mm long x 20 mm wide x 10 mm deep).
- To qualitatively assess the decellularised osteochondral tissues using histology, to determine the histoarchitecture, GAG distribution and cellularity.
7.3 Methods

7.3.1 Dissection of porcine medial condyles

Whole porcine (6 months old) right legs (n=3) were dissected as described in Section 2.2.2.1 and 2.2.2.2. Following exposure of the femoral component of the knee, the femur was clamped and a hand saw used to cut into the bone between the two condyles. Another cut was made across the femur, from the base of the femoral groove to the back of the condyle, to release the condyle in its entirety. Using a hand drill with 5 mm diameter drill bit, the cancellous bone was drilled into from the underside and bone cutters used to hollow out the condyle, leaving approximately 10 mm subchondral bone underneath the cartilage surface. Condyles were stored frozen as described in Section 2.2.2.4 until required.

7.3.2 Dissection of bovine medial groove plates

Bovine femurs (n=3) were dissected as described in Section 2.2.2.1 and 2.2.2.2. Using callipers, a plate 20 mm wide x 40 mm long was measured and marked out on the cartilage surface using a scalpel. Using a hand saw, the shape was cut into the groove, and a final cut was made parallel to the cartilage surface through the bone to release the plate. A purpose built clamp was used to hold the plate and the subchondral bone was accurately trimmed to 10 mm depth using a hack saw (Figure 7.1).
Figure 7.1 Preparation of bovine osteochondral plates. a) a 20 mm x 40 mm rectangle was marked out on the medial femoral groove and cut out using a saw. b) the plate was secured in a purpose designed holder and the bone was cut to 10 mm deep using a saw.

7.3.3 Porcine condyle decellularisation

Porcine condyles (n=3) were decellularised one per 150 ml pot with 125 ml decellularisation solution as described in Section 5.3.2.2. Condyles were subject to additional 4 x 24 h PBS washes at 42 °C with agitation following the extended 48 - 60 h PBS wash.

7.3.4 Bovine plate decellularisation

Bovine osteochondral plates (n=3) were decellularised one per 150 ml pot with 125 ml decellularisation solution as described in Section 4.4.2.14. Plates were subject to additional 4 x 24 h PBS washes at 42 °C with agitation following the extended 48 - 60 h PBS wash.
7.3.5 Histological analysis

7.3.5.1 Slide preparation

Whole decellularised porcine condyles and bovine plates were fixed in 10 % (v/v) NBF and decalcified as described in Section 2.2.3.1 and 2.2.3.2. A 5 - 8 mm thick slice was taken through the centre of the porcine condyle (medial to lateral side) which was then processed, wax embedded and sectioned as described in Section 2.2.3.3 and 2.2.3.4. Slices (5-8 mm thick) were also taken through the centre of the bovine plate across the plate width which were then processed, wax embedded and sectioned.

7.3.5.2 Reverse processing

Wax embedded bovine tissue blocks were melted in an oven at 60 °C overnight. Under vacuum with agitation in the Leica TP 120 automated tissue processor, tissues were dewaxed through 2 x 2 h cycles of xylene and dehydrated in 2 x 2 h cycles of 100 % methylated spirits. Tissues were placed in 70 % methylated spirits for 15 min prior to being re-processed as described in Section 2.2.3.3.

7.3.5.3 Wax block decalcification

Wax embedded bovine tissue blocks were trimmed down to expose the sample using a microtome (Leica RM2125RTF). Blocks were then immersed in a 12.5 % (w/v) EDTA solution (Section 2.2.3.2.1) overnight at room temperature without agitation to decalcify the exposed surface to be sectioned.

7.3.5.4 Sectioning and slide preparation

Wax embedded tissues were sectioned and transferred to slides as described in Section 2.2.3.4.

7.3.5.5 Histological staining

Tissue sections were stained with H&E (Section 2.2.4.1), Alcian blue (Section 2.2.4.3), van Gieson (Section 2.2.4.4) and DAPI (Section 2.2.4.6) prior to being mounted and imaged microscopically.
7.4 Results

7.4.1 Porcine whole medial condyles

Porcine whole medial condyles (n=3) were decellularised as described in Section 7.3.3. Macroscopically, following decellularisation, cartilage appeared firm and smooth over the whole surface of the condyle, even following the prolonged PBS washes. The cartilage appeared to be intact. Histologically, the decellularised porcine osteochondral tissues appeared to be largely devoid of cells (Figure 7.2 – 7.7), however, a few isolated nuclei were still present in the calcified cartilage regions of the condyle (Figure 7.7b). The cartilage of all three decellularised condyles did not show the increased porosity characteristic of damaged cartilage (Chapter 5), however a slight increase in chondron lacunae size in the superficial zone and surface roughening could be seen (Figure 7.2 – 7.4). The minimal disruption to collagens at the cartilage surface could be more clearly visualised with van Geison staining (Figure 7.8 – 7.10). Alcian blue staining showed low levels of GAGs throughout the superficial and middle cartilage zones (Figure 7.11 & 7.13) and also in the deep zone (Figure 7.12) across the whole cross section of the condyle.

7.4.2 Bovine osteochondral plates

Bovine osteochondral plates were decellularised as described in Section 7.3.4. Following decellularisation, cartilage appeared smooth, firm and white and appeared to be intact. Initially, as a result of technical error, samples were incompletely processed, attempts were made to reverse process the tissues and correctly reprocess as described in Sections 7.5.3.1 and 7.5.3.2. Incomplete processing rendered the tissues dry, crumbly and difficult to section, so blocks were directly decalcified to ease sectioning (Section 7.3.5.3). Samples were successfully sectioned, however as a result of sample preparation, histological stains appeared more intense than in tissues which had been processed correctly. Histologically, bovine tissues showed incomplete decellularisation, with nuclei remaining in the deep calcified regions (Figures 7.14 – 7.19). The cut edges and cartilage surface
of all three bovine osteochondral cuts appeared porous, showing signs of damage, however the internal middle and deep zones were not damaged as shown by intense van Gieson staining of cartilage collagens (Figure 7.20 – 7.22). The cut edges and surface also showed reduced intensity of alcian blue staining showing loss of GAGs in these regions directly in contact with the decellularisation solutions (Figure 7.23 – 7.25). Bovine osteochondral plates showed larger areas of damage than whole porcine condyles following decellularisation.
Figure 7.2 H&E stained sections of decellularised porcine condyle 1. a) full condyle, scale bar = 1000 µm, b) and c) scale bar = 500 µm.
Figure 7.3  H&E stained sections of decellularised porcine condyle 2. a) full condyle, scale bar = 1000 μm, b) scale bar = 500 μm.
Figure 7.4 H&E stained sections of decellularised porcine condyle 3. a) full condyle, scale bar = 1000 µm, b) and c) scale bar = 500 µm.
Figure 7.5 DAPI stained sections of decellularised porcine condyle 1. Blue = DAPI. Green = collagen autofluorescence. a) full condyle, scale bar = 1000 µm, b) and c) scale bar = 500 µm.
Figure 7.6. DAPI stained sections of decellularised porcine condyle 2. Blue = DAPI. Green = collagen autofluorescence. a) full condyle, scale bar = 1000 µm, b) and c) scale bar = 500 µm.
Figure 7.7. DAPI stained sections of decellularised porcine condyle 3. Blue = DAPI. Green = collagen autofluorescence. a) full condyle, scale bar = 1000 µm, b) and c) scale bar = 500 µm.
Figure 7.8. van Gieson stained sections of decellularised porcine condyle 1. a) full condyle, scale bar = 1000 µm, b) and c) scale bar = 500 µm.
Figure 7.9. van Gieson stained sections of decellularised porcine condyle 2. a) full condyle, scale bar = 1000 µm, b) and c) scale bar = 500 µm.
Figure 7.10. van Gieson stained sections of decellularised porcine condyle 3. a) full condyle, scale bar = 1000 µm, b) and c) scale bar = 500 µm.
Figure 7.11. Alcian blue stained sections of decellularised porcine condyle. a) full condyle, scale bar = 1000 µm, b) and c) scale bar = 500 µm.
Figure 7.12. Alcian blue stained sections of decellularised porcine condyle 2. a) full condyle, scale bar = 1000 µm, b) and c) scale bar = 500 µm.
Figure 7.13. Alcian blue stained sections of decellularised porcine condyle. 3. a) full condyle, scale bar = 1000 µm, b) and c) scale bar = 500 µm.
Figure 7.14 H&E stained sections of decellularised bovine groove plate 1. Cross section of plate, scale bar = 500 µm.
Figure 7.15 H&E stained sections of decellularised bovine groove plate 2. Cross section of plate, scale bar = 500 µm.
Figure 7.16 H&E stained sections of decellularised bovine groove plate 3. Cross section of plate, scale bar = 500 µm.
Figure 7.17 DAPI stained sections of decellularised bovine femoral groove plate 1. Cross section of plate, blue = DAPI, green = collagen autofluorescence, scale bar = 500 µm.
Figure 7.18 DAPI stained sections of decellularised bovine femoral groove plate 2. Cross section of plate, blue = DAPI, green = collagen autofluorescence, scale bar = 500 µm.
Figure 7.19 DAPI stained sections of decellularised bovine femoral groove plate 3. Cross section of plate, blue = DAPI, green = collagen autofluorescence, scale bar = 500 µm.
Figure 7.20 van Gieson stained sections of decellularised bovine femoral groove plate 1. Cross section of plate, scale bar = 500 µm.
Figure 7.21 van Gieson stained sections of decellularised bovine femoral groove plate 2. Cross section of plate, scale bar = 500 µm.
Figure 7.22 van Gieson stained sections of decellularised bovine femoral groove plate 3. Cross section of plate, scale bar = 500 µm.
Figure 7.23 Alcian blue stained sections of decellularised bovine femoral groove plate 1. Cross section of plate, scale bar = 500 µm.
Figure 7.24. Alcian blue stained sections of decellularised bovine femoral groove plate 2. Cross section of plate, scale bar = 500 µm.
Figure 7.25 Alcian blue stained sections of decellularised bovine femoral groove plate 3. Cross section of plate, scale bar = 500 µm.
7.1 Discussion

Previous attempts to decellularise porcine osteochondral tissues (Chapter 5) resulted in the cartilage becoming damaged, showing a large reduction in GAGs and increase in water content. Macroscopically, damaged tissues appeared soft, shrinking in from the edges of the bone plug and presented with a roughened dull surface. Histologically, cartilage showed increased porosity, with the smooth collagen network becoming a more mesh-like matrix full of holes and the size of chondron lacunae appeared increased. The work in Chapter 5 attempted to systematically elucidate the cause of cartilage damage, the high temperature of incubations in the decellularisation solutions was found to be a contributing factor, but it was not possible to identify a protocol that fully eradicated the cartilage damage. Furthermore, the work carried out in Chapter 6 to determine the biocompatibility of decellularised bovine osteochondral scaffolds showed that increasing the duration of the terminal PBS wash cycles to greater than 60 h also resulted in damage to bovine cartilage, which had not previously been seen.

To further investigate the cause of cartilage damage, the preliminary work carried out in this Chapter used large osteochondral plates (2 mm x 4 mm, bovine) or whole condyles (porcine) to investigate the effect of having a reduced ratio of cut edges to cartilage area on cartilage stability during decellularisation. As reviewed in Chapter 1 and by Mow et al. (2005) the fibres of the cartilage collagen network have a characteristic orientation (Jeffery et al., 1991) and are under tension, providing the tensile strength of the tissue. Proteoglycans are dispersed throughout the collagen network and bind water in the tissue, providing the compressive strength of the tissue (Roughley, 2006). It was hypothesised that preparing osteochondral tissues for decellularisation by cutting around the circumference of the pin had severed collagen fibres and resulted in loss of tension, particularly in fibres orientated parallel to the surface in the cartilage superficial zone. With the loss of tension, the collagen network would have reduced ability to counter the repulsive forces of negatively charged proteoglycans being held in close
proximity to one another, resulting in an increase in porosity allowing GAGs to be removed, more water to enter the tissue and the collagen fibres to therefore clump together.

Porcine osteochondral blocks (50 mm in length, 10 mm in width and 10 mm in depth) with a larger area to cut edge ratio than 9 mm diameter pins were successfully decellularised by Kheir *et al.* (2011) using a similar protocol to that described here. The osteochondral blocks were subject to four cycles of freeze thaw (the final two in hypotonic solution) and then incubated through 6 cycles of 24 h in hypotonic solution followed by 24 h in 0.1 % (w/v) SDS in hypotonic solution, both containing aprotinin and EDTA at 45 °C with agitation. Blocks were then treated with nuclease and sterilised using 0.1 % PAA (v/v). The process was completed by 2 x 30 min PBS washes and a short 24 h final PBS wash. Following use of this protocol, porcine osteochondral tissues were fully decellularised and remained intact. Histological analysis showed full removal of cell nuclei, however GAG content was greatly reduced. No collagen clumping, characteristic of cartilage damage was seen, however some loss of tension could be seen at the surface and superficial zone of cartilage sections stained specifically using an antibody against collagen type II.

In contrast, Shwartz *et al.* (2012) used much smaller 5 mm diameter discs of human and porcine nasal septum cartilage with a large cut edge to area ratio. The decellularised tissue had the characteristic collagen clumping and loss of GAG seen in severely damaged cartilage in this study (Chapter 5), differing greatly to decellularisation results seen by Kheir *et al.* (2011).

The size of osteochondral tissues used for decellularisation in this Chapter was therefore increased to minimise the ratio of cut edge to cartilage area. For bovine osteochondral tissues, the ratio of cut edge to cartilage area was reduced by using larger osteochondral plates (2 mm x 4 mm) as opposed to small pins (9 mm diameter). For porcine osteochondral tissues, only one cut edge was produced when separating the condyle from the groove, all other edges were natural cartilage edges on the condyle.

Following decellularisation the bovine tissues were not fully decellularised and showed some damage, especially at the cut edge and cartilage surface.
The central cartilage regions retained GAG content and did not show the porous structure characteristic of damage histologically. The cartilage did not detach from the bone or appear damaged macroscopically, with the exception of the cut edges.

Porcine tissues did not appear damaged macroscopically following decellularisation, but upon histological analysis, characteristic damage could be seen in the superficial zone of the cartilage and at the surface, however this damage appeared less severe than that seen in bovine cartilage. The tissues were almost completely decellularised apart from a few nuclei still present at the cartilage-bone interface and the tissue remained intact following extended washing in PBS.

It was therefore demonstrated that reducing the ratio of cut edges to cartilage area greatly improved cartilage stability during the decellularisation processes, but did not completely eradicate cartilage damage. Additional steps should be taken to ensure complete decellularisation of porcine whole condyles and bovine blocks. It would then be possible to harvest acellular osteochondral pins of clinically relevant size from the least damaged regions of these decellularised large osteochondral constructs.

Further biochemical and biomechanical testing is required to determine the degree of damage caused to decellularised whole condyles and determine whether the resultant tissues retained adequate biological and biomechanical function for use as a cartilage substitution biomaterial.
Chapter 8
Discussion

8.1 General Discussion

The underpinning hypothesis of this study was that it was possible to generate an acellular xenogeneic osteochondral graft material of future clinical utility in osteochondral lesion repair. A novel regenerative cartilage repair material such as this is required, as joint damage is common in the active population. Osteochondral lesions as a result of trauma can in themselves cause pain, swelling and mechanical problems, reducing the quality of life for patients (Minas & Nehrer, 1997). Furthermore, some instances of osteoarthritis have been found to originate from initially minor cartilage lesions (Ding et al., 2005) which, due to their avascular nature are unable to undergo healing and repair, so degenerate over time with regular joint loading and articulation (Chaing & Jiang, 2009). Currently, for osteoarthritic joints, the only effective treatment is total joint arthroplasty. In the case of osteoarthritis which originates from cartilage lesions, early intervention therapies are available to prevent or delay the disease onset by repair of the initial lesion. Surgical therapies currently performed to preserve cartilage such as lavage and debridement (Chang et al., 1993; Moseley et al., 2002) and marrow stimulation techniques (Steinwachs et al., 2008) however have variable long term outcomes and often result in inferior fibrocartilage like repair materials. Similarly, perichondrial/periosteal grafting, mosaicplasty (Koulalis et al., 2004) and autologous chondrocyte implantation (Grande et al., 1989) may result in donor site morbidity amongst other complications (Minas & Nehrer, 1997; Lützner et al., 2009).

As current surgical techniques have been unable to restore fully functional hyaline cartilage, tissue engineering is seen as the way forward in developing adequate cartilage repair therapies with good long term prognosis, by optimising and balancing scaffold biomaterials with bioactive factors and cells (Vinatier et al., 2009). Biomaterials can be broadly labelled
as natural or synthetic. Synthetic materials currently being researched include polylactic acid (PLA; Giurea et al., 2003) and polyglycolic acid (PGA; Liu et al., 2002), they are easy to manufacture and their specific properties can be accurately controlled to mimic the natural ECM. The biomechanical properties of such materials can be tailored, however, often there are issues with tissue integration, a lack of biological cues and detrimental degradation products (Atala, 2007, Puppi et al., 2010). Natural biomaterials such as collagens (Frenkel et al., 1997) or hyaluronic acid (Grigolo et al., 2001) have the advantage of low toxicity and biological signalling, however there are issues regarding batch variation and they do not have adequate biomechanical properties (Puppi et al., 2010).

The aforementioned biomaterials, although promising, do not show the same level of structural and biological complexity found in healthy cartilage which is key to the function and regeneration potential of the natural tissue. Recent tissue engineering approaches have been to decellularise or devitalise natural ECM scaffolds, thereby retaining the natural ECM constituents and structure as well as biological cues such as growth factors whilst removing immunogenic materials which would result in graft rejection (Crapo et al., 2011, Benders et al., 2013).

This tissue engineered acellular ECM approach is already being used clinically for a number of tissues including heart valves (Bach et al., 2005) and trachea (Macchiarini et al., 2008). Decellularised small intestinal submucosa (SIS) has been used clinically for over a decade to repair tissues such as skin, muscle and tendon (eg. Restore™, DePuy Orthopaedics; Surgisis®, Cook Biotech Inc.). For musculoskeletal tissue repair, decellularised ECM biomaterials including tendon (Ingram et al., 2007; Martinello et al., 2012) and meniscus (Stapleton et al., 2008) are undergoing development. The current study was performed with the aim of producing a decellularised osteochondral ECM scaffold from xenogeneic tissues which could be used either seeded with cells or as a standalone off-the-shelf product to enable effective long term repair of osteochondral lesions.

This tissue engineered approach for cartilage or osteochondral repair has previously been researched in a number of studies and the current research
position was well summarised by Benders et al. (2013). Some approaches have focused on homogenising cartilage tissue by lyophilisation and grinding (Yang, Z et al., 2010) or shattering into fragments and centrifuging to produce microfilaments (Yang, Q et al., 2008) prior to decellularisation. Scaffolds produced from decellularised homogenised cartilage have the benefit of retaining bioactive cues for cell growth and are also more customisable, however, they lack the 3D architecture and structure of native cartilage and therefore have reduced biomechanical function. Other approaches have been to research the decellularisation of cell derived cartilage ECM (Elder et al., 2009). Decellularised cell-derived-ECM scaffolds have no issues of biocompatibility, however the need to culture cells renders the process time consuming and this approach requires cell harvesting from the patient, increasing the number of invasive procedures performed and potentially causing donor site morbidity. Yet another approach has been to develop methods to decellularise intact, native 3D cartilage tissues (Elder et al., 2010, Schwarz et al., 2012). The decellularisation of intact native cartilage tissues has the potential to produce an ECM with bioactive cues, and with the natural biological, biochemical and biomechanical composition and structure.

Although protocols for whole native 3D cartilage decellularisation are currently under development, there are to date no reports of the clinical relevance/success of such products. Such approaches do not provide a subchondral bone component for integration and stability of a cartilage repair biomaterial upon implantation.

Kheir et al. (2011) developed a process to decellularise porcine osteochondral tissues, whereby intact, full thickness, zonal cartilage, subchondral bone plate and trabecular bone were fully acellular and biocompatible and retained similar biological and biochemical composition compared to native tissue. In this decellularisation protocol, osteochondral blocks (50 mm in length, 10 mm in width and 10 mm in depth) were subject to four cycles of freeze thaw (the final two in hypotonic solution) and then incubated through 6 cycles of 24 h in hypotonic solution followed by 24 h in 0.1 % (w/v) SDS in hypotonic solution, both containing aprotinin and EDTA at 45 °C with agitation. Blocks were then treated with nuclease and sterilised
using 0.1 % PAA (v/v). The process was completed by 2 x 30 min PBS
washes and a short 24 h final PBS wash. The decellularised matrix had
reduced GAG content compared to native cartilage which resulted in inferior
biomechanical properties. Additionally the size and shape of the acellular
osteochondral matrix was not appropriate for current surgical applications,
such as mosaicplasty.

The current study was intended to improve and progress the work of Kheir et
al. (2011), Firstly by addressing the xenogeneic source material used for
decellularisation, to identify a joint region and species which had similar
biological, biochemical and biomechanical properties to human cartilage,
and potentially find a tissue with high GAG content, to reduce the detrimental
effect of GAG loss following decellularisation. The source material would
also be shaped to a 9 mm diameter osteochondral pin, to enable the
biomaterial to be used with current surgical techniques and equipment.
Secondly, alterations to the established decellularisation protocol (Stapleton
et al., 2008; Kheir et al., 2011) were investigated to minimise GAG loss and
produce a biologically, biochemically and biomechanically acceptable
osteochondral scaffold. The improved decellularised scaffold could then be
investigated in terms of biocompatibility and regenerative capacity.

A variety of source tissues have been used for articular cartilage
decellularisation including immature 4 week old calf cartilage from the distal
femur (Elder et al., 2010), 7 – 8 month old pig knee cartilage (Schwartz et
al., 2012) and osteochondral tissues from the femoral groove of 4 month old
pigs (Kheir et al., 2011). It has been well documented that the biomechanical
properties of cartilage vary topographically (Shepherd & Seedhom, 1999b),
as does the cartilage GAG content (Rogers et al., 2006) and thickness
(Shepherd & Seedhom 1999a). It therefore seems sensible to constrain a
decellularisation source cartilage to a specific joint region, rather than taking,
for example, distal femoral knee cartilage as a whole, in order to avoid
unnecessary variation in the properties of the final scaffold. Similarly,
cartilage properties are also known to vary between species (Athanasiou et
al., 1991; Kääb et al., 1998) and with age (Julkunen et al., 2009; Rieppo et
al., 2009; Hamann et al., 2013). The properties of a decellularised scaffold
will relate to those of the source material, so it was logical that donor
species, age and joint region should be investigated to determine the optimum starting material to produce a scaffold with acceptable properties for use in human patients.

In the current study, cartilage from three common species were investigated; pig (6 month), cow (18 month) and sheep (aged ~12 month or > 4 year). Tissues were compared from the acetabulum and femoral head of the hip and the groove, medial and lateral condyles and medial and lateral tibial plateau of the knee of each species. Ovine cartilage, being from the smallest species, was thinnest (femoral groove cartilage was 0.86 mm in young sheep and 0.63 mm in old sheep) much thinner than values reported for human cartilage (1.76-2.59 mm, Shepherd & Seedhom 1999b). If implanted into a human knee joint, the ovine osteochondral pin would not be able to simultaneously align at the surface and at the cartilage-bone interface with the surrounding human tissue which would be problematic for integration and function. For this reason, ovine tissues were discounted as a potential source material. Additionally, mature ovine tissues showed signs of degeneration, being yellowed and stiffened suggesting the presence of advanced glycation end products (Bank et al., 1998). Both porcine and bovine tissues had comparable cartilage thickness to human (2.04 mm and 1.52 mm respectively), and also showed high levels of sulphated proteoglycans, 78 ± 30 μg.mg⁻¹ and 68 ± 8 μg.mg⁻¹ GAG per wet weight cartilage respectively, compared to the 54.7 mg.g⁻¹, 38.6 mg.g⁻¹ (Vilim et al., 1991) and 25 – 40 μg.mg⁻¹ (Hollander et al., 1991) quoted for human femoral head cartilage. The biomechanical properties of each species differed, with bovine cartilage being stiffer than porcine on the whole, with an equilibrium elastic modulus of 0.66 MPa and 0.81 MPa respectively for cartilage from the lateral condyle, compared to an equilibrium aggregate modulus of 0.70 MPa for human cartilage (Athanasiou et al., 1991). The relatively low stiffness of porcine cartilage was in contrast with the high GAG content measured which should infer compressive resistance, this highlighted the possible effects of cartilage maturity. Proteoglycans within the relatively immature porcine cartilage may not be as well developed and functional as those of mature tissues. Differences were seen between the properties of different joint regions, with cartilage from the femoral knee (condyles and
groove) tending to be thickest and having higher GAG content than other areas, the tissue in these regions also tended to be stiffer.

Regrettably, biotribological analysis of cartilage was not performed in the current study. It has been reported that the coefficient of friction of cartilage is increased with GAG depletion (Katta et al., 2009), likely due to reduced biphasic and surface lubrication. Varying levels of proteoglycans, therefore, may have an impact on the frictional properties of cartilage from different species and/or joint regions. Information in the literature regarding this is limited for comparison due to the variation in testing conditions.

A major limitation of this aspect of the current study is that animals of different ages were compared. This was due to the restricted availability of animals bred from the food chain for human consumption. In this case, it is difficult to discern which properties can be attributed to the species and which to the stage of cartilage maturation. This raises an intriguing point for consideration, is a mature or immature source tissue more appropriate for an acellular natural ECM scaffold? As highlighted previously, underdeveloped, immature cartilage may not have the same biomechanical function as mature human cartilage, which may restrict the success of such a material upon implantation. However, beneficially, immature cartilage is still undergoing growth and remodelling, it is more metabolically active than fully developed mature cartilage. It stands to reason that the growth factors and biological signals which are present, guiding tissue growth, may be advantageous if retained in a decellularised scaffold, to potentially improve recellularisation and neomatrix deposition by infiltrating host cells. Furthermore, immature porcine cartilage had an underdeveloped subchondral bone plate and vascularisation of the cartilage. These features may aid in the migration of host cells (including mesenchymal stem cells) from the surrounding bone marrow and cartilage.

As the benefits of using a mature versus an immature tissue were unknown, it was decided that two tissues, immature porcine medial condyle and mature bovine medial groove osteochondral tissues should both be taken forward as source materials for the development of a decellularisation
protocol, based on the biological, biochemical and biomechanical properties investigated.

Following a number of iterations, a protocol was optimised to decellularise bovine osteochondral pins. The method, based on that of Stapleton et al. (2008) and Kheir et al. (2011), comprised a novel incubation of osteochondral pins in PBS with proteinase inhibitors for 18 h followed by use of a water pik to physically remove bone marrow. In accordance with previous methods, tissues underwent four freeze/thaw cycles (two of which were in hypotonic buffer) to produce ice crystals to physically open up the matrix and produce an osmotic tension to lyse cells. Tissues were then subject to two cycles of washing in hypotonic buffer followed by 0.1% (w/v) SDS in hypotonic buffer to again lyse cells and solubilise cellular membranes, all solutions contained aprotinin (10 KIU.ml⁻¹) to inhibit protease activity. One cycle of hypotonic and SDS washes was not sufficient to remove cells in the dense calcified cartilage and subchondral bone plate, however only two cycles were required, fewer than that of Kheir et al. (2011) and Stapleton et al. (2008), likely due to the smaller size of tissue used. Osteochondral pins were then treated with nucleases to digest nucleic acids and sterilised using 0.1% (v/v) PAA. Following a final extended PBS wash the decellularised tissue had no visible whole cell nuclei, and a dry weight cartilage DNA content of 39 ng.mg⁻¹. An undesirable effect of the decellularisation process was that the cartilage GAG content of the acellular scaffold was severely reduced from that of the native bovine cartilage. Porcine tissues were also successfully decellularised using the same method, however only a single incubation in SDS was required to fully remove cellular debris and reduce the DNA content to below 50 ng.mg⁻¹. Again, GAG loss was seen, but the reduction was only 60%.

A decellularised matrix, as classified by Crapo et al. (2011), should have a DNA content of < 50 ng.mg⁻¹. Reduction of DNA content is important as the phosphate backbone of residual nucleic acids can act as a nucleation site for calcification of implants (Schoen & Levy, 2005). Whereas this would not be a problem for the bone component of the composite scaffold, calcification of cartilage may severely alter the equilibrium elastic modulus of the tissue and have detrimental effects on the friction coefficient. Such calcified cartilage
could cause severe damage to the opposing articulating surface and accelerate joint degeneration, rather than prevent or delay cartilage damage, as was the original intention of the cartilage lesion repair.

It is also important to take into account the fragment length of any residual DNA in decellularised matrix. Keane et al. (2012), used DNA fragment length as an indication of decellularisation efficacy, to quantify potential residual cellular debris. It was shown that decellularised tissues with a larger residual DNA fragment length (500 – 1500 bp) had tended towards an inflammatory M1 macrophage response from the host. Whereas a constructive M2 macrophage response was seen with more effectively decellularised porcine SIS with residual DNA of < 500 bp fragment length, although values were not significant in vivo. There was a significantly higher number of infiltrating macrophages in the SIS with large DNA fragment size. However, the study only used DNA quantification to test decellularisation efficacy and did not make any attempt to quantify other antigenic cell remnants such as intracellular cytoplasmic proteins and membrane components by any other means. Stapleton et al. (2008) assessed the fragment length of residual DNA in decellularised porcine meniscal tissue via agarose gel electrophoresis. Fresh tissue showed a clear band around 10,000 base pairs, no band could be seen for DNA extracted from decellularised meniscus. However, they later report a significant reduction in DNA concentration following decellularisation, so the lack of a band present for decellularised tissue may not be that the fragment length is too short to be detected, but that the amount of DNA is too small to be seen, as the same volume of extracted DNA was loaded into the gel, despite the fact that the concentration of DNA differed between native and decellularised tissues.

It is also important to ensure DNA fragment size is minimal so to ensure that functional genomic material is not transferred to the host, which could be problematic, for example, the potential transfer of latent viral DNA. The risks from latent viral DNA are minimal however, as infection-independent horizontal gene transfer of porcine endogenous retrovirus (PERV) from porcine cells to phagocytic human fibroblasts has been shown to be inefficient, with the virus only transmitted to 0.22 % of co-cultured cells, and transient, with no detectable virus after 4 weeks. The virus was unable to
insert into the human genome and no viral replication occurred (Bisset et al., 2007). A major concern when using porcine xenogeneic tissues is the transmission of PERV, as pigs are asymptomatic carriers (Bucher et al., 2005). Whilst transmission of PERV has been seen in vitro (Patience et al., 1997), it has not been observed in vivo (Irgang et al., 2003). As viruses are obligate intracellular parasites, destruction and removal of porcine cells from the xenograft should result in the complete removal of PERV’s also.

In the current decellularisation protocol, nucleases (DNase and RNase) were used to cleave nucleic acids exposed following rupture of cytoplasmic and nuclear membranes (Stapleton et al., 2008; Kheir et al., 2011). Cleavage of nucleic acids into small fragments enabled effective wash out, and the DNA content of decellularised cartilage was significantly reduced, however the fragment length of residual DNA was not quantified. Initial attempts were made to measure fragment length of residual DNA isolated from decellularised tissues using agarose gel electrophoresis, however potentially due to the low levels of DNA present, no DNA could be detected. It may be necessary in future studies to concentrate DNA extracts to enable better detection.

Sterilisation of the natural scaffold is an important step in the decellularisation process to avoid disease transfer from viral, bacterial and fungal agents.

Another concern regarding the medical use of animal materials is the risk of bovine spongiform encephalopathy (BSE) transmission (Laurencin & Al-Amin, 2008). Transmissible spongiform encephalopathys (TSE) are chronic degenerative diseases characterised by the accumulation of the abnormal cellular glycoprotein known as prion protein. Bovine spongiform encephalopathy appeared in UK cattle in the mid 1980’s. This TSE is transmissible between species and the infectious prion protein in humans causes fatal Creutzfeldt-Jakob disease. Transmission has been seen to occur through human consumption of contaminated beef products, and so caution should be taken when using biological materials, especially bovine products for medical applications (Dormont, 1996; Wickham, 1996). Terminal sterilisation techniques currently used for xenotransplants are
ineffective against prion proteins and processes which are able to inactivate the protein would cause damage to delicate biological tissues (Dormont, 1996). It is possible to minimise the risk of spongiform encephalopathy transmission from xenotransplant as different tissues have different levels of infectiousness. The brain and central nervous system are highly infectious, spleen tissue is infectious but $10^6$ times less so than the brain. However, other tissues such as heart and bone have not been shown to transmit any TSE agent (Dormont, 1996). Therefore it is unlikely that any bovine osteochondral tissues, even from an animal with BSE, would be capable of transmitting spongiform encephalopathy to humans following transplantation. To eradicate the risk, it is a regulatory requirement to use tissues from certified BSE free cattle, as outlined in the Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (EMEA/410/01 REV 3) published in the official journal of the European Union (2011), however this would greatly increase the manufacturing costs.

Terminal sterilisation is a more viable alternative to screening source tissues and sterile tissue processing. The current protocol used 0.1% (v/v) PAA to sterilise the decellularised osteochondral scaffolds. This method, although shown to be effective for other tissues (Lomas et al., 2003), has not been validated for osteochondral tissues. It may be that the dense nature of bone and cartilage inhibits penetration of PAA. Mirsadraee et al. (2006) developed a methodology to validate the sporicidal activity of 0.1 % (v/v) PAA on decellularised human pericardium. Bacillus Atrophaeus spores were applied directly to the surface of the decellularised tissue, or to filter paper which could only be accessed by permeation of the sterilisation solution through a layer of pericardium. Tissues and filter papers were macerated and cultured on tryptone soya agar plates, the number of colony forming units was compared between PAA treated and untreated tissues. A similar method was performed by Pruss et al. (1999) to confirm virus inactivation by PAA in bone. These methods could be modified and used to validate PAA sterilisation of osteochondral tissues. Additionally, sterilised osteochondral tissues could be macerated and cultured on a variety of plates (agar, blood agar etc.) to observe the presence/absence of residual microbes. Other sterilisation methods such as ethylene oxide
treatment or γ irradiation could potentially provide more acceptable results in terms of tissue penetration, however the level of irradiation would need to be optimised in order to minimise tissue damage (Vangsness et al., 2003). Although bovine and porcine osteochondral tissues were successfully decellularised, a significant structural change was seen in the cartilage matrix. The GAG content of porcine cartilage was reduced by 60 %, and bovine cartilage was almost devoid of GAGs, with only 1 % of the original content remaining following decellularisation. SDS has been linked to GAG reduction, as the anionic detergent can disrupt protein-protein interactions (Seddon et al., 2004). It is hypothesised that ionic interactions with SDS disrupt link protein, a key attachment protein which links hyaluronan to aggrecan. This link protein disruption essentially would cleave the proteoglycan network of cartilage and enable wash out of aggrecan. The loss of GAGs from the cartilage tissue would lead to increased cartilage deformation as the fluid phase of load support was reduced. The effect of GAG loss on the biotribology of cartilage is currently unknown. It is unclear what the effect of altered biomechanical and biotribological properties of the osteochondral graft would be post implantation. However, previous studies have shown that the reduction of cartilage GAG content had deleterious effects on the coefficient of friction (Katta et al., 2009). It may be that ingress of host cells into the biomaterial post implantation would begin to produce proteoglycans to replenish those lost during decellularisation, without the temporarily reduced GAG concentration having adverse effects in the short term. Alternatively, it may be necessary to take a MACI-type approach and recellularise scaffolds prior to implantation with extracted autologous cells, this would potentially begin the production of proteoglycans ex vivo, improving or restoring tissue function. Furthermore, to produce an off the shelf product, without the need to culture a patient’s own cells, it may be possible to restore cartilage biochemistry using self-assembling peptides. Many peptides in nature undergo self-assembly; “the spontaneous association of molecules under equilibrium conditions into stable, structurally well-defined aggregates joined by noncovalent bonds” (Whitesides et al., 1991). Synthesised, designed self-assembling peptides are not naturally occurring but are produced from natural building blocks. A number of fibrous
self-assembling peptide hydrogels have been identified as potential candidates for use as tissue engineering biomaterials. It is possible to alter the amino acid sequence to control peptide properties such as assembly (to control hydrogel pore size, fibre thickness and mechanical properties), chemical properties (to determine pH and ionic sensitivity and surface properties) or even to include biologically relevant amino acid sequences to alter the biological properties (Maude et al. 2013). A set of self-assembling peptides currently undergoing research and development are the P₁₁ group of peptides. These peptides are of particular interest in tissue engineering approaches as their assembly can be triggered by a change in pH, ionic strength or, for some peptides, upon addition of a complementary counterpart, so they can be applied as a liquid and undergo gelation in situ and some peptides have shown to be cytocompatible (Maude et al. 2013). Such peptides in liquid form could be applied to the decellularised cartilage matrix alongside or covalently bonded to chondroitin sulphate or other GAGs, self-assembly could then be triggered, trapping the GAGs in the tissue. Research into such an approach is already underway to restore GAG content in spinal discs (Miles et al., 2012).

The biocompatibility of the decellularised osteochondral xenograft required investigation prior to pre-clinical evaluation. ISO 10993 is in place to ensure thorough and appropriate testing of all medical devices is completed during product development. Initial basic in vitro biocompatibility testing should be performed prior to in vivo testing and use in animal models to ensure the scaffold has no immediate toxic effects before more advanced immune responses can be investigated.

SDS used in the decellularisation process has been reported to have cytotoxic effects when not effectively removed from decellularised tissue matrices (Rieder et al, 2004). Residual SDS within the scaffold may prevent ingrowth of cells in vivo and result in graft failure. Quantification of residual SDS using detection of C¹⁴ labelled SDS in decellularised bovine osteochondral tissues revealed a relatively high concentration of residual SDS, above cytotoxic levels, even though most SDS had been washed out into the wash solutions. Surprisingly, no cytotoxic effects were observed when decellularised osteochondral tissues were cultured with the 3T3 cell
line, however, when cultured with BHK cells, contact cytotoxicity was seen with one sample. This largely corroborates with the results of Kheir et al. (2011), in which three times as many SDS washes were applied to a larger tissue section with the same duration of terminal wash cycles as the current study, but no cytotoxicity was seen. It is likely that the three dimensional dense nature of the osteochondral tissue may give false positives for cytotoxicity and further testing should be performed to assess the biocompatibility of soluble extracts of the scaffold, or attempts to 3D culture cells with the scaffold. It did however appear that the acellular tissue was having some cytotoxic effect and alterations to the decellularisation protocol were required to improve biocompatibility.

As porcine osteochondral tissues required a less extensive protocol for decellularisation, the protocol was altered in an attempt to retain a higher percentage of the cartilage GAG content. Upon changing the duration/concentration of the SDS wash it was found that cartilage was sustaining damage, and in cases completely disintegrating and dissolving away from the bone into the wash solutions. Analysis of damaged tissues revealed quantitatively a huge increase in water content, a large reduction in cartilage GAG content, however collagen denaturation was not occurring. Histologically, tissues appeared highly porous, had a roughened surface and the connection of cartilage to the bone in the hypertrophic cartilage region was stretched and tenuous. Furthermore, upon increasing the duration of the final PBS wash to improve biocompatibility of decellularised bovine cartilage, the same damage was also witnessed in bovine tissues.

Such damage to articular cartilage has not been reported previously using SDS based decellularisation methods (Elder et al., 2010; Kheir et al., 2011). However, similar characteristics were witnessed when using more harsh protocols on nasal septum cartilage (Schwarz et al., 2012). Numerous iterations of the current decellularisation protocol were carried out to elucidate the cause of cartilage damage. None of the individual wash cycles of the protocol were found to be solely responsible for cartilage degradation, and surprisingly, incubation in PBS alone for the duration of the protocol lead to damage. The effect of solution osmolality and protease activity were ruled out, as were the number of pins decellularised per pot and the age/stage of
maturation of the source tissue. Only a decrease in incubation temperature was shown to reduce the extent of cartilage damage, however not to eliminate it.

That porcine cartilage had sustained damage, simply with incubation in PBS with agitation at 42 °C, was surprising. This finding highlighted the relative fragility of cartilage when extracted from its natural environment.

The stretching of the porcine cartilage matrix at the hypertrophic zone and detachment of the cartilage at the interface with bone suggests that this area is a weak spot. The number of large cells in this region makes it highly porous and removal of cells from this region may have further weakened the structure. The difference in structure in this region between porcine and bovine cartilage may provide a possible explanation as to why porcine cartilage was damaged more readily than bovine. Bovine deep cartilage has linear columns of chondrocytes aligned perpendicularly to the bone, with defined areas of cartilage matrix between columns, and has fewer cells, rather than the clusters of large numbers of hypertrophic cells seen in porcine cartilage. Therefore the attachment of cartilage to bone may be stronger in the more mature bovine tissues. It has been widely accepted that collagen fibres are continuous between cartilage and bone to anchor the two tissues securely together (Mow et al., 2005). Other studies, however contradict this idea (Clark & Huber, 1990), so it may be that the attachment of cartilage to bone is not as secure as is commonly assumed.

It was unexpected that neither immature porcine cartilage (4 months old), nor mature cartilage (2.4 – 4.2 years old) became damaged during the decellularisation process, whereas cartilage of 6 month old pigs did. Also of note was the relative inconsistency with which 6 month old porcine tissue became damaged. It is possible that at this transitional maturation stage porcine cartilage is comparatively unstable. Rieppo et al. (2009) reported that in porcine cartilage during maturation, the collagen structure was altered from fibres being aligned mostly parallel to the surface (4 months old) to developing the characteristic anisotropic fibre orientation of mature (21 month old) cartilage. The current study highlighted other major characteristics of immature cartilage which are lost during maturation;
vascularisation and cartilage thickness. Cartilage also decreases in cellularity following maturation. It is clear that maturing tissues are undergoing major metabolic changes, greatly altering the structure of the tissue (Hamann et al., 2013). Following the anabolic processes of tissue formation, catabolic processes ensue to reduce cellularity, vascularisation and cartilage thickness as well as re-orientate collagen fibrils, during this transitional period prior to mature tissue homeostasis, it is possible that the tissue is more susceptible to damage, providing a possible explanation for why cartilage from 6 month old pigs was more readily, yet inconsistently damaged during decellularisation.

It was hypothesised that by decellularising an osteochondral pin, the production of a cut edge was detrimental to cartilage stability; reducing tension in the collagen fibres aligned parallel to the cartilage surface and exposing the middle and deep zones of the cartilage, allowing solutions to enter more freely. This loss of tension in the collagen network would allow for swelling of the tissue with the ingress of decellularisation solutions. This would result in the subsequent wash out of proteoglycans, which are no longer confined and compressed by the collagen matrix. This would explain the hugely increased water content and absolute loss of GAGs seen in damaged tissues.

If this theory is correct, a further explanation for the differences seen between the damage to porcine and bovine cartilage could be the relative permeability of each tissue. Despite having a higher GAG content, porcine cartilage was shown to be more permeable than bovine, so the rate at which solutions entered the structure and washed out the GAGs may have been higher.

In an attempt to test this theory, larger osteochondral blocks (bovine) and entire condyles (porcine) were decellularised. Bovine tissues sustained damage at the cut edge, showing increased porosity and GAG loss, but did retain GAGs within the central region. Porcine condylar cartilage did still show some GAG reduction, as seen in successful pin decellularisation, however the structure appeared mainly intact, with only a slight increase in
matrix porosity seen at the cartilage surface, suggesting that the cut edge effect is important to consider during osteochondral decellularisation.

Future work to fully elucidate the cause of cartilage damage should be undertaken if osteochondral decellularisation is to be further optimised. It would not be acceptable to put a decellularised product into large scale manufacture for clinical use without understanding the reason behind the damage highlighted in this study and having appropriate safeguards put in place to avoid damage occurring. To this end, it will be necessary to find improved, more definitive methods of detecting whether damage has occurred, either by quantitative analysis of tissues themselves, or by quantification of components lost into wash solutions. Additionally, more in depth immunohistochemical analysis may identify specific factors which are lost which can then potentially be quantified using ELISA or other quantitative means. Once such methods are applied, it may be possible to more accurately follow damage progression and identify when/how it is initiated.

What is clear is that some level of structural alteration will occur with decellularisation, and it will be necessary to identify the tissue condition at which damage has occurred, but will not progress and is not detrimental to the cartilage function. It may therefore also be useful to characterise human osteochondral tissues, to identify the range of tissue properties with which the decellularised graft will need to be compatible, if it is to function correctly within the host post implantation.
8.2 Further future work

Prior to in vivo testing, methods of graft rejection should be considered to reduce chances of failure. Intracellular cytoplasmic proteins and membrane components of cells in allografts and xenografts may elicit an immune response and lead to graft rejection. Xenogeneic grafts additionally have a cell surface sugar, galactose α1-3 galactose (α-gal), which is present in all mammals except for humans and old world monkeys (Galili et al., 1987). Due to the presence of a sugar similar in shape to α-gal on some human gut bacteria, humans have a high titre of pre-formed antibodies reactive against α-gal. Implantation of a graft material containing cells presenting α-gal results in hyperacute rejection of the graft (Bucher et al., 2005). Usually, initial exposure to circulating anti-α-gal antibodies occurs with endothelial cells of vascularised xenografts. The binding of antibodies to α-gal on endothelial cells results in activation of the complement cascade; this recruits neutrophils and macrophages to the site and results in inflammation and blood clotting. Blood vessels in the xenograft become blocked, resulting in hypoxia and necrosis of the implanted tissue (Janeway, et al., 2005; Knight & Ingham, 2006).

Acellular xenografts will not undergo the same mechanism of rejection as cellular vascularised grafts as described above, as hypoxia and necrosis are not relevant, however the presence of α-gal may well negatively impact on the degradation/remodelling of the implanted scaffold. Antibody binding to the α-gal epitope may activate the classical complement cascade, and soluble α-gal may also activate the alternative complement pathway. Complement triggered release of C5a and C3a will recruit polymorphonuclear cells (PMNs; such as neutrophils) and monocytes to the xenograft. PMNs perform phagocytosis as well as releasing pro-inflammatory mediators and reactive oxygen species (ROS) which may result in destruction of the scaffold matrix. Monocytes recruited to the implanted material may differentiate into proinflammatory (M1) macrophages which will release pro-inflammatory cytokines (IL-1β and TNF-α), degradative enzymes and ROS (Boehler et al. 2011; Franz et al. 2011). The
continued release of proinflammatory cytokines may perpetuate the response and continued destruction of the implant.

A further mechanism of adverse scaffold degradation is ‘frustrated phagocytosis’. As macrophages are unable to phagocytose particles larger than 5 µm, a number of cells can fuse to form foreign body giant cells (FBGCs) in response to foreign biomaterials. If unable to phagocytose the foreign material, FBGCs display a change in activity, with reduced phagocytosis activity and enhanced degradative capacity. The increased release of enzymes and ROS may result in the scaffold being resorbed, and only once all foreign material is remodelled will the inflammatory response subside (Franz et al. 2011). This adverse remodelling of acellular xenografts would impair functionality and minimise the regenerative capacity of such medical devices. Furthermore, indiscriminate release of degradative products could damage surrounding healthy host tissues and exacerbate the original tissue disorder.

Immunohistochemical analysis of α-gal in osteochondral tissues by Kheir et al. (2011) revealed that chondrocytes of porcine cartilage did not positively stain for the epitope, however α-gal was present on cells of the subchondral bone. Stone et al. (1997) quantified low levels of α-gal in porcine and bovine cartilage.

Due to the avascular nature of cartilage and the tissue density, cartilage is relatively immunoprivileged, so exposure of α-gal to circulating antibodies would be minimal with xenogeneic cartilage implantation. Stone et al. (1997) have reported that hyperacute rejection of porcine and bovine cartilage does not occur when implanted into old world monkeys, however tissues were subject to chronic inflammatory rejection. Upon implantation, the primate immune system increased the anti-α-gal activity 10 – 300 fold in response to α-gal containing cartilage tissues (Galili et al., 1997). Treatment of cartilages with α-galactosidase to remove the epitope resulted in anti-α-gal titres less than two fold greater than basal levels upon implantation (Stone et al., 1998), however an immune response was still seen to other cartilage-specific antigens. Of note was that these cartilage tissues were not implanted in situ into the avascular joint space, but into a suprapatellar
pouch, so would have had higher exposure to vascularisation and therefore circulating antibodies/lymphocytes.

Analysis of the immune response to acellular porcine small intestinal submucosa (SIS) containing high levels of α-gal implanted into α1,3 galactosyltransferase knockout mice was performed by Raeder et al. (2002). They found that the presence of α-gal delayed tissue remodelling and increased the inflammation duration in mice without the α1,3 galactosyltransferase gene (GTKO mice), however the tissue was effectively remodelled to the same degree. A small number of acute inflammatory cells did remain in implants in the knockout mice. The time frame for complete tissue remodelling may well be increased in dense cartilage tissues from that seen with SIS, and inflammation may not subside as quickly. Therefore, it is imperative to ensure that the α-gal epitope is thoroughly removed from tissues to avoid adverse tissue damage as a result of prolonged inflammatory reactions. Tests to determine α-gal within the decellularised osteochondral tissues should be performed in future, prior to in vivo testing, if the scaffold is to be considered biocompatible.

To overcome the issues with α-gal and afore mentioned BSE transmission, it may be possible to use human cadaveric osteochondral tissues as an allogeneic source material for decellularisation in the future. A protocol developed for mature bovine tissue would likely be successful for mature human tissue also. It would require careful selection of donor material, as stiffened, aged cartilage may not be a suitable repair material for young active patients.

After the removal of α-gal from decellularised osteochondral tissues has been confirmed, in vivo testing should be performed to assess the immunogenicity of the scaffold. The approach commonly used is to subcutaneously implant small pieces of the decellularised tissue into a mouse model. Histological and immunohistochemical analysis of explants could then be performed to determine the presence or absence of immune cells (Stapleton et al. 2010)

Prior to clinical trials in humans, the acellular osteochondral graft should be implanted in situ into a large animal model, such as a sheep or goat. This
would give a better indication of the immune response to the graft when it is implanted into the appropriate environment, and would also reveal whether integration of the graft with the surrounding recipient tissue has been successful.
8.3 Detailed approach to future work

Four key objectives should be met in the further development of an acellular osteochondral graft in order to enable progress to a clinical therapy. Firstly, the mechanism of cartilage damage should be fully elucidated. Secondly, human host tissues should be characterised to determine the acceptable range of properties which an acellular graft may possess. Thirdly, the decellularisation process should be further optimised for use with large osteochondral tissue segments to produce a scaffold with levels of damage acceptable for implantation. Finally, the regenerative capacity of the scaffold should be investigated in vitro and in vivo prior to the commencement of human clinical trials.

8.3.1 Mechanism of cartilage damage

To fully understand the mechanism of cartilage damage it will first be necessary to further understand the damage which occurs during the decellularisation process by performing more in depth characterisation of damaged tissues. Analysis of collagen loss/damage could be achieved through immunohistochemical (IHC) analysis of minor collagens. Transmission electron microscopy (TEM) and atomic force microscopy (AFM) would allow more detailed qualification of the tissue collagens and differential scanning calorimetry (DSC) would enable quantitative assessment of collagen denaturation.

The solutions used for decellularisation could be assessed using quantitative biochemical assays and TEM to determine what is being removed from tissues during damage development. Additionally, biomechanical testing and analysis of cartilage biotribology would potentially provide sensitive detection of subtle changes in tissue function due to low levels of structural damage.

Once sensitive and quantitative methods for tissue damage detection and monitoring have been developed, the progression of damage over time could be observed and by determining the nature and progression of damage, the mechanism could potentially be elucidated. Once the mechanism is understood, alterations to the decellularisation process to avoid damage could be made accordingly.
8.3.2 Human tissue characterisation

An acellular osteochondral scaffold should ideally mimic the structure and function of the surrounding recipient tissue to provide a successful repair material. Therefore it will be necessary to characterise human osteochondral tissues from appropriately aged donors using the biological, biochemical and biomechanical methods described in Chapter 3. Furthermore, assessment of cartilage biotribology using methods such as pin on plate friction testing or, preferably, whole natural joint friction testing would give further understanding of host tissue biomechanical properties. The properties of acellular osteochondral tissues produced using variations of the decellularisation process could then be compared to the range of properties observed for human tissues to determine which process, if any, provides an acceptable cartilage substitute biomaterial.

8.3.3 Decellularisation of large osteochondral tissue segments

Once the mechanism of cartilage damage has been determined, the processes to decellularise large osteochondral tissue segments could be optimised, as described in Chapter 4 and 5, to fully decellularise tissues whilst minimising damage. Such a process should produce a scaffold with acceptable levels of damage and properties comparable to those determined for human recipient cartilage.

8.3.4 Regenerative capacity of acellular osteochondral scaffolds

Before the acellular osteochondral scaffold could be tested in clinical trials, the biocompatibility and regenerative capacity of the biomaterial would require assessment in vitro and in vivo.

8.3.4.1 In vitro assessment

The methods described in Chapter 6 could be used for basic in vitro compatibility testing. The presence of residual cytotoxic SDS in the tissue should be quantified either using radiolabelled SDS or using a colourimetric assay with Stains-all dye. Contact cytotoxicity of the scaffold should be determined and the cytotoxicity of scaffold extracts could be measured by culturing with appropriate cell lines and their proliferative capacity measured using, for example, the ATPLite-M® assay.
In addition, as discussed previously, methods to assess the sterility of tissue scaffolds should be employed, such as incubating sterilised scaffold samples on agar plates or in nutrient broth. The scaffolds should also be inoculated with *Bacillus atrophaeus* spores and the efficacy of PAA for effectively killing the microbes determined.

To predict the detrimental effects of α-gal on the *in vivo* response to the implanted scaffold, qualitative immunohistochemistry and quantitative antibody absorption assays followed by ELISA to detect α-gal in decellularised scaffolds should be performed. The capacity of the scaffold to bind antibodies to α-gal in human sera and activate complement could be determined. If the scaffold is found to be cytotoxic, non-sterile or to contain unacceptable levels of α-gal the decellularisation process will need to be re-optimised to address these issues before *in vivo* studies are performed.

Other *in vitro* predictions of scaffold performance could be performed. The presence of growth factors such as TGF-β within the scaffold could be assessed using ELISA and IHC to indicate how inductive the scaffold might be to recellularisation and regeneration. Testing of the scaffold in a whole natural joint simulator could give an idea of how the graft would perform biomechanically/biotribologically upon implantation.

**8.3.4.2 In vivo assessment**

Subcutaneous implantation of the acellular osteochondral scaffold into mice would allow assessment of the immune response to the biomaterial. Histological and immunohistochemical analysis of explants would allow characterisation of cell infiltrates. If there is an adverse response to the tissue, further alterations to the decellularisation protocol should be made to produce an immune-compatible scaffold.

A proof of concept study should be performed in an appropriate large animal model, such as the sheep, to predict the clinical performance of the scaffold. Implantation of the scaffold *in situ* should give a more accurate representation of the local immune response and regeneration of the scaffold following histological and IHC analysis of explants. Furthermore, implantation into the joint using the appropriate surgical procedures would
provide details on the clinical utility of the scaffold and the performance of the implant in terms of joint function could be observed.

Subject to acceptable performance in the proof of concept study, the acellular osteochondral scaffold could then be taken forward to pre-market medical device clinical trials to assess safety and clinical utility in humans.
8.4 Conclusion

In conclusion, this study represents a major step forward in the understanding of cartilage decellularisation. The methods presented here are currently the most effective known for decellularisation of intact bovine cartilage, with no visible cell nuclei present and a residual DNA content of < 50 ng.mg\(^{-1}\) in the decellularised cartilage matrix. However, issues with cartilage disintegration need to be better understood and fully overcome before further necessary testing of the biocompatibility and regenerative capacity of such a scaffold material can be performed. This study has increased knowledge and understanding of the effects of decellularisation on osteochondral tissues which will form the basis for future development of a bioactive, acellular, natural tissue engineered repair material for osteochondral lesions to prevent or delay the onset of osteoarthritis.
Chapter 9
References


European Commission (EMA) No, 410/01 rev.3 of 5 March 2011  Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products.


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## Appendix A
### Materials

**Table 1** Equipment used throughout the study.

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<th>Equipment</th>
<th>Model</th>
<th>Supplier</th>
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<td>Micro-plate</td>
<td>Multiscan Spectrum 1500</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Instrument</td>
<td>Model/Part Number</td>
<td>Manufacturer/Supplier</td>
</tr>
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<td>----------------------------</td>
<td>----------------------------</td>
<td>-------------------------------------------------</td>
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<tr>
<td>Spectrophotometer</td>
<td></td>
<td>Microscopes, Medical Diagnostics Systems and Olympus Patient Systems Ltd</td>
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<tr>
<td>Microscope (Inverted)</td>
<td>Olympus IX71</td>
<td>Microscopes, Medical Diagnostics Systems and Olympus Patient Systems Ltd</td>
</tr>
<tr>
<td>Microscope (upright)</td>
<td>Olympus BX51</td>
<td>Microscopes, Medical Diagnostics Systems and Olympus Patient Systems Ltd</td>
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<tr>
<td>Microtome</td>
<td>RM2125 RTR</td>
<td>Leica Microsystems</td>
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<tr>
<td>Nanodrop Spectrophotometer</td>
<td>ND-1000</td>
<td>Labtech Int</td>
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<tr>
<td>Neubauer Haemocytometer</td>
<td>MNK-420-010N</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Orbital Shaker</td>
<td>IKA KS130 basic</td>
<td>Jencons PLC</td>
</tr>
<tr>
<td>Oven (hot air)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH meter</td>
<td>Jenway 3010</td>
<td>VWR International</td>
</tr>
<tr>
<td>Piezo-electric force transformer</td>
<td>060-1896-02</td>
<td>Electrosence</td>
</tr>
<tr>
<td>Pipette boy</td>
<td>Acu</td>
<td>Integra biosciences</td>
</tr>
<tr>
<td>Plate Shaker</td>
<td>IKA KS130 basic</td>
<td>Jencons PLC</td>
</tr>
<tr>
<td>Slide holder</td>
<td>E102</td>
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<tr>
<td>Tissue Processor</td>
<td>TP11020</td>
<td>Leica Microsystems</td>
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<tr>
<td>Vortexer</td>
<td>Topmix FB15024</td>
<td>Fisher Scientific</td>
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<td>Water Bath</td>
<td>Grant</td>
<td>Jencons PLC</td>
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<td>Water Pik Ultra Water Flosser</td>
<td>WP100</td>
<td>WaterPik</td>
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<td>Water purifier</td>
<td>Option 7</td>
<td>ELGA</td>
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<tr>
<td>Wax oven</td>
<td>Windsor E18/31</td>
<td>Scientific Laboratory Supplies</td>
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**Table 2** Chemicals/reagents used throughout the study.

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<tr>
<td>1,9 dimethylene blue</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>(^{14})C labelled SDS</td>
<td>Hartmann Analytical</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>Thermo Fisher Scientific Ltd</td>
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<tr>
<td>Acetone</td>
<td>European Bios</td>
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<tr>
<td>Alcian blue</td>
<td>Atom Scientific</td>
</tr>
<tr>
<td>Aprotinin (10,000 KUI)</td>
<td>Mayfair house</td>
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<tr>
<td>ATP-Lite-M(^*) assay</td>
<td>Perkin Elmer Ltd</td>
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<tr>
<td>Bovine Serum Albumin</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>VWR International</td>
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<tr>
<td>Calf thymus DNA</td>
<td>Sigma-Aldrich</td>
</tr>
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<td>Chloramine T</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Chondroitin sulphate B</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Citric acid</td>
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<td>Cyanacyrlylate</td>
<td>Sigma-Aldrich</td>
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<td>Sigma-Aldrich</td>
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<td>Sigma-Aldrich</td>
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<tr>
<td>DAPI</td>
<td>Sigma-Aldrich</td>
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<td>Dimethyl sulfoxide (DMSO)</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>di-sodium hydrogen orthophosphate</td>
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<td>Sigma-Aldrich</td>
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<tr>
<td>DNase</td>
<td>Sigma-Aldrich</td>
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<td>Qiagen</td>
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<tr>
<td>DNeasy blood and tissue kit</td>
<td>Qiagen</td>
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<td>D PX mountant</td>
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<td>Dulbecco’s modified Eagle’s medium (DMEM)</td>
<td>Gibco Life Technologies Ltd.</td>
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<td>Invitrogen Ltd</td>
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<tr>
<td>Dulbecco’s PBS tablets</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Eosin</td>
<td>VWR International</td>
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<tr>
<td>Ethanol</td>
<td>Thermo Fisher Scientific Ltd</td>
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<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>VWR International</td>
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<td>Fast green</td>
<td>Sigma</td>
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<td>Chemical</td>
<td>Supplier</td>
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<tr>
<td>--------------------------------</td>
<td>------------------------------------</td>
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<td>Sigma-Aldrich</td>
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<tr>
<td>Gentamycin</td>
<td>Sigma-Aldrich</td>
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<td>Gibco Life Technologies Ltd.</td>
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<td>Glycerol</td>
<td>Sigma-Aldrich</td>
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<td>Glycerol</td>
<td>Sigma-Aldrich</td>
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<td>Haematoxylin (Gill’s no. 3)</td>
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<td>Haematoxylin (Mayers)</td>
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<td>Haematoxylin (Weigert’s)</td>
<td>Atom Scientific</td>
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<td>Hirudin</td>
<td>Sigma-Aldrich</td>
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<td>Hydrochloric acid</td>
<td>VWR International</td>
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<td>Hydrogen peroxide</td>
<td>Sigma-Aldrich</td>
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<td>ImmEdge Hydrophobic Barrier Pen</td>
<td>Vector laboratories</td>
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<td>Isopropanol</td>
<td>Thermo Fisher Scientific Ltd</td>
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<td>L-cystine hydrochloride</td>
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<td>L-glutamine</td>
<td>Lonza</td>
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<td>Mannitol</td>
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<td>MicroScint™-20</td>
<td>Perkin Elmer Ltd</td>
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<td>Monosodium citrate</td>
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<td>Neutral buffered formalin (10% v/v)</td>
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<tr>
<td>PBS without Ca$^2+/Mg^{2+}$</td>
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<td>Penicillin</td>
<td>Lonza</td>
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<tr>
<td>Peracetic acid</td>
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<td>Perchloric acid</td>
<td>BDH</td>
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<tr>
<td>Periodic acid</td>
<td>Sigma-Aldrich</td>
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<td>pH standards (4, 7, 10)</td>
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<td>Piric acid</td>
<td>Sigma-Aldrich</td>
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<td>Chemical Name</td>
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<td>RNase</td>
<td>Sigma-Aldrich</td>
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<td>Sigma-Aldrich</td>
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<td>Acros</td>
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<td>sodium di-hydrogen orthophosphate</td>
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<td>Sodium hydrogen carbonate</td>
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<tr>
<td>Tween 20</td>
<td>Sigma-Aldrich</td>
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<td>Thermo Fisher Scientific Ltd</td>
</tr>
<tr>
<td>Ultra Vision One Large Volume Detection System, DAB plus substrate System</td>
<td>Thermo Fisher Scientific Ltd</td>
</tr>
<tr>
<td>Virkon</td>
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<tr>
<td>Xylene</td>
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<tr>
<td>α-chymotrypsin</td>
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### Table 3 General consumables used throughout the study.

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<tr>
<th>Item</th>
<th>Model/Size</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>Cryovials</td>
<td>Cryolife™</td>
<td>Nunc International Corporation</td>
</tr>
<tr>
<td>Glass coverslips</td>
<td>MIC3228</td>
<td>Scientific Laboratory Supplies Ltd</td>
</tr>
<tr>
<td>Microtome Blades</td>
<td>SD3050835</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Parafilm M</td>
<td>-</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Scalpel blade</td>
<td>Size 22A</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Superfrost Plus microscope slide</td>
<td>MIC3022</td>
<td>Scientific Laboratory Supplies Ltd</td>
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<tr>
<td>Syringe needles</td>
<td>-</td>
<td>Thermo Fisher Scientific Ltd</td>
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</table>

### Table 4 Plasticware used throughout the study.

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<th>Item</th>
<th>Model/Size</th>
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<td>Bijous</td>
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<td>Scientific Laboratory Supplies Ltd</td>
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<tr>
<td>Cell culture flasks</td>
<td>T75, 75 cm²</td>
<td>Thermo Fisher Scientific Ltd</td>
</tr>
<tr>
<td>Disposable plastic syringes</td>
<td>1 ml, 2 ml, 5 ml, 10 ml, 20 ml, 50 ml</td>
<td>Scientific Laboratory Supplies Ltd</td>
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<tr>
<td>Pipette tips (filtered and non-filtered)</td>
<td>10 μl, 20 μl, 200 μl, 1000 μl</td>
<td>Star Labs</td>
</tr>
<tr>
<td>Optiplate™</td>
<td>96-well</td>
<td>PerkinElmer™</td>
</tr>
<tr>
<td>Sterile containers</td>
<td>60 ml, 150 ml, 250 ml</td>
<td>Scientific Laboratory Supplies Ltd</td>
</tr>
<tr>
<td>Stripette (disposable pipettes)</td>
<td>1 ml, 2 ml, 5 ml, 10 ml, 25 ml</td>
<td>Sigma-Aldrich Ltd</td>
</tr>
<tr>
<td>Universals</td>
<td>25 ml</td>
<td>Scientific Laboratory Supplies Ltd</td>
</tr>
<tr>
<td>Well plates, Nunc® (flat bottomed)</td>
<td>6-well, 12-well, 24-well, 48-well, 96-well plates</td>
<td>Nunc International Corporation</td>
</tr>
</tbody>
</table>
Appendix B
Publications


Appendix C
Presentations


