Design and fabrication of scaffolds for cartilage applications through photopolymerization of hydrogels

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<td>1D</td>
<td>One dimension</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimensions</td>
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<td>2PP</td>
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<td>Three dimensions</td>
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<tr>
<td>3DP</td>
<td>Three-dimensional printing</td>
</tr>
<tr>
<td>ASTM</td>
<td>American Society for Testing and Materials</td>
</tr>
<tr>
<td>BASCs</td>
<td>Brown adipose-derived stem cells</td>
</tr>
<tr>
<td>CAD</td>
<td>Computer aided design</td>
</tr>
<tr>
<td>CaP</td>
<td>Calcium phosphates</td>
</tr>
<tr>
<td>CGCaP</td>
<td>Collagen–glycosaminoglycan</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEF</td>
<td>Diethyl fumarate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GelMA</td>
<td>Gelatin methacrylate</td>
</tr>
<tr>
<td>hADSCs</td>
<td>Human adipose-derived stem cells</td>
</tr>
<tr>
<td>hBMSCs</td>
<td>Human bone marrow mesenchymal stem cells</td>
</tr>
<tr>
<td>HCAECs</td>
<td>Human coronary artery endothelial cells</td>
</tr>
<tr>
<td>hMSCs</td>
<td>Human mesenchymal stem cells</td>
</tr>
<tr>
<td>hUVECs</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>hUVSMCs</td>
<td>Human umbilical vein smooth muscle cells</td>
</tr>
<tr>
<td>IPN</td>
<td>Interpenetrating Polymer Networks</td>
</tr>
<tr>
<td>MC</td>
<td>Methylcellulose</td>
</tr>
<tr>
<td>MeCol</td>
<td>Methacrylate collagen</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBT</td>
<td>Poly(butylene terephthalate)</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly-ε-caprolactone</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermal gravimetric analysis</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIPS</td>
<td>Thermally induced phase separation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TPU</td>
<td>Thermoplastic polyurethane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet Light</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>ACAN</td>
<td>Aggrecan gene</td>
</tr>
<tr>
<td>ACI</td>
<td>Autologous chondrocyte implantation</td>
</tr>
<tr>
<td>AlgMA</td>
<td>Alginate methacrylate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbello’s modified eagle medium</td>
</tr>
<tr>
<td>DMMB</td>
<td>DMMB Dimethylmethylene blue</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GelMA</td>
<td>Gelatin-methacrylate</td>
</tr>
<tr>
<td>NMR (1H NMR)</td>
<td>Nuclear magnetic resonance (Proton NMR)</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
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<tr>
<td>HA-MA</td>
<td>Hyaluronic acid methacrylate</td>
</tr>
<tr>
<td>Hep-MA</td>
<td>Heparin methacrylate</td>
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<tr>
<td>KS</td>
<td>Keratan sulfate</td>
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<tr>
<td>MAAh</td>
<td>Methacrylic anhydride</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stromal cell</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis/osteoarthritic</td>
</tr>
<tr>
<td>PABS</td>
<td>Plasma-Assisted Bio-extrusion System</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
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</tbody>
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# List of Notations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\dot{\gamma}$</td>
<td>Shear rate ($s^{-1}$)</td>
</tr>
<tr>
<td>$G'$</td>
<td>Storage modulus (Pa)</td>
</tr>
<tr>
<td>$G''$</td>
<td>Loss modulus (Pa)</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Flow coefficient or consistency (Pa s$^n$)</td>
</tr>
<tr>
<td>$\tan\delta$</td>
<td>Loss factor</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Apparent viscosity (Pa·s)</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Shear stress (Pa)</td>
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</table>
Abstract

Articular cartilage is a load-bearing tissue that covers the ends of bone joints, acting as a low-friction bearing surface and a mechanical damper for the bones. Articular cartilage is a matrix-rich tissue with specialized cells (chondrocytes) that maintain the structural and functional integrity of the extracellular matrix (ECM). An important feature of cartilage is lacking self-repair ability and as a consequence, the zonal structure and cartilage functions are often irreversibly lost following trauma and disease. As a result, cartilage defects are prone to develop into predominant cartilage disease osteoarthritis (OA), characterized by loss of cartilage, pain and debilitation. The most common treatment for OA is joint replacement surgery, in which damaged joint components are replaced by artificial prosthesis. This typically reduces the pain and increases the mobility. However, over time such implants often fail and require revision surgeries.

Tissue engineering has emerged as an alternative strategy to overcome the limitations of traditional therapies by replacing the damaged part of cartilage tissue with a biofabricated cartilage tissue for long-lasting periods and holds great promise as an effective treatment for cartilage repair. However, most of the approaches in this field focus on the use of single material structures, not able to mimic the real structure of cartilage. This research project focus on the design and fabrication of novel multi-material cartilage replacement by using two different hydrogels (alginate, gelatin) and a hybrid system based on the combination of both alginate and gelatin. These materials were successfully functionalized with methacrylate anhydride allowing them to be processed through UV photopolymerization. The effect of polymer concentration, methacrylated anhydride concentration and functionalization reaction time was investigated allowing to tailor rheological and viscoelastic properties. Pre-polymerized polymeric solutions were also prepared considering different concentrations of photoinitiators. Mechanical, swelling and degradation characteristics of these different systems were determined allowing to identify the most suitable compositions for each single polymer system. Additionally, the ability of each system to be used as a cartilage bioink was further investigated through live/dead assay using human chondrocyte and mesenchymal stem cells. Finally, the novel hybrid system proposed by this research was further investigated in terms of its
composition (ratio between gelatin and alginate) and a detailed biological study was preformed not only to investigate the ability of this system to support cell growth and cell-cell network formation but also the ECM formation through the quantification of GAGs, Collagen and aggrecan. Results show that the developed systems can be used as bioinks, their properties can be easily tailored and that the novel hybrid system, which overcomes the main limitations of each individual system, is a promising bioink material to support cartilage formation.
Declaration

I declare that no portion of the work referred to in the dissertation has been submitted in support of an application for another degree or qualification of this university or any other university or other institute of learning.

Hussein Mishbak

2019
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I am grateful to all colleagues in my research group: Cian, Salam, Enes, Mohammed, Vag, Alex, Pinar and Tom. Their daily support, friendship and our discussion helped me to move forward. My time at Manchester was made enjoyable in large part because of you, thanks for being part of my life. Big thanks to all my friends, old and new, who have made the last few years so enjoyable.

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Last but not least, I would like to acknowledge from the bottom of my heart the endless love and support of small family, my lovely supportive wife (Najlaa) who was always in my side in the good and bad days, my children (Bilal and Layaan). Special thanks to my parents who supported me through my whole life during this journey since the
primary school till now. My brothers and Sisters and all my big family. They all kept me moving forward and without them it would be very difficult to successfully complete my PhD.
إلى والديّ الغاليين، زوجتي الحبيبة ومرّةً فؤادي أولادي بلال و ليان.
Graphical abstract illustrating this research work focusing on material preparation and characterization, and bioprinting of multi-material cell laden constructs for cartilage tissue engineering.
Chapter 1

Introduction
1.1 Background

Millions of people around the world have experienced cartilage disease through accidents, such as a tear to the anterior cruciate ligament (ACL), trauma, joint injuries and abnormal joint loading, or due to ageing such as, degenerative joint diseases, that can develop into osteoarthritis. Osteoarthritis (OA) is the most common disease affecting cartilage tissue (1). When the hyaline cartilage is completely damaged, this means that the joint surface may no longer be smooth to facilitate bone movement. Despite new therapies have been developed to treat cartilage diseases, articular cartilage repair is still a major clinical challenge. This is particularly critical since cartilage presents very limited capacity to naturally heal due to its avascular nature and relatively low cellular mitotic activity. Moreover, there are no available therapies for long term treatment of articular cartilage damages. This is a very active field of research and new therapeutic techniques are being developed not only to treat damaged or diseased joint cartilage tissue but also to achieve and restore functional normal cartilage that will give long-lasting improvements and allow patients to return to a fully active lifestyle. Particularly relevant to this field is the concept of tissue engineering (TE), combining the knowledge of engineers, biologists, material scientists, and clinicians towards the development of biological substitutes that restore, maintain, or improve the function of tissues (2). For cartilage applications, the combined used of biodegradable and biocompatible materials, cells and growth factors and the use of additive biomanufacturing systems, present high potential to create customised cartilage replacements. In this case, three-dimensional biocompatible and biodegradable structures (hydrogel-based scaffolds) are normally used and designed considering the following requirements (3):

- The designed structure must allow cells to accommodate, proliferate and differentiate.
- Support the metabolic activity of cells serving as a template for cells to produce their own extra-cellular matrix (ECM);
- Provide an adequate mechanical and biological environment promoting tissue regeneration.

In this context, the aim of hydrogel-based 3D constructs is to direct cartilage cells (chondrocytes) to grow, mature, and build their own ECM mimicking the native cartilage tissue (4, 5). Besides chondrocytes, non-differentiated stem cells are also
used together with specific growth factors (transforming growth factor beta superfamily) to promote chondrogenesis (6).

Two approaches have been considered for cartilage tissue engineering, the use of scaffolds seeded with cells or the use of bioinks (hydrogels encapsulating cells) (7). Both approaches strongly depend on both the characteristics of the biomaterials being considered and the fabrication processes. The scaffold-based approach is the most commonly used but requires a pre-culture phase in a bioreactor before implantation, while the fabrication of cell laden constructs allow the development of in situ printing strategies (5).

The use of additive manufacturing for cartilage applications is a relatively new approach. Most of the studies focused on the use of extrusion-based or pressure-assisted additive manufacturing systems with or without cells (8, 9). Interesting results were obtained in terms of the design of scaffolds (it is clear from the literature that porous scaffolds for cartilage applications require small pores in comparison to bone scaffolds) and cell differentiation (positive effect of hypoxia conditions) (9). Additionally, most of these studies used hard materials (e.g. polycaprolactone) or very soft materials (e.g. collagen), not able to completely simulate the mechanical behaviour of cartilage and compromising the stability of any constructs. Besides this, up till now, very few studies investigated the combined used of different materials to create a structure mimicking cartilage. Naturally derived polymers such as alginate, agarose, gelatin and collagen have been investigated as a single material system. Most of these polymers are non-covalently crosslinked or ionically crosslinked, which could be considered a major challenge since the produced hydrogels are weak and the gelling conditions complex. Hybrid systems combining naturally derived and synthetic polymers have also been investigated to overcome some mechanical and degradation limitations of natural polymers (10, 11). Hybrid systems based on natural polymers with tailored properties represents a novel area of research.

1.2 Research aim and objectives

The common problem with all available clinical strategies for cartilage repair is their limitation to fully repair damaged cartilage providing long-term treatment. Usually, they are expensive strategies and not appropriated to be applied in children. Thus, tissue engineering is an alternative approach to overcome the problems of traditional
therapies by replacing the damaged part of cartilage tissue with a biofabricated engineered cartilage tissue for long-lasting periods of time.

The aim of this research project is to design and evaluate a novel cell-laden multi-hydrogel able to provide a temporospatial environment that could form functional cartilage. This cell-laden, produced using additive manufacturing, is based on biodegradable and biocompatible hydrogels resembling the structure of cartilage, with tuneable mechanical and biodegradability properties able to support cells, promote cell-cell interactions and the formation of ECM and processed under mild and physiological conditions.

This construct is based on two different polymer materials (alginate and gelatin), one for the superficial zone and the other for the deeper region of cartilage) and a hybrid system obtained by mixing both alginate and gelatin for the transitional zone. Alginate and gelatin were selected due to their biocompatibility, biodegradability properties, easy to process under mild conditions and low cost.

Both alginate and gelatin were functionalized with methacrylate anhydride (MA) to introduce photoreactive sites into the polymer backbone to produce photocrosslinkable constructs. Ultraviolet (UV) photopolymerization was selected to induce rapid gelation, allowing to control the polymeric network properties such as crosslinking density, matrix stiffness and cell encapsulation by tuning the photopolymerization parameters (e.g. light intensity, wavelength, photoinitiator and polymer concentration, and exposure time). Key research challenges considered to design the cartilage constructs are related to the mechanical properties of produced constructs, photoinitiator concentration, biological effect of the unsaturated degree, cell exposure to UV radiation and cytotoxicity of both photoinitiators and unreacted polymers.

To achieve the research aim, the following research objectives were considered:

✓ **Objective 1:** understand cartilage structure and its role, main clinical problems and treatment strategies; investigate current biofabrication technologies, suitable materials and design requirements to produce a bioink for cartilage regeneration;

✓ **Objective 2:** select and functionalize polymeric materials and develop a suitable approach for cartilage regeneration by investigating two different hydrogels and the combination of both polymers into a hybrid system able to functionally mimic the articular cartilage structure, composition and
properties. Variations in polymer concentration, methacrylate anhydride concentration and degree of substitution can provide hydrogels with tunable and controllable properties;

✓ **Objective 3**: establish a correlation between process conditions and morphology, mechanical and biological properties of 3D polymeric constructs and the capacity of producing a functional engineered cartilage replacement;

✓ **Objective 4**: quantify the optimal composition, conditions, biomechanical, biophysical and biochemical properties of tailored polymeric constructs for better ECM matrix production.

✓ **Objective 5**: develop a new tissue engineering approach to enable the neocartilage formation fully anchored to the underlying subchondral bone.

In order to achieve the research aims and objectives four main research phases were considered:

❖ **Material design (phase 1)** – Selected polymeric systems must be functionalized in order to be photopolymerizable. Functionalization corresponds to the introduction of unsaturations (C=C sites) into the hydrogel structure through the use of methacrylate. Solutions containing different concentrations of alginate or gelatin mixed with different concentrations of methacrylate during different reaction times were prepared. Proton nuclear magnetic resonance $^1$HNMR is used to assess the equivalent unsaturation level and to confirm the functionalization process. The level of unsaturation has an impact on the processability of these materials and crosslinked density, which determines the mechanical properties and free volume available for cell encapsulation, viability and proliferation.

❖ **Photopolymerization process (phase 3)** – Low light intensity values and low concentrations of free-radical photoinitiators are considered. Photocurable polymers prepared in phase 1 are mixed with different concentrations of photoinitiator and polymerised at different light intensity values, by changing the distance between the material and the light source. The curing kinetics and mechanical properties are assessed as a function of light intensity and photoinitiator concentration.

❖ **Printing optimisation (phase 2)** – The rheological and viscoelastic properties of the prepared solutions are critical. High viscous materials and Newtonian formulations require high pressures during the printing process, which might
induce significant shear stresses on encapsulating cells. During this phase a general understanding on the effect of the material design characteristics on the rheological properties is obtained.

❖ **Biological assessment (phase 4)** – Human chondrocytes and/or human mesenchymal stem cells (hMSC) were encapsulated on those three different bioink systems (alginate, gelatin and hybrid systems), before photopolymerization. The production of glycosaminoglycan, Collagen secretion, and the formation of cell networks were studied. Histological analysis is used to semi quantify the gene expression in the deposited ECM in the hydrogel constructs.

### 1.3 Thesis outlines

This thesis, based on published and submitted papers and book chapters, comprises eight Chapters, which progress in accordance with the identified research objectives. Besides this introductory Chapter, which presents the current research context, research aims and objectives, the remaining chapters can be summarized as follows:

**Chapter II: State-of-art in cartilage tissue engineering;**

This Chapter, focusing on objective 1, comprises two sections. First, it overviews the problem being addressed by this research, describing the cartilage structure and composition, main clinical problems, and most commonly used clinical approaches. It discusses also the concept of tissue engineering, presenting the key requirements of cartilage tissue engineering constructs. The second Section provides an overview on recent advances in the design and engineering of naturally derived photocrosslinkable hydrogels for cartilage tissue engineering. Hydrogel properties, synthesis routes, crosslinking methods, and strategies for hydrogel bio-functionalization are described and future research perspectives discussed.

**Chapter III: Development and characterisation of a photocurable alginate bioink for 3D bioprinting**

This Chapter, focusing on the research objectives 2 and 3, investigates the preparation of alginate-based systems for UV bioprinting applications. The material is characterised before and after the polymerisation (curing) process and the effect of both functionalization time and photoinitiator concentration on the rheological, mechanical, morphological, swelling and degradation properties are investigated.
Chapter IV: Photocurable alginate bioink development for cartilage replacement bioprinting
This Chapter, focusing on the research objectives 2 and 3, investigates the use of the photocurable alginate presented in Chapter 3 as a carrier of chondrocyte cells for cartilage repair. Different methacrylate concentration and reaction times are considered and their effects on the degree of substitution, rheological and biological properties investigated. Alginate methacrylate cell laden constructs were produced, and preliminary cytotoxicity and cell proliferation tests performed using the live/dead assay.

Chapter V: Photocrosslinkable gelatin methacrylate (GelMA) hydrogels: towards 3D cell culture and bioprinting for cartilage applications
This Chapter, focusing on the research objectives 2 and 3, investigates the use of photocurable gelatin to encapsulate human adipose-derived stem cells. Gelatin is functionalised and the level of functionalisation is investigated. Material preparation and characterisation is discussed. The influence of the functionalisation time, polymer concentration, degree of modification and viscoelastic behaviour on the mechanical strength and cell viability is also discussed.

Chapter VI: Development and characterisation of a photocurable alginate-gelatin bioink for cartilage applications
This Chapter, addressing the research objectives 2 and 4, investigate a novel hybrid alginate/gelatin photocrosslinking hydrogel system. The synthesis, morphological, rheological, mechanical and biological characterisation is detailed.

Chapter VII: Hybrid PCL/Hydrogel scaffold fabrication and in-process plasma treatment using PABS;
This Chapter, addressing the research objectives 4 and 5, investigates the use of a novel plasma-assisted bio-extrusion system (PABS), to produce multi-material scaffolds for the interface between the subchondral bone and cartilage, consisting in a synthetic biopolymer (polycaprolactone) and a hybrid natural hydrogel system (previously described in Chapter 6). The Plasma-Assisted Bio-extrusion System (PABS), developed at the University of Manchester, is also used for the scaffold surface modification (N2 plasma modification).

Chapter VIII: Conclusions and future work.
This Chapter provides an overall summary of the thesis and the main conclusions that can be drawn from the research work carried out. Possible future directions for research following on from this thesis are also presented.

The structure of this thesis is illustrated in Figure 1.1

**Figure 1.1:** Framework structure of this thesis.
Chapter 2
State-of-the-art of Cartilage Tissue Engineering
This Chapter, which provides the current state-of-the-art on cartilage tissue engineering comprises two main sections. The first section overviews the problem being addressed by this research, describing the cartilage structure and composition, and the most commonly used clinical approaches presenting their main advantages and limitations. It discusses also the concept of tissue engineering, presenting the key requirements of cartilage tissue engineering constructs. The second section provides an overview on recent advances in the design and engineering of naturally derived photocrosslinkable hydrogels for cartilage tissue engineering. Hydrogel properties, synthesis routes, crosslinking methods, and strategies for hydrogel biofunctionalisation are described and future research perspectives discussed. Therefore, these two Sections covered the two main cartilage tissue engineering approaches, scaffold-based and cell laden (bioprinting), covered by this research.

2.1 Biological perspectives and current strategies in osteochondral tissue engineering †

Articular cartilage and the underlying subchondral bone are crucial in human movement and when damaged through disease or trauma results in a severe impact on quality of life. Cartilage has a limited capacity to regenerate due to the avascular composition of the tissue and limited efficacy of current therapeutic interventions. Worldwide the numbers of patients requiring therapy for osteochondral indications is rising due to an ageing population, thus increasing the pressure on healthcare systems. Subsequently, research into new therapies especially using the principles of tissue engineering is increasing. However, a basic understanding of the biological composition, structure, and mechanical properties is required. Furthermore, consideration of material design, architecture, biomanufacturing strategies, and the role of experimental standardisation is needed to help develop tissue engineering therapies that can be successfully translated into the clinic.

This Section provides an overview of the biological properties of osteochondral tissue and the role of tissue engineering principles in developing new therapies is provided.

† This Section is based on the paper: Hussein Mishbak, Cian Vyas, Glen Cooper, Ruben Pereira, Chris Peach, Paulo Bartolo. “Osteochondral tissue: a biological and mechanical review for tissue engineering”, Biomanufacturing Reviews, Springer, in Press.
2.1.1 Introduction

Osteochondral tissue is composed of articular cartilage, a specialized tissue that covers the distal ends of the bones in articulating joints, and the subchondral bone which anchors the cartilage to the underlying bone (12-16). Articular cartilage has a highly flexible and lubricated surface to reduce frictional forces during movement and facilitate smooth articulation. The tissue enables the transmission of mechanical loads from movement to the skeleton (17-20). Osteochondral tissue is composed of distinct regions with articular cartilage, comprising the majority of the structure, and an underlying subchondral bone phase.

Articular cartilage is avascular and aneural with low metabolic activity and thus trauma or diseases (e.g. osteoarthritis and rheumatoid arthritis) are difficult to treat due to the inherent inability of articular cartilage to self-regenerate in comparison to the greater healing capacity of bone (Figure 2.1) (20). A thorough understanding of the composition and structure of the tissue will enable superior tissue engineering approaches to be explored to solve the clinical challenges of osteochondral defects.

Current clinical approaches, typically palliative, are ineffective and the tissue either continues to degrade, resulting in total replacement with an implant, or fibrocartilage is formed. Tissue engineering approaches have been widely explored to develop new approaches to repair and regenerate the tissue. However, to date these have been unsuccessful in producing mature articular cartilage. The current fundamental approaches based on scaffolds (acellular and cellular), biomaterials, and cells may need to be revaluated to understand why these approaches consistently fail in producing a relatively simple and thin tissue, although exhibiting a highly complex hierarchical organisation (21). Adult articular cartilage tissue takes over 20 years to mature and the tissue generated once produced does not turnover or regenerate unlike other tissues in the body. The cartilage tissue produced during childhood is the same throughout a person’s lifetime. Malda et al. suggest that new approaches must appreciate this fundamental aspect of cartilage tissue physiology which may require the incorporation of permeant non-degradable structures to support the major mechanical properties required of the tissue and the restoration of the microenvironment (cytokines, growth factors, and mechanical loading profiles) in the early stages of development (fetal and childhood) to recapitulate the developmental processes which generate the correct articular cartilage tissue (21). Furthermore, elucidating the underlying processes of cartilage development will provide a
mechanistic understanding that can be utilised in tissue engineering approaches for cartilage regeneration.

Figure 2.1: Comparison between the physiology and healing capacity of bone and cartilage. Cartilage is avascular tissue so lacks ready access to a supply of circulating stem cells and nutrients so relies on the synovial fluid for nourishment. This combined with its largely acellular composition and low metabolic activity results in a nearly complete lack of innate regenerative capacity. Consequentially, defects fail to heal, and clinical interventions typically result in the formation of inferior fibrocartilage. In contrast, bone has a greater ability for self-repair due to the constant tissue remodelling by osteoblasts and osteoclasts. Bone is also a highly vascularised tissue which allows a ready supply of nutrients and proteins that stimulate bone repair. Furthermore, there is a large source of stem cells in the bone marrow and periosteum which are able to differentiate into bone producing cells. This allows bone to heal defects up to a certain critical size after which vascularisation becomes an issue. Image from reference (20).

2.1.2 Composition of articular cartilage
There are three forms of cartilage in the human body which are fibrocartilage (22), elastic cartilage, and hyaline cartilage each with their own specific biological, mechanical, and structural properties (23). Hyaline cartilage is a thin connective tissue present at synovial joints such as the knee, elbow, shoulder, and hip where it covers the bearing surface of the underlying bone and is termed articular cartilage (24). Thus,
Hyaline cartilage provides an efficient load bearing surface that has a low friction coefficient thus lubricating the movement of joints and can support load transfer of up to six times the human body weight in the knees (17). The complex, nonlinear, viscoelastic, anisotropic, and heterogeneous structure and composition of cartilage enable these vital properties (25, 26).

Adult articular cartilage consists of predominately extracellular matrix (ECM), approximately 95–99% of the total volume, which itself consists of 80% water and 20% solid contents (27). The solid content is mostly comprised of collagens (50-75%), proteoglycans (15-30%), and a small amount of non-collagen proteins (24, 28). Cells account for a small percentage of the total volume (1-5%) and consist of only a single cell type, chondrocytes (29). The composition of articular cartilage changes as the tissue matures from initial formation during embryogenesis to final maturation (18-21 years old) (30).

The mature tissue has a low density of chondrocytes in a low proliferative and metabolic state, and are isolated from each other, so lack cell-cell interactions due to being encapsulated in the pericellular matrix (19). This is partly responsible for the low healing capacity of mature articular cartilage. However, the mature chondrocyte has an important role in the tissue homeostasis by coordinating and producing the ECM components. The morphology, orientation, and phenotypic expression of chondrocytes are depth and biomechanically dependent as the cells are influenced through mechanotransduction (31, 32). This results in the wide range of morphologies observed, which range from rounded, elongated, flattened, and hypertrophic, all within the same tissue (33).

The collagen network in articular cartilage is highly organised and primarily composed of collagen type II (~50% dry weight of articular cartilage) (25). The organisation is depth dependent in the tissue and is partly responsible for the biomechanics, especially the tensile and compressive properties (26). The fibre diameter increases from the articular surface through the depth of the tissue (superficial zone ~ 55 nm, middle zone ~ 87 nm, and deep zone ~ 108 nm) (34). Although collagen type II is the main collagen (95% of total collagen), there are other collagens present such as type I, II, VI, IX, X, and XI. Collagen type I is present in small amounts only in the superficial zone but can be found abundantly in fibrocartilage so can be used as a useful indicator of fibrocartilage formation (35-37). Subsequently, the ratio between collagen I and collagen II can be used as a marker to
assess the status of the cartilage tissue as chondrocytes cultured in vitro monolayer express higher levels of collagen type I indicating that the chondrocytes have undergone dedifferentiation (32). Collagen IX and XI are found throughout the tissue in small amounts and are involved in crosslinking between fibrils, regulation of fibril size, and interactions with other biomolecules (25). Collagen X is found in the deep and calcified zones and is believed to have a role in the mineralisation between the cartilage and the subchondral bone (38, 39). A major non-collagenous component of the ECM is proteoglycans and glycosaminoglycan’s (GAGs) (17). Proteoglycans consist of a core protein that is heavily bound with covalently attached polysaccharide chains, GAGs. Aggrecan is a main proteoglycan within articular cartilage and is covalently attached to negatively charged GAGs, keratan sulphate and chondroitin sulphate. This is able to bind multiple times to a hyaluronic acid backbone to form aggrecan-hyaluronan aggregates which are highly negatively charged (40, 41). The negative charge on this proteoglycan aggregate, and other proteoglycans, causes an osmotic pressure to be generated as water is taken in and entrapped which causes swelling (38). This turgidity produced by the network of proteoglycans and combined with the structural confinement caused by the organisation of collagen results in a high compressive modulus (14, 32). Subsequently, as the concentration of proteoglycans increases with depth in articular cartilage towards the subchondral bone region, the water content and swelling pressure rises, thus the compressive modulus of the tissue increases (42-45). This enables articular cartilage to have a high mechanical load bearing capability which can transfer and distribute loads effectively (46).

2.1.3 Structure of osteochondral tissue
Articular cartilage has a hierarchical organisation from the nanoscale to the macroscale with a distinct zonal structure. Each zone has its own specific ECM composition, biomolecule orientation, chondrocyte shape and organisation (47), imparting specific biomechanical characteristics (Figures 2.2 a, b and c). These zones are termed, descending from the articulating surface, the superficial or tangential zone, the middle or transitional zone, the deep or radial zone, and the calcified zone, before the articular cartilage gives way to the subchondral bone region. Furthermore, there is a microscale radial organisation surrounding the chondrocyte termed the chondron which has a unique composition depending on the distance from the chondrocyte (Figure. 2.2b).
2.1.4 Zonal structure of articular cartilage

On the articular surface and above the superficial zone is a thin layer, ranging from a few hundred nanometres to a micrometre, termed the *lamina splendens* which is acellular and composed of proteins (48). Although the structural role is unclear it is thought that the gradual build-up of proteins from the synovial fluid acts as a protective and low friction interface for the articular cartilage surface (35).

Immediately below the lamina splendens is the **superficial zone** (10-20% of cartilage thickness) which comprises small diameter collagen fibres (predominately type II and IX) that are organised parallel to the articular surface and densely packed. This allows a low coefficient of friction, which enables smooth movement of the joint and imparts the ability to withstand both the high tensile and shear stresses that the articular cartilage encounters under loading. The chondrocytes are densely packed and arrange themselves along the collagen fibres parallel to the surface, displaying a flattened morphology. Chondrocytes also secrete proteins such as superficial zone protein (SPZ, also known as lubricin or PGR 4) and collagen I which act as lubricants (25, 49-51).

![Figure 2.2: Hierarchical and graded composition, structure, and properties of osteochondral tissue. a) Procollagen polypeptides bind together to form collagen that assemble into microfibrils and then fibrils which can be crosslinked together by collagen type IX. Collagen fibrils exhibit a characteristic banding pattern of ~67 nm due to the staggered packing arrangement of collagen. Image from reference (36). b) Molecular composition and arrangement of the chondron depicting the pericellular, territorial, and interterritorial matrix with increasing distance from the chondrocyte. Image from reference (37). c) Zonal structure and properties of osteochondral tissue. Image adapted from (52).](image)
SPZ is a potential marker to identify this zone. The amount of proteoglycans is low compared to the other zones which increases the permeability, thus resulting in compressive strains of up to 50% and high fluid flow which influences the compressive properties of the entire cartilage tissue (25, 26, 40, 52).

Below the superficial zone is the **middle zone** (40–60% of cartilage thickness), which is predominately composed of collagen II fibres randomly arranged and displaying a larger diameter than the superficial zone. Chondrocytes are present at a lower density, have a rounded morphology, and also express large quantities of collagen II and aggrecan. A marker for this zone is the cartilage intermediate layer protein which is expressed throughout this zone (39). This zone also has the highest concentration of proteoglycans especially aggrecan (52, 53).

The **deep zone** (20-50% of cartilage thickness) is comprised of the largest diameter collagen fibres that are oriented perpendicular to the subchondral bone region with the chondrocytes organised along the collagen fibres in columns with an elongated morphology. The cells themselves are present in lower density compared to the other zones and express lower levels of collagen II (53, 54).

The final zone before the subchondral bone region is the **calcified zone** which is distinguished from the deep zone by the presence of a tidemark which demarks the boundary between calcified and non-calcified regions (55, 56). The zone anchors the collagen fibres of the deep zone to the subchondral bone thus integrating the cartilage to the underlying bone. This also provides an interface between the hard phase of bone and the soft phase of cartilage since the presence of hydroxyapatite reduces the mechanical gradient between the phases (25, 41, 57). The calcified zone also has the highest number of chondrocytes which are in a hypertrophic state.

The final zone of the osteochondral tissue is the **subchondral bone** which lies directly below the calcified zone and separates the articular cartilage from the bone marrow. This zone consists of the bony lamella (cortical endplate) and the subarticular spongiosa, the supporting trabeculae and bone components, which is separated from the calcified zone of articular cartilage by a cement line (58, 59). The subchondral bone differs markedly in composition and structure to the articular cartilage. The subchondral trabeculae are highly vascularised which enables transportation of nutrients, gases, and waste through channels which cross the subchondral bone plate and enter the calcified zone, which is entirely reliant on the synovial fluid as a source of nutrients if these channels are absent (58, 59). The main collagen is type I due to
the tissue being mineralised bone. These collagen fibres do not cross between the calcified cartilage and subchondral bone so does not act as an anchor as occurs with collagen fibres that cross the tidemark and connect the non-calcified and calcified cartilage. Furthermore, the subchondral trabecular structure and mechanical properties are anisotropic and can dynamically remodel itself to respond to applied forces (60-62). The main function of the subchondral bone is to maintain joint shape and provide mechanical support since it has a high compressive modulus and is impermeable so is able to stabilise the tissue and distribute the applied mechanical forces (25, 40, 58, 63, 64). Furthermore, the subchondral bone acts as a nutrient source for articular cartilage which is not vascularised itself (43).

2.1.5 Current osteochondral treatments
The zonal structure of osteochondral tissue and the innate inability of articular cartilage for self-regeneration poses a problem for clinical interventions. Subsequently, a variety of both clinical treatments are available with different degrees of success and tissue engineering approaches are currently in clinical trials (65, 66).

The type of treatment used will depend on the defect category, stage, size, and location. Osteochondral defects are commonly classified by the Outerbridge classification system which indicates the severity of a lesion (67). This system classifies defects from grade I-IV, where a grade 0 defect is normal healthy articular cartilage; grade I indicates swelling and softening of the tissue; grade II indicates a partial thickness defect with a diameter less than 1.5 cm; grade III defect has a diameter greater than 1.5 cm and presents as a full thickness lesion up to the subchondral bone; and grade IV is a full thickness defect that exposes the subchondral bone.

The main treatments currently used clinically are classified into (1) microfracture, (2) autologous chondrocyte implantation (ACI), (3) matrix-induced autologous chondrocyte implantation (MACI), and (4) osteochondral auto- and allografts as shown in Figure 2.3 (18, 44, 68). These approaches mainly utilise in vivo methods to repair and regenerate the tissue in situ.

Microfracture is a technique that has been used clinically since the 1980s to produce fibrocartilage in articular cartilage defects (69, 70). The technique recruits MSCs from the bone marrow by drilling into the subchondral bone. The defect area is debrided to ensure a clean and stable margin before drilling into the subchondral bone. This
induces bleeding into the defect area which forms a fibrin clot that contains MSCs which subsequently remolds into fibrocartilaginous tissue over a period of 12-16 months. This long period of remodelling requires a lengthy postoperative rehabilitation period with limited mechanical loading (51, 71).

**Figure 2.3:** Current treatment options for cartilage regeneration. a) A full-thickness chondral lesion (Grade III). b) Debridement of the lesion is used as precursor to more invasive procedures as it removes damaged cartilage and bone to create a healthy border which enables improved integration of neotissue with native tissue. In smaller cartilage defects debridement may be used without further procedures as a minimally invasive treatment option. c) Microfracture is treatment that drills into the subchondral bone to create channels that allow MSCs to migrate into the defect. d) Autologous chondrocyte implantation (ACI) uses a population of 12-48 million autologous chondrocytes to which are inserted into the defect which is then covered with either a periosteal patch or a collagen membrane. e) Matrix-induced autologous chondrocyte implantation (MACI) is a development of the ACI technique. The autologous chondrocytes are cultured in vitro and seeded onto an absorbable 3D scaffold (collagen or hyaluronic acid) before being implanted into the defect and fixed to the defect with fibrin glue. Image from reference (37).

Although considered the gold standard by the FDA and some clinicians, the long-term outcomes for joint functionality using microfracture technique show limited improvement (49, 50). This is due to the inferior biomechanical and biochemical properties of fibrocartilage compared to hyaline cartilage, which creates a mismatch between the native tissue and the neotissue (52, 72). The treatment provides a short-term benefit to the patient but only postpones cartilage degeneration as the repair tissue typically deteriorates approximately 18-24 months after surgery (52). Subsequently, five years after surgery the likelihood of treatment failure is high irrespective of the cartilage defect (49, 50). This technique can also be combined with other treatment
methods and an advancement on the technique utilises a collagen matrix that is inserted into the defect to promote MSC differentiation into chondrocytes (53).

In the early 1990s a new technique called autologous chondrocytes implantation (ACI) was developed as an improvement on the microfracture technique (18, 44, 54, 73). The technique is a two-stage surgical procedure that first involves harvesting autologous chondrocytes from a minimal load bearing region through a biopsy punch and expanding these cells *in vitro* to obtain a population of approximately 12-48 million cells. In the second surgery, the defect area is debrided, and the cell suspension is seeded into the defect area and confined to the defect by membrane coverage. This membrane is typically a periosteal patch; however, a major cause of treatment failure is hypertrophy of the patch (56). Subsequently, synthetic collagen or hyaluronic acid patches have been utilised, showing reduced failure rates (5 to 26%) due to patch hypertrophy (57). However, these synthetic patches are considered as off-label in the USA due to the sourcing of the material from allogenic sources which may increase the chance of an immune response (44). ACI has been shown to be effective through clinical trials which demonstrated positive long-term functional and clinical outcomes. This is potentially due to the use of autologous chondrocytes which are having a greater inherent ability to form hyaline cartilage than MSCs. However, the technique has limitations as fibrocartilaginous tissue was shown to develop in the majority of patients. This may be a result of the *in vitro* culturing stage as studies have demonstrated that chondrocytes dedifferentiate into fibro chondrocytes in 2D culture however other studies have shown that by culturing the cells in a 3D and hypoxic environment this can be reversed (58, 59). There are further limitations to ACI which include the need for two invasive surgical procedures combined with *in vitro* culturing and the subsequent long recovery period (6-12 months) needed to ensure successful neotissue formation.

A derivative of the ACI technique is MACI, which can be considered as a tissue engineering approach as it utilises a scaffold to assist in cell attachment, distribution, proliferation, and to guide matrix formation. MACI is similar to ACI in that it requires the initial isolation of autologous chondrocytes from the patient, *in vitro* cell expansion and seeding onto the scaffold, which can be made of collagen or hyaluronic acid. The cell-seeded scaffold is then cultured *in vitro* before implantation into the debrided defect and fixation with fibrin glue. The clinical advantage of MACI over other techniques remains to be confirmed as current clinical trial data has shown that MACI
either has similar or better functional results. MACI however has the same limitations as ACI in that two surgical procedures are required, slow tissue maturation, and long recovery periods. However, the long recovery periods may happen with any treatment due to the nature of the limited regenerative capacity of articular cartilage. There are a number of benefits to using a scaffold based treatment such as easier fitting of the graft into the defect, improved graft stability, and better control in preventing dedifferentiation of the chondrocytes due to fact that cells are cultured in a 3D matrix which reduces the formation of fibrocartilaginous tissue (44, 60).

A further treatment option is the use of osteochondral auto- and allografts, mosaicplasty, which involves the transplantation of osteochondral tissue from either patient (autograft) or a cadaveric donor (allograft) (61). A biopsy is taken from a non-loading or minimal loading region, in an autograft transplant, which also includes a portion of the subchondral bone. This is then transplanted into the defect, which has been prepared with only healthy tissue remaining, and the graft is then aligned with the native tissue. Mosaicplasty has been shown to have better results than microfracture however there are a number of limitations to the technique (49, 62, 63). The use of autografts is limited due to the need to restrict donor site morbidity which results in only small defect sizes (<4 cm²) being treated. Furthermore, the use of allografts raises the issue of disease transmission and immunogenicity which is potentially an issue, however the size of defect treated can be larger as the tissue is derived from cadavers. Finally, graft failure has been observed in 15-55% of patients after 10 years due to poor integration with the host tissue which is also a major limitation with the other techniques as well (64).

Although there are a number of clinical approaches none as of yet have fully repaired either an articular cartilage defect or a full osteochondral defect over the long-term. All the approaches have significant limitations which fail to recapitulate the native structure and result in mechanical mismatch with failure over the long-term. Subsequently, new tissue engineering approaches are required that enable rapid weight bearing neotissue that develops into hyaline cartilage and that is fully anchored to the underlying subchondral bone to enable vertical integration.

Patients with clinical conditions causing cartilage loss present either discrete cartilage loss in a joint surface or full thickness cartilage degeneration as shown in Figure 2.4 (74). Discrete areas of cartilage loss e.g. osteochondritis dissecans, often caused by trauma affects the younger population, whereas full thickness cartilage loss in the
whole joint affect more elderly patients and is a result of a systemic disease such as rheumatoid or osteoarthritis (75).

When considering discrete small areas of cartilage loss, patients present with pain due to exposed bone and mechanical symptoms (e.g., joint locking), due to the disruption in the smooth low friction joint surface.

![Figure 2.4: a) Discrete cartilage defect; b) End stage arthritis; c) Full thickness area of cartilage loss.](image)

Current solutions haven’t addressed the goal of restoration of the hyaline cartilage surfaces of the joint (76). The most common surgical procedure to address small areas of cartilage loss is arthroscopic (keyhole) surgery, called debridement, which uses mechanical shavers to remove debris from the joint and loose cartilage material which solves some of the mechanical symptoms (77). The aim of the surgery is to remove all loose material and roughen the surface of the exposed bone enough to allow new tissue to adhere and form in the base.

Due to the unpredictable results from debridement techniques were then developed to create more biological healing in the cartilage defect. Microfracture, popularised by Steadman (70), involves making 3mm perforations in the subchondral bone, ensuring that the structural integrity of that bone is not compromised. This allows a super clot to form in the space creating an environment for pluripotential marrow cells to differentiate into stable tissue. In one study biopsies after microfracture treatment noted that 11% had formed predominantly hyaline cartilage and 17% a mixture of fibrocartilage and hyaline cartilage within them (Figure 2.5). The remaining patients formed either predominantly fibrocartilage or no tissue at all (78, 79).
With continued poor results from microfracture due to poor differentiation of new tissue, allografting techniques were developed. Mosaicplasty involves tacking small osteochondral allograft plugs from the periphery of weightbearing joints and transplanted into the defect. There are issues of donor site morbidity with this technique and the lack of integration of the periphery of the plugs with each other or the native hyaline cartilage (80).

Lastly, autologous chondrocyte implantation techniques have been in use since the first reported series in 1987 (81). Cartilage is harvested and at a second surgery, culture-expanded autologous chondrocyte cells are injected into the cartilage defect underneath a patch of periosteum that prevents cell migration from the site. Clinical results are promising in the early years following this treatment and hyaline-like tissue has been identified in some specimens. However, the tissue is not morphologically or histochemical identical to normal hyaline cartilage and fibrocartilage is often found in samples.

These are promising techniques however, rarely last more than 5 years and results often gradually deteriorate over this period. However, as no other superior alternatives are available to clinicians, these procedures continue to represent the mainstay of management despite their failure to recreate the hyaline cartilage that has been damaged.

Whole joint osteoarthritis represents a different clinical challenge. Not only is the articular cartilage damaged, but the subchondral bone becomes deformed and the periarticular capsule, muscles and tendons become affected. Current treatment strategies are dominated by joint replacement surgery (82). Since the development of the successful low friction arthroplasty by the late Sir John Charnley, the metal on
high molecular weight polyethylene bearing surfaces has been widely used in total joint prostheses. In the UK over 160,000 hip and knee replacements are carried out each year (83). However, the challenge continues to overcome the mechanical loosening of these prostheses over time. In addition, cellular regeneration strategies are unlikely to overcome the structural changes in all of the tissues types that are involved (84). Revision surgery to replace failed prostheses causes not only significant morbidity to the patient but also the results of the second or subsequent joint replacement being significantly inferior to primary procedures. There is an associated substantial financial burden to the healthcare system due to increased cost of perioperative investigations, blood transfusions, surgical instrumentation, implants and operating time, as well as an increased length of stay in hospital which accounts for most of the actual costs associated with surgery. Innovation has been able to reduce the incidence of prosthetic failure by improving the techniques employed to ensure good integration with the host bone, e.g. coating implants with hydroxyapatite and using uncemented implants.

2.1.6 Tissue engineering: requirements and challenges

Tissue engineering is a multidisciplinary field of research conducted to meet clear clinical requirements of therapies to promote the regeneration and repair of diseased and damaged tissues. Tissue engineering approaches in cartilage regeneration and repair has great potential and provides an alternative to current available therapies which are inadequate, however, challenges remain (18, 20, 44, 85, 86). Engineered cartilage constructs are comprised of biomaterials, cells, and stimulatory factors (e.g. growth factors and biomechanical stimulation), which are considered key to the design of functional cartilage tissue (Figure 2.6) (87, 88). However, the current paradigm still lacks in the development of long-term phenotypically stable articular cartilage tissue which exhibits integration with the surrounding tissue, mechanical stability, and withstands inflammatory factors, especially in a diseased environment such as osteoarthritis.
Figure 2.6: Tissue engineering strategies for articular cartilage regeneration. Image from reference (85).

2.1.6.1 Biomaterials design and selection

Biomaterials are the backbone of 3D engineered constructs and support tissue growth and formation by providing a similar environment to the native tissue and structural integrity during maturation to allow cell proliferation, cell to cell communication, and ECM formation. The ideal design specifications of 3D tissue engineered scaffolds from the biomaterials perspective include: i) biocompatibility, cell viability with a desired cellular behaviour; ii) biodegradability, the scaffold degrades at a controlled rate which matches tissue formation; iii) provides mechanical and biochemical cues to promote a desired cellular response. An alternative strategy to the use of biomaterials is cell self-assembly approaches such as spheroid formation which do not require supporting materials.

Biomaterial scaffolds can be produced from different sources such as naturally derived polymers, synthetic polymers and ECM derived materials.

Natural derived biopolymers have been explored extensively for cartilage tissue engineering applications (86, 89, 90). The most associated advantages with naturally derived polymer are their capacity of supporting cell attachment, viability, proliferation, attachment, and differentiation, and, in some cases, maintenance of cell phenotype (91). This is mediated in protein derived biopolymers through binding motifs present in the polymer. Despite these key advantages, naturally derived polymeric materials present significant limitations such as poor degradation kinetics.
and mechanical properties, however, these limitations may be improved through the modification of the backbone of the polymer chain or via crosslinking mechanisms (91).

Naturally derived polymeric materials can be classified into two main categories:

1- Polysaccharides: gel forming polysaccharides such as alginic acid and mucopolysaccharides (glycosaminoglycans), storage polysaccharides which include starch and glycogen, and structural polysaccharides such as cellulose and chitin.

2- Protein based polymeric material composed of amino acid groups such as collagen, gelatin, and silk fibroin. Proteins can be classified by their shape, size, solubility, composition, and function.

Synthetic polymers have shown potential in tissue engineering due to their improved mechanical and degradation properties with the capacity to be more easily chemically modified or engineered to tune their properties (92-94). The hydrolytic and enzymatic degradation of the polymer can be controlled through modification of the polymer (95). However, due to lack of biologically functional domains, which can reduce the risk of immune response, synthetic polymers may not facilitate cell phenotype expression or cell attachment as occurs in naturally derived protein-based polymers.

Decellularized extracellular matrix (dECM) based biomaterials have also been explored to create 3D constructs. The native ECM is ideal for tissue engineering as it is identical to the desired matrix structure required and helps controls cell behaviour (96-100). Thus, the use of dECM is suitable as it is biodegradable, does not produce antagonistic immune responses, provides cues for cell differentiation, and presents bioactive molecules that determine tissue homeostasis and tissue regeneration (101, 102). The decellularisation process comprises mechanical and chemical manipulation to remove the cellular components (103). This protocol from harvesting, decellularisation, and sterilisation to creating the dECM based scaffolds affects the hydration status and 3D configuration of the proteins and ECM, and hence strongly influences biomechanical and biological behaviour properties which may not be suitable anymore (104, 105). The replication of the ECM microenvironment has provided inspiration to use the ECM from articular cartilage as matrix for tissue regeneration (101, 106). However, concerns remain about potential immunogenicity and poor biomechanical and biological performance.
2.1.6.2 Bioprinting cartilage tissue constructs

The advancement of 3D printing or additive manufacturing in tissue engineering has allowed the fabrication of scaffolds and biological models that more accurately reflect the complex organisational structure and material properties of tissues and organs (107, 108). 3D bioprinting uses biomaterials, cells (encapsulated or seeded), and biomolecules, typically referred to as a bioink, which are precisely deposited in a layer-by-layer process to build-up a 3D structure. The ability to print multiple biocompatible materials with greater design freedom compared with conventional fabrication techniques has enabled the development of 3D structures that resemble the complex 3D biophysical and biochemical environment in tissues. The use of 3D bioprinting within cartilage tissue engineering is becoming widespread as an enabler technology to fabricate complex multi-material structures that mimic the biological and mechanical properties of cartilage tissue (109, 110). Currently, 3D bioprinting predominately uses inkjet, extrusion, and laser-assisted systems to fabricate 3D structures (Figure 2.7). However, stereolithography based systems are gaining attention due to novel visible light photo crosslinkers with improved cytocompatibility and advancements in the technology which promises faster fabrication times and increased structure complexity (111, 112).

Figure 2.7: 3D bioprinting technologies; a) Inkjet bioprinting; b) Laser-assisted bioprinting; c) Extrusion bioprinting (113).

The development of bioinks has become a key factor in 3D bioprinting in particular biomaterials with controllable mechanical, biological, and biophysical characteristics which can modulate cell behaviour combined with printability (Figure 2.8) (114, 115). Printing resolution, structure fidelity, material viscoelasticity are crucial parameters in determining the printability of bioinks and its relationship to the final mechanical and biological properties of the structure. Developing advanced bioinks requires
consideration of pre-functionalisation processes to incorporate biological functional groups and crosslinking moieties, the rheological behaviour of the bioink to ensure printability and fidelity, and the crosslinking method to ensure rapid gelation of the hydrogel (116-119). Depending on the biofabrication process and material properties, the bioink polymers will have various chemical and physical characteristics that will determine the corresponding application (120). These properties can be determined by rheological characterization, mechanical assessment and crosslinking properties of the hydrogel (120, 121). Shear-thinning behaviour, a decrease in the viscosity as a function of increasing shear rate, is crucial for bioprinting applications, since the material will flow with an applied force during printing and the lower the applied force the higher the cell viability (119, 122, 123). The viscoelastic behaviour characterised by the material response during printing needs to be optimised as low viscous materials will deform and collapse during printing, unless a rapid crosslinking process can be initiated. On the contrary high viscosity materials can be difficult to print as they can block the printing nozzle, require high deposition force, and restrict cell attachment and spreading which can negatively impact cell viability (124, 125).

Figure 2.8: Bioink properties for successful 3D bioprinting require a suitable a) biofabrication window which balances printability and biocompatibility whilst providing a variety of b) suitable characteristics (126).

2.1.6.3 Biochemical, biophysical and biomechanical stimulation

Biochemical, biophysical, and biomechanical stimulation of 3D bioengineered constructs is crucial in the formation of functional neocartilage tissue. The stimulation of cells can be achieved at specific stages: cell expansion, differentiation, and the maturation of the tissue construct in vitro. This can be attained through supplementation of the cell culture media, incorporation of biomolecules within the
3D structure, engineering the physical ECM environment, and mechanical stimulation of the construct.

A key approach to direct cell behavior and facilitation of neocartilage tissue formation is the use of biological signaling molecules (e.g. growth and transcription factors) during cell culture, tissue maturation, and utilization via direct inclusion, encapsulation or binding to the biomaterial matrix of the construct (127-132). A range of growth and transcription factors have been identified and investigated including transforming growth factors (TGFs), bone morphogenetic proteins (BMPs), insulin growth factors (IGFs), fibroblastic growth factors (FGFs), platelet-derived growth factors (PDGFs), and sex determining region Y (SRY)-box (SOXs).

Growth factors are proteins that have a key role in cell behavior and regulate cellular growth, proliferation, differentiation, and migration and are grouped into families with shared amino acid sequences and superfamilies with shared structural folds (130, 133). In articular cartilage tissue engineering, 3D engineered constructs have been used to deliver these biological factors (130). For instance, TGF-β1 stimulates the synthesis of cartilage ECM, maintain chondrocytes phenotypes, synthesis of proteoglycans, aggrecan and type II collagen and can enhance the repair of cartilage defects (134, 135). IGF-1 has been reported showing high anabolic effects and decrease in catabolic responses in articular cartilage metabolism in vitro (135, 136). Other studies have reported using different growth factors which were successful in producing several features that resembled typical articular cartilage (137-139). Despite of the capacity to promote cartilage matrix formation, growth factors have shown some drawbacks with IGF-1 associated with a loss of chondrocyte phenotype and extracellular matrix breakdown (136). Furthermore, IGF-1 in human MSCs inhibited collagen II expression and overexpression can induce hypertrophic differentiation and mineralization (140).

Biomechanical stimulation is key in the development and homeostasis of functional cartilage tissue (141-145). The importance of mechanical loading and physical movement on embryonic chondrogenesis has demonstrated in chicken embryos which when physically impaired exhibited poor development of cartilage tissue (146-148). Mechanical loading is required for healthy tissue; however, excessive loading can lead to trauma and disease progression (141). Thus, mechanical stimulation of cells and tissue constructs via compression, shear and hydrostatic pressure is important to promote chondrogenic differentiation, maintain a chondrogenic phenotype, and
generation of functional tissue in vivo. Mechanical stimulation of MSCs under varied loading regimes have been shown to increase the deposition and expression of collagen II, GAG, TGF-β1/β3, and SOX9 and modulated secretory factors such as stromal-derived factor-1 (SDF-1), matrix metalloproteinase-2 (MMP-2), FGF, vascular endothelial growth factor (VEGF), activated leukocyte cell adhesion molecule (ALCAM), nitric oxide, urokinase receptor (uPAR), macrophage inflammatory protein 3α (MIP3α). The advancement of bioreactors in tissue engineering has enabled the use of mechanical stimulation as a key capability in engineering cartilage tissue formation (149-151). Dual compressive and shear mechanical stimulation of human articular chondrocytes encapsulated in gelatin methacrylate and hyaluronic acid methacrylate hydrogel have been demonstrated by Meinert et al (152). Cartilage specific marker genes and ECM were upregulated with significant increase in collagen II synthesis. Combining compressive and shear stimulation has been investigated using multi-axial loading bioreactor which mimics the movement of articulating joint (153, 154). Vainieri et al investigated a chondrocyte seeded hybrid fibrin-polyurethane scaffold implanted in an osteochondral defect model that was mechanically stimulated using a joint mimicking bioreactor (154). The results showed increased production of chondrogenic specific markers, proteoglycan 4 and cartilage oligomeric matrix protein, and the improved collagen II to I ratio. Alternatively, tensile stimulation of a self-assembled scaffold-free neocartilage construct has shown to increase the tensile strength and modulus of the construct and once implanted in vivo in a mice model had similar mechanical properties and collagen content of native tissue (155). The development of improved mechanically stimulating bioreactors and osteochondral models will provide a valuable tool in understanding cartilage development and will aid the screening of biomaterials and tissue engineering.

Challenges remain in the use of stimulatory processes to guide cell behavior and tissue development. A complete understanding of chondrogenic development is still developing so the entire milieu of factors that influence tissue formation incomplete. However, tissue engineering strategies will most likely, to be successful, use a combination of growth factors, a controlled biophysical environment, and a mechanical stimulation to promote tissue formation with phenotypic stability. As the use of soluble factors alone in the differentiation of chondrocytes and MSCs in vitro
typically results in the expression of hypertrophic chondrocyte phenotype. The design of these stimulatory environments will aim to recapitulate the native environment during early stage development of the tissue. A successful strategy will need to determine the combination, dosage, and delivery profile of growth factors. The design of the physical matrix surrounding the cells by controlling parameters such as crosslinking density, ECM protein selection, and oxygen tension. Finally, the timing, type, and loading conditions of mechanical stimulation will be key in promoting an ECM which is mechanically compliant. However, the complexity of this environment and the actual implementation of a multi-stimulatory strategy is a serious challenge for researchers.

2.1.7 Conclusion and future perspectives
This Section discusses articular cartilage structure, composition, and current clinical therapies. Tissue engineering strategies are discussed with a focus on selection of biomaterials, stimulatory factors, and bioprinting to provide an overview of approaches to generate osteochondral tissue.

The clinical size and market of cartilage and osteochondral problems is expanding due to the ageing worldwide population and the most common treatment approaches are ineffective at stopping the progression of degeneration of the tissue. Thus, tissue engineering strategies are key in solving this pressing clinical problem. The design specification of any biomaterial-based 3D construct must fulfil a stringent criterion requiring suitable biomaterial selection, scaffold architecture, fabrication technique, stimulatory factors, and maturation of the tissue.

Key research challenges are the maintenance of phenotype in the engineered tissue construct and the prevention of hypertrophic or fibrocartilage phenotypes being expressed. The tissue constructs also require strategies to promote integration with the surrounding healthy tissue in an osteochondral defect, although in total replacement the strategy would primarily to be to anchor the neocartilage to underlying bone. Furthermore, the underlying biological behaviour of the tissue, the early-stage developmental biology, and haemostatic processes in adult tissue need further understanding. This knowledge may unlock key aspects of the tissue which may guide tissue engineering strategies. This could require a strategy of multiple stimulatory factors (e.g. growth factors and mechanical stimulation) over an extended maturation time of up to many years to mimic the underlying biological
development of the tissue and even then, the incorporation of permanent mechanical structures may be necessary. Subsequently, future studies should focus on a multi-stimulatory environment, long-term studies to determine phenotypic alterations and tissue formation, and the development of novel bioreactor systems that can more accurately resemble the in vivo environment. Finally, a clear and considered route in the development process of the materials, structures, and strategy should be evaluated prior and during the research phase to expedite clinical and regulatory approval. This will allow faster and more successful access to animal trials and eventually human clinical trials with the prospect of a successful therapy being developed.
2.2 Engineering natural-based photocrosslinkable hydrogels for cartilage applications†

Articular tissue is an avascular tissue at the ends of articulating joints which provides lubrication and transmission of compressive forces during movement. The tissue has poor regenerative capacity and low cellular metabolic activity thus disease or trauma can result in significant clinical issues characterised by pain in the joints and restriction of movement. This can affect patient’s quality of life and impose substantial socioeconomic costs on society. Currently no treatment can prevent the long-term degradation of the tissue, so new and improved therapies are required. Tissue engineering based strategies are a promising approach to repair and regenerate damaged tissue. Natural hydrogel-based scaffolds have been widely developed to promote cartilage regeneration due to their biocompatibility, resemblance to the native extracellular matrix, and capacity for cell encapsulation. However, a key challenge is the engineering of biomimetic hydrogels to promote the development of articular cartilage rather than fibrocartilage. Hydrogels can be designed through the selection of appropriate materials, crosslinking mechanisms, mechanical properties and the incorporation of biofunctional moieties that can regulate biodegradation, cell attachment and differentiation, and the delivery of biomolecules. The range of parameters to engineer provides the opportunity to fabricate biomimetic structures that can facilitate cartilage regeneration. However, the complexity of the challenge is daunting and requires an interdisciplinary approach to successfully fabricate hydrogel-based scaffolds that can promote long-term regeneration of articular cartilage.

2.2.1 Introduction

The desire to regenerate and replace damaged or dysfunctional human tissues to improve quality of life is as old as human history. This aspiration has been expressed in numerous cultures throughout history such as the myth of Prometheus with his eternally regenerating liver and Mary Shelley’s ‘Frankenstein’ in which the scientist created new life from the rejuvenation of dead tissue. This human fascination to regenerate the body has in recent decades developed into the rapidly expanding scientific field of tissue engineering (TE). TE holds the promise to offer a paradigm

shift in clinical treatment and understanding of a host of disease states. This shift will enable a science fiction like prospect of infinite replacement human body parts which will have tremendous positive impact on human health. The concept of TE has developed into the currently accepted definition put forward by Langer and Vacanti in 1993 that states:

“Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function.” (156).

Due to an ageing population worldwide and increasing numbers of age-related and lifestyle linked diseases such as osteoarthritis, diabetes, and cardiovascular disease, the importance of TE is enormous. TE will enable the development of superior clinical treatments to the benefit of patients but also relieve the strain on healthcare systems. Furthermore, TE has applications beyond tissue regeneration and replacement such as the development of three-dimensional (3D) culture and tissue constructs that allow pharmaceutical testing, disease modelling, toxicology testing, and improved understanding of cell biology (157).

There are multiple strategies employed in TE to enable tissue regeneration and replacement. A scaffold based or top-down approach, typically utilises a scaffold which is a supporting construct that outlines the spatial requirement of the tissue, enables cell attachment, and provides environmental cues to promote a desired cellular response (158-160). This approach can be typically split into either acellular or cellular techniques. Cellular approaches using a traditional in vitro TE strategy require cells to be isolated, expanded in culture, and then seeded onto a scaffold in vitro to enable maturation and remodelling of the construct before subsequent implantation into a patient. An alternative method is to seed the scaffold with cells isolated at the point of surgical implantation which avoids the in vitro maturation phase and requires a single surgical intervention. The acellular in vivo TE scaffold approach bypasses the need for cell seeding and maturation completely as a bio-functional scaffold is directly implanted into the patient. This approach relies on the recruitment of cells in vivo to initiate tissue repair and remodelling of the scaffold. An alternative approach is the scaffold-free strategy which is a bottom-up process utilising cell spheroids, cell sheets, and self-assembly to generate new tissue and typically does not require exogenous scaffolds or cell seeding (161). These approaches utilise the ability of cells to fuse into
larger constructs and in self-assembly necessitates the recapitulation of embryonic and developmental pathways of tissue and organ genesis (162-164). The scaffold-free approach using cells, their own extracellular matrix (ECM) and signalling molecules aims to generate new tissues and organs with high level of biomimicry, thus requiring a deep understanding of the underlying biological and physical mechanisms of these pathways. Finally, a new hybrid approach aims to combine both scaffold and scaffold-free TE in a synergistic approach to benefit from the inherent advantages of both techniques (165).

Hydrogels are a specific class of materials highly relevant for both the fabrication of acellular scaffolds and cell-laden constructs. They are biocompatible and can be designed to be biodegradable with a structure that closely mimics the native in vivo ECM (166-168). This is a result of hydrogels typically being composed of a three-dimensional (3D) polymer network which swells on contact with water (169). This enables storage of large quantities of water which resembles soft biological tissues and allows for encapsulation of cells and bioactive molecules. Furthermore, hydrogels are typically biocompatible and allow for diffusion of oxygen, nutrients, and watersoluble compounds. Hydrogel properties can be tuned through chemical, physical, and biological modification which allows a variety of cell behaviours such as adhesion, proliferation, and differentiation to be optimised. Moreover, hydrogels can be tuned to respond to stimuli such as temperature, light, pH, and biomolecules which enables hydrogels to have a variety of properties such as self-healing, controlled degradation, and drug delivery (170). A pioneering example of a cell-responsive hydrogel is Hubbell et al. demonstration that inclusion of specific peptides in a hydrogel could enhance matrix metalloproteinases (MMP) activity and thus control the rate of scaffold degradation (171). The ability to fabricate hydrogels with specific properties has enabled precise control of cell behaviour which is a benefit in creating scaffolds that will allow tissue engineering to be successfully translated.

Photocrosslinkable or photocurable hydrogels are particularly relevant for TE applications due to the rapid gelation, control over spatiotemporal formation, and the ability to tune polymer network properties such as crosslinking density and matrix stiffness (172). This is often achieved by controlling operating parameters such as light intensity, exposure time, and illumination area. When used in combination with bioprinting techniques, photopolymerization enables the fast and automated crosslinking of complex scaffold architectures during and post-printing. However,
photocrosslinking conditions need to be optimized to overcome concerns regarding cell exposure to UV radiation and potential cytotoxicity from photoinitiators, by-products, and unreacted reagents.

This Section outlines the recent advances in the design of naturally derived photocrosslinkable hydrogels for cartilage tissue engineering. A brief description of the structure and composition of cartilage tissue will be firstly provided, followed by an overview of hydrogel properties, synthesis routes, and strategies for hydrogel biofunctionalisation within cartilage tissue engineering (Figure 2.9).

**Figure 2.9:** Schematic diagram of photocrosslinkable 3D hydrogel constructs and approaches for use in cartilage tissue engineering.

### 2.2.2 Articular cartilage

Articular cartilage is a load-bearing and non-vascularised tissue that covers the ends of bone joints (Figure 2.10a), acting as a low-friction bearing surface and a mechanical damper for the bones (173, 174). It is a matrix-rich tissue with specialized cells (chondrocytes) that maintain the structural and functional integrity of the ECM (175-178). However, cartilage lacks inherent self-repair ability and remains a significant clinical and economic challenge throughout the world, hence the zonal structure and function are often irreversibly lost following trauma and disease. Thus, cartilage defects are prone to develop into osteoarthritis (OA), which is a major disease
associated with cartilage tissue and is characterized by a loss of healthy and functional cartilage tissue, pain, and debilitation thus is a significant clinical and economic challenge (179, 180).

2.2.2.1 Composition and zonal organization

Cartilage is characterized by a distinct zonal structure comprising, in descending order, the articular or superficial surface, the middle (or transitional) zone, the deep zone, a tidemark (separates the non-calcified and calcified regions) (18, 181), and the calcified zone (Figure 2.10b). These zones differ with respect to the molecular composition and organization of the cartilage ECM, the shape, density, alignment of the resident chondrocytes and the mechanical properties of each zone. The subchondral bone acts as a shock absorber, the loading forces experienced by the joint are transmitted to this region, and the bone tissue contains blood vessels that supply nutrients, oxygen, and removes waste from the lower regions of the avascular cartilage tissue (182). Surrounding each individual chondrocyte are three distinct ECM regions which depending on the distance from the cells are termed the pericellular, territorial, and interterritorial region. The highly organised and hierarchical properties of the tissue are responsible for its remarkable mechanical properties (183-187).

Figure 2.10: a) Articular cartilage covers the surfaces of bones in diarthrodial joints (e.g. knee and elbow); b) Zonal structure and composition of articular cartilage (40)

Articular cartilage is typically composed of:

**Chondrocytes:** responsible for production and maintenance of the cartilage ECM and essential for tissue formation and functionality. They represent about 5-10% of the total volume of cartilage (17, 187). However, they have limited proliferation capability and low metabolic activity, which is partially responsible for the limited capacity of cartilage to recover from trauma or disease (188, 189).
**Collagen:** the main component of cartilage with up to 60% of the dry weight being collagen with type II the most common type representing 90-95% of total collagen in the ECM which can form fibrils and fibres entwined with proteoglycan aggregates.

**Proteoglycans:** molecules responsible for the resistance to compression of cartilage tissue (190). Proteoglycans (PG), corresponding to around 35% of the cartilage dry weight, are made up of repeating disaccharide units, glycosaminoglycans (GAGs), with two main types; chondroitin sulphate and keratin sulphate (190). Proteoglycans have a turnover rate of three months and weave between the collagen fibres creating a mesh responsible for retaining water and providing elasticity to the tissue (191).

**Water:** represents 65-80% of the total cartilage mass (192). This content reduces with aging and is linked to a reduction of strength and elasticity of the tissue (193).

**Synovial fluid:** a highly viscous liquid secreted by synovial lining cells, they are responsible for secreting hyaluronic acid (the intrinsic component of synovial fluid) (194, 195). The main function of the synovial fluid is to distribute nutrients and gases to the upper regions of the cartilage tissue whilst providing lubrication at the interface between articulating surfaces in the joint to allow facilitation of movement (196).

2.2.2.2 Clinical treatments and challenges

Cartilage disease and traumas are a serious clinical problem that can lead to debilitating pain, swelling, inflammation, and can result in drastically impaired mobility. An ageing worldwide population is resulting in increased number of patients suffering from osteoarthritis, a leading cause of cartilage damage (197). Additionally, tissue degeneration through disease or significant trauma results in steady degradation of the tissue with limited successful clinical interventions to prevent this long-term decline which eventually results in a total joint replacement (Figure 2.11). The limited regenerative capacity of cartilage is due to the avascular nature of the tissue and low metabolic activity of the resident chondrocytes and is in marked contrast to other tissues such as bone and the liver which have remarkable healing properties in comparison (163, 198). Current clinical interventions have limited success in regenerating or replacing articular cartilage with most therapies producing inferior fibrocartilage or only delaying the progression of tissue degeneration (199, 200).
The most commonly used cartilage repair strategies are debridement and microfracture, autologous chondrocyte implantation (ACI), matrix-assisted autologous chondrocyte transplantation/implantation (MACT/MACI), autologous matrix-induced chondrogenesis (AMIC), and osteochondral auto- and allografts (Figure 2.12) (201, 202). Each of these approaches has major disadvantages such as the formation of inferior fibrocartilage, requirement of two surgeries, donor site morbidity, and limited prevention of continued tissue degeneration. Therefore, new approaches are required to address these unmet clinical challenges. TE has the potential for a breakthrough approach by synergistically incorporating chondrocytes/stem cells, biomaterials, 3D scaffolds, stimulatory growth factors, mechanical stimulation, and bioreactors for the fabrication of tissue constructs. If this approach is successful, then we will approach our final goal of the predictable regeneration of articular cartilage (110, 203-207).
Figure 2.12: Current techniques for articular cartilage repair and future scaffold-based approaches. a) Debridement and microfracture; 1-4 debridement of damaged tissue, 5 microfracture into the subchondral bone, 6 blood clot formation containing mesenchymal stem cells (MSCs), and 7 formation of inferior fibrocartilage. b) ACI: 1 cartilage biopsy from a non-load bearing site, 2 in vitro expansion of chondrocytes to obtain sufficient numbers for implantation, 3 a second operation cleans the damaged area and is covered with the periosteal flap and the expanded chondrocytes are injected under this or the expanded chondrocytes are seeded into a matrix (e.g. collagen membrane) and fixed with fibrin glue into the defect area (MACI/MACT). c) AMIC; a microfracture is created and a membrane placed over the top and fixed with a fibrin glue or sutures. The membrane protects the clot formation and allows chondrogenic differentiation of the MSCs in the blood to aid the regeneration process. d) Osteochondral auto/allograft transplantation; cylindrical plug of fresh cartilage and subchondral bone is taken from the patient or a cadaver donor, 1 a guide pin is placed perpendicular to the joint surface in the defect, 2 implantation of the graft plug, and 3 securing the plugs with the insertion of screws. e) Acellular scaffold only requiring a single surgery and in vivo recruitment and stimulation of cells for tissue regeneration. f) Cellular-based scaffold involving two surgeries of initial cartilage biopsy, chondrocyte expansion, and seeding into the scaffold before implantation into the defect. Adapted with permission from the ICRS (200)
2.2.3 Engineering hydrogels for cartilage tissue engineering

Hydrogels are three-dimensional (3D) insoluble crosslinked polymer networks able to retain a large amount of water and biological fluids (between 10-200%) in their swollen state (208-211). The 3D network of the hydrogel (Figure 2.13) is maintained by polymer crosslinking among the polymer chains, with water taken up and contained within the polymeric structure (212, 213).

Figure 2.13: Engineering 3D hydrogels for cartilage application through a) cell-encapsulation, b) optimizing crosslinking density, and c) functionalizing with biomolecules (e.g. RGD) to guide cell behavior.

Hydrogels have a range of useful characteristics such as their hydrophilic capacity to absorb a volume of water that is significantly beyond their weight when dehydrated (214), potential printability and ability to create a self-supporting 3D structure to suit various biological engineering needs (cell encapsulation, cell support and biodegradability) (215), uniform cell distribution, as well as providing both chemical and biological signalling (216). The ability of the 3D network to resist dissolution in water is related to the presence of the crosslinked polymer molecules which will determine the quality and stability of the hydrogel. Due to these properties and characteristics, a wide range of different hydrogels have been used for ECM formation and cartilage regeneration applications. Currently, natural based hydrogels represent the most relevant group of materials to produce constructs for cartilage applications (Table 2.1). Depending on the source, they can mimic the ECM structure and composition of articular cartilage presenting relevant swelling, degradation, mechanical and lubrication characteristics. Natural based hydrogel materials such as alginate (217), agarose (218), gelatin (219), collagen (220-222), hyaluronic acid (HA) (223), chitosan (224), and interpenetrating network/hybrid systems have been extensively explored for cartilage applications (225).
### Table 2.1: Photocrosslinkable natural polymers used in cartilage tissue engineering.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Crosslinking conditions</th>
<th>Biological properties</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>Methacrylate anhydride, photoinitiators (VA-086, Irgacure 2959) and 254-365 nm exposure wavelength. Simple preparation.</td>
<td>Chondrocytes showed ECM formation with ~80% similarity to native cartilage. &gt;85% viability</td>
<td>(226-228)</td>
</tr>
<tr>
<td></td>
<td>Typically, physical crosslinked (thermal), however, can be functionalized with methacrylate group,</td>
<td>Stem cells/ MSCs/ hASCs: could be regulated via the introduction of soluble factors and biophysical cues in 3D cell culture systems</td>
<td></td>
</tr>
<tr>
<td>Agarose</td>
<td>Methacrylate and acrylic anhydride, photoinitiators (VA-086, Irgacure 2959) and 254-365 nm exposure wavelength. Simple preparation.</td>
<td>Chondrocytes: no results reported (for chondrocyte cells encapsulated in photocurable agarose).</td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>Glucuronic acid carboxylic acid, the primary and secondary hydroxyl groups, and the N-acetyl group (following deamidation) thiols, methacrylate, tyramines Photoinitiators (VA-086, Irgacure 2959) and 254-365 nm exposure wavelength</td>
<td>Chondrocytes showed ECM formation similar to native cartilage. &gt;85-97% viability, cell morphology could be controlled by the stiffness of hydrogels.</td>
<td>(232-235)</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td></td>
<td>Chondrocytes, epithelial cells and murine fibroblasts</td>
<td></td>
</tr>
<tr>
<td>Chitosan (CS)</td>
<td>Vinylated CS Macromers, Azido-Functionalized CS azido, and (meth)acrylated CS</td>
<td>Structurally analogous to cartilage glycosaminoglycans, Biocompatible, showed low cytotoxicity at various concentrations for chondrocytes, cell viability between 82-96%</td>
<td>(236, 237)</td>
</tr>
<tr>
<td>Dextran</td>
<td>Methacrylate groups (glycidyl methacrylate) introduced in dextran to prepare photocrosslinkable dextran (photoinitiator Irgacure 2959 to UV light. 254-365 nm wavelength)</td>
<td>Chondrocytes, endothelial cells and stem cells, the dextran-based IPN hydrogel provides cell-adhesive and enzymatically degradable properties</td>
<td>(238, 239)</td>
</tr>
<tr>
<td>Gellan Gum</td>
<td>Methacryl anhydride, the aqueous solution of methacrylated gellan is added with 0.08 mg/mL calcium chloride and 0.5% (w/v) Irgacure 2959</td>
<td>Fibroblasts ~90% cell viability.</td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>Collagen I: dual crosslinking with visible light, Rose Bengal, and chemical crosslinking using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide.</td>
<td>Promoted the retention of chondrocyte phenotype and matrix synthesis of encapsulated porcine chondrocytes and enhanced cell infiltration and tissue repair in rabbit osteochondral defect model at 2-week post-surgery</td>
<td>(233)</td>
</tr>
<tr>
<td>Collagen II: amidation reaction between amino groups on the collagen lysine and methacryl anhydride resulting in collagen methacrylamide. Irgacure 2959 used as photoinitiator and exposed to UV (8 W cm^-2) for 30 s. Triple helical structure of collagen was preserved after functionalization.</td>
<td>BMSCs differentiated into a chondrogenic phenotype with lacuna-like cartilage formation. High cell viability. Increase in chondrogenic gene expression (AGG, SOX9, COL II, COL X). In vitro subcutaneous implantation in a mouse model demonstrated the secretion and deposition of cartilage specific matrix.</td>
<td>(220)</td>
<td></td>
</tr>
</tbody>
</table>
Although pure natural hydrogels have been widely explored in cartilage applications, they often have significant limitations relating to the suitability of their biomechanics, degradation profiles, and cell-material biological interactions. Subsequently, significant research into new hydrogel systems that have tuneable mechanical and degradation properties, provide specific control over cellular behaviour, allow incorporation of novel materials, and display suitable material processing properties for different fabrication routes such as 3D printing have been developed (126, 242-245). The incorporation of multiple materials, hybrid hydrogels, to produce a system with a mixture of two or more of distinct classes of molecules (e.g. a blend of natural and synthetic hydrogels) or different structures (e.g. blend of organic polymer hydrogels and inorganic particles/metals or synthetic nanoparticles) have been developed to improve single material-based systems (Figure 2.14) (246-249).

**Figure 2.14:** Hybrid hydrogel network systems (96). Multi-material bioink networks are two different component polymers or more crosslinked together. Interpenetrating networks (IPN) is a 3D structure of two or more polymeric networks, which are partially or fully chemically bonded but not covalently bonded, or a network of embedded linear polymers entrapped within the original hydrogel (semi-IPN) (97, 98). Nanocomposites bioink network can be produced by adding nanoparticles to the polymeric hydrogels (99). Supramolecular bioink network is composed of short repeating units with functional groups that can interact non-covalently with other functional units, forming large, polymer-like entanglements (100).
The use of photocrosslinkable hybrid hydrogels is a burgeoning research field especially within tissue engineering. Photocurable hydrogels are particularly relevant for biomedical applications due to the advantage of being able to encapsulate cells, fast gelation time, tuneable mechanical properties and crosslinking density, and the cytocompatibility processing conditions that allow their in situ crosslinking into the defect site during a surgical procedure (250-252). The use of vat photopolymerization and light curing processes in additive manufacturing also enables the fabrication of high resolution and complex structures that are not possible through conventional methods (111, 172). This approach facilitates the development of more biomimetic structures that can recapitulate the complexities of native tissues.

Typically, polymers are unable to initiate a photopolymerization reaction alone and can require both functionalisation with photocrosslinkable groups and the addition of light sensitive molecules, called photoinitiators. The photoinitiators typically used within tissue engineering are normally excitable within the ultraviolet (UV) (e.g. 1-4-(2-hydroxyethoxy)-phenyl-2-hydroxy-2-methyl-1-propane-1-one, Irgacure 2959, and 2,2'-azobis-2-methyl-N-(2-hydroxyethyl)propionamide, VA-086), UV-visible (e.g. lithium phenyl 2,4,6-trimethylbenzoylphosphinate (LAP), and diphenyl(2,4,6-trimethylbenzoyl) phosphine oxide, TPO), and visible light range (e.g. ruthenium, Bengal rose, riboflavin, eosin-Y, and camphorquinone). Upon irradiation, an initiation step begins and the photoinitiator undergoes an excitation mechanism which results in the formation of highly reactive species (free radicals). The next step is propagation, free radicals attach at specific sites on the monomer/oligomer structure (unsaturation sites) starting a chain reaction that creates a permanent crosslinked network. Finally, a termination step results in the completion of the polymer chain and crosslinking with free radical extinction. The degree of photopolymerization (curing percentage) of the resulted hydrogels is highly affected by the functionalisation process and polymer solution concentration, light wavelength and intensity, photoinitiator type and concentration, and curing time (253). The number of parameters to control also provides an opportunity to highly engineer the resulting hydrogels properties and when used in conjunction with bioprinting processes can result in tuneable and biomimetic structures.

Photocrosslinking is an advantageous process, however, specific issues need to be addressed especially in biomedical applications. The chemical processes used can generate toxic by-products that can result in cytocompatibility residues in the hydrogel
Thus, the hydrogel is usually subjected to a purification step prior to its application, for example, the modified macromer may require dialysis prior to use. Furthermore, the photoinitiators themselves can be toxic. Thus, specific care is required when designing the photocuring system such as using a low concentration of photoinitiator. Finally, the wavelength and intensity of light can induce genetic damage within the cells resulting in reduced viability and function, thus this requires careful consideration.

Crosslinking mechanism, biofunctionalization, controlled delivery of biochemical factors and hydrogel physical characteristics are the most crucial parameters that determine hydrogel suitability for the target application.

2.2.4 Crosslinking mechanisms of hydrogels

The crosslinking mechanisms of hydrogels are broadly classified into two categories (Figure 2.15): chemically crosslinking (permanent crosslinking) and physical crosslinking (reversible crosslinking) (260). Chemically crosslinking hydrogels are formed by covalent bonding that creates a permanent 3D polymer network (261). Physical crosslinking hydrogels are produced through the entanglement mechanism of the polymer chains (262). For the fabrication of both scaffolds and cell-laden constructs, chemical crosslinking is the most suitable mechanism (254, 263, 264).

Crosslinking reactions can be induced by using a variety of stimuli such as light, pH stimulation, ionic exchange, enzymes, and temperature (254). The crosslinking density strongly determines the characteristics of the hydrogels, such as mechanical, degradation, porosity, pore size, swelling and biological properties (266, 267).
Light initiated crosslinking reactions correspond to the most common approach, allowing the fabrication of complex structures under relatively mild conditions. Two different reaction mechanisms are being considered: free radical polymerisation and click chemistry.

Free radical photopolymerization is a chain growth reaction started by the absorption of light by photoinitiators. The photoinitiator concentration determines the polymerisation kinetics and consequently the properties of the polymerised structures. Radical photopolymerisable hydrogels can be functionalised with cell adhesive moieties and degradation sites in a relatively easy and reproducible manner (268).

Major limitations are associated to the relatively poor control over the crosslinking kinetics, the presence of unreacted double bonds which can react with biological substances, and the generation of heterogeneities due to the random chain reaction process. As an alternative, orthogonal click reactions (e.g. thiol-norbornene) proceed under mild reaction conditions with higher efficiency and faster curing kinetics compared to free radical photopolymerization (269). This step-growth reaction allows high spatio-temporal control and the fabrication of 3D networks with minimal defects (270).

2.2.5 Biofunctionalization of hydrogels

Hydrogels for cartilage tissue engineering must be designed to closely capture the composition, architecture, biophysical properties and biochemical cues of the native tissue. This is essential to provide a cell-instructive 3D microenvironment that guides the fate of embedded cells and morphogenesis. In the case of acellular networks, hydrogel characteristics such as mechanical properties and porosity must ensure adequate load transfer and eventually promote cell recruitment. Multiple approaches have been explored to develop photocrosslinkable hydrogels that perform fundamental functions of the ECM, including the (i) material selection and hydrogel structure, (ii) modulation of hydrogel viscoelasticity, (iii) bioconjugation of bioactive peptides, and (iv) tethering of biochemical factors (271).

2.2.5.1 Hydrogel composition and structure

Important design criteria for hydrogels include appropriate mechanical properties, ensuring nutrient/waste transport and maintenance of cell viability. Several studies have modulated the mechanical properties of hydrogels by varying the macromer concentration or crosslinker type/content using a single polymer network. These
works demonstrated that hydrogel stiffness has a tremendous impact on matrix deposition with highly crosslinked networks restricting new tissue formation and eventually leading to matrix calcification (272, 273). In addition to the matrix stiffness, it was also reported that the molecular weight (50 to 1100 kDa) and macromer concentration (2 to 20 % wt ) of methacrylated hyaluronic acid hydrogels determine the amount and distribution of new ECM via modulation of the gel structure (e.g., compressive modulus, swelling ratio) (274). Another important consideration of hydrogels is to balance the mechanical integrity and the degradation rate. Gel networks should support tissue growth as the hydrogel degrades, maintaining the structural integrity of the new ECM. One interesting approach to control the degradation rate of hydrogels for cartilage applications involves the synthesis of photocrosslinkable macromers bearing hydrolytically labile linkages. This strategy was explored to design hydrolytically degradable hydrogels via thiol-ene photopolymerization of norbornene-modified Poly(ethylene glycol) (PEG) containing caprolactone segments with hydrolytically labile ester linkages and PEG dithiol crosslinker in the presence of a photoinitiator (275). Although hydrogels based on a single polymer are relatively simple to design, their limited mechanical properties and potential reduced cell viability due to high crosslink density constitute important concerns. To tackle these limitations, more complex hydrogels have been developed based on the combination of multiple polymers. Depending on the selected materials and crosslinking chemistries, different hydrogel structures have been designed, including interpenetrating networks (IPNs) (276), semi-IPNs (277), double networks (278) and guest-host networks (279). Wei et al (280) synthetized a two-component guest-host hydrogel of adamantane-functionalized hyaluronan (HA) as guest polymers and monoacrylated β-cyclodextrin as host monomers, rapidly crosslinked through UV photopolymerization. Hydrogels displayed self-healing properties, supported robust chondrogenesis of embedded human MSCs and stimulated cartilage formation in a rat model. In addition to the dynamic nature of the network and/or superior mechanical properties often achieved by more complex hydrogel systems, these gels can also address key compositional and biological properties of the native tissue by the incorporation of major components of the cartilage ECM (281). One strategy consists of the chemical incorporation of ECM components bearing a photocrosslinkable moiety into the hydrogel network via photopolymerization. This strategy was explored by Wang et al (282) to evaluate the influence of methacrylated ECM molecules
(chondroitin sulfate, heparan sulfate and hyaluronic acid) on the in vivo cartilage formation using ECM-containing hydrogels with tuneable stiffness and controllable biochemical composition. Methacrylated ECM molecules were incorporated into the network of PEG dimethacrylate hydrogels through photopolymerization in the presence of adipose derived stem cells and neonatal chondrocytes. Cellular hydrogels with varying matrix stiffness and biochemical cues showed increased collagen type II deposition in both chondroitin sulfate- and hyaluronic acid-containing hydrogels, while heparan sulfate-containing hydrogels promoted a fibrocartilage phenotype and failed to retain newly deposited matrix. This study revealed the importance of biochemical composition of hydrogels and matrix stiffness on the phenotype of neocartilage formation.

2.2.5.2 Hydrogel viscoelasticity
Traditional hydrogels obtained by photopolymerization reactions are made of strong covalent bonds, leading to almost purely elastic gel networks. In contrast to native cartilage tissue, which is viscoelastic and exhibit partial stress relaxation when a constant strain is applied, chemical hydrogels store cellular forces and resist deformation. Purely elastic hydrogels with embedded chondrocytes were found to limit cell proliferation and restrict cartilage matrix deposition to the pericellular space, probably due to the elastic nature of hydrogels (283). Recent findings have shown that viscoelastic hydrogels, in which the stress is relaxed over time, support the spreading, proliferation and differentiation of embedded mesenchymal stem cells in 3D culture without requiring matrix degradation (284). It has also been reported that cell-laden hydrogels exhibiting faster stress relaxation support significantly more new bone growth in vivo when compared to slow stress-relaxing gel networks (285). As stress relaxation is suggested as a key design parameter of hydrogels to both better recapitulate the viscoelastic behaviour of natural ECM and perform important biological behaviours, efforts have been focused on the design of viscoelastic hydrogels with tuneable stress relaxation for cartilage tissue engineering. In a recent work, Lee et al, (286), developed a series of viscoelastic alginate hydrogels exhibiting tuneable stress relaxation to evaluate the effects of viscoelasticity on embedded chondrocytes in 3D culture. Stress relaxation of calcium-crosslinked hydrogels was controlled by varying the molecular weight of alginate and covalent coupling of short PEG spacers. It was found that hydrogels with faster stress relaxation supported the
formation of increased cartilage matrix with deposition of both collagen and GAGs (Figure 2.16), while slower relaxing gel networks led to the up-regulation of genes associated to cartilage degradation and cell death. An alternative approach to engineer hydrogels with tuneable viscoelastic properties consists on the formation of covalent adaptable networks with the ability to reorganize the network connectivity to dissipate local stress. As an example, hydrazone crosslinked PEG hydrogels were synthesised as viscoelastic gels for cartilage applications (287). After 4 weeks of culture, porcine chondrocytes embedded within hydrogels exhibiting average relaxation times of 3 days secreted an interconnected articular cartilage-specific matrix with increased deposition of collagen (e.g., collagen type II) and sulfated glycosaminoglycans (e.g., aggrecan) compared to predominantly elastic hydrogels with slow average relaxation times (~ 1 month). The authors suggested that a balance between slow and fast relaxing crosslinks is essential to preserve gel network integrity and support the formation of high-quality neocartilaginous tissue.

Figure 2.16: Influence of hydrogel stress relaxation on cartilage matrix production and formation. Immunohistochemical staining of chondrocytes embedded within 3 kPa alginate hydrogels for 21 days (scale bar: 25 µm) (170).
Articular cartilage is an intricate meshwork rich in collagen and proteoglycans. Rather than a static milieu, the ECM is a highly dynamic microenvironment that establishes reciprocal interactions with neighbouring cells. Cartilage ECM components provide biochemical motifs for cell adhesion and degradation sites for cell-mediated matrix degradation, which are essential to allow cell adhesion, proliferation, migration and tissue formation. As most cell types require adhesion sites to survive and perform their functions, several bioconjugation strategies have been explored for the functionalization of hydrogels with bioactive peptides that impart cell adhesion domains (288). The fibronectin-derived adhesion peptide RGD (arginine-glycine-aspartic acid) is often bond to the hydrogel network to promote integrin-mediated cell binding and allow cell attachment and spreading (276, 289). Other sequences derived from collagen and decorin have also been immobilized into hydrogels with beneficial outcomes regarding chondrogenic differentiation of MSCs and matrix retention (290, 291). Since chondrocytes in native ECM display a round morphology, the benefit of RGD functionalization in chondrocytes is still controversial. For example, chondrogenesis of bone marrow stromal cells embedded within RGD-functionalized alginate hydrogels was inhibited in response to TGF-β1 and dexamethasone regarding gene expression and matrix synthesis (292). Another work showed that photocrosslinked RGD-modified PEG hydrogels enhanced cartilage-specific gene expression and matrix synthesis by bovine chondrocytes, but only in the presence of dynamic mechanical stimulation (293). In fact, most of the hydrogel systems used for chondrocyte encapsulation did not require cell-adhesion domains to support tissue formation (277, 286). Overall, this data demonstrates that the immobilization of cell-adhesion domains in hydrogels perform a key role in terms of cell phenotype and chondrogenesis, further studies are necessary to elucidate the cell-specific influence of peptide ligands.

Natural ECM is continuously remodelled by cell-secreted enzymes, which is required to create space in the matrix for cell migration, proliferation and tissue deposition. Thus, the development of hydrogels susceptible to hydrolytic and/or cell-driven degradation is becoming increasingly recognized as a key feature to improve the ECM biomimicry (294). Hydrolytic degradation of hydrogels is commonly obtained by hydrolysis of ester linkages, leading to changes in the overall network properties. While enzymatic degradation depends on the cell type and levels of circulating...
enzymes, hydrolytic degradation is spontaneous and occurs via bulk and/or surface erosion mechanisms. Cell-driven hydrogel degradation via cleavage of protease-sensitive peptides occurs in a more localized manner, at the pericellular space, better recapitulating the remodelling of natural ECM. Hydrogels based on animal polymers such as fibrin, collagen and hyaluronic acid are naturally degraded, whereas some plant-based polymers (e.g., alginate, pectin) and synthetic polymers are often modified to make them susceptible to enzymatic degradation (288). This has been accomplished by the incorporation of peptide sequences recognized by specific proteases. These peptides can be grafted into the polymer backbone or used as crosslinker agents with a dual function – allow gel formation and promote matrix degradation. As an example, MMP7-degradable hydrogels based on recombinant Streptococcal collagen-like 2 (Scl2) proteins and functionalized with peptides that bind to hyaluronic acid and chondroitin sulphate were synthetized for cartilage tissue engineering (295). Cell-degradable hydrogels with embedded hMSCs and functionalized with GAG-binding peptides significantly enhanced chondrogenic differentiation and gene expression of COL2A1, ACAN, and SOX9, compared to non-functionalized hydrogels.

2.2.5.4 Tethering and controlled delivery of biochemical factors
Cartilage formation is regulated by a coordinated spatiotemporal delivery of biochemical factors that interact with cells to induce chondrogenesis. To this end, a major role of ECM is to serve as a storage depot for growth factors, regulating their spatiotemporal and tissue-specific presentation to the cells. Its ability to locally bind, store, and release growth factors is essential to regulate their bioavailability, bioactivity and stability in order to elicit a biological response (296). The binding of growth factors to the ECM, for example, via electrostatic interactions to heparan sulfate, is fundamental to enhance their activity in the vicinity of cells, prolong their action and protect them from degradation (297).

To recreate the dynamic presentation of bioactive cues available to the cells in native cartilage, hydrogels have been designed with the ability to sequester and/or deliver biochemical cues such as growth factors. This has been achieved through different strategies including the covalent immobilization (298), affinity binding (299), and encapsulation within carrier vehicles (300). Members of the transforming growth factor beta (TGF-β) superfamily such as TGF-β1 play a role in regulating chondrogenesis during development. To allow the immobilization into the hydrogel
network towards improved bioactivity and cell presentation, growth factors are usually modified with photoreactive groups. In one example, TGF-β1 was reacted with 2-iminothiolane, yielding thiolated TGF-β1 that can homogeneously bond throughout the gel network of non-degradable PEG hydrogels during photopolymerization without impairing its bioactivity (298). Thiolated TGF-β1 was covalently linked to the norbornene end groups of PEG hydrogels crosslinked by photoinitiated step-growth polymerization using an MMP-degradable peptide (KCGPQGIWGQCK) as a crosslinker (301). The ability of chondrocytes to promote in situ hydrogel degradation was firstly confirmed using a fluorogenic peptide sensor to determine the extent of MMP-sensitive sequence cleavage (Figure 2.17). In this hydrogel, GAG and collagen deposition was found to be restricted to the pericellular space, which was attributed to the limited chondrocyte degradation of the gel network to allow diffuse matrix production. When hydrogels were used to co-encapsulate chondrocytes and hMSCs, a higher degradation rate was observed, along with increased GAG and collagen deposition at 14 days of culture compared to non-degradable hydrogels. This data highlights the importance of cell-degradable motifs and localized presentation of biochemical cues in photocrosslinked hydrogels to support the formation of cartilage specific ECM.
Figure 2.17: Effect of chondrocytes embedded within cell-degradable hydrogels. A) Schematic illustration of cell-laden hydrogel formation with tethered MMP fluorescent sensor (Dab-GGPQG↓IWGQK-Fl-AhxC), and TGF-β1; hydrogels are crosslinked using either an MMP-degradable peptide sequence (KCGPQG↓IWGQCK) or non-degradable (3.5 kDa PEG dithiol) linker. B) Determination of in situ cleavage of fluorescent sensor by chondrocytes. C) GAG staining of sections from cell-laden hydrogels after 28 days of culture: nuclei stained black and GAGs stained red. D) Collagen staining of sections obtained from cell-laden hydrogels at day 28: nuclei stained black and collagen stained blue (scale bars: 100 μm) (185).

2.2.6 Conclusions and future perspectives

Naturally derived hydrogels are suitable materials for cartilage applications. They are biocompatible and biodegradable with a chemical and physical structure that resembles the ECM structure of natural tissues. Different light-mediated
photopolymerization strategies have been explored for the fabrication of scaffolds with micro- and nano-scale resolutions. These strategies proceed under biocompatible conditions in the presence of cells and biochemical signals allowing the fabrication of cell-laden bioconstructs. In order to improve the biological performance of both scaffolds and cell-laden constructs different biofunctionalisation strategies have been explored. Many naturally derived hydrogels degrade through enzymatic and/or hydrolytic mechanisms, adding another layer of complexity to control their degradation rate. This is an important limitation in terms of the design of suitable constructs for cartilage tissue engineering.

Another critical challenge in cartilage tissue engineering is the integration and engineering of hydrogels that are specific for each region of the osteochondral tissue. The structure and properties of constructs for osteochondral applications must address the characteristics of the two comprising tissues, articular cartilage and subchondral bone, which still represents a major research challenge. The section of such constructs related to the cartilage zone must present adequate mechanical properties to resist mechanical loading and friction whilst promoting ECM formation and chondrogenic expression of mesenchymal stem cells or chondrocytes, inhibiting hypertrophic differentiation and mineralization of chondrocytes in the upper regions of the tissue. Contrary, the section of the constructs corresponding to subchondral bone must promote the formation of a blood vessel network, stimulate osteoblast proliferation, and osteogenic differentiation of mesenchymal stem cells whilst remaining biomechanically suitable until successful tissue regeneration is complete. How to engineer hydrogels to promote or inhibit specific cellular behavioural pathways is a major challenge, for example, a osteochondral hydrogel must promote hypertrophic chondrocytes in the calcified zone and flatter and aligned chondrocytes in the superficial surface. This will demand the development of multi-material and multi-scale hydrogels that are specifically biofunctionalized to promote the complex behaviour and organisation of chondrocyte cells. Furthermore, top-down fabrication techniques will need to improve their resolution and multi-material processing capabilities thus driving demand for hybrid biomanufacturing systems that incorporate multiple fabrication technologies into a single system. Whilst bottom-up fabrication processes will require further understanding of the fundamental developmental biology of osteochondral tissue.
Chapter 3
Development and characterisation of a photocurable alginate bioink for 3D bioprinting†

† This Chapter is based on the paper: Hussein Mishbak, Glen Cooper, Paulo Bartolo, “Development and characterisation of a photocurable alginate bioink for 3D bioprinting”, International Journal of Bioprinting, 5(2): 189, 2019 http://dx.doi.org/10.18063/ijb.v5i2.189. A preliminary version of this manuscript was also presented at the International Conference on Engineering Science and Applications (Tokyo, Japan, 2017) and was awarded as the best paper.
Alginate is a biocompatible material suitable for biomedical applications, which can be processed under mild conditions upon irradiation. This Chapter investigates the preparation and the rheological behaviour of different pre-polymerised and polymerised alginate-methacrylate systems for 3D photopolymerisation bioprinting. The effect of the functionalization time on the mechanical, morphological, swelling and degradation characteristics of crosslinked alginate hydrogel is also discussed. Alginate was chemically modified with methacrylate groups and different reaction times considered. Photocurable alginate systems were prepared by dissolving functionalized alginate with 0.5-1.5% photoinitiator solution and crosslinked by ultraviolet (UV) light (8 mW/cm²).

3.1 Introduction

Three different approaches are being explored for tissue engineering applications (302, 303). The first approach, cell therapy, is based on harvesting cells, sorting, expanding and implanted them. This is a simple process but presents limited outcomes as it is difficult to keep the cells in the desired region for clinically relevant periods of time (289). The second approach, scaffold-based approach, is based on the use of three-dimensional support structures that provide the necessary environment for cell attachment, differentiation and proliferation (304). In this approach, scaffolds can be directly implanted after fabrication or seeded with cells and pre-cultured in a bioreactor before implantation (305). Finally, the third approach (bioprinting) uses bioinks (hydrogels and cells) to create cell-laden constructs. This is a highly relevant approach allowing in situ printing (172, 306). Techniques such as inkjet bioprinting, extrusion-based and photopolymerization-based process are being explored. Among them, photopolymerization is a very versatile method allowing a rapid crosslinking under biocompatible reaction conditions without the use of solvents (172, 302). Additionally, photocurable hydrogels are particularly relevant for biomedical applications due to the advantage of being able to encapsulate cells and the mild processing conditions that allow their in-situ crosslinking within a patient during a surgical procedure.

Suitable hydrogels for bioprinting must be biocompatible, biodegradable, present appropriate mechanical properties, which depend on the type of tissue, good printability (307) and shear thinning properties to facilitate the printing process (308). In the case of photopolymerization bioprinting systems the amount and type of
photoinitiator is also critical as determines the crosslinking density, cytotoxicity, mechanical properties and biocompatibility (309). The increase of crosslinking density is usually associated to an increase of printability and mechanical properties and a decrease of biocompatibility due to the reduction of free space to accommodate cell proliferation (310).

Alginate is a suitable material for bioprinting (311). It is a natural water-soluble linear polysaccharide derived from alginic acid, extracted from several species of brown algae, such as Laminaria Hyperborea, Ascophyllum Nodosum and Macrocystis Pyrifera (312-314). Its structure contains 1,4-linked β-D-mannuronic (M) and α-L-guluronic (G) acid residues (Figure 3.1), arranged in a non-regular and block-wise fashion along the chain (315-317). Alginate shows good biocompatibility, low cytotoxicity and high-water content (high swelling ratio), mimicking the structure of the natural extra-cellular matrix (318-322). These properties make alginate a suitable material for wound dressings, drug delivery systems and soft tissue engineering applications (323).

This Chapter investigates the preparation of alginate-based systems for UV bioprinting applications. The material is characterised both before and after the curing process and the effect of functionalization time on the rheological, mechanical, morphological, swelling and degradation properties investigated. The effect of photoinitiator concentration in terms of rheological and mechanical properties is also assessed and discussed.

**Figure 3.1:** Alginate showing a linkage between the mannuronic and guluronic acid (324).
3.2 **Materials and methods**

3.2.1 **Synthesis of methacrylate alginate**

Photocurable alginates were prepared through a functionalization mechanism with methacrylate anhydride (MA) (325). Briefly, sodium alginate powder (W201502) 1%, 2% and 3% w/v (Sigma-Aldrich, UK) was dissolved in Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma-Aldrich, UK) and then mixed with methacrylate anhydride (MA) (Sigma-Aldrich, UK) at 15 mL MA/g of alginate under vigorous stirring. The pH of the solution was kept around 7.4-8.0 during the reaction time by adding 5M of NaOH. Maintaining a higher pH during the reaction is crucial since higher pH enhances the reaction among the amine and hydroxyl groups, which lead to higher degree of modification (326). Two different reaction times (8 and 24 hours) were considered to assess the effect of the reaction time on the degree of functionalisation. After the chemical modification reaction, the polymeric solution was precipitated and totally mixed in 100 mL of ethanol (100% ethanol) and dried in an oven overnight at 50 ºC. The precipitate polymer was dissolved in distilled water (diH₂O), loaded in the dialysis tubes membranes (SnakeSkin Dialysis Tubing 3.5K MWCO from Thermo Fisher Scientific, UK), sealing both sides and dialyzed the solution against NaCl for 7 days with periodic water changes every day. The solution was freeze at -80ºC and the polymer recovered by lyophilisation.

3.2.2 **Characterisation of pre-polymerised methacrylate alginate**

3.2.2.1 Nuclear magnetic resonance

The chemical structure of functionalized alginate was assessed through Nuclear Magnetic Resonance Spectroscopy (¹HNMR), using the B400 Bruker Avance III 400 MHz (Billerica, Massachusetts, USA). Polymeric materials were dissolved in Deuterium oxide (D₂O) (Sigma-Aldrich, UK), transferred to NMR tubes and the spectra acquired with 128 scans.

3.2.2.2 Rheological characterisation

The rheological tests were performed to characterise the viscoelastic behaviour of both pre-polymerised and polymerised alginate. The rheological assessment of pre-polymerised alginate systems was carried out using the DHR2 TA Instrument (USA). Samples were placed between two parallel plates and two different tests (rotational and oscillation) were considered. Rotational tests were performed to evaluate the viscosity and the material strength. In these tests it is assumed that the material flows
by applying a stress, being the response measured alongside time (temperature was not considered in this work). A controlled stress was applied, and the resulting movement measured. Oscillation tests were considered to evaluate the viscoelastic behaviour of the material and dynamic moduli. The viscoelastic behaviour was characterized by measuring the energy stored (storage modulus, $G'$) in the material during shearing and the energy subsequently lost (loss modulus, $G''$). Shear strain was controlled by varying the oscillation amplitude.

To assess the rheological changes during the photopolymerization process, the rheological tests were carried out using the Bohlin Gemini system (Malvern Instruments) equipped with the OmniCure® S1000 light source irradiating in the range of 254-450 nm wavelength, and oscillation tests were considered. The light intensity was 10 mW/cm².

The rheological behaviour of the materials is described by the following equation:

$$\tau = \eta \dot{\gamma}^n$$

where $\tau$ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s⁻¹), $\eta$ the consistency index or equivalent viscosity (Pa.s) and $n$ is the power law index (dimensionless) that varies as follows:

- $n<1$: shear-thinning system;
- $n=1$: Newtonian system;
- $n>1$: shear-thickening system.

### 3.2.3 Hydrogel formation

Photocrosslinked alginate methacrylate hydrogels were prepared by dissolving 2% w/v alginate-methacrylate with different photoinitiator concentration solutions 0.5% w/v, 1% w/v and 1.5% w/v of VA-086 photoinitiator solution (2,2'-Azobis[2-methyl-N-(2-hydroxyethyl) propionamide azo initiator (Wako Pure Chemical Industries, USA). Crosslinked disks were produced by pipetting the alginate solution in an acrylic mould with 8 mm diameter and 4 mm height. Photopolymerization was conducted using a 365nm UV light (Model Dymax 2000-EC, Dymax Europe GmbH, Wiesbaden, Germany) irradiating at 8 mW/cm² during 8 min.

The photopolymerization process of alginate-methacrylate is a radicular polymerization process started by the absorption of UV light by the photoinitiators, followed by the generation of free radicals and a crosslinking chain reaction (327). The mechanism is briefly presented in Figure 3.2.
Figure 3.2: Schematic representation of the photopolymerization process of alginate-methacrylate. a) After exposing the polymer solution to UV radiation, the photoinitiators generated free radicals that react with the vinyl methylene starting the crosslinking reaction; b) The reaction propagates with macro-radicals reacting with unreacted carbon-carbon double bonds; c) At the end through a bimolecular termination mechanism a 3D network of crosslinked hydrogel is formed. Adapted from (328).

3.2.4 Characterisation of photocrosslinked hydrogels

3.2.4.1 Morphological characterisation

The morphology of the internal structure of the hydrogels was investigated through Scanning Electron Microscopy (SEM), using the Hitachi S3000N VPSEM system. Alginate samples were produced using cylindrical moulds (8 mm diameter and 4 mm high). After hydrogel formation, samples were extensively washed in distilled water, frozen at -80°C and lyophilized. Samples were fixed on stubs using a double-sided adhesive tape and sputter coated with platinum sputter-coating.

3.2.4.2 Mechanical characterisation

Compression tests were performed at constant strain rate using the Instron 3344 machine equipped with a 10-N load cell (Instron, Buckinghamshire, UK). Crosslinked alginate hydrogel disks were prepared as described in Section (3.2.3) and maintained in distilled water at 37°C following the protocol described by Jeon (325). After 24 hours of incubation, swollen alginate-methacrylate hydrogel disks were measured using callipers to determine both the diameter and thickness, and unconfined
compression tests were performed on the hydrogel disks at room temperature, 0.5 mm/min of speed at a rate of 20% strain. Compressive modulus was determined from the slope of stress versus strain plots and limited to the first 10% of strain as recommended for cartilage applications (329).

3.2.4.3  Swelling and degradation characterisation

Alginate methacrylate hydrogel disks were frozen at -80 °C, then, lyophilized and the dry weights (Wi) were measured. Afterwards, the dried hydrogel samples were immersed in distilled water (diH2O) and the same number of samples were also immersed in Dulbecco’s Modified Eagle’s Medium - high glucose (DMEM) (Sigma-UK) diluted with 10% FBS (Fetal Bovine Serum) (Thermofisher, UK) at pH 7 and incubated at 37 °C to reach an equilibrium swelling state. The distilled water and DMEM were replaced every 1-2 days. Over the course of 3 weeks, samples were removed from the DMEM/diH2O and the swollen hydrogel sample weights (Ws) measured. The swelling ratio (Q) was calculated according to the following equation:

\[ Q = \frac{W_s}{W_i} \]  

(3.2)

where \( W_i \) is the dry weight and \( W_s \) is the weight of the swollen hydrogel sample. After this, the swollen hydrogels were lyophilized and weighed again. The percentage of mass loss was calculated as follows:

\[ \frac{(W_i-W_d)}{W_i} \times 100 \]  

(3.3)

where \( W_d \) is the weight after lyophilisation (N=3 for each time point).

3.3  Results and discussion

3.3.1  Alginate functionalization

The alginate modification with methacrylate anhydride was performed under standard conditions, allowing the introduction of photo-reactive methacrylate groups into the polymer backbone, as confirmed by \(^1\)HNMR analysis (Figures 3.3 to 3.5). Results indicate the present of new characteristic peaks of methacrylate (MA) at 5.63 ppm and 6.09 ppm attributed to the methylene group in the vinyl bond, and a peak at 1.82 ppm assigned to the methyl group, which are not present in the non-modified polymer, showing that the polymers were successfully functionalized. Two different functionalisation reaction times (8 and 24 hours) were also considered and the degree of modification determined by dividing the relative integrations of methylene to carbohydrate protons (330, 331). Results, presented in Table 3.1, show that the degree
of modification increases by increasing the reaction time, reaching a maximum value of 33% at 24 hours.

Table 3.1: Effect of reaction time on the modification degree of alginate methacrylate.

<table>
<thead>
<tr>
<th>Composition (% w/v)</th>
<th>Reaction time (h)</th>
<th>Degree of modification (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2%</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>2%</td>
<td>24</td>
<td>33</td>
</tr>
</tbody>
</table>

The functionalization is confirmed by the presence of new peaks in the spectra at 5.63 ppm and 6.09 ppm attributed to the methylene group and a peak at 1.82 ppm that corresponds to the methyl group.
Figure 3.4: a) Non-functionalized alginate 2% w/v, b) Functionalized alginate (2% w/v) after 8 hours of reaction, c) Functionalized alginate 2% w/v after 24 hours of reaction. The functionalization is confirmed by the presence of new peaks in the spectra at 5.63 ppm and 6.09 ppm attributed to the methylene group and a peak at 1.82 ppm that corresponds to the methyl group.
Figure 3.5: a) Non-functionalized alginate 3% w/v; b) Functionalized alginate 2% w/v after 8 hours of reaction; c) Functionalized alginate 2% w/v after 24 hours of reaction. The functionalization is confirmed by the presence of new peaks in the spectra at 5.63 ppm and 6.09 ppm attributed to the methylene group and a peak at 1.82 ppm that corresponds to the methyl group.

3.3.2 Rheological behaviour of pre-polymerised alginate methacrylate systems

Figures 3.6 to 3.9 show the stress versus shear rate behaviour of solutions containing 1, 2 and 3% w/v of alginate-methacrylate reacted for 8 and 24 hours. Key rheological parameters are presented in Tables 3.2 and 3.3. A non-linear behaviour is observed for all samples. Samples containing 2% w/v obtained after 24 hours of reaction show a clear Bingham behaviour (332). Results also show a decrease of the equivalent viscosity by increasing the alginate concentration for samples obtained after 24 hours of reaction, while no trend was observed for samples obtained after 8 hours of reaction. The flow behaviour is also closer to a Newtonian fluid for samples containing high concentrations of alginate. Based on these results the system containing 2% w/v of
alginate was selected as presents less variation of viscosity with the reaction time and a clear shear-thinning behaviour being also less dependent with the reaction time compared to the other systems.

**Table 3.2:** Rheological constants for solutions containing 15 mL of methacrylate and different alginate concentrations, 24 h.

<table>
<thead>
<tr>
<th>Composition (%w/v)</th>
<th>Equivalent viscosity (Pa. s)</th>
<th>Power law constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.36 ± 0.012</td>
<td>0.23</td>
</tr>
<tr>
<td>2</td>
<td>0.54 ± 0.014</td>
<td>0.68</td>
</tr>
<tr>
<td>3</td>
<td>0.16 ± 0.020</td>
<td>0.72</td>
</tr>
</tbody>
</table>

**Table 3.3:** Rheological constants for solutions containing 15 mL of methacrylate and different alginate concentrations, 8h.

<table>
<thead>
<tr>
<th>Composition (%w/v)</th>
<th>Equivalent viscosity (Pa. s)</th>
<th>Power law constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.09 ± 0.011</td>
<td>0.81</td>
</tr>
<tr>
<td>2</td>
<td>0.48 ± 0.012</td>
<td>0.48</td>
</tr>
<tr>
<td>3</td>
<td>0.08± 0.010</td>
<td>0.74</td>
</tr>
</tbody>
</table>

**Figure 3.6:** Stress versus shear rate profiles for alginate-methacrylate solutions containing different alginate-methacrylate concentrations, reacted for 8 hours. a) 1% w/v of alginate; b) 2% w/v of alginate; c) 3% w/v of alginate, and d) comparison of all compositions.
Figure 3.7: Stress versus shear rate for solutions containing different alginate-methacrylate concentrations reacted for 24 hours. a) 1% w/v of alginate, b) 2% w/v of alginate, c) 3% w/v of alginate, and d) comparison of all compositions.

Figure 3.8: Viscosity versus shear rate for solutions containing different alginate-methacrylate concentrations reacted for 8 hours. a) 1% w/v; b) 2% w/v; c) 3% w/v d) comparison of all compositions.
Figure 3.9: Viscosity versus shear rate for solutions containing different alginate-methacrylate concentrations reacted for 24 hours. a) 1% w/v; b) 2% w/v; c) 3% w/v d) comparison of all compositions.

Figure 3.10 and 3.11 show the variation of storage modulus (G'), loss modulus (G''), complex modulus and tanδ as a function of frequency and strain for systems containing 2% w/v alginate-methacrylate concentrations and different reaction times. The results show that both the storage and loss modulus are higher in samples obtained after high functionalisation times. In both cases the complex modulus increases with frequency, while the variation of G' and G'' with strain shows a shift point after which G'' becomes higher than G'. This shift occurs at high frequencies for alginate samples obtained with high functionalisation times.
Figure 3.10: 2% wt. Alginate solution reacted for 8 hours. a) Storage and loss modulus vs strain b) Storage and Loss modulus vs frequency; c) Complex modulus vs frequency; d) tan δ vs frequency.

Figure 3.11: 2% wt. Alginate solution reacted for 24 hours. a) Storage and loss modulus vs strain; b) Storage and Loss modulus vs frequency; c) Complex modulus vs frequency; d) tan δ vs frequency.
3.3.3 Rheological changes during the photopolymerization process

Based on the characterisation of the pre-polymerised samples only systems containing 2% w/v of alginate were considered for photopolymerization studies. The curing kinetics was assessed by monitoring the variation of $G'$ and $G''$ at room temperature through a controlled frequency of 1Hz. Photo-rheology was used to characterise the curing process (photopolymerization) of functionalised alginate polymers obtained after 8 and 24 hours of reaction time, mixed with 0.5% w/v, 1% w/v and 1.5% w/v of VA-086 photoinitiator.

As observed from Figures 3.12 and 3.13 by increasing the curing time $G'$ increases becoming significantly higher than $G''$ showing the liquid to solid phase change of the material. It is also possible to observe that by increasing the photoinitiator concentration the gelation time, which corresponds to the time point where the $G'$ curve crosses the $G''$ curve, decreases. It is also possible to observe that by increasing the amount of photoinitiator $G'$ seems to tend to a plateau which corresponds to a vitrification stage of the curing process (333, 334). The possible explanation for this observation is that, for the layer thickness considered in this study (~100 µm), the photoinitiator concentration is approaching a critical value. By increasing the photoinitiator concentration above the critical values, the polymerization occurs very fast at the polymer surface, reducing the light penetration and, consequently, the overall polymerization reduces.

![Figure 3.12: 2% wt. methacrylate alginate with different concentration of VA-086 photoinitiators functionalized for 8 hours](image-url)
Figure 3.13: 2% wt. methacrylate alginate with different concentration of VA-086 photoinitiators functionalized for 8 hours a) 0.5% w/v of PI; b) 1% w/v of PI; c) 1.5% w/v of PI.

3.3.4 Viscoelastic properties of formed hydrogels

Hydrogel disks (4 mm of height and 8 mm of diameter) were produced using an acrylic machined mould. Samples of 2% w/v of alginate-methacrylate obtained after both 8 and 24 hours of reaction time containing different concentrations of photoinitiator (0.5, 1 and 1.5% w/v) were polymerised during 8 minutes under a light intensity of 8 mW/cm². Produced disks were then assessed and both $G'$ and $G''$ measured at room temperature through a controlled frequency of 1Hz as presented in Figures 3.14 and 3.15. In the case of alginate-methacrylate samples obtained after 8 hours of reaction time it’s possible to observe that there is no significant change of both $G'$ and $G''$ (the storage modulus is always higher than the elastic modulus) with the increase in the photoinitiator concentration. In the case of alginate-methacrylate samples obtained after 24 hours of reaction time, results show that by increasing the photoinitiator concentration $G'$ and $G''$ increases. In this case, it is also possible to observe that the difference between $G'$ and $G''$ increases with the increase of photoinitiator concentration, which is associated to the high crosslinked density.

The viscoelastic nature of the crosslinked disks is also observed from the $G'$ and $G''$ versus strain graphs. In this case, it is possible to observe for all samples that, till a critical strain value, the storage modulus is higher than the loss modulus. After the strain critical value, the loss modulus become more significant due to the break of the crosslinked network. In the case of alginate-methacrylate samples obtained after 24 hours of reaction time, the storage modulus is always higher than the storage modulus of alginate-methacrylate samples obtained after 8 hours of reaction time. This is due to the high-crosslinking density of the alginate-methacrylate samples obtained after 24 hours of reaction time.
Figure 3.14: 2% wt methacrylate alginate hydrogel with different concentration of VA-086 functionalized for 8 hours a) 0.5% w/v of PI; b) 1% w/v of PI; C) 1.5% w/v of PI.
Figure 3.15: 2% wt methacrylate alginate with different concentration of VA-086 functionalized for 24 hours. a) 0.5% w/v of PI; b) 1% w/v of PI; C) 1.5% w/v of PI.

3.3.5 Internal morphology of alginate hydrogels

The internal structure of crosslinked alginate is presented in Figure 3.16. SEM images were obtained for samples containing 2% w/v of alginate prepared during 8 and 24 hours of reaction time and 1% wt of photoinitiator. Results show that the reaction time influences the hydrogel morphology, with high reaction times being associated to structures presenting both small pore size and number of pores. In addition, results seem to indicate that long reaction times generate structures with more closed pores. Results also show associated porosity of 94.5% ± 0.75 (292.5 µm ± 10 pore size) and 90.8% ± 0.21(252.5 µm ± 20 pore size) for 8 and 24 h respectively.
Figure 3.16: SEM images of crosslinked methacrylate-alginate hydrogel structures obtained from alginate-methacrylate at different reaction times: 8 and 24 hours.

3.3.6 Mechanical characterisation

The mechanical performance of crosslinked alginate structures is presented in Figures 3.17 and 3.18. As observed, high compression moduli were obtained for crosslinked disks produced with high functionalization times (13.16 kPa for 24 hours of reaction time and 2.63 kPa for 8 hours of reaction time) and 0.5% w/v photoinitiator concentration. These results can be explained by the high crosslinking density that characterizes the structures obtained from alginate samples functionalized during long reaction times and the corresponding internal morphology characterized by small size and low number of pores. It also possible to observe that the mechanical properties increase by increasing the photoinitiator concentration. For samples containing 1.5% w/v of photoinitiator, compression moduli were obtained (75.4 kPa for 24 hours of reaction time and 7.23 kPa for 8 hours of reaction time).

Figure 3.17: Compression tests for alginate methacrylate samples (8h) with different photoinitiator concentrations a) 0.5% w/v; b) 1% w/v; and c) 1.5% w/v.
Figure 3.18: Compression tests for alginate methacrylate samples (24h) with different photoinitiator concentrations a) 0.5% w/v; b) 1% w/v; and c) 1.5% w/v.

3.3.7 Swelling and degradation kinetics

The swelling and degradation behaviour of crosslinked alginate hydrogel disks are presented in Figure 3.19. Results show that samples absorb both DMEM and distilled water until reaching a state of equilibrium. This state is accomplished when the osmotic pressure from the swelling and the elasticity of the hydrogel network is equal. It is also possible to observe that crosslinked samples prepared with alginate-methacrylate obtained after 24 hours of functionalization present low swelling ratio, which shows that the degree of crosslinking controls the swelling properties of the hydrogel. In all samples, the equilibrium status was reached at day 3. Crosslinked alginate structures based on functionalized alginate prepared during 24 hour of reaction time swelled up to 155%, while alginate structures based on functionalized alginate prepared during 8 hour of reaction time swelled up to 190%. Moreover, it is also possible to observe that the level of functionalization of the pre-polymerised alginate not only determines the internal morphology of the crosslinked structures but also the degradation process as shown in Figures 3.19b and d.
Figure 3.19: Swelling and degradation rate for 2% wt. functionalized alginate (24 and 8) hours reaction time in a) distilled water b); degradation rate in distilled water; c) DMEM; d) degradation rate in DMEM.

3.4 Conclusion

This Chapter describes the synthesis and characterisation of alginate systems for UV-based bioprinting applications. The alginate was successfully functionalized in the presence of methacrylate in order to introduce the necessary number of unsaturation allowing its crosslinking upon photopolymerization. Two different functionalization reaction times were considered and photocurable systems containing different photoinitiator concentrations prepared. From the results it is possible to conclude that high functionalization reaction times originates crosslinked structures with less porosity, smaller pores and a larger number of closed pores, less swelling, higher degradation properties and higher mechanical stiffness. By increasing photoinitiator concentration it was possible to observe an increase of mechanical properties and gelation time. Moreover, for high values of photoinitiator concentration the reaction tends to reach verification.
Chapter 4

Photocurable alginate bioink development for cartilage replacement bioprinting†

Bioink design and assessment for tissue engineering replacement is a key topic of research. This Chapter investigates suitable photocurable alginate bioink macromers for bioprinting of 3D constructs for cartilage replacements. Alginate chemically modified with methacrylate anhydride groups was assessed through different techniques. 2% Alginate methacrylate (AlgMA) solutions containing different contents of methacrylate at different reaction times were assessed. The methacrylate functionalization and the degree of modification through various reaction times were assessed through nuclear magnetic resonance (NMR), showing the ability to tune the unsaturation degree by changing the reaction conditions. AlgMA synthesized using different reaction times were also characterized by rheological analysis to investigate the effect of reaction time on the rheological behavior. Results show that all Alginate methacrylate samples exhibit a shear thinning behavior and the initial viscosity is affected by the reaction time and methacrylate concentrations. These results indicate that both the reaction conditions and methacrylate concentrations are of paramount importance to control the extent methacrylation and rheological properties, which is critical to design bioinks with appropriate properties of extrusion printing. Biocompatibility and ecotoxicity were assessed through chondrocyte encapsulation.

4.1 Introduction

Articular cartilage is a non-vascularized tissue, covering the distal ends of the bones, formed by a low density of cells called chondrocytes that maintain the structural and functional integrity of the extracellular matrix (335-337). Cartilage is characterized by a distinct zonal structure comprising, in descending order, the articular or superficial surface, the middle (or transitional) zone, the deep zone, and the tidemark that separates the non-calcified and calcified regions (181). However, it lacks inherent self-repair capacity and hence the zonal structure and function are often irreversibly lost following trauma and disease (338). As a result, cartilage defects are prone to develop into osteoarthritis (OA), which is the predominant cartilage disease, characterized by loss of cartilage, pain and debilitation (339). Despite the current therapeutic strategies such microfracture, autologous chondrocyte implantation (ACI), matrix-assisted autologous chondrocyte transplantation/implantation (MACT/MACI), autologous matrix-induced chondrogenesis (AMIC), and osteochondral auto- and allografts (340, 341), articular cartilage repair is still a major clinical challenge. Tissue engineering strategy emerged as a potential therapy through the fabrication of constructs
mimicking the ECM structure of articular cartilage (342). Through this approach different biocompatible and biodegradable materials with or without cells were explored.

This Chapter focus on the use of photocurable alginate as a carrier of chondrocyte cells for cartilage repair. Alginate material considered in this Chapter was previously characterized in Chapter III and the rheological properties were further assessed in order to guarantee good printability. Two different methacrylate concentration (15 and 25 mL for each gram of alginate) and two different reaction times (8 and 24 hrs) were considered for each methacrylate concentration. Finally, cell-laden constructs were produced, and cytotoxicity and cell proliferation tests performed.

4.2 Materials and methods

4.2.1 Synthesis of methacrylated alginate

Methacrylate alginate was prepared following a published protocol (343). In this study, 2% wt of sodium alginate powder (W201502) was completely dissolved in Dulbecco's Phosphate-Buffered Saline (DPBS) (Sigma-Aldrich, UK). Afterwards, methacrylate anhydride (Sigma-Aldrich, UK) was added to alginate solution. The pH of the solution was kept around 7-8.0 during the reaction time by adding 5M of NaOH. After the chemical modification, the polymer solution was precipitated with 100% ethanol, dried in an oven overnight at 40-50 C°, then diluted with distilled water and purified through dialysis for 6 days (SnakeSkin™ Dialysis Tubing, 3.5K MWCO-Thermo fisher, UK). The solution was frozen at -80°C and the polymer recovered by lyophilization. Two different methacrylate concentration (15 and 25 mL for each gram of alginate) were used and two different reaction times (8 and 24 hrs) were considered for each methacrylate concentration. Crosslinked discs were produced by dissolving the functionalized alginate (AlgMA) polymer in a 1% w/v photoinitiator solution (PI) VA-086, 2,2'-Azobis[2-methyl-N-(2-hydroxyethyl) propionamide azo initiator (Wako Pure Chemical Industries, USA). The photocurable material was then pipetting into a custom-made cylindrical Teflon mould (diameter: 8 mm; height 4 mm). Photopolymerization was conducted using a 365 nm UV light (Dymax 2000-EC, Dymax, Germany) irradiating at 8 mW/cm² for 8 min.

4.2.2 HNMR

\(^1\)HNMR was used to characterize the chemical modification of alginate. Methacrylate alginate solution was dissolved in deuterium oxide D₂O and placed in an NMR tube.
The $^1$HNMR spectra were recorded using the B400 Bruker Avance III 400 MHz (Billerica, Massachusetts, USA) and the spectra acquired with 128 scans.

### 4.2.3 Rheological characterization

The rheological tests were carried out using the DHR2 TA Instrument (USA). Rotational tests were considered, and all measurements were conducted at room temperature.

### 4.2.4 Cell viability and proliferation

The cytotoxicity of the hydrogels was investigated through the encapsulation of human chondrocyte cells (Cell Applications, USA) and a live/dead assessment (Thermofisher Scientific, UK). Chondrocyte passage 4 were encapsulated at a density of $0.75 \times 10^6$ cell/mL on sterilized hydrogel precursor solution. 160 µL of gel-cell solution was pipetted into a 24-well custom mould and a UV radiation (365 nm of wavelength emitted by a Dymax 2000-EC Lamp (Dymax 2000-EC, Dymax, Germany) during 8 min with a light intensity of 8 mW/cm². After 3 and 7 days the cytotoxicity was analyzed by removing cell culture media, washing gently in DPBS adding a live dead stain solution, and incubating for 30 min. The hydrogels were then observed using a confocal fluorescence microscope (TCS-SP5, Leica, Germany). The Alamar blue assay was also used to evaluate cell metabolism and proliferation after 1, 3, 5 and 7 days of culture.

### 4.3 Result and discussion

#### 4.3.1 Chemical modification through $^1$HNMR

$^1$HNMR results of non-functionalized and functionalized alginate are show in Figure 4.1. In the case of functionalized alginate results show the appearance of characteristic peaks of methacrylate (MA) at 5.53 ppm and 6.10 ppm attributed to the methylene group in vinyl bond, and a peak at 1.82 ppm associated to the methyl group. These peaks are not presented in the nonmodified alginate (Figure 4.1a). The effect of reaction time and methacrylate anhydride concentration on the degree of modification (i.e., on the extent of methacrylation) determined by dividing the relative integrations of methylene to carbohydrate protons (344), is shown in Table 4.1. In the case of 15 ml MA, Results show that the degree of modification increases by increasing the reaction time, reaching a maximum value of 72.1% at 24 hours. However, by increasing the methacrylate anhydride concentration up to 25 ml MA, the degree of modification decreases, reaching 40.3% for 24h reaction time. We hypothesized that
the reduction on the degree of modification can be a result of the unreacted methacrylate, hydrolysis in aqueous conditions and elevated temperature (40°C) during the reaction as well as possible reactions between methacrylate groups during dialysis, as previously reported (234). These results suggest that the modification efficiency with methacrylic anhydride is significantly affected by the methacrylate concentration.

**Figure 4.1:** $^1$H NMR spectra of a) unmodified alginate and; b) 2% wt methacrylate alginate (15 mL MA) after 8 hrs of reaction; c) 2% wt methacrylated alginate (25 mL MA) after 8 hrs of reaction; d) 2% wt methacrylate alginate (15 mL MA) after 24 hrs of reaction.
Table 4.1: Effect of reaction time on the degree of modification.

<table>
<thead>
<tr>
<th>Composition (2 % w/v)</th>
<th>Reaction time (hours)</th>
<th>DoM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlgMA Low MA (15 ml)</td>
<td>8</td>
<td>55.9</td>
</tr>
<tr>
<td>AlgMA Low MA (15 ml)</td>
<td>24</td>
<td>72.1</td>
</tr>
<tr>
<td>AlgMA High MA (25 ml)</td>
<td>8</td>
<td>49.6</td>
</tr>
<tr>
<td>AlgMA High MA (25 ml)</td>
<td>24</td>
<td>40.35</td>
</tr>
</tbody>
</table>

4.3.2 Rheological characterization of alginate methacrylate

Figure 4.2 shows the variation of both the stress versus shear rate and the variation of viscosity versus shear rate considering different reaction rates and methacrylate concentration. Results show that all samples present a shear thinning behavior making them suitable for printing. It is also possible to observe that by increasing the reaction time the maximum stresses supported by the non-polymerised AlgMA increases. For longer reaction times and high concentration of methacrylate mechanical properties increases. Therefore, low methacrylate concentration (15 mL/g of alginate) will be considered.
Figure 4.2: Rheological characterization of 2 % wt methacrylate alginate samples. a) Stress vs shear rate profile for samples functionalized for 8 hrs reaction time with different methacrylate concentrations; b) Stress vs shear rate profile for samples functionalized for 24 hrs reaction time with different methacrylate concentrations; c) Viscoelastic behaviour low methacrylate concentration (15 mL); d) Viscoelastic behaviour high methacrylate concentration (25 mL).

4.3.3 Cell viability assessment

Cell viability assessment was examined using a live/dead assay (Figures 4.3 a, b, c and d). As observed for both the 3D cell laden constructs and 2D cell culture after 3 and 7 days of cell encapsulation, cell viability is higher than 85% with only few dead cells being observed. The results demonstrate that the functionalized hydrogel presents low cytotoxicity being a suitable bioink materials. The metabolic and proliferation of human chondrocyte cells was also assessed using the Alamar blue assay (Figure 4.3e). Results show that the encapsulated cells with AlgMA hydrogels present good proliferation rate at day 1 and 3, while after day 5 cell proliferation starts to decrease. This can be explained by the confined space available for cells due to the absence of any degradable sites. Moreover, these results show that photocrosslinkable AlgMA hydrogels are biocompatible and adequately support the encapsulated cells.
Figure 4.3: Live/dead results at a) day 3, in AlgMA hydrogels; b) day 3, in the control; c) day 7, in AlgMA hydrogels; d) day 7, in the control; and Alamar blue results e) Fluorescence intensity as a function of time for different time point (1, 3, 5 and 7 days). Note: in the live/dead results green corresponds to live cells and red to dead cells.

4.4 Conclusions

A bioink for cartilage applications is presented. Alginate was functionalized with methacrylate groups and the effect of reaction time and methacrylation concentrations on the degree of substitution and rheological properties were assessed through NMR and rheological analysis, respectively. NMR data clearly show the ability to tailor the methacrylation degree by changing the reaction time and methacrylate anhydride concentration, enabling to tailor the properties of the bioink. Successful cell laden constructs were produced, showing that the bioink is able to support cell proliferation after 7 days of photopolymerization. No cytotoxicity emerging from both the alginate and the photoinitiator was observed.
Chapter 5
Photocrosslinkable gelatin methacrylate (GelMA) hydrogels: Towards 3D cell culture and bioprinting for cartilage applications†

† This Chapter based on the paper: Hussein Mishbak, Glen Cooper, Paulo Bartolo, “Photocrosslinkable Gelatin Methacrylate (GelMA) Hydrogels: towards 3D cell culture and bioprinting for cartilage applications”, Journal of Applied Polymer Science, submitted.
This Chapter investigates the use of gelatin methacrylate (GelMA) as a potential photocurable bioink for cartilage applications. The methacrylate functionalisation process is discussed, and the level of functionalisation investigated. Photocurable constructs are produced using ultraviolet radiation (8 mW/cm²) and 8 min of irradiation time. The influence of the functionalisation time, polymer concentration, degree of modification and viscoelastic behaviour on the mechanical strength and cell viability is also discussed. Results show that the increase of polymer concentration, photoinitiator concentration and reaction time increase the mechanical properties. Moreover, compression results show acceptable values for cartilage applications (up to 76 kPa). Rheology shows that the GelMA presents shear thinning behaviour and proper crosslinking structure to support encapsulated cells during the printing process. High cell viability (85-90%) is also observed.

5.1 Introduction

Osteoarthritis (OA) is a critical clinical irreversibly cartilage defect developed after trauma or disease (175-178, 345, 346). Currently, OA is treated through a wide range of therapies including chondrocyte implantation (347), bone marrow stimulation (347), mosaicplasty (348), osteochondral implantation (349) and autologous and allogeneic tissue transplantation (350). However, these therapies present several limitations such as the formation of inferior fibrocartilage, donor site morbidity, limited prevention of continued tissue degeneration and in some cases two surgeries are required (351-353). Cell laden approaches have the potential to solve these limitations. In this case additive manufacturing technologies are used to create the constructs using bioinks made of suitable hydrogels and encapsulated cells. However, the design of these bioinks is complex requiring complex mechanical-rheological-degradation-and biological relationships to be addressed. Moreover, selected hydrogels must present a structure resembling the structure of the native tissue and good bio affinity to support cell encapsulation and sustain cell differentiation, proliferation, cell-cell networks and extra-cellular matrix (ECM) formation (354). Cell laden cartilage constructs can also be produced using both chondrocytes or mesenchymal stem cells (MSCs) (355). Several crosslinking methods have been explored to create cell laden constructs using different types of bioinks and successful results were obtained for different types of tissues such as skin, (356, 357), bone (358), cardiac (359), and liver (360). Among these methods, photoinitiated crosslinking is
particularly relevant as it is a relatively simple process, fast and can be performed under physiological conditions (361). For cartilage, different photocrosslinkable hydrogels were investigated (362, 363).
Among the different possible natural hydrogel materials, gelatin obtained from partial hydrolysis of collagen and presenting arginyl-glycyl-aspartic acid (RGD) cell adhesion motifs and matrix-metalloproteinase (MMP) degradation sites (356, 365), is an ideal candidate for cartilage bioinks.
This Chapter investigates the use of gelatin as a potential photocurable bioink for cartilage applications. The methacrylate functionalisation process is discussed, and the level of functionalisation investigated. Photocurable constructs are produced using ultraviolet radiation (8 mW/cm^2) and 8 min of irradiation time. The influence of the functionalisation time, polymer concentration, degree of modification and viscoelastic behaviour on the mechanical strength and cell viability is also discussed.

5.2 Experimental

5.2.1 Synthesis of methacrylated gelatin
Photocrosslinkable gelatin was prepared through methacrylate functionalization (Figure 5.1) following a protocol previously reported by Van den Bulcke et. al (366). Briefly, powder of gelatin bovine skin type B (G9382) (Sigma-Aldrich, UK) was dissolved at a concentration of 12.5% wt in Dulbecco's Phosphate Buffered Saline DPBS (Sigma-Aldrich, UK) under a constant temperature of 50 °C. Then, 10% v/v (10x molar excess) of methacrylate anhydride (MA) (Sigma-Aldrich, UK) was added under vigorous stirring. The pH of the gelatin solution was kept around 7.4-8.0 during the reaction period. Maintaining a higher pH during the reaction time is crucial to enhance the reaction between the amine and hydroxyl groups, which lead to high degree of modification. Different reaction times (2, 4 and 6 hrs) were considered. Afterwards, the solution was diluted with of DPBS at 40 °C, loaded at high permeability dialysis tubes (12-14 kDa) (Spectra/Por 2, US) and purified against distilled water for 7 days at 45 °C, changing water every day to remove the methacrylic acid and other impurities. Finally, the pH of the final product was adjusted to 7–7.4 and frozen at -80 °C and the polymer recovered by lyophilization for 72 hours. The final product (modified polymer) was frozen at -20 °C until further use.
Figure 5.1: Schematic crosslinking mechanism of gelatin and methacrylate.

5.2.2 Photocurable hydrogel solutions

After gelatin functionalisation, 10 and 15% wt of gelatin methacrylate (GelMA) concentrations were diluted with a photoinitiator solution (VA-086) (2,2'-Azobis[2-methyl-N-(2-hydroxyethyl) propionamide azo initiator (Wako Pure Chemical Industries, USA). Two different photoinitiator concentrations were considered (1 and 1.5% wt). Crosslinked discs were produced by pipetting the photocrosslinkable GelMA solution in an acrylic mould with a diameter of 8 mm, and 4 mm of height, then irradiated with a UV light source (365 nm wavelength) using a UV lamp model Dymax 2000-EC (Dymax Europe GmbH, Wiesbaden, Germany). Light intensity at the material surface was fixed at 8 mW/cm² and the material was irradiated for 8 minutes.

5.2.3 HNMR characterization

The chemical structure of functionalized gelatin was assessed using the B400 Bruker Avance III 400 MHz. 150 mg of GelMA was dissolved in 600 μL of deuterium oxide (D₂O) (Sigma-Aldrich, UK), and then transferred to NMR tubes (VWR, UK) and the spectra acquired with 128 scans.

5.2.4 Rheological characterization

The rheological assessment of non-polymerised gelatin solutions were carryout using the discovery hybrid rheometer system DHR2 (TA Instrument, USA). Samples were placed between the test pad and cone geometry (40 mm diamet) fixed at the rheometer head. Two different tests (rotational and oscillation) were considered and performed at room temperature Rotational tests were considered to evaluate viscosity and material strength. It is assumed that the material flows by applying a stress being the response measured alongside time. A controlled stress was applied, and the resulting movement measured at constant frequency (1 Hz). Oscillation tests (amplitude and frequency sweep tests) were considered to evaluate the viscoelastic behaviour of the material and the dynamic modulus. During the frequency sweep samples were exposed to small-deformation oscillations covering a range of frequencies (0.01-1000 Hz) to assess short and long-time scale responses to deformations. While, during an
amplitude sweep, the shear stress is varied, and the frequency is kept constant (1Hz). The elastic or storage modulus (G’) and the viscous or loss modulus (G’’), were measured at room temperature. The viscoelastic behaviour was characterized by measuring the energy stored (storage modulus, G’) in the material during shearing and the energy subsequently lost (loss modulus, G’’). Shear strain was controlled by varying the oscillation amplitude. For photocrosslinked hydrogels, the viscoelastic properties were examined. Crosslinked hydrogels discs were placed between two parallel plates and oscillatory tests were conducted using the DHR2 rheometer with a 20 mm diameter plate.

5.2.5 Scanning Electron Microscopy
The internal morphology of the hydrogel structure was observed using the Hitachi S3000N VPSEM scanning electron microscopy system (Hitachi, Japan). Two different hydrogel compositions were considered (10 and 15% wt of GelMA concentrations), and both were irradiated at 8 mW/cm² for 8 min. Gelatin methacrylate hydrogels specimens were frozen at -80 °C for 3 days following lyophilization. Finally, all samples were sputter-coated with gold by sputter-coating.

5.2.6 Mechanical characterization
Compression tests were conducted using the Instron 3344 machine (Instron, UK) equipped with a 10-N load cell. Crosslinked gelatin hydrogel discs were prepared as previously described and maintained in distilled water at 37 °C. After 24 hrs of incubation, swollen gelatin hydrogel discs were measured using callipers to determine both the diameter and thickness. Unconfined compression tests were performed on the hydrogel discs at room temperature and wet conditions, using a constant crosshead speed of 0.5 mm/min, at a rate of 10% strain. Compressive young modulus was determined from the slope of stress versus strain plots and limited to the first 10% of strain as recommended for cartilage applications (329).

5.2.7 Swelling and degradation characterization
Crosslinked gelatin methacrylate hydrogel discs were frozen at -80 °C, lyophilized and then the dry weights (Wᵢ) were measured. Then, dried hydrogel samples were immersed in Dulbecco’s Modified Eagle’s Medium - high glucose (DMEM) (Sigma-UK) diluted with 10% FBS (Fetal Bovine Serum) (Thermofisher, UK) at pH 7.4 and incubated at 37 °C to reach an equilibrium swelling state. The DMEM was replaced every 2 days. Over the course of 3 weeks, samples were removed from the DMEM
and the swollen hydrogel sample weights (W_s) measured. The swelling ratio (Q) was calculated as follows:

\[ Q = \frac{W_s}{W_i} \]  

(5.1)

After weighting the swollen hydrogels, the samples were freeze-dried and weighted again to measure the weight after lyophilization (W_d). The percentage of mass loss ratio was calculated according to the following equation:

\[ \left( \frac{W_i - W_d}{W_i} \right) \times 100 \]  

(5.2)

Measurements were performed in triplicate.

5.2.8 hADSCs Cell encapsulation

The gelatin methacrylate hydrogels (GelMA) were cytotoxicity assessed, considering both live dead and alamar blue assays. Human adipose-derived stem cells (hADSCs, passage 4) (Stempro, Invitrogen, Waltham, MA, USA) were encapsulated with hydrogel solution at 150000 cell/mL. 200 µL of the gel/cell (cell suspension) solution was pipetted into custom-made cylindrical teflon moulds (diameter: 10 mm; height 4 mm), then exposed to 365 nm UV light, irradiating at 8 mW/cm² for 8 min to cast the encapsulated cell-hydrogel discs. Cell laden constructs were transferred into 24-well plate and cell culture media (600 µl) was added until the hydrogel discs are fully covered. The number of encapsulated cells was 30000 cell/well as recommended by the well-plate manufacturer. The cytotoxicity was assessed at day 3, 7 and 19 by removing cell culture media, washing gently in DPBS, and then live/dead stains (Thermofisher Scientific, UK) were added. The Live/dead stain was prepared by adding 5µl of the supplied calcein AM and 15µl of 2mM Ethidium homodimer (EthD-1) stock solution to 10 mL of sterile, tissue culture-grade Dulbecco's phosphate-buffered saline (DPBS), vortexing to ensure thorough mixing. Afterwards, 600 µl of stain were added to each well to ensure that the hydrogel discs were fully covered. Samples were kept in the incubator for 30 min prior to imaging. An inverted fluorescence microscope (Leica DMI6000 B, Leica Microsystems, Wetzlar, Germany) was used to obtain images of the hydrogel constructs. Images were processed and analysed using ImageJ software. The Alamar blue assay was used to evaluate cell bioactivity after 1, 3, 5 and 7 days of culture. For each time point, samples were washed twice in DPBS and alamar blue solution 0.01% v/v (Sigma-Aldrich, UK) was added to each well (600 µl of cell culture medium) and incubated for 4 hrs under standard conditions (37 ºC, 5% of CO₂ and 95% of humidity). After incubation, 150
µL of each sample solution was transferred to a 96-well plate and the fluorescence intensity was measured at 540 nm excitation wavelength and 590 nm emission wavelength with a spectrophotometer (Sunrise, Tecan, Männedorf, Zurich, Switzerland). 2D cell culture was assessed as a control point. The experiments were performed in triplicate (n=3).

5.2.9 Data analysis

All data are represented as mean ± standard deviation. Cell proliferation results were subjected to one-way analysis of variance (one-way ANOVA) and post hoc Tukey’s test using GraphPad Prism software version 8.0. Significance levels were set at p < 0.05.

5.3 Results and discussion

5.3.1 Characterization of gelatin and GelMA by HNMR

Figure 5.2a shows the resonance characteristic peaks of non-functionalized gelatin. The following peaks were identified: methyl group of amino acids, valine, leucine and isoleucine (Peak 1 at 0.5-0.7 ppm), methylene residues of threonine (Peak 2 at 0.9-1.1 ppm), alanine (Peak 3 at 1.1-1.3 ppm), lysine (Peak 4 at 1.2-1.4 ppm, and Peak 7 at 2.6-2.7 ppm), arginine (Peak 5 at 1.6-1.8 ppm, and Peak 8 at 2.8-3.0 ppm), methylene resonances of aspartate (Peak 6 at 2.2-2.5 ppm), and proline (Peak 9 at 3.2-3.4 ppm). These peaks are also present in the functionalized gelatin (Figures 5.2b and c). New peaks at 1.6-2.19 ppm, 5.69 ppm and 5.93 ppm correspond to methylene protons (H₂C=C(CH₃)) and confirm the successful functionalization with methacrylic anhydride. After confirming the chemical functionalization of gelatin, the degree of functionalization (DoF) was determined following the procedure previously described by Billet et al (366), and the results are presented in Table 5.1. As observed the degree of functionalization increases with the increase of the functionalization reaction time. After 2 hours GelMA presents low levels of C=C bonds on the material backbone, which will originate less dense crosslinked polymer networks upon irradiation, while after 6 hours GelMA presents high levels of C=C bonds on the backbone, which will originate more dense crosslinked networks. No significant increase on the degree of functionalization was observed between 4 and 6 hours of reaction. Therefore, only samples obtained after 2 and 6 hrs of functionalization were considered for further analysis.
Table 5.1: Effect of reaction time on the degree of functionalization.

<table>
<thead>
<tr>
<th>Reaction time (hrs)</th>
<th>Degree of functionalization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>52</td>
</tr>
<tr>
<td>6</td>
<td>54</td>
</tr>
</tbody>
</table>

Figure 5.2: $^1$H NMR spectra for non-functionalized and functionalized gelatin. (a) Non-functionalized gelatin (b) GelMA functionalized for 2 hrs; (c) GelMA functionalized for 4 hrs; (d) GelMA functionalized for 6 hrs.
5.4 Rheological characterization

5.4.1 Prepolymer rheology

The stress versus shear rate and the variation of viscosity with shear rate for prepolymerised GelMA samples are shown in Figure 5.3. As observed, the materials exhibit a shear-thinning behaviour. Results also show that the viscosity of the polymer solution is dependent of the shear rate, decreasing by increasing the shear rate. This is an important characteristic for 3D bioprinting applications as less pressure is required during the printing process. It’s also possible to observe that the polymer concentration has an impact on the maximum stress for a specific shear rate with high values being obtained for long functionalization reaction times. Results show that for both GelMA compositions viscosity decreases by increasing the functionalization time. For the same functionalization time and different material compositions results show no significant differences of viscosity for low shear rates, but for high shear rates, the viscosity is significantly low in the case of high concentration of gelatin methacrylate.

Figure 5.3: Rheological behaviour of 10 and 15% wt of GelMA samples considering 2 and 6 hrs of functionalization time. (a) 10% wt of GelMA functionalized for 2 hrs; (b) 10% wt of GelMA functionalized for 6 hrs; (c) 15% wt of GelMA functionalized for 2 hrs; (d) 15% wt of GelMA functionalized for 6 hrs.
Since the polymers exhibit a shear-thinning behaviour, a power law model can be used to fit the experimental data:

\[ \tau = \eta \dot{\gamma}^n \]  

(5.3)

where \( \tau \) is the shear stress (Pa), \( \dot{\gamma} \) is the shear rate (s\(^{-1}\)), \( \eta \) the consistency index or equivalent viscosity (Pa. s) and \( n \) is the power law index (dimensionless).

Table 5.2 shows the values of the power law coefficients for the different materials. A good approximation (\( R^2 \) around 0.97) was obtained between the power law and the experimental data.

**Table 5.2:** Power law coefficients for different reaction times and GelMA concentration.

<table>
<thead>
<tr>
<th>Polymer concentration - Reaction time (h)</th>
<th>n value</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% wt - 2h</td>
<td>0.4978</td>
<td>0.9652</td>
</tr>
<tr>
<td>10% wt - 6h</td>
<td>0.5613</td>
<td>0.9834</td>
</tr>
<tr>
<td>15% wt - 2h</td>
<td>0.825</td>
<td>0.999</td>
</tr>
<tr>
<td>15% wt - 6h</td>
<td>0.5519</td>
<td>0.9868</td>
</tr>
</tbody>
</table>

Viscoelastic changes for pre-polymerised solutions were assessed using oscillation tests (Figure 5.4).

For both concentrations, results show that both the storage and loss modulus are high when the reaction time is long (6 hrs).

Results for GelMA samples functionalized during 2 hrs show that at low frequencies the storage modulus is higher than the loss modulus. While for high frequencies, the loss modulus increases and become higher than the storage modulus. For GelMA samples functionalised during 6 hrs it is possible to observe that the loss modulus is higher than the storage modulus for all frequencies.
Figure 5.4: Oscillatory tests: storage and loss modulus of 10% wt GelMA for 2 and 6 hrs; (a) Amplitude sweep for 10 and 15% wt GelMA functionalized for 2hrs; (b) Frequency sweep for 10 and 15% wt GelMA functionalized for 2hrs; (c) Amplitude sweep for 10 and 15% wt GelMA functionalized for 6hrs (d) Frequency sweep for 10 and 15% wt GelMA functionalized for 6hrs.

Figures 5.5 and 5.6 show the variation of $G'$ and $G''$ as a function of strain rate and frequencies of crosslinked hydrogels. Amplitude sweep results show that for the two different functionalisation times, $G'$ is higher in the case of samples obtained from solutions containing higher concentration of GelMA. For the same GelMA concentration, $G'$ also increases by increasing the functionalization time. The increase of $G'$ is associated with the increase in the crosslinking density. In all cases it is possible to observe a critical strain rate value. Below this threshold strain, $G'$ is higher than $G''$, while above the threshold $G''$ becomes higher than $G'$. In the case of frequency sweep, results show that the $G'$ is always higher than $G''$ showing a dominant solid like behaviour.
Figure 5.5: Oscillatory results for GelMA hydrogel samples functionalized for 2 hrs (a) Storage and loss modulus as a function of strain for 10 and 15% wt GelMA and 1% wt of PI; (b) Storage and loss modulus as a function of frequency for 10 and 15% wt GelMA and 1% wt of PI; c) Storage and loss modulus as a function of strain for 10 and 15% wt GelMA and 1.5% wt of PI; d) Storage and loss modulus as a function of frequency for 10 and 15% wt GelMA and 1.5% wt of PI.
Figure 5.6: Oscillatory results for GelMA hydrogel samples functionalized for 6 hrs
(a) Storage and loss modulus as a function of strain for 10 and 15% wt GelMA and 1% wt of PI; (b) Storage and loss modulus as a function of frequency for 10 and 15% wt GelMA and 1% wt of PI; (c) Storage and loss modulus as a function of strain for 10 and 15% wt GelMA and 1.5% wt of PI; (d) Storage and loss modulus as a function of frequency for 10 and 15% wt GelMA and 1.5% wt of PI.

5.4.2 Morphological characterization
The internal pore size is crucial as it determines the internal free space to encapsulate cells and to support cell growth and proliferation. In the case of cartilage bioinks for 3D bioprinting the references are chondrocytes cells and human adipose-derived stem cells presenting around 20 µm of diameter (367, 368). Figure 5.7 shows the internal morphology of crosslinked GelMA hydrogels. Results show that crosslinked structures obtained from solutions containing 10% wt of GelMA (2 and 6 hrs of reaction time) and 1% wt of photoinitiator present pore sizes with 100 to 150 µm of diameter (Figures 5.7 a and b). The increase of photoinitiator concentration reduces both pore size and number of pores Figures 5.7c and d as it contributes to high crosslinking density. Polymer solutions containing 15% wt of GelMA result in high density crosslinked structures with small pores (23 to 25 µm) (Figures 5.7e and f) or almost no pores (Figures 5.7g and h). As observed the most suitable system for cell
encapsulation seems to be the one based on solutions containing 10% of GelMA and 1% wt of photoinitiator as it allows more free space to accommodate cell encapsulation and proliferation. Therefore, this is the composition further considered.

**Figure 5.7:** Cross-section images of GelMA crosslinked structures considering different GelMA concentrations, reaction times and PI concentration; (a) 10% of GelMA, 2 hrs of functionalization time and 1% wt of PI; (b) 10% of GelMA, 6 hrs of functionalization time and 1% wt of PI; (c) 10% of GelMA, 2 hrs of reaction time and 1.5% wt of PI; (d) 10% GelMA, 6 hrs of reaction time and 1.5% wt of PI; (e) 15% of GelMA, 2 hrs of reaction time and 1% wt of PI; (f) 15% of GelMA, 6 hrs of reaction time and 1% wt of PI; (g) 15% of GelMA, 2 hrs of reaction time and 1.5% wt of PI; (h) 15% of GelMA, 6 hrs of reaction time and 1.5% wt of PI.
5.4.3 Mechanical characterization

Compressive modulus of crosslinked GelMA discs were determined one day after equilibrium swelling and the results are presented in Figure 5.8. As observed, high compression modulus is obtained with high functionalisation times and high photoinitiators concentrations. For the same functionalisation time and photoinitiator concentration, compressive modulus increases by increasing the gelatin concentration. These results can be explained by the high crosslinking density that characterises the structures obtained from solutions containing high GelMA concentration, functionalised during long reaction times and containing high concentration of photoinitiators and the corresponding internal structure characterised by small size and low numbers of pores.

![Graph](image)

**Figure 5.8:** Unconfined compression tests for gelatin methacrylate hydrogels samples with different photoinitiator concentrations a) 10% wt GelMA functionalized for 2h containing 1% wt of PI; b) 10% wt GelMA functionalized for 2h containing 1.5% wt of PI; c) 10% wt GelMA functionalized for 6h containing 1% wt of PI; d) 10% wt GelMA functionalized for 6h containing 1.5% wt of PI.

5.4.4 Swelling and degradation characterization

The swelling and degradation behaviour of crosslinked GelMA disks are presented in Figure 5.9. Results show that all samples absorb DMEM until reaching a state of equilibrium. This state is accomplished when the osmotic pressure from the swelling
and the elasticity of the hydrogel network is equal. In all samples, the equilibrium status was reached at day three. It is also possible to observe that crosslinked samples prepared with GelMA obtained after 6 hrs of functionalization present low swelling ratio, which shows that the degree of crosslinking controls the swelling properties of the hydrogel. Crosslinked GelMA structures based on GelMA prepared during 6 hours of reaction time swelled up to 14%, while GelMA structures based on functionalized gelatin prepared during 2 hour of reaction time swelled up to 20% (Figure 5.9a). Moreover, it is also possible to observe that the level of functionalization of the prepolymerised gelatin not only determines the internal morphology of the crosslinked structures but also the degradation process (Figure 5.9b). Results show that samples produced using GelMA obtained considering high reaction times and solutions containing high polymer concentration (15% wt) show lower degradation rates (20% of mass loss for crosslinked GelMA obtained from gelatin functionalised during 6 hrs and solutions containing 15% wt of GelMA, and 35% of mass loss for crosslinked GelMA obtained from gelatine functionalized during 2 hrs and solutions containing 10% wt of gelatin).

![Figure 5.9](image)

**Figure 5.9:** Swelling and degradation profiles as a function of functionalization time for different functionalized gelatin methacrylate systems; a) Swelling in DMEM ; b) degradation rate in DMEM.

### 5.4.5 Cell viability and proliferation

The live/dead assay was used to investigate the potential toxicity of both GelMA hydrogels, photoinitiator concentration and the effect of irradiation conditions on the encapsulated cells. Results after 3, 7 and 19 days of cell-encapsulation are presented in Figure 5.10. Results show that most of the hADSCs are alive (green) with few dead cells (red) at day 3, indicating good biocompatibility for the produced materials. At day 7, cells show an elongated cell morphology and a cell-cell networking can be observed at day 19, which is more intensive in samples prepared with GelMA obtained
at high functionalization times. This might be attributed to the higher functionalization degree of GelMA obtained after 6 hrs of functionalization.

Cell proliferation was assessed using the alamar blue assay and the results are presented in Figure 5.11, confirming the bioactivity of encapsulated hADSCs. At day 1, the 6 hrs GelMA hydrogel shows higher fluorescence intensity in comparison to the 2 hrs hydrogel group. However, this difference reduces after day 1. At day 5, both GelMA groups shares similar fluorescence intensity and 2hrs GelMA hydrogel group shows higher intensity at day 7. This might be attributed to the faster degradation of 2hrs hydrogel group which releases more cells attaching to the cell culture plate. These results, which are aligned with the results presented in Figure 5.10c, shows that 6 hrs hydrogel presents better cell affinity for a long cell culture time.

![Figure 5.10: Live/dead images of adipose-derived mesenchymal stem cells encapsulated in samples based on 10% wt GelMA dissolved at 1% wt of PI solution and exposed to UV light (Light intensity of 8 mW/cm²) for 8 min a) After 3 days of encapsulation; b) After 7 days of encapsulation; c) After 19 days of encapsulation. Note: Green corresponds to live cells and red to dead cells.](image-url)
Figure 5.11: Alamar blue fluorescence intensity at different time points (1, 3, 5 and 7 days) considering samples functionalized at two different reaction times (2 and 6 hrs)

5.5 Conclusion

Photocrosslinkable hydrogels have shown great potential for cartilage repair, due to their unique properties. GelMA systems were fully characterised in terms mechanical, photopolymerization process, degradation, swelling and biological properties. Results show the ability to tune these characteristics by controlling the functionalization process. Moreover, the biological affinity between gelatin and cells were confirmed being possible to observe that both material systems and processing conditions do not induce any negative effect on cell survival. High cell attachment and proliferation was observed. Results demonstrates the viability of using GelMA as a bioink for cartilage applications.
Chapter 6
Development and characterisation of a photocurable alginate-gelatin for cartilage applications†

This Chapter focuses on the design of three different photocrosslinkable hydrogels based on alginate and gelatin for cartilage applications. The synthesis, morphological, rheological, mechanical, and biological characterisation of AlgMA, GelMA, and hybrid alginate-gelatin Alg-Gel(MA) methacrylate hydrogels are detailed. Results show that the hybrid hydrogels display superior mechanical properties than the single component hydrogels and present a more stable degradation behaviour. The hybrid hydrogels also demonstrate high cell viability, improved cell adhesion, and increased production of cartilage tissue markers such as collagen and glycosaminoglycan GAGs. Both AlgMA and GelMA materials considered in this Chapter were functionalized as described in Chapters III and V.

6.1 Introduction

Articular cartilage is a tissue which covers the ends of bones at joints and provides a smooth and lubricated surface for articulation during movement and mechanical load transmission (17, 369). The tissue has low metabolic activity and lacks a vasculature; thus, disease or trauma can severely impact tissue functionality (369). Articular cartilage heals poorly, typically forming fibrocartilage, and current therapies have limited clinical efficacy in halting the degradation of the tissue which ultimately can result in the requirement of a total joint replacement implant (370, 371). This has motivated the development of tissue engineering strategies utilizing biomaterials and scaffolds to promote cartilage regeneration (85, 110, 372, 373). A promising class of biomaterials are hydrogels, three-dimensional (3D) hydrated polymer networks, which have properties that resemble the extracellular matrix (ECM) of biological tissues (120, 374-377). Hydrogels have been investigated for tissue engineering applications due to their biocompatibility, oxygen and nutrient permeability, and the ability to tailor their properties (372, 378-381). Biodegradable and biocompatible synthetic and natural based hydrogels have been explored to promote the repair and regeneration of different tissues such as skin, nerve and cartilage (382-384).

Synthetic hydrogels have been widely investigated as a potential suitable biomaterial for articular cartilage tissue engineering applications due to their ability to be engineered to mimic the ECM and tailored to have similar properties in terms of mechanical, morphological and biological properties (385-388). However, synthetic hydrogels have limitations regarding their biocompatibility and biodegradability due to the lack of inherent adhesion and degradation sites which requires the incorporation
of motifs such as the tripeptide Arg-Gly-Asp (RGD) sequences and matrix metalloproteinases (389).

Alternatively, natural based hydrogels are attractive biomaterials due to their biocompatibility and biodegradability and have been explored for cartilage tissue engineering applications (390-393). Particular relevant are alginate and gelatin. Alginate has been investigated due to its low cost, biocompatibility, hydrophilic structure, stiff polymer backbone, ease of modification and processing (394, 395). Alginate hydrogel based scaffolds can be produced via ionic-crosslinking, phase transition, free radical polymerisation, and click conjugation (396). Free radical photopolymerization has gained tremendous attention due to the ease of use and rapid gelation kinetics. Furthermore, photopolymerisation enables polymer solutions (pre-polymerised monomers) containing cells or biomolecules to be printed using 3D bioprinting technologies for the development of scaffolds or using in vivo minimally invasive delivery technique (e.g. injection) with subsequently crosslinking through light exposure (113, 383, 397). However, alginate has significant limitations which limits its applicability as a single material for tissue engineering applications such as the lack of cell binding motifs and slow gelation speed which affects gel uniformity and strength (396). Alternatively, gelatin, obtained from the hydrolysis of collagen, has been investigated due to its biocompatibility, biodegradability, promotion of cell adhesion and proliferation (398, 399). The main advantages of gelatin are its biocompatibility since it has high cell affinity and cell adhesion sites are abundant (RGD peptides) and its ability to be degraded by native enzymatic processes (400, 401). However, gelatin has limitations such as poor mechanical properties (brittle) and short biodegradation profile (401). Due to their limitations both alginate and gelatine are not totally suitable for tissue engineering applications and for cartilage applications in particular. However, the combination of both materials has the potential to overcome their individual limitations.

This Chapter investigates the potential of using a hybrid AlgMA and GelMA photocrosslinking hydrogel system for cartilage applications. The synthesis, morphological, rheological, mechanical, and biological characterisation of AlgMA, GelMA, and hybrid alginate-gelatin Alg-Gel(MA) methacrylate hydrogels are detailed.
6.2 Materials and methods

6.2.1 Photocrosslinkable polymer preparation
Photocrosslinkable polymers were synthesised by reacting alginate and gelatin with methacrylic anhydride to introduce methacrylate moieties into the polymer backbone (326, 402, 403).

6.2.2 Alginate methacrylate preparation (AlgMA)
Alginate methacrylate AlgMA was prepared by dissolving sodium alginate (Sigma-Aldrich, UK) at a concentration of 2% wt. in an aqueous solution containing Dulbecco's phosphate buffered saline (DPBS) and dimethyl sulfoxide (DMSO), (Sigma Aldrich, UK) at a ratio of 85:15. The dissolved solution was then mixed with 20% v/v methacrylic anhydride (MA) (Sigma Aldrich, UK) for 24 h, at room temperature (RT), as previously described (404). The pH of the solution was maintained between 7.4-8.0 by adding 5M NaOH (Sigma Aldrich, UK). After chemical modification, the polymer solution was precipitated with 100% ethanol (absolute ethanol ≥99.8%, AnalaR NORMAPUR®, WVR, UK), dried in an oven overnight at 40-50°C, and purified through dialysis tubes (3.5 kDa) (Spectra/Por 2, Fisher Scientific, USA) for 5 days. The solution was frozen at -80°C for 2 days and the polymer recovered by lyophilisation for 3 days.

Similarly, GelMA was prepared by dissolving gelatin bovine skin type B (Sigma Aldrich, UK) at a concentration of 12.5% wt. in DPBS at a temperature of 45°C. After complete dissolution of gelatin a 10% v/v MA solution was added under vigorous stirring. The pH was kept at 8 during the reaction time of 6 h. The solution was purified through dialysis tubes (12-14 kDa) (Spectra/Por 2, Fisher Scientific, USA) for 5 days. Then, the solution was frozen at -80°C for 2 days and the polymer was recovered by lyophilisation for 3 days.

6.2.3 Hydrogel formation

6.2.3.1 Photoinitiator solution preparation
Photoinitiator solution was prepared by dissolving the photoinitiator powder, 2,2’-Azobis[2-methyl-N-(2-hydroxyethyl) propionamide azo initiator (VA-086) (Wako Pure Chemical Industries, USA) in DPBS (Sigma-Aldrich, UK) at 40°C for 30 min, to a final concentration of 1% wt. The PI concentration was previously investigated for both alginate and gelatin single systems and the results presented in chapters III and V.

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6.2.3.2 Single hydrogel systems

The AlgMA and GelMA hydrogels are produced by dissolving 2% wt. and 10% wt. respectively of the lyophilised polymer in the 1% wt. photoinitiator solution. Crosslinked hydrogel discs were produced by pipetting the AlgMA and GelMA solutions into custom-made cylindrical acrylic moulds (diameter: 8 mm, height: 4 mm) and irradiated with ultraviolet (UV) light (365 nm wavelength) using a UV lamp (Dymax 2000-EC, Dymax Europe GmbH, Germany). The light intensity at the material surface was fixed at 8 mW/cm² and samples were irradiated during 8 min. The exposure conditions were selected using 365 nm and between 5–20 mW/cm² for 2–20 min since these low UV light intensities have been reported to have a lower impact on cell viability, proliferation and gene expression (405) (Figure 6.1).

![Diagram of photocrosslinking mechanism](image)

**Figure 6.1**: Photocrosslinking mechanism to form a) AlgMA and b) GelMA hydrogels using UV irradiation.

6.2.3.3 Hybrid hydrogel system

The hybrid hydrogel systems were synthesised by mixing 2% wt. AlgMA and 10% wt. GelMA solutions at ratios of 75:25 AlgMA/GelMA, 50:50 AlgMA/GelMA, and 25:75 AlgMA/GelMA. These alginate-gelatin methacrylate hybrid hydrogels (Alg-Gel(MA)) were crosslinked as previously described (Figure 6.2).
Figure 6.2: Photocrosslinking of hybrid alginate-gelatin systems when exposed to UV irradiation.

6.2.4 Structural conformation of the hybrid hydrogels

Proton nuclear magnetic resonance (NMR) spectroscopy (Bruker 400, Bruker Corporation, USA) was used to assess the degree of methacrylate modification for all three systems (AlgMA, GelMA, and Alg-Gel(MA)). Samples were prepared by dissolving 150 mg of each polymer in 750 µl deuterium oxide (D₂O) (Sigma Aldrich, UK) and then transferred to NMR tubes (VWR, UK). The spectra were acquired at 400 MHz with 128 scans. The degree of methacrylation was calculated following the procedure previously described by Billet et al (234).

6.2.5 Rheological characterisation

Rheological characterisation was used to determine the general flow behaviour and viscoelastic characteristics of the functionalized pre- and polymerized hydrogels. Rotational and oscillatory rheology (Discovery Hybrid Rheometer, TA Instruments, USA) was considered. All samples (n=3) were measured at room temperature (RT). Rotational tests were performed by loading the polymer samples using a cone and plate geometry (60 mm diameter and 200 µm gap size), applying a torque to the top plate exerts a rotational shear stress on the material to flow and the resulting shear rate and movement are measured. The rotational speed depends on the viscosity computed by means of stress and shear rate. By means of the flow curve it is possible to measure the viscosity as a function of shear rate (0.01 – 1000 s⁻¹).

The oscillatory tests were performed to measure the viscoelastic behaviour and the stiffness of the specimens and dynamic moduli. Amplitude and frequency sweeps were considered. A range of frequencies (0.01-100 Hz) was used to assess short and long-time scale responses to deformations at a constant 20% strain, while the strain rate varies during the amplitude sweep test, and the frequency was constant at 1 Hz. The storage (G’) and the loss (G”) modulus were measured.

For photocrosslinked hydrogels, oscillatory tests were performed to evaluate the hydrogel stiffness by subjecting the hydrogel to a shear stress. The hydrogel samples
were carefully placed onto the surface of the plate (20 mm in diameter) lowered to a 500 µm gap distance, shear stress was applied, and then, $G'$ and $G''$ were measured as a function of strain (0.1-500%).

### 6.2.6 Structure morphology and porosity

The hydrogel’s internal morphology was investigated using scanning electron microscopy (SEM, S3000N, Hitachi, Japan). Cylindrical hydrogels samples (8 mm diameter, 4 mm height) were frozen at -80°C for 2 days then lyophilised to recover the dried hydrogel constructs. The samples were cut to obtain a cross-section and sputter coated with gold prior to imaging.

Porosity is an important property of the hydrogel’s structure due to its influence on the mechanical performance of the scaffold; fluid permeability; the supply of oxygen, nutrients and removal of waste products; and the free volume provided for cells to migrate and proliferate. The percentage of porosity of the hydrogels (n=3) was calculated using a gravimetric method. Dry samples (n=3) were obtained by lyophilisation and then the samples were placed in a desiccator under vacuum (VWR, UK) for three days and the volume was determined by using a calliper. The porosity was determined using the following equation:

$$\text{Porosity} \, (\%) = \left( 1 - \frac{P_{\text{hydrogel}}}{P_{\text{material}}} \right) \times 100$$  \hspace{1cm} (6.1)

where $P_{\text{hydrogel}}$ is the experimental density of the 3D hydrogel construct and $P_{\text{material}}$ is the theoretical density of the polymer. The theoretical density for the hybrid systems was calculated as follows:

$$P_{\text{material}} = X_{\text{AlgMA}} P_{\text{AlgMA}} + X_{\text{GelMA}} P_{\text{GelMA}}$$  \hspace{1cm} (6.2)

where $X_{\text{AlgMA}}$ and $X_{\text{GelMA}}$ indicate the volume fractions and the densities of alginate, and gelatin, $P_{\text{AlgMA}}$ and $P_{\text{GelMA}}$, are 1.64 g/cm$^3$ and 0.98 g/cm$^3$, respectively.

The experimental apparent hydrogel density, $P_{\text{hydrogel}}$, can be determined according to the following equation:

$$P_{\text{hydrogel}} = \frac{\text{mass}}{\text{volume}}$$  \hspace{1cm} (6.3)

where mass is the dry weight of the specimen and the volume is based on a measurement of the dried sample.

The average pore size of the hydrogels was semi-quantified using ImageJ software analysis of the SEM images through calculation of the Feret's diameter, which is an approximation method to calculate the pore size diameter (406).
6.2.7 Swelling and degradation kinetics of the hybrid hydrogels

The hydrogel discs were produced as previously mentioned in section (6.2.3) (n=3, at each timepoint), frozen at -80°C for 2 days, and then lyophilised for 3 days to recover dry hydrogel structures. After lyophilisation, the initial dry disc weights were measured (W_i). The dry hydrogel samples were immersed in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma Aldrich, UK) supplemented with 10% fetal bovine serum (FBS) (Thermofisher, UK) at pH 7, and incubated at 37°C to reach an equilibrium swelling state. The swelling weights (W_s) were measured over the course of 20 days and the DMEM was changed every two days.

The swelling ratio (Q) was calculated as follows:

\[ Q = \frac{W_s}{W_i} \]  

(6.4)

Where Wi is the initial dry weight and Ws is the swollen hydrogel sample weight.

For the degradation kinetics, the swollen hydrogels samples were frozen and lyophilised at each timepoint and the dry weight (W_d) measured. The mass loss percentage was calculated using the following equation:

\[ \text{Mass loss (\%)} = \left( \frac{W_i - W_d}{W_i} \right) \times 100 \]  

(6.5)

6.2.8 Mechanical assessment

The mechanical behaviour of the crosslinked hydrogels was characterised through unconfined compression testing using Instron 3344 single column system with a 10 N load cell (Instron 3344, Instron, UK). Hydrogel discs (n=3) were prepared as previously described using a custom-made cylindrical acrylic mould with a 1:1 dimension ratio (diameter 10 mm and height 10 mm) and incubated in DPBS at 37°C for 24 h. The hydrogel discs were measured after 24 h to determine the diameter and thickness, and unconfined compression tests were performed on the hydrogel discs at RT. The compressive strain rate was fixed at 0.5 mm/min, and samples were loaded up to a maximum strain of 20%. The compressive Young’s Modulus (E) was calculated from the slope of the initial linear region of the stress-strain curve (elastic deformation region) by calculating the tangent modulus from the curve fit equation at 20 % strain.

6.2.9 Biological assessment

The photocrosslinked hydrogels were biologically evaluated through the encapsulation of human chondrocyte cells (Cell Applications, USA) and assessed for
cell viability, proliferation, and ECM production. The AlgMA, GelMA, and Alg-Gel(MA) 50:50 photocrosslinked hydrogels were selected for evaluation.

6.2.9.1 Cell culture and encapsulation

Human chondrocyte cells were cultured until passage four in a complete human chondrocyte growth medium (Cell Applications, USA). Cells were harvested and encapsulated at density of 0.6 x 10^6 cells/mL in the hydrogel solution. 160 µL of the hydrogel/cell solution was pipetted into a custom-made cylindrical acrylic mould (diameter 8 mm and height 4 mm) and exposed to UV light (wavelength 356 nm), irradiated at 8 mW/cm² for 8 min to cause gelation of the encapsulated hydrogel/cell solution. Cell-laden hydrogels (n=3) were transferred into 24-well plates and cell culture media added.

6.2.9.2 Cell viability, proliferation, and metabolic activity

The cytotoxicity of the hydrogels was observed using a live/dead assay kit (Thermofisher Scientific, UK). Cell viability was observed on day 3, 7 and 16 by removing cell culture media, washing gently in DPBS, adding a 2 µM calcein-AM and 4 µM ethidium homodimer-1 solution, and incubating for 25 min. The hydrogels were then observed using a confocal fluorescence microscope occupied with fluorescence filter sets (A, I3 and N2.1) (TCS-SP5, Leica, Germany). Images were processed and analysed using ImageJ software (n=3) (407).

Cell proliferation and metabolic activity was evaluated by using the resazurin (Alamar blue) assay at day 1, 3, 5, 7 and 14 days of culture. At each time point, samples (n=3) were washed twice in DPBS and 75 µL of Alamar blue solution 0.01% (wt.) (Sigma-Aldrich, UK) was added to each well in 750 µl of cell culture medium and incubated for 4 h under standard conditions (37°C, 5% CO₂, and 95% humidity). After incubation, 150 µL of each sample solution was transferred to a 96-well plate and the fluorescence intensity was measured at 540 nm excitation wavelength and 590 nm emission wavelength with a plate reader (Infinite 200, Tecan, Männedorf, Zurich, Switzerland). 2D tissue culture plastic (TCP) was assessed as a positive control for both cell viability and proliferation.

6.2.10 Histological analysis

Histological analysis of the cell encapsulated hydrogels was investigated at day 14, 21, 28, and 35 to assess the amount of deposited ECM. Three histological stains were used: Gomori trichrome, aggrecan immunohistochemistry, and safranin O.
Gomori trichrome is used to quantify the total collagen secretion in the hydrogel as collagen is a main structural component of cartilage representing more than 60% of its dry weight (17). Gomori trichrome bind to the nuclei (dark purple) and collagen (green or blue). Aggrecan immunohistochemistry is used to quantify the proteoglycan, which represents more than 20% of the cartilage dry weight (408). Aggrecan, is specific proteoglycan in cartilage tissue, and its crucial in a cartilage functioning (17, 409). Safranin O used to identify the chondrocytes and the detection cartilage formation, and it stains cell nuclei (dark red).

At each time-point, the crosslinked hydrogel systems were fixed with 10% (v/v) formalin (Sigma Aldrich, UK) for 20 min at RT. After fixation the samples were processed in a Tissue-Tek Vacuum Infiltration Processor (Bayer Diagnostics, Newbury, UK). The processed tissue was embedded in Paraplast Plus paraffin medium (Sigma Aldrich, UK) and sectioned at 5 µm before mounting on glass slides.

Gomori trichrome staining sections were de-waxed in xylene for 5 mins then hydrated through descending grades of ethanol down to water. They were stained with Mayers haematoxylin (Sigma Aldrich, UK) for 5 min, washed with water for 5 min and then stained with Gomori (Sigma, UK) for 5 min. After rinsing with 0.2% acetic acid, sections were blotted dry and dehydrated in ethanol before immersing in xylene and finally cover-slipped.

Aggrecan immunohistochemistry staining sections were de-waxed in xylene for 5 min then hydrated through descending grades of ethanol down to water. They were then stained using Aggrecan (LSBio - LS-C359243) antibody on the Thermo Autostainer 480S Immunohistochemistry stainer. Briefly, antigen retrieval was performed using citrate buffer pH 6. The primary antibody (Aggrecan, 1: 500) was added for 30 min then the UltraVision™ Quanto Detection System HRP DAB (Thermo Scientific, UK) was used. Sections were counterstained with Mayers Haematoxylin (Sigma Aldrich, UK) then dehydrated in ethanol, immersed in xylene (Fisher Scientific, UK) and cover slipped.

Safranin O staining sections were de-waxed in xylene for 5 min then hydrated through descending grades of ethanol down to water. They were stained with Weigerts haematoxylin (Fisher Scientific, UK) for 10 min, washed in water for 5 min and then stained with 0.05% Fast Green (Sigma Aldrich, UK) for 5 min. After briefly rinsing with 1% acetic sections were then stained with Safranin O (Sigma Aldrich, UK) for 5
min, dehydrated in ethanol, immersed in xylene (Fisher Scientific, UK) and cover slipped.
All sections were imaged on a Leica DM2700M microscope (Leica Biosystems, UK) at x10 magnification and images were processed using ImageJ analysis software (407).

6.2.11 Glycosaminoglycan quantification
Glycosaminoglycan (GAGs) is a key marker of articular cartilage matrix production. A dimethylmethylene blue (DMB) assay kit (Biocolor Ltd, UK) was used according to the manufacturer’s instructions using a papain digestion method to quantify the production of GAGs in the hydrogel samples (n=3, at each timepoint) and TCP control at day 7, 14, 21, 28, and 35.
The papain extraction reagent was prepared by mixing 50 mL of prepared 0.2M sodium phosphate buffer (pH 6.4) (Sigma Aldrich, UK) with 400 mg sodium acetate, 200 mg EDTA disodium salt, and 40 mg cysteine HCl (Sigma Aldrich, UK). Then, 5 mg of papain (Sigma, UK) was dissolved in 250 µL of deionised water before addition to the extraction solution.
Cell culture media was removed from the samples (n=3), washed twice with DPBS, and the samples digested for 3.5 h at 65°C by adding 1 mL of the papain digestion solution to each well. After digestion, 50 µL of supernatant was added to 50 µL deionised water, 1 mL DMB dye was added, and then mixed for 30 min on a mechanical shaker. Samples were centrifuged at 12,000 rpm for 10 min and the supernatants removed. The precipitated GAGs were dissociated by adding 0.5 mL dissociation reagent, vortexed, and centrifuged at 12,000 rpm for 5 min.
The GAGs were measured by transferring the sample solution to a 96-well plate. A microplate reader (Infinite 200, Tecan, Männedorf, Zurich, Switzerland) was used and the absorbance measured at 656 nm.

6.3 Statistical analysis
The statistical analysis of cell proliferation, histology, and biochemical characterisation (GAGs quantification and collagen content) were performed using GraphPad Prism software version 8.0.1 (GraphPad Software, California, USA). Significance levels were set at p < 0.05. Metabolic activity and proliferation were subjected to one-way analysis of variance (one-way ANOVA) and post hoc Tukey’s test.
6.4 Results and discussion

6.4.1 Functionalisation confirmation and rheological characterisation

Methacrylate amino groups were successfully bonded to the alginate and gelatin polymer backbones as confirmed by HNMR (Table 6.1 and Figure 6.3). Results show that the reaction with methacrylate anhydride was successful and the polymers were functionalized with methacrylate groups grafted onto the alginate and gelatin structure (Figures 6.3 c and d). AlgMA shows methacrylation moieties at 6.2 ppm and 5.7 ppm, corresponding to the protons on the alkene of the methacrylate, and at 1.7 ppm corresponding to the methyl group on the methacrylate (CH\textsubscript{3}). GelMA shows new peaks at 1.7-2.2 ppm, 5.69 ppm and 5.93 ppm corresponding to methylene protons (H\textsubscript{2}C=\text{C(CH}}\textsubscript{3}), confirming the successful functionalisation with methacrylic anhydride (Fig. 4d). The reaction between both polymers has resulted in the appearance of new characteristic peaks of MA at 5.63 ppm and 6.09 ppm attributed to the methylene group in the vinyl bond, and a peak at 1.82 ppm assigned to the methyl group, which are not present in the non-modified polymers. The hybrid systems were also successfully functionalized as shown in Figures 6.3 e, f, and g.

The degree of methacrylation was calculated and results are presented in Table 6.1. The results show that all groups were reacted with methacrylic anhydride, and the degree of carbon-carbon double bond substitution ranged between 55-79%. AlgMA shows a high degree of methacrylation compared to other systems, which could be due to using a high concentration of methacrylate anhydride (20%) reacted for long time (24 h). GelMA shows the lowest substitution of methacrylate groups (55%). While in the case of the hybrid systems, these values range between 65 and 71.5%.

Table 6.1: Degree of functionalization for all functionalized group polymers.

<table>
<thead>
<tr>
<th>Sample (group)</th>
<th>Degree of methacrylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate methacrylate(AlgMA)</td>
<td>79.0%</td>
</tr>
<tr>
<td>Gelatin methacrylate (GelMA)</td>
<td>55.0%</td>
</tr>
<tr>
<td>Hybrid IPN (Alg-Gel)MA 75:25</td>
<td>65.0%</td>
</tr>
<tr>
<td>Hybrid IPN (Alg-Gel)MA 50:50</td>
<td>71.5%</td>
</tr>
<tr>
<td>Hybrid IPN (Alg-Gel)MA 25:75</td>
<td>70.0%</td>
</tr>
</tbody>
</table>
6.4.2 Rheological Characterization

The rheological behaviour of the polymers was investigated to determine their viscoelastic properties which influences cell behaviour and their printability for the development of a bioink. The viscoelastic behaviour of the polymers is directly related to the molecular structure, percentage dilution, and functionalization processes. The results show that all three systems (AlgMA, GelMA, and Alg-Gel(MA)) present shear-thinning behaviour which is characterised by a decrease in the viscosity with the increase of shear rate (Figure 6.4). This viscoelastic behaviour is important for bioprinting cell laden constructs as the material will flow once a pressure is applied and the lower pressure required to process the bioink through the printing nozzle will improve cell viability (410). The cells also respond to the shear rate/time-dependent
properties of their substrates since viscoelastic polymers encourage cell aggregation and promote ECM formation (286, 411, 412).

**Figure 6.4:** Viscosity vs share rate profiles of AlgMA, GelMA, and Alg-Gel(MA) (25:75, 50:50, and 75:25) samples.

The measurement of the amplitude sweep dependence of the $G'$ and $G''$ allows quantification of the linear viscoelastic region (LVER) that determines the stability of the polymer solution. The viscoelastic behaviour is observed to be independent of stress or strain values by monitoring the moduli ($G'$ and $G''$) versus strain curve and the critical strain point when $G'$ crosses $G''$. The results show that the polymer specimens behave as a rigid body, when low deformation is applied to the polymer samples, $G'$ and $G''$ are constant ($G'$ is higher than $G''$) and the sample structure is undisturbed (Figure 6.5 a, c and e). At higher strain rates both $G'$ and $G''$ decrease and the structure of the polymer solution is disturbed. Once the critical strain point is identified, $G'$ crosses $G''$, the LVER ends and the polymer behaves as a fluid after this point. The material behaves as a rigid body at low strain rates but flows as a viscous fluid at high strain rates.

After identifying the critical strain point, where $G'$ crosses $G''$, frequency sweep measurements were performed to determine the material frequency dependence over a range of oscillation frequencies (0.1-100 Hz). The results present the effect of frequency on the $G'$ and $G''$ for all hydrogel systems (Figure 6.5 b, d, and f). For all
hydrogels, $G'$ is higher than $G''$, showing that in all cases there is a prevalence of the elastic behaviour in comparison to the viscous one, which can also allow for high print fidelity after the printing process. It can also be seen that the $G'$ and $G''$ is not affected by increasing the frequency, showing a plateau behaviour. The results also show that the AlgMA system presents lower elasticity ($G'$) and viscous properties ($G''$) than GelMA and consequently lower printability characteristics. The gelatin and hybrid systems present higher mechanical properties with higher $G'$ in the LVER which is also independent of strain to higher values with a shift in the $G'$ and $G''$ crossing point (Figure s 6.5 c and e).

**Figure 6.5:** Oscillatory results for pre-polymerized systems a) Storage and loss modulus as a function of strain for AlgMA systems; b) Storage and loss modulus as a function of frequency for AlgMA systems; c) Storage and loss modulus as a function of strain for GelMA systems; d) Storage and loss modulus as a function of frequency for GelMA systems; e) Storage and loss modulus as a function of strain for hybrid systems; f) Storage and loss modulus as a function of frequency for hybrid systems.
Hydrogel rheological characterisation was performed on the photocrosslinked hydrogel discs by means of oscillation rheology test. Amplitude sweep results (G' and G'' plotted as a function of shear strain) show that all hydrogel systems have the same trend of G' being higher than G'' at low strain rates. However, the hydrogel crosslinking bonds begin to break at higher strain rates, when the G' crosses G'' and both decrease (Figure 6.6). The hybrid systems have higher mechanical elastic properties (G') compared to the single material only systems (AlgMA and GelMA). The hybrid hydrogel containing equal percentage of each polymer (50:50 AlgMA-GelMA) presents the highest mechanical properties among all hybrid systems and withstands deformation at higher strains compared to other hydrogel systems, indicating improved stability (413).

**Figure 6.6:** Oscillatory results for polymerized systems a) Storage and loss modulus as a function of strain for AlgMA systems; b) Storage and loss modulus as a function of frequency for AlgMA systems; c) Storage and loss modulus as a function of strain for GelMA systems; d) Storage and loss modulus as a function of strain for GelMA systems; e) Storage and loss modulus as a function of strain for hybrid systems; f) Storage and loss modulus as a function of frequency for hybrid systems.
6.4.3 **Structure morphology and porosity**
The hydrogels morphology was assessed using SEM (Figure 6.7). The photocrosslinked hydrogel groups are characterised by a highly porous cellular structure with an average pore size of ~250 µm for all systems. AlgMA has larger pore size than GelMA, and different pore sizes were obtained for the hybrid systems as shown in Table 6.2. This porous structure provides a route for nutrient supply, gas exchange and metabolic activity as well as supporting and maintaining the phenotype of the chondrocyte cells for articular cartilage applications. Accordingly, Mei Lien et al. (414) reported that a pore size between 250-500 µm provides better ECM production (62-64). All hydrogels provide highly porous structures (> 61%). The AlgMA hydrogel have the highest porosity (90.8%) with an average pore size of 252.5 µm ± 20. The GelMA has the lowest porosity (61.0%) and an average pore size of 239.5 µm ± 30. The results show that the hybrid hydrogel samples present higher porosity with higher amounts of AlgMA and that the porosity decreases by increasing the incorporation of GelMA, which can be related to the inherent densities of the biomaterials. These results are highly relevant for cartilage tissue engineering applications since the highly porous structures produced, promote the secretion of articular cartilage specific ECM and allows chondrocyte interactions and the creation of a cell-cell network through the surrounding ECM.

![Figure 6.7: Cross-section images of different hydrogel systems a) AlgMA, b) GelMA, c) Alg-Gel(MA) 75:25, d) Alg-Gel(MA) 50:50, and e) Alg-Gel (MA) 25:75. Scale bar: 200 µm.](image-url)
Table 6.2: Experimental density, porosity, and average pore size for the different material systems

<table>
<thead>
<tr>
<th>Sample</th>
<th>Experimental density (g/cm³)</th>
<th>Porosity (%)</th>
<th>Average pore size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlgMA</td>
<td>0.149±0.003</td>
<td>90.8%±0.21</td>
<td>252.5±20</td>
</tr>
<tr>
<td>GelMA</td>
<td>0.382±0.011</td>
<td>61.0%±1.16</td>
<td>239.5±30</td>
</tr>
<tr>
<td>Alg-Gel(MA) 75:25</td>
<td>0.226±0.001</td>
<td>84.5%±0.13</td>
<td>251±24</td>
</tr>
<tr>
<td>Alg-Gel(MA) 50:50</td>
<td>0.263±0.001</td>
<td>79.9%±0.13</td>
<td>247±30</td>
</tr>
<tr>
<td>Alg-Gel(MA) 25:75</td>
<td>0.340±0.005</td>
<td>70.2%±0.47</td>
<td>240±15</td>
</tr>
</tbody>
</table>

6.4.4 Swelling and degradation kinetics and hydrogel

The swelling profile of all hydrogel groups is depicted in Figure 6.8. Results show that the AlgMA hydrogel presents the highest swelling ratio, 50%, and GelMA hydrogels present the lowest swelling, 18%. However, all hybrid systems show swelling ratios ranging from 32%, 40%, and 17% for Alg-Gel(MA) 75:25, 50:50, and 25:75, respectively. The swelling equilibrium was reached by day 3 for all hydrogels as evidenced by a plateauing of the swelling curve. Moreover, results show that adding AlgMA to the hybrid system improves the swelling ratio, indicating that the hydrogel composition (ratio of biomaterials) controls the swelling properties.

The degradation profile of a hydrogel-based scaffold should match the development and growth of new tissue with the ECM formation being able to maintain the mechanical integrity of the structure (415). The degradation rates (percentage mass loss) at day 22, for AlgMA was 23%, 27% for GelMA, and 17%, 20% and 47% for the hybrid systems Alg-Gel(MA) 75:25, 50:50, and 25:75, respectively (Figure 6.8b). However, these degradation rates can be tailored by increasing the crosslinking density either by increasing the polymer solution/photoinitiator concentration and by increasing the exposure time to the UV light source. The hydrogels containing gelatin underwent higher degradation than alginate as gelatin undergoes faster rate of hydrolytic degradation (416).
Figure 6.8: Swelling and degradation profiles of all hydrogel groups. a) Swelling ratio profile and b) degradation (mass loss profile).

6.4.5 Mechanical characterisation

The mechanical properties of the hydrogels were determined using unconfined uniaxial compression testing (Figure 6.9). The hydrogel discs (cell-free constructs) were measured on day 1 after incubation for 24 h at 37°C. The results show that AlgMA and GelMA present an average compressive Young’s modulus of 28.1± 3.5 and 36.4± 6.0 kPa, respectively. The hybrid hydrogels exhibit an average of compressive Young’s moduli 43.8 ± 3.1, 50.2 ± 4.4, and 66.2 ± 9.8 kPa for Alg-Gel(MA) 25:75, 50:50, and 75:25 respectively. The Young’s modulus is highly influenced by hydrogel composition, polymer concentration, photoinitiator concentration, and exposure time as previously reported (417).

Figure 6.9: Mechanical properties of different photocrosslinked hydrogel, a) stress-strain curve; b) Young modulus. Legend: A-alginate; G-gelatin; H-Hybrid system.

The mechanical compression test results are important to indicate the mechanical stability of the hydrogels under potential physiological loading conditions. The reported results within 20% strain are less than cartilage stiffness (400 to 600 kPa) (418), however, it has been reported for other polymeric systems that similar mechanical properties are appropriated to support the ECM formation and to maintain...
chondrogenic differentiation (419-421). Moreover, the proposed hybrid system must also be anchored to the subchondral bone using a stiffer polymeric material.

6.4.6 Hydrogel encapsulated chondrocyte viability and proliferation

The viability of the encapsulated cells in the hydrogel was assessed using a live/dead assay (Figure 6.10). At day 3, AlgMA shows a few live cells and the encapsulated chondrocytes are dying due to lack of cell binding motifs such as RGDs which are present in fibronectin, collagen, and laminin to which mammalian cells can bind (422, 423). However, GelMA and the hybrid systems present high cell viability. At later timepoints (day 7 and 16) the alginate viability improves potentially due to the binding of proteins from the cell culture media and ECM formation of the surviving cells. These results show that cell encapsulation conditions were suitable. The photoinitiator concentration, UV wavelength (365 nm), light intensity (8 mW/cm²), and exposure time (8 min) had limited cytotoxic effect on the cells. Preliminary evaluation was performed to assess the cell morphology. In the case of alginate encapsulated chondrocyte cells, the surviving cells retained a round morphology due to high crosslinking density of alginate and lack of binding motifs. However, the encapsulated cells in gelatin system have shown to exhibit phenotype drift, development of hypertrophic chondrocytes which could be due to low crosslinking density. Cells encapsulated in the hybrid system had a cell morphology with a more spherical shape for the entire duration of the in vitro studies compared to the AlgMA, GelMA and 2D TCP systems. The chondrocytes cultured on 2D TCP have an elongated shape and fibroblastic morphology.
Figure 6.10: Cell viability of human chondrocytes encapsulated in AlgMA, GelMA, Alg-Gel(MA) 50:50, and seeded on a TCP control at day 3, 7, and 16. Scale bar: 200 µm.

Cell proliferation and metabolic activity in the hydrogels was assessed using the Alamar blue assay at day 1, 3, 5, 7, and 14 (Figure 6.11). The results show that the cellular metabolic activity increases for all hydrogels up to day 3 before decreasing with the AlgMA hydrogel exhibiting the largest decrease in metabolic activity. The large decrease in AlgMA may be attributed to the lack of cell binding sites in alginate thus poor viability, as previously shown, and the degradation of the hydrogel.
preventing cell maintenance and proliferation. The GelMA and hybrid hydrogel show higher cell metabolic activity up to day 14 compared to AlgMA, potentially due to the presence of cell binding sites in gelatin allowing cell attachment. Furthermore, the reduction and plateauing of chondrocyte metabolic activity after day 5 may be due to the low metabolic and proliferative activity of chondrocytes when present in an environment that mimics the native ECM environment of articular cartilage tissue.

Figure 6.11: Fluorescence intensity indicating the metabolic activity of human Chondrocytes in AlgMA, GelMA, Alg-Gel (MA) 50:50 and TCP control at days 1, 3, 5, 7 and 14.

6.4.7 Histological assessment of hydrogel ECM formation

Histological evaluations were performed to visualise and semi-quantify the cartilaginous specific ECM formation in the photocrosslinked hydrogels at day 14, 21, 28, and 35 (Figure 6.12). The Gomori trichrome staining results indicate that in all experimental groups, most cells were positive, and collagen was produced, however, the hybrid system presented higher collagen content (Figure 6.12 and 6.13). Semi-quantitative analysis shows clear difference in the amount of collagen detected. The amount of collagen in all hydrogels increased until day 21. At day 28 and 35, the amounts of collagen started to decrease. A possible interpretation is due to the degradation of the hydrogel, since at least a minimum of 20% of the hydrogel has
degraded by this timepoint. Thus, cells are migrating to attach to the TCP and amount of collagen production is reduced and being removed during standard cell culture procedures. This shows the importance of developing a hydrogel which maintains structural stability long enough for sufficient ECM tissue formation to support the cells.

The expression of aggrecan is investigated through immunohistological staining. The results show that aggrecan was deposited within all the hydrogel systems. Aggrecan was distributed throughout the hydrogels and was highest in the Alg-Gel(MA) hybrid system at all timepoints whilst lowest in the AlgMA (Figure 6.12 and 6.13). The peak aggrecan content is at day 28 before decreasing potentially due to the degradation of the hydrogels.

The safranin-O staining is present in all hydrogels with the most proteoglycans detected in the hybrid system with considerably stronger staining than AlgMA and GelMA.

The histological staining and semi-quantification showed that the composition, content, and distribution of the ECM varied among the hydrogels. The AlgMA had the poorest capacity to support cartilage specific ECM formation, as shown by the weak intensity of staining in the samples, due to the poor cell binding capacity. The GelMA and the hybrid hydrogel enabled the deposition of ECM by the encapsulated chondrocytes and had the highest qualitative and semi-quantitative staining. This indicates that the hybrid hydrogel is supportive of maintaining a chondrocyte phenotype. The hybrid Alg-Gel(MA) is stiffer than the single component hydrogels support the importance of stiffness as factor in hydrogel design as a stiffer hydrogel system provides better chondrocyte stimulation to maintain chondrogenic phenotype after encapsulation in 3D, these results align with Li et al. (424) demonstration of different chondrocyte phenotypes based on the stiffness of a gelatin hydrogel.
Figure 6.12: Histological analysis. a) Gomori; b) Aggrecan; c) Safranin-O production at days 14, 21, 28 and 35 in Alginate methacrylate hydrogels, Gelatin methacrylate hydrogels and hybrid system (50:50 Alg-Gel)MA.
Figure 6.13 (Cont.): Histological analysis. a) Gomori; b) Aggrecan; c) Safranin-O production at days 14, 21, 28 and 35 in Alginate methacrylate hydrogels, Gelatin methacrylate hydrogels and hybrid system (50:50 Alg-Gel)MA.
Figure 6.14 (Cont.): Histological analysis. a) Gomori; b) Aggrecan; c) Safranin-O production at days 14, 21, 28 and 35 in Alginate methacrylate hydrogels, Gelatin methacrylate hydrogels and hybrid system (50:50 Alg-Gel)MA.

Figure 6.15: Semi-quantification of the relative area of histological staining of the AlgMA, GelMA, and Alg-Gel(MA) hydrogels at day 14, 21, 28, and 35 for a) Gomori trichrome and b) aggrecan.
6.4.8 GAGs quantification

GAGs are a major component of articular cartilage and an indicator of cartilage formation. The secretion of GAGs was detected in the AlgMA, GelMA, and hybrid Alg-Gel(MA) hydrogels at day 7, 14, 21, 28, and 35 (Figure 6.14). The results show a clear difference in GAGs production in the three different hydrogel systems. The AlgMA hydrogels show the lowest GAG production due to poor cell viability and cell attachment. However, the GelMA and hybrid hydrogels produced larger quantities of GAGs with the hybrid hydrogel having the largest production of GAGs by day 35. The 2D TCP control produced similar amounts to the AlgMA hydrogel even though a larger number of cells are present in the TCP control. This discrepancy can be attributed to the elongated chondrocyte cell morphology present in 2D cell culture and the dedifferentiation and phenotypic changes that occur as the chondrocytes proliferate in 2D cell culture (425). GAGs are crucial ECM factors in cartilage tissue due to their ability to strongly bind water, create osmotic pressure, and enable attachment of proteins. The high quantity of GAGs produced in the hybrid system indicates that the hydrogel can support the maintenance of the chondrocyte phenotype and secretion of a cartilage specific marker.

![Figure 6.16: Assessment of GAG secretion in the photocrosslinked AlgMA, GelMA, and Alg-GelMA hydrogels at day 7, 14, 21, 28, and 35.](image)
6.5 Conclusions

The AlgMA, GelMA, and hybrid Alg-Gel(MA) solutions and hydrogels were extensively characterised aiming at understanding their suitability to be used as bioink materials for cartilage applications. All polymers were successfully functionalised with MA as observed by HNMR. Photopolymerisation was successfully used to polymerised functionalized hydrogels. Porous structures with appropriate pore sizes for cartilage applications were obtained. The hybrid system demonstrates that the combination of both AlgMA and GelMA improves the viscoelastic behaviour of pre-polymerised materials (improved printability) and mechanical properties of polymerised hydrogels.

The hybrid system also presents high cell viability, proliferation, and production of ECM proteins, and GAGs. The results show that the developed hybrid hydrogel is a suitable bioink material for cartilage applications. It is able to support chondrocytes proliferation, cell-cell interaction and ECM formation. Moreover, the properties of this system can be easily tailored by changing the ration between AlgMA and GelMA.
Chapter 7
Hybrid PCL/Hydrogel scaffold fabrication and in-process plasma treatment using PABS†

† This Chapter based on the paper: Fengjuan Liu, Hussein Mishbak, Paulo Bartolo, “Hybrid polycaprolactone/hydrogel scaffold fabrication and in-process plasma treatment using PABS”, International Journal of Bioprinting, 5(1): 174, 2019, http://dx.doi.org/10.18063/ijb.v5i1.174. A preliminary version of this manuscript was also presented at the International Conference on Progress in Additive Manufacturing (Singapore, 2018) and was awarded as the best paper.
The development of a new tissue engineering approach to enable the neocartilage formation fully anchored to the underlying subchondral bone is critical for tissue engineering. In Chapters III to VI, two different hydrogel materials (alginate and gelatin) and a hybrid system based on both alginate and gelatin were characterised and their potential for cartilage bioinks explored. These different polymeric systems will enable to create a cartilage construct replicating the zonal structure of cartilage. However, it is also important to guarantee a good interface between the subchondral bone and the cell laden construct to guarantee the stable formation of neocartilage tissue and to enable vertical integration. This interface must be created using both hard and soft materials.

For bone tissue engineering scaffolds, a wide range of polymeric, ceramic and polymer-ceramic materials have been investigated (426). Among these materials polycaprolactone (PCL) has been one of the most popular material. However, due to its hydrophobicity produced scaffolds are limited in terms of cell-seeding and proliferation efficiency (427). Moreover, non-uniform cell distribution along the scaffolds with limited cell attachment in the core region is a common problem. The absence of commercially available additive manufacturing systems able to produce multi-type-material and gradient scaffolds makes this problem difficult to address. This Chapter investigates the use of a novel Plasma-Assisted Bio-extrusion System (PABS), to produce multi-material scaffolds for the interface between the subchondral bone and cartilage consisting in a synthetic biopolymer (PCL) and hybrid natural hydrogel system (previously described in Chapter VII). The PABS system is also used for the scaffold surface modification ($N_2$ plasma modification).

### 7.1 Introduction

Tissue engineering is promising for organ replacement which minimizes the side effects of organ transplantation (428, 429). Biomanufacturing is the major strategy of tissue engineering aiming at the development of biological substitutes that restore, maintain, or improve tissue function, and it requires the combined use of additive manufacturing (AM), biocompatible and biodegradable materials, cells and biomolecular signals (304).

The scaffolds-based strategies (Figure 7.1) for tissue engineering has been most commonly used, depending strongly on materials and manufacturing processes. For
materials, five types of biomaterials have been used: acellular tissue matrices, synthetic polymers, natural polymers, ceramics and polymer/ceramic composites (430-434). The most commonly used biomaterial for producing scaffolds are synthetic polymers, such as polycaprolactone (PCL). Polymeric scaffolds play a pivotal role in tissue engineering through cell seeding, proliferation, and new tissue formation in three dimensions, showing great promise in the research of engineering a variety of tissues. Moreover, Scaffolds made from collagen are being rapidly replaced with ultraporous scaffolds from biodegradable polymers.

**Figure 7.1:** scaffold-based approach for tissue engineering.

Biodegradable polymers are attractive candidates for scaffolding materials because they degrade as the new tissues are formed, eventually leaving nothing foreign to the body. The major challenges in scaffold manufacturing lies in the design and fabrication of customizable biodegradable constructs with properties that promote cell adhesion and cell porosity, along with sufficient mechanical properties that match the host tissue, with predictable degradation rate and biocompatibility (435, 436).

For the fabrication methods, AM techniques have been commonly applied in scaffold fabrication due to the superior ability in controlling pore size, pore shape and pore distribution, and thus creating interconnected porous structures (304, 437). When combined with clinical imaging data, these fabrication techniques can be used to
produce constructs that are customized to the shape of the defect or injury (438). In terms of tissue and organ manufacturing, the additive nature ensures minimal waste of scarce and expensive building material, namely cells, growth factors and biomaterials (439-441). Among the AM techniques, Material Extrusion has been mostly applied in the bioengineering field due to the flexibility in material selection based on the use of pneumatic (442, 443), piston (444) and screw-assisted (445-447) extrusion systems enabling a wider range of materials to be applied. Some processes operate at room temperature, thus allowing for cell encapsulation and biomolecule incorporation without significantly affecting viability. However, cell-seeding and proliferation efficiency is currently a big challenge due to the following limitations (448-451):

• Most AM techniques are limited to single-material fabrication, which is difficult to provide appropriate environment for cells due to the inadequate chemical, physical and biological cues provided during AM processes.

• Additionally, non-uniform cell distribution, especially rare cell adhesion in the core region of scaffolds, is often caused by the tortuosity of the constructs.

• Moreover, the synthetic biopolymers, most commonly used, are hydrophobic, and the cell colonization.

Different strategies have been explored to solve the above problems. Multi-material have been developed and utilized to produce multiple-material scaffolds (442, 452). However, most of these systems can only form one type of biomaterials, either soft hydrogels containing cells or bio-signals in the scale of KPa, or rigid biopolymers and composites in the scale of MPa, which fails in the mimicry of natural tissues. Additionally, low temperature plasma modification is capable of improving the hydrophilicity of biopolymers by inducing certain functional groups on the surface to change the chemistry, wettability and energy without altering the bulk properties (453, 454). However, most plasma treatment can be conducted after scaffolds printed and the penetration depth is limited, which results in non-uniform cell distribution along the scaffold.

A novel plasma-assisted bioprinting system (PABS) has been developed in the University of Manchester, allowing processing soft-hard biomaterial integration and plasma surface modification layer by layer during the fabrication process in the same chamber. This paper utilized the plasma-assisted bioextrusion system (PABS) to produce PCL/Hydrogel hybrid scaffolds and plasma fully treated scaffolds. The
hydrogel is assessed with the preparation process, functionalization process and rheology properties, while the PCL fully treated scaffolds are both morphologically and biologically assessed.

7.2 Materials and Methods

7.2.1 Polycaprolactone (PCL)

PCL (CAPATM6500, Mw = 50,000 g/mol), purchased from Perstorp (Cheshire, UK) in the form of 3 mm pellets, was used to produce the scaffolds. PCL is an easy-to-process semi-crystalline polymer with a density of 1.1 g/cm³, a melting temperature between 58-60 °C, and a glass transition temperature of -60 °C.

7.2.2 Hybrid hydrogel methacrylate anhydride (Alg-Gel)MA preparation

Hybrid hydrogel system platform were considered in this paper. Hydrogel which made of mixing alginate methacrylate and gelatin methacrylate at 50:50% v/v.

Functionalization process for both polymer (alginate and gelatin) is necessary to introduce the carbon-carbon double bond into the polymer chains that eventually convert the polymer into photopolymerized polymer. According to published protocols (455), alginate was functionalized with methacrylate groups with minor modifications to be photopolymerized. The 2% wt powder alginate (Sigma-Aldrich, UK) was dissolved in Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma-Aldrich, UK), and then mixed with methacrylate anhydride (MA) (Sigma-Aldrich, UK) at 23% v/v of alginate solution under vigorous stirring. The pH of the solution was kept around 7.4 during the reaction time by the addition of 5M NaOH. The reaction time was 24 hours. After the chemical modification, the polymer solution was precipitated with cold ethanol, dried in an oven overnight at 45 °C and purified through dialysis for 6 days. The solution was then frozen at -80 °C and recovered by lyophilization.

Gelatin was functionalized by dissolving gelatin bovine skin type B (Sigma-Aldrich, UK) at a concentration of 12.5%, in Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma-Aldrich, UK) at a temperature of 45°C. After gelatin dissolution, MA (10x molar excess) was added under vigorous. The pH of the solution was kept around 7.0-8.0 during the reaction. The reaction time was 6 hours. The solution was purified through dialysis for 7 days, frozen at -80°C and the polymer recovered by lyophilization.
7.2.3 Plasma-assisted Bioextrusion System set up

Scaffolds were produced using a hybrid additive manufacturing system called PABS, being developed at University of Manchester. PABS (Figure 7.2 a) comprises two main units, a multi-extrusion unit and a three-inlet plasma modification unit (Figure 7.2b). The multi-extrusion unit consists of two pressure-assisted extruders and one screw-assisted extruder, allowing operating a range of biomaterials, such as synthetic biopolymers, hydrogels and biopolymer/ceramic composites. The extrusion unit has four movements: one rotational movement (C1) for selecting the required extrusion heads, a second rotational movement (C2) for driving and controlling the screw rotational speed of the screw-assisted extruder, and two linear movements (X and Y) in the X-Y plane. All these four movements are controlled by stepper motors and CNC drives. The build platform moves in the z-direction and constitutes the sixth controllable axis. The build platform was fabricated using 7076 aluminium plate (250 mm x 200 mm x 7.5 mm) and is attached to the z-axis rail guide with an L-shape support underneath.

The plasma modification unit is mounted on the X-Y platform which is co-planar with the extrusion platform. Both platforms share common cylindrical guide rails in the X-direction. The Y-direction movement (V axis) for the plasma modification unit is independently driven by a stepper motor parallel to the Y axis of the extrusion unit. A quartz capillary (outside diameter of 7 mm; inner diameter r of 5 mm; length of 70 mm) with three gas inlets serves as the reaction jet. A tungsten rod (inner diameter of 2 mm) and one copper film (10 mm of width) wrapped around the quartz tube serve as the high-voltage and ground electrodes, respectively. The electrode is connected to a high-voltage DC power supply (applied voltage of 10 kV; frequency of 50 kHz). The plasma is generated from the top central electrode, expanding to the surrounding air inside and outside the nozzle.
Figure 7.2: a) Set-up of plasma-assisted bio-extrusion system; b) system diagram: multi-extrusion system, plasma modification system and control system.

The control system consists of a motion control system, temperature control system and gas supply control system. The motion control system utilizes a Geo Brick LV motion controller (Delta Tau Data Systems, Inc) to manipulate all motors. The built-in software allows for complete machine logic control, including G code execution.
The temperature control system consists of four digital temperature controllers (P.I.D. Omron E5CN), which are used to precisely control the temperature in the extruder heating chamber with polyimide thermos-foil heating elements (Omega Online/Kapton Heaters), and thermocouples (Omega). The gas supply control system includes an air compressor which works as the main gas supply, regulators and gauges (SMC, UK), which enables pressure on/off control and manually adjustment. Power supply (230V ac, PHOENIX CONTACT) offers 24v output voltage and 40A current for all the control system. Safety considerations were taken into account regarding the electrical parts, including fuses, main circuit breakers (MCBs), main filter, push buttons and estops (all supplied by OneCall Electronics Company, UK), and circuits. The control software was developed in MATLAB as previously reported, and G code files was generated containing all the instructions for the fabrication process (456). This configuration enables multi-material dispensing and plasma modification in a sequential mode. The system is able to achieve a maximum linear velocity of 20 mm/s and resolution of 0.05 mm.

7.2.4 Scaffolds printing strategies

Scaffolds with a cross-section of 10x10 mm and a height of 3 mm were fabricated using the single laydown pattern of 0/90° and a filament distance of 1 mm with pore size of 1 mm (slice thickness of 0.5 mm; heating temperature of 90°C; extrusion screw rotational speed of 15 rpm; and nozzle tip size of 0.5 mm.). The strategies for printing hybrid scaffolds and plasma treated scaffolds are as follows (Figure 7.3):

- Hybrid PCL/hydrogel scaffolds fabrication (Figure 7.3a): After the scaffold being printed with screw-assisted extruder, the extruder selection unit rotated with 120°, and then the hydrogel solution was printed in the pores using the pressure-assisted extruder.
- Full layer treated scaffolds fabrication (Figure 7.3b): The N₂ plasma modification was performed after one-layer PCL was deposited, and the process was repeated until the last layer of PCL was complete. The treatment was conducted at a pressure of 0.689 bar and a flow rate of 5 L/mm. The deposition speed of the plasma jet was 3 mm/s and each layer was subjected to the plasma treatment for one minute. The distance from the bottom of the jet to the surface of the PCL filaments was 10 mm.
Figure 7.3: a) Sequence of operations to fabricate hybrid PCL/hydrogel scaffolds; b) Sequence of operations to fabricate full-layer plasma treated scaffolds.

7.2.5 Nuclear Magnetic Resonance characterisation
Hybrid hydrogel were characterized by means of $^1$HNMR to confirm the functionalization process. The chemical structure of functionalized hybrid hydrogel system was assessed through Nuclear Magnetic Resonance Spectroscopy ($^1$HNMR), using the B400 (Bruker Avance III, USA) 400 MHz. Polymeric materials were dissolved in Deuterium oxide (D$_2$O), transferred to NMR tubes and the spectra acquired with 128 scans.

7.2.6 Rheological characterisation
To assessing the mechanical properties of the hybrid hydrogel quantitatively, a deformation rheology tests are performed on polymeric hydrogels discs. The rheological analyses were carried out using the DHR2 Rheometer (TA Instrument, USA). Small amplitude oscillatory shear measurements tests were considered, the shear storage modulus, $G'$, loss modulus, $G''$ were quantified. It is assumed that the material flows by applying a stress being the response measured. A controlled stress was applied, and the resulting movement measured. Small strain oscillations have been used to measure viscoelastic properties without destroying the sample structure the oscillatory test provides a mechanical spectrum for the material.

7.2.7 Morphology Characterization
Scanning electron microscopy (SEM) was used to assess the morphology and surface characteristic of printed scaffolds. The scaffolds were gold/palladium coated using a Q150T turbo-pumped sputter coater (Quorum technologies, UK) and imaged at 10 kV (Hitachi S3000N, Japan). These images were then analysed using ImageJ software.
7.2.8 Wettability measurement

Water Contact Angle (WCA) measurements on the flat surfaces of untreated and plasma-treated PCL scaffolds were carried out with a commercial KSV CAM 200 system (KSV Instruments, Finland). The system is equipped with a CCD video camera and a micrometric liquid dispenser to drop 2 µL of distilled water on the surface of the scaffold. The measurements of the contact angles are automatically calculated with the instrument software.

7.2.9 Biological tests

*In vitro* biological assessments were conducted with human adipose-derived stem cells (hADSCs) (STEMPRO, Invitrogen, Waltham, MA, USA). Before cell seeding, scaffolds were sterilized by soaking in 70% ethanol for 2 hours. After sterilization, samples were rinsed twice in phosphate buffered saline (PBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA), transferred to 24-well plates and air-dried for 24 hours at room temperature. 50,000 cells were seeded on each sample, including plasma-treated and untreated scaffolds.

Cell viability/proliferation behavior and the percentage of cells attached to the scaffolds (cell-seeding efficiency) were assessed through Alamar Blue assay (also termed the Resazurin assay, reagents from Sigma-Aldrich, UK). Cell viability/proliferation was measured at 1, 3, 7 and 14 days after cell seeding. For each measurement, cell-seeded scaffolds were transferred to a new 24-well plate and 0.7 ml of Alamar Blue solution was added to each well, the plate was incubated for 4 hours under standard condition (37 °C, 5% CO₂ and 95% humidity). After incubation, 150 µL of each sample solution was transferred to a 96-well plate and the fluorescence intensity measured at 540 nm excitation wavelength and 590 nm emission wavelength with a spectrophotometer (Sunrise, Tecan, Männedorf, Zurich, Switzerland).

Cell attachment and distribution are assessed using laser confocal microscopy, with cell nuclei stained. At day one of cell culture and after 14 days, scaffolds were removed from 24-well cell culture plate, rinsed twice in phosphate-buffered saline (PBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA), fixed with 10% neutral buffered formalin (Sigma-Aldrich, Dorset, UK) for 30 minutes at room temperature. After fixation, samples were rinsed twice with PBS for the removal of formalin, then permeabilised with 0.1 % Triton-X100 (Sigma-Aldrich, Dorset, UK) in PBS at room temperature for 10 minutes, rinse twice for the removal of Triton-X100. Cell nuclei
were stained blue by soaking scaffolds in a PBS solution containing 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, Dorset, UK) at the manufacturer recommended concentration. Samples were left in the staining solution for 10 min prior to removal, rinsed twice thoroughly with PBS. Confocal images were obtained using a 3D rendering mode on a Leica TCS SP5 (Leica, Milton Keynes, UK) confocal microscope, and cell colonization was quantified using a standard z-stack method. All images were taken at the centre area of the scaffolds and the experiments were performed three times in triplicate.

**7.2.10 Data analysis**

All data are represented as mean ± standard deviation. Biological results were subjected to one-way analysis of variance (one-way ANOVA) and post hoc Tukey’s test using GraphPad Prism software. Significance levels were set at p < 0.05.

**7.3 Results**

**7.3.1 Hybrid Scaffold Fabrication**

The hybrid scaffold, consisting of PCL and hydrogel solution, was successfully fabricated using PABS. Figure 7.4a shows the image of the hybrid scaffold, where the porous structures hold the hydrogel solution without any deformation. The position of the deposited hydrogel was precisely controlled.

**7.3.2 Full-layer treated PCL scaffold**

Figure 7.4b shows that the printed full-layer N₂ plasma treated scaffolds has a well-bonded interconnected structure with uniform pore distribution and pore size in the range of ~500 µm (Figure 7.4c). The N₂ plasma treatment was conducted at a pressure of 0.689 bar and a flow rate of 5 L/mm. The deposition speed of the plasma jet was 3 mm/s and each layer was subjected to the plasma treatment for one minute. The distance from the bottom of the jet to the surface of the PCL filaments was 10 mm. Figure 7.4d presents the filament surface after N₂ plasma modification, where lines in the direction of plasma movement can be observed making the surface roughness increased.
Figure 7.4: (a) Hybrid polycaprolactone (PCL)/hydrogel scaffold; (b) Scanning electron microscopy (SEM) image of top view of PCL/hydrogel scaffold; (c) SEM image of side view of PCL/hydrogel scaffold; (d) photo of a full-layer N2 plasma-treated PCL scaffold; (E) SEM image of top view of full-layer N2 plasma treated PCL scaffold; (f) SEM image of filament surface of full-layer N2 plasma-treated PCL scaffold.

7.3.3 Wettability assessment of full-layer plasma modified scaffold

WCA measurements were performed on untreated and fully treated plasma PCL scaffold surfaces to determine the effect of plasma modification on the surface wettability. Table 7.1 highlights the WCA results at different time points after the droplet was dropped on the surface of the scaffolds. The results show that in the case of an untreated PCL scaffold, there are no significant changes in the WCA values with time with values varying between 83.2±2.0° and 80.9±2.7°. For treated scaffolds, the WCA value, at 0 s, was lower (63.0±3.1°), leading to a fully wetting value of 26.7±0.9° at 0.5 s. At 3 s, the droplet was fully absorbed.
Figure 7.5: a) Temporal variation of fluorescence intensity of cell-seeded PCL scaffolds with and without N2 plasma treatment; b) Confocal microscope images of untreated and full-layer treated of cell seeded scaffolds, 1 day and 14 days after cell culture. Scale bar 250 µm.

Table 7.1: Temporal variation of water contact angles for treated and untreated scaffolds.

<table>
<thead>
<tr>
<th>Time</th>
<th>PCL scaffolds</th>
<th>N2 plasma fully treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0s</td>
<td>83.2±2.0 °</td>
<td>63.0±3.1 °</td>
</tr>
<tr>
<td>0.5s</td>
<td>82.9±1.2 °</td>
<td>26.7±0.9 °</td>
</tr>
<tr>
<td>3s</td>
<td>80.9±2.7 °</td>
<td>Fully absorbed</td>
</tr>
</tbody>
</table>
7.3.4 Biological assessment of full-layer plasma modified scaffold

The adhesion and proliferation of hADSCs cells on plasma full-layer modified PCL scaffolds was studied and compared with untreated ones. The biological characterization was assessed using Alamar Blue Assay. The fluorescence intensity of cell seeded scaffolds measured at four different culture time points (Day 1, 3, 7 and 14) is shown in Figure 7.5a. Higher fluorescence intensity corresponds to more metabolically active cells. As observed, cell proliferation increases with time in all types of scaffolds, suggesting that they are suitable structures for cell attachment and proliferation. However, a fast proliferation rate is observed in the case of plasma treated scaffolds. The different performance is statistically significant after day 3. Confocal microscopy images (Figure 7.5b) present the cell attachment and distribution after cell seeding (day 1) and proliferation (day 14). It can be observed that plasma treated scaffolds presented higher numbers of cells than untreated scaffolds. Additionally, it is also possible to observe that plasma surface treated scaffolds presented best cell attachment and dispersion.

7.4 Discussion

The printed hybrid PCL scaffolds filled with hydrogel present the printability of PBAS, enabling the soft-hard material integration. The photo and SEM images of the whole printed structure indicate that the plasma modification process does not affect the physical appearance of the structure and potentially no effect on the mechanical performances (457). Moreover, SEM image of the filament surface confirms the increased surface roughness is due to the etching process (458), which results in the stripping off the topmost layer of the polymer filament. From this study, the increase surface roughness is also resulted from the linear scratches attributed to the gas flow by the plasma jetting directly after the PCL deposition when the material is still in the molten status. As the printed material is not totally cooled down, when the plasma modification occurs, the gas flow effect is stronger, significantly influencing the surface topography. Moreover, the increase of surface roughness is beneficial for cell colonization in the scaffolds (459).

The wettability results reveal that the hydrophilicity of the surface is dramatically improved due to the ionisable groups introduced on the surface by the N\textsubscript{2} plasma, which enhances the hydrogel bonding with water. Compared with the results published in (454,460), the absorption speed is dramatically increased, due to chemical
heterogeneity on the surface of each layer because of the plasma modification layer upon layer. However, the effect of plasma modification can last within a certain period of time depending on the density of chemical regents deposited on the surface, and the effect also decreases with time since the structural rearrangement of polymer chains resulting in the decreased surface energy (461).

In addition, it is important to control hydrogels printability window (injection, deposition and extrusion) during printing process to mimic the native organ structures. To achieve precise printable properties, hydrogels have been designed to achieve adjustable viscoelastic behaviour, and the SEM images confirm the liquid-like-gel structure before printing and quick gelation kinetics to produce structure with good fidelity. Hybrid hydrogel system presents an adjustable viscoelastic behaviour that could be changed/ tailored with temperature, shear thinning and polymer concentrations. The rheological assessment result confirms that the hybrid hydrogel system has clear linear viscoelastic region (LVE) which characterize by both storage moduli $G'$ and loss moduli $G''$ are independent of shear strain and the system is solid-like-gel ($G' \gg G''$). These characteristics make it easy to inject hybrid hydrogel system (Alg-Gel)MA.

Consistent with the other studies (462, 463), a significant increase in the biological behavior is observed after plasma modification. Since the hydrophilicity has been improved with the plasma modification process, the environment is changed to be more suitable for cell colonization and proliferation. Also, with the full-layer plasma modification capability, the plasma active species can reach the walls of the pores on each layer of the scaffold, leading to the uniform cell distribution along the scaffold. This will enable higher rate of tissue formation in the clinical research.

7.5 Conclusions

This paper presents a novel additive manufacturing system comprising a multi-material printing unit and a plasma jet unit. To assess the system, hybrid PCL/hydrogel scaffolds and full-layer plasma treated PCL scaffolds were produced. The effect of plasma treatment on PCL samples was examined. WCA results confirmed that the hydrophilic character of the PCL samples increased due to the nitrogen groups introduced by the plasma jetting on the scaffold filaments. It was also possible to observe that the plasma treatment positively influences cell attachment and proliferation. Applications that may benefit from this technology include hybrid tissue,
which has compositional variations depending on the region or organ-like structure that require continuous vascular network to facilitate nutrient diffusion.
Chapter 8
Conclusions and future work
8.1 Conclusions

Cartilage is an avascular tissue with limited regenerative capabilities. Its structure, role and main diseases and clinical therapies were detailed in Chapter II (research objective 1). Despite different treatments have been proposed and being clinically used they still present several limitations such as high cost, poor clinical outcomes, and limited temporal efficacy. In order to address these limitations cartilage tissue engineering approaches based on the use of biocompatible and biodegradable materials and additive manufacturing technologies to produce scaffolds for cartilage repair or cell laden constructs based on the use of bioinks have been proposed. The scaffold-based approach is based on the use of 3D structures that will provide the substrate for cell adhesion, proliferation and differentiation. In this approach scaffolds can be used as acellular structures and implanted into the defect, recruiting cells from the surrounding tissue; or seeded with cells and pre-cultured in a bioreactor prior implantation. The success of this approach has been limited and the scaffolds being proposed so far are not able to recapitulate the zonal structure of cartilage. Cell laden constructs seems to be the ideal strategy not only because it allows to print a cartilage-like construct encapsulating relevant cartilage cells and materials with a structure similar to cartilage but also because it allows the development of future in situ printing approaches. Bioprinting approaches to produce cell laden constructs for cartilage repair have been proposed. However, few limitations still exist related to materials (e.g. tailoring viscoelasticity), printing process (e.g. shear stresses and shape fidelity) and risk of contamination during the bioprinting process. The development of novel constructs for cartilage applications is critical and requires the:

- Design of suitable materials, able to be printed and presenting suitable mechanical, swelling, degradability and morphology and able to sustain cell growth and ECM formation;
- Design of a multi-layer construct able to replicate the zonal structure of cartilage;
- Selection of the best additive manufacturing technique (e.g. crosslink mechanism).

As discussed in Chapter 2, natural-based hydrogels are ideal candidates for cartilage applications and photocrosslinking reactions a suitable method to produce cell laden constructs. However, only two photocurable hydrogels are commercially available
(ChonDuxTM and GelrinC). This an area that requires more research not only in terms of design the hydrogels (e.g. level of C=C in the pre-polymer chain) but also on investigating the effect of the curing process on both printability and cell survival. However, this is not a simple task as high levels of C=C bonds increase the crosslinking density, improving mechanical properties and shape fidelity during the fabrication process but decreases biocompatibility as the free space available to support cell growth is reduced. Similarly, high levels of photoinitiator increase the curing kinetics and crosslinking density but might also induces some toxicity. Light intensity and irradiation time must also be optimised in order to avoid any negative effects on cells. Some of these correlations were investigated in this research work and the results presented in Chapters III to VI.

Based on the aims and research objectives presented in Chapter I, the main novelty of this research is the development of a novel multi-material cell laden construct for cartilage based on two different polymer materials (alginate and gelatin) and a hybrid system based on the combination of both alginate and gelatin. Single type materials are considered for both the superficial zone and the deeper region of cartilage, while the hybrid system is considered for the transitional zone. Both alginate and gelatin have been explored before for different tissue engineering applications but their combination to create a zonal construct such the one proposed by this dissertation was never investigated before. Each material was investigated in detail as a single material and the results presented in Chapters III and IV for alginate and Chapter V for gelatin. Each material was successfully functionalized with methacrylate anhydride as observed through NMR and it was possible to control the functionalization process by changing the polymer concentration, methacrylate anhydride concentration and reaction time (research objective 2). Key conclusions emerging from this part of the work are:

- By increasing the polymer concentration, keeping the reaction time and the methacrylate anhydride concentration constant, the degree of modification (level of unsaturation) decreases;
- By increasing the methacrylate anhydride concentration, keeping polymer concentration and reaction time constant, the degree of modification decreases;
• By increasing the reaction time, keeping both methacrylate anhydride and polymer concentration constant, the degree of modification increases.

The effect of the functionalization process on the rheological, printability, mechanical, morphological, swelling and degradation properties was also investigated for both materials (Chapters III and V) (research objective 3). Results show that is possible to tailor the properties of each single material and the main conclusions are:

• The increase of the degree of modification increases the crosslinking density, printability and mechanical properties;
• The increase of the degree of modification decreases the swelling properties and slow down the degradation process;
• The increase of the degree of modification produces structures with high number of pores with small pore sizes.

Based on the results presented in Chapters III to V the following single material systems were considered (research objective 3):

• AlgMA (2% wt of alginate and 20% v/v of methacrylate anhydride) functionalized during 24 h and dissolved in a solution containing 1% wt of photoinitiator;
• GelMA (12.5% wt of gelatin and 10% v/v of methacrylate anhydride) functionalized during 6 h and dissolved in a solution containing 1% wt of photoinitiator;

These systems were used to encapsulate mesenchymal stem cells (Chapter V) and human chondrocytes (Chapter IV) and cell laden constructs were produced using a UV lamp (8 mW/cm² of light intensity) and 8 min of irradiation time (research objective 3). Results show that:

• All material showed no toxicity effects;
• All materials were able to support cell growth. However, fast cell proliferation was observed for GelMA, which can be attributed to the presence of specific cell adhesion motifs as discussed in Chapter V.

Chapter VI investigates hybrid systems obtained through the combination of different ratios between alginate and gelatin. These materials were investigated before and after photopolymerisation and the effect of material composition in terms of rheological, mechanical, swelling, degradation properties and biological properties was investigated (research objective 4). Results show that:
• AlgMA and GelMA hybrid systems are able to support cell growth and ECM formation;
• The addition of AlgMA increases the mechanical properties, swelling characteristics and slow down the degradation process;
• The levels of collagen, aggrecan and GAGs formation were significantly higher in the hybrid systems in comparison to the single material systems. Moreover, GelMA presents higher levels of ECM formation in comparison to AlgMA.

Finally, Chapter VII investigates the use of a scaffold produced with a novel additive manufacturing system and comprising two main materials (PCL and hybrid alginate/gelatin system). This construct was investigated aiming to define a proper vertical integration between the subchondral bone and cartilage (research objective 5). Due to hydrophobic the nature of PCL (a material commonly used for bone tissue engineering scaffolds) plasma treatment was used. Results showed that it was not only possible to create a construct using these two different polymeric systems but also that the plasma treatment positively influenced cell attachment and proliferation.

8.2 Future works

This thesis discusses the use of hydrogel-based constructs scaffolds for tissue engineering produced for cartilage replacements through additive manufacturing. An extensive in vitro work was conducted to design and assess the materials and the 3D produced scaffolds. Despite the contributions to the current knowledge provided by this research work, it was possible also to open new research questions that will be addressed in the future. Moreover, additional work should be conducted to complete the current research. Planned activities include:

(1) A zonal construct containing layers of alginate, gelatin and alginate/gelatin will be produce encapsulating chondrocytes but also stem cells and growth factors. The construct will be characterized in terms of overall mechanical properties, adhesion between layers and the ability to sustain cell adhesion, proliferation and differentiation. The zonal construct will be produced using the materials investigated in these research work;

(2) Investigate new functionalization methods for both alginate and gelatin by introducing C=C on the hydroxyl (e.g. oxidation, sulfation and copolymerization)
and carboxyl (esterification and amidation) groups in order to improve both mechanical, rheological, biological and degradability characteristics;

(3) Investigate the effect of light intensity, irradiation time and photoinitiator concentration on the different properties of the printed constructs;

(4) The lubrication properties of cartilage, particularly the upper zone of cartilage, is an important property not considered in this research. The coefficient of friction, under both dynamic and static loading conditions, of the different materials as a function of functionalization and processing conditions will be assessed;

(5) Cartilage is an avascular tissue and as a consequence chondrogenesis improves under hypoxia conditions. The effect of the functionalization process and printing conditions in terms of oxygen permeability will be investigated allowing to design constructs creating a more suitable biological environment;

(6) In order to have a deeper understanding on the interactions between the hydrogel and cells, additional protein and gene expression tests will be conducted. This will include the quantification of the transcription factor SOX-6 and SOX-9, Collagen II, and cartilage oligomeric protein (COMP) of chondrocytes after long periods of culture;

(7) Free radical polymerization was used in this research. This is a chain-growth reaction allowing the spatiotemporal control of the polymeric network but producing heterogeneous networks. In the future, other chemistries such as photoclick reactions (step-growth mechanism) will be explored to allow fast and more efficient reactions with minimal network defects;

(8) Scaffolds will be also characterized in vivo. Initially, constructs will be subcutaneously implanted into the flanks of nude mice to investigate swelling, degradability and neocartilage formation. Later, more detailed studies will be performed considering osteochondral defects created in rabbits.
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