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Uncovering a novel hydrogel based therapeutic approach to tackle articular osteoarthritis

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UNCOVERING A NOVEL HYDROGEL BASED THERAPEUTIC APPROACH TO TACKLE ARTICULAR OSTEOARTHRITIS

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Publications

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Cruz Ramirez:

"How did you know you could do it?"

Lightning McQueen:

"I don't know. I just never thought I couldn't."



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Abstract

Osteoarthritis (OA) is an irreversible heterogenous degenerative disorder of articular joints. The main associated symptoms for affected individuals include pain and hindered mobility, as direct results of articular cartilage degradation. The mechanism for such occurrences arises due to wear and tear, promoting inflammatory-related catabolic cues and the subsequent ossification of cartilage.

To alleviate OA symptoms and improve joint function, when conservative therapies fail, replacing the degraded articular cartilage is considered necessary by both the scientific and medical communities as the optimal approach. As a result, tissue engineering and regenerative medicine have emerged as promising strategies for treating OA patients. An approach which unifies these fields is the use of decellularised extracellular matrix (dECM). This is a widely studied approach where an acellular extracellular matrix (ECM) scaffold is retrieved via obliterating cellular materials while maintaining the matrix components. However, a consequence of detergent-based decellularisation techniques is the depletion of the essential glycosaminoglycan (GAG) chondroitin sulphate (CS), vital for the cartilage microenvironment and chondrogenesis.

The hypothesis of this project is that CS functionalisation will restore the native cartilage environment by replenishing lost GAGs, while quercetin will further enhance chondrogenesis due to its stimulatory properties. Therefore, the aim is to develop a novel matrix-orientated hydrogel from decellularised bovine articular cartilage, functionalised with CS and quercetin (quercetin hydrate). The focus is on solubilising matrix components and optimising a functionalisation strategy to create a proof-of-concept hydrogel.

To overcome CS deficiency-induced chondrogenic impairment, CS is reconstituted via developing a dECM-based hydrogel (originating from bovine cartilage) functionalised with shark-sourced CS. Firstly, a decellularisation protocol is optimised with deoxyribonucleic acid (DNA) being efficiently removed. However, 73% of sulphated GAGs are depleted, highlighting the need to reconstitute the depleted GAGs. After solubilising the material, CS functionalisation with CS or aldehyde modified chondroitin sulphate (mCS) is performed. All the tested hydrogels exhibit low rheological stiffness, showing CS functionalisation does not improve rheological properties. In relation to turbidity experiments to assess gelation kinetics, the mCS-functionalised variant depicts slower gelation kinetics. To aid the chondrogenic ability of mCS functionalised hydrogels, a novel approach with quercetin is investigated. Incorporation of 0.3 mg/mL quercetin in 0.4 mg/mL mCS functionalised hydrogels results in a significant increase in rheological stiffness. Cell viability and chondrogenic differentiation are assessed. Results show similar cell viability in dECM and CS modified hydrogels at 1 and 3 days of culture, with no significant changes in gene expression of chondrogenic and hypertrophic genes. In quercetin-incorporated hydrogels, human dermal fibroblast (HDF) viability remains unchanged, while chondrocytes show enhanced chondrogenic gene expression, es-

pecially collagen type II alpha 1 chain (COL2A1), with 6.6- and 2.2-fold increases for 0.15 mg/mL and 0.3 mg/mL quercetin, respectively.

Taken together, the results presented here support functionalising dECM hydrogels as a method to modify their properties. Solely utilising CS is insufficient to promote chondrogenesis without quercetin integration. In the future, investigating a broader range of CS, including its modifications and concentrations, as well as the concentrations of quercetin, may allow the identification of an optimal hydrogel formulation for further study.

Resumo

A osteoartrose (OA) é uma doença degenerativa, heterogénea e irreversível das articulações, caracterizada por dor e redução de mobilidade. Com origem na degradação progressiva da cartilagem articular associada ao desgaste, a sua evolução é acompanhada por um aumento de mediadores inflamatórios e catabólicos e pela subsequente ossificação da cartilagem.

Nos últimos anos, tem vindo a ser discutida e estudada, entre as comunidades científica e médica, a substituição da cartilagem articular degradada como potencial estratégia para o alívio dos sintomas da OA. Especialistas das áreas de engenharia de tecidos e da medicina regenerativa têm apostado no uso de matriz extracelular descelularizada (dECM) como abordagem promissora da regeneração da cartilagem. A técnica de descelularização consiste em recuperar matriz extracelular (ECM) através da obliteração de materiais celulares com recurso a detergentes. No entanto, uma consequência notável desta técnica é a depleção do glicosaminoglicano essencial (GAG) sulfato de condroitina (CS), vital para o microambiente da cartilagem e para a condrogénese. Para compensar a deficiência de CS induzida pela deficiência condrogénica, o CS é reconstituído através do desenvolvimento de um hidrogel à base de dECM (originário da cartilagem bovina) funcionalizado com CS de origem de tubarão em conjunto com a incorporação de quercetina, para melhorar as propriedades do hidrogel e a condrogénese.

A investigação assenta na hipótese de que a funcionalização com CS restaurará o ambiente nativo da cartilagem, repondo os GAGs perdidos, enquanto a quercetina potencializará a condrogénese devido às suas propriedades estimulantes. Assim, o objetivo deste projeto é desenvolver um novo hidrogel a partir de cartilagem articular bovina descelularizada, funcionalizada com CS e quercetina (hidrato de quercetina). O foco incide na solubilização dos componentes da matriz e na otimização de uma estratégia de funcionalização para desenvolver um hidrogel protótipo para validação de conceito.

Um protocolo de descelularização é otimizado com a remoção eficiente do ácido desoxirribonucleico (DNA). No entanto, observa-se uma redução de 73% na quantidade de GAGs sulfatados, o que reforça a necessidade da sua reconstituição. Após solubilizar o material, é realizada a funcionalização CS com CS ou sulfato de condroitina modificado com aldeído (mCS). Todos os hidrogéis testados exibem baixa rigidez reológica, indicando que a funcionalização com CS não melhora as propriedades reológicas. Nas experiências de turbidez com a finalidade de avaliar a cinética de gelificação, a variante funcionalizada com mCS apresenta uma cinética de gelificação mais lenta. Para melhorar a capacidade condrogénica dos hidrogéis funcionalizados com mCS, é investigada uma nova abordagem com quercetina. A incorporação de 0.3 mg/mL de quercetina em 0.4 mg/mL de hidrogel funcionalizado com mCS resulta num aumento significativo da rigidez reológica. Além disso, a viabilidade celular e a diferenciação condrogénica são avaliadas. Os resultados indicam

uma viabilidade celular semelhante em dECM e hidrogel modificado com CS após 1 e 3 dias de cultura, sem alterações significativas na expressão génica de genes condrogénicos e hipertróficos. Nos hidrogéis incorporados com quercetina, a viabilidade dos fibroblastos de derme humanos (HDF) permanece inalterada, enquanto os condrócitos apresentam uma expressão génica condrogénica aumentada, especialmente cadeia alfa 1 do colágeno tipo II (COL2A1), com aumentos de 6.6 e 2.2 vezes para 0.15 mg/mL e 0.3 mg/mL de quercetina, respetivamente.

Em conjunto, os resultados aqui apresentados comprovam que a funcionalização dos hidrogéis dECM pode ser utilizada para modificar as suas propriedades. No entanto, a utilização exclusiva de CS é insuficiente para promover a condrogénese. No futuro, a investigação da CS, incluindo as suas modificações e concentrações, bem como as concentrações da quercetina, poderá permitir a identificação da formulação ideal de hidrogel para estudos adicionais.

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List of Abbreviations and Symbols

2D	two-dimensional
3D	three-dimensional
ACAN	aggrecan
ACTB	β -actin
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AKT	protein kinase B
ALPL	alkaline phosphatase
ATMP	Advanced Therapy Medicinal Product
ATR	attenuated total reflectance
bMSC	bone mesenchymal stem cell
Ca²⁺	calcium ions
COL1	collagen type I
COL1A1	collagen type I alpha 1 chain
COL2	collagen type II
COL2A1	collagen type II alpha 1 chain
COL5	collagen type V
COL9	collagen type IX
COL10	collagen type X
COL10A1	collagen type X alpha 1 chain
COL11	collagen type XI
COMP	cartilage oligomeric matrix protein
CS	chondroitin sulphate
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dECM	decellularised extracellular matrix
DMSO	dimethyl sulfoxide
DS	dermatan sulphate
dsDNA	double strand deoxyribonucleic acid
DSJ	diarthroidal synovial joint
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
EthD-1	ethidium homodimer-1
FCS	fetal calf serum
FCT	Fundação para a Ciência e a Tecnologia
FJ	facet joint
FTIR	fourier transform infrared spectroscopy
GAG	glycosaminoglycan
GaINAc	N-acetylgalactosamine
GlcA	D-glucuronic acid
Gly	glycine
G'	storage modulus
G''	loss modulus
G*	complex modulus
HA	hyaluronic acid
HB	hypotonic buffer
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
HDF	human dermal fibroblast
hTERT	human telomerase reverse transcriptase
Hyp	hydroxyproline
HE	haemotoxylin and eosin
IL	interleukin
ISO	International Organization for Standardization
IVD	intervertebral disc
kDa	kilodalton
KS	keratan sulphate
LPXTG	leucine-proline-any amino acid-threonine-glycine
LVER	linear viscoelastic region
mCS	aldehyde modified chondroitin sulphate
MMP	matrix metalloproteinase
MQ	Milli-Q®

MSC	mesenchymal stem cells
NaIO₄	sodium periodate
NaOH	sodium hydroxide
NHS	N-hydroxysuccinimide
OA	Osteoarthritis
P38	p38 mitogen-activated protein kinase
Pen/Strep	penicillin-streptomycin
PBS	phosphate buffered saline
PG	proteoglycan
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
Pro	proline
RANKL	receptor activator of nuclear factor kappa-B ligand
RGD	Arginine-Glycine-Aspartic acid
RNA	ribonucleic acid
RT	room temperature
RT-PCR	Real-time polymerase chain reaction
SAP	surface active phospholipids
SDC	sodium deoxycholate
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
Ser	serine
sGAG	sulphated glycosaminoglycan
siRNA	short interfering ribonucleic acid
SOX9	SRY-box 9
SP	substance P
TGF-β	tumour growth factor- β
TNF	tumour necrosis factor
ToS	transmittance of solids
t_{1/2}	half-time gelation turbidity
VEGF	vascular endothelial growth factor
-HG	high glucose
-LG	low glucose

Chapter 1:

Introduction

This chapter reviews the structure and biochemistry of the articular joint and how these change in osteoarthritis. It focuses on the interaction between cartilage matrix and cells, and how mechanical stress, matrix remodelling, and inflammation contribute to disease progression. This provides the foundation for later chapters on cartilage repair biomaterials.

Next, an overview of bioamaterials such as hydrogels is given, emphasising their chemical and physical properties support cells, and their potential for tissue engineering applications.

Finally, the chapter discusses enhancing cartilage regeneration by functionalising solubilised decellularised extracellular matrix. It explains the role of chondroitin sulphate modified with aldehyde groups and quercetin in scaffolds. This sets up the approach using these components to develop new proof-of-concept treatments for osteoarthritis.

1.1 Articular Joints: Diarthrodial Synovial Joints

A joint is defined as a region where two bones meet and can be classified either functionally or histologically (Juneja et al., 2025). Structurally, joints are divided into three main types: fibrous, cartilaginous, and synovial (see Table 1.1) (Alves et al., 2018; Hickman et al., 2022; Rice, 2008). This thesis focuses on the synovial type, specifically the diarthrodial synovial joint (DSJ), as the cartilage used for decellularisation was obtained from these joints in the bovine leg. The knee and spinal facet joint (FJ) are typical examples of DSJ. The term “diarthrodial” indicates that these joints are freely movable, while “synovial” refers to the presence of a synovial membrane and fluid (Davies, 1945). These joints are essential for everyday mobility and form part of the group known as articular joints, which are made up of different tissues working together to allow movement while maintaining skeletal stability (Archer et al., 1994; Khan et al., 2007; Tecklenburg et al., 2006).

Table 1.1: Structural joint types | The main types of articular joints in the body are shown below. Each joint type has specific structural features that determine its degree of mobility (Alves et al., 2018; Hickman et al., 2022; Rice, 2008).

Type	Description	Location
Fibrous	Immobile Bones joined by fibrous tissue	Skull sutures
Cartilaginous	Slightly mobile Bones joined by cartilage	Intervertebral discs
Synovial	Freely movable Fluid-filled cavity	Knees Shoulder Hip

At the ends of the articulating bones lies articular cartilage, a smooth and lubricated connective tissue that lacks both nerves and blood vessels (Fox et al., 2009). It is less rigid than bone and provides a low-friction surface that minimizes resistance during motion (Eschweiler et al., 2021; Loparic et al., 2010). In DSJs, the cartilage is of the hyaline type, identifiable by its pearl-grey color and firm texture. It has an ECM containing large amounts of biopolymers such as collagen type II (COL2) and hyaluronic acid (HA), which confer the compressive, tensile, and shear strength required for joint function (Jaumard et al., 2011; O’Leary et al., 2017). As illustrated in Figure 1.1, the articular cartilage covers the proximal and distal bone surfaces within the joint capsule (Chijimatsu and Saito, 2019). The capsule itself is composed of dense collagenous connective tissue and functions to stabilise the joint while allowing free movement (Aparisi Gómez et al., 2019; Blalock et al., 2015; Ralphs and Benjamin, 1994).

The synovial cavity contains synovial fluid, produced by the synovial membrane (Schmid, 1968). This thin membrane has an outer layer of fibroblast-like synoviocytes and a deeper layer that includes blood and lymphatic vessels, adipose tissue, and nerves (Smith, 2011). Synovial cells synthesise key components, including HA and surface active phospholipids (SAP), which help regulate the composition of the fluid. The synovial fluid mainly consists of

HA, lubricin (also secreted by surface chondrocytes), and various glycoproteins (Jay et al., 2001; Nygaard and Firestein, 2020; Smith, 2011; Watkins and Reesink, 2020). It acts as both a lubricant and a nutrient medium, supplying chondrocytes with essential molecules, while also facilitating the removal of their waste byproducts through diffusion (Zhou et al., 2004).

The underlying bone is a specialised connective tissue that is crucial for providing mechanical support to the joint and serves as the rigid framework required for movement (Addison et al., 2015; Pasteris et al., 2004). It contains osteoclasts, responsible for resorption; osteoprogenitor cells or bone mesenchymal stem cells (bMSCs), which differentiate into osteoblasts; and osteocytes, which help maintain bone tissue (Bernhardt et al., 2020; Leitao et al., 2020). The remaining bone ECM consists mainly of inorganic calcium salts and collagen type I (COL1) fibers (Boskey et al., 2002). Together these form the structural base beneath the articular cartilage and synovial cavity (Chijimatsu and Saito, 2019). In growing bones, a layer of cartilage known as the growth plate separates the epiphysis from the diaphysis, enabling longitudinal bone growth until it is replaced by bone at skeletal maturity (Xie et al., 2020).

1.1.1 Hyaline Cartilage

The physical properties of the hyaline cartilage ECM are largely governed by electrostatic interactions between its molecular components (Rojas et al., 2014). The mechanical resilience of cartilage arises from the contribution of the ECM components which in tandem act as an energy-dissipating medium capable of absorbing and dispersing mechanical loads. Cartilage exhibits elastic and viscous (viscoelastic) behaviour, ensuring the ability to deform under stress and recover its shape, while dissipating part of the applied energy as heat (June and Fyhrie, 2010).

A key decisive player of this viscoelastic response is the high-water content bound to negatively charged HA and proteoglycan (PG) chains (Ebrahimi et al., 2020; Petitjean et al., 2023; Rojas et al., 2014). This confined water increases the tissue's incompressibility, while providing additional load-bearing capacity under both compressive and hydrostatic stresses (Ebrahimi et al., 2020; Jaumard et al., 2011; Petitjean et al., 2023; Rojas et al., 2014). Energy dissipation further depends on the poroelastic nature of the ECM and its ability to undergo reorganisation as consequence of being under load. During compression or shear, fluid moves within the porous matrix, while coincidentally rearranging collagen fibres and PGs generating internal friction contributing to the diffusion of mechanical energy (Nia et al., 2011).

Physiological cartilage lacks a direct blood supply (due to being an avascular tissue) resulting in chondrocytes having an inefficient repair capability since these have reduced metabolic activity causing insufficient synthesis and matrix deposition (Mapp and Walsh, 2012). These limitations provide this tissue a great disadvantage, since it tends to be replaced by fibrocartilage during cartilage repair (following injury) (Sardroud et al., 2025). This in contrast to hyaline cartilage, possesses inferior biomechanical properties exhibiting significantly re-

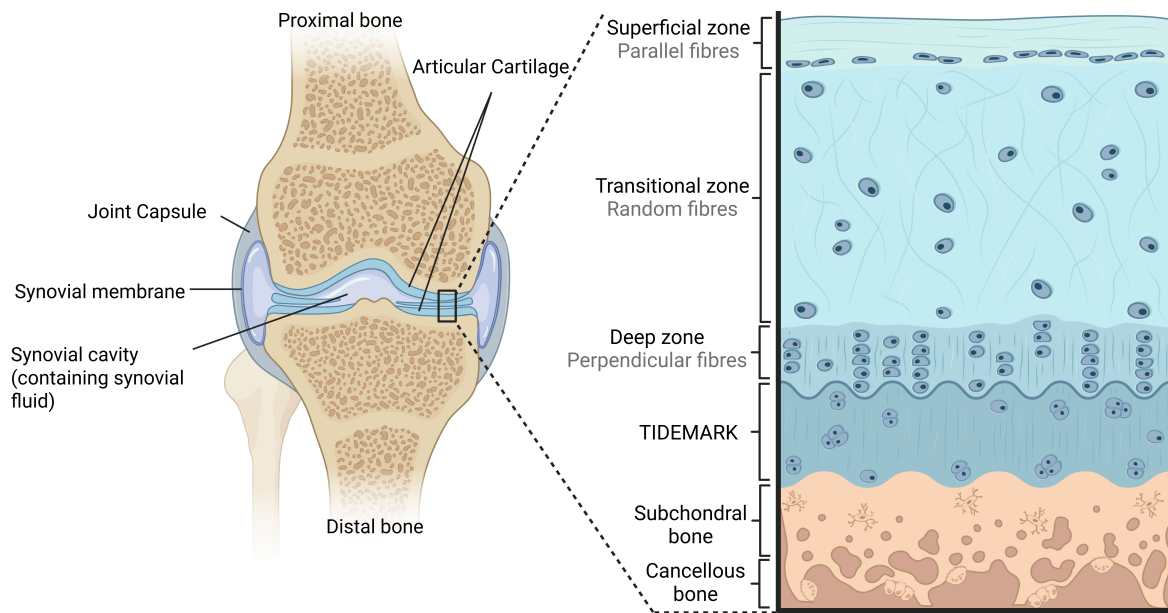


Figure 1.1: Diarthrodial synovial joint and organisation of the cartilage | The diarthrodial joints are formed of various components which together ensure joint stability and movement. The synovium ensures diffusion of nutrients, minerals and proteins to the chondrocytes within the articular cartilage along with lubricants within the fluid. Any waste or cellular debris can be diffused from the articular cartilage into the synovial fluid to be then removed by the synoviocyte cells within the synovial membrane. All cartilage zones contain chondrocytes that are aligned in a specific conformation complementary to the zones they are located. Its important to note the chondrocytes in the tidemark zone are hypertrophic, explained further in subsection 1.1.2 (Buckwalter et al., 1994; Chaudhary et al., 2015; Smith, 2011). Created in <https://BioRender.com>

duced capacity to endure repetitive mechanical stress (Schinagl et al., 1997).

The structure of cartilage tissue with its characteristic layers/zones is shown in figure 1.1. The components that constitute the cartilage tissue are arrayed into specific structures that prove complimentary to its properties within the joint (Buckwalter et al., 1994; Chaudhary et al., 2015). Cartilage matrix components are essential for homeostasis contributing to its inherent properties to hinder mineralisation (section 1.1.2 explains this occurrence related to the OA pathology) (Aisenbrey and Bryant, 2019; Lian et al., 2019). Below are several of these and an outline of how they commit to providing a surface that can bear loads and movement.

1.1.1.1 Collagen

Hyaline cartilage contains a high abundance of collagen fibres, especially those consisting of COL2, which contribute to the tissue's three-dimensional structure providing strength and support to cartilage (Shoulders and Raines, 2009). Collagen is a protein which tends to not include the amino acid, cysteine (Kadler et al., 1996). Collagen monomers are formed from three parallel polypeptide strands, each in a left-handed polyproline II-type helical conformation (Cai et al., 2021a; Shoulders and Raines, 2009). These three strands intertwine to form

a single right-handed triple helix. This is a quaternary structure being stabilised by hydrogen bonds with the unique amino acid sequence of glycine (Gly)-X-Y amino acids, where X and Y are often proline (Pro) or hydroxyproline (Hyp) (Cai et al., 2021a). Collagen fibres assembled from the linear arrangements of monomers have diameters ranging from 20 to 500 nm (Siadat et al., 2021). Notably, collagen only reveals its inherent Arginine-Glycine-Aspartic acid (RGD) sequence following denaturation to gelatin (Pawelec et al., 2016).

As previously mentioned, the predominant collagen type is COL2 (90-95% abundance), this is followed by collagen type IX (COL9) and collagen type XI (COL11), together these the principal collagens responsible for the properties of cartilage (Fox et al., 2009). COL2 is described further below. COL9 is found on the surface of COL2 fibrils in hyaline cartilage and contains both collagenous and non-collagenous domains essential for ECM interactions (Olsen, 1997; Parsons et al., 2011). It stabilises the cartilage matrix through covalent crosslinking to COL2 and itself, influencing fibril spacing and cartilage biomechanics (Diab et al., 1996). Deficiency in COL9 can worsen arthritis by promoting collagen-targeting antibodies (Carlsen et al., 2006). Similarly, COL11 plays a regulatory and structural role, associating with COL2 and COL9 to maintain matrix stability and regulate fibril growth and organisation (Sun et al., 2020; Wu and Eyre, 1995). The ratio of COL11 to COL2 affects fibril thickness, impacting mechanical properties (Holmes and Kadler, 2006).

Minor collagens located within cartilage include collagen type V (COL5), collagen type VI and collagen type X (COL10) (Alcaide-Ruggiero et al., 2021). Notably, COL5 plays a regulatory role in relation to the collagen fibrils. This includes the assembly and diameter control of collagen fibrils, including COL1 and COL2 (Alcaide-Ruggiero et al., 2021; Kadler et al., 2008; Wenstrup et al., 2004). This collagen hybridises with COL11 to form a hybrid which is subsequently essential for COL11 mediated nucleation and regulation of COL2 fibrillogenesis (Alcaide-Ruggiero et al., 2021; Kadler et al., 2008).

Collagen type II

The COL2 protein fibres are a structural motif, formed of three parallel polypeptide strands in a polyproline II-type helical conformation coil. These have the molecular composition of $[\alpha 1(\text{II})]$ (Shoulders and Raines, 2009). These fibres can interact with PG, and HA to form the cartilage matrix. Essentially, COL2 acts as a structural component, providing the support for the ECM, via the formation of a highly organised dense network of fibres. This provides the tissue's resilience and mechanical strength (Aszodi et al., 2001; Fox et al., 2009).

COL2 has unique amino acid sequences, which binds to the chondrocyte's extracellular integrins. Therefore, in addition to its structural role within the ECM, COL2 play an important role as a signalling molecule. Notably, activating the aiding in the regulation of chondrocyte behaviour: proliferation, metabolism, and differentiation (see subsection 1.1.2) (Wu et al., 2021; Xin et al., 2015).

Loss or degradation of COL2 is detrimental to cartilage being associated with pathologies such as OA, see section 1.1.3 (Poole et al., 2002). Furthermore, there is evidence, COL2

has the ability to suppress OA, demonstrating it is a vital component of cartilage homeostasis (Lian et al., 2019).

1.1.1.2 Proteoglycans

The PGs are highly glycosylated molecules and are comprised of a core protein which is covalently conjugated to at least one long GAG chain (Iozzo and Schaefer, 2015). PGs are one of the principal constituents of cartilage ECM being highly hydrated (due to the GAG's highly negatively charged sulphate and carboxyl groups - see subsection 1.1.1.3) (Hernandez et al., 2015). These structures are highly hydrated, their bound water molecules, provides the tissue a load distributing ability to resist compressive forces while maintaining hydration and ECM integrity (Fox et al., 2009). PG examples include aggrecan, biglycan and decorin (Han et al., 2021; Watanabe et al., 1997).

Aggrecan

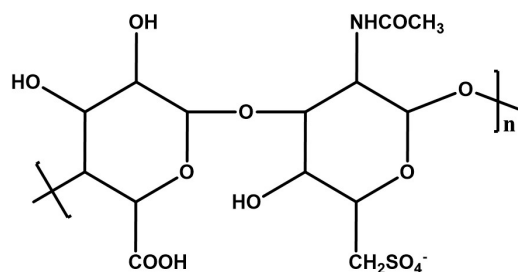
Aggrecan is a major PG embedded in the highly organised cartilage ECM, having a vital role in enabling cartilage to withstand compressive forces. Thus, preserving normal joint function. It forms large aggregates within the ECM via binding to HA at its N-terminal G1 domain, with such interaction believed to be stabilised by the link protein (see subsection 1.1.1.4) (Watanabe et al., 1997). Also, this PG contains an interglobular domain, being a short extended segment which provides a complementary site for proteolytic cleavage by proteinases including matrix metalloproteinase (MMP)s (Struglics and Hansson, 2012; Watanabe et al., 1997). Another domain, G2, is involved in a proteolytic target for aggrecanases and may be involved in product processing (Kiani et al., 2003). The GAG chains (such as CS and keratan sulphate (KS)) that aggrecan contains are linked to specific serine residues on the core protein. These sites are located at consensus sequence that includes a serine (Ser)-Gly dipeptide. These ensure a PG structure being highly hydrated due to bound water (Kiani et al., 2002; Li et al., 1993).

1.1.1.3 Glycosaminoglycans

The GAGs are long linear polysaccharides composed of repeating disaccharide units. As previously mentioned, these are highly negatively charged due to carboxyl and sulphate groups (Hernandez et al., 2015). These are typically covalently linked to the PG's core protein (Iozzo and Schaefer, 2015). Therefore, the GAGs provide load bearing strength and have the ability (negative charges) to attract both water and cations (Hernandez et al., 2015). As a result, this allows for an osmotic swelling pressure which is considered to aid the ECM's ability to maintain its resilience and spacing between strands/fibrils (Han et al., 2011). Furthermore, the biosynthesis of these biopolymers is correlated with chondrogenic differentiation (Vincent et al., 2025). Various GAGs exist in cartilage, including CS, dermatan sulphate (DS) and HA

(Fox et al., 2009).

Chondroitin sulphate



Chondroitin Sulphate

Figure 1.2: Chemical Structure of Chondroitin-6-Sulphate monomer | Here shows the repeating disaccharide monomer composed of both β -D-glucuronic acid (GlcA) and β -D-N-acetylgalactosamine (GalNAc). The GalNAc is sulphated at either the 4th or 6th carbon atom of the carbon ring (in this figure chondroitin-6-sulphate is shown) (Shen et al., 2023).

As a quintessential GAG, CS is highly prevalent in connective tissues, constituting a fundamental component of cartilage, where its elevated abundance is integral to the tissue's structural resilience and physiological function (Shen et al., 2023). These polymers vary in molecular weight (thus, the polymer length) with humans being up to 45 kilodalton (kDa) while murine sourced can be approximately up to 70 kDa (Henrotin et al., 2010).

As per figure 1.2, two disaccharides form the CS monomer unit, being D-glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc), with the latter having the notable sulphate group ($-\text{SO}_4^-$) (Cunha et al., 2015; Yang et al., 2021a). Its predominantly synthesised by the golgi apparatus of chondrocytes by a variety of enzymes, commencing with the glycosyltransferases: CS N-acetylgalatosaminyltransferase 1 and 2 (Adhikara et al., 2021; Vertel et al., 1989; Watanabe et al., 2010). These initiate and then elongate the polysaccharide chain via the addition of GalNAc, with the chondroitin sulphate glucuronyltransferase adding the subsequent GlcA residues to form the GalNAc and GlcA linked monomer (Adhikara et al., 2021; Izumikawa et al., 2008; Watanabe et al., 2010). To sulphate these monomers at the GalNAc residue, which requires sulphotransferases including chondroitin 4-O- and chondroitin 6-O sulphotransferase to catalyse the formation of chondoritin 4- and chondroitin 6-sulphate respectively. These function via the addition of the sulphate group at these specific positions of the carbon ring (Yamada et al., 2004).

Hyaluronic acid

The negatively charged HA is one of the major hyaline cartilage components (Gupta et al., 2019). It is a relatively short (15-20 kDa) non-sulphated unbranched GAG consisting entirely of a repeating disaccharide of GlcA and GalNAc. It is synthesised by three transmembrane synthase proteins HAS1, HAS2 and HAS3. This polymer is present in both the synovial fluid and, more notably, within the articular cartilage, where it localises around chondrocyte cells and forms a protective coat. The HA's main role is to provide cartilage its resilience by

absorbing water. A previous study has demonstrated HA is the primary mediator of chondrocyte matrix interactions (Knudson and Loeser, 2002). While, evidence provided by various studies has demonstrated the ability for this biopolymer to downregulate cytokines (Wang et al., 2006b), free radicals (Berdiaki et al., 2023) and catabolic enzymes (Ozawa et al., 2015; Wang et al., 2006a). Together, this can potentially protect cartilage from becoming pathogenic (Gupta et al., 2019).

1.1.1.4 Glycoproteins

Glycoproteins are proteins with short covalently-linked branched oligosaccharide chains along with being a main cartilage component (Han et al., 2022). Glycoproteins ensure chondrocytes anchor to the ECM, contributing to the overall structural integrity and biochemical signalling microenvironment (Cui and Zhang, 2022). Two examples of glycoproteins include cartilage oligomeric matrix protein (COMP) and link protein. These contribute substantially to matrix stability and integrity, alongside the other key components (previously mentioned) which mediate both cartilage formation and function (Cui and Zhang, 2022; Hardingham, 1979).

Cartilage oligomeric matrix protein

The pentameric glycoprotein, cartilage oligomeric matrix protein (COMP), predominantly resides within the ECM and belongs to the thrombospondin family, with each of its subunits comprising a series of sophisticated domains (Riessen et al., 2001; Verma and Dalal, 2013). These include epidermal growth factor-like repeats and via its N-terminal coiled-coil domain, with extending arms being adorned with functional domains essential for calcium ion coordination, ensuring the proper folding and molecular stability for the COMP molecule (Cui and Zhang, 2022).

This glycoprotein appears to be fundamental for ECM structural integrity and resilience via interactions, with various ECM components especially the collagens (Gebauer et al., 2018). Studies have shown COMP to have a role in fibrillogenesis, mediating chondrocyte adhesion and proliferation along with mesenchymal stem cells (MSC) chondrogenic differentiation (Chen et al., 2005; Halász et al., 2007; Haleem-Smith et al., 2012). Furthermore, is vital in fortifying the tissue to ensure mechanical robustness of cartilage (Wang et al., 2017).

Link protein

Link protein specifically binds to the aggrecan core protein along with interacting with HA, stabilising the aggrecan-HA aggregates (ternary complex) (Shi et al., 2004). Their mechanical properties provide cartilage to function as a shock absorber, necessitate these aggregates to be larger, more uniform and stable. Essentially, the primary role of link protein is to aggregate and stabilise the proteoglycans (Mow et al., 1989).

1.1.2 Cartilage cells

Cells located within the cartilage have dual roles of forming cartilage ECM along with maintaining its homeostasis (Pang et al., 2025). The MSCs (located in the superficial cartilage zone and synovial membrane) differentiate into chondroblasts (located in the perichondrium), being the precursor cells prior to mature chondrocyte differentiation (Sousa et al., 2014). However, following fetal and early postnatal stages the perichondrium begins to disappear since the cartilage matures (Fox et al., 2009). Another cell type that is found scarcely in physiological cartilage are senescent chondrocytes, with these being further explained in subsection 1.1.3 (Huang and Cai, 2025).

Chondroblasts and Chondrocytes

During cartilage development, MSCs have the ability to condense together followed by differentiating into chondroblasts which are immature cartilage-forming cells (Schwarzl et al., 2023). These are located at the periphery of growing cartilage, specifically beneath the perichondrium and at the epiphyseal plate sites (Ballock and O’Keefe, 2003; Von Der Mark and Von Der Mark, 1977). These cells have the ability to produce and secrete the ECM components (including COL2 and PGs (CS, etc.)). As a consequence of producing and secreting the surrounding ECM, these mature into chondrocytes (Fox et al., 2009; Karlsen et al., 2011; Sousa et al., 2014).

Chondrocytes are the mature cartilage producing cells located within mature cartilage (Galéra et al., 1992). These cells are located specifically at various zones within cartilage called lacunae and have a oval morphology with a visibly observed nucleus. These cells continuously maintain and reorganise the ECM to ensure homeostasis (Fox et al., 2009; Hall, 2019). Overall, mature chondrocytes are exempt from proliferation; however, it has been reported that these cells can undergo cellular division when required to repair cartilage (Nalesso et al., 2011).

Hypertrophic chondrocytes

Chondrocytes shift to a hypertrophic stage which proceeds these having undergone proliferation and/or maturation. Such hypertrophic differentiation is characterised by an extremely enlarged chondrocyte morphology (up to 20 fold enlargement) and heightened metabolic activity (Aisenbrey and Bryant, 2019; Hallett et al., 2021; Lian et al., 2019). Furthermore, they transition from a typical chondrogenic expression of COL2 and commence overexpressing COL10 among other unique markers (see subsection 1.1.3.1) (Chen et al., 2023).

Hypertrophic chondrocytes have the inherent ability to transition into osteoblasts or chondroclasts, the latter being chondrocytes involved in producing catabolic mediators to aid in the resorption of cartilage (Aghajanian and Mohan, 2018; Yang et al., 2014). These cells physiologically aid in bone growth, being normally located in the growth plate during endochondral ossification (long bone growth) (Chagin and Chu, 2023). They mediate this by having the ability to promote ECM mineralisation via modifying the surrounding matrix (by catabolising

the ECM) to ensure its susceptible to calcification. This occurs since they produce factors (receptor activator of nuclear factor kappa-B ligand (RANKL)) which ensures cartilage is catabolised since such factors promote the formation of chondroclasts and osteoclasts (Li et al., 2023). To convert avascular cartilage to vascularised bone, the production and secretion of vascular endothelial growth factor (VEGF) occurs resulting in angiogenesis. This is required to ensure the conversion of cartilage to bone is aided further (Carlevaro et al., 2000). The newly formed bone tissue produces COL1, COL10 and alkaline phosphatase (ALPL) which are further explained in subsection 1.1.3.1 (Aghajanian and Mohan, 2018).

1.1.3 Osteoarthritis

OA is a degenerative joint disease that affected an estimated 595 million people worldwide in 2020. It is a major cause of reduced work capacity and a key contributor to musculoskeletal pain, particularly in the lower back (FJ), knee, etc. (Hsu and Siwiec, 2023; Jaumard et al., 2011). The condition is characterised by progressive degeneration of articular joints and loss of cartilage, leading to pain that ranges from mild discomfort to severe, disabling symptoms as the disease advances (Neogi, 2013).

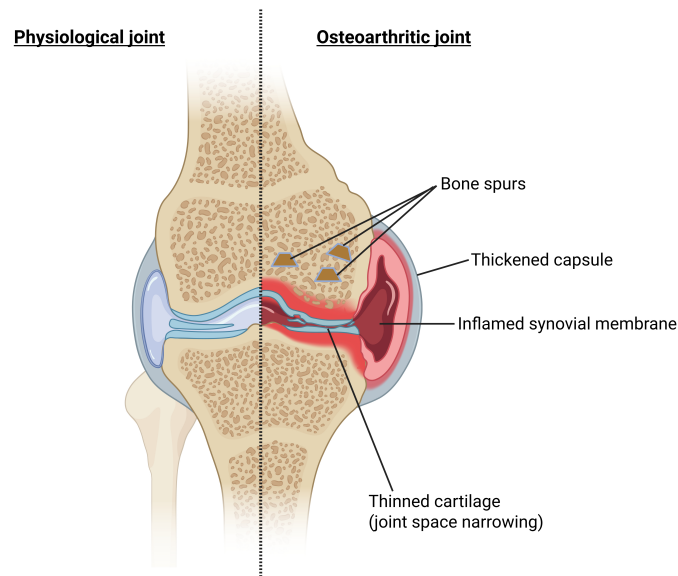


Figure 1.3: Physiological joint vs osteoarthritic joint | Here illustrated is intact cartilage and normal joint space of the physiological articular joint. Versus cartilage degradation, joint space narrowing, and formation of osteophytes (bone spurs) in the osteoarthritic joint. Notably cartilage is not solely affected, with the joint capsule becoming thickened along with the presence of the inflamed synovial membrane (Yuan et al., 2014). Created in <https://BioRender.com>

The pathology involves a gradual breakdown of the cartilage matrix, where tissue homeostasis is disrupted (Elder et al., 2009; Kim et al., 2015). As the protective capacity of the cartilage declines, bone-on-bone contact occurs, intensifying structural damage and pain (Halmandge et al., 2025). This is accompanied by subchondral bone remodeling, synovial inflammation, and deterioration of adjacent tissues (Li et al., 2013; Yuan et al., 2014). Such processes

exacerbate cartilage erosion, amplifying symptoms and disease progression (O'Neill and Felson, 2018).

Unlike rheumatoid arthritis, which has an autoimmune origin and a rapid onset, OA typically develops over years due to mechanical stress and localised inflammation (Chauhan et al., 2025; Hsu and Siwiec, 2023; Im, 2022; Zhang et al., 2024b). OA can be divided into primary and secondary forms. Primary OA results from degenerative cartilage associated with ageing, along with wear and tear, and tends to affect single, weight-bearing joints. Secondary OA arises from a known cause, such as physical trauma, inflammatory conditions, etc., and typically affects atypical joints (Hsu and Siwiec, 2023). In contrast, rheumatoid arthritis usually presents rapidly affecting multiple joints (Chauhan et al., 2025).

The onset of OA results from complex molecular and cellular interactions that disrupt cartilage homeostasis (Corciulo et al., 2017). Excessive mechanical loading increases water content in the superficial zone of articular cartilage, followed by degradation of glycosaminoglycans (GAGs) and proteoglycans (PGs). As the matrix swells, chondrocytes in deeper zones undergo hypertrophic differentiation (see subsection 1.1.2) (Goldring and Goldring, 2010). This process promotes chondrocyte senescence, leading to functional decline. Both hypertrophic and senescent chondrocytes secrete degradative enzymes such as aggrecanases, chondroitinases, and (MMPs), which accelerate breakdown of the extracellular matrix (ECM) and trigger inflammation (Jung et al., 2019; Liu et al., 2016a).

At the molecular level, dysregulated signalling pathways including tumour growth factor- β (TGF- β)/Smad, Wnt/ β -catenin, and HIF-2 α disrupt the balance between cartilage synthesis and degradation, promoting hypertrophy, dedifferentiation, and tissue damage (Wu et al., 2012). Metabolic stress further shifts chondrocyte activity toward catabolism while reducing new matrix formation, a process aggravated by oxidative stress and mitochondrial dysfunction (Tan et al., 2025).

Hypertrophic and senescent chondrocytes also produce aggrecanases (a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and ADAMTS-5), which degrade aggrecan, while chondroitinases break down PG-linked GAG chains, leading to ECM disintegration (Bai et al., 2009; Miwa et al., 2006; Rim et al., 2020). Alongside these, MMPs intensify cartilage loss and local inflammation (Jung et al., 2019; Liu et al., 2016a). In particular, MMP-13, secreted predominantly by hypertrophic chondrocytes, contributes to cartilage calcification and osteophyte formation (see subsection 1.1.2) (Jung et al., 2019; Liu et al., 2016b; Struglics and Hansson, 2012).

Inflammation plays a central role in OA progression. Pro-inflammatory cytokines (interleukin (IL)-1 β , tumour necrosis factor (TNF)- α , IL-6), produced by chondrocytes, synoviocytes, and immune cells, activate degradative enzymes and reactive oxygen species (Corciulo et al., 2017). Cartilage-derived damage-associated molecular patterns stimulate immune receptors such as toll-like and nod-like receptors, promoting chronic inflammation. Activation of the NOD-, LRR-, and pyrin domain-containing protein 3 inflammasome induces pyroptosis

and further release of IL-1 β , perpetuating tissue destruction.

Cartilage degradation by MMPs exposes nerve endings within the joint, contributing to pain sensitisation. These enzymes, together with inflammatory mediators, activate nociceptors and enhance signalling through neuropeptides such as substance P (SP). Released by sensory nerve fibres, SP amplifies inflammation and pain by inducing IL-1 β production, oxidative stress, and cell death. Synovial inflammation and subchondral remodelling further increase SP levels, reinforcing a cycle of pain and inflammation (Mapp and Walsh, 2012; Mathiessen and Conaghan, 2017; Morgan et al., 2022; Sakai et al., 1998).

Together, these interconnected molecular, cellular, and inflammatory processes disrupt cartilage integrity, drive progressive joint damage, and sustain the chronic pathology of OA.

1.1.3.1 Main Extracellular Matrix Hallmarks of Osteoarthritis

Hypertrophic chondrocytes produce a bone-like ECM once the OA affected cartilage being resorbed, via three main bone matrix components including COL1, COL10 and ALPL (Aghajanian and Mohan, 2018). Together, apart from being associated with chondrocyte hypertrophy processes, these are also the main OA matrix hallmarks, explained below:

Collagen type I

The predominant collagen within bone is COL1, being the scaffold where mineralisation by calcification (via ALPL) occurs. These collagen fibres form strong rod structures within the ECM, providing structural support along with tensile strength (Amirrah et al., 2022). Also, when overexpressed is a notable hallmark of chondrocyte hypertrophy or during repair where fibrous cartilage is deposited instead of hyaline cartilage (Hosoya et al., 1998).

Collagen type X

Notably, COL10 is the main hallmark of chondrocyte hypertrophy during the endochondral ossification process (Chen et al., 2023; He et al., 2014). This protein fibre is less fibrous in comparison to COL1 and is upregulated during repair processes (Chen et al., 2023). This collagen tends to be located in the hypertrophic zone of cartilage, at sites transitioning to mineralised tissue (He et al., 2014).

Alkaline Phosphatase

The ALPL enzyme is expressed by hypertrophic chondrocytes and osteoblasts, it manages to catalyse the mineralisation of COL1 to produce the typical calcified bone ECM. This enzyme facilitates the hydrolysis of the effective mineralisation inhibitor, inorganic pyrophosphate (PPi) (present in OA cartilage and synovial fluid), to form inorganic phosphate (Pi). The formation of the latter results in the surrounding ECM being enriched with Pi which is necessary to ensure the nucleation and deposition of hydroxyapatite crystals (Vimalraj, 2020). This is the main mineral within bone, with the chemical formula of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, depicting a high content of calcium ions (Ca^{2+}) (Bogdanoviciene et al., 2006). Together, with its action

upon mineralising COL1 ensures cartilage calcification is successful (Hosoya et al., 1998).

1.1.3.2 Treatment Options

Traditionally, OA treatment has focused on symptom relief, via managing pain using supplements such as CS, non-steroidal anti-inflammatory Drugs (ibuprofen), with medical professionals also prescribing antidepressants approved for pain management (duloxetine) (Leaney et al., 2022; Richard et al., 2023). Also, medical professionals imply preventive measures play an increasingly prominent role in alleviating OA onset and symptoms. Primarily, ensuring an individual's healthy diet and regular physical activity to maintain joint mobility (lifestyle modifications) along with promoting appropriate posture, resulting in a reduction of mechanical stress upon joints (Dhollander et al., 2016).

When the previously mentioned approaches are insufficient or pain becomes severe, surgical interventions are necessary. For spinal OA affecting the intervertebral disc (IVD) and/or FJ, spinal fusion reduces painful motion by immobilising affected vertebrae. Modern medicine has ensued advances in joint replacement surgeries (robotic-assisted ensuing minimally invasive techniques) have improved these treatments due to decreasing long-term pain while enhancing mobility (O'Leary et al., 2018).

Despite such approaches, no effective cure currently exists, with recent strategies targeting OA's underlying pathological mechanisms (Cai et al., 2021a). In regards to potential future molecular and biologic therapies appear promising. Gene-silencing techniques, such as specific short interfering ribonucleic acid (siRNA) which successfully target complementary genes implicated in mineralisation, joint inflammation and pain (Yan et al., 2019; Zhou et al., 2021). Regenerative medicine approaches, particularly those involving MSCs, have demonstrated potential for joint repair (Wu et al., 2024). An ever more applied approach is platelet rich plasma therapy representing an efficient cost-effective option, has managed to demonstrate pain relief, though its long-term benefits require further study since certain considerations are required (adequate dose of platelets) etc. (Schwitzer et al., 2025).

1.2 Biomaterials

Biomaterials are defined as "a material designed to take a form that can direct, through interactions with living systems, the course of any therapeutic or diagnostic procedure" (Zhang et al., 2019). Biomaterials can be derived from natural or synthetic sources. Natural biomaterials tend to better mimic native ECM and have enhanced capabilities in supporting cellular functions essential for tissue regeneration. As a result, these generally offer superior biocompatibility, reduced immune rejection, and better promote tissue integration. Synthetic biomaterials offer tunable mechanical properties (stability) and degradation rates but may lack necessary biological cues unless functionalised with certain natural biopolymers (Riaz

et al., 2025).

This thesis project utilises hydrogels based on natural biomolecules, namely dECM, CS, and quercetin. Below is an introduction to these matrices, focusing especially on their production and applications in cartilage tissue engineering.

1.2.1 Background – What is a Hydrogel?

The concept of a hydrogel originated due to the pioneering endeavours undertaken by Otto Wichterle and Drahoslav Lim in 1960, whom managed to successfully utilise such porous hydrated scaffolds for biomaterial applications. Their published work established the basis for identifying hydrogels as hydrophilic, three-dimensional (3D) polymeric networks which have the capability of absorbing large portions of water while maintaining structural integrity. They managed to synthesise a water-absorbing polymer, poly(2-hydroxyethyl methacrylate), to form hydrophilic gels which identifiably were stable, had both low stiffness and transparent properties (optically clear), while being biocompatible. Such characteristics ensured these would be ideal biomaterials for direct contact with the eye, being applied as contact lenses to substitute eye spectacles (Wichterle and Lim, 1960).

The polymers within the hydrogel can undergo physical or chemical crosslinking. Physical crosslinking tends to occur due to electrostatic attractions between the negative and positive charges. These electrostatic attractions, in the case of this doctoral work occurred optimally at 37 °C (figure 4.2A). When there are imbalances between the charged molecules, there may be discrepancies in their rheological behaviour and gelation kinetics (Wilbanks et al., 2025). While, chemical crosslinking involves the introduction of covalent links between complementary functional chemical groups of two or more polymers, see section 1.3.1 (Chuang et al., 2018).

In this thesis project, hydrogels are produced from dECM. The following sections present the approaches used for dECM retrieval, hydrogel production, and the characterisation of their rheological and gelation kinetics properties. Additionally, the functionalisation of dECM-based hydrogels is examined through the incorporation of CS and its aldehyde modified derivative (mCS), as well as quercetin. The role of quercetin in promoting chondrogenesis is examined, within an mCS functionalised hydrogel. In combination, these represent the constituents of the hydrogels analysed here.

1.2.1.1 An Overview of Decellularised Matrices with a Focus on Cartilage Decellularised Matrixes

Utilising dECMs holds significant biomedical value while supporting sustainability by converting animal by-products from the meat industry into valuable biomaterials. This reduces biological waste and prevents harmful substances from leaching into groundwater, thus pro-

protecting the environment and public health (Corridon et al., 2025; Salim et al., 2021). The first decellularisation approach producing acellular biomaterials was introduced in 1948 by We Poel, focusing on decellularised muscle homogenates (Poel, 1948). The field advanced notably with Stephen Badylak's 1995 work on acellular scaffolds derived from small intestinal submucosa for tissue repair, enabling wider biomaterial applications (Badylak et al., 1995).

Developing dECM-based scaffolds involves three main steps: decellularisation of donor tissue, recellularisation with appropriate cells, and application to mimic the original tissue. The scaffold may be ground and moulded to prevent cavities or left as a ground form for implantation (Golebiowska et al., 2024). Alternatively, it can be processed into hydrogels using acid/base solutions and salt gradients (see section 1.2.1) (Bordbar et al., 2020). Decellularisation is crucial to avoid immune reactions (Gvaramia et al., 2022), but typically reduces mechanical strength (Bordbar et al., 2020), making reinforcement necessary, especially when thermo-mechanical properties and cell-matrix interactions influence cell fate. Although no standard operating protocol exists globally, effective dECM must meet criteria such as less than 50 ng double strand deoxyribonucleic acid (dsDNA) per mg dry weight, DNA fragments below 200 base pairs, and absence of nuclear material by staining (Hengel et al., 2025). Scaffolds can be recellularised with various cells, including MSCs, providing a 3D culture microenvironment that enhances proliferation and cell viability (Zhang et al., 2024a).

The impact of decellularisation on the biointerface depends on the type and concentration of detergents, as well as temperature, since these factors influence ECM composition, mechanical properties, and cell interactions (Fernández-Pérez and Ahearne, 2019; Kostelnik et al., 2022; Mendibil et al., 2020). Common detergents include sodium deoxycholate (SDC), sodium dodecyl sulphate (SDS), and Triton X-100 (see table 1.2) (Mendibil et al., 2020). Protocols often combine detergents like SDS and Triton X-100 for efficacy (Cai et al., 2021b). Freeze-thaw cycles can precede detergent exposure to lyse cells, as this approach may reduce the required detergent exposure, thereby enhancing ECM preservation (Fernández-Pérez and Ahearne, 2019). Vacuum-assisted decellularisation employing negative pressure enhances reagent penetration and shortens processing time compared to traditional methods. This proves effective for tissues such as the nucleolus pulposus and organs such as the trachea (Botelho et al., 2025; Den Hondt et al., 2017). Ultrasonic methods combined with detergents such as SDS mitigate ECM damage (Lin et al., 2021). Overall, modern approaches strive to remove cells without compromising ECM integrity.

Cartilage decellularisation was first reported by Schwarz et al. in 2012, establishing it as a promising biomatrix for cartilage tissue engineering (Schwarz et al., 2012). Cartilage-derived dECM retains key structural and biochemical components including COL2 and GAGs (Sun et al., 2018), which support chondrocyte attachment and matrix synthesis. The rigid cartilage matrix complicates complete decellularisation without damaging matrix integrity (Bordbar et al., 2020). Optimizing cartilage decellularisation requires balancing efficient cell removal with preservation of extracellular matrix architecture and bioactivity (Bordbar et al., 2020; Yari et al., 2024). Achieving this is vital to produce functional scaffolds or hydrogels replicating native

Table 1.2: Detergents utilised in decellularisation | Main detergent types used in decellularisation approaches with their advantages and disadvantages (Fernández-Pérez and Ahearne, 2019; Kostelnik et al., 2022; Mendibil et al., 2020).

Detergent	Description
SDC	Ionic bile salt detergent effective at removing cytoplasmic proteins with low ECM damage.
SDS	Ionic detergent significantly effective at removing cellular and nuclear material but detrimental to ECM components, especially GAGs.
Triton X-100	Non-ionic detergent that solubilises cell membranes and preserves most ECM structure and proteins, though less effective at removing nuclear material than SDS.

cartilage environments. Moreover, combining dECM with polymers like polyvinyl alcohol has enhanced its potential as cartilage tissue engineering scaffolds (Yari et al., 2024).

1.2.1.2 Decellularised extracellular matrix-based hydrogels

Hydrogels derived from dECM were first reported in 2008 by *Freytes et al.*, who successfully used a pepsin 0.01 M hydrochloric acid (HCl)-based solution to solubilise urinary bladder dECM. This solution was then neutralised with 0.1 N sodium hydroxide (NaOH) and exposed to 10X phosphate buffered saline (PBS). Evaluation of the produced hydrogels revealed their suitability for therapeutic approaches, providing scaffolds with biological and biomechanical mimicry of physiological conditions, alongside minimal immunogenicity (Freytes et al., 2008). Overall, dECM offers a platform for reconstituting and repairing damaged tissues (Bordbar et al., 2020; Freytes et al., 2008).

The feasibility of using these hydrogels as biomaterials depends on how the dECM was attained. It has been shown that decellularisation protocols influence the properties of the resulting hydrogels. A study focusing on detergent-based protocols using SDC, SDS, and Triton X-100, with or without freeze-thaw cycles, reported that SDS-mediated decellularisation of porcine corneas produced cytotoxic hydrogels (Fernández-Pérez and Ahearne, 2019). However, other publications demonstrate the feasibility of using SDS, depending on the tissue type and the intended biomaterial application (Bordbar et al., 2020; Botelho et al., 2025; Lee et al., 2024; Turan Sorhun et al., 2023).

A current concern with dECM-based hydrogels is their lack of mechanical mimicry (see subsection 1.2.1.3) relative to native cellularised ECM properties. This issue parallels observations in solid-state scaffolds, where solubilisation strategies may exacerbate the loss of mechanical properties (also discussed in subsection 1.2.1.3).

Cartilage-derived dECM hydrogels represent a promising platform for cartilage tissue engi-

neering. These hydrogels originate from solubilised native articular cartilage ECM, characterised by a high content of COL2, PGs, and GAGs (see subsection 1.1.1). These components provide unique biochemical cues supporting chondrogenic maintenance and differentiation. The hydrogels display viscoelastic properties and structural features that partially reproduce the cartilage tissue's biological microenvironment (Bordbar et al., 2020).

Typically, cartilage-derived hydrogels exhibit shear-thinning behaviour and thermoresponsive gelation, enabling their application as injectable scaffolds for cartilage repair. Their biochemical composition fosters chondrocyte viability and extracellular matrix deposition, while modulating inflammatory responses *in vivo*. Despite their relative softness compared to native cartilage, the biological functionality of these hydrogels enhances tissue integration and regenerative potential (Bordbar et al., 2020). To enhance their mechanical strength and stability, functionalisation with natural or synthetic molecules is performed (see subsection 1.3.1) (Dawlee et al., 2005; Liu et al., 2017; Sousa et al., 2022; Strehin et al., 2010).

Importantly, dECM-based scaffolds facilitate three-dimensional culture environments, promoting stronger mimicry of the *in vivo* microenvironment. Such an improved environment enhances chondrogenic differentiation due to better cell–cell and cell–matrix interactions (Chu et al., 2025).

1.2.1.3 Rheology & Gelation Kinetics for Decellularised Cartilage-based hydrogels

Rheology is the study of the deformation and flow characteristics of gels and viscous liquids, and the behaviour of gels is dictated by their responses to such deformation and flow. These responses depend on the interplay between structural resilience (solid-like features) and fluid adaptability (liquid-like behaviour), which vary with mechanical forces and temperature. Rheological analysis of hydrogels therefore provides key insights into their mechanical performance and suitability for biological applications (Malkin et al., 2023).

Hydrogel stiffness is a critical property, as the mechanical characteristics of the microenvironment influence biomechanical cues perceived by encapsulated cells. The phenotype of dedifferentiated chondrocytes is particularly dependent on substrate stiffness, which helps regulate cell morphology, cytoskeletal organisation, and mechanotransduction signalling pathways. Together, these processes maintain the chondrogenic phenotype through modulation of gene expression and extracellular matrix production (Camarero-Espinosa and Cooper-White, 2019; Frith et al., 2012; Han et al., 2019; Markway et al., 2010). Stiffness also determines mechanical suitability by ensuring that hydrogels can withstand physiological compressive stresses (Xiao et al., 2013).

The storage modulus (G') and loss modulus (G'') indicate the elastic and viscous behaviour of hydrogels, respectively. This allows them to be described as solid-like ($G' > G''$) or liquid-like ($G'' > G'$) under given strain, frequency, time, and temperature conditions. These moduli are especially relevant in cartilage tissue engineering, where constructs must mimic the native

tissue's ability to absorb shock during movement and return to their original shape (Malkin et al., 2023; Xiao et al., 2013). In regards to knee cartilage-sourced dECM-based hydrogels, it has been reported that these exhibit viscoelastic properties, with G' being greater than G'' , indicating that they are solid-like at 37 °C (Bordbar et al., 2020).

Pre-gel solutions are disorganised polymeric suspensions which, upon gelation, form a structured hydrogel network (Gulrez et al., 2011). Gelation kinetics can be determined either via rheological analysis or turbidity measurements (Chuang et al., 2018; Freytes et al., 2008). In this study, turbidity-based monitoring (see subsection 3.11) was used to compare functionalised and non-functionalised hydrogels. Samples were heated to 37 °C in a plate reader to track turbidity changes, allowing the determination of the time to reach maximum turbidity and the half-maximum opacity time (half-time gelation turbidity ($t_{1/2}$)). Gelation kinetics reveal the rate of network formation, which is critical for designing hydrogels that must gel rapidly in situ. The gelation rate correlates with the network structure, reflected in the G' , and can influence cell distribution during encapsulation: slow gelation allows cell settling, whereas excessively rapid gelation may expose cells to uneven physical stresses. Optimising these parameters enables precise tuning of hydrogels for particular biomedical applications (Freytes et al., 2008).

Hydrogels derived from dECM typically display weaker mechanical properties than native cartilage (Beck et al., 2016; Bordbar et al., 2020). These limitations arise primarily from the decellularisation and solubilisation procedures, which can denature, reducing viscosity and storage modulus (García-García et al., 2024). This compromises mechanical stability and limits compatibility with fabrication processes such as bioprinting (Panwar and Tan, 2016). Moreover, certain detergents used in decellularisation (for instance, SDS) prolong the lag phase of fibrillogenesis and lower fibril density, leading to slower gelation and diminished mechanical strength. Enzymatic digestion, although necessary for solubilisation and injectability, further disrupts the native ECM integrity (Fernández-Pérez and Ahearne, 2019). Mechanical reinforcement, for example via polymer additives (methacryloyl groups, etc.), can provide methods to reduce such drawbacks (Kafili et al., 2025). Several studies show dECM-based hydrogels exhibit shear-thinning behaviour, with viscosity decreasing under shear, indicating injectability (Antich et al., 2021).

Overall, combining rheological and gelation analyses provides a comprehensive understanding of how these hydrogels behave under biologically relevant conditions.

1.3 Chondroitin Sulphate Functionalisation Strategies

1.3.1 Functionalised Biomaterials

Functionalisation strategies for polymers have proven vital in the production of novel biomaterials with enhanced scaffold properties promoting efficient tissue regeneration (Tallawi et

al., 2015). This is mostly due to their bioactive signalling or mechanical cues coinciding with various beneficial tissue regeneration processes including the mediation of vascularisation, inflammatory responses and tissue regeneration (Zhang et al., 2025). Functionalisation approaches tend to involve site-specific incorporations of functional chemical groups (including amines, carboxyls, etc.). Polymers can possess specific reactive sites within each monomer unit that enable chemical functionalisation (Rossi and Van Griensven, 2014). Achieving uniform incorporation of functional chemical groups throughout a polymer typically requires the use of chemical reagents in excess, along with extended reaction times to ensure a thorough diffusion and reaction at all available sites. If lower reagent concentrations or shorter treatment periods are used, functionalisation will occur only at accessible or more reactive sites. This leads to a more random or heterogeneously distributed incorporation of functional groups (Dawlee et al., 2005).

Selecting a suitable functionalisation-site is paramount to ensure bioactivity is maintained or enhanced, a crucial factor when determining such sites to exploit. Notably, if a site that can be exploited is in the region of a polymer, which is vital for bioactivity or function (protein folding), a different approach should be explored (Wang and Wu, 2018). However, if due to random functionalisation, a suitable site located in a non-bioactive area of the polymer is also present in the bioactive area, it may be necessary to prevent functionalisation of the bioactive site. For proteins, this can involve engineering recombinant proteins with a cysteine tag that is selectively functionalised via incorporation of the desired functionality on a short oligoglycine or leucine-proline-any amino acid-threonine-glycine (LPXTG)-containing peptide, which is then covalently attached to the protein using the sortase enzyme through a site-specific transpeptidation reaction (Guimaraes et al., 2013). This approach allows precise modification while preserving the protein's bioactive regions by targeting only the engineered tag.

1.3.1.1 Chondroitin Sulphate Functionalisation Strategies

Usage of CS in tandem with dECM as a biomaterial has been previously well documented in experimental tissue engineering approaches (dECM sourced from nucleus pulposus, adipose tissue and kidney) (Cheung et al., 2014; Quijano et al., 2022; Rafighdoust et al., 2015). Applying CS functionalisation strategies have provided novel scaffolds with microenvironments reported to have enhanced capabilities for tissue regeneration. For glycan-based polymers including CS, functionalisation strategies differ and may involve selective chemical modification of specific hydroxyl or carboxyl groups to maintain bioactivity (Perez et al., 2023). Notably, the main regions to be undisturbed for CS comprise the sulphated position along with the carboxyl groups, since these offer this polymer's negative charge. Also, the CS length is vital to be maintained since impacting the length may result in reduced bioactive signalling (Shen et al., 2023).

Among the currently reported strategies, click chemistry enables site-specific, bioorthogonal

conjugation and methacrylation strategies are frequently used to introduce photocrosslinkable groups within CS, enabling light-induced covalent network formation (Liu et al., 2017; Sousa et al., 2022). Click chemistry approaches tend to necessitate a key step involving N-hydroxysuccinimide (NHS), which involves activating the CS's carbonyl group using carbodiimide chemistry with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sousa et al., 2022; Strehin et al., 2010). Also, the introduction of NHS within CS allows for these to react with amines located on other polymers. Another method involves oxidising CS (explained further below) to introduce aldehyde groups which can be combined with other modifications (methacrylated) for dual-bonded approaches (Dawlee et al., 2005; Liu et al., 2017). Notably, such approaches, if undertaken optimally, appear to effectively preserve biological function. Furthermore, CS hydrogels necessitate modifications to provide functional groups which ensure crosslinking of the polymer can occur to form an organised hydrogel network (Duran-Mota et al., 2025; Rashidi et al., 2022).

Alternatively, approaches that do not require chemical modification of CS are increasingly attractive. These use ionic interactions, or supramolecular assembly to embed non-modified CS to functionalise materials. This preserves the natural structure and biofunction to a higher degree (native inherent characteristics) compared to CS which has undergone chemical modifications while ensuring these have simple integration strategies for scaffolds. Such approaches circumvent potential loss of bioactivity encountered with chemical modifications and simplify the experimental techniques applied, as has been previously reported (Fang et al., 2022).

Overall, combining certain chemical approaches with strategies preserving key CS functional groups, or alternatively employing non-covalent functionalisation methods (using non-modified CS), supports scaffold designs that balance mechanical stability with biological effectiveness in tissue engineering applications.

Aldehyde-modified chondroitin sulphate

As mentioned in chapter 2, this thesis focuses on oxidising CS to produce an aldehyde modified variant, mCS. This is a chemically altered form of CS, which introduces aldehyde groups via selective oxidation of hydroxyl groups on the polysaccharide backbone, using sodium periodate (NaIO_4), as shown in figure 1.4.

This modification facilitates the covalent crosslinking via Schiff base chemistry with amine-containing biomolecules forming imine bonds (figure 1.5). Furthermore, aldehydes react with alcohol containing biomolecules, which in the case of the research work undertaken, are located in both CS and mCS to form hemiacetals (Azofra et al., 2012; Mihajlovic et al., 2022). It is important to note, both imine and hemiacetal bonds respectively, are reversible reactions. Upon exposure to low pH, imine and hemiacetal bonds cleave, reverting to their original functional chemical groups (Gambaro et al., 2020; Han et al., 2024).

Such chemical reactions enable the formation of hydrogels (non-modified CS cannot undergo gelation) and adhesive biomaterials for tissue engineering applications (Reyes et al., 2005).

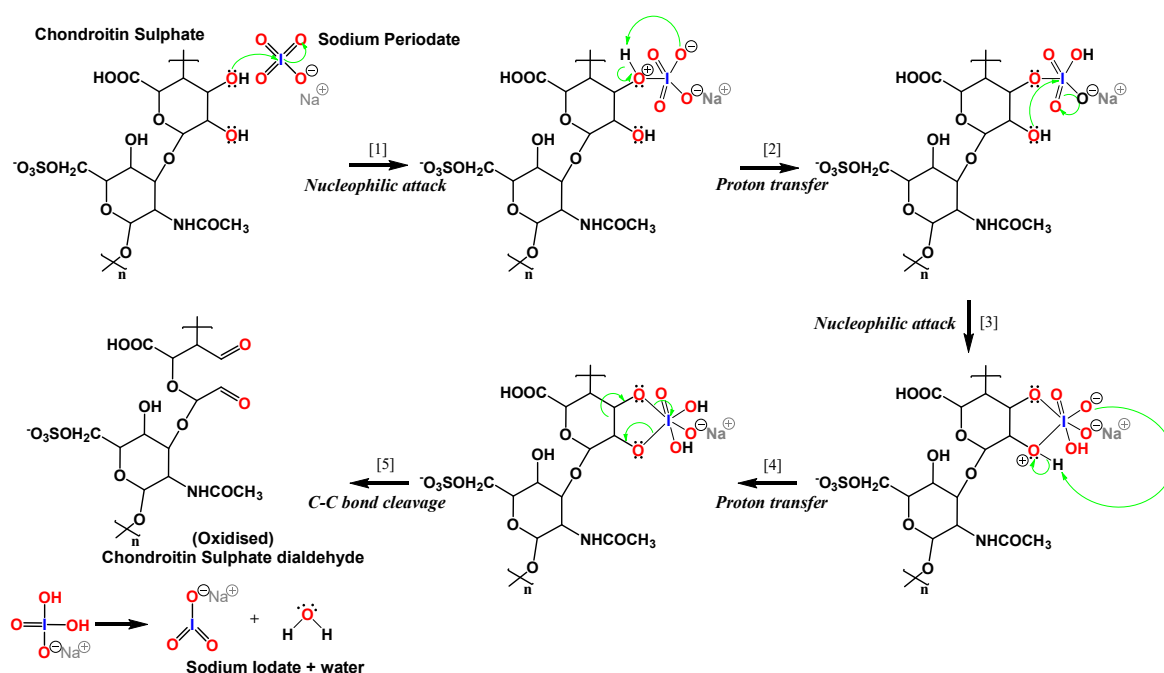


Figure 1.4: Action of sodium periodate upon chondroitin sulphate | A schematic of diol cleavage in the chondroitin sulphate monomer. [1] The hydroxyl oxygen attacks sodium periodate, forming an O–I bond. [2] A cyclic periodate ester intermediate forms. [3] Proton transfer restores neutrality at the attacking oxygen. [4] The adjacent hydroxyl oxygen attacks iodine, producing a 5-membered cyclic intermediate. [5] The proton previously transferred in [3] may be lost (deprotonation) with additional proton transfers occurring to stabilise the structure. [6] The ester cleaves the vicinal C–C bond, yielding two aldehydes as electrons redistribute, while sodium periodate is reduced from iodine(VII) to iodine(V) (iodate ion formation). Electrons from the broken C–C σ bond and original O–I bonds redistribute to forming aldehyde carbonyl double (π) bonds (Clayden et al., 2012a; Ziegler-Borowska et al., 2018)

The oxidation approach is explained in section 3.5 and as discussed in chapter 5, their several experimental concerns were taken into account when selecting this approach. Notably the extent of oxidation should be limited to not produce a polymer which is significantly different to unmodified CS via limiting the amount of oxidiser provided (Dawlee et al., 2005; Fang et al., 2022; Reyes et al., 2005). It has been previously reported that this may be due to the glycosidic bond cleavage causing the length of the CS polymer to diminish (Dawlee et al., 2005; Fang et al., 2022). Oxidation of CS was deemed acceptable and feasible for the application of functionalising dECM since this results in an aldehyde modifications, while managing to preserve key features (sulphate functional group, polymer length) essential for aiding in cartilage tissue engineering. Such modifications can then react with complementary reactive groups (amines and hemiacetals) within the components of the dECM (Dawlee et al., 2005; Fang et al., 2022; Reyes et al., 2005).

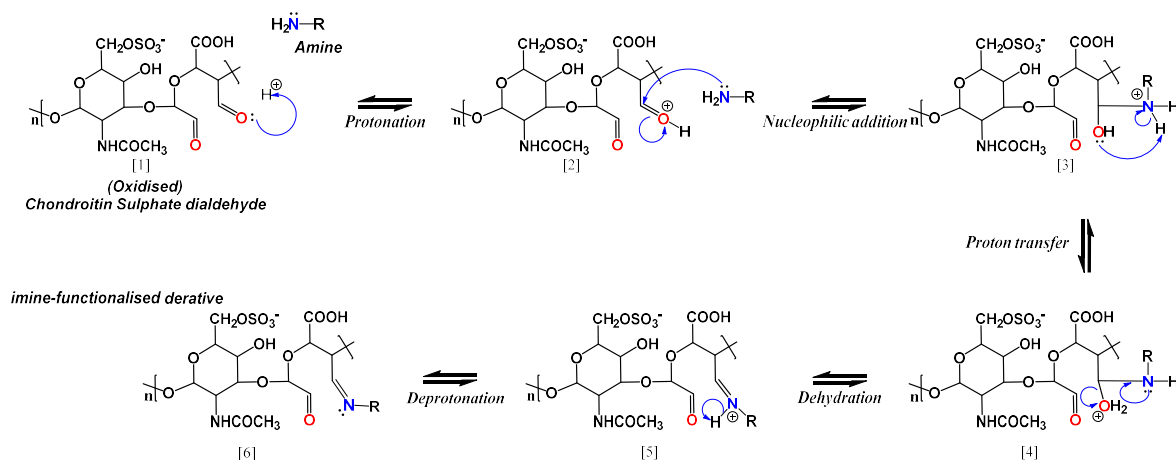


Figure 1.5: Formation of imine derivative at physiological pH [1] The aldehyde carbonyl oxygen is protonated, increasing electrophilicity of the carbonyl carbon to facilitate nucleophilic attack. [2] Nucleophilic Addition: The primary amine attacks the electrophilic carbonyl carbon, forming a tetrahedral intermediate called the carbinolamine (an amino alcohol). [3] Proton Transfer: Intramolecular proton transfer occurs to stabilise the carbinolamine intermediate, often transferring a proton from nitrogen to oxygen. Protonation of the Hydroxyl Group: The hydroxyl group ($-\text{OH}$) of the carbinolamine is protonated to form a better leaving group (OH_2^+). [4] Elimination of Water: The protonated hydroxyl group leaves as a neutral water molecule, forming an iminium ion intermediate ($\text{C}=\text{N}^+$). [5] Deprotonation of Nitrogen: The iminium ion loses a proton on the nitrogen. [6] Yields the neutral imine (Schiff base) product ($\text{C}=\text{N}-\text{R}$). R symbolises the rest of the molecule. (Clayden et al., 2012b; Kulchat et al., 2017; Rufino and Pliego Jr, 2020)

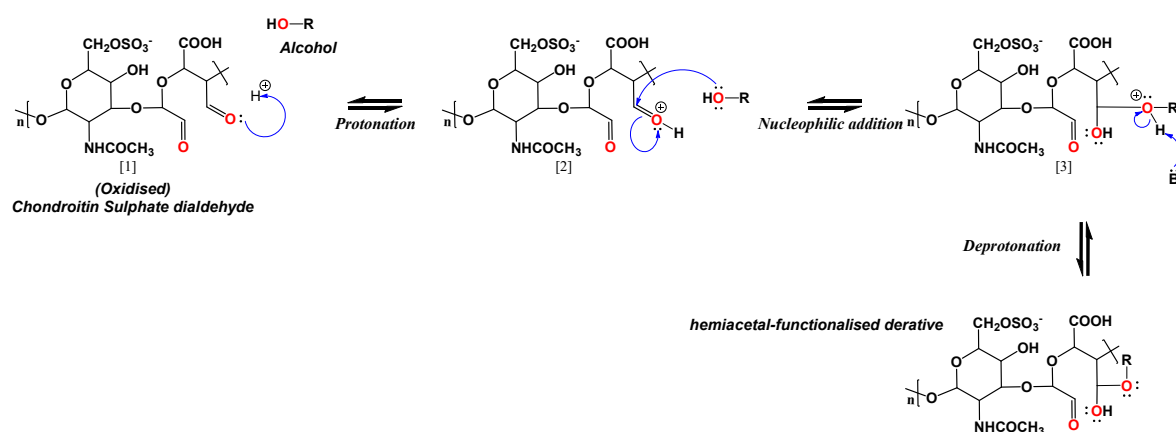


Figure 1.6: Formation of hemiacetal derivative [1] Protonation of the carbonyl oxygen. [2] Nucleophilic attack by an alcohol: neutral alcohol attacks the electrophilic carbonyl carbon, forming protonated hemiacetal intermediate (a tetrahedral intermediate with an $-\text{OH}$ and $-\text{OR}$ group attached). [3] Deprotonation of the protonated hemiacetal: A base (often water) deprotonates the protonated hemiacetal intermediate, forming a stable (neutral) hemiacetal. R symbolises the rest of the molecule and B symbolises any generic base. (Azofra et al., 2012; Clayden et al., 2012c)

1.3.2 Quercetin: A Background

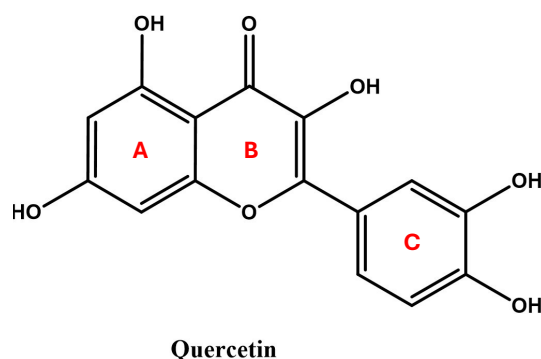


Figure 1.7: Chemical structure of Quercetin | Shown above is the structural formula for quercetin (molecular formula is $C_{15}H_{10}O_7$); characterised by five hydroxyl groups on its flavone core structure located at the 3-, 3'-, 4'-, 5-, and 7-positions.

Quercetin, with its structure shown in figure 1.7, is a naturally occurring plant flavanoid, sub-classed as a flavanol. It is considered among the most prevalent polyphenolic compound widely distributed in the plant kingdom, being also a majorly abundant dietary flavonoid (vegetables, fruits, grains, etc.). Isolating the flavanol involves drying and pulverising the source material. This is followed by solvent extraction with ethanol or ethyl acetate-based solutions providing an intermediate extract. This can be then purified via adsorption chromatography on a silica gel column to retrieve quercetin while discarding any remaining compounds (Baquer et al., 2024).

In relation to biomaterials, quercetin incorporation has been applied to these for tissue engineering (in relation to chondrogenesis see subsection 1.3.2.1), drug delivery, and wound healing (Lotfi et al., 2024; Mi et al., 2022; Ren et al., 2017). It provides crosslinking capabilities for scaffolds (see subsection 1.3.2.2), along with having enhanced bioavailability (anti-inflammatory and antioxidant capabilities) upon encapsulation (Greco et al., 2018; Wang et al., 2025).

Table 1.3: Key properties of quercetin | This table shows the main properties of quercetin (Carrillo-Martinez et al., 2024; Goyal, 2024)

Property	Description
Chemical Formula	$C_{15}H_{10}O_7$
Structure	3,3',4',5,7-pentahydroxyflavone
Appearance	Yellow solid crystalline powder
Density	1.799 g/cm^3
Melting Point	316°C
Solubility	Poor in water
Key Functional Groups	Five hydroxyl groups

Quercetin is chemically known as 3,3',4',5,7-pentahydroxyflavone and has a tricyclic arrangement (Frenț et al., 2024). Its structure shown in figure 1.7, denotes a core structure compris-

ing of three rings: two benzene rings (A and C) and a heterocyclic pyrone ring (B). Quercetin is most commonly found naturally as glycosides (including rutin, quercitrin, and isoquercitrin), where the non-saccharide form of a compound (aglycone), being quercetin itself, is bound to one or more sugar moieties. Notably, these alter the molecule's solubility and bioavailability (Aghababaei and Hadidi, 2023; Michala and Pritsa, 2022; Vollmannová et al., 2024).

Eventhough these molecules contain five hydroxyl groups, quercetin dissolves poorly in water but better in organic solvents (lipophilic). The hydrated form of quercetin, quercetin hydrate, contains a water molecule within its crystalline structure. The inclusion of the water molecule has been shown to not improve the solubility since the monohydrate and anhydrous quercetin are practically insoluble in water at room temperature (RT). While, the dihydrate form is slightly more soluble, at 25 °C being up to 2.5 times higher, while anhydrous quercetin is 0.00215 g/L, being still a minimal difference (Abraham and Acree Jr, 2014; Srinivas et al., 2010).

Upon quercetin being in aqueous solvent, quercetin can undergo oxidation forming various metabolites (Zhou and Sadik, 2008). Furthermore, within the body quercetin is transformed into various metabolite forms which still have antioxidant properties (Morand et al., 1998). It may not be possible to determine the form of quercetin influencing the bioactivity and microenvironment without complex analysis (Morand et al., 1998; Zhou and Sadik, 2008).

1.3.2.1 Quercetin Promotes Chondrogenesis and Preserves Extracellular Matrix Integrity

Quercetin while still being mostly used experimentally shows promising bioactive effects (Yu et al., 2020). Extensive research has been conducted on the application of quercetin in the treatment of various diseases, along with its potential to support tissue engineering and regenerative medicine (Ivan et al., 2024; Li et al., 2024; Papan et al., 2020; Yamaura et al., 2023; Yu et al., 2020). Notably, quercetin has been proven to be advantageous in both *in vitro* and *in vivo* studies for chondrogenesis (Yu et al., 2020; Zhi-Peng et al., 2020).

in vitro studies utilising chondrocytes in two-dimensional (2D) cultures determined cell viabilities following quercetin exposure. In one study a concentration up to 20 μ M, provided low cytotoxicity (and cell proliferation at 7 days) while 100 μ M quercetin demonstrated after 24 hours culture, increased cytotoxicity (highest concentration showed moderate cytotoxicity). Furthermore, concentrations of 50 to 100 μ M, depicted cell numbers decreased significantly compared to the control (chondrocytes exposed to 0.1% dimethyl sulphoxide (DMSO)), demonstrating reduced rate of cell proliferation. Relative to the expression of chondrogenic specific genes (aggrecan (ACAN), COL2A1 and SRY-box 9 (SOX9)), quercetin promoted their expression, with the ACAN expression being significantly increased from day 4, with an increase continuously observed for the remaining genes versus the control. The concentration of 1.5 μ M quercetin proved to have an optimal effect in relation to these genes. Favourably,

the authors of the study managed to utilise selective inhibitors (PD98059, SB202190 and LY294002 respectively) against extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (P38) and protein kinase B (AKT) signalling pathways to determine these were vital for quercetin's advantageous chondrogenic effect upon chondrocytes (Zhi-Peng et al., 2020). However, another study by *Yu et al.* demonstrated similar results using rat chondrocytes with concentrations up to 20 μ M (Yu et al., 2020). However, they did not utilise DMSO solvent to dissolve quercetin and did not analyse chondrogenic genes in 2D cultures. Therefore, it appears in such *in vitro* cultures it is possible to ensure excellent cell viability upon utilising low concentrations of quercetin.

While, *in vivo* studies have presented the chondrogenic role of quercetin via incorporating this in agarose hydrogels mixed with injectable sodium alginate/bioglass hydrogel (Yu et al., 2020) or on silk fibrin scaffolds (Zhi-Peng et al., 2020) in rat cartilage defects. In a rat degenerative chondrocyte model, *Yu* and colleagues gathered evidence for quercetin upregulates SOX9, ACAN and COL2A1 expression (Yu et al., 2020). While, *Zhi* and colleagues via histological analysis of quercetin-loaded silk protein scaffolds demonstrated enhanced cartilage-like tissue formation (Zhi-Peng et al., 2020).

Furthermore, quercetin has been evaluated to determine how this small molecule can interact with cellular pathways to alleviate the progression of nucleolus pulposus tissue degradation. *Liu* and colleagues employed an *in vivo* rat intervertebral disc degeneration model to assess the genes being influenced due to interaction of quercetin. The authors of the study determined Quercetin reduced liposaccharide-induced M1 macrophage polarisation while decreasing ECM degradation in nucleus pulposus cells. Furthermore, determined quercetin can interfere with the CEBPB-HMGA1 interaction, resulting in decreased transcription of the pro-inflammatory gene TNFA (Liu et al., 2025).

1.3.2.2 Quercetin: An Alternative Crosslinker to Genipin

Demonstration of the interaction between quercetin and collagen first arose in 2009 due to a study undertaken by Yang and colleagues (Yang et al., 2009). While, a direct study evaluating this flavonoid against the typically utilised small molecule crosslinker, genipin; has been previously undertaken by *Greco et al.* (further information is shown in table 1.4). Notably the study demonstrated quercetin is a potential alternative to genipin in applications where lower crosslinking density and enhanced biological signalling (skewed towards regenerative cues) are necessary (Greco et al., 2018).

Quercetin is considered to have pro-oxidant and apoptosis-inducing effects, impacting cell viability, especially of cancerous cells (Kusaczuk et al., 2022; Ren et al., 2017; Sohn et al., 2018). Utilising suitable concentrations appears to be beneficial for regeneration and cell viability. Thus, applying quercetin always requires the determination of a suitable concentration and exposure period to ensure viability for cells (Yu et al., 2020; Zhi-Peng et al., 2020).

With genipin this is also pertinent, while due to its lack of anti-oxidant and anti-inflammatory properties ensure it is an easier approach to maintain cell viability (Greco et al., 2018).

Table 1.4: Comparisons of genipin and quercetin in tissue scaffolds | This table shows various comparisons being previously reported in relation to the characteristics of these crosslinkers in tissue scaffolds (Greco et al., 2018).

Genipin	Quercetin
Higher degree of crosslinking in tissue scaffolds enhancing mechanical strength and stiffness	Lower degree of crosslinking and stiffness, resulting in more flexible and less rigid scaffolds.
Scaffolds with better biomechanical stability and enzymatic resistance, making it suitable for long-term implantation and mechanical support roles.	Provides a degree of stability to scaffolds
Exhibits low cytotoxicity and good biocompatibility for dermal fibroblasts and other cells, widely used in tissue engineering applications.	Has additional antioxidant and anti-inflammatory properties, which support cell viability, reduce oxidative stress, and aid tissue regeneration.

Genipin has been previously used to crosslink cartilage-derived dECM scaffolds, demonstrating enhanced stabilisation and increased stiffness of the rigid solid scaffold. These characteristics were linked to improved chondrogenic expression of COL2A1, a gene critical for type II collagen production, thereby enhancing chondrogenic properties via scaffold rigidity (Cheng et al., 2013). In contrast, quercetin provides a moderate degree of crosslinking and stabilisation, yet it uniquely promotes tissue regeneration through its potent pro-chondrogenic bioactivity and antioxidative effects (Greco et al., 2018; Yu et al., 2020). Furthermore, quercetin could be particularly suitable for hydrogels, as its lower degree of crosslinking preserves the necessary flexibility that hydrogels require (Greco et al., 2018; Kopka et al., 2023). This potentially positions quercetin as a multifunctional molecule capable of supporting cartilage regeneration while maintaining the flexibility of hydrogel scaffolds.

Originally, it was unclear how quercetin would affect chondrogenesis when integrated within a dECM scaffold functionalised with CS, especially in a (flexible) hydrogel scaffold. This approach, as described in the next chapters, is therefore a novel one. Because quercetin can promote chondrogenesis and has potential as an alternative crosslinker to genipin, combining quercetin with CS-functionalised dECM-based hydrogels may offer a new proof-of-concept hydrogel biomaterial to be explored.

Chapter 2:

Hypothesis, Aims and Objectives

Having previously presented the background and central themes of this doctoral thesis, this chapter presents the hypothesis, aim and objectives. The hypothesis proposes a testable explanation of the project, while the aim addresses the research problem by establishing a suitable chondrogenic microenvironment. While the objectives set out the precise steps for evaluating the hypothesis underpinning this investigation.

2.1 Hypothesis

The hypothesis that underlies this work is that functionalising decellularised cartilage with CS will recreate a native-like cartilage microenvironment by replenishing GAGs lost during decellularisation, thereby supporting cell differentiation and matrix synthesis. It is further hypothesised that the addition of quercetin will synergistically enhance this effect through its antioxidant and pro-chondrogenic properties, leading to improved hydrogel stability and bioactivity. Collectively, the study will evaluate whether the combined use of functionalised dECM and quercetin can yield a sustainable, matrix-responsive biomaterial capable of promoting cartilage tissue engineering.

2.2 Aims

The aim of this project is a matrix-oriented approach focused on developing a novel decellularised bovine articular cartilage-based hydrogel functionalised with CS and incorporating quercetin (quercetin hydrate). This work prioritises the acquisition and solubilisation of matrix components along with functionalisation strategies to create a proof-of-concept hydrogel. The evaluation mainly assesses the hydrogel's biochemical, histological, gelation and rheological properties relevant to cartilage tissue engineering, rather than focusing on cellular pathways. The CS functionalisation aims to restore key matrix components lost during detergent-based decellularisation to enhance scaffold bioactivity. While the integration of quercetin into CS functionalised hydrogels introduces a novel research direction investigating how matrix composition and mechanics affect gene expression and protein production during chondrogenic differentiation.

2.3 Objectives and Scope of the Thesis

Osteoarthritis (OA) is a degenerative joint disease for which current treatments—spanning medication, physiotherapy, and surgery—primarily serve to alleviate symptoms rather than repair the damaged cartilage (Dhollander et al., 2016). This underscores the pressing need for regenerative solutions capable of restoring cartilage structure and function. Hydrogels derived from dECM and tailored to promote chondrogenesis hold significant promise in this regard.

In this study, the specific objectives were as follows:

Objective 1: To prepare a dECM hydrogel from bovine cartilage obtained from abattoir sources, involving tissue decellularisation and subsequent solubilisation to obtain a pre-gel hydrogel solution.

Objective 2: To functionalise the dECM pre-gel with CS using two distinct approaches: (i) self-assembly and (ii) covalent modification through mCS, facilitating imine bond formation with amine groups in the dECM.

Objective 3: To incorporate quercetin, a pro-chondrogenic and antioxidant flavonoid, into CS-functionalised hydrogels and analyse its influence on hydrogel structure, mechanical performance, and biological behaviour.

Objective 4: To evaluate the physicochemical, rheological, and histological characteristics of the hydrogels, as well as their biological performance in terms of cell viability, morphology, and matrix-related gene expression relevant to chondrogenesis.

2.4 Overview

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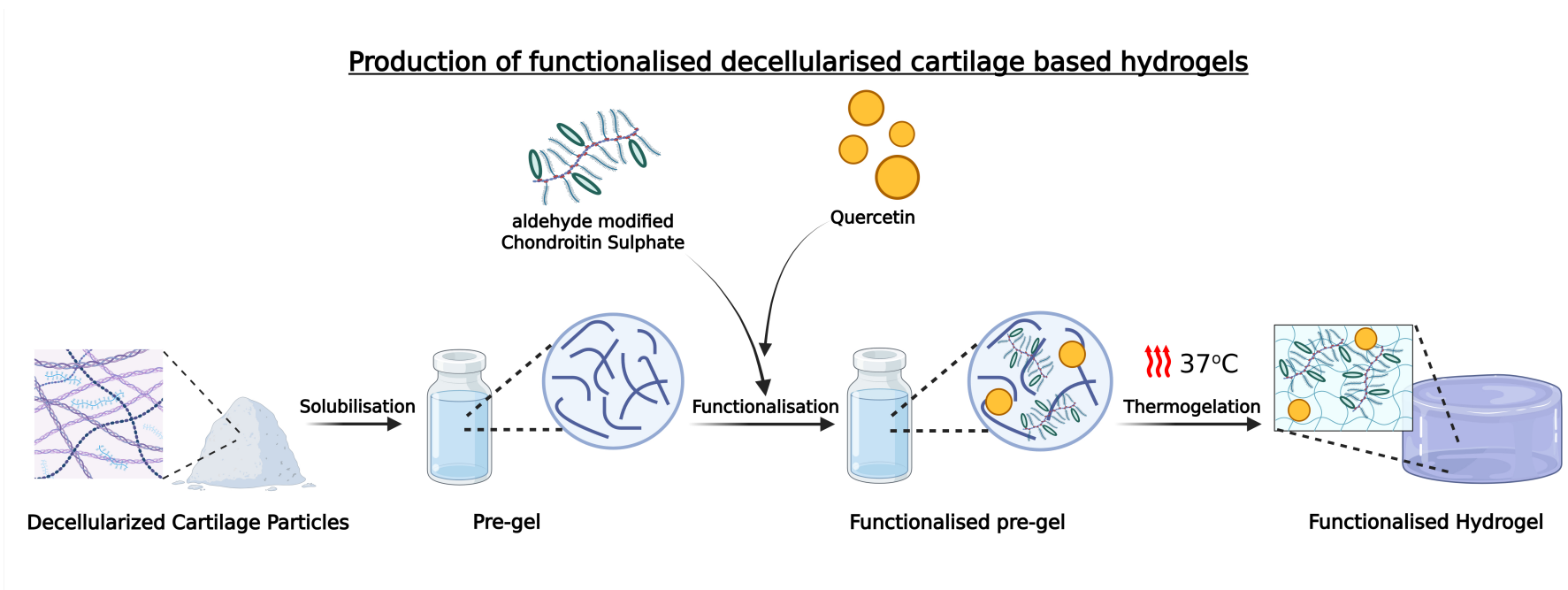


Figure 2.1: Graphical abstract. Decellularised cartilage particles were solubilised to form a pre-gel solution. This was functionalised with aldehyde modified chondroitin sulphate and quercetin hydrate. Following stirring, a functionalised pre-gel was produced that underwent thermogelation at 37 °C to produce a hydrogel. Created in BioRender.

Chapter 3:
Experimental methods

This chapter introduces the experimental methods being based on those shown in chapter 9.

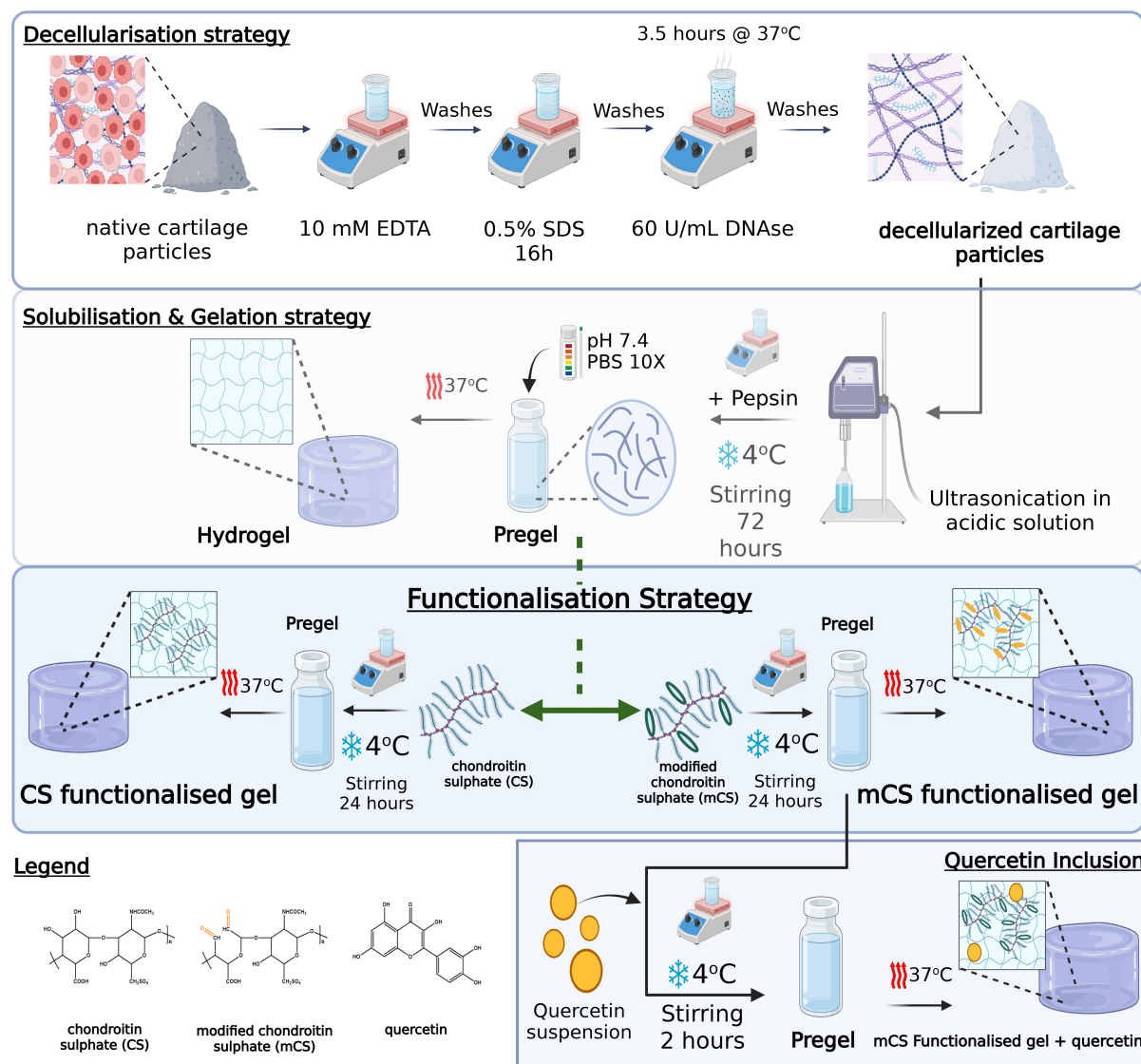


Figure 3.1: Schematic of functionalised decellularised cartilage hydrogel production. Cartilage matrix was decellularised, milled, filtered and solubilised into a pre-gel. This was used as a hydrogel or functionalised with either chondroitin sulphate (CS) or aldehyde modified chondroitin sulphate (mCS). Quercetin was also incorporated in the 0.4 mg/mL mCS hydrogel. Created in BioRender.

3.1 Harvesting Bovine Cartilage and Particle Production

Freshly cut bovine legs (approximately 1 year old) were obtained from Carnes Landeiro (Portugal), disinfected with 70% ethanol, with skin, ligaments and muscle removed. Cartilage was harvested from articular joints with a scalpel and placed in cold 1X PBS containing 10% penicillin-streptomycin (Pen/Strep) (Gibco, USA) and 5% Fungizone (Capricorn Scientific, Germany). Tissue slices were placed in a holder with optical temperature cutting compound (Kalttek srl, Italy), frozen in liquid nitrogen-cooled 2-methylbutane, and stored at -80 °C until use. When required, these were thawed at RT and washed in 1X PBS for 6 cycles of 30 minutes. Excess solution was removed and the slices were placed at -80 °C, then freeze dried. The lyophilised slices were milled and filtered through an 800 μ m sieve. The sieved dECM particles were collected and stored at -20 °C until use.

3.2 Decellularisation Protocol

Native cartilage particles were thawed at RT and placed in Milli-Q® (MQ) water containing 1% Pen/Strep (Gibco, USA), 0.5% Fungizone (Capricorn Scientific, Germany), 0.1% Gentamycin (Gibco, Waltham, USA). The particles, at a concentration of 6 mg/mL, were stirred at RT for 15 minutes. The solution was removed at all stages of the protocol via centrifugation at 1300 G for 4 minutes, with the supernatant being removed. A 10 mM Tris based hypotonic buffer (HB) containing 10 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.8 was then added and allowed to stir for 22 hours at RT. Following its removal, 1X PBS was added for 3 cycles of 1 hour each. MQ water was then added to remove any excess salts for 5 minutes. A HB solution containing 0.5% SDS was introduced and allowed to stir for 16 hours. To wash and remove the solution, 6 cycles for 15 minutes each of HB were required. To remove any excess salts, MQ water wash for 5 minutes. After centrifugation, 60 U/mL of DNase enzymes in a solution containing 20 mM Tris, 2 mM MgCl₂ (pH 7.8) was employed for 3.5 hours (37 °C). This was then removed and washed for 3 cycles of 10 minutes with 1X PBS, followed by 3 cycles of 10 minutes with MQ water to remove salts. The remaining supernatant was removed, with the particles being then frozen and lyophilised to form the dECM particles.

3.3 dsDNA quantification

Lyophilised ECM and dECM particles underwent DNA extraction using the PureLink Genomic DNA Mini Kit (Invitrogen, USA) per manufacturer instructions. Particles were digested with Proteinase K in digestion buffer at 55 °C for 4 hours with occasional vortexing. The lysate was centrifuged to remove RNA, then mixed with ethanol and applied to a silica spin column.

After washing, purified DNA was eluted. dsDNA was quantified using the PicoGreen assay per manufacturer's instructions, with fluorescence measured on a Synergy™ Mx microplate reader (BioTek, USA) at 480 nm excitation and 520 nm emission. Samples were run in duplicate with blanks and dual standard curves for DNA concentration, normalised to dry weight. The data was presented as ng DNA/mg dry weight.

3.4 Sulphated Glycosaminoglycan Quantification

The blyscan sulphated glycosaminoglycan (sGAG) (Biocolor, UK) assay was utilised to quantify sGAGs. The dry ECM and dECM particles were placed in a digestion solution (0.1 M sodium acetate, 0.01 M EDTA disodium salt, 0.005 M cysteine HCl, pH 6.4) containing 0.1 mg/mL of crystallised papain (Merck, USA). The samples digested for 12 hours at 65 °C, being stirred at 250 rpm. The kit quantifies sGAG by dye binding, pellet formation, and dye release for measurement. Absorbances were measured at 625 nm using a Synergy™ Mx microplate reader (BioTek, USA). Deionised water served as reference and blank. sGAG concentration was determined from a standard curve, normalised for dilution and initial dry weight. The data was presented as μg sGAGs/mg dry weight.

3.5 Aldehyde Modified Chondroitin Sulphate

Within an amber vial, 60.06 mg/mL of shark CS salt (Merck, USA) and 7.48 mg/mL of Sodium periodate (NaIO_4 , Honeywell, USA) were dissolved in MQ water. Once dissolved, were stirred for 6 hours in the dark at RT, then dialysed in a 1 kDa SpectraPor® bag (Repligen, USA) against MQ water for 4 days with 3 daily changes of the solvent. Then filtered (0.22 μm), frozen and freeze-dried with the mCS stored at -20 °C until use.

3.6 dECM Solubilisation Procedure and Gelation Approach

dECM particles were suspended in an amber vial with cold 3% acetic acid added at 80% FV (final concentration: 35 mg/mL). Were sonicated in ice using an ultrasonic liquid processor (FisherScientific, USA) with an amplitude of 20% and ON/OFF cycle of 2/3 seconds (total ON cycle: 1 hour). Then $\geq 3,200$ units/mg pepsin powder (Merck, USA) was added (final concentration: 5 mg/mL), residual enzyme suspended in the remaining 20% of cold 3% acetic acid, ensure desired final concentration reached. Mixture was stirred with a suitable magnet and rpm (prevent foaming) for a total of 72 hours.

Under cold conditions and utilising cold solutions throughout, 10X PBS (10% FV) was added and vortexed gently, stirred at low rpm for 30 minutes. 10 M NaOH was added until the pH

was approximately pH 7, mixture filtered to remove the undigested particles and allowed to centrifuge at 100G for 1 minute (4 °C) ensuring maximum recovery. Then altered to pH 7.4 using 5 M NaOH.

To undergo gelation, place the pre-gel at 37 °C until complete gelation.

3.7 Pre-Gel Functionalisation procedure

To fabricate the functionalised hydrogels, working stock mixtures produced were 1.0 mg/mL CS and 0.4 mg/mL mCS. Under cold conditions throughout, pre-gel solutions with either CS/mCS were stirred overnight (suitable rpm to prevent foaming). Diluted to 0.2 and 0.4 mg/mL, vortexed gently and stirred for 5 hours to ensure homogeneity prior to use.

3.8 Inclusion of quercetin within functionalised hydrogels

Quercetin (quercetin hydrate, MedChemExpress, USA) dissolved in cold 1X PBS at a concentration of 12 mg/mL and then diluted to 6 mg/mL. Each were then added to 0.4 mg/mL mCS pre-gel at 2.5% FV (final concentration: 0.3 and 0.15 mg/mL). These were allowed to stir for 2 hours at 4 °C prior to use.

3.9 Fourier Transform Infrared Spectroscopy Analysis

A fourier transform infrared spectroscopy (FTIR)-RAMAN Spectrometer 2000 (Perkin Elmer, USA) with: Universal attenuated total reflectance (ATR) sampling accessory and transmittance of solids (ToS) modules was used with 32 scans and a resolution of 4 cm⁻¹. To obtain the spectrum of CS and quercetin powders, these were dried overnight at RT under vacuum. Approximately 0.5 mg to 2 mg of each sample with 200 mg of potassium bromide were compressed into a pill being then read using the ToS module. The mCS and hydrogel-based solutions were read using the ATR module. Spectra were processed using the advanced baseline correction function provided by Spectragryph software.

3.10 Rheological Analysis

Pre-gel samples were placed in a pre-warmed 37 °C, kinexus rheometer (Malvern Instruments, USA) with drops of water surrounding the centre platform, to ensure humidity. The samples were allowed to stabilise and undergo gelation for 25 minutes. 10 samples per decade recorded; firstly, a frequency sweep from 0.1 Hz to 10 Hz with a strain of 1%, (linear

viscoelastic region (LVER) can be observed); secondly, with a fresh pre warmed sample, a strain amplitude analysis with 1 Hz (within LVER) frequency and strain from 0.01% to 100%.

3.11 Turbidity Measurements

Under cold conditions, pre-gel samples were prepared in duplicates and placed in a pre-cooled 96 well plate, with this being left on ice until further use. A Synergy™ Mx multi-mode microplate reader (BioTek, USA) was pre-warmed to 37 °C. 100 μ L of pre-gel solution of each condition were added as duplicates. The plate was left on a cold metal block until being placed in the plate reader with absorbances read every 31 seconds at a wavelength of 410 nm.

To normalise the absorbances as percentage turbidity, the method was adapted from a previous publication (Freytes et al., 2008). For the $t_{1/2}$ calculations, linear interpolation between measured time points was undertaken when required.

3.12 CryoSEM Preparation and Analysis

Pre-gels (50 μ L) underwent full gelation at 37 °C for 25 minutes. Then 200 μ L MQ water at RT was delicately added above each hydrogel, being then placed at 7 °C for 24 hours. As per figure 3.2, these observations were performed using a high-resolution scanning electron microscopy (SEM) with X-Ray Microanalysis with the following CryoSEM: JEOL JSM 6301F (JEOL Ltd., Japan), Oxford INCA Energy 350 (Oxford Instruments, UK), Gatan Alto 2500 (Gatan Inc., USA). After removing excess water, hydrogels were quickly cooled in sub-cooled nitrogen, transferred under vacuum to the chilled preparation chamber. The specimens were fractured and sublimated for 180 seconds at -90 °C followed by being coated via Gold/Palladium sputtering for two 54 second cycles. Then samples transferred into the SEM chamber (-150 °C) with the images being then captured.

3.13 Viability Analysis

Human MSC immortalised with human telomerase reverse transcriptase (hTERT) via lentiviral transfer were kindly provided by Prof. Matthias Schieker's group, Munich, Germany. Human dermal fibroblasts HDF were obtained commercially (ATCC, USA). The hTERT - MSCs or HDF were monolayer cultured in Dulbecco's modified eagle medium (DMEM) low glucose (-LG) medium (Gibco, USA), containing 10% fetal calf serum (FCS) (Gibco, USA or Corning, USA or Biowest, France), 100 U/mL penicillin, and 100 μ g/mL streptomycin (Gibco, USA); being maintained at 37 °C and a 5% CO₂ humidified environment. Either cell types

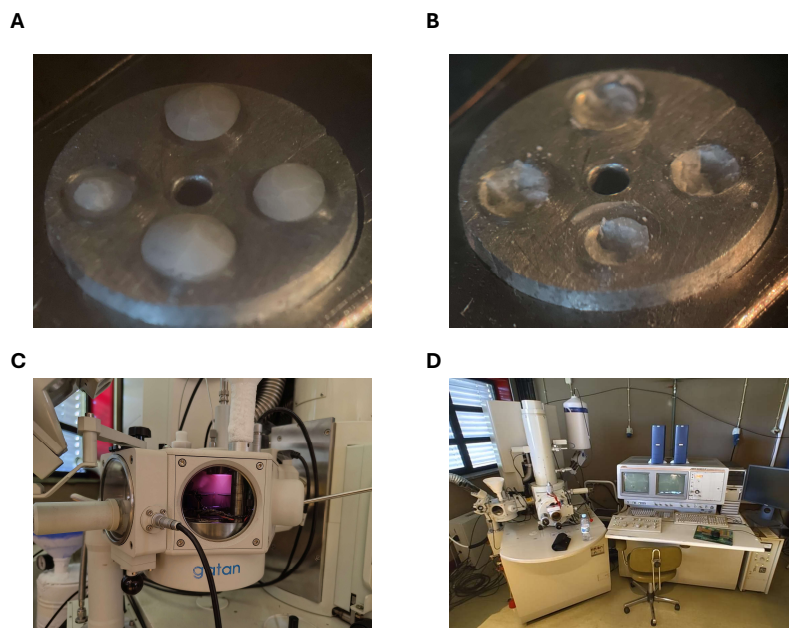


Figure 3.2: CryoSEM analysis undertaken at CEMUP to assess hydrogel morphology | (A) Specimens after exposure to sub-cooled liquid nitrogen. (B) Fractured specimens. (C) Surface coating applied by Gold/Palladium sputtering in two cycles of 54 seconds each. (D) CryoSEM imaging analysis performed using JEOL JSM 6301F equipped with Oxford INCA Energy 350 and Gatan Alto 2500.

were trypsinised and encapsulated within the hydrogel.

Cell seeding undertaken via 4×10^5 cells/mL in a volume matching 2.5 % FV of the pre-gel solution. Both solutions were combined under cold conditions and mixed using the pipette tip, up-down technique. $50 \mu\text{L}$ (containing 2×10^4 cells at a concentration of 4×10^5 cells/mL) of the mixed pre-gel solution for each condition was added as duplicates to a 96 well culture plate, undergoing gelation for approximately 8.5 minutes at 37°C within the incubator. Renewed with warm supplemented culture media and allowed to incubate for 75 minutes. The plates were then collected, media renewed and placed in the incubator overnight.

Stainings were conducted on day 1 and 3 after cell encapsulation. The cell media was replaced with culture media containing $2.4 \mu\text{M}$ Calcein AM (Thermofisher Scientific, USA) and $4.8 \mu\text{M}$ ethidium homodimer-1 (EthD-1) (Biotium, USA or Merck, USA), followed by incubating for 30 minutes. The media was carefully removed, and warm 1X PBS was added being then removed to wash away excess media. This was repeated, with excess liquid removed prior to imaging using a Keyence BZ-X810 microscope (Keyence Corporation, USA) or Operetta CLS (Perkin Elmer, USA). The day 3 plate should have media renewed on day 1 and repeat the same assay on day 3. Imaging was undertaken in 2 replicate gels per each condition, for each independent experiment, quantified using Image J software and results for replicate hydrogels were averaged.

3.14 Chondrocyte Isolation

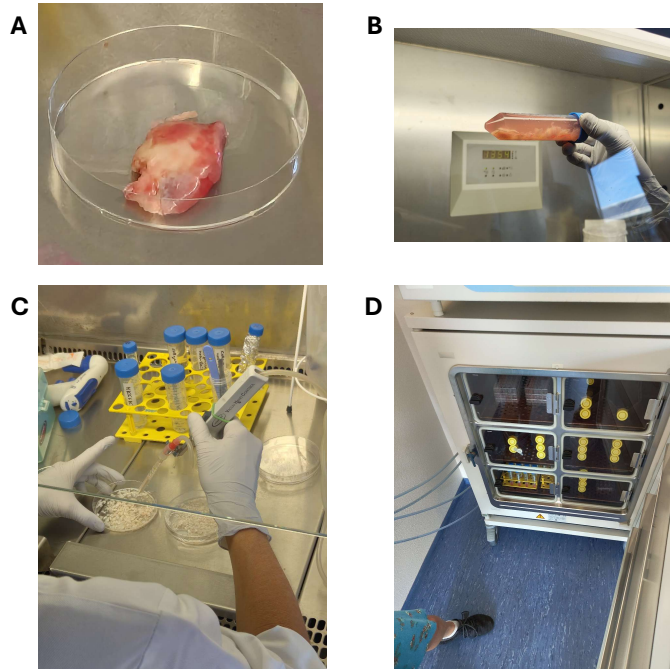


Figure 3.3: Chondrocyte isolation undertaken at the TFO group | (A) Human distal femur and tibia plateau fragments were retrieved from endoprosthetic knee arthroplasties at the Hospital Carl Gustav Carus Dresden. (B) Cartilage was harvested utilising a scalpel to carefully remove this tissue without collecting mineralised cartilage or bone. (C) Cartilage was minced. (D) Allowed to digest in HBSS containing 1 mg/mL collagenase II overnight on a shaker at 37 °C.

Process adapted from a previous protocol (Gossila et al., 2021), describing the process to obtain human chondrocytes. Briefly, these were isolated from the cartilage of human distal femur and tibia plateau fragments procured during endoprosthetic knee arthroplasties at the Hospital Carl Gustav Carus Dresden. Both donors were female aged 62. Prior to surgical intervention, patients provided written informed consent and cell isolation approved by the ethics commission of TU Dresden.

Cartilage was harvested, sliced and placed in HBSS (Gibco, USA) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, USA) along with 2.5 µg/mL amphotericin B (Merck, USA). Here, using a scalpel, pieces were minced and placed in HBSS containing 1 mg/mL collagenase II (Merck, USA), being placed overnight on a shaker at 37 °C. Solution was filtered through a 70 µm cell strainer (Corning, USA) with cells collected by centrifugation (5 minutes, 423 g). Cell pellet was resuspended in expansion media consisting of DMEM-LG with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin (all from Gibco, USA). Also, 2.5 µg/mL amphotericin B was included only at this stage. These were then added to a cell culture flask and placed in an incubator (37 °C, humidified atmosphere and 5% CO₂). These

were replenished twice a week with the expansion media and expanded to passage 3.

3.15 Chondrogenic Differentiation

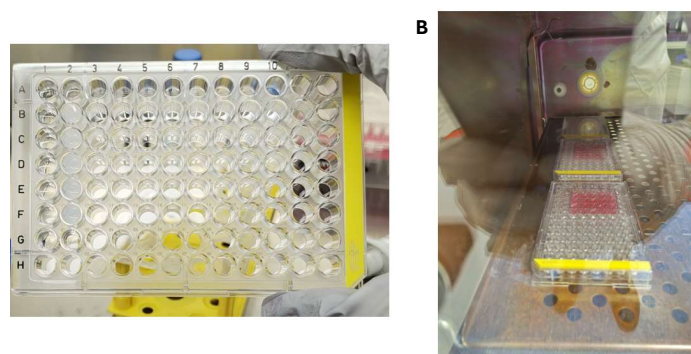


Figure 3.4: Hydrogels seeded with cells | (A) Hydrogels seeded with cells (B) Hydrogels seeded with cells with chondrogenic medium and incubated in an incubator (37 °C, humidified atmosphere and 5% CO₂).

All chondrogenic experiments were undertaken as three independent experiments. Dedifferentiated chondrocytes at passage 3 were trypsinised and placed at a concentration of 2.4×10^6 cells/mL where it constituted 2.5% FV (pre-gel and cell suspension mixture) in chondrogenic medium. This medium contained DMEMhigh glucose (-HG) (Gibco, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, 1% ITS-X-Mix (Gibco, USA), 40 µg/mL proline, 50 µM ascorbic acid-2-phosphate, 10^{-7} M dexamethasone (all sourced from Merck, USA) and 10 ng/ml TGF- β 3 (Milteny Biotec, Germany).

97.5% FV of the pre-gel solution and 2.5% FV of cell suspension was mixed using the positive displacement pipette. 50 µL of the mixed solution (containing 1.2×10^5 cells: concentration of 2.4×10^6 cells/mL), each condition was added to a 96 well culture plate and underwent gelation for approximately 10 minutes at 37 °C. Followed by this warm supplemented DMEM-HG was added and allowed to incubate for 75 minutes. The media was renewed and placed in the incubator overnight. On day 1, 3, 5 and 7/8 post encapsulation the media was renewed, being then renewed twice a week. On day 21, hydrogels were processed for further analysis (section 3.16 to 3.18).

3.15.1 *in vitro* Chondrogenesis

In this study, the use of these *in vitro* cell cultures have various characteristics which are mentioned here.

In relation to chondrocyte 2D culture expansion, these were expanded in a cell medium containing DMEM-LG and 10% FCS. The DMEM, includes a concoction of amino acids and minerals, with these providing the necessary nutrients required to maintain cells, with the low glucose content being fundamental for expanding chondrocyte population (Arora, 2013; Heywood et al., 2014). The FCS is necessary for ensuring cells are exposed to nutrients, proteins, etc. that support robust cellular proliferation and metabolic activity (Sedelaar and Isaacs, 2009). While ensuring cellular proliferation increases, there is a drastic effect upon the chondrocytes since they transition, becoming dedifferentiated; at this stage, they are considered to have adopted an MSC-like phenotype (Lee et al., 2017b).

An effective *in vitro* method to ensure differentiation via the chondrogenic lineage is supplementing the cell media with various growth factors along with increasing the glucose concentration (DMEM-HG). While maintaining the basis of utilising the DMEM solution, the higher glucose concentration ensures the heightened cellular activity is sustained throughout the differentiation process. The ITS-X-mix includes insulin, transferrin and selenium; which ensure cell survival along with stimulating and maintaining metabolic homeostasis. The ascorbic acid 2 phosphate (vitamin C) promotes collagen synthesis and deposition of ECM. Inclusion of the glucocorticoid, dexamethasone, enhances the differentiation efficiency by modulating gene expression pathways which are complementary to chondrocyte maturation. The inclusion of TGF- β 3, acts as a stimulus for triggering the Smad signalling pathway which is necessary for chondrocytes to produce the ECM (Heng et al., 2004).

3.16 Histology

dECM and ECM were fixed in 4% paraformaldehyde in HBSS. Hydrogels with encapsulated cells, fixed in 4% phosphate buffered formaldehyde. These were paraffinised, sliced into 4 μ m or 1.2 μ m slices, respectively, being deparaffinised and stained using haematoxylin and eosin (HE) or alcian blue staining. For DAPI staining these were exposed to Spectral DAPI solution (AYOKA Biosciences, Marlborough, MA, USA), incubated for 5 minutes within a humidified chamber, followed by subsequent washing and mounting.

3.17 RNA Extraction and cDNA Synthesis

Cell media was removed, hydrogels incubated with 3 mg/mL Collagenase type II (Merck, USA) in HBSS at 37 °C for 1 hour. Samples were centrifuged for 2 minutes at 250 G, supernatant was removed and cells collected to undergo lysis. The lysis and RNA purification was undertaken following the manufacturer's protocol, either peqGOLD Microspin (VWR, USA) or ReliaPrepTM miRNA Cell and Tissue Miniprep System (Promega, USA) kits. The amount of RNA was determined using a Nanodrop spectrometer (Thermofisher Scientific, USA). Re-

verse transcriptase reaction was performed from 300 ng of total ribonucleic acid (RNA), utilising High capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) as per the manufacturer's instructions.

3.18 Real Time Reverse Transcription-Polymerase Chain Reaction

Real-time polymerase chain reaction (RT-PCR) used for quantifiable expression of chondrogenic and hypertrophic markers. Was evaluated using selected TaqMan Gene Expression Assay primers (Table 1) for chondrogenic markers (COL2A1, ACAN, COMP). Along with hypertrophic markers (collagen type I alpha 1 chain (COL1A1), collagen type X alpha 1 chain (COL10A1) and ALPL). The housekeeping gene utilised was β -actin (ACTB). Gene expression levels were assessed by quantifiable (Q) RT-PCR reaction via the cDNA from each sample with Taqman Fast Advanced Master Mix (Applied biosystems, USA). All samples were run as duplicates for each gene while the normalised quantification was performed by using the comparative $\Delta\Delta C_t$ method. Sample size was 6 for figure 4.5, being 3 independent experiments with 2 biological replicates per condition; while for figure 4.6, the sample size was 9, including 3 independent experiments with 3 biological replicates per condition.

Table 3.1: Taqman primers utilised for the housekeeping, chondrogenic and hypertrophic genes

Gene Name	Gene Symbol	Taqman Assay ID
Aggrecan	ACAN	Hs00153936_m1
β -Actin	ACTB	Hs01060665_g1
Alkaline Phosphatase	ALPL	Hs01029144_m1
Cartilage oligomeric matrix protein	COMP	Hs00164359_m1
Collagen Type I alpha 1	COL1A1	Hs00164004_m1
Collagen Type II alpha 1	COL2A1	Hs00264051_m1
Collagen Type X alpha 1	COL10A1	Hs00166657_m1

3.19 Statistical analysis

Statistics was performed using GraphPad Prism (v8) and Excel software. Three independent experiments (minimum) were undertaken with the Shapiro-Wilk normality test used to assess normal (gaussian) distribution. dsDNA and GAG quantification analysed using paired t-test. $t_{1/2}$ and rheological data analysed using RM one-way ANOVA with Tukey correction (normally distributed data). Turbidity comparisons and cell viability analysis utilised RM two-way ANOVA with the Geisser-Greenhouse correction with Tukey's multiple comparisons test. Cell viability comparisons, same time point utilised RM two-way ANOVA followed by Tukey's

multiple comparisons test. Comparisons between time points used RM two-way *ANOVA* followed by Bonferroni's multiple comparison test. For the RT-PCR results, gene expression was analysed as technical replicates generated from either three duplicate samples sourced from three (50 μL) cell seeded hydrogels (Figure 4.5); or three triplicate samples, each from two (50 μL) cell seeded hydrogels (Figure 4.6). The statistical analysis utilised the ΔCt data. All data groups were compared to each other using ordinary one-way *ANOVA* followed by Tukey multiple comparison (normally distributed data) or Kruskal-Wallis test followed by Tukey multiple comparison, for (non-parametric data). The data is shown as fold changes ($\Delta\Delta\text{Ct}$) compared to the control (dECM) with upper and lower limits. Cell viability, gelation and rheology spectra, analysed using ordinary two-way *ANOVA* with Tukey's multiple comparison test.

Chapter 4:

Results

This chapter includes the results in relation to the experimental work. The results here are based on the Results section of the research article in chapter 9.

4.1 Cartilage Decellularisation

To obtain a dECM-based hydrogel, I started by decellularising native ECM particles using a combination of physical and enzymatic methods. After decellularisation, both native ECM and dECM particles were evaluated to ascertain their DNA and GAG contents, providing insight into the decellularisation efficiency in removing cellular material, while preserving ECM components. Since cartilage is extremely compact, cartilage particles (ca. 0.8 mm) were produced to increase the surface area and aid decellularisation. Cartilage particles were subjected to combined chemical and enzymatic decellularisation (figure 3.1), resulting in the removal of virtually all cellular material (figure 4.1A-B). This was confirmed by observing a porous structure within dECM particles that lacked or contained only remnants of cellular contents (figure 4.1A). In relation to retaining the matrix components, the alcian blue results (figure 4.1A, left images) appear to have a lighter blue stain observed in dECMs, showing loss of acidic polysaccharides including sGAGs.

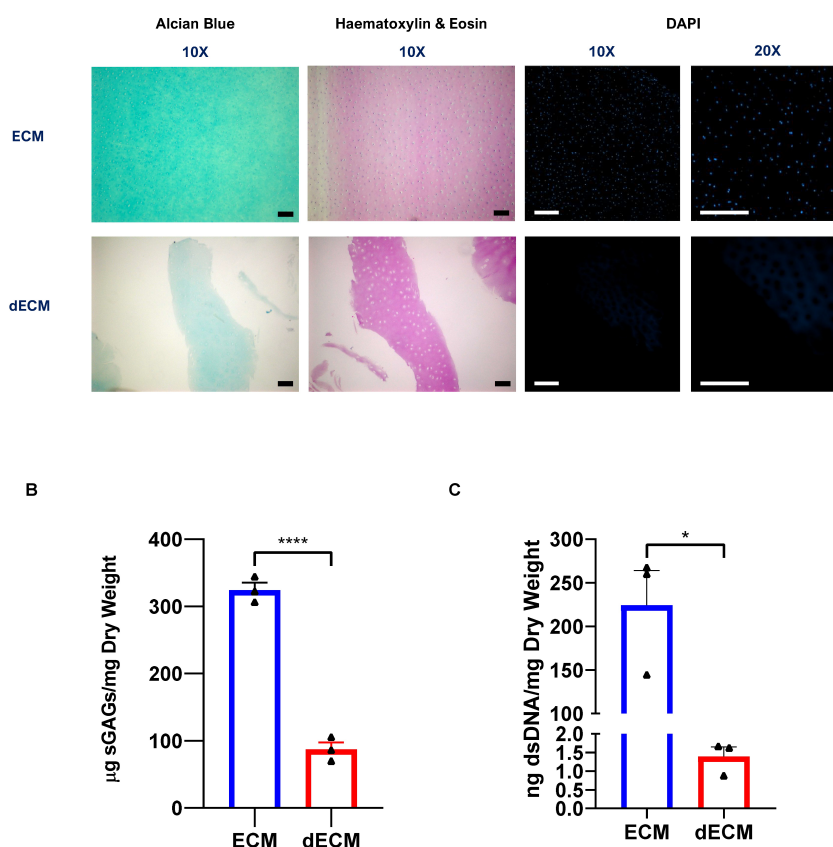


Figure 4.1: Characterisation of decellularised cartilage extracellular matrix. (A) Images of native and decellularised ECM cartilage particles stained with Alcian blue (GAGs), haematoxylin & eosin (matrix and cells), and DAPI (DNA). Images at 10X and 20X magnification with scale bars (100 µm for Alcian blue and H&E; 200 µm for DAPI). (B) Quantification of double-stranded DNA (dsDNA) in ECM and dECM particles. (C) Sulphated glycosaminoglycans (sGAGs) content quantified. Data shown as mean ± SEM (n=3), with paired t-test significance indicated: $p < 0.05$ (*), $p < 0.0001$ (****). Symbols on the graph represent individual data points for each N.

DAPI staining showed the cell nuclei in native cartilage particles, which were lost in dECM (figure 4.1A, right images). Results for dsDNA quantification, depicted in figure 4.1B, showed a significant decrease in the dECM, with 1.4 ng/mg, compared to the ECM particles with 224.2 ng/mg. The subsequent sGAG quantification (Figure 4.1C) showed a significant loss of 73% to approximately 87.3 $\mu\text{g}/\text{mg}$ sGAG remaining in the dECM particles, compared with 324.2 $\mu\text{g}/\text{mg}$ in the native ECM, caused by the decellularisation protocol.

Together, these results support the decellularisation was effective and the dECM was then used for further functionalisation and incorporation.

4.2 Production of Aldehyde Modified Chondroitin Sulphate

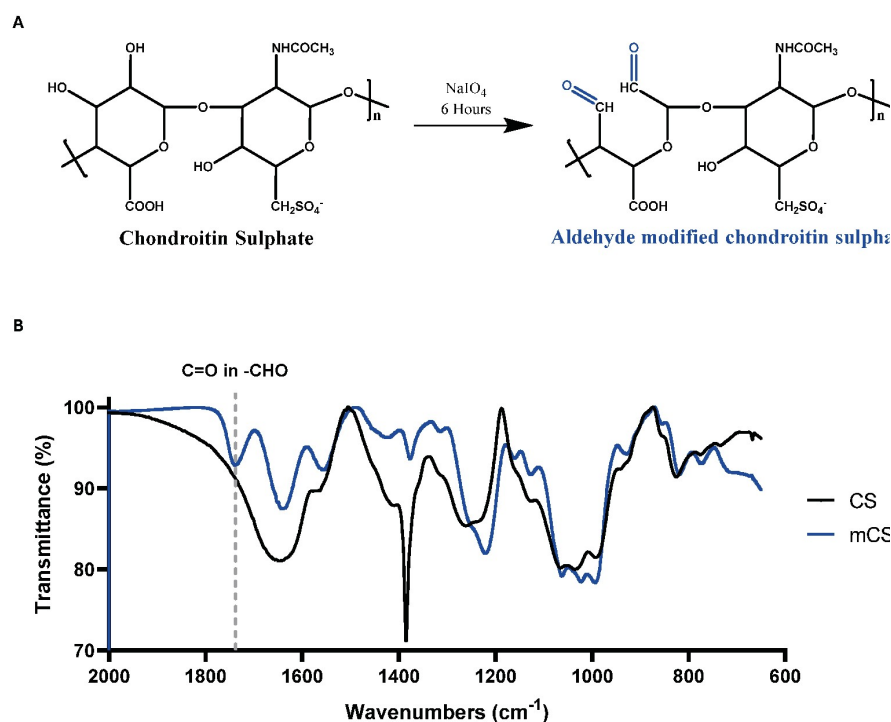


Figure 4.2: Partial aldehyde modification of chondroitin sulphate. (A) Chemical structure of chondroitin sulphate salt before and after oxidation with 0.3 molar equivalents of sodium periodate (NaIO_4) for 6 hours being then dialysed for 4 days, filtered (0.22 μm) and freeze-dried. (B) FTIR spectrum for chondroitin sulphate (CS) and aldehyde modified chondroitin sulphate (mCS). Spectra were zoomed in allowing for the aldehyde peak to be observed at 1738 cm^{-1} .

Then, to re-introduce the GAGs lost during the decellularisation, I proceeded to incorporate CS in the decellularised ECM. I started by using NaIO_4 mediated oxidation of CS ensured the opening on the neighbouring diols (-OH) located on the N-acetylgalactosamine residue, since the diols were converted producing a dialdehyde functionalised derivative (Figure 4.2A). To preserve the CS original structure and properties, 0.3 molar equivalents of NaIO_4 was used, to prevent excessive oxidation. To ensure formation of aldehyde groups, FTIR analysis (Figure 4.2B) of the mCS and natural CS salt was performed. The new peak observed

at 1738 cm^{-1} (Figure 4.2B) corresponded to the C-O stretch, suggesting the presence of aldehyde groups. The produced mCS was used to functionalise the dECM hydrogel to form the mCS hydrogels.

4.3 Characterisation of dECM-based and Functionalised Hydrogels

To develop the ECM-based hydrogels, including functionalised hydrogels, a pre-gel solution was first obtained. After collecting the lyophilised dECM particles, the material was solubilised via treatment with 3% acetic acid under sonication. Following the addition of 10X PBS and neutralisation with NaOH, along with filtration, a homogenous pre-gel without visible particles was produced, as depicted in figure 4.3A. Also, this image shows that following thermogelation at 37°C , the pre-gel forms an opaque hydrogel. FTIR showed alterations within protein secondary structure following gelation, especially in the Amide I (1647 cm^{-1}), Amide II (1551 cm^{-1}) and Amide A stretching ($3200\text{-}3500\text{ cm}^{-1}$) regions, as shown in figure 4.3B.

4.3.1 Characterisation of Functionalised Hydrogels with CS or mCS

CryoSEM imaging was used, since this approach prevents loss of the original gelatinous network structure, which occurs during normal SEM sample preparation. The representative images in figure 4.3C indicates a fibrous network within the hydrogels, providing further evidence for having successfully produced a hydrogel.

Turbidity measurements allowed for gelation kinetics to be determined for the hydrogels developed following functionalisation with different mCS concentrations (figure 4.3D). The dECM hydrogel had the most efficient gelation kinetics, requiring a shorter period (6.0 minutes) to reach 50% turbidity as shown in figure 4.3E. In relation to the functionalised hydrogels, the 0.2 mg/mL mCS hydrogels required significantly more time to reach $t_{1/2}$, with 6.8 minutes.

Rheological analysis was performed to examine the viscoelastic properties and strength of the gelatinous network. When comparing the functionalised hydrogels to the control, it was observed both these had viscoelastic properties and solid-like hydrogel profiles (supplementary figure 2). This was deduced due to the spectra showing G' being greater than the G'' . As per figure 4.3F-H, the G' and G'' of the hydrogels did not show any significant differences to each other at 0.1% strain. The lowest G' value (figure 4.3F) recorded was 252.6 Pa for the 0.4 mg/mL mCS hydrogel, however this was not significantly different to the dECM with a G' of 415.8 Pa . The G'' (figure 4.3G) and complex modulus (G^*) (figure 4.3H) also did not show any significant differences amongst the hydrogels. Overall, functionalising the dECM pre-gel

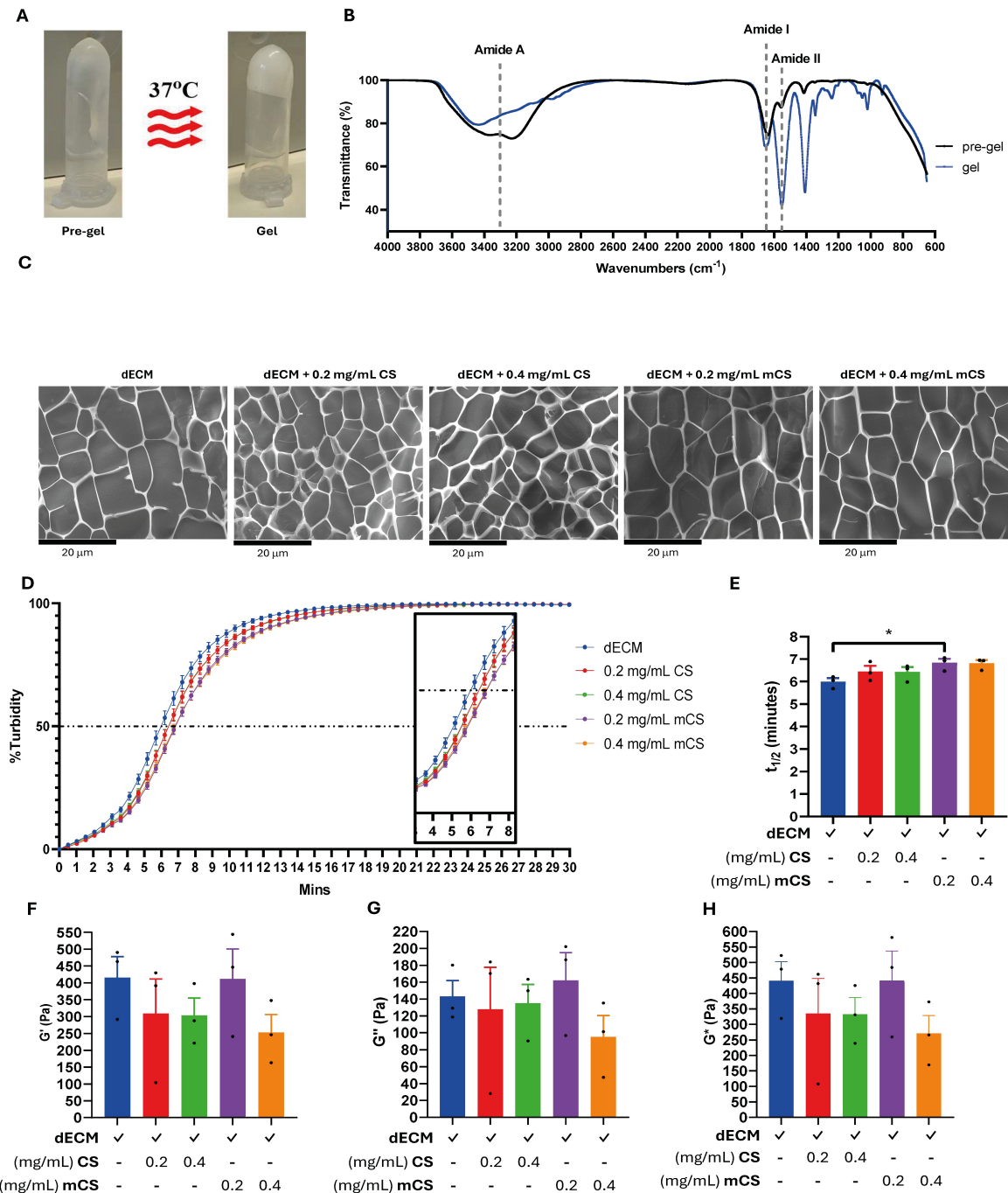


Figure 4.3: Characterisation of decellularised cartilage matrix and chondroitin sulphate-functionalised hydrogels. (A) Images of pre-gel solution and hydrogel. (B) FTIR spectra showing secondary structure formation. (C) CryoSEM analysis of decellularised matrix and functionalised hydrogels with either chondroitin sulphate (CS) and aldehyde modified chondroitin sulphate (mCS). Representative Images acquired utilising x2500 magnification with scale bars on the images. (D) Percentage turbidity of dECM with or without CS/mCS functionalisation over time (minutes). (E) Time (minutes) to reach 50% maximum turbidity for each hydrogel. (F-H) Rheological results show at 0.1% strain (F) The average storage modulus (G'), (G) the loss modulus (G'') and (H) complex modulus (G^*) of the hydrogels. Data presented as average $\pm$ SEM ($n = 3$) with significant differences shown as $p < 0.05$ (*) with the p values being compared to dECM. Symbols on the graph represent individual data points for each N.

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with CS or mCS did not significantly alter the rheological characteristics with all hydrogels having a low G' and G'' modulus following CS functionalisation.

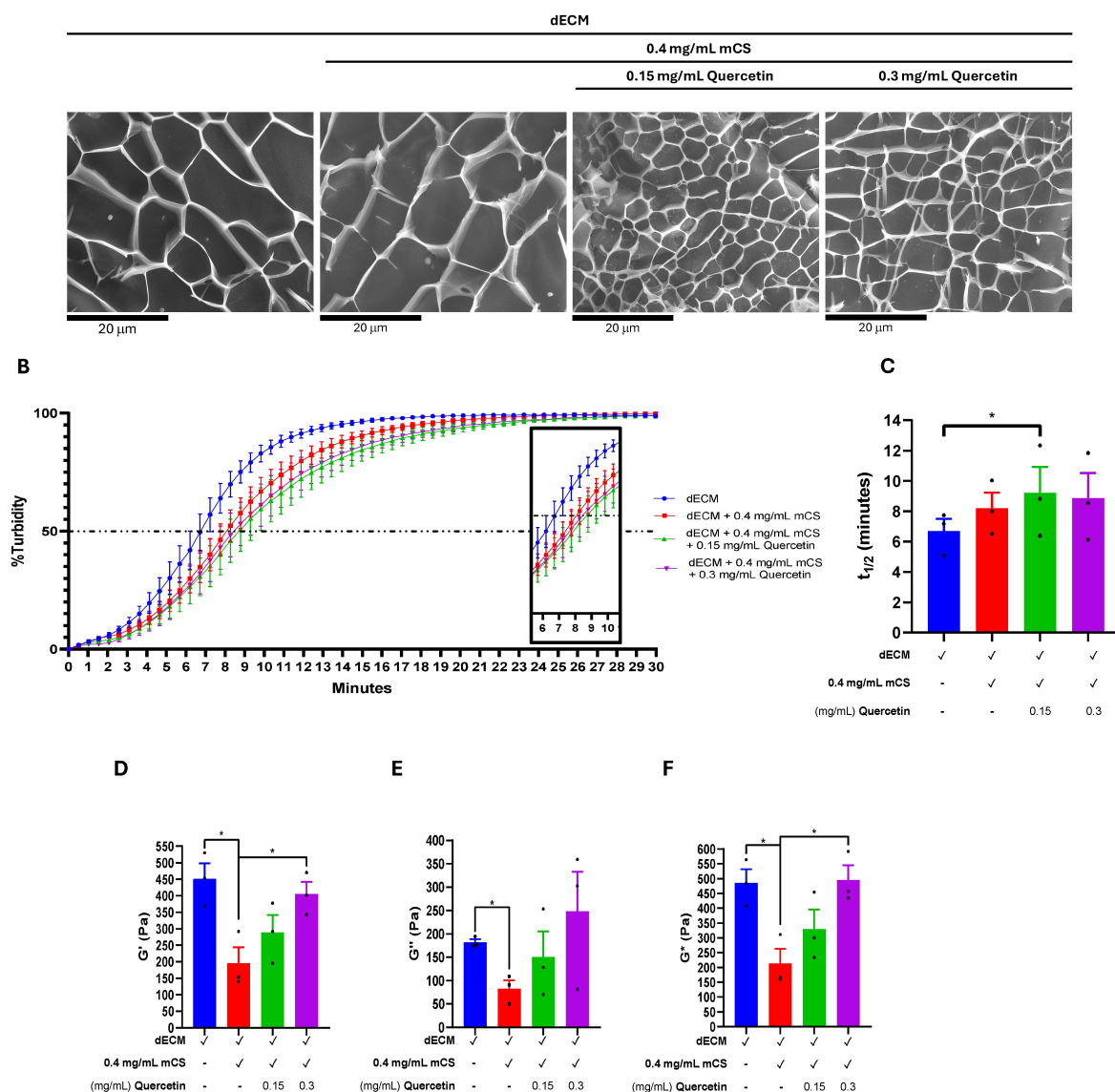


Figure 4.4: Characterisation of decellularised cartilage matrix and 0.4 mg/mL aldehyde-modified chondroitin sulphate hydrogels with or without quercetin. (A) CryoSEM analysis with representative images acquired utilising x2500 magnification (scale bars on images). (B) Percentage turbidity of decellularised matrix (dECM) functionalised with aldehyde-modified CS (mCS) with/without two quercetin concentrations (0.15 or 0.3 mg/mL) over time (minutes). (C) Time (minutes) to reach 50% maximum turbidity for the different hydrogels. Rheological results show at 0.1% strain (D) The average storage modulus (G'), (E) The loss modulus (G'') and (F) Complex modulus (G^*) of the different hydrogels. Data presented as mean $\pm$ SEM ($n = 3$). Depending on the normal distribution, the statistical analysis undertaken either with 1) One-Way ANOVA test followed by Tukey's multi-comparison test 2) Kruskal-Wallis test followed by Tukey's multi-comparison test $p < 0.05$ (*). In (B) statistical significance is against the dECM condition. Symbols on the graph represent individual data points for each N.

The 0.4 mg/mL mCS hydrogel was selected to further understand the impact of incorporating quercetin. The CryoSEM representative images in figure 4.4A, showed the organised

fibrillar network within the hydrogels. The turbidity results in figure 4.4B-C show that dECM hydrogels required 6.9 minutes to reach $t_{1/2}$ while in comparison the 0.15 mg/mL quercetin-incorporated hydrogels required significantly more time, taking 9.2 minutes. No significantly different $t_{1/2}$ times were observed when comparing the quercetin-incorporated hydrogels to 0.4 mg/mL mCS. In addition, comparing the quercetin concentrations also showed no significant differences.

The hydrogels had viscoelastic properties and were solid-like hydrogels at low strains and frequencies (supplementary figure 3). Relative to 0.1% strain, the rheological data (figure 4.4D), demonstrated the G' of dECM hydrogel was 451.0 Pa, being significantly higher than the 0.4 mg/mL mCS with a G' of 195.7 Pa. Both quercetin-incorporated hydrogels had a G' that was not significantly different to each other and the dECM hydrogel. While the 0.3 mg/mL quercetin hydrogel had a G' of 405.0 Pa that was significantly different to the 0.4 mg/mL mCS hydrogel. In relation to the G'' (figure 4.4E), the 0.4 mg/mL mCS hydrogel had an average value of 83.3 Pa which was significantly different to the dECM gel with 182.2 Pa. With no significant differences when comparing either hydrogel to either quercetin-incorporated hydrogel. To determine the strength of the hydrogels, the G^* values at 0.1% strain were observed (figure 4.4F). The 0.3 mg/mL quercetin hydrogel had a G^* of 495.7 Pa which was significantly different to 0.4 mg/mL mCS with a G^* of 213.9 Pa. The latter had a G^* that was significantly different to the dECM hydrogel (486.9 Pa). However, there were no significant differences for 0.15 mg/mL quercetin hydrogel in comparison to the rest of the hydrogels (figure 4.4D-F).

Overall, the characterisation analysis, showed 0.4 mg/mL mCS hydrogel to show significantly lower stiffness compared to dECM hydrogel. Also, the results show quercetin provides 0.4 mg/mL hydrogels with significantly increased strength at 0.1% strain.

4.4 Biointerface Studies

Having produced a family of hydrogels, the impact on cell viability and chondrogenic differentiation was subsequently investigated.

To ascertain the viability of MSC within the developed hydrogels with the two concentrations of CS and mCS, a Calcein-AM and EthD-1 staining was undertaken at 1- and 3-days of MSC culture (figure 4.5A-C). Representative images of the obtained results are illustrated in figure 4.5A. The quantitative results presented in figure 4.5B show the percentage of live cells in the total of live and dead cells for the different conditions. The dECM cell viability was around 70% and it remained consistent since the impact of the hydrogel functionalisation or time point provided no significant differences. Albeit, the hydrogels containing mCS show a slight decrease in cell viability at day 3 of culture (figure 4.5B). To determine the impact of the developed hydrogels on chondrogenesis, human chondrocytes were cultured within the different hydrogels for 21 days. Histological stainings were performed with representative re-

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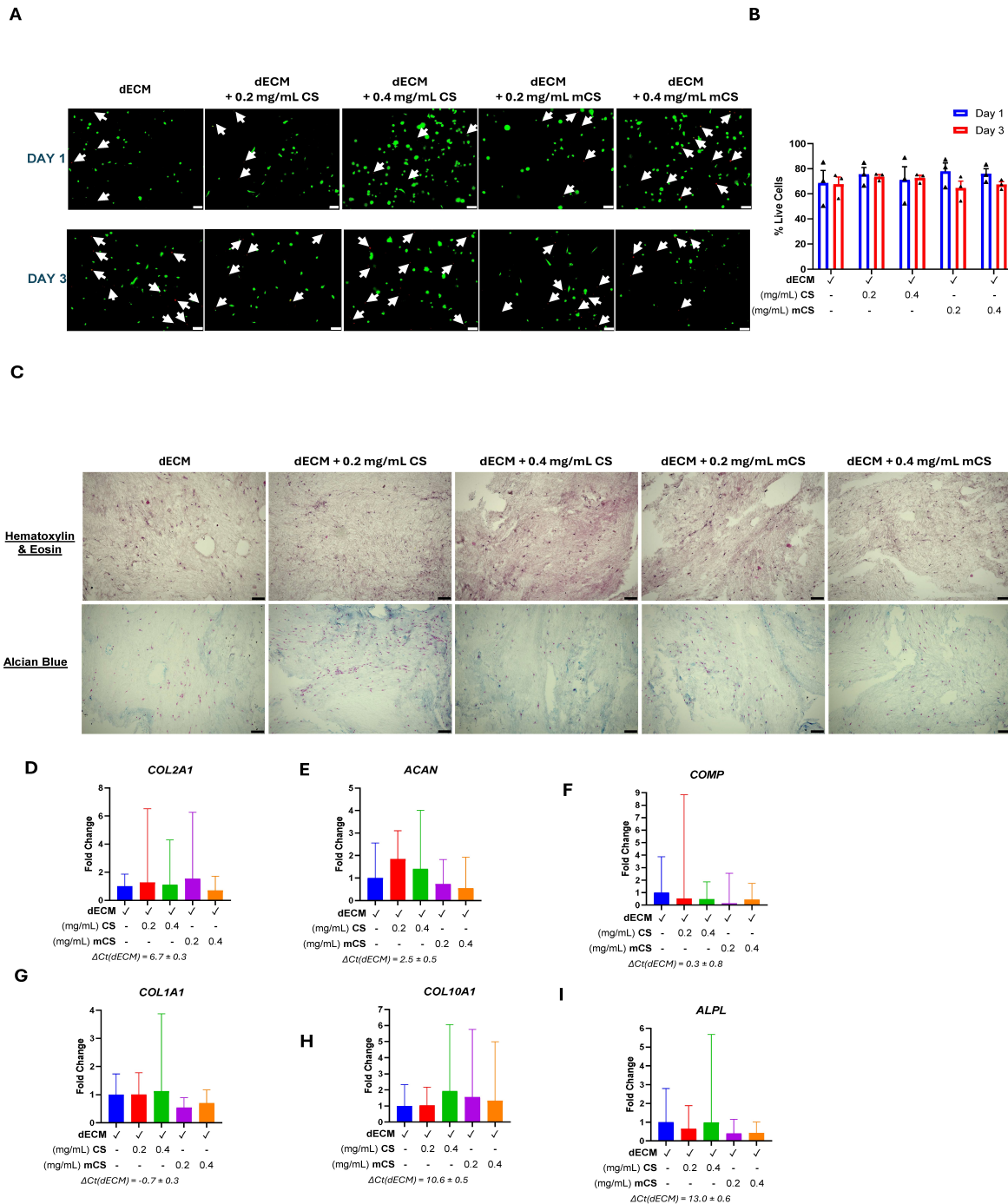


Figure 4.5: MSC viability and chondrogenesis in both non- and functionalised hydrogel types. (A) Viability of hTERT-MSCs in decellularised matrix (dECM) and functionalised hydrogels, stained with Calcein AM (green, live) and Ethidium Homodimer-1 (red, dead) at days 1 and 3, with arrows pointing at examples of dead cells (10X magnification, 100 μ m scale). (B) Percentage of viable cells shown as mean $\pm$ SEM (n=3). (C) Haematoxylin & Eosin and Alcian blue staining of chondrocyte seeded hydrogels after 21 days in chondrogenic media (scale bars 100 μ m). Symbols on the graph represent individual data points for each N. (D–I) qRT-PCR analysis of ECM gene expression (COL2A1, ACAN, COMP, COL1A1, COL10A1, ALPL) in hydrogels, seeded with chondrocytes, after 21 days. Hydrogels tested included dECM with or without 0.2 or 0.4 mg/mL chondroitin sulphate (CS) or aldehyde-modified CS (mCS). Fold change ($\Delta\Delta$ CT) was calculated relative to dECM control. Error bars represent upper and lower limits (n = 6).

sults presented in figure 4.5C showing cells are widely distributed throughout the hydrogels and appear to maintain a relatively round morphology. The alcian blue staining shows similar staining intensities as the different conditions (figure 4.5E). To gain a deeper understanding, both chondrogenic markers and hypertrophic marker related genes were analysed. All conditions were compared to the control sample, the dECM hydrogel. The gene expression results for cartilage matrix proteins (COL2A1, ACAN and COMP) are depicted in figure 4.5D-F, while hypertrophic markers (COL1A1, COL10A1 and ALPL), are depicted in figure 4.5G-I. These showed relatively high variations between experiments but no statistically significant differences between dECM hydrogels and CS hydrogels.

Overall, there were no significant differences in cell behaviour, upon functionalisation with either CS or mCS in dECM hydrogels.

To ascertain the ability of the developed hydrogels with incorporated quercetin to support cell culture, HDF cell viability was evaluated along time, using the Calcein-AM and EthD-1 staining (figure 4.6A). Representative images show some dead cells in all experimental conditions, even in dECM (figure 4.6A). Quantitative results are presented in figure 4.6B and show no significant differences for cells cultured in 0.4 mg/mL mCS hydrogel between day 1 (66.0%) and 3 (65.7%). Also, cells cultured in hydrogels with 0.15 mg/mL quercetin showed similar viability as in the control hydrogel. While on day 1, the cells cultured in 0.3 mg/mL quercetin containing hydrogels showed a viability of 58.6%, which on day 3, reached a viability of 68.8%. However, the differences in cell viability were not significant.

Histological staining analysis showed well dispersed chondrocytes with a round morphology, while alcian blue staining did not show any significant differences when comparing to those with incorporated quercetin (figure 4.6C). Then, the effect of quercetin incorporation in 0.4 mg/mL mCS on chondrogenic gene expression of human chondrocytes was evaluated. Results indicate that incorporation of this compound, upregulated COL2A1 expression, when compared with 0.4 mg/mL mCS hydrogels, with results showing 6.6-fold increase for the lower concentration while the higher concentration provided a 2.2-fold increase (figure 4.6D). No significant differences were observed in ACAN and COMP expression (figure 4.6E, F).

In relation to the hypertrophic markers, as per figure 4.6G and I, both COL1A1 and ALPL gene expression were not significantly different amongst the hydrogels. For COL10A1 expression, the 0.3 mg/mL quercetin concentration, with a fold change of 4.0 (figure 4.6H), showed a statistically significant increase when compared with dECM and 0.4 mg/mL mCS (0.9-fold). The lowest concentration of quercetin showed no significant differences, when compared to the other conditions. This demonstrates, 0.3 mg/mL quercetin and not the lower concentration of 0.15 mg/mL quercetin, in tandem with 0.4 mg/mL mCS can increase hypertrophic gene expression (COL10A1).

Overall, 0.15 mg/mL quercetin in 0.4 mg/mL mCS hydrogels enhances chondrogenic gene (COL2A1) expression.

CHAPTER 4. RESULTS

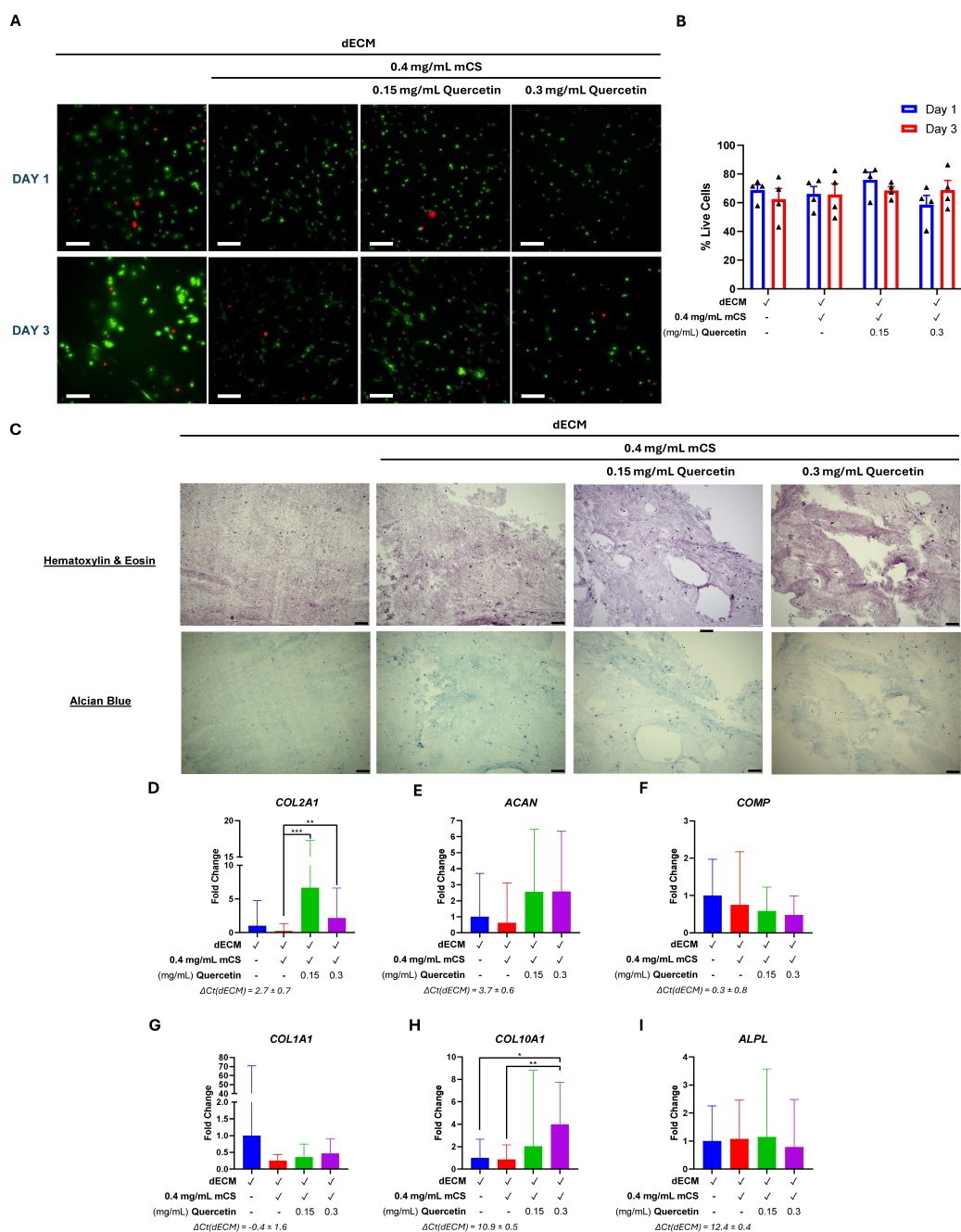


Figure 4.6: Cell viability and differentiation upon culture in non-functionalised and 0.4 mg/mL aldehyde modified chondroitin sulphate functionalised hydrogels with or without quercetin hydrate. (A) Viability of seeded human dermal fibroblasts (HDF) seeded in the different indicated hydrogels and stained with Calcein AM (green, live) and Ethidium Homodimer-1 (red, dead) at days 1 and 3. Scale bars = 200 μ m. (B) Percentage of viable cells shown as mean $\pm$ SEM (n = 4). Symbols on the graph represent individual data points for each N. (C) Haematoxylin & Eosin and Alcian blue staining of chondrocyte seeded hydrogels after 21 days in chondrogenic media (scale bars 100 μ m). (D–I) qRT-PCR analysis of ECM gene expression (COL2A1, ACAN, COMP, COL1A1, COL10A1, ALPL) in chondrocyte seeded hydrogels after 21 days. Hydrogels observed included that derived from dECM along with 0.4 mg/mL aldehyde modified chondroitin sulphate incorporated with quercetin at 0.15 and 0.3 mg/mL. Fold change ($\Delta\Delta CT$) was calculated relative to dECM control. Error bars represent upper and lower limits (n = 9). Depending on the normal distribution, the statistical analysis undertaken either with 1) One-Way ANOVA test followed by Tukey's multi-comparison test 2) Kruskal-Wallis test followed by Tukey's multi-comparison test p < 0.05 (*), p < 0.01 (**), p < 0.001 (***).

Chapter 5:

General Discussion

This chapter includes a general discussion in relation to the aspects introduced in the first chapters. The text here is based on the discussion section of the research article in chapter 9.

Potential biomaterials for cartilage regeneration require extensive evaluation to ensure their solubility, biocompatibility, and mechanical efficacy. Within this context, the functionalised hydrogels developed in this study (dECM, CS, and quercetin) may be categorised either as a medical device when acting primarily as a scaffold, or as a drug–device combination when intended for the pharmacological release of quercetin (Tataru et al., 2025). The latter classification was not evaluated in this study, as the current doctoral research focused on producing a hydrogel-based scaffold seeded with dedifferentiated chondrocytes. According to European regulatory frameworks, such constructs are defined as Advanced Therapy Medicinal Product (ATMP). Consequently, this work considers several factors related to device classification in accordance with International Organization for Standardization (ISO) standards (Kakroodi et al., 2025).

New biomaterials capable of restoring cartilage structure and function are needed for OA patients. An ideal biomaterial should provide a microenvironment which closely mimics native cartilage. Therefore, cartilage dECM demonstrates potential for hydrogel and scaffold preparation, with benefits reported both *in vitro* and *in vivo* studies (Bordbar et al., 2020; Zeng et al., 2022). Producing these biomaterials requires efficient ECM decellularisation to minimise immunogenicity. Furthermore, detergent-based cell removal may disproportionately reduce certain GAGs, notably decreasing CS more extensively compared to the GAG HA (Tan et al., 2023; Uhl et al., 2020).

To optimise the decellularisation protocol to be employed in the current study, three times of exposure to SDS were assessed: 2, 16 and 24 hours. Histological Haematoxylin & Eosin staining revealed that a 2-hour exposure did not efficiently decellularise the cartilage matrix, with cellular material still present (Supplementary Figure 7.1A). In contrast, no significant differences were observed between 16-hour and 24-hour exposures (Supplementary Figure 7.1B). Therefore, the 16-hour exposure was selected for further evaluation. An undesirable result of decellularisation is GAG loss, associated with the use of detergent-based solutions (e.g. SDS) (Bordbar et al., 2020). This class of biopolymers, especially CS, is vital for chondrogenesis (Wang and Yang, 2017). Thus, the ability to retain this polymer is paramount to ensure biomimicry (Yazdanpanah et al., 2023). Therefore, in this study, CS was reintroduced via functionalising dECM, compensating for the loss occurring during decellularisation. The decellularisation approach resulted in a retention of 87.3 $\mu\text{g}/\text{mg}$ of sGAGs, a 73% decrease compared to the native cartilage (figure 4.1C). In accordance with the literature, a reduced quantity of GAGs was to be expected, with Bordbar et al., reporting a decrease with 11 $\mu\text{g}/\text{mg}$ of sGAGs remaining (Bordbar et al., 2020). A subsequent paper by Guo and colleagues reported bovine articular cartilage contains approximately 40% GAGs, their dECM particle production utilising SDS detergent resulted in a 62% decrease (Guo et al., 2023). This study utilised a different sGAG quantification technique, 1,9-dimethylmethylene blue assay, showing higher initial sGAG levels than my results and Bordbar et al. (Bordbar et al., 2020; Guo et al., 2023). Despite GAG loss, dsDNA reduction to residual levels (figure 3.1B) allowed progression to subsequent experiments aimed at compensating for this

loss.

Considering the importance of sGAGs in chondrogenesis, reconstituting these appeared advantageous for enhancing hydrogel biomimicry (Singh et al., 2023). This was explored via functionalising dECM pre-gel with CS and evaluating physical versus covalent functionalisation approaches. Physical functionalisation entailed solely adding CS salt (shark sourced), allowing the polymers to self-assemble into an organised network during gelation, as previously reported (Rafighdoust et al., 2015). While, covalent functionalisation requires a CS modification, allowing the introduction of a complementary specific functional group to form a covalent bond with a chemical group within the dECM (Fang et al., 2022; Wang et al., 2007). An aldehyde modification was selected, as CS oxidation provides dialdehyde groups, enable multiple functional sites to be introduced (Dawlee et al., 2005; Fang et al., 2022). These can react with primary amines (imine bonds) or alcohols (hemiacetals) (Dawlee et al., 2005). As previously reported, such modifications may impact the biopolymer's bioactivity and stability, along with high oxidiser concentrations associated with increased polymer chain cleavage (Dawlee et al., 2005; Fang et al., 2022). Thus, the CS was exposed to low oxidiser levels to compare CS and mCS in cartilage dECM-based hydrogels.

Prior studies focused on CS functionalisation of solid dECM scaffolds from tissues other than cartilage, using methacrylated CS hydrogels or CS impregnation, rather than hydrogels (Cheung et al., 2014; Quijano et al., 2022; Rafighdoust et al., 2015). This is different to the approach I used, which involves directly functionalising the dECM-based pre-gel solution with CS or mCS. To my knowledge, CS functionalisation of dECM-derived hydrogels has not been previously shown, making this study a proof-of-concept.

Herein, the mCS modification was confirmed via FTIR analysis, with results aligning with previous reports (Fang et al., 2022). The spectra revealed a new peak at 1738 cm^{-1} , corresponding to the aldehyde group (Figure 4.2B). Additionally, FTIR analysis (Figure 4.3B) indicated secondary structure formation, characteristic of the transition from pre-gel to hydrogel. This observation was consistent across all CS and mCS-functionalised hydrogels (Supplementary Figure 7.2), demonstrating that secondary structure formation was retained in these.

To convert a solid scaffold into a soluble pre-gel, a typical approach is performed using an acidic pepsin-containing solution. Furthermore, since it was necessary to maximise the retrieval of ECM components, ultrasonic waves were applied through a sonication process since Akram and colleagues had already demonstrated its ability to aid in maximising the retrieval of collagen type II from cartilage (Akram and Zhang, 2020). Evidence from the literature shows ultrasonic waves result in mechanical oscillation and cavitation within the samples, disrupting protein structure (Akram and Zhang, 2020; Jin et al., 2021). Such disruption can expose concealed protein regions, aiding in digestion (Jin et al., 2021). Future studies should be undertaken to determine a more effective solubilisation strategy, since difficulties were encountered in the experiments to ensure full solubilisation of the dECM. Therefore,

filtering was necessary to remove the insoluble material.

A concerning issue with dECM scaffolds is high batch-to-batch variability due to tissue source differences between animals of the same species (Bejleri and Davis, 2019). Also, the solubilisation process can introduce variability in the final product due to incomplete digestion leading to heterogeneous hydrogels (Almalla et al., 2023). To ensure consistency, standardised decellularisation and solubilisation protocols are needed to reduce batch-to-batch variability (Almalla et al., 2023; Bejleri and Davis, 2019). In the current study, variability in characterisation and biointerface results (figure 4.3 to figure 4.6) reflects the use of different dECM batches.

Hydrogel mechanical properties were measured to assess the functionalisation effect on viscoelasticity. The overall results show hydrogels with a low G' modulus, with dECM hydrogel having an initial modulus that was significantly different to the functionalised hydrogel, of 416 Pa (figure 4.3F). According to a previous publication by Beck and colleagues (Beck et al., 2016), the compressive modulus for native articular cartilage is 1.8 MPa. While the compressive and G' moduli measure different mechanical properties, the high level of difference shows the hydrogel is considerably weaker than cartilage.

The utilisation of dECM in this study provided a biomaterial whose inherent mechanical properties are considerably lower than those of native cartilage. Both the decellularisation process—particularly when detergents such as SDS are used—and the subsequent solubilisation stage can negatively affect the ECM structure, leading to partial degradation or loss of essential components. These adverse effects are further exacerbated by the acid–pepsin digestion solution, which can denature structural proteins within the ECM. Consequently, the resulting hydrogels display mechanical properties that fall below the stiffness range desirable for cartilage applications. As a result, the produced hydrogels may not yet be suitable for implantation or preclinical evaluation, as they do not meet the mechanical performance requirements outlined in ISO 6721 (ISO, 2025).

Although no studies with dECM-based hydrogels functionalised with CS could be found in the literature, these results appeared to correlate with a study undertaken by Stuart et al. (Stuart and Panitch, 2008), who observed decreased G'' modulus following the presence of CS in collagen hydrogels (Stuart and Panitch, 2008). While the G'' of the 0.4 mg/mL mCS hydrogel (figure 4.4D) is significantly decreased versus the dECM hydrogel, supporting that mCS hydrogels may follow this trend.

An objective of this research was also to determine how the network forms over time. Turbidity measurements showed decreased gelation kinetics for the functionalised hydrogels compared to the dECM hydrogel. The average $t_{1/2}$ (figure 4.3E) showed the dECM hydrogel had the fastest gelation kinetics (6 minutes), with 0.2 mg/mL mCS hydrogels being significantly slower in comparison. Clearly, the mCS functionalisation within these dECM hydrogels is hindering the network organisation, slowing down gelation efficiency.

The viability of cells in the CS modified hydrogels was evaluated at day 1 and day 3 of cul-

ture. These early time points were selected to deduce the effect of cells being exposed to a hydrogel containing 0.5 M sodium acetate. The presence of this salt is a direct result of utilising NaOH to neutralise the 3% acetic acid pepsin containing solution used during the dECM solubilisation. Such a concentration of the resulting 0.5 M sodium acetate may lead to lower cellular viability according to previous studies (Zhao et al., 2021). Secondly, these time points allowed for ascertaining the cell viability of mCS functionalised hydrogels, which contained aldehyde modified CS. Such functional groups can interact with cellular components, disrupting cellular processes, resulting in toxicity which impacts cellular viability (LoPachin and Gavin, 2014). Overall, these time points showed there is only mild cytotoxicity to the seeded cells, at both day 1 and day 3 for all hydrogels (figure 4.5A, B), with no significant differences when comparing the conditions. Therefore, cell viability of 70% is a potential limitation within this study. Also, the number of cells appears to show some variations (figure 4.5A) especially between day 1 and 3. Previous studies have reported at week 6, a beneficial impact in cell viability when CS was provided to 3D cell cultures (Ingavle et al., 2012). No similar significant effects were observed within this study at day 1 and 3. Perhaps the composition or low stiffness of this dECM hydrogels nullifies the beneficial effect upon cell viability provided by CS. Furthermore, since it was necessary to decellularise the cartilage ECM, this can modify the biochemical and structural integrity of the ECM components (Fernández-Pérez and Ahearne, 2019). This may be associated with the cytotoxicity observed within the hydrogels including the dECM-based hydrogel. A study by Bordbar and colleagues reported that rabbit bone marrow stem cells exhibited lower metabolic activity (an indirect measurement of cell viability) at day 7 within cartilage dECM hydrogels compared to dECM solid scaffolds. Also, all conditions showed higher activity at day 7 compared to day 3, with the dECM hydrogel showing the slowest cell growth Bordbar et al., 2020. While, in this study, cell viability was directly measured (counting live and dead cells), with mild cytotoxicity observed in the dECM hydrogel. Evidently, in both the previous study and these results show no significant differences at 3 days. Although later time points were not investigated, the early-stage data provided valuable insight into initial cell responses to these hydrogels and functionalisation strategies.

Hydrogel mechanical cues, especially stiffness, can influence MSC chondrogenic differentiation (Ai et al., 2023). However, in contrast to the initial hypothesis on the effect of CS functionalisation, the expression of chondrogenic and hypertrophic genes showed no significant differences overall (figure 4.5D-I). A previous study by Bachmann and colleagues (Bachmann et al., 2020), showed a stiffness of 30 kPa compared to 1 kPa, resulted in increased collagen type II and aggrecan synthesis (Bachmann et al., 2020). Since in this study, hydrogels presented a low stiffness, this may play a role in the results obtained in suppressing cartilage matrix production. Finally, it should be ascertained in future studies if hydrogel stiffness or CS have a preponderant impact on chondrogenesis induced by dECM hydrogels, since Ai et al. (Ai et al., 2023) have shown the optimal CS concentration for chondrogenesis was 6% (w/v) regardless of the stiffness of hydrogels, but for the same CS

concentration stiffer hydrogels tend to have better performance (Ai et al., 2023).

Furthermore, the approach utilised a chondrogenic supplemented culture medium. This was due to having MSC or dedifferentiated chondrocytes which necessitate supplements to undergo re-differentiation to express cartilage specific genes (Lee et al., 2017a). Consequently, any changes in the expression of chondrogenic genes is not only influenced by the biochemical and biomechanical microenvironment provided by the hydrogel, but also these factors. To assess the direct impact of the hydrogels, removing chondrogenic supplementation, as demonstrated by Roncada et al., could provide valuable insights. (Roncada et al., 2022). However, supplementation is considered necessary for the activation of the chondrogenic signalling pathways (Futrega et al., 2021) and thus was selected.

This study investigated quercetin's impact on hydrogel mechanical and biological behavior, building on existing evidence that quercetin can crosslink polymers and promote chondrogenesis (Greco et al., 2018; Yu et al., 2020). Notably, no previous studies have examined quercetin incorporation within dECM-based hydrogels, highlighting the novelty of this work and reinforcing the crucial roles of quercetin, CS, and dECM in cartilage tissue engineering. Incorporation of quercetin within these hydrogels demonstrates its value as a key component for enhancing cartilage regeneration. In a previous study involving genipin crosslinking of decellularized cartilage solid scaffolds, which provided increased rigidity along with improved chondrogenic gene expression notably of COL2A1 (Cheng et al., 2013). Quercetin showed a similar outcome, implying there may now be more than one strategy to improve chondrogenesis using cartilage dECM, with quercetin offering a method that maintains a flexible hydrogel scaffold (all these aspects are further evaluated below).

Rheological data for quercetin-incorporated hydrogels demonstrated its ability to improve the G' modulus. This aligns with previous studies on evaluating quercetin's crosslinking ability (Greco et al., 2018). The G^* analysis in figure 4.4F, provides evidence for 0.3 mg/mL quercetin-incorporated within 0.4 mg/mL mCS, significantly increases the hydrogel's strength (0.3 mg/mL quercetin versus 0.4 mg/mL mCS), while not showing significant differences to the dECM hydrogel. This can imply, incorporated quercetin within the dECM and 0.4 mg/mL mCS hydrogel, restored the mCS functionalised hydrogels to their original strength prior to functionalisation (since no significant differences between quercetin concentrations and dECM). Thus, depicting the importance of quercetin for improving mCS hydrogel's rheological properties. However, no significant difference was observed between quercetin-incorporated hydrogels gels and its corresponding hydrogel, 0.4 mg/mL mCS. In a previous publication by Greco et al. (Greco et al., 2018), incorporation of 1 mg/mL quercetin (in PBS) in decellularised dermal scaffolds, stiffness increased, though less than with genipin. (Greco et al., 2018). The results I obtained may suggest that quercetin can overcome the hindrances which mCS provides to the hydrogel network, allowing for improved rheological properties.

Quercetin exhibits anti-cancer cytotoxicity against hTERT-mutated cells (Kusaczuk et al., 2022; Ren et al., 2017). Therefore, HDF cells, which tolerate quercetin, were used in these

experiments (Sohn et al., 2018). Results in figure 4.6A-B showed that quercetin incorporation within the 0.4 mg/mL mCS-functionalised hydrogels did not lead to significant increases in cytotoxicity (as can potentially occur with hTERT cell lines). However, these all showed low to moderate cytotoxicity when observing both day 1 and day 3, which constitutes a potential limitation of the current study. A previous study by Zawani and colleagues used quercetin-embedded gelatin–elastin hydrogels, and showed HDF cell viability at day 1, was optimal with 0.125 mg/mL quercetin (Zawani et al., 2022). Their results report cell viability via a cell metabolic assay rather than directly quantifying the percentage of live cells as in this study. My quercetin-incorporated hydrogels that had the highest viability at day 1 was 0.15 mg/mL (75.9%), which corresponds to mild cytotoxicity and was not statistically different to the rest of the conditions. Notably, the results shown here could possibly be associated with the inherent properties of the dECM hydrogel contributing to the observed cytotoxicity. Also, Greco and colleagues reported that 1 mg/mL quercetin is optimal for crosslinking, while Zawani and colleagues found that cell viability is preferable at concentrations below 0.5 mg/mL (Greco et al., 2018; Zawani et al., 2022). To minimise cytotoxicity, the highest quercetin concentration was limited to below 0.5 mg/mL. Despite this, cytotoxicity was still observed, which may have influenced the chondrogenesis related results.

The gene expression results show quercetin inclusion provides statistically significant advantages (especially when comparing the 0.4 mg/mL mCS hydrogel +/- quercetin) relative to the expression of COL2A1. The 0.15 mg/mL quercetin-incorporated hydrogel showing the highest significant increase (6.6-fold change) vs 0.4 mg/mL mCS hydrogel, (0.2-fold change) seen in figure 4.6D. Increased COL2A1 has been previously reported in rat chondrocytes effected by quercetin-bioglass hydrogels [41]. Both quercetin concentrations significantly increased COL2A1 expression. There was a significant increase in the hypertrophic gene COL10A1 with 0.3 mg/mL quercetin showing 4.0-fold increase (figure 4.6H) verse both the dECM hydrogel and 0.4 mg/mL mCS-functionalised dECM hydrogel. Since the percentage of live cells at day 1 for 0.3 mg/mL quercetin showed moderate cytotoxicity, this could have implications in the results (COL10A1). Also, this research project for the first time showed an instance where hypertrophic gene expression may occur. The expression of COL10A1, appears to be normal with expression indicating both maturation and differentiation status (Bian et al., 2021). Together, these results show 0.4 mg/mL mCS hydrogels have a positive influence upon chondrogenesis (> hypertrophy) when quercetin is incorporated especially at 0.15 mg/mL. Therefore, providing a basis for future studies to apply quercetin for enhancing chondrogenic regenerative capability of CS functionalised dECM.

In summary, this study introduces a highly novel hydrogel system by combining quercetin with CS-functionalised dECM-based scaffolds to create a flexible biomaterial designed to restore cartilage ECM. Leveraging quercetin's unique pro-chondrogenic bioactivity and moderate crosslinking ability, this approach promotes chondrogenesis. The hydrogel developed here offers a promising new direction for cartilage tissue engineering by creating a supportive and bioactive microenvironment that more closely mimics native cartilage.

Chapter 6:

Conclusion and Future Work

This chapter includes a an overall conclusion of the thesis. Finally, a discussion is presented on future work aimed at answering certain scientific queries that arose during the project and how this coincides in producing a successful hydrogel. The text here is based on the discussion and conclusion section of the research article in chapter 9.

6.1 Conclusion

The work undertaken in this doctoral project focused on developing a proof-of-concept functionalised hydrogel as a potential therapeutic approach for OA. All components used in the production of these hydrogels are sourced from natural materials without the use of synthetic materials. Hydrogel characterisation and biointerface studies revealed no significant differences between CS- and mCS-functionalised hydrogels. However, incorporating quercetin within the 0.4 mg/mL mCS-functionalised formulation enhanced both mechanical performance and chondrogenic activity. Notably, COL2A1 expression increased by 6.6-fold and 2.2-fold at quercetin concentrations of 0.15 and 0.3 mg/mL, respectively. Thus, functionalising decellularised cartilage-based hydrogels with mCS and quercetin provides a novel biomaterial for cartilage regeneration and demonstrates the relevance of quercetin in scaffolds for this purpose.

6.2 Future Work

As mentioned in the previous chapter, in comparison with previous studies, the decellularisation process used in this work resulted in a higher loss of sGAGs (Bordbar et al., 2020; Guo et al., 2023). A potential optimisation could involve vacuum-assisted decellularisation, which applies negative pressure to the tissue during processing. This facilitates deeper penetration of detergents and enzymes into the ECM, improving efficiency while reducing the need for high concentrations of decellularising agents. Such methods have been reported to better preserve ECM integrity and improve the biological performance of the resulting biomaterial, including cell viability (Butler et al., 2017). Another promising alternative involves utilising cartilage-derived dECM particles incorporated into HA-tyramine hydrogels, which avoid solubilisation steps and thereby retain key cartilage matrix components, resulting in improved mechanical performance (Guo et al., 2023).

An ongoing challenge in this field is mitigating damage to dECM components during decellularisation while achieving full solubilisation of these rigid tissues. As noted in subsection 4.1, cartilage was difficult to solubilise, even when ultrasonic treatment was employed to promote protein disruption via mechanical oscillation and cavitation (Akram and Zhang, 2020; Jin et al., 2021). Future studies should therefore focus on optimising the solubilisation process, potentially by refining pulverisation methods or combining mechanical and enzymatic pre-treatments, to ensure a homogeneous, fully solubilised pre-gel solution suitable for hydrogel formation (Bordbar et al., 2020; Golebiowska et al., 2024).

Enhancing the hydrogel's mechanical properties to more closely mimic native cartilage remains a key objective. It will also be important to investigate whether functionalisation with negatively charged CS introduces charge imbalances that influence gelation kinetics or stiffness (Nagayasu et al., 2012). If these effects are detrimental, supplementation with positively

charged biomolecules, such as collagen or collagen peptides, could help restore equilibrium (Gachon and Mesquida, 2021; Sim et al., 2024). Furthermore, modulating the molar concentration of NaIO_4 to generate mCS with increased carbonyl functional groups could further enhance crosslinking and stiffness (Dawlee et al., 2005). To improve understanding of the effects of mCS in combination with quercetin, it may be necessary to perform quantitative analysis of pore size using CryoSEM imaging. While this study focused on acquiring qualitative images, further quantitative evaluation would help elucidate how these molecules influence the hydrogel's physical network and subsequent mechanical properties (Jayawardena et al., 2023). Furthermore, this will allow to determine if these effects can effectively enhance the microenvironment's chondrogenic potential.

In vivo approaches would provide physiologically meaningful assessments of biomaterial performance, particularly regarding host immune response and cartilage regeneration. Static *in vitro* studies may miss critical dynamic interactions; thus, protein- or peptide-radiolabelled conjugates could be employed to visualise molecular and cellular mechanisms underlying immune polarisation during the regenerative process (Calabrese et al., 2016; Szychlińska et al., 2020). Specifically, future work should prioritise evaluation in an *in vivo* OA model (Xu et al., 2024). The STR/ort mouse model offers a particularly relevant system, as it closely represents human OA pathology and enables analysis of genetic, molecular and biomechanical mechanisms (Staines et al., 2017). Within such models, non-invasive imaging including Single-Photon Emission Computed Tomography combined with micro-Computed Tomography could be used to track hydrogel-mediated modulation of inflammation through targeted antibody–radioprobe conjugates, such as those addressing the macrophage mannose receptor (Put et al., 2013; Rix et al., 2025).

Regarding the cell viability results, these are relevant since the hydrogels were seeded with cells. It is also necessary to measure cell cytotoxicity to determine whether the hydrogels meet the ISO 10993 standard (ISO, 2009). This norm states that cell viability needs to be above 70%, which, in several conditions observed in these results, is lower. As detailed in section 3.13, a cell viability test was performed using live and dead cell counts, while previous publications used indirect viability methods such as metabolic activity assays (Bordbar et al., 2020; Zawani et al., 2022). Bordbar et al. reported improved viability in dECM solid scaffolds compared to hydrogels (Bordbar et al., 2020). This may be linked to the solubilisation technique, combined with prior decellularisation detergent exposure, which may cause damage to ECM components (Almalla et al., 2023; Fernández-Pérez and Ahearne, 2019). Further investigation may be required to understand the underlying causes and identify methods to reduce cytotoxicity observed in cartilage dECM-based hydrogels.

In relation to further evaluating the chondrogenic potential of the functionalised hydrogels, future studies should include molecular analysis of key chondrogenic markers, such as SOX9, via quantitative RT-PCR. Analysing this marker would provide evidence of transcriptional activation driving cartilage-specific matrix production (Bordbar et al., 2020), thereby complementing viability and morphological data.

It is possible that the decellularisation and solubilisation strategies implemented could expose RGD peptides present in the collagen fibres and influence the hydrogel biointerface (Huang and Cai, 2025; Taubenberger et al., 2010). Typically, these peptides are not exposed; however, the bioactive sequence may enable cells to adhere within the hydrogel, potentially influencing cell behaviour, as this peptide domain is bioactive (Taubenberger et al., 2010; Yang et al., 2021b). Therefore, increased exposure of residues that are usually hidden in physiological cartilage tissue could have non-physiological effects on the cells (Kumar et al., 2023; Taubenberger et al., 2010). This area may require more detailed investigation in the future, since RGD concentration, if not optimised, can favour bone repair and formation (Yang et al., 2021b).

Finally, because conventional scaffold-based and cell-seeded systems may overlook molecular modulators of repair, integrating or modulating specific microRNAs offers a promising therapeutic angle. MicroRNAs regulate key pathways involved in chondrogenic differentiation, matrix synthesis and inflammatory signalling (Zhang et al., 2023). Evaluating these strategies through *in vivo* studies, supported by molecular and imaging biomarkers, would provide a comprehensive approach to assess the regenerative and immunomodulatory capacity of novel biomaterials (Rix et al., 2025).

Chapter 7:
Supplementary Information

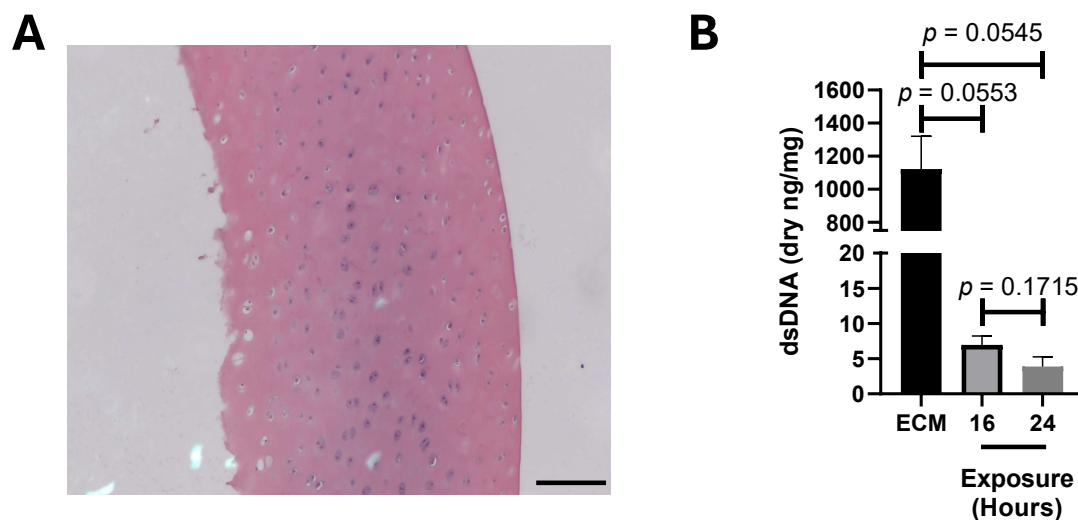


Figure 7.1: Characterising removal of cellular and dsDNA contents related to 0.5% SDS exposure durations | (A) Image of dECM cartilage particles stained with haematoxylin & eosin (matrix and cells) after 2 hours exposure to 0.5% SDS. Image obtained at 10X magnification with 100 μ m scale bars. (B) dsDNA quantification for ECM particles verse 0.5% SDS exposure for either 16 or 24 hours. Data presented as mean $\pm$ SEM ($n = 3$). The statistical analysis was undertaken with RM One-Way ANOVA test followed by Tukey's multi-comparison test.

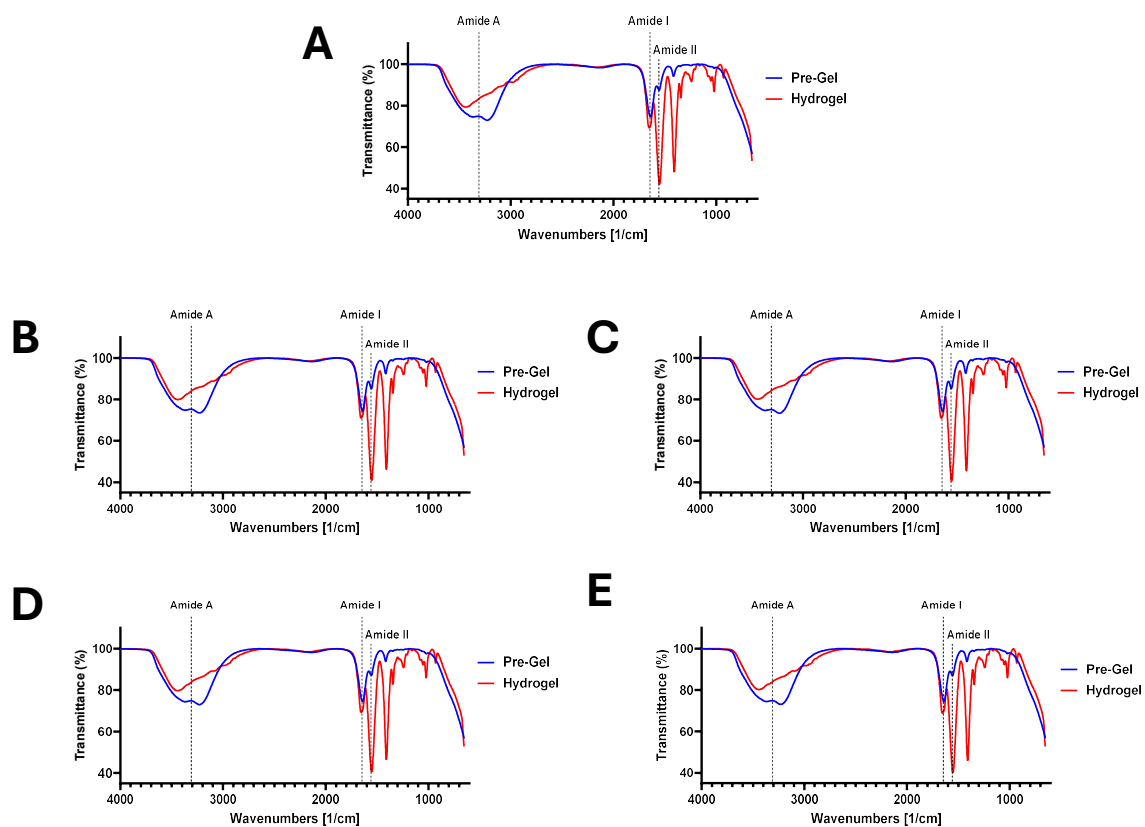


Figure 7.2: FTIR of dECM and functionalised pre-gels and hydrogels | All diagrams show the pre-gel and hydrogel spectra for: (A) dECM (B) 0.2 mg/mL nmCS (C) 0.4 mg/mL nmCS (D) 0.2 mg/mL mCS and (E) 0.4 mg/mL mCS

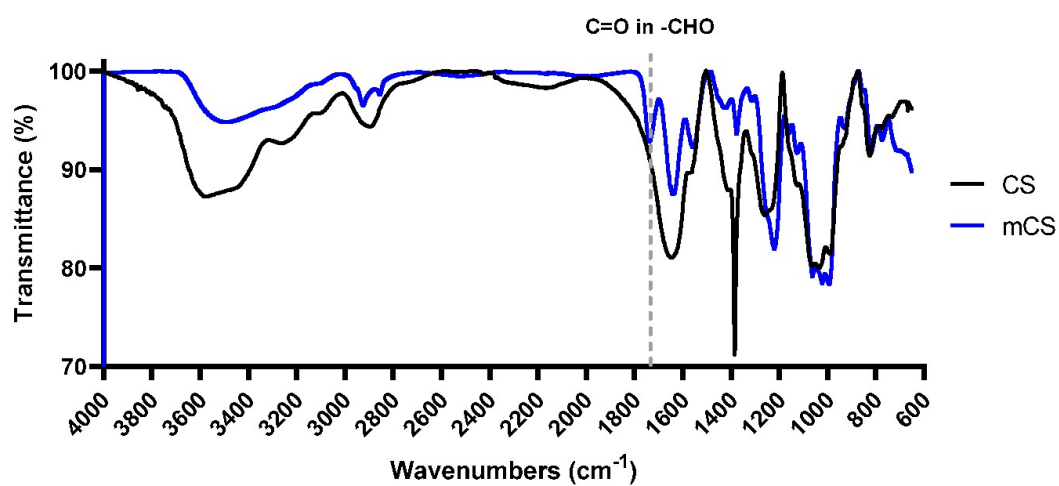


Figure 7.3: FTIR of aldehyde modified chondroitin sulphate. FTIR spectrum for both chondroitin sulphate (CS) and aldehyde modified chondroitin sulphate (mCS). Aldehyde peak was observed at 1738 cm^{-1} . For the research article in chapter 9, this is Figure S1

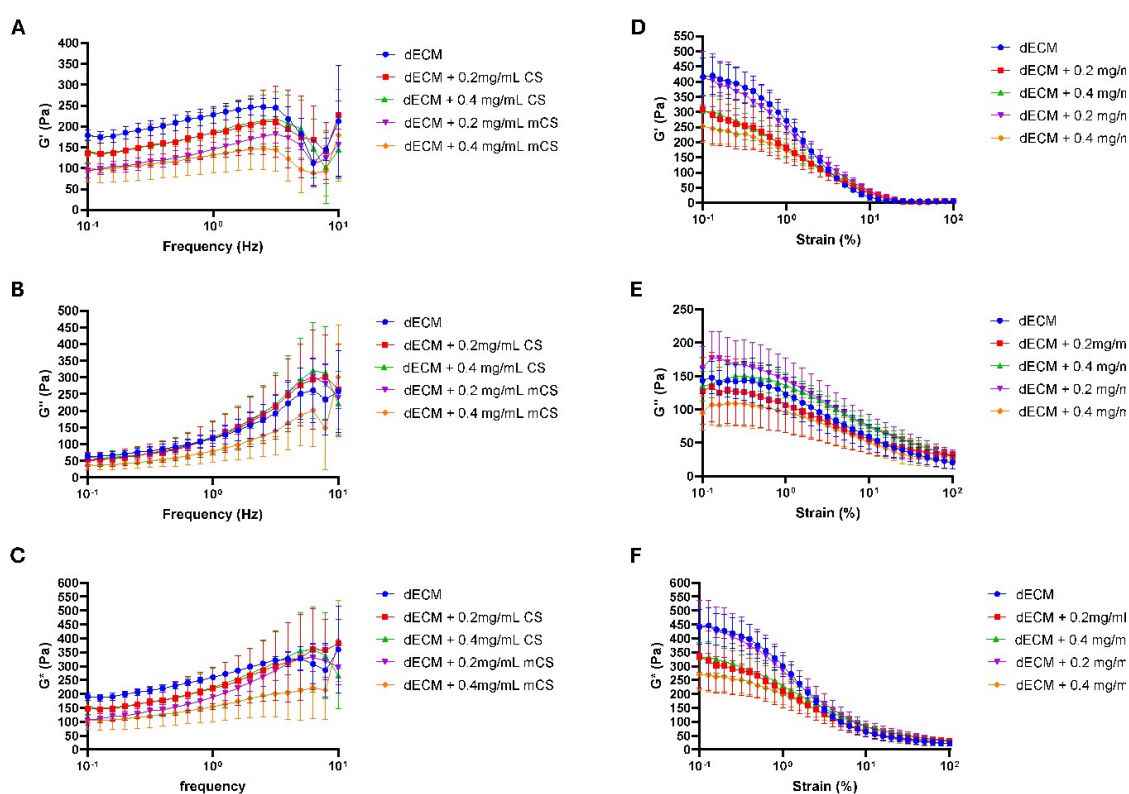


Figure 7.4: Rheological analysis spectra for decellularised cartilage-based hydrogels and with modified/non-modified chondroitin sulphate functionalised gels. (A,D) G' , (B,E) G'' , (C,F) G^* . (A-C) Frequency analysis from 0.1 to 10 Hz with 1% strain. (D-F) Strain amplitude of 0.1% to 100% with 1 Hz frequency. For the research article in chapter 9, this is Figure S2

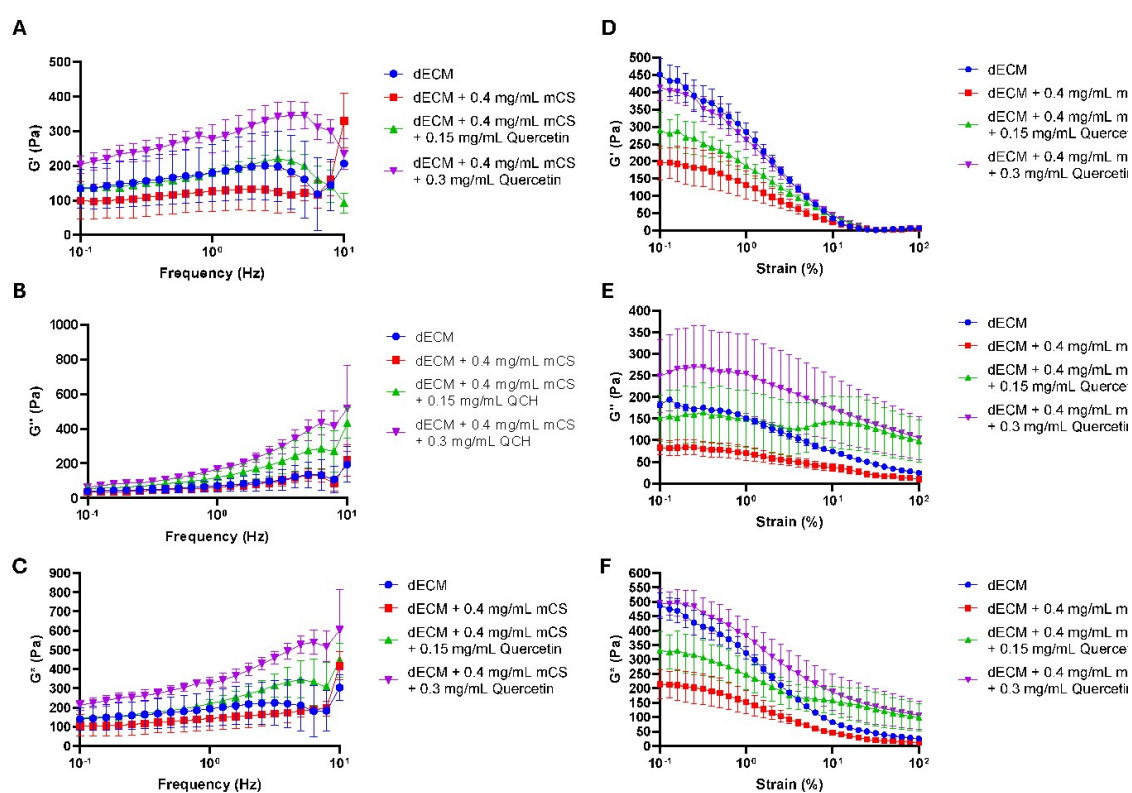


Figure 7.5: Rheological analysis spectra for dECM, aldehyde modified chondroitin sulphate functionalised hydrogels and quercetin incorporated functionalised hydrogels. (A,D) G' , (B,E) G'' , (C,F) G^* . (A-C) Frequency analysis from 0.1 to 10 Hz with 1% strain. (D-F) Strain amplitude of 0.1% to 100% with 1 Hz frequency. For the research article in chapter 9, this is Figure S3

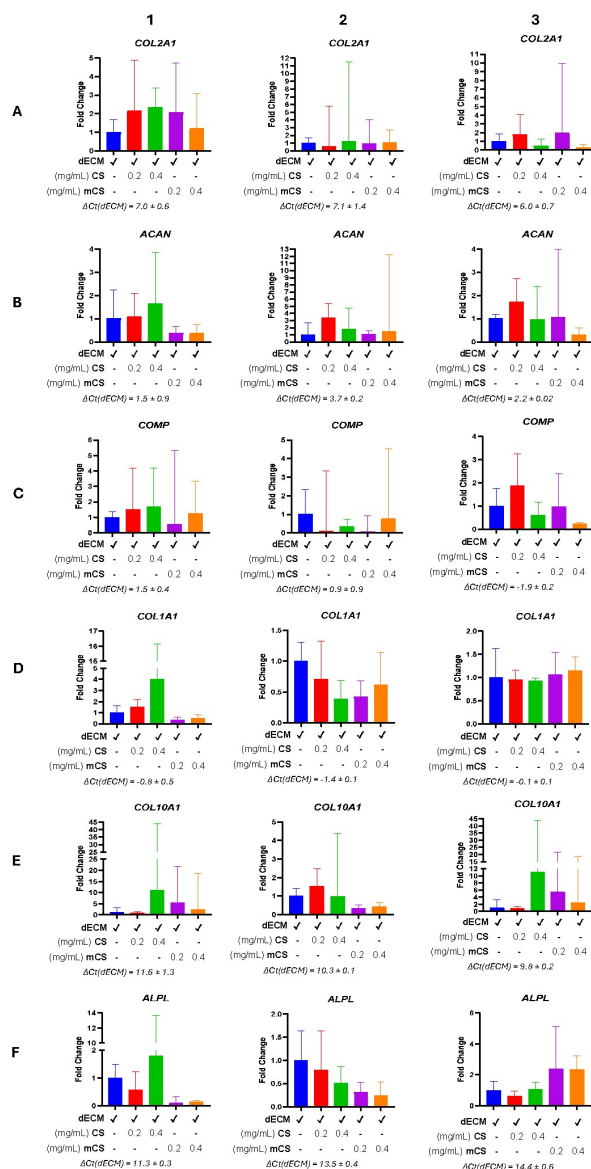


Figure 7.6: *in vitro* gene expression of extracellular matrix components. mRNA expression level of (1-6A) Collagen type 2 (COL2A1), (1-6B) Aggrecan (ACAN), (1-6C) Cartilage oligomeric matrix protein (COMP), (1-6D) Collagen type 1 (COL1A1), (1-6E) Collagen type 10 (COL10A1), (1-6F) Alkaline phosphate (ALPL) by quantitative real-time PCR of cell seeded gels after 21 days of *in vitro* culture. (1-3) Hydrogels observed included the dECM hydrogel along with non-modified (CS) and modified (mCS) functionalised gels with concentrations of 0.2 mg/mL and 0.4 mg/mL. (4-6) Hydrogels observed included the dECM hydrogel along with 0.4 mg/mL mCS with this hydrogel being then incorporated with quercetin at concentrations of 0.15 mg/mL and 0.3 mg/mL. Fold change ($\Delta\Delta C_t$) to control samples, being the dECM hydrogel. Error bars represent upper and lower limits ($n = 2$). For the research article in chapter 9, this is Figure S4

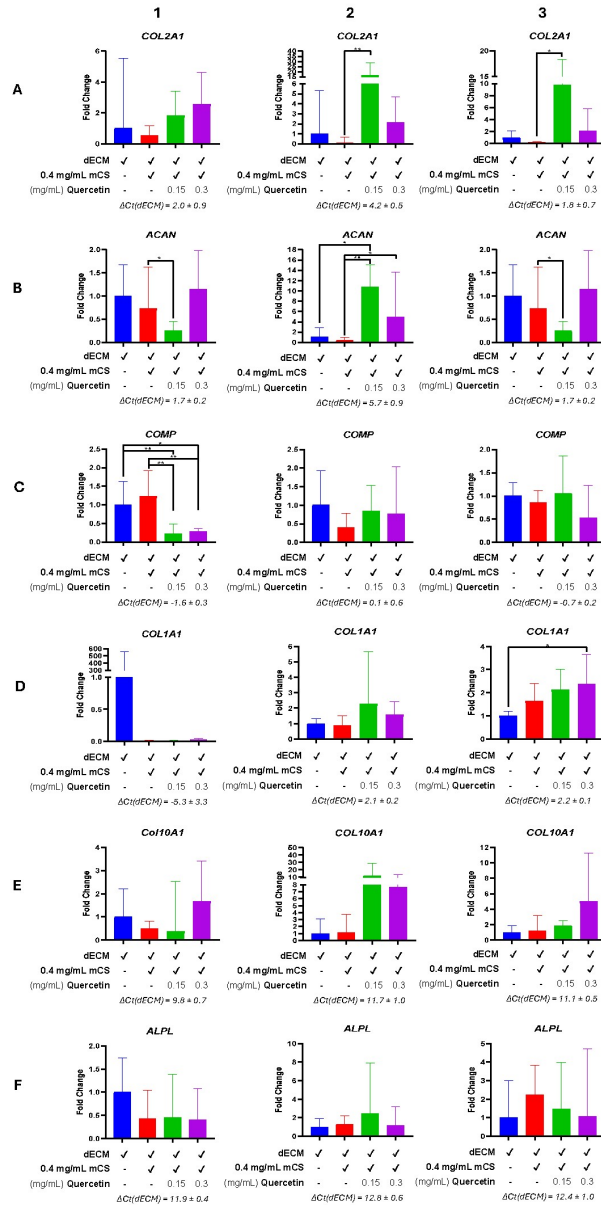


Figure 7.7: *in vitro* gene expression of extracellular matrix components. mRNA expression level of (1-3A) Collagen type 2 (COL2A1), (1-3B) Aggrecan (ACAN), (1-3C) Cartilage oligomeric matrix protein (COMP), (1-3D) Collagen type 1 (COL1A1), (1-3E) Collagen type 10 (COL10A1), (1-3F) Alkaline phosphatase (ALPL) by quantitative real-time PCR of cell seeded hydrogels after 21 days of *in vitro* culture. Hydrogels observed included the dECM hydrogel along with 0.4 mg/mL mCS hydrogel with this being then incorporated with quercetin at concentrations of 0.15 mg/mL and 0.3 mg/mL. Fold change ($\Delta\Delta C_t$) to control samples, being the dECM hydrogel. Error bars represent upper and lower limits ($n = 3$). Depending on the normal distribution, the statistical analysis undertaken either with 1) One-Way ANOVA test followed by Tukey's multi-comparison test 2) Kruskal-Wallis test followed by Tukey's multi-comparison test $p < 0.05$ (*), $p < 0.01$ (**). For the research article in chapter 9, this is Figure S5

G'		
Strain (%)	Conditions	Significance
0.13	dECM vs. dECM + 0.4 mg/mL mCS	*
2.00	dECM vs. dECM + 0.4 mg/mL mCS	*
2.52	dECM vs. dECM + 0.4 mg/mL mCS	*
G*		
Strain (%)	Conditions	Significance
0.10	dECM vs. dECM + 0.4 mg/mL mCS	*
0.13	dECM vs. dECM + 0.4 mg/mL mCS 0.4 mg/mL mCS vs. dECM + 0.4 mg/mL mCS + 0.30mg/mL Quercetin	*
3.99	dECM vs. dECM + 0.4 mg/mL mCS	*
5.02	dECM vs. dECM + 0.4 mg/mL mCS	*
6.31	dECM vs. dECM + 0.4 mg/mL mCS	*
7.95	dECM vs. dECM + 0.4 mg/mL mCS	**
10.00	dECM vs. dECM + 0.4 mg/mL mCS	*
12.59	dECM vs. dECM + 0.4 mg/mL mCS	*
15.85	dECM vs. dECM + 0.4 mg/mL mCS	*

Table 7.1: Statistical analysis of G', G'' and G* over strain amplitude for supplementary figure 7.5. The statistical analysis undertaken with RM two-way ANOVA with the Geisser-Greenhouse correction followed by Tukey's multiple comparisons test $p < 0.05$ (*), $p < 0.01$ (**). For the research article in chapter 9, this is Table S1

G'		
Frequency (Hz)	Conditions	Significance
5.012	dECM + 0.4 mg/mL mCS vs. 0.4 mg/mL mCS + 0.3 mg/mL Quercetin	*
G''		
Frequency (Hz)	Conditions	Significance
1.585	dECM + 0.4 mg/mL mCS vs. 0.4 mg/mL mCS + 0.3 mg/mL Quercetin	*
1.995	dECM + 0.4 mg/mL mCS vs. dECM + 0.4 mg/mL mCS + 0.3 mg/mL Quercetin	*
G*		
Frequency (Hz)	Conditions	Significance
0.7943	dECM + 0.4 mg/mL mCS + 0.15 mg/mL Quercetin vs. dECM + 0.4 mg/mL mCS + 0.3 mg/mL Quercetin	*
3.981	dECM + 0.4 mg/mL mCS vs. dECM + 0.4 mg/mL mCS + 0.3 mg/mL Quercetin	*
5.012	dECM + 0.4 mg/mL mCS vs. dECM + 0.4 mg/mL mCS + 0.3 mg/mL Quercetin	*

Table 7.2: Statistical analysis of G', G'' and G* over Frequency for supplementary figure 7.5. The statistical analysis undertaken with RM two-way ANOVA with the Geisser-Greenhouse correction followed by Tukey's multiple comparisons test $p < 0.05$ (*). For the research article in Chapter 9, this is Table S2

Chapter 8:
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This chapter includes the bibliography relevant to all the citations.

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Chapter 9:

ANNEX 1: Research Article

This chapter includes the research article corresponding to this thesis:

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RESEARCH ARTICLE OPEN ACCESS

Decellularised Cartilage-Based Hydrogels Functionalised With Chondroitin Sulphate and Quercetin: The Impact on Chondrogenesis

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ABSTRACT

Tissue engineering and regenerative medicine approaches are being actively developed for degenerative disorders, including osteoarthritis (OA). Decellularized matrix (dECM) is a promising biomaterial; however, glycosaminoglycan (GAG) loss during decellularization limits its chondrogenic potential. In this study, we aimed to overcome this by developing a dECM hydrogel originating from cartilage, functionalized with the GAG chondroitin sulphate (CS), to replenish those originally depleted and incorporating quercetin to enhance hydrogel properties and chondrogenesis. An optimized decellularization protocol efficiently removed DNA, but with a significant loss of GAGs (73%). After dECM solubilization, functionalization with CS or aldehyde modified CS (mCS) was performed. CS-functionalized hydrogels maintained low stiffness compared to non-functionalized hydrogel, while 0.2 mg/mL mCS hydrogels exhibited significantly slower gelation kinetics. To aid the hydrogel's chondrogenic ability, a novel approach using quercetin was investigated. Incorporation of 0.3 mg/mL quercetin in 0.4 mg/mL mCS-functionalized hydrogels resulted in increased gel stiffness. The impact on cell viability and chondrogenic differentiation was evaluated. Results showed similar cell viability in dECM and CS-functionalized hydrogels at 1 and 3 days of culture, with no significant changes in gene expression of chondrogenic and hypertrophic genes. In quercetin-containing hydrogels, the viability of human dermal fibroblasts was not significantly different from non-functionalized hydrogels, while human chondrocytes showed a significant upregulation of collagen type II, with 6.6- and 2.2-fold increases for 0.15 and 0.3 mg/mL quercetin, respectively. These results provide an initial proof-of-concept for dECM functionalization strategies that restore lost CS while incorporating quercetin, creating a microenvironment favorable for cartilage repair.

1 | Introduction

The loss of cartilage and the onset of bone growth are notable during osteoarthritis (OA), a degenerative joint disease affecting

an estimated 595 million people globally in 2020 [1]. This pathological cartilage remodeling contributes to the disease being both painful and debilitating for the affected individuals [2, 3], having a high impact upon the workforce and healthcare

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systems [1]. Therefore, the development of new therapeutic tools to repair and regenerate cartilage lesions is urgently required.

Physiologically, the cartilage matrix allows the joint to provide movement for the skeletal system with low frictional resistance [4, 5]. Notably, hyaline cartilage, the main cartilage affected by OA, is rich in collagen type II and proteoglycans including aggrecan, a glycosylated protein containing glycosaminoglycan (GAG). These are vital for the hydration of the extracellular matrix (ECM), a feature that is essential for the transmission of mechanical strains during movement [6, 7]. The cartilage degradation, along with its ossification, leads to pain and loss of joint movement [8, 9].

Cartilage is primarily non-innervated and avascular, with the latter being considered as the main contributor towards the lack of physiological regeneration [10, 11]. Cartilage matrix proteins and GAGs are essential for homeostasis, contributing to chondrocyte resistance to hypertrophy [12, 13]. However, as OA progresses, cartilage matrix degradation intensifies, with the disease managing to overcome the tissue's hindrance for vascularization, innervation, and osteogenesis [14]. Together, these can exacerbate the disease, further preventing cartilage repair [14, 15]. Chondrocyte hypertrophy leads to apoptosis and cartilage calcification onset [16]. Cartilage physiological limitations hinder halting pathological OA progression and cartilage regeneration, key to effective treatment [17].

The ubiquitous presence of the GAG, especially chondroitin sulphate (CS), is widespread in tissues and vital *in vitro/in vivo*, driving interest in this natural polysaccharide for health [18]. In cartilage ECM, it can constitute up to 50% at birth and 15% in adulthood (depending on species, age, and gender) [19, 20]. Notably, the monomer structure contains a sulphate group along with two sugar units: D-glucuronic acid and N-acetylgalactosamine. CS sulphation varies, mainly as 4- or 6-sulphate variants [21, 22]. The sulphate group is the main contributor to the biopolymer's negative charge, enabling interaction with positive substrates and water [23]. If required, CS can be modified to contain aldehyde groups, proving essential for tissue bioadhesiveness or functionalizing biomaterials. This can be achieved via conjugation to an amine (imine bond) or alcohol group (hemiacetal) [24]. Such modifications can alter the biopolymer properties and should be evaluated for bioengineering applications.

Cartilage-mimicking scaffolds have been developed, including solid implants and injectable hydrogels [25, 26]. Both two-dimensional (2D) and three-dimensional (3D) cultures have been used to ascertain the biological impact of these materials, with the latter having proven to be the most effective in mimicking the *in vivo* 3D environment [27]. In relation to chondrogenic differentiation studies, the utilization of 2D monolayer cultures has proven subpar in stem cell differentiation and chondrocyte activity. The onset of 3D cell culture approaches appears to have provided the necessary biotechnology platform for *in vitro* experimentation, enabling the development of approaches focusing on novel proof-of-concept treatments for cartilage regeneration [28].

Previous studies, using different tissues, focused on decellularized extracellular matrix (dECM) scaffolds demonstrating their enhanced biochemical interactions, biodegradability, and

biocompatibility [29, 30]. Briefly, obtaining dECM requires the removal of cellular content while retaining the original ECM components [31, 32]. Such approaches rely on the use of physical and chemical processes, which often have the drawback of removing/damaging the GAG content, including CS [33, 34]. Furthermore, when producing dECM-based hydrogels, the required acidic solubilization can cause further alterations to these polymers [35]. Previous studies have determined the ability of cartilage-sourced dECM to ensure differentiated stem cells have a chondrogenic phenotype with the expression of several genetic markers. These include the main collagen of hyaline cartilage, collagen type II, along with other markers, namely aggrecan and cartilage oligomeric matrix protein [32]. The latter is a glycoprotein whose role is to aid in the proliferation of chondrocytes and collagen fibrillogenesis [36]. These all work in tandem to ensure a chondrogenic microenvironment while limiting the onset of chondrocyte hypertrophy, a major complication associated with certain hydrogels as observed when using collagen type I hydrogels [37]. Biomaterials from natural sources generally offer higher biocompatibility and biodegradability and lower immunogenicity compared to synthetic biomaterials like polyethylene glycol [38, 39]. Thus, dECM use in clinics is promising due to reduced host rejection [40]. However, the critical role of GAGs in the chondrogenic microenvironment makes restoring their levels vital to improve the efficacy of dECM in cartilage repair [24, 41]. Considering this, functionalization strategies can be applied to reconstitute the GAG CS within dECM-based hydrogels, offering a novel bioengineering approach that is explored in the current study.

To complement a strategy based on dECM hydrogels, inclusion of the natural flavonoid, quercetin, has been shown *in vivo* to aid in chondrogenesis [42]. This molecule has been reported to influence cartilage tissue regeneration, exhibiting beneficial effects upon cells along with modulating inflammation [43, 44]. Also, it has been shown to provide a degree of crosslinking to collagen polymers, as an alternative to genipin [45, 46]. In a previous study by Greco et al. [45], the authors determined within decellularized dermal scaffolds (non-hydrogel), quercetin crosslinks collagen via interactions involving quercetin's hydroxyl groups and the amino groups of collagen [45]. Also, its inclusion within injectable alginate-based/bioglass hydrogels aided in chondrogenesis, along with inducing cartilage regeneration in an *in vivo* model [42]. To the best of our knowledge, the role of quercetin within dECM-based hydrogels or these functionalized with CS has not been reported, providing a novel avenue for experimentation.

Since the OA joint has a wide array of biochemical cues that may limit the success of any cell-seeded scaffolds, necessary functionalization approaches should be applied [47]. Notably, the significant loss of GAGs, including CS, is prominent in OA joints [12]. The absence of GAGs reduces the chondrogenic capacity of the ECM microenvironment [48]. Therefore, it adversely impacts the feasibility of using these biomaterials for tissue engineering approaches to tackle OA [48, 49]. Previous studies have applied functionalization strategies to produce biomaterials for a variety of applications [48, 50].

In the current study, we aimed to develop a novel hydrogel based on cartilage dECM functionalized with CS and containing

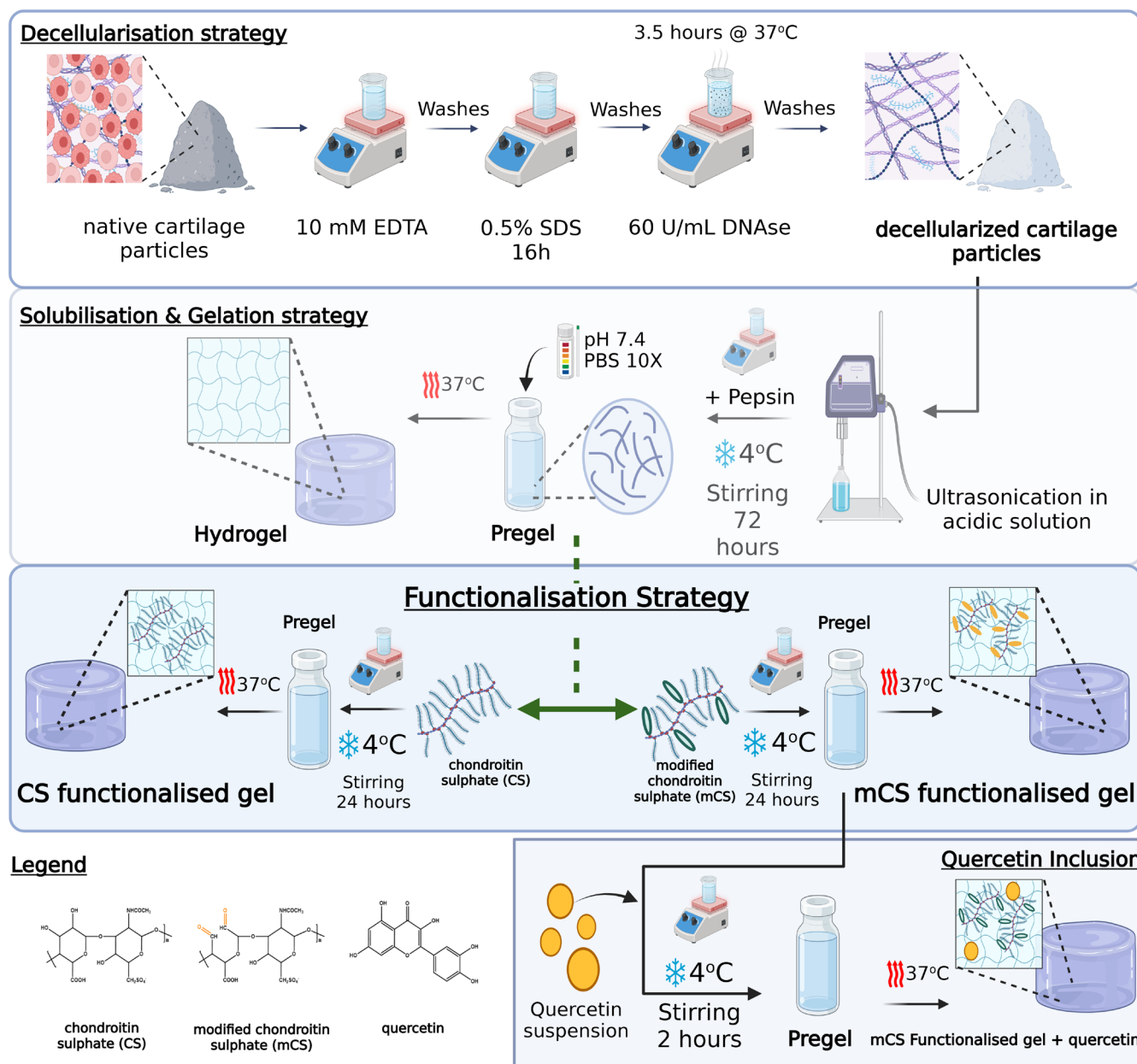


FIGURE 1 | Schematic of functionalized decellularised cartilage hydrogel production. Cartilage matrix was decellularised, milled, filtered, and solubilised into a pre-gel. This was used as a hydrogel or functionalized with either chondroitin sulphate (CS) or aldehyde modified chondroitin sulphate (mCS). Quercetin was also incorporated in the 0.4 mg/mL mCS hydrogel. Created in BioRender. Granja, P. (2025) <https://BioRender.com/k5adszs>.

quercetin (quercetin hydrate) to improve hydrogel properties and enhance chondrogenesis. The dECM pre-gels were functionalized using two approaches: (i) self-assembly with CS and (ii) modification of CS with aldehydes to provide a covalent functionalized hydrogel via formation of imine bonds. To further improve the hydrogel properties, we used quercetin, a pro-chondrogenic molecule (Figure 1). Using the different developed hydrogels led to similar cell viability as in dECM. While CS functionalization led to no significant changes in chondrogenic and hypertrophic gene expression, chondrocyte culture in quercetin-containing hydrogels significantly upregulated collagen type II. These results support dECM functionalization with aldehyde-modified chondroitin sulphate (mCS) and quercetin

may promote a chondrogenic microenvironment for cartilage repair.

2 | Materials and Methods

2.1 | Harvesting Bovine Cartilage and Particle Production

Freshly cut bovine legs (approximately 1 year old) were obtained from Carnes Landeiro (Portugal), disinfected with 70% ethanol, with skin, ligaments, and muscle removed. Cartilage was harvested from articular joints with a scalpel and placed in

cold 1× phosphate buffered saline (PBS) containing 10% penicillin–streptomycin (Gibco, USA) and 5% Fungizone (Capricorn Scientific, Germany). Tissue slices were placed in a holder with optical temperature cutting compound (Kalttek srl, Italy), frozen in liquid nitrogen-cooled 2-methylbutane, and stored at -80°C until use. When required, these were thawed at room temperature (RT) and washed in 1× PBS for 6 cycles of 30 min. Excess solution was removed, and the slices were placed at -80°C , then freeze dried. The lyophilized slices were milled and filtered through an $800\mu\text{m}$ sieve. The sieved dECM particles were collected and stored at -20°C until use.

2.2 | Decellularization Protocol

Native cartilage particles were thawed at RT and placed in Milli-Q (MQ) water containing 1% Pen/Strep (Gibco, USA), 0.5% Fungizone (Capricorn Scientific, Germany), and 0.1% Gentamycin (Gibco, Waltham, USA). The particles, at a concentration of 6 mg/mL, were stirred at RT for 15 min. The solution was removed at all stages of the protocol via centrifugation at $1300\times g$ for 4 min, with the supernatant being removed. A 10 mM Tris-based hypotonic buffer (HB) containing 10 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.8 was then added and allowed to stir for 22 h at RT. Following its removal, 1× PBS was added for 3 cycles of 1 h each. MQ water was then added to remove any excess salts for 5 min. A HB solution containing 0.5% sodium dodecyl sulphate (SDS) was introduced and allowed to stir for 16 h. To wash and remove the solution, 6 cycles of 15 min each of HB were required. To remove any excess salts, an MQ water wash was performed for 5 min. After centrifugation, 60 U/mL of DNase enzymes in a solution containing 20 mM Tris and 2 mM MgCl_2 (pH 7.8) was employed for 3.5 h (37°C). This was then removed and washed for 3 cycles of 10 min with 1× PBS, followed by 3 cycles of 10 min with MQ water to remove salts. The remaining supernatant was removed, with the particles then being frozen and lyophilized to form the dECM particles.

2.3 | dsDNA Quantification

Lyophilized ECM and dECM particles underwent DNA extraction using the PureLink Genomic DNA Mini Kit (Invitrogen, USA) per manufacturer instructions. Particles were digested with Proteinase K in digestion buffer at 55°C for 4 h with occasional vortexing. The lysate was centrifuged to remove RNA, then mixed with ethanol and applied to a silica spin column. After washing, purified DNA was eluted. Double-strand deoxyribonucleic acid (dsDNA) was quantified using the PicoGreen assay per manufacturer's instructions, with fluorescence measured on a Synergy Mx microplate reader (BioTek, USA) at 480 nm excitation and 520 nm emission. Samples were run in duplicate with blanks and dual standard curves for DNA concentration, normalized to dry weight. The data were presented as ng deoxyribonucleic acid (DNA)/mg dry weight.

2.4 | Sulphated Glycosaminoglycan Quantification

The blyscan sulphated glycosaminoglycan (sGAG) (Biocolor, UK) assay was utilized to quantify sGAGs. The dry ECM and

dECM particles were placed in a digestion solution (0.1 M sodium acetate, 0.01 M EDTA disodium salt, 0.005 M cysteine HCl, pH 6.4) containing 0.1 mg/mL of crystallized papain (Merck, USA). The samples digested for 12 h at 65°C , being stirred at 250 rpm. The kit quantifies sGAG by dye binding, pellet formation, and dye release for measurement. Absorbances were measured at 625 nm using a Synergy Mx microplate reader (BioTek, USA). Deionized water served as a reference and blank. sGAG concentration was determined from a standard curve, normalized for dilution and initial dry weight. The data were presented as μg sGAGs/mg dry weight.

2.5 | Aldehyde Modified Chondroitin Sulphate

Within an amber vial, 60.06 mg/mL of shark CS salt (Merck, USA) and 7.48 mg/mL of sodium periodate (NaIO_4 , Honeywell, USA) were dissolved in MQ water. Once dissolved, they were stirred for 6 h in the dark at RT, then dialyzed in a 1 kDa SpectraPor bag (Repligen, USA) against MQ water for 4 days with 3 daily changes of the solvent. Then filtered ($0.22\mu\text{m}$), frozen, and freeze-dried with the mCS stored at -20°C until use.

2.6 | dECM Solubilization and Gelation

dECM particles were suspended in an amber vial with cold 3% acetic acid added at 80% FV (final concentration: 35 mg/mL). They were sonicated in ice using an ultrasonic liquid processor (FisherScientific, USA) with an amplitude of 20% and an ON/OFF cycle of 2/3 s (total ON cycle: 1 h). Then, 3200 units/mg pepsin powder (Merck, USA) was added (final concentration: 5 mg/mL), and the residual enzyme was suspended in the remaining 20% of cold 3% acetic acid to ensure the desired final concentration was reached. The mixture was stirred with a suitable magnet and rpm (to prevent foaming) for a total of 72 h.

Under cold conditions and utilizing cold solutions throughout, 10× PBS (10% FV) was added and vortexed gently, stirred at low rpm for 30 min. 10 M sodium hydroxide (NaOH) was added until the pH was approximately pH 7, the mixture was filtered to remove the undigested particles and allowed to centrifuge at 100 G for 1 min (4°C), ensuring maximum recovery. Then it was altered to pH 7.4 using 5 M NaOH . To undergo gelation, place the pre-gel at 37°C until complete gelation.

2.7 | Pre-Gel Functionalization

To fabricate the functionalized hydrogels, working stock mixtures produced were 1.0 mg/mL CS and 0.4 mg/mL mCS. Under cold conditions throughout, pre-gel solutions with either CS/mCS were stirred overnight (suitable rpm to prevent foaming). Diluted to 0.2 and 0.4 mg/mL, vortexed gently and stirred for 5 h to ensure homogeneity prior to use. 3.8 Inclusion of quercetin within functionalized hydrogels Quercetin (quercetin hydrate, MedChemExpress, USA) was dissolved in cold 1× PBS at a concentration of 12 mg/mL and then diluted to 6 mg/mL. Each was then added to 0.4 mg/mL mCS pre-gel at 2.5% FV (final concentration: 0.3 and 0.15 mg/mL). These were allowed to stir for 2 h at 4°C prior to use.

2.8 | Fourier Transform Infrared Spectroscopy Analysis

A fourier transform infrared spectroscopy (FTIR)-RAMAN Spectrometer 2000 (Perkin Elmer, USA) with: Universal attenuated total reflectance (ATR) sampling accessory and transmittance of solids (ToS) modules was used with 32 scans and a resolution of 4 cm^{-1} . To obtain the spectrum of CS and quercetin powders, these were dried overnight at RT under vacuum. Approximately 0.5–2 mg of each sample with 200 mg of potassium bromide were compressed into a pill and then read using the ToS module. The mCS and hydrogel-based solutions were read using the ATR module. Spectra were processed using the advanced baseline correction function provided by Spectragryph software.

2.9 | Rheological Analysis

Pre-gel samples were placed in a pre-warmed 37°C kinexus rheometer (Malvern Instruments, USA) with drops of water surrounding the centre platform to ensure humidity. The samples were allowed to stabilize and undergo gelation for 25 min. Ten samples per decade were recorded; first, a frequency sweep from 0.1 to 10 Hz with a strain of 1% (linear viscoelastic region (LVER) can be observed); second, with a fresh pre-warmed sample, a strain amplitude analysis with 1 Hz (within LVER) frequency and strain from 0.01% to 100%.

2.10 | Turbidity Measurements

Under cold conditions, pre-gel samples were prepared in duplicates and placed in a pre-cooled 96-well plate, with this being left on ice until further use. A Synergy Mx multi-mode microplate reader (BioTek, USA) was pre-warmed to 37°C . 100 μL of pre-gel solution of each condition was added as duplicates. The plate was left on a cold metal block until being placed in the plate reader with absorbances read every 31 s at a wavelength of 410 nm.

To normalize the absorbances as percentage turbidity, the method was adapted from a previous publication [51]. For the half-time gelation turbidity ($t_{1/2}$) calculations, linear interpolation between measured time points was undertaken when required.

2.11 | CryoSEM Preparation and Analysis

Pre-gels (50 μL) underwent full gelation at 37°C for 25 min. Then 200 μL MQ water at RT was delicately added above each hydrogel, being then placed at 7°C for 24 h. These observations were performed using a high-resolution scanning electron microscopy (SEM) with X-Ray Microanalysis with the following CryoSEM: JEOL JSM 6301F (JEOL Ltd., Japan), Oxford INCA Energy 350 (Oxford Instruments, UK), Gatan Alto 2500 (Gatan Inc., USA). After removing excess water, hydrogels were quickly cooled in sub-cooled nitrogen, transferred under vacuum to the chilled preparation chamber. The specimens were fractured and sublimated for 180 s at -90°C followed by being coated via Gold/

Palladium sputtering for two 54 s cycles. Then samples were transferred into the SEM chamber (-150°C) with the images being then captured.

2.12 | Viability Analysis

Human mesenchymal stem cells (MSC) immortalized with human telomerase reverse transcriptase (hTERT) via lentiviral transfer were kindly provided by Prof. Matthias Schieker's group, Munich, Germany. Human dermal fibroblasts (HDF) were obtained commercially (ATCC, USA). The hTERT-MSCs or HDF were monolayer cultured in Dulbecco's modified eagle medium (DMEM) low glucose (-LG) medium (Gibco, USA), containing 10% fetal calf serum (FCS) (Gibco, USA or Corning, USA or Biowest, France), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Gibco, USA), being maintained at 37°C and a 5% CO_2 humidified environment. Either cell type was trypsinised and encapsulated within the hydrogel.

Cell seeding was undertaken via 4×10^5 cells/mL in a volume matching 2.5% FV of the pre-gel solution. Both solutions were combined under cold conditions and mixed using the pipette tip, up-down technique. 50 μL (containing 2×10^4 cells at a concentration of 4×10^5 cells/mL) of the mixed pre-gel solution for each condition was added as duplicates to a 96 well culture plate, undergoing gelation for approximately 8.5 min at 37°C within the incubator. The media was renewed with warm supplemented culture media and allowed to incubate for 75 min. The plates were then collected, the media renewed, and placed in the incubator overnight.

Stainings were conducted on days 1 and 3 after cell encapsulation. The cell media was replaced with culture media containing 2.4 μM Calcein AM (ThermoFisher Scientific, USA) and 4.8 μM ethidium homodimer-1 (EthD-1) (Biotium, USA or Merck, USA), followed by incubation for 30 min. The media was carefully removed, and warm $1 \times$ PBS was added, then removed to wash away excess media. This was repeated, with excess liquid removed prior to imaging using a Keyence BZ-X810 microscope (Keyence Corporation, USA) or Operetta CLS (Perkin Elmer, USA). The day 3 plate should have media renewed on day 1 and repeat the same assay on day 3. Imaging was undertaken in 2 replicate gels per condition, for each independent experiment, quantified using Image J software, and results for replicate hydrogels were averaged.

2.13 | Chondrocyte Isolation

Process adapted from a previous protocol [52], describing the process to obtain human chondrocytes. Briefly, these were isolated from the cartilage of the caput femoris procured during endoprosthetic total hip arthroplasties at the Hospital Carl Gustav Carus Dresden. Both donors were female aged 62. Prior to surgical intervention, patients provided written informed consent and cell isolation approved by the ethics commission of TU Dresden.

Cartilage was harvested, sliced, and placed in Hanks' balanced salt solution (HBSS) (Gibco, USA) supplemented with 100 U/mL

penicillin and 100 µg/mL streptomycin (Gibco, USA) along with 2.5 µg/mL amphotericin B (Merck, USA). Here, using a scalpel, pieces were minced and placed in HBSS containing 1 mg/mL collagenase II (Merck, USA), being placed overnight on a shaker at 37°C. The solution was filtered through a 70 µm cell strainer (Corning, USA) with cells collected by centrifugation (5 min, 423 g). The cell pellet was resuspended in expansion media consisting of DMEM-LG with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Gibco, USA). Also, 2.5 µg/mL amphotericin B was included only at this stage. These were then added to a cell culture flask and placed in an incubator (37°C, humidified atmosphere, and 5% CO₂). These were replenished twice a week with the expansion media and expanded to passage 3.

2.14 | Chondrogenic Differentiation

All chondrogenic experiments were undertaken as three independent experiments. Dedifferentiated chondrocytes at passage 3 were trypsinised and placed at a concentration of 2.4×10^6 cells/mL where it constituted 2.5% FV (pre-gel and cell suspension mixture) in chondrogenic medium. This medium contained DMEM high glucose (-HG) (Gibco, USA), 100 U/mL penicillin, 100 g/mL streptomycin, 1% ITS-X-Mix (Gibco, USA), 40 µg/mL proline, 50 µM ascorbic acid-2-phosphate, 10–7 M dexamethasone (all sourced from Merck, USA) and 10 ng/mL transforming growth factor β3 (Milteny Biotec, Germany).

97.5% FV of the pre-gel solution and 2.5% FV of cell suspension was mixed using the positive displacement pipette. 50 µL of the mixed solution (containing 1.2×10^5 cells: concentration of 2.4×10^6 cells/mL), each condition was added to a 96 well culture plate and underwent gelation for approximately 10 min at 37°C. Following this, warm supplemented DMEM-HG was added and allowed to incubate for 75 min. The media was renewed and placed in the incubator overnight. On days 1, 3, 5, and 7/8 post encapsulation, the media was renewed, being then renewed twice a week. On day 21, hydrogels were processed for further analysis (Sections 2.16–2.18).

2.15 | Histology

dECM and ECM were fixed in 4% paraformaldehyde in HBSS. Hydrogels with encapsulated cells were fixed in 4% phosphate buffered formaldehyde. These were paraffinized, sliced into 4 µm or 1.2 µm slices, respectively, deparaffinized, and stained using haemotoxylin and eosin (HE) or alcian blue staining. For DAPI staining, these were exposed to Spectral DAPI solution (AYOKA Biosciences, Marlborough, MA, USA), incubated for 5 min within a humidified chamber, followed by subsequent washing and mounting.

2.16 | RNA Extraction and cDNA Synthesis

Cell media was removed, and hydrogels were incubated with 3 mg/mL Collagenase type II (Merck, USA) in HBSS at 37°C for 1 h. Samples were centrifuged for 2 min at 250×g, the

supernatant was removed, and cells were collected to undergo lysis. The lysis and RNA purification were undertaken following the manufacturer's protocol, either peqGOLD Microspin (VWR, USA) or ReliaPrep™ miRNA Cell and Tissue Miniprep System (Promega, USA) kits. The amount of RNA was determined using a Nanodrop spectrometer (ThermoFisher Scientific, USA). The reverse transcriptase reaction was performed from 300 ng of total ribonucleic acid (RNA), utilizing the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) as per the manufacturer's instructions.

2.17 | Real Time Reverse Transcription-Polymerase Chain Reaction

Real-time polymerase chain reaction (RT-PCR) was used for quantifiable expression of chondrogenic and hypertrophic markers. It was evaluated using selected TaqMan Gene Expression Assay primers (Table 1) for chondrogenic markers (collagen type II (COL2A1), aggrecan (ACAN), cartilage oligomeric matrix protein (COMP)), along with hypertrophic markers (collagen type I (COL1A1), collagen type X (COL10A1), and alkaline phosphatase (ALPL)). The housekeeping gene utilized was β-actin (ACTB). Gene expression levels were assessed by quantifiable (Q) RT-PCR reaction via the cDNA from each sample with Taqman Fast Advanced Master Mix (Applied Biosystems, USA). All samples were run as duplicates for each gene while the normalized quantification was performed by using the comparative ΔΔCt method. The sample size was 6 for Figure 6, being 3 independent experiments with 2 biological replicates per condition, while for Figure 7, the sample size was 9, including 3 independent experiments with 3 biological replicates per condition.

2.18 | Statistical Analysis

Statistics were performed using GraphPad Prism (v8) and Excel software. Three independent experiments (minimum) were undertaken with the Shapiro–Wilk normality test used

TABLE 1 | Taqman primers utilized for the housekeeping, chondrogenic and hypertrophic genes.

Gene name	Gene symbol	Taqman assay ID
Aggrecan	ACAN	Hs00153936_m1
Beta-actin	ACTB	Hs01060665_g1
Alkaline phosphatase	ALPL	Hs01029144_m1
Cartilage oligomeric matrix protein	COMP	Hs00164359_m1
Collagen Type I alpha 1	COL1A1	Hs00164004_m1
Collagen Type II alpha 1	COL2A1	Hs00264051_m1
Collagen Type X alpha 1	COL10A1	Hs00166657_m1

to assess normal (Gaussian) distribution. dsDNA and GAG quantification were analyzed using paired t-test. $t_{1/2}$ and rheological data were analyzed using RM one-way ANOVA with Tukey correction (normally distributed data). Turbidity comparisons and cell viability analysis utilized RM two-way ANOVA with the Geisser–Greenhouse correction and Tukey's multiple comparisons test. Cell viability comparisons at the same time point utilized RM two-way ANOVA followed by Tukey's multiple comparisons test. Comparisons between time points used RM two-way ANOVA followed by Bonferroni's multiple comparison test. For the RT-PCR results, gene expression was analyzed as technical replicates generated from either three duplicate samples sourced from three (50 μ L) cell-seeded hydrogels (Figure 6) or three triplicate samples, each from two (50 μ L) cell-seeded hydrogels (Figure 7). The statistical analysis utilized the Δ Ct data. All data groups were compared to each other using ordinary one-way ANOVA followed by Tukey multiple comparison (normally distributed data) or Kruskal-Wallis test followed by Tukey multiple comparison for (non-parametric data). The data are shown as fold changes ($\Delta\Delta$ Ct) compared to the control (dECM) with upper and lower limits. Cell viability, gelation, and rheology spectra were analyzed using ordinary two-way ANOVA with Tukey's multiple comparison test.

3 | Results

3.1 | Cartilage Decellularization

To obtain a dECM-based hydrogel, we started by decellularizing native ECM particles using a combination of physical and enzymatic methods. After decellularization, both native ECM and dECM particles were evaluated to ascertain their DNA and GAG contents, providing insight into the decellularization efficiency in removing cellular material, while preserving ECM components. Since cartilage is extremely compact, cartilage particles (ca. 0.8 mm) were produced to increase the surface area and aid decellularization. Cartilage particles were subjected to combined chemical and enzymatic decellularization (Figure 1), resulting in the removal of virtually all cellular material (Figure 2A,B). This was confirmed by observing a porous structure within dECM particles that lacked or contained only remnants of cellular contents (Figure 2A). In relation to retaining the matrix components, the alcian blue results (Figure 2A, left images) appear to have a lighter blue stain observed in dECMs, showing loss of acidic polysaccharides including sGAGs. DAPI staining showed the cell nuclei in native cartilage particles, which were lost in dECM (Figure 2A, right images). Results for dsDNA quantification, depicted in Figure 2B, showed a significant decrease in the dECM, with 1.4 ng/mg, compared to the ECM particles with 224.2 ng/mg. The subsequent sGAG quantification (Figure 2C) showed a significant loss of 73% to approximately 87.3 μ g/mg sGAG remaining in the dECM particles, compared with 324.2 μ g/mg in the native ECM, caused by the decellularisation protocol.

Together, these results support that the decellularization was effective and the dECM was then used for further functionalization and incorporation.

3.2 | Production of Aldehyde Modified Chondroitin Sulphate

Then, to reintroduce the GAGs lost during the decellularization, we proceeded to incorporate CS in the decellularised ECM. We started by using sodium periodate (NaIO_4) mediated oxidation of CS, which ensured the opening of the neighboring diols ($-\text{OH}$) located on the N-acetylgalactosamine residue, since the diols were converted, producing a dialdehyde functionalized derivative (Figure 3A). To preserve the CS original structure and properties, 0.3 M equivalents of sodium periodate (NaIO_4) were used to prevent excessive oxidation. To ensure the formation of aldehyde groups, FTIR analysis (Figure 3B) of the mCS and natural CS salt was performed. The new peak observed at 1738 cm^{-1} (Figure 3B) corresponded to the C–O stretch, suggesting the presence of aldehyde groups. The produced mCS was used to functionalise the dECM hydrogel to form the mCS hydrogels.

3.3 | Characterization of dECM-Based and Functionalised Hydrogels

To develop the ECM-based hydrogels, including functionalized hydrogels, a pre-gel solution was first obtained. After collecting the lyophilized dECM particles, the material was solubilized via treatment with 3% acetic acid under sonication. Following the addition of 10 $\times$ PBS and neutralization with NaOH, along with filtration, a homogenous pre-gel without visible particles was produced, as depicted in Figure 4A. Also, this image shows that following thermogelation at 37°C , the pre-gel forms an opaque hydrogel. FTIR showed alterations within protein secondary structure following gelation, especially in the Amide I (1647 cm^{-1}), Amide II (1551 cm^{-1}), and Amide A stretching ($3200\text{--}3500\text{ cm}^{-1}$) regions, as shown in Figure 4B.

3.3.1 | Characterization of Functionalised Hydrogels With CS or mCS

Then, CryoSEM imaging was performed, since this approach prevents loss of the original gelatinous network structure, which occurs during normal SEM sample preparation. The representative images in Figure 4C indicate a fibrous network within the hydrogels, providing further evidence for having successfully produced a hydrogel.

Turbidity measurements allowed for gelation kinetics to be determined for the hydrogels developed following functionalization with different mCS concentrations (Figure 4D). The dECM hydrogel had the most efficient gelation kinetics, requiring a shorter period (6.0 min) to reach 50% turbidity, as shown in Figure 4E. In relation to the functionalized hydrogels, the 0.2 mg/mL mCS hydrogels required significantly more time to reach $t_{1/2}$, with 6.8 min.

Rheological analysis was performed to examine the viscoelastic properties and strength of the gelatinous network. When comparing the functionalized hydrogels to the control, it was observed both these had viscoelastic properties and solid-like hydrogel

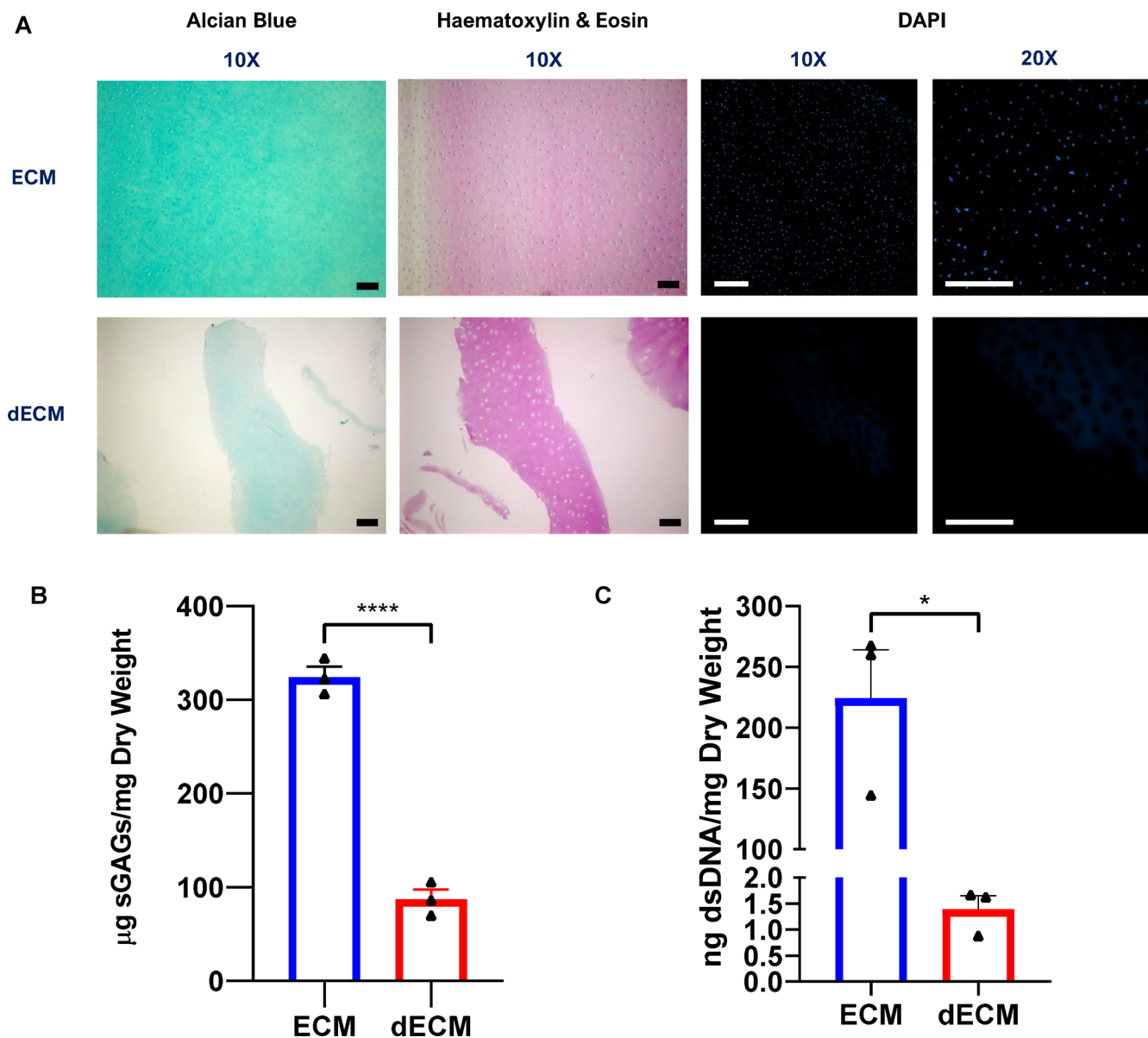


FIGURE 2 | Characterization of decellularized extracellular matrix. (A) Images of native and decellularized ECM cartilage particles stained with Alcian blue (GAGs), hematoxylin & eosin (matrix and cells), and DAPI (DNA). Images at 10× and 20× magnification with scale bars (100 µm for Alcian blue and H&E; 200 µm for DAPI). (B) Quantification of double-stranded DNA (dsDNA) in ECM and dECM particles. (C) Sulphated glycosaminoglycans (sGAGs) content quantified. Data shown as mean ± SEM ($n = 3$), with paired t-test significance indicated: $p < 0.05$ (*), $p < 0.0001$ (****). Symbols on the graph represent individual data points.

profiles (supplementary Figure S2). This was deduced due to the spectra showing storage modulus (G') being greater than the loss modulus (G''). As per Figure 4F–H, the G' and G'' of the hydrogels did not show any significant differences to each other at 0.1% strain. The lowest G' value (Figure 4F) recorded was 252.6 Pa for the 0.4 mg/mL mCS hydrogel; however, this was not significantly different to the dECM with a G' of 415.8 Pa. The G'' (Figure 4G) and complex modulus (G^*) (Figure 4H) also did not show any significant differences amongst the hydrogels. Overall, functionalizing the dECM pre-gel with the CS variants did not significantly alter the rheological characteristics with all hydrogels having a low G' and G'' modulus following CS functionalization.

The 0.4 mg/mL mCS hydrogel was selected to further understand the impact of incorporating quercetin. The CryoSEM

representative images in Figure 5A, showed the organized fibrillar network within the hydrogels. The turbidity results in Figure 5B,C show that dECM hydrogels required 6.9 min to reach $t_{1/2}$ while in comparison the 0.15 mg/mL quercetin-incorporated hydrogels required significantly more time, taking 9.2 min. No significantly different $t_{1/2}$ times were observed when comparing the quercetin-incorporated hydrogels to 0.4 mg/mL mCS. In addition, comparing the quercetin concentrations also showed no significant differences.

The hydrogels had viscoelastic properties and were solid-like hydrogels at low strains and frequencies (supplementary Figure S3). Relative to 0.1% strain, the rheological data (Figure 5D) demonstrated that the G' of dECM hydrogel was 451.0 Pa, being significantly higher than the 0.4 mg/mL mCS, with a G' of 195.7 Pa.

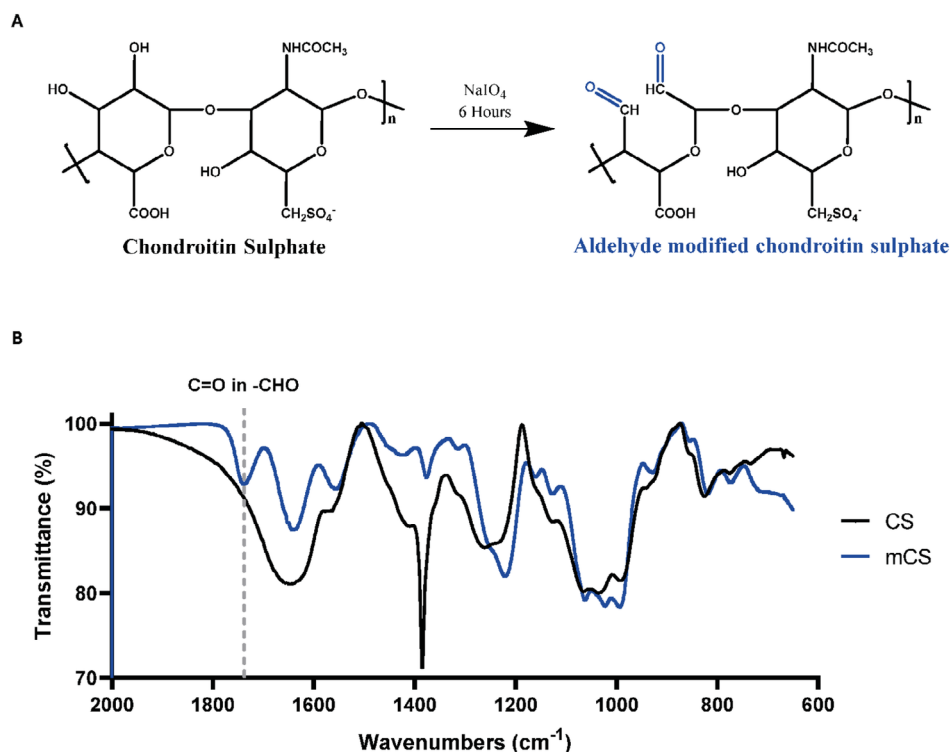


FIGURE 3 | Partial aldehyde modification of chondroitin sulphate. (A) Chemical structure of chondroitin sulphate salt before and after oxidation with 0.3 M equivalents of sodium periodate (NaIO₄) for 6 h, being then dialysed for 4 days, filtered (0.22 μm), and freeze-dried. (B) FTIR spectrum for chondroitin sulphate (CS) and aldehyde modified chondroitin sulphate (mCS). Spectra were zoomed in, allowing for the aldehyde peak to be observed at 1738 cm⁻¹.

Both quercetin-incorporated hydrogels had a G' that was not significantly different from each other and the dECM hydrogel. While the 0.3 mg/mL quercetin hydrogel had a G' of 405.0 Pa that was significantly different from the 0.4 mg/mL mCS hydrogel. In relation to the G'' (Figure 5E), the 0.4 mg/mL mCS hydrogel had an average value of 83.3 Pa, which was significantly different from the dECM gel, with 182.2 Pa. With no significant differences when comparing either hydrogel to either quercetin-incorporated hydrogel. To determine the strength of the hydrogels, the G^* values at 0.1% strain were observed (Figure 5F). The 0.3 mg/mL quercetin hydrogel had a G^* of 495.7 Pa, which was significantly different from 0.4 mg/mL mCS, with a G^* of 213.9 Pa. The latter had a G^* that was significantly different from the dECM hydrogel (486.9 Pa). However, there were no significant differences for the 0.15 mg/mL quercetin hydrogel in comparison to the rest of the hydrogels (Figure 5D–F).

Overall, our characterization analysis, showed 0.4 mg/mL mCS hydrogel to show significantly lower stiffness compared to dECM hydrogel. Overall, our characterization results showed that the 0.4 mg/mL mCS hydrogels have significantly lower stiffness, compared to dECM hydrogels.

3.4 | Biointerface Studies

Having produced a family of hydrogels, we proceeded to ascertain their impact on cell viability and chondrogenic differentiation.

To ascertain the viability of MSC within the developed hydrogels with the two concentrations of CS and mCS, a

Calcein-AM and EthD-1 staining was undertaken at 1- and 3-days of MSC culture (Figure 6A–C). Representative images of the obtained results are illustrated in Figure 6A. The quantitative results presented in Figure 6B show the percentage of live cells in the total of live and dead cells for the different conditions. The dECM cell viability was around 70% and it remained consistent since the impact of the hydrogel functionalisation or time point provided no significant differences. Albeit, the hydrogels containing mCS show a slight decrease in cell viability at day 3 of culture (Figure 6B). To determine the impact of the developed hydrogels on chondrogenesis, MSC were cultured within the different hydrogels for 21 days. Histological stainings were performed with representative results presented in Figure 6C showing cells are widely distributed throughout the hydrogels and appear to maintain a relatively round morphology. The alcian blue staining shows similar staining intensities in the different conditions (Figure 6E). To gain a deeper understanding, both chondrogenic markers and hypertrophic marker related genes were analyzed. All conditions were compared to the control sample, the dECM hydrogel. The gene expression results for cartilage matrix proteins (COL2A1, ACAN and COMP) are depicted in Figure 6D–F, while hypertrophic markers (COL1A1, COL10A1, and ALPL), are depicted in Figure 6G–I. These showed relatively high variations between experiments but no statistically significant differences between dECM hydrogels and CS hydrogels.

Overall, there were no significant differences in cell behavior, upon functionalization with either CS or mCS in dECM hydrogels.

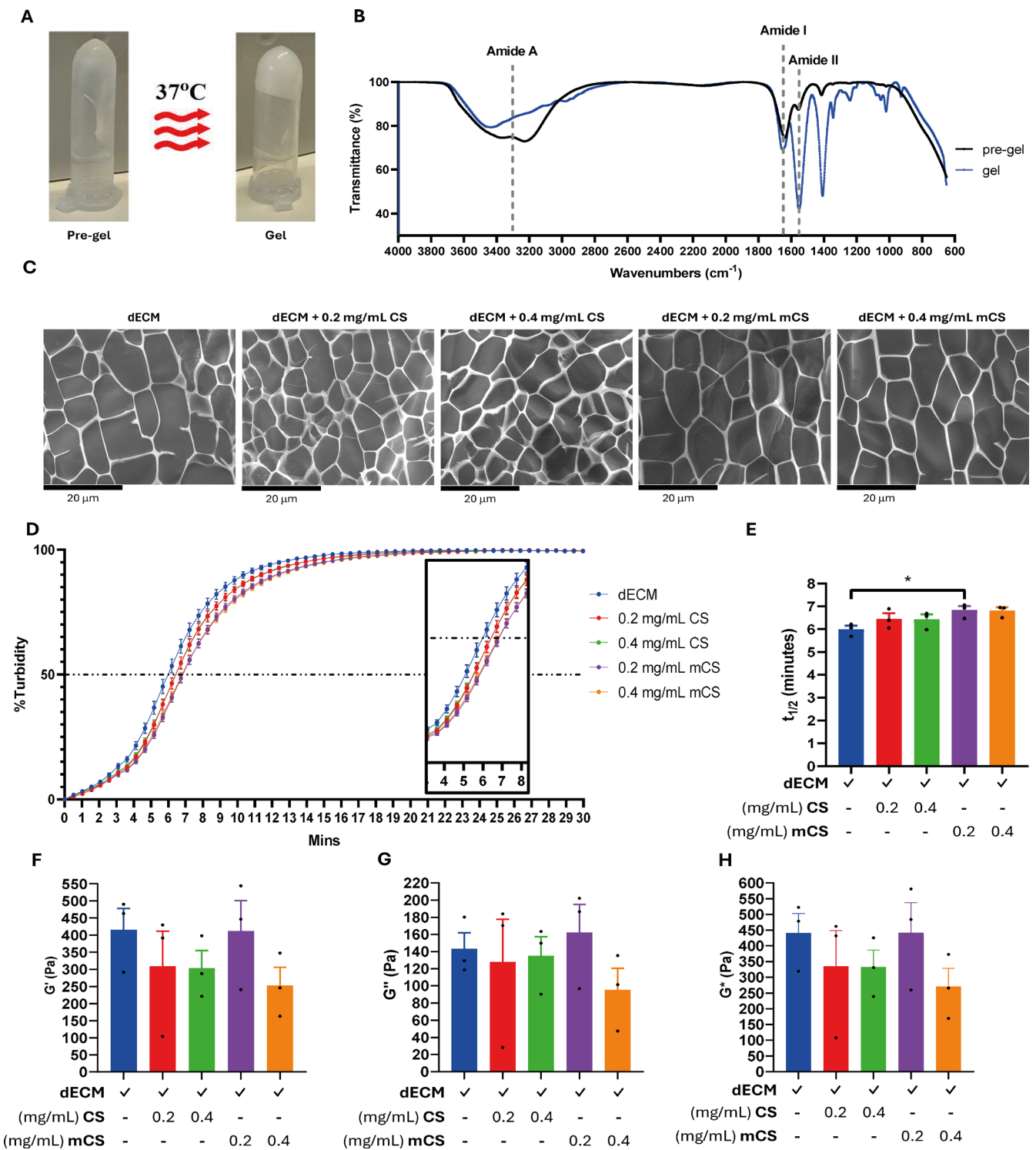


FIGURE 4 | Characterization of decellularized cartilage matrix and chondroitin sulphate-functionalized hydrogels. (A) Images of pre-gel solution and hydrogel. (B) FTIR spectra showing secondary structure formation. (C) CryoSEM analysis of decellularized matrix and functionalized hydrogels with either chondroitin sulphate (CS) and aldehyde modified chondroitin sulphate (mCS). Representative Images acquired utilizing $\times 2500$ magnification with scale bars on the images. (D) Percentage turbidity of dECM with or without CS/mCS functionalization over time (minutes). (E) Time (minutes) to reach 50% maximum turbidity for each hydrogel. (F–H) Rheological results show at 0.1% strain (F) The average storage modulus (G'), (G) the loss modulus (G'') and (H) complex modulus (G^*) of the hydrogels. Data presented as average $\pm$ SEM ($n = 3$) with significant differences shown as $p < 0.05$ (*) with the p values being compared to dECM. Symbols on the graph represent individual data points.

To ascertain the ability of the developed hydrogels with incorporated quercetin to support cell culture, HDF cell viability was evaluated along time, using the Calcein-AM and

EthD-1 staining (Figure 7A). Representative images show some dead cells in all experimental conditions, even in dECM (Figure 7A). Quantitative results are presented in Figure 7B

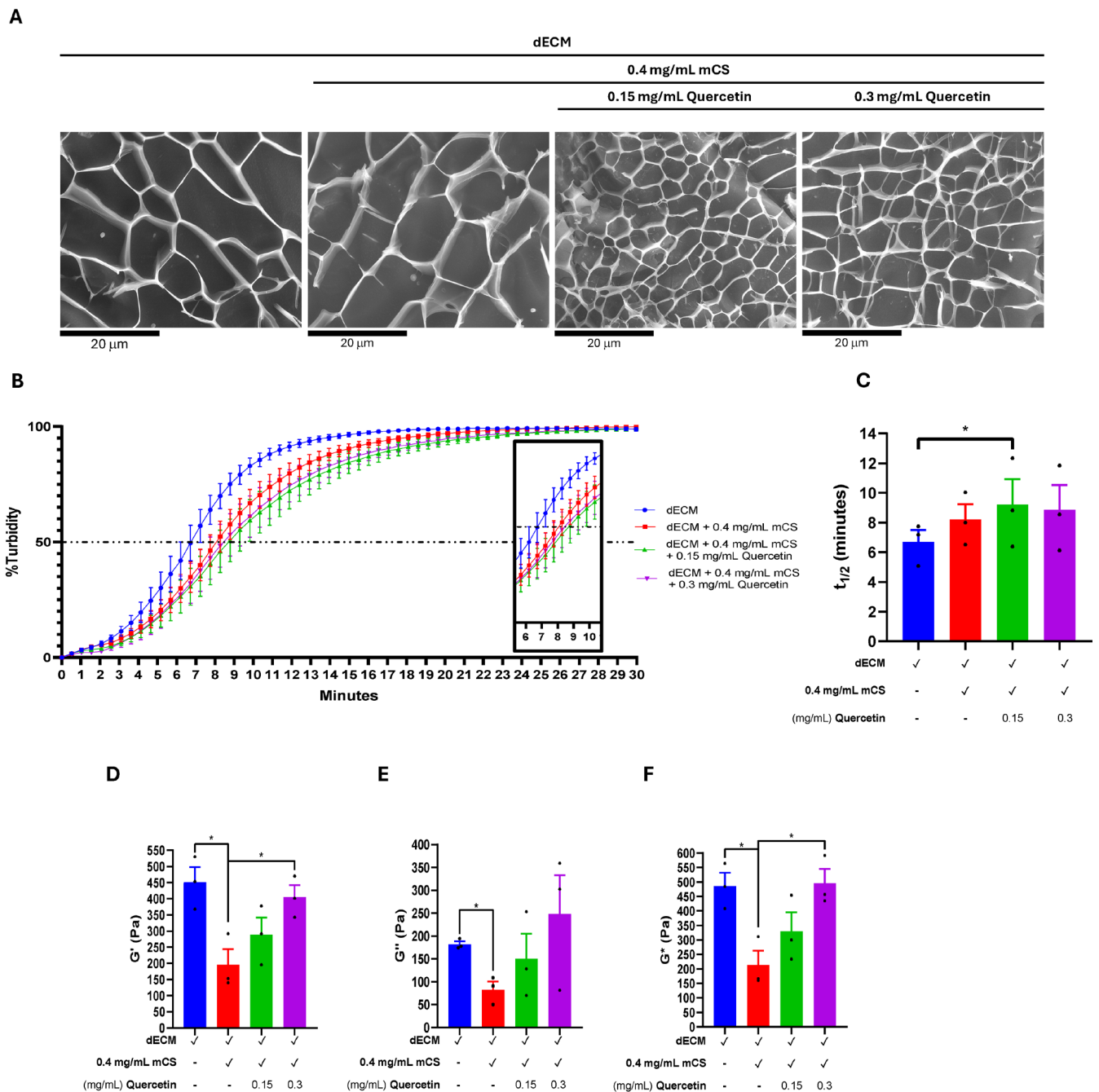


FIGURE 5 | Characterization of decellularized cartilage matrix and 0.4 mg/mL aldehyde-modified chondroitin sulphate hydrogels with or without quercetin. (A) CryoSEM analysis with representative images acquired utilizing $\times 2500$ magnification (scale bars on images). (B) Percentage turbidity of decellularized matrix (dECM) functionalized with aldehyde-modified CS (mCS) with/without two quercetin concentrations (0.15 or 0.3 mg/mL) over time (minutes). (C) Time (minutes) to reach 50% maximum turbidity for the different hydrogels. Rheological results show at 0.1% strain (D) the average storage modulus (G'), (E) The loss modulus (G'') and (F) Complex modulus (G^*) of the different hydrogels. Data presented as mean $\pm$ SEM ($n = 3$). Depending on the normal distribution, the statistical analysis undertaken either with (1) One-Way ANOVA test followed by Tukey's multi-comparison test and (2) Kruskal–Wallis test followed by Tukey's multi-comparison test $p < 0.05$ (*). In (B) statistical significance is against the dECM condition. Symbols on the graph represent individual data points.

and show no significant differences for cells cultured in 0.4 mg/mL mCS hydrogel between day 1 (66.0%) and 3 (65.7%). Also, cells cultured in hydrogels with 0.15 mg/mL quercetin showed similar viability as in the control hydrogel. While on day 1, the cells cultured in 0.3 mg/mL quercetin containing hydrogels showed a viability of 58.6%, which on day 3, reached

a viability of 68.8%. However, the differences in cell viability were not significant.

Histological staining analysis showed well dispersed chondrocytes with a round morphology, while alcian blue staining did not show any significant differences when comparing to those

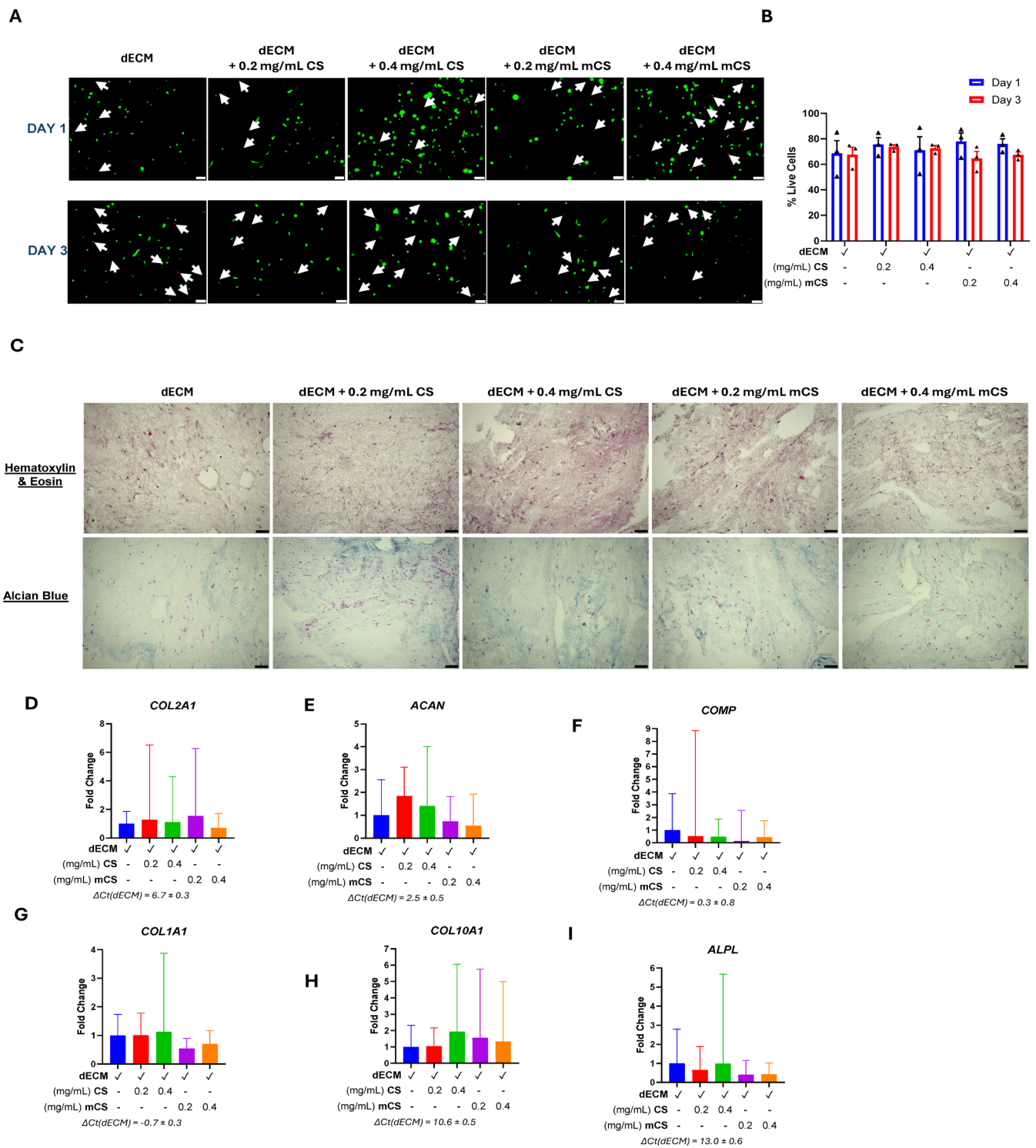


FIGURE 6 | MSC viability and chondrogenesis in both non- and functionalized hydrogel types. (A) Viability of hTERT-MSCs in decellularized matrix (dECM) and functionalized hydrogels, stained with Calcein AM (green, live) and Ethidium Homodimer-1 (red, dead) at days 1 and 3, with arrows pointing at examples of dead cells (10× magnification, 100 μm scale). (B) Percentage of viable cells shown as mean ± SEM ($n = 3$). (C) Hematoxylin & Eosin and Alcian blue staining of seeded hydrogels after 21 days in chondrogenic media (scale bars 100 μm). Symbols on the graph represent individual data points. (D–I) qRT-PCR analysis of ECM gene expression (COL2A1, ACAN, COMP, COL1A1, COL10A1, and ALPL) in seeded hydrogels after 21 days. Hydrogels tested included dECM with or without 0.2 or 0.4 mg/mL chondroitin sulphate (CS) or aldehyde-modified CS (mCS). Fold change ($\Delta\Delta C_T$) was calculated relative to dECM control. Error bars represent upper and lower limits ($n = 6$).

with incorporated quercetin (Figure 7C). Then, the effect of quercetin incorporation in 0.4 mg/mL mCS on chondrogenic gene expression of human chondrocytes was evaluated. Results indicate that incorporation of this compound, upregulated COL2A1 expression, when compared with 0.4 mg/mL mCS hydrogels, with results showing 6.6-fold increase for the lower

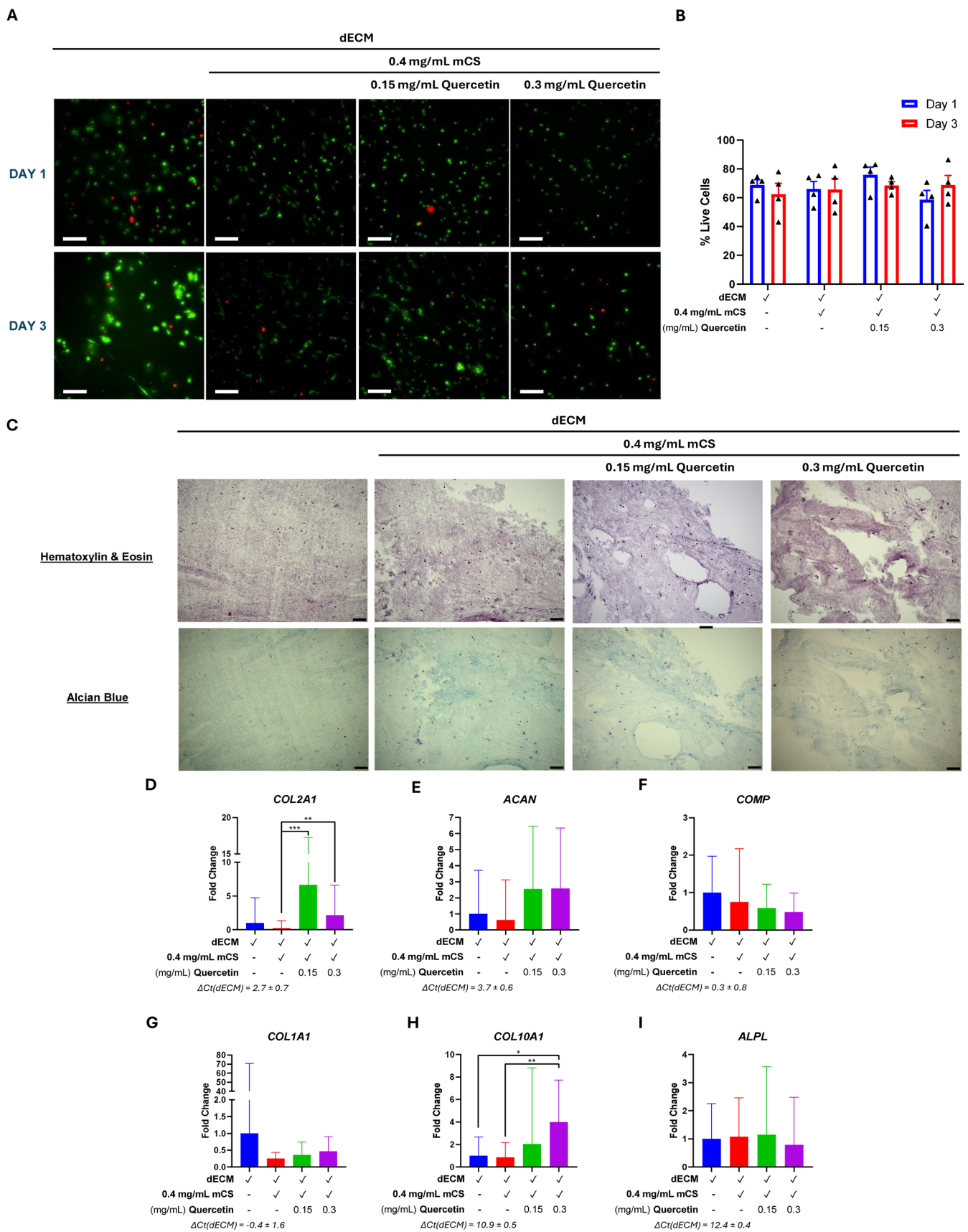


FIGURE 7 | Legend on next page.

FIGURE 7 | Cell viability and differentiation upon culture in non-functionalized and 0.4 mg/mL aldehyde modified chondroitin sulphate functionalized hydrogels with or without quercetin hydrate. (A) Viability of seeded human dermal fibroblasts (HDF) seeded in the different indicated hydrogels and stained with Calcein AM (green, live) and Ethidium Homodimer-1 (red, dead) at days 1 and 3. Scale bars = 200 μ m. (B) Percentage of viable cells shown as mean $\pm$ SEM ($n = 4$). Symbols on the graph represent individual data points. (C) Hematoxylin & Eosin and Alcian blue staining of seeded hydrogels after 21 days in chondrogenic media (scale bars 100 μ m). (D–I) qRT-PCR analysis of ECM gene expression (COL2A1, ACAN, COMP, COL1A1, COL10A1, and ALPL) in seeded hydrogels after 21 days. Hydrogels observed included that derived from dECM along with 0.4 mg/mL aldehyde modified chondroitin sulphate incorporated with quercetin at 0.15 and 0.3 mg/mL. Fold change ($\Delta\Delta$ CT) was calculated relative to dECM control. Error bars represent upper and lower limits ($n = 9$). Depending on the normal distribution, the statistical analysis undertaken either with (1) One-Way ANOVA test followed by Tukey's multi-comparison test and (2) Kruskal–Wallis test followed by Tukey's multi-comparison test $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

concentration while the higher concentration provided a 2.2-fold increase (Figure 7D). No significant differences were observed in ACAN and COMP expression (Figure 7E,F).

In relation to the hypertrophic markers, as per Figure 7G,I, both COL1A1 and ALPL gene expression were not significantly different amongst the hydrogels. For COL10A1 expression, the 0.3 mg/mL quercetin concentration, with a fold change of 4.0 (Figure 7H), showed a statistically significant increase when compared with dECM and 0.4 mg/mL mCS (0.9-fold). The lowest concentration of quercetin showed no significant differences, when compared to the other conditions. This demonstrates, 0.3 mg/mL quercetin and not the lower concentration of 0.15 mg/mL quercetin, in tandem with 0.4 mg/mL mCS can increase hypertrophic gene expression (COL10A1).

Overall, 0.15 mg/mL quercetin in 0.4 mg/mL mCS hydrogels enhances chondrogenic gene (COL2A1) expression.

4 | Discussion

New biomaterials capable of restoring cartilage structure and function are needed for OA patients. An ideal biomaterial should provide a microenvironment which closely mimics native cartilage. Therefore, cartilage dECM demonstrates potential for hydrogel and scaffold preparation, with benefits reported both in vitro and in vivo studies [32, 53]. Producing these biomaterials requires efficient ECM decellularization to minimize immunogenicity. Furthermore, detergent-based cell removal may disproportionately reduce certain GAGs, notably decreasing CS more extensively compared to the GAG hyaluronic acid (HA) [54, 55].

An undesirable result of decellularization is GAG loss, associated with the use of detergent-based solutions (e.g., SDS) [32]. This class of biopolymers, especially CS, is vital for chondrogenesis [56]. Thus, the ability to retain this polymer is paramount to ensure biomimicry [57]. Therefore, in this study, we re-introduced CS via functionalizing dECM, compensating for the loss occurring during decellularization. Our decellularization approach resulted in a retention of 87.3 μ g/mg of sGAGs, a 73% decrease compared to the native cartilage (Figure 2C). In accordance with the literature, a reduced quantity of GAGs was to be expected, with Bordbar et al., reporting a decrease with 11 μ g/mg of sGAGs remaining [32]. A subsequent paper by Guo and colleagues reported bovine articular cartilage

contains approximately 40% GAGs, their dECM particle production utilizing SDS detergent resulted in a 62% decrease [58]. This study utilized a different sGAG quantification technique, 1,9-dimethylmethylene blue assay, showing higher initial sGAG levels than our results and Bordbar et al. [32, 58]. Despite GAG loss, dsDNA reduction to residual levels (Figure 1B) allowed progression to subsequent experiments aimed at compensating for this loss.

Considering the importance of sGAGs in chondrogenesis, reconstituting these appeared advantageous for enhancing hydrogel biomimicry [59]. This was explored via functionalizing dECM pre-gel with CS and evaluating physical versus covalent functionalization approaches. Physical functionalization entailed solely adding CS salt (shark sourced), allowing the polymers to self-assemble into an organized network during gelation, as previously reported [60]. While, covalent functionalization requires a CS modification, allowing the introduction of a complementary specific functional group to form a covalent bond with a chemical group within the dECM [24, 61]. An aldehyde modification was selected, as CS oxidation provides dialdehyde groups, enable multiple functional sites to be introduced [61, 62]. These can react with primary amines (imine bonds) or alcohols (hemiacetals) [62]. As previously reported, such modifications may impact the biopolymer's bioactivity and stability, along with high oxidiser concentrations associated with increased polymer chain cleavage [61, 62]. Thus, we exposed CS to low oxidiser levels to compare CS and mCS in cartilage dECM-based hydrogels.

Prior studies focused on CS functionalization of solid dECM scaffolds from tissues other than cartilage, using methacrylated CS hydrogels or CS impregnation, rather than hydrogels [60, 63, 64]. This is different to our approach, which involves directly functionalising the dECM-based pre-gel solution with CS or mCS. To our knowledge, CS functionalisation of dECM-derived hydrogels has not been previously shown, making our study a proof-of-concept.

Herein, the mCS modification was confirmed with FTIR analysis, and results are in agreement with previous reports [61]. Spectra showed a new peak at 1738 cm^{-1} correlating with the aldehyde (Figure 3B). Future studies may use varying amounts of oxidiser to produce different mCS variants and determine their corresponding hydrogel characteristics and biological behavior.

To convert a solid scaffold into a soluble pre-gel, a typical approach is performed using an acidic pepsin-containing solution.

Furthermore, since we wanted to maximize the retrieval of ECM components, ultrasonic waves were applied through a sonication process since Akram and colleagues had already demonstrated its ability to aid in maximizing the retrieval of collagen type II from cartilage [65]. Evidence from the literature shows ultrasonic waves result in mechanical oscillation and cavitation within the samples, disrupting protein structure [65, 66]. Such disruption can expose concealed protein regions, aiding in digestion [66]. Future studies should be undertaken to determine a more effective solubilization strategy, since difficulties were encountered in our experiments to ensure full solubilization of the dECM. Therefore, filtering was necessary to remove the insoluble material.

A concerning issue with dECM scaffolds is high batch-to-batch variability due to tissue source differences between animals of the same species [67]. Also, the solubilization process can introduce variability in the final product due to incomplete digestion leading to heterogeneous hydrogels [68]. To ensure consistency, standardized decellularization and solubilization protocols are needed to reduce batch-to-batch variability [67, 68]. In the current study, variability in characterization and biointerface results (Figures 4–7) reflects the use of different dECM batches.

Hydrogel mechanical properties were measured to assess the functionalization effect on viscoelasticity. The overall results show hydrogels with a low G' modulus, with dECM hydrogel having an initial modulus that was significantly different to the functionalized hydrogel, of 416 Pa (Figure 4F). According to a previous publication by Beck and colleagues [69], the compressive modulus for native articular cartilage is 1.8 MPa. While, the compressive and G' moduli measure different mechanical properties, the high level of difference, shows the hydrogel is considerably weaker than cartilage. Although no studies with dECM-based hydrogels functionalized with CS, could be found in the literature, our results appeared to correlate with a study undertaken by Stuart et al. [70], who have observed decreased G'' modulus following the presence of CS in collagen hydrogels [70]. While the G'' of the 0.4 mg/mL mCS hydrogel (Figure 5D), is significantly decreased versus the dECM hydrogel, supporting mCS hydrogels may follow this trend.

An objective of this research was also to determine how the network forms over time. Turbidity measurements showed decreased gelation kinetics for the functionalized hydrogels compared to the dECM hydrogel. The average $t_{1/2}$ (Figure 4E) showed the dECM hydrogel had the fastest gelation kinetics (6 min), with 0.2 mg/mL mCS hydrogels being significantly slower in comparison. Clearly, the mCS functionalization within these dECM hydrogels is hindering the network organization, slowing down gelation efficiency.

The viability of cells in the CS modified hydrogels was evaluated at day 1 and day 3 of culture. These early time points were selected to deduce the effect of cells being exposed to a hydrogel containing 0.5 M sodium acetate. The presence of this salt is a direct result of utilizing NaOH to neutralize the 3% acetic acid pepsin containing solution used during the dECM solubilization. Such a concentration of the resulting 0.5 M sodium acetate may lead to lower cellular viability according to previous studies

[35]. Second, these time points allowed for ascertaining the cell viability of mCS functionalized hydrogels, which contained aldehyde modified CS. Such functional groups can interact with cellular components, disrupting cellular processes, resulting in toxicity which impacts cellular viability [71]. Overall, these time points showed there is only mild cytotoxicity to the seeded cells, at both day 1 and day 3 for all hydrogels (Figure 6A,B), with no significant differences when comparing the conditions. Therefore, cell viability of 70% is a potential limitation of our study. Also, the number of cells appears to show some variations (Figure 6A) especially between day 1 and 3. Previous studies have reported at week 6, a beneficial impact in cell viability when CS was provided to 3D cell cultures [50]. No similar significant effects were observed within our study at day 1 and 3. Perhaps the composition or low stiffness of our dECM hydrogels nullifies the beneficial effect upon cell viability provided by CS. Furthermore, since we had to decellularize the cartilage ECM, this can modify the biochemical and structural integrity of the ECM components [72]. This may be associated with the cytotoxicity observed within the hydrogels including the dECM-based hydrogel. A study by Bordbar and colleagues reported that rabbit bone marrow stem cells exhibited lower metabolic activity (an indirect measurement of cell viability) at day 7 within cartilage dECM hydrogels compared to dECM solid scaffolds. Also, all conditions showed higher activity at day 7 compared to day 3, with the dECM hydrogel showing the slowest cell growth [32]. While, in our study, cell viability was directly measured (counting live and dead cells), with mild cytotoxicity observed in the dECM hydrogel. Evidently, in both the previous study and our results show no significant differences at 3 days. Although later time points were not investigated in our study, the early-stage data provided valuable insight into initial cell responses to these hydrogels and functionalization strategies.

Hydrogel mechanical cues, especially stiffness, can influence MSC chondrogenic differentiation [73]. However, in contrast to our initial hypothesis on the effect of CS functionalisation, the expression of chondrogenic and hypertrophic genes showed no significant differences overall (Figure 6D–I). A previous study by Bachmann and colleagues [74] showed a stiffness of 30 kPa compared to 1 kPa that resulted in increased collagen type II and aggrecan synthesis [74]. Since in our study we have hydrogels presenting low stiffness, this may play a role in the results obtained in suppressing cartilage matrix production. Finally, it should be ascertained in future studies if hydrogel stiffness or CS has a preponderant impact on chondrogenesis induced by dECM hydrogels. A study by Ai et al. [73] has shown the optimal CS concentration for chondrogenesis was 6% (w/v) regardless of the stiffness of hydrogels, but for the same CS concentration stiffer hydrogels tend to have better performance [73]. Perhaps investigating further CS concentration and improving dECM hydrogel stiffness via crosslinking may allow CS functionalization to effectively enhance the microenvironment's chondrogenic ability.

Furthermore, our approach utilized a chondrogenic supplemented culture medium. This was due to having MSC or de-differentiated chondrocytes which necessitate supplements to undergo re-differentiation to express cartilage specific genes [75]. Consequently, any changes in the expression of chondrogenic genes are not only influenced by the biochemical and

biomechanical microenvironment provided by the hydrogel, but also by these factors. To assess the direct impact of the hydrogels, removing chondrogenic supplementation, as demonstrated by Roncada et al., could provide valuable insights [76]. However, supplementation is considered necessary for the activation of the chondrogenic signaling pathways [77], and thus was selected.

This study also examined quercetin's influence upon hydrogel mechanical and biological behavior, since it has been shown to crosslink collagen and support chondrogenesis [42, 45]. However, no previous studies have been published focusing on its use within dECM-based hydrogels.

Rheological data for quercetin-incorporated hydrogels demonstrated its ability to improve the G' modulus. This aligns with previous studies on evaluating quercetin's crosslinking ability [45]. The G^* analysis in Figure 5F provides evidence that 0.3 mg/mL quercetin incorporated within 0.4 mg/mL mCS significantly increases the hydrogel's strength (0.3 mg/mL quercetin vs. 0.4 mg/mL mCS), while not showing significant differences to the dECM hydrogel. This can imply that incorporated quercetin within the dECM and 0.4 mg/mL mCS hydrogel restored the mCS functionalized hydrogels to their original strength prior to functionalisation (since no significant differences between quercetin concentrations and dECM). Thus, it depicts the importance of quercetin for improving mCS hydrogel's rheological properties. However, no significant difference was observed between quercetin-incorporated hydrogels and its corresponding hydrogel, 0.4 mg/mL mCS. In a previous publication by Greco et al. [45], incorporation of 1 mg/mL of quercetin (in PBS) in decellularized dermal scaffolds increased their stiffness, though less than crosslinking with genipin [45]. Our results may suggest that quercetin can overcome the hindrances that mCS provides to the hydrogel network, allowing for improved rheological properties. In our current study, we are limited to observing the influence of quercetin in a specific mCS hydrogel. It may be necessary and of great importance to analyze the influence in the remaining CS gels and dECM-based hydrogel.

Quercetin exhibits anti-cancer cytotoxicity against hTERT-mutated cells [78, 79]. Therefore, HDF cells, which tolerate quercetin, were used in these experiments [80]. Results in Figure 7A,B showed that quercetin incorporation within the 0.4 mg/mL mCS-functionalized hydrogels did not lead to significant increases in cytotoxicity (as can potentially occur with hTERT cell lines). However, these all showed low to moderate cytotoxicity when observing both day 1 and day 3, which constitutes a potential limitation of the current study. A previous study by Zawani and colleagues used quercetin-embedded gelatin-elastin hydrogels and showed HDF cell viability at day 1 was optimal with 0.125 mg/mL quercetin [81]. Their results report cell viability via a cell metabolic assay rather than directly quantifying the percentage of live cells as in this study. Our quercetin-incorporated hydrogels that had the highest viability at day 1 were 0.15 mg/mL (75.9%), which corresponds to mild cytotoxicity and was not statistically different from the rest of the conditions. Notably, the results in our study could possibly be associated with the inherent properties of the dECM hydrogel contributing to the observed cytotoxicity. Also, Greco and colleagues reported that 1 mg/mL quercetin is optimal for crosslinking, while Zawani and colleagues found that cell viability

is preferable at concentrations below 0.5 mg/mL [45, 81]. To minimize cytotoxicity, we limited the highest quercetin concentration to below 0.5 mg/mL. Despite this, cytotoxicity was still observed, which may have influenced the chondrogenesis-related results.

The gene expression results show quercetin inclusion provides statistically significant advantages (especially when comparing the 0.4 mg/mL mCS hydrogel +/- quercetin) relative to the expression of COL2A1. The 0.15 mg/mL quercetin-incorporated hydrogel showed the highest significant increase (6.6-fold change) vs. 0.4 mg/mL mCS hydrogel (0.2-fold change) seen in Figure 7D. Increased COL2A1 has been previously reported in rat chondrocytes affected by quercetin-bioglass hydrogels [41]. Both quercetin concentrations significantly increased COL2A1 expression. There was a significant increase in the hypertrophic gene COL10A1, with 0.3 mg/mL quercetin showing a 4.0-fold increase (Figure 7H) versus both the dECM hydrogel and 0.4 mg/mL mCS-functionalized dECM hydrogel. Since the percentage of live cells at day 1 for 0.3 mg/mL quercetin showed moderate cytotoxicity, this could have implications in the results (COL10A1). Together, these results show 0.4 mg/mL mCS hydrogels have a positive influence upon chondrogenesis (> hypertrophy) when quercetin is incorporated, especially at 0.15 mg/mL. Therefore, providing a basis for future studies to apply quercetin for enhancing the chondrogenic regenerative capability of CS functionalised dECM.

5 | Conclusion

Cartilage dECM-derived hydrogels showed no significant differences in rheological behavior, gelation kinetics, or biointerface effects compared to CS/mCS-functionalized hydrogels. The inclusion of quercetin in 0.4 mg/mL mCS-functionalized dECM hydrogels appears to enhance COL2A1 expression. Therefore, this study presents a proof-of-concept hydrogel that combines mCS and quercetin to enhance chondrogenesis. Future work should investigate CS and further mCS concentrations for dECM hydrogel functionalization along with quercetin's interaction with CS/mCS. This will allow for the identification of an optimal formulation for chondrogenesis and clinical use.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** FTIR of aldehyde modified chondroitin sulphate. FTIR spectrum for both chondroitin sulphate (CS) and aldehyde modified chondroitin sulphate (mCS). Aldehyde peak was observed at 1738 cm⁻¹. **Figure S2:** Rheological analysis spectra for decellularised cartilage-based hydrogels and with modified/non-modified chondroitin sulphate functionalised gels. (A, D) *G'*, (B, E) *G''*, (C, F) *G**. (A–C) Frequency analysis from 0.1 to 10 Hz with 1% strain. (D–F) Strain amplitude of 0.1% to 100% with 1 Hz frequency. **Figure S3:** Rheological analysis spectra for dECM, aldehyde modified chondroitin sulphate functionalised hydrogels and quercetin incorporated functionalised hydrogels. (A, D) *G'*, (B, E) *G''*, (C, F) *G**. (A–C) Frequency analysis from 0.1 to 10 Hz with 1% strain. (D–F) Strain amplitude of 0.1% to 100% with 1 Hz frequency. **Figure S4:** in vitro gene expression of extracellular matrix components. mRNA expression level of (1–6A) Collagen type 2 (COL2A1), (1–6B) Aggrecan (ACAN), (1–6C) Cartilage oligomeric matrix protein (COMP), (1–6D) Collagen type 1 (COL1A1), (1–6E) Collagen type 10 (COL10A1), (1–6F) Alkaline phosphatase (ALPL) by quantitative real-time PCR of cell seeded gels after 21 days of in vitro culture. (1–3) Hydrogels observed included the dECM hydrogel along with non-modified (CS) and modified (mCS) functionalised gels with

concentrations of 0.2 mg/mL and 0.4 mg/mL. (4–6) Hydrogels observed included the dECM hydrogel along with 0.4 mg/mL mCS with this hydrogel being then incorporated with quercetin at concentrations of 0.15 mg/mL and 0.3 mg/mL. Fold change ($\Delta\Delta CT$) to control samples, being the dECM hydrogel. Error bars represent upper and lower limits ($n=2$). **Figure S5:** In vitro gene expression of extracellular matrix components. mRNA expression level of (1-3A) Collagen type 2 (COL2A1), (1-3B) Aggrecan (ACAN), (1-3C) Cartilage oligomeric matrix protein (COMP), (1-3D) Collagen type 1 (COL1A1), (1-3E) Collagen type 10 (COL10A1), (1-3F) Alkaline phosphatase (ALPL) by quantitative real-time PCR of cell seeded hydrogels after 21 days of in vitro culture. Hydrogels observed included the dECM hydrogel along with 0.4 mg/mL mCS hydrogel with this being then incorporated with quercetin at concentrations of 0.15 mg/mL and 0.3 mg/mL. Fold change ($\Delta\Delta CT$) to control samples, being the dECM hydrogel. Error bars represent upper and lower limits ($n=3$). Depending on the normal distribution, the statistical analysis undertaken either with (1) One-Way ANOVA test followed by Tukey's multi-comparison test (2) Kruskal-Wallis test followed by Tukey's multi-comparison test $p < 0.05$ (*), $p < 0.01$ (**). **Table S1:** Statistical analysis of G' , G'' and G^* over strain amplitude for supplementary Figure S3. The statistical analysis undertaken with RM two-way ANOVA with the Geisser–Greenhouse correction followed by Tukey's multiple comparisons test $p < 0.05$ (*), $p < 0.01$ (**). **Table S2:** Statistical analysis of G' , G'' and G^* over Frequency for supplementary Figure S3. The statistical analysis undertaken with RM two-way ANOVA with the Geisser–Greenhouse correction followed by Tukey's multiple comparisons test $p < 0.05$ (*).