Decellularized cartilage as a chondroinductive material for cartilage tissue engineering

By

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ABSTRACT

Cartilage defects, whether caused by osteoarthritis, joint trauma, or other disease, have provoked a wide variety of tissue engineering scaffold strategies in recent years. Traditionally, cartilage tissue engineering scaffolds have utilized synthetic polymer components to form hydrogels or other porous matrices. However, components found within the extracellular matrix (ECM) such as collagen, glycosaminoglycans (GAGs), and ECM-based matrices have emerged as an essential subset of biomaterials for tissue engineering scaffolds. The objective of this research was to develop and evaluate decellularized cartilage (DCC) as a chondroinductive material for cartilage tissue engineering applications. This work was successful in developing a decellularization method for hyaline cartilage fragments that removed 99% of cells, while retaining 87% of GAGs and also in determining a method to produce a homogenous nanopowder of DCC. Additionally, this research was the first to examine the ability of DCC to induce chondrogenesis in stem cells by quantifying gene expression of chondrogenic markers. The results demonstrate for the first time that DCC can indeed upregulate chondrogenic markers and may be a new chondroinductive material that can provide microenvironmental cues and signaling to promote stem cell differentiation in cartilage regeneration.

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LIST OF ABBREVIATIONS

DCC – decellularized cartilage

GAG – glycosaminoglycan

ECM – extracellular matrix

CG – collagen-GAG

HA – hyaluronic acid

CS – chondroitin sulfate

PEG – poly(ethylene glycol)

PEG-DA – poly(ethylene glycol) diacrylate

PLGA – poly(lactic-co-glycolic acid)

PLCL – poly(lactide-co-ε-caprolactone)

PLA – poly(lactic acid)

PLLA – poly(L-lactic acid)

PGA – poly(glycolic acid)

HAp – hydroxyapatite

B-TCP – beta tricalcium phosphate

SIS – porcine small intestinal submucosa

DBM – demineralized bone matrix

VEGF – vascular endothelial growth factor

PDGF – platelet derived growth factor

TGF-β1 – transforming growth factor beta-1

TGF-β3 – transforming growth factor beta-3

BMP-2 – bone morphogenetic protein-2

PS – phosphatidylserine

RT-PCR – reverse transcriptase polymerase chain reaction

FTIR – Fourier transform infrared spectroscopy

TEM – transmission electron microscopy

BMSC – bone marrow stem cell

RGD – argine-glycine-aspartic Acid

FBS – fetal bovine serum

DMSO – dimethyl sulfoxide

ELISA – enzyme linked immunosorbent assay

ACI – autologous chondrocyte implantation

P4 – passage 4

EDTA – ethylenediaminetetraacetic acid

DMMB – dimethlymethylene blue

Sox9 – sex determining region-box 9

CHAPTER 1: Introduction

The overall objective of this thesis was to determine the feasibility and potential of decellularized hyaline cartilage tissue as a chondroinductive material for cartilage tissue engineering applications. To achieve this objective, three phases were recognized: the decellularization phase, material characterization phase, and cellular response phase. In the decellularization phase, the objective was to identify conditions and tissue preparation methods in order to obtain cartilage extracellular matrix with greater than 97% cellular removal, while obtaining glycosaminoglycan content concentrations similar to native cartilage tissue. In the material characterization phase, the objective was to further elucidate properties of the acellular cartilage ECM, including methods for grinding the tissue into a fine powder, characterizing particle size, and chemical composition. In the cellular response phase, decellularized cartilage powder was evaluated in two different in vitro cell culture environments – pellet culture and encapsulated within an agarose hydrogel. Pellet culture was used to quantify gene expression in response to the decellularized cartilage powder and encapsulation within a hydrogel was used to quantify DNA content in gels over a 3-week culture period.

To achieve the overall objective, two specific aims were designed: (1) to decellularize and characterize hyaline cartilage, and (2) to evaluate cellular response to decellularized cartilage powder.

The organization of the remaining chapters is as follows:

Chapter 2 serves to provide a complete review of the literature in the area of using raw materials as components of tissue engineering scaffolds, which is pertinent to subsequent chapters. Also provided in Chapter 2 is the background literature on the limited use of decellularized cartilage in regenerative medicine solutions to lead into the study presented in Chapter 3.

Following the establishment of pertinent background information, Chapter 3 serves to satisfy the aforementioned Specific Aims. The deliverables include biochemical analyses of decellularized cartilage, chemical composition analysis by Fourier transform infrared spectroscopy, particle size analysis by transmission electron microscopy, and cellular response characterized by gene expression and cell proliferation.

Chapter 4 presents the conclusion where findings from all experiments are summarized. Possible future research directions are also presented.

CHAPTER 2: Leveraging ''Raw Materials'' as Building Blocks and Bioactive Signals in Regenerative Medicine[1]

CHAPTER PURPOSE:

This chapter serves as a review of the recent literature surrounding the use of raw materials in tissue engineering scaffolds. For this article, a raw material was defined as a material that is found naturally within the body.

2.1 ABSTRACT

Components found within the extracellular matrix (ECM) have emerged as an essential subset of biomaterials for tissue engineering scaffolds. Collagen, glycosaminoglycans (GAGs), bioceramics, and ECM-based matrices are the main categories of "raw materials" used in a wide variety of tissue engineering strategies. The advantages of raw materials includes their inherent ability to create a microenvironment that contains physical, chemical, and mechanical cues similar to native tissue, which prove unmatched by synthetic biomaterials alone. Moreover, these raw materials provide a head start in the regeneration of tissues by providing building blocks to be bioresorbed and incorporated into the tissue as opposed to being biodegraded into waste products and removed. This article reviews the strategies and applications of employing raw materials as components of tissue engineering constructs. Utilizing raw materials holds the potential to provide both a scaffold and a signal, perhaps even without the addition of exogenous growth factors

[1] Published as **Amanda N. Renth** and Michael S. Detamore, "Leveraging "raw materials" as building blocks and bioactive signals in regenerative medicine," Tissue Engineering Part B, 18(5): 341-362, 2012.

or cytokines. Raw materials contain endogenous proteins that may also help to improve the translational success of tissue engineering solutions to progress from laboratory bench to clinical therapies. Traditionally, the tissue engineering triad has included cells, signals, and materials. Whether raw materials represent their own new paradigm or are categorized as a bridge between signals and materials, it is clear that they have emerged as a leading strategy in regenerative medicine. The common use of raw materials in commercial products as well as their growing presence in the research community speak to their potential. However, there has heretofore not been a coordinated or organized effort to classify these approaches, and as such we recommend that the use of raw materials be introduced into the collective consciousness of our field as a recognized classification of regenerative medicine strategies.

2.2 INTRODUCTION

As the intertwined fields of tissue engineering and regenerative medicine continue to grow and evolve, the search for a "perfect" scaffold inevitably continues. This ongoing quest to search for new materials and fabrication techniques has led researchers anywhere from insect cuticle[1, 2] to precious metals and minerals[3] over the past decade. Researchers are continuously finding new materials and technology for fabricating scaffolds with heightened mechanical integrity, porosity, biocompatibility, and biodegradability. Hollister[4] described biomaterials used in tissue engineering scaffolds as the distinct "lynch pin" for finding effective regenerative solutions. Most

attribute the lack of efficacy of biomaterials to the inability of materials to mimic the extracellular matrix (ECM) when compared to natural tissues and organs of the body.[5] Recent trends in the field suggest that it may be appropriate to ask the question, "Have we looked too far for the ideal, synthetic biomaterial and missed the actual building blocks needed for scaffolds in this process?" Utilization of materials that occur naturally within the human body such as collagen, chondroitin sulfate, and calcium phosphates have gained immense attention within the tissue engineering community.

This review seeks to indicate the emergence of raw materials as components of tissue engineering scaffolds. For the purpose of this review, *we define raw materials as those found naturally within the human body, such as collagen, glycosaminoglycans (GAGs), bioceramics, and ECM-based matrices*. Several comprehensive reviews of non-mammalian, natural polysaccharides such as alginate, chitosan, dextran and gelatin have been detailed extensively in the literature.[5-9] In this review, we intend to instead highlight the most widely used mammalian raw materials and the strategies behind using these materials as building blocks for tissue engineering scaffolds. In addition, we seek to review the connection made to formulate scaffolds based upon components of native extracellular matrix, which has been used as a strategy by many in the field, but has not been collectively been brought to the attention of our field as a classification of strategies, but which perhaps should become part of our collective consciousness.

Traditionally, the most common strategy to develop a tissue-engineered construct is through a combination of the factors described in the tissue engineering

triad (Fig. 1): scaffolds, signals, and cells. Scaffold development has attracted immense attention among researchers to design biomaterials with highly specific properties. The primary objective of a tissue engineering scaffold is to emulate the natural environmental conditions of the target tissue, while contributing to the synthesis of

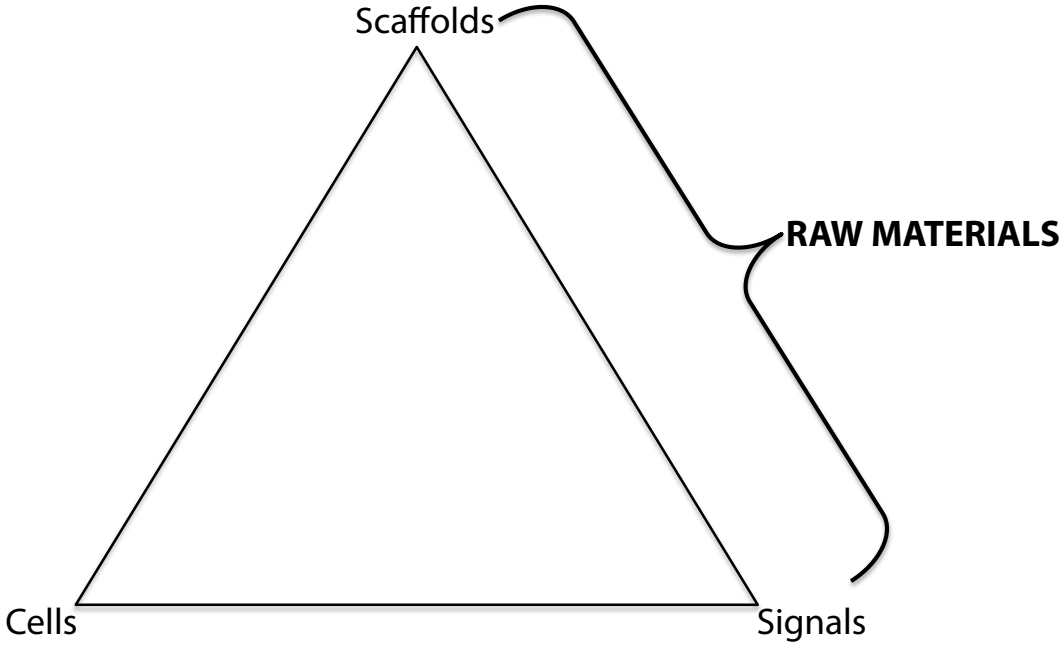

Figure 1. Schematic of the traditional tissue engineering triad illustrating the potential of raw materials to bridge the gap between scaffolds and signals.

new tissue.[8, 10] Sokolsky-Papkov *et al*.[11] outlined the ideal criteria for tissue engineering constructs: (a) sufficient mechanical properties, (b) low toxicity, (c) mimic the native extracellular matrix, (d) support cell adhesion and migration, and (e) degradation rate that is approximately equal to the growth rate of new tissue. Selection of the appropriate biomaterial(s) to construct a scaffold must take into consideration the differences that exist between the components, types, and organization of both the cells and the surrounding extracellular matrix of the tissue.[12] One of the main

advantages of raw materials is the ability of the body to recognize and metabolize these scaffold components in the local microenvironment, which mitigates toxicity or chronic inflammatory response that may be observed with synthetic polymers.[13]

Ultimately, the scaffold materials will influence multiple interactions in the microenvironment surrounding an implanted scaffold, which is crucial to the success or failure of tissue regeneration. Investigations of biomaterial-based "physical" signals propose that cell-biomaterial components and orientation at the micro/nanoscale level may affect cell survival, differentiation, and motility through interactions between cell receptors and ECM molecules.[12, 14] Toh *et al*.[15] as well as Marklein and Burdick[16] have suggested the importance of optimizing scaffold materials and fabrication processes to modulate these interactions. The researchers noted that physical cues, such as the scaffold formulation and/or geometry, and mechanical cues, such as matrix elasticity, should be controlled to aid in the proliferation and differentiation of stem cells.[15, 16] In addition, inherent adhesive cues or peptides and immobilized cues can also be incorporated into the biomaterial to manipulate the cell-matrix interface.[15, 16] Adopting a methodology that focuses on cell-scaffold interactions provides an effective strategy for utilizing material selection and fabrication to bridge two components of the tissue engineering triad – scaffolds and signals (Fig. 1). For example, selecting a collagen microparticle scaffold could affect three of these cues through high surface area and porosity (scaffold and geometric cue),[14] soft matrix rigidity (mechanical cue), and inherent adhesive RGD (Arg-Gly-Asp) sequences.[17] All of these scaffold attributes can collectively influence the local scaffold microenvironment

before the addition of growth factors or cytokines, which is a common theme in many tissue engineering strategies.[18] Raw materials such as collagen can effectively deliver microenvironmental cues without additional materials or fabrication steps that may be needed in a synthetic polymer scaffold. Therefore, the selection of raw materials as scaffold components could potentially bridge the gap between scaffolds and signals in the traditional tissue engineering triad, suggesting that the two are not modulated as separate components, but rather as integrated factors that contribute to the local scaffold microenvironment (Fig. 1). Employing raw materials that are natural components of tissue's ECM within scaffolds can act not only as a substrate for cell proliferation and attachment, but also a physical signaling environment for differentiation.

The following sections will highlight four main categories of raw materials that are commonly used in recent tissue engineering scaffold strategies: collagen, GAGs, bioceramics, and ECM-based materials. Within each section, the most frequently used materials for tissue repair and regeneration purposes will be highlighted. For organizational purposes, although raw materials have been grouped by material type in the following sections, due to the overlap of multiple raw materials in several approaches, tables are arranged by the target tissue application. Specifically, raw materials used in bone tissue engineering applications *in vitro* and *in vivo* can be found in Tables 1 and 2, respectively. Strategies employed in cartilage tissue engineering *in vitro* and *in vivo* can be found in Tables 3 and 4, respectively. Additional target tissue

applications can be found in Table 5 and strategies utilizing small intestinal submucosa (SIS) can be found in Table 6.

2.3 COLLAGEN

Collagen is the most prevalent protein in the body, making up approximately 30 percent of proteins in mammals, and is responsible for both tensile strength and structural support in the ECM of many tissues.[19] Collagen type I is the most universal type, found in bone, skin, tendons, ligaments, and other tissues, and its ubiquity has made it one of the most frequently used raw materials in tissue engineering over the past decade.[20] Hyaline cartilage and nucleus pulposus are the main tissues that contain little collagen type I in their native ECM, but are rich in collagen type II.[10, 21, 22] The main advantages of utilizing collagen as a part of a tissue engineering scaffold include its intrinsic cell adhesion motif RGD, biocompatibility, and bioresorbability.[17, 23] Questions concerning immunogenicity are considered negligible with the development of enzymatic digestion procedures to remove telopeptides.[23] Poor mechanical properties and rapid degradation are the main drawbacks when considering collagen as a scaffold component.[17, 20, 23-25] The following sections will discuss the use of collagen type I and II in tissue engineering scaffolds and applications of collagen as a component of constructs (Tables 1-5). The reader is also directed to an extensive review on the use of collagen scaffolds in tissue engineering[23] and collagen nanofibers for bone tissue engineering applications.[24]

Table 1. Recent Applications of Raw Material Strategies for *In Vitro* Bone Tissue Engineering Applications

Reference(s)	*Raw material(s)*	*Additional material(s)*	*Scaffold formulation*	*Cell type*	*Growth factor supplementation*
Chan *et al.*[44]	Collagen type I (rat)	–	Microspheres	Human and murine MSCs	–
Koegh *et al.*[46]	Collagen type I (bovine), CS	–	Porous composite	Human fetal osteoblasts	TGF-β1
Kruger *et al.*[34]	Collagen type I (bovine)	PLGA	Porous matrix	Human MSCs	–
Shen *et al.*[42]	Collagen type I (porcine), HAp	–	Nanocomposite hydrogel	–	–
Sionkowska and Kozlowska[29]	Collagen type I, HAp	–	Nanocomposite hydrogel	–	–
Thein-Han and Xu[35]	Collagen type I (rat)	CaP cement, alginate	Injectable microbead hydrogel	Human UCMSCs	–
Wang and Stegemann[40]	Collagen type I (bovine)	Chitosan	Composite hydrogel	Human MSCs	–
Akkouch *et al.*[27]	Collagen type I[b], HAp	PLCL	Porous composite	Human osteosarcoma	HEGF
Chicatun *et al.*[57]	Collagen type I (rat)	Chitosan	Dense collagen composite hydrogel	MC3T3-E1	–
Marelli *et al.*[43]	Collagen type I (rat)	Bioactive glass	Composite hydrogel	MC3T3-E1	–
Bae *et al.*[80]	HA	–	Hydrogel	MC3T3-E1	Simvastatin[a]
Chen *et al.*[81]	HA, collagen type I[b]	PCL	Porous matrix	hMSC-TERT	–
Chen *et al.*[82]	HA, collagen type I (bovine)	Bioactive glass, PS	Porous composite	MC3T3-E1	–
Liao *et al.*[83]	HA	HA-CPN	Injectable, thermoresponsive hydrogel	Canine MSCs	TGF-β1
Li *et al.*[104]	HAp	Chitosan, PLLA	Porous composite	MC3T3-E1 cells	–
Liu *et al.*[109]	nHAp, collagen type I[b]	PLA	Porous composite	Rabbit DPSCs	BMP-2
Peng *et al.*[107]	HAp	PLLA	Nanofibrous composite	Rat osteosarcoma cells	–
Prosecka *et al.*[110]	HAp, collagen type I (bovine)	–	Porous composite	Porcine MSCs	–
Haimi *et al.*[122]	TCP	PLA, bioactive glass	Porous composite	Human ASCs	–
Lee *et al.*[117]	TCP, collagen type I (porcine)	PCL	Porous composite	MG63	–
Lin *et al.*[131]	TCP	–	Porous matrix	–	–
Rai *et al.*[118]	TCP	PCL	Porous composite	hMSCs	–
Yanoso-Scholl *et al.*[123]	TCP	PLA	Porous composite	None	BMP-2, VEGF[a]
Yeo *et al.*[120]	TCP, collagen type I (porcine)	PCL	Nanofibrous composite	MG63	–
Zhang *et al.*[126]	TCP, collagen type I[b]	–	Microfibrous composite	MG63	–
Honsawek *et al.*[141]	SIS, human DBM	–	Composite matrix	Human periosteal cells	–
Supronowicz *et al.*[155]	Human DBM	–	Porous matrix	Human ASPSCs	–
Thomas *et al.*[160]	Bovine DBM	PL	Composite matrix	Murine MSCs	–
Lee *et al.*[156]	Human DBM, HAp	–	Porous composite	Human MSCs	–
Liu *et al.*[152]	pDBM	–	Porous matrix	UCB-BMSCs	TGF-β1
Chen *et al.*[153]	Bovine DBM	Heparin	Porous matrix	HUVECs	VEGF[a]
Jayasuriya *et al.*[162]	Human DBM	PLGA	Composite film	Murine MSCs	–
Kang *et al.*[158]	Human DBM	Fibrin glue	Composite glue	Porcine SDMSCs	–

[a]Denotes incorporation of the molecules into the scaffold. All other entries indicate the addition of the growth factor to culture medium; dexamethasone, β-glycerophosphate, and ascorbic acid were considered standard osteogenic medium components and not factored in for growth factor supplementation.

[b]Collagen species not specified.

CS, chondroitin sulfate; HAp, hydroxyapatite; HA, hyaluronic acid; TCP, β-tricalcium phosphate; SIS, porcine small intestinal submucosa; DBM, demineralized bone matrix; pDBM, partially demineralized porcine trabecular bone; PLGA, poly(lactic-co-glycolic acid); CaP, calcium phosphate; PLCL, poly(lactide-co-ε-caprolactone); HA-CPN, hyaluronic acid-g-chitosan-g-poly(N-isopropylacrylamide); PCL, poly(ε-caprolactone); PLA, poly(L-lactic acid); PLLA, poly(L-lactic acid); PL, polylactide; MSCs, mesenchymal stem cells; UCMSCs, umbilical cord mesenchymal stem cells; MC3T3-E1, murine calvarial osteoblasts; hMSC-TERT, human mesenchymal stem cell–telomerase reverse transcriptase gene-transduced; DPSCs, dental pulp stem cells; ASCs, adipose stem cells; MG63, human osteoblast-like cells; ASPSCs, adipose-derived side population stem cells; UCB-BMSCs, human umbilical cord blood–derived mesenchymal stem cells; HUVECs, human umbilical vein endothelial cells; SDMSCs, skin-derived mesenchymal stem cell–like cells; TGF-β1, transforming growth factor beta-1; HEGF, human epidermal growth factor; BMP-2, bone morphogenetic protein-2; VEGF, vascular endothelial growth factor.

Table 2. Recent Applications of Raw Material Strategies for *In Vivo* Bone Tissue Engineering Applications

Reference(s)	*Raw material(s)*	*Additional material(s)*	*Scaffold formulation*	*Animal model*	*Highlighted finding*
Bae *et al.*[80]	HA	Simvastatin	Hydrogel	New Zealand white rabbits, parietal bone defect model	Hydrogels loaded with simvastatin significantly increased new bone formation after 9 weeks
Chen *et al.*[82]	HA, collagen type I (bovine)	Bioactive glass, phosphatidylserine	Porous composite	New Zealand white rabbits, radial defect model	Composite scaffold promoted new bone formation and new blood vessel formation after 12 weeks compared to the control (sham) which displayed little new bone formation
Xu *et al.*[78]	HA, collagen type I (bovine)	Bioactive glass, phosphatidylserine	Porous composite	Sprague-Dawley rat, femoral defect model	Composite scaffolds seeded with MSCs enhanced greater new bone formation compared with control scaffolds with no seeded cells after 6 weeks
Patterson *et al.*[79]	HA	HA-GMA	Hydrogel	Sprague-Dawley rat, critical-sized calvarial defect model	Codelivery of BMP-2 and VEGF from hydrogels showed improved new bone formation after 6 weeks compared with either of the proteins when delivered alone
Rentsch *et al.*[99]	CS, collagen type I (porcine)	PCL	Porous composite	Athymic nude mice, critical-sized femoral defect model	PCL meshes coated with CS/collagen type I and seeded with rat MSCs showed significantly increased new bone formation and new blood vessel formation after 12 weeks
Lee *et al.*[156]	HAp, human DBM	–	Porous composite	Athymic nude rat, intramuscular abdominal pouch model	DBM/HA putty induced ectopic mineralized bone formation after 8 weeks, HA granules alone (control) showed limited mineralization
Liu *et al.*[109]	nHAp, collagen type I[a]	PLA	Porous composite	New Zealand white rabbits, segmental critical-sized alveolar bone defect model	nHAp–collagen type I–PLA scaffolds seeded with rabbit DPSCs and cultured with BMP-2 prior to implantation promoted new bone formation after 12 weeks
Teixeira *et al.*[102]	HAp, collagen type I (bovine)	–	Porous matrix	Immune-deficient mice, subcutaneous implantation model	HAp scaffolds seeded with human MSCs showed that those with a collagen coating tended to have a negative effect on bone formation regardless of collagen crosslinking method after 6 weeks
Yeo *et al.*[119]	TCP	PCL, NaOH	Porous composite	New Zealand white rabbits, calvarial defect model	PCL-TCP scaffolds treated with NaOH for 48 hours to increase surface roughness showed superior bone formation after 8 weeks
Yanoso-Scholl *et al.*[123]	TCP	PLA	Porous composite	C57BL/6 mice, intramuscular quadriceps implantation	Scaffolds loaded with BMP-2 and VEGF promoted new blood vessel formation but limited mineralization after 8 weeks
Rojbani *et al.*[174]	TCP, HAp	–	Porous matrix	Wistar rat, calvarial defect model	TCP promoted greater bone regeneration after 8 weeks, the addition of simvastatin increased bone formation in all groups
Rai *et al.*[118]	TCP	PCL	Porous composite	CBH/Rnu rats, critical-sized femoral defect model	Composites seeded with human MSCs showed new bone formation after 3 weeks compared with limited bone formation in scaffolds seeded with no cells
Hao *et al.*[125]	TCP, collagen type I (bovine)	PLGA	Composite hydrogel	Japanese white rabbits, critical-sized segmental radial defect	Higher cell numbers of rabbit ASCs encapsulated within the scaffold led to enhanced osteogenesis and bone union after 24 weeks
Ghanaati *et al.*[115]	TCP	–	Granules with varying size and porosity	Wistar rat, subcutaneous implantation model	Higher porosity led to greater new blood vessel formation near the center of the construct over 60 days

(*continued*)

Table 2. (Continued)

Reference(s)	*Raw material(s)*	*Additional material(s)*	*Scaffold formulation*	*Animal model*	*Highlighted finding*
Ghanaati *et al.*[129]	TCP, HA	Methylcellulose	Injectable bone paste	Wistar rat, subcutaneous implantation model	The addition of HA and methylcellulose resulted in a formable material to fill in bone defects and led to higher vascularization after 60 days
Cao *et al.*[124]	TCP, HAp	PGA	Porous composite	Sprague-Dawley rat, critical-sized femoral defect	PGA–TCP scaffolds in a 1:3 ratio provided the greatest new bone formation after 90 days
Eleftheriadis *et al.*[157]	TCP, human DBM	Hydroxyl sulfate	Porous composite	New Zealand white rabbits, mandibular defect model	TCP–hydroxyl sulfate scaffolds resorbed more slowly than DBM putty over 8 weeks, making them a potential candidate for larger, critical-sized defects
Fujita *et al.*[127]	TCP	Gelatin	Sponge	Nihon white rabbits, segmental bone defect model	No significant difference was found in new bone regeneration between gelatin–TCP sponge and the BMP-2-loaded gelatin–TCP sponge after 8 weeks
Tadokoro *et al.*[128]	TCP	Gelatin	Sponge	Fisher rats, subcutaneous implantation model	TCP–gelatin sponges loaded with BMP-2 and seeded with MSCs showed significant new bone formation compared with nonloaded scaffolds and scaffolds with no cells after 4 weeks
Abbah *et al.*[116]	TCP	PCL	Porous composite	Yorkshire pigs, spinal interbody fusion model	PCL–TCP scaffolds seeded with autogenous MSCs showed new bone formation after 3 months and fusion was observed after 6 months, no fusion occurred in control samples with no seeded cells
Chen *et al.*[153]	Bovine DBM	–	Porous matrix	Sprague-Dawley rat, subcutaneous implantation model	Heparin-crosslinked DBM loaded with VEGF promoted new blood vessel formation superior to unloaded and noncrosslinked scaffolds after 3 weeks
Kang *et al.*[158]	Human DBM	Fibrin glue	Composite glue	Miniature pig, maxillary sinus floor implantation model	Enhanced new bone activity was observed in the cell-seeded scaffold sites compared with the scaffold-only regions after 4 weeks
Rhee *et al.*[154]	Human DBM	PLA	Porous composite	Sprague-Dawley rat, critical-sized calvarial defect model	DBM seeded with SVF cells promoted greater new bone formation than groups containing PLA and those without cells after 8 weeks
Supronowicz *et al.*[155]	Human DBM	–	Porous matrix	Athymic nude rat, intramuscular abdominal pouch model	DBM seeded with human ASPSCs provided significantly greater new bone formation after 14 days

[a]Collagen species not specified.
nHAp, nanohydroxyapatite; HA-GMA, glycidyl methacrylate modified hyaluronic acid; NaOH, sodium hydroxide; PGA, poly(glycolic acid); SVF, human stromal vascular fraction.

Table 3. Recent Applications of Raw Material Strategies for *In Vitro* Cartilage Tissue Engineering Applications

Reference(s)	*Raw material(s)*	*Additional material(s)*	*Scaffold formulation*	*Cell type*	*Growth factor supplementation*
Li *et al.*[45]	Collagen type I (rat)	–	Microspheres	Human MSCs	TGF-β3
Lu *et al.*[26]	Collagen type I (porcine)	–	Sponge	Bovine chondrocytes	–
Lu *et al.*[33]	Collagen type I (porcine)	PLGA	Porous composite	Bovine chondrocytes	–
Ng *et al.*[32]	Collagen type I, collagen type II (porcine)	–	Porous matrix	Porcine MSCs and murine ECCs	TGF-β1
Ohyabu *et al.*[31]	Collagen type I,[a] HAp, CS	–	Porous composite sponge	Rabbit MSCs	TGF-β3
Yan *et al.*[28]	Collagen type I (bovine), chitosan	Chitosan	Composite hydrogel	Rabbit chondrocytes	–
Berendsen *et al.*[36]	Collagen type I (rat), collagen type II (chicken sternum)	–	Hydrogel	Goat articular chondrocytes	–
Zhang *et al.*[67]	Collagen type I (bovine), HA, CS	–	Composite hydrogel	Rabbit articular cartilage	–
Mueller-Rath *et al.*[59]	Collagen type I (rat)	–	Dense collagen hydrogel	Human articular chondrocytes	–
Chang *et al.*[60]	Collagen type II,[a] CS	PCL	Coated porous mesh	Rat chondrocytes	–
Francioli *et al.*[61]	Collagen type II (porcine)	–	Porous matrix	Human articular chondrocytes	TGF-β1, TGF-β3, FGF-2
Vickers *et al.*[22]	Collagen type II (porcine), GAG	–	Composite hydrogel	Carpine MSCs	FGF-2, TGF-β1
Wu *et al.*[62]	Collagen type II (bovine)	Exogenous GAGs	Composite hydrogel	Human articular chondrocytes	–
Park *et al.*[77]	HA	Fibrin	Composite hydrogel	Rabbit MSCs	TGF-β1
Fan *et al.*[76]	HA, CS	PLGA, gelatin	Porous composite	Rabbit MSCs	TGF-β3[b]
Correia *et al.*[66]	HA	Chitosan	Porous composite	Bovine chondrocytes	TGF-β3
Nguyen *et al.*[54]	CS, HA	PEG, MMP-pep	Multilayered hydrogel	Murine MSCs	TGF-β1
Coburn *et al.*[97]	CS	PCL, PVAMA, CSMA, PEGDA	Fiber–hydrogel composite	Goat MSCs	–
Liang *et al.*[56]	Concentrated CS, collagen type I (bovine)	–	Porous composite	Human MSCs	TGF-β1, FGF-2
Kinneberg *et al.*[53]	CS, collagen type I (bovine)	–	Sponge	Rabbit MSCs	–
Wang *et al.*[159]	Human DBM	Gelatin, fibrin glue	Composite sponge	Rabbit articular chondrocytes	–

[a]Collagen species not specified.
[b]Denotes incorporation of the protein into the scaffold. All other entries indicate the addition of the growth factor to culture medium; nonessential amino acids, ascorbic acid, and dexamethasone were considered standard chondrogenic medium components and were not factored in for growth factor supplementation.

GAG, glycosaminoglycan; PEG, poly(ethylene glycol); MMPs, matrix metalloproteinase–sensitive peptides; PVAMA, poly-(vinyl alcohol)-methacrylate; CSMA, chondroitin sulfate-methacrylate; PEGDA, poly(ethylene glycol)-diacrylate; ECCs, P19 embryonal carcinoma cells; FGF-2, fibroblast growth factor-2.

2.3.1 Collagen Type I

Collagen type I scaffold formulations have included sponges,[25-33] fibers,[19, 34, 35] hydrogels[36-43] and microspheres.[44, 45] Applications of collagen type I span target areas

of bone,[27, 29, 34, 35, 40, 42, 44, 46] tendon,[19, 47] peripheral nerves,[41, 48] cartilage,[26, 28, 31-33, 37, 45] skin,[25, 39, 49, 50] and bladder tissue engineering.[51] To address limitations associated with collagen, researchers have often chosen to use different crosslinking agents and/or composites of collagen with other materials.[20] In many approaches, blends of collagen I with chondroitin sulfate (CS), hyaluronic acid (HA), bioceramics, and synthetic polymers have been utilized to enhance mechanical properties, reduce susceptibility to degradation, and encourage mineralization.[7] Seo *et al*.[52] provided a comprehensive review of the reinforcement of collagen and other raw materials by synthetic polymers. Akkouch *et al*.[27] presented an interesting approach of employing a reinforced natural material scaffold composed of collagen-hydroxyapatite-poly(lactide-co-ε-caprolactone) (PLCL) for bone tissue engineering (Table 1). In this case, PLCL offered a solution to enhance the inherent poor mechanical stability that collagen and hydroxyapatite lacked when used without a reinforcing material.[27] This composite material showed the innovative use of both a synthetic and bioceramic material additives to a collagen type I matrix to overcome limitations associated with each of the materials when used alone.

Other strategies have combined collagen with GAGs for additional applications. For example, scaffolds of type I collagen and chondroitin-6-sulfate, termed in the literature more generally as collagen-GAG or CG scaffolds, represent a common raw material blend for bone,[46] cartilage,[53, 54] tendon,[47] and skin[38, 55, 56] tissue engineering. One particularly innovative raw material technique used a CG core-shell fabrication strategy to enhance mechanical integrity while maintaining a highly

porous structure.[47] The scaffold consisted of a high density CG shell to promote tensile strength and a low density CG core scaffold with high porosity (Table 5). This study was representative of a scaffold that combined an innovative formulation approach and raw materials for tendon tissue engineering.

Table 4. Recent Applications of Raw Material Strategies For *In Vivo* Cartilage Tissue Engineering Applications

Reference(s)	*Raw material(s)*	*Additional material(s)*	*Scaffold formulation*	*Animal model*	*Highlighted finding*
Chang *et al.*[37]	Collagen type I (porcine)	–	Hydrogel	Lee-Sung miniature pigs, osteochondral defect model	Undifferentiated collagen gels seeded with porcine MSCs were superior to those that were differentiated using TGF-β3 prior to implantation based on gross appearance and histological evaluation after 6 months
Lu *et al.*[26]	Collagen type I (porcine)	–	Funnel-like sponge	Athymic nude mice, subcutaneous dorsa model	After 3 weeks, funnel-like collagen sponges outperformed control collagen sponges in cell number and GAG production
Lu *et al.*[33]	Collagen type I (porcine)	PLGA	Funnel-like hybrid sponge	Athymic nude mice, subcutaneous dorsa model	Funnel-like hybrid sponges (collagen type I–PLGA) outperformed collagen-only sponges in the expression of collagen type II and aggrecan genes after 7 weeks of implantation
Fan *et al.*[76]	HA, CS	PLGA, gelatin with immobilized TGF-β3	Porous composite sponge	New Zealand white rabbits, full-thickness osteochondral defect model	After 8 weeks, TGF-β3-immobilized scaffolds seeded with autologous MSCs promoted significant cartilage formation when compared with control (no TGF-β3)
Yagihashi *et al.*[164]	Bovine DDM	–	Powder	New Zealand white rabbits, full-thickness osteochondral defect model	After 9 weeks, defects filled with 100 mg of DDM had filled in with hyaline-like cartilage, with incomplete cartilage formation in the control (sham) group

DDM, demineralized dentin matrix.

Another method for overcoming the inherent poor mechanical properties of collagen included plastic compression of collagen type I hydrogels to produce dense collagen.[43, 51, 57-59] This approach has been employed for applications in bone,[43, 57] cartilage,[59] and bladder[51] tissue engineering with favorable outcomes. Chicatun *et al.*[57]

fabricated a dense collagen and chitosan scaffold that retained an open, interconnected pore structure that attempted to mimic the osteoid of native bone (Table 1). This strategy demonstrated an excellent example of the use of a raw material to mimic not only a component of native bone tissue but also the inherent pore structure and ECM structure. The ubiquity of collagen type I in the body and the versatility of scaffold formulations have promoted widespread use in tissue engineering scaffolds. Relatively new fabrication methods, such as dense collagen techniques help to mitigate mechanical limitations without the need for additional materials. However, crosslinking and composite strategies still remain the most common approach for enhancing construct properties, while maintaining the benefits associated with cell adhesion capability of collagen.

2.3.2 Collagen Type II

Collagen type II has been used much less frequently in raw material strategies for tissue engineering constructs, mostly likely due to its presence in considerably fewer extracellular matrices of tissues in the body. Scaffold formulations reported recently in the literature of collagen type II include hydrogels,[21, 36] sponges,[22, 60-62] and microspheres.[14] These scaffolds have been mainly utilized for cartilage[22, 36, 60-63] and nucleus pulposus[21] tissue engineering. Hyaline cartilage and the nucleus pulposus have the greatest amount of collagen type II present in their extracellular matrix with little to no collagen type I, so this material strategy may be beneficial for these limited applications. One group utilized a collagen type I/calcium phosphate layered with an

interfacial layer connecting to a collagen type II/CS layer to mimic native constituents involved in the transition of tissue types at the osteochondral interface.[63] Calderon *et al*.[21] utilized a similar strategy to formulate a scaffold for nucleus pulposus tissue engineering that consisted entirely of raw materials. They used collagen type II and hyaluronic acid in a ratio equivalent to the native tissue ECM of the nucleus pulposus and noted that with sufficient crosslinking, this raw material scaffold would be a potential candidate for regeneration of the nucleus pulposus (Table 5).[21] Far fewer approaches utilize collagen type II in raw material scaffolds, however, the strategy of mimicking native ECM composition has increased its utility in hyaline cartilage and nucleus pulposus applications.

Summary

Overall, collagen type I has been explored in numerous areas of tissue engineering with growing interest in areas of new fabrication techniques and composite strategies. Collagen type II, however, has been utilized much less frequently and may require more in-depth studies to verify its potential. It is unclear whether the limited use of collagen II is due more to its high cost and limited availability, the absence of compelling data thus far to support its use, a limited awareness of the idea to use collagen II, or a combination of the above. There is no question, however, that using collagen I or collagen II can allow for scaffold bioresorbability and cell adhesion unmatched by synthetic polymers.

Table 5. Recent Applications of Raw Material Strategies for Additional Tissue Engineering Applications

Target tissue	*Reference(s)*	*Raw material(s)*	*Additional material(s)*	*Scaffold formulation*	*Biological model(s)*
Skin	Gaspar *et al.*[25]	Collagen type I (bovine)	Agarose	Composite sponge	*In vitro:* composite sponges seeded with L929 mouse fibroblasts and cultured for 48 hours
	Hartwell *et al.*[38]	Collagen type I (rat), CS	PVA	Composite hydrogel	*In vitro:* primary human fibroblasts were encapsulated within the hydrogel and cultured for 10 days
	Wang *et al.*[50]	Collagen type I (bovine), HA, CS	–	Porous composite	*In vitro:* 9 different ratios of collagen type I, HA, and CS were tested and seeded with rat dermal fibroblasts *In vivo:* composite seeded with rat dermal fibroblasts and cultured for 7 days before implantation into full-thickness skin defect model in Sprague-Dawley rats for 6 weeks
	Wong *et al.*[39]	Collagen type I (rat)	Pullulan	Composite hydrogel	*In vitro:* murine MSCs and human foreskin fibroblasts were seeded onto separate hydrogel scaffolds and cultured for 7 days *In vivo:* hydrogel implanted into subcutaneous wound model and stented excisional wound model in C57BL/6 mice for 21 days
	Ghezzi *et al.*[58]	Collagen type I (bovine)	Silk fibroin	Dense collagen multilayered composite	*In vitro:* composites seeded with rat MSCs and cultured for 7 days
	Zhang *et al.*[75]	HA	Gelatin	Composite hydrogel	*In vitro:* hydrogels seeded with L929 mouse fibroblasts and cultured for 7 days
	Emami *et al.*[98]	CS	Gelatin, chitosan	Composite hydrogel	*In vitro:* hydrogels seeded with L929 mouse fibroblast and cultured for 14 days
	Liang *et al.*[56]	CS, collagen type I (bovine)	–	Porous composite (concentrated CS)	*In vitro:* composite seeded with human foreskin keratinocytes and cultured for 7 days
Osteochondral	Harley *et al.*[63]	Collagen type I (bovine), collagen type II (porcine), CS, CaP	–	Multilayered porous composite	–
	Sundararaghavan and Burdick[92]	HA	RGD peptide	Gradient fibrous composite	*In vitro:* scaffolds seeded with aortic arch explants from chick embryos and cultured for 7 days
	Zhou *et al.*[103]	HAp, collagen type I[a]	–	Porous, layered composite	*In vitro:* scaffold seeded with human MSCs and cultured in both osteogenic and chondrogenic medium for 14 days

Table 5. (Continued)

Target tissue	*Reference(s)*	*Raw material(s)*	*Additional material(s)*	*Scaffold formulation*	*Biological model(s)*
	Haaparanta *et al.*[121]	TCP	PLA	Porous composite	–
	Niyama *et al.*[130]	TCP	–	Porous matrix with chondrocyte cell sheet	*In vitro:* porcine articular chondrocyte attached to TCP block and cultured for 21 days
Nucleus Pulposus	Calderon *et al.*[21]	Collagen type II (bovine), HA	–	Composite hydrogel	*In vitro:* rat MSCs injected into hydrogels and cultured for 21 days
	Park *et al.*[90]	HA	Silk fibroin	Composite hydrogel	*In vitro:* human chondrocytes encapsulated in hydrogel and cultured for 28 days
Nerve	Suri *et al.*[41]	Collagen type I[a], HA	Laminin	Hydrogel	*In vitro:* rat neonatal Schwann cells were encapsulated in hydrogel and cultured for 14 days
Tendon, meniscus, ligament	Caliari *et al.*[47]	Collagen type I (bovine), CS	–	Core-shell composite	*In vitro:* composites seeded with horse tendon cells and cultured for 14 days
	Freymann *et al.*[88]	HA	PGA	Porous composite	*In vitro:* composites seeded with human adult meniscus-derived cells and cultured for 21 days
Vascular	Duffy *et al.*[55]	Collagen type I,[a] CS	–	Porous composite	*In vitro:* composites seeded with rat MSCs or rat aortic endothelial cells and cultured for 28 days
	Perng *et al.*[30]	Collagen type I (bovine), HA	–	Porous composite	*In vivo:* composites implanted into inferior epigastric skin flap of nude mice as angiogenesis model for 28 days
	Ekaputra *et al.*[84]	Collagen type I (bovine), HA	PCL	Fiber–hydrogel composite	*In vitro:* composites seeded with HUVECs and IMR90 human lung fibroblast cells and cultured for 14 days
	Seidlits *et al.*[85]	HA	Fibronectin	Composite hydrogel	*In vitro:* HUVECs encapsulated in hydrogels and cultured for 6 days
	Tedder *et al.*[150]	Adult swine pericardium	–	Trilayered construct with adhesive	*In vivo:* constructs implanted subdermally into Sprague-Dawley rats for 35 days
Urogenital	Engelhardt *et al.*[51]	Collagen type I (rat)	PLAC	Porous composite (dense collagen)	*In vitro:* human SMCs and urothelial cells encapsulated within composite and cultured for 14 days *In vivo:* precultured constructs (14 days) were implanted subcutaneously on the backs of nude mice for 6 months

[a]Collagen species not specified.
PVA, poly(vinyl alcohol); RGD, (arginine-glycine-aspartic acid); PLAC, poly(lactic acid-co-ε-caprolactone); SMCs, smooth muscle cells.

Table 6. Recent Tissue Engineering Strategies Utilizing Porcine Small Intestinal Submucosa

Target tissue	*Reference(s)*	*Additional material(s)*	*Scaffold formulation*	*Biological model(s)*
Bone	Kim *et al.*[142]	–	Sponge	*In vitro:* sponges seeded with rat MSCs and cultured for 14 days *In vivo:* sponges seeded with cells and implanted into a cranial defect model in Fisher rats for 28 days
	Honsawek *et al.*[141]	Human DBM	Tissue/ composite matrix	*In vitro:* scaffolds seeded with human periosteal cells and cultured for 10 days *In vivo:* composites were implanted intramuscularly into Wistar rats for 42 days
	Zhao *et al.*[143]	–	Hydrated SIS matrix	*In vivo:* SIS scaffolds seeded with rabbit MSCs and implanted into radial bone defects of critical size in New Zealand white rabbits for 12 weeks
Skin	Zhou *et al.*[145]	–	Hydrated SIS matrix	*In vitro:* scaffolds seeded with murine ADSCs and cultured for 7 days before digestion *In vivo:* scaffolds seeded with murine ADSCs and cultured for 1 week and then implanted into cutaneous and subcutaneous wound models in C57 mice for 28 days
Nerve	Kang *et al.*[144]	PLGA	Porous composite	*In vivo:* composite scaffolds seeded with rat ADSCs and implanted into complete spinal cord transaction in Fisher rat model for 8 weeks
Vascular	Liu *et al.*[86]	Collagen type I–HA–CS (comparison study between SIS and polymer composite)	Tissue scaffold and polymer composite	*In vivo:* SIS and polymer composite scaffolds seeded with murine ADSCs and implanted into full-thickness cutaneous defects in C57BL/6 mice for 21 days
	Mondalek *et al.*[87]	HA-PLGA nanoparticles	Porous composite	*In vivo:* composite scaffolds implanted into canine bladder model of Beagle dogs for 10 weeks to evaluate angiogenic potential
	Crapo *et al.*[137]	–	Gel	*In vitro:* SIS gel seeded with rat neonatal cardiomyocytes and cultured for 13 days
	Okada *et al.*[138]	–	Gel	*In vivo:* SIS gel injected into infarct cardiac tissue in NON-SCID mice for 6 weeks
	Peng *et al.*[139]	–	Hydrated SIS matrix	*In vitro:* SIS tissue seeded with lamb hair follicle MSCs and cultured for 14 days under uniaxial strain conditions
	Tan *et al.*[140]	–	Hydrated SIS matrix	*In vivo:* SIS sheets seeded with rabbit MSCs and implanted to patch infarct myocardial tissue model in New Zealand White rabbits for 28 days
Urogenital	Heise *et al.*[146]	–	Hydrated SIS matrix	*In vitro:* SIS sheets seeded with rat MSCs and subjected to a period of static culture for 7 days followed by dynamic culture with cyclic strain for an additional 7 days
	Qin *et al.*[147]	–	Hydrated SIS matrix	*In vitro:* SIS sheets seeded with rat intestinal SMCs and implanted into jejunal interposition model of adult Lewis rats for 8 weeks
	Wu *et al.*[148]	–	Hydrated SIS matrix	*In vitro:* SIS sheets seeded with human UDSCs and cultured under static and dynamic conditions for 14 days. Cultured sheets were sectioned for *in vitro* characterization and implantation *In vivo:* precultured SIS sheets were implanted subcutaneously into the flanks of athymic nude mice for 1 month
	Zhang *et al.*[149]	–	Hydrated SIS matrix	*In vivo:* SIS sheets implanted into abdominal wall defect model in adult Sprague-Dawley rats for 8 weeks

ADSCs, adipose-derived stem cells; NON-SCID, nonobese diabetic severe combined immunodeficiency; UDSCs, urine-derived stem cells.

2.4 GLYCOSAMINOGLYCANS

Over the past decade, GAGs have emerged as additional raw material strategy for multiple tissue engineering applications. Two of the most widely used GAGs include HA and CS. HA is well known for its role in the regulation of cell behaviors, such as adhesion, proliferation, differentiation and migration.[64] However, limitations including water solubility, fast resorption, and negative charge have caused researchers to adopt specific concentration limits and fabrication methods.[65, 66] CS functions as a structural component of native ECM and strategies have utilized CS in tissue engineered constructs often with additional raw materials, such as HA and collagen, respectively.[53, 67] The main motivation for blending CS with additional raw materials or synthetic polymers lies in its innate capability to be readily water-soluble.[9] Some of the approaches used to overcome weaknesses and incorporate these raw materials will be discussed in the following sections. For more in-depth reviews of all natural polysaccharides used in tissue engineering, the reader is directed to articles by Baldwin *et al*.[6] and Oliviera *et al*.[9] An exceptional review of hyaluronic acid is also available from Murano *et al*.[64] Hydrogels that are fabricated from biopolymers have also been reviewed extensively, and the reader is directed to articles by Van Vlierberghe *et al*.,[68] Slaughter *et al*.,[69] Spiller *et al*.,[70] Hunt *et al*.,[71] and Burdick and Prestwich.[72]

2.4.1 Hyaluronic Acid

HA is the only non-sulfated GAG and is found in the ECM of many tissues in the body. HA is well-known for its viscoprotective capabilities and has been used in ophthalmology applications for over thirty years.[73] Supplementation of HA for synovial fluid viscosity in arthritic joints has also been used for over a decade.[73] In addition, HA interacts with specific protein receptors on the surface of cells, such as CD44 and RHAMM, to modulate cell adhesion, proliferation, motility, and other signaling cascades.[74] For these reasons, HA has been utilized in recent tissue engineering strategies for skin,[50, 75] cartilage,[14, 66, 76-78] bone,[79-83] angiogenesis,[30, 84-87] meniscus,[88] nerve,[41, 89] and nucleus pulposus[21, 90] applications. Methacrylated HA that is crosslinked to form hydrogels[21, 41, 72, 79, 80, 83, 89-91] has been the most common formulation as a tissue engineering construct, however, electrospun fibers,[92] porous composite coatings and sponges have also been tested.[91] For an exceptional review on the use of HA in cartilage tissue engineering, the reader is directed to Kim *et al*.[91]

Many different strategies have been employed to overcome the fast resorption, mechanical integrity, and water solubility of HA. An approach most frequently employed for formulating tissue engineering constructs consists of crosslinking HA by photopolymerization[79, 80, 85, 92-94] or thermal[76, 83, 90] mechanisms to form hydrogels in which cells can be encapsulated.[89, 91] Crosslinking can function to increase mechanical strength, while also prolonging degradation of HA.[65] Zhang *et al*.[67] engineered a hydrogel scaffold by thermal crosslinking for cartilage tissue engineering comprised solely of components found in the ECM of cartilage tissue using bovine collagen type I,

HA, and CS (Table 3). Freeze drying is a common fabrication method to form composite porous matrices containing HA and other materials for tissue engineering constructs.[66, 75, 78, 81, 82, 88, 95] Zhang *et al*.[75] assembled highly macroporous composite scaffolds of HA and gelatin for soft tissue engineering applications using a freeze drying technique (Table 5). It is also important to note that HA must be utilized in relatively low concentrations to avoid limited cell adhesion that can occur at higher concentrations due to its negative charge.[66] Fabricating composites with HA and neutral or positively charged materials can help mitigate this charge limitation. One specific example of a composite HA strategy by Sundararaghavan and Burdick[92] created dual-gradient, electrospun fiber scaffolds incorporating HA with RGD peptide sequences to promote cell adhesion. This example demonstrated both an exceptional raw material and scaffold formulation approach, while also providing a recent example of a gradient scaffold that incorporated a raw material.[92]

The versatility and biocompatibility of HA has attracted attention for the delivery of growth factors and other biological molecules in tissue engineering scaffolds.[18] Recent approaches have included the delivery of signaling molecules such as simvastatin,[80] vascular endothelial growth factor (VEGF),[79, 84] platelet derived growth factor (PDGF),[84] transforming growth factor beta-1 (TGF-β1),[77] transforming growth factor beta-3 (TGF-β3),[76] bone morphogenetic protein-2 (BMP-2),[79] phosphatidylserine (PS),[78] and fibronectin.[85] Bae *et al*.[80] fabricated HA hydrogels loaded with simvastatin prior to photocrosslinking to entrap the molecule within the entangled gel matrix. Most researchers utilized the ability to control molecule delivery within HA scaffolds

by modulating properties such as molecular weight, crosslinking, and scaffold formulation, accordingly. Overall, the ubiquity of HA in the body has been mirrored by tissue engineers in a wide variety of applications. Chemical modifications, crosslinking, and blending of HA with other materials are the most common methods used to apply this raw material for regenerative constructs and innovative approaches continue to be developed for several different applications.

2.4.2 Chondroitin Sulfate

CS is a GAG that is found mainly attached to proteoglycans in connective tissue matrices or conjugated to proteins such as aggrecan in articular cartilage.[6] The different forms of CS depend on the sulfation site, typically at either the 4 or 6 carbon, however, chondroitin-6-sulfate is used in tissue engineering most frequently.[6] The presence of CS in native tissues has led to its use in cartilage,[31, 53, 54, 56, 60, 76, 96, 97] skin,[38, 50, 56, 98] bone,[46, 99], and blood vessel[55, 86] tissue engineering scaffolds. In addition to the aforementioned CG scaffolds, CS has been blended with many synthetic polymers and raw materials. A study by Kinneberg *et al*.[53] employed CS within a collagen hydrogel to investigate a potential increase in the linear stiffness of the gel constructs by helping to link discontinuous collagen fibrils in the gel network. Nyugen *et al*.[96] designed a three-layer hydrogel scaffold with varying compositions of CS, HA, and polyethylene glycol (PEG) to simulate the mechanical properties of each zone of articular cartilage. This triphasic construct demonstrated another approach for mimicking native tissue using raw materials and synthetic polymers in a spatially varying scaffold architecture.

Additionally, Coburn *et al*.[97] pioneered a fiber-hydrogel composite fabricated with methacrylated poly(vinyl-alcohol) and chondroitin sulfate fibers encapsulated within a PEG hydrogel. The fibers were hypothesized to mimic the nature of native protein networks, while the hydrogel served to simulate the polysaccharide-based ground substance that are both characteristic of the ECM of tissue.[97] Liang *et al*.[56] investigated the differences in scaffold properties with varying concentrations of collagen and CS in CG scaffolds for both cartilage and skin tissue engineering. This strategy showed the tunability of CG scaffolds with respect to water uptake, pore size, and elastic modulus to tailor properties for necessary properties for each target tissue.[56] A combination of HA, CS, and gelatin were fabricated into tri-*co*-polymer sponges and incorporated into a poly(lactic-co-glycolic acid) (PLGA) framework.[76] Additionally, the scaffolds were loaded with immobilized transforming growth factor beta-3 (TGF-β3) and implanted in full-thickness cartilage defects in New Zealand white rabbits (Table 4).[76] Wang *et al*.[50] employed a strategy of using solely raw materials to mimic the ECM of the dermis for skin tissue engineering grafts. The scaffold matrix consisted of collagen, CS, and HA with different ratios of each component, and were tested for optimal construct properties (Table 5).[50] This study, along with several others, embodied the emerging raw material approach for tissue engineering scaffolds. Overall, CS can be used to enhance mechanical integrity of a scaffold while also helping to mimic native ECM in connective tissues as well as articular cartilage. Skin, cartilage, and bone tissue engineering have utilized CS most frequently, however, this raw material is poised to become an effective scaffold component in many other target tissue applications.

Summary

The use of GAGs in tissue engineering strategies continues to become more sophisticated in fabrication techniques and raw material approaches. The combination of HA and CS has recently became evident as a conceivable raw material approach in both cartilage and skin tissue engineering applications. As the use of these native molecules continues to spread to additional applications, the potential of achieving clinical success using these raw materials appears limitless.

2.5 BIOCERAMICS

Mineralization of scaffolds plays a major role in bone as well as osteochondral interface tissue engineering. Calcium phosphate ceramics are biocompatible and their ability to be bioactive in the body stems from their similarity in composition and structure to the mineral phase of bone.[100] Some of the advantages of using bioceramics as part of a tissue engineering scaffold include increased mechanical strength, biocompatibility, and osteoconductivity.[3, 100] However, the brittle nature and slow degradation times of these ceramics can prove unattractive for tissue engineering constructs.[3] Researchers have blended synthetic polymers and/or several of the aforementioned raw materials with bioceramics to help to overcome the limitations of calcium phosphate materials for bone and cartilage tissue engineering constructs. Additionally, advances in fabrication methods to produce highly macroporous bioceramic scaffolds has helped to facilitate faster degradation rates. Two of the most widely used bioceramic materials in tissue engineering scaffolds,

hydroxyapatite (HAp) and beta-tricalcium phosphate (β-TCP), will be highlighted in the following sections. For comprehensive reviews on ceramic materials and their use in tissue engineering, the reader is directed to articles by Dorozhkin *et al*.,[100] Li *et al*.,[3] and Porter *et al*.[101]

2.5.1 Hydroxyapatite

Hydroxyapatite (HAp) is the main inorganic phase of bone and these crystals bind to collagen type I fibers in the ECM of native tissue.[101] Since collagen regulates the size and orientation of the HAp crystals, the structural relationship of this organic-inorganic matrix contributes largely to the mechanical properties of bone.[3, 101] In its non-porous and highly crystalline form, HA is known to remain unchanged for 5-7 years in the body with little to no resorption.[100] However, most tissue engineering strategies have incorporated synthetic HAp into porous scaffolds along with raw materials and/or synthetic polymers to best mimic the native ECM and properties of bone. The need for blends of polymeric materials with HAp stems from the brittle nature of HAp as a macroporous scaffold, and biopolymer incorporation can help to tune the elasticity of the scaffold as well as the degradation properties.[101] Texiera *et al*.[102] employed a raw material blend consisting of a collagen type I coating on a porous HAp matrix to mimic native bone composition and aid in cell adhesion. As a composite matrix, this material combination provided a microstructure that attempted to mimic native bone and provided a suitable microenvironment for new bone formation *in vivo* (Table 2).[102] Zhou *et al*.[103] demonstrated a similar strategy by

formulating bi-layered osteochondral scaffolds that consisted of a collagen type I layer on the top of the construct with a collagen/HAp layer on the bottom to imitate the transition from cartilage to bone tissue structure at this interface. The biphasic scaffolds were seeded with human mesenchymal stem cells and cultured separately in chondrogenic and osteogenic medium (Table 5).[103] Li *et al*.[104] constructed a composite of poly(L-lactic) acid (PLLA), chitosan, and HAp microspheres as a hybrid bone tissue engineering composite and studied the cellular response to these constructs *in vitro* using murine calvarial osteoblasts (Table 1). Approaches by each of these groups demonstrated the growing tendency of raw materials to be utilized as building blocks in bone tissue engineering scaffolds.

A longstanding debate in the bone tissue engineering literature is the use of micro versus nanoscale HAp in constructs.[3, 17] Employing a nanoscale HAp approach is hypothesized to allow the scaffold to better mimic the nanostructure of bone and encourage the differentiation of stem cells.[105, 106] Peng *et al*.[107] investigated the use of microscale versus nanoscale HAp powders incorporated with PLLA electrospun fibers. After a 10-day culture period, the composite scaffolds containing microscale HAp particles showed the best cell performance, but both particle sizes exhibited satisfactory cell viability and signaling.[107] Nanoscale HAp formulations have included nanoparticles[108-110] or nanofibers[111, 112] in combination with other materials. Zhang *et al*.[112] created a nanofibrous composite scaffold of HAp, collagen type I, and chitosan to mimic the nanostructure of native bone. A similar nano-composite approach was employed by Liu *et al*.[109] for treatment of periodontal bone defects using nano HAp,

collagen type I, and poly(lactic acid) (PLA). Overall, collagen type I has been one of the most widely utilized raw materials for creating HAp composites due to its ability to promote cell adhesion, which is limited in pure HAp constructs (Table 1). An exceptional review by Wahl *et al*.[113] detailed collagen-HAp composites for bone regeneration. The results of the debate between micro or nanoscale HAp formulations may suggest the need for additional studies to examine multiple size ranges simultaneously or differences that exist between fabrication methods that can help enhance mechanical integrity while also modulating cell differentiation.

2.5.2 Beta-Tricalcium Phosphate

The tunability of resorption rates of β-TCP has attracted great attention within the bone and osteochondral interface tissue engineering communities.[101, 114] While β-TCP can be resorbed too quickly for some applications *in vivo*, the ability to blend the material with polymers and control the granule size[115] offers methods to modulate resorption rate while utilizing the advantage for tissue in-growth when compared to the prolonged degradation of crystalline HAp. Synthetic polymers such as poly(ε-caprolactone) (PCL),[116-120] PLA,[121-123] poly(glycolic acid) (PGA),[124] and PLGA[125] are used most often to fabricate composite scaffolds with β-TCP. The main drawback of composites with β-TCP and synthetic polymers is poor cell attachment and proliferation. However, collagen,[117, 120, 126] gelatin,[127, 128] and hyaluronic acid[129] have also been employed with β-TCP and/or synthetic polymers to aid in cell adhesion and viability. Yeo *et al*.[120] presented an innovative approach composed of a PCL/β-TCP

composite embedded in collagen nanofibers to create a hierarchical structure similar to native bone. Niyama *et al*.[130] formulated an osteochondral scaffold using a β-TCP porous block covered with a scaffold-free chondrocyte matrix to induce both types of tissue formation. Tadokoro *et al*.[128] utilized a gelatin and β-TCP sponge loaded with BMP-2 in an *in vivo* subcutaneous model and observed the presence of new bone formation.

The microscale versus nanoscale debate has been investigated using powders of β-TCP, although the issue is much less controversial than that of HAp. Lin *et al*.[131] found that nanoscale β-TCP ceramics degraded slower than those fabricated from microscale powders. Furthermore, ceramics made from nanoscale β-TCP had twice the mechanical strength of those fabricated from microscale powder, and the nanoscale β-TCP ceramic reached a compressive strength in the upper range of native cancellous bone.[131] The combination of mechanical properties and fast resorption of β-TCP made from nanoscale powder provide tissue engineers another attractive bioceramic formulation option. Another group investigated granule size and morphology of β-TCP granules in a subcutaneous rat model (Table 2) and found that the greatest vascularization occurred in the group with polygonal morsel-shaped granules ranging from 63 to 250 microns in size.[115] Depending on defect size, healing time, and/or target application of the bone tissue engineering construct, the size and shape of β-TCP particles used in the raw material strategy must be considered and characterized.

Summary

Overall, the raw material approach to use of bioceramics in bone tissue engineering constructs appears to be shifting more away from hydroxyapatite and more toward β-TCP due to the ability to finely tune resorption rates to match newly forming bone and allow for incorporation of the scaffold into new bone tissue (summarized more in-depth in the discussion section). HAp may still be an effective raw material strategy in cases where new bone formation is expected to take more time. Advances in particle size and formulations of each bioceramic material has allowed for many new insights into considerations for fabricating bone tissue engineering scaffolds.

2.6 ECM-BASED MATERIALS

In addition to native ECM components, raw materials include those derived from mammalian tissue, which have been used in several tissue engineering applications from skin to heart valves.[132-135] Decellularized matrices, such as small intestinal submucosa (SIS), as well as heart valves and arteries, are additional sources of collagen and endogenous proteins.[132] Demineralized bone matrix (DBM) and decellularized cartilage are additional ECM-based strategies for retaining organic components of native tissue, while removing cells and/or mineralized crystals. Both decellularizing and demineralizing strategies can potentially weaken mechanical integrity of the matrix. However, many approaches have been employed to modulate mechanical stability of SIS and DBM. The following sections will review the use of SIS,

DBM, and decellularized cartilage as components of tissue engineering scaffolds and strategies to blend each with additional materials or cells for enhanced properties.

2.6.1 Small Intestinal Submucosa (SIS)

Of all the potential sources, porcine small intestinal submucosa (SIS) has been one of the most studied and utilized ECM-based raw materials in a wide variety of applications.[132, 135] Studies have shown that SIS contains over 90% collagen by dry weight, with a majority being collagen type I.[136] Depending on the type of decellularization method used, SIS can maintain GAGs and growth factors present in the native tissue.[132, 135] In addition to these native ECM molecules, the collagen fiber orientation that is maintained after the decell process has also attracted attention.[132] Both of these inherent properties have sparked strategies employing SIS as scaffolds in the fields of cardiovascular,[137-140] bone,[141-143] nerve,[144] soft tissue,[86, 87, 145] and urogenital[146-149] tissue engineering (Table 6). Currently, SIS is FDA approved for several urogenital applications, including hernia repair.[132] The presence of aligned collagen fibers and endogenous growth factors remaining in the acellular SIS matrix has sparked interest within the bone tissue engineering community as well. Kim *et al*.[142] and Honsawek *et al*.[141] showed that SIS scaffolds promoted new bone formation in a rat model. Zhao *et al*.[143] found similar results in a rabbit model when SIS was seeded with mesenchymal stem cells. Composite scaffolds fabricated with SIS and synthetic polymers or other raw materials have also been employed. Mondalek *et al*.[87] utilized all three types of materials by fabricating a SIS scaffold combined with hyaluronic acid-

poly(lactide-co-glycolide) (HA-PLGA) nanoparticles to enhance angiogenesis in the implanted scaffold when compared to SIS only (Table 6).

Urinary bladder matrix as well as heart valves and arteries from both xenogeneic and allogeneic sources have also been used in several other applications.[150] For a more comprehensive review on decellularized matrices and their role in tissue engineering, the reader is directed to articles by Badylak *et al*.,[132] Hoshiba *et al*.,[135] and Piterina *et al*.[136] Overall, utilizing SIS may offer a new dimension to raw material scaffolding by inherently combining aligned collagen fibers with remaining GAG molecules and growth factors. This complex tissue arrangement presents a suitable option for many different tissue engineering applications.

2.6.2 Demineralized Bone Matrix (DBM)

Demineralized bone matrix (DBM) mimics the strategy behind SIS, and has been studied for over 3 decades for use in bone grafting procedures.[151] DBM is formulated through acidic washing and defatting of human allograft cortical bone, which leaves an acellular organic matrix that mimics the microstructure of bone tissue.[151] Native concentrations of organic materials as well as mechanical integrity following the demineralization process are directly proportional to the extent of acidic washing.[152] Therefore, as more mineral is removed, the mechanical properties weaken and the presence of organic components decreases.[152] Nevertheless, the presence of organic components and proteins has led to the use of DBM in both bone and cartilage tissue engineering solutions. After the demineralization process, the

remaining acellular matrix is composed mainly of collagen with associated bone morphogenetic proteins (BMPs) and GAGs, which is an osteoinductive network that can aid in cell attachment, migration, and differentiation.[153] However, the inherently poor mechanical performance of DBM, along with the variance in quality and concentration of the organic materials from donor to donor, present barriers for utilizing DBM as a single-component construct.[151] To address these limitations and construct DBM composite constructs, studies have seeded DBM with stem cell sources or blended DBM with both synthetic and raw materials. Researchers have used DBM as a sole scaffold component in conjunction with seeded umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs)[152] and adipose-derived stem cells (ADSCs).[154, 155] Combination of DBM with additional raw materials such as SIS,[141] HAp,[156] and β-TCP[157] as well as fibrin glue,[158, 159] and heparin[153] have been employed in bone and cartilage tissue engineering. In addition, blends of DBM with synthetic PLA,[160] reverse-thermo responsive polymers,[161] and PLGA[162] allow for increased stability and modulation of mechanical properties.[163] Demineralized dentin matrix (DDM) has also gained attention for use in osteochondral tissue engineering. As an example, Yagihashi *et al*.[164] investigated the potential of DDM to promote osteochondral regeneration in full-thickness cartilage defects of New Zealand white rabbits and observed the formation of hyaline-like cartilage and new bone formation (Table 4). Both DBM and DDM can serve as effective raw materials to be incorporated into both bone and tissue engineering scaffolds without the need for additional exogenous growth factors or

cytokines. Endogenous organic components allow these raw materials to signal surrounding cells and tissue in ways unmatched by purely synthetic scaffolds.

2.6.3 Decellularized Cartilage

One scarcely explored tissue in the area of ECM-based materials is the notion of decellularizing hyaline cartilage. In theory, acellular hyaline cartilage would be expected to provide a scaffold rich in collagen type II, aggrecan, and endogenous growth factors following the decellularization process. Some groups have attempted to render hyaline cartilage acellular as an intact explant,[165, 166] while others have sliced or shattered explanted cartilage prior to this process due to the compact nature of cartilage tissue that does not allow complete penetration of decellularization solutions.[165, 167-169] Once the tissue had all of the cellular components removed, the remaining cartilage powder or solution was freeze dried to obtain an acellular, porous matrix.[167, 168] Gong *et al*. made a sandwich model of porcine acellular cartilage sheets with porcine chondrocytes seeded in between each layer of cartilage sheets.[169] This raw material strategy appears to have potential in the area of ECM-based materials, but will warrant future investigation both *in vitro* and in animal models.

Summary

ECM-based matrices offer a distinct advantage of retaining the composition of native materials and proteins as well as their inherent spatial arrangements in some cases. Both SIS and human DBM are FDA approved for clinical applications.[132, 170]

Composites utilizing ECM-based materials may also have the potential to translate into the clinical setting considering all of the current research attempting to develop these raw material hybrids.

2.7 BIOACTIVE SIGNALING OF RAW MATERIALS

In addition to providing building blocks for fabricating tissue engineering scaffolds, raw materials also hold the potential to present signals to cells. As previously mentioned, biomaterial-based signaling can arise from physical, chemical, adhesive, and mechanical properties of the construct. While many have exploited the inherent adhesive RGD peptide present in collagen, many others have examined the signaling potential of other raw materials used in tissue engineering constructs. Park *et al*.[77] investigated the chondrogenic potential of HA/fibrin glue composite hydrogels with encapsulated rat mesenchymal stem cells (MSCs) when treated with or without TGF-β1. Results suggested that treatment with exogenous growth factors was not essential for chondrogenic differentiation of rat MSCs in the HA-fibrin glue gel.[77] The authors hypothesized that the chondroinductive signaling potential of this composite gel most likely stemmed from the interaction of cells with the scaffold ECM via integrins on the cell surface.[77] This interaction was thought to induce intracellular signaling for regulation of many cell functions, including differentiation and matrix synthesis.[77] Another study aimed to elucidate the osteoinductive potential of collagen type I/HAp scaffolds for bone regeneration.[110] The porous composite constructs were seeded with porcine MSCs and cultured for 28 days. Results demonstrated osteogenic

differentiation of seeded MSCs by relative gene expression analysis using common osteogenic markers.[110] These studies suggested that mimicking the ECM components of native tissue may be a suitable alternative for the promotion of bioactive signaling without the addition of exogenous proteins. Similarly, ECM-based materials also offer evidence of bioactive signaling potential that stems from inherent native materials and growth factors. For example, Kim *et al*.[142] compared the regenerative potential of rat MSCs seeded on either a PGA mesh or an SIS sponge to repair full thickness bilateral bone defects in rat crania. SIS sponges showed significantly greater new bone regeneration when compared to PGA meshes 4 weeks after implantation.[142] Additionally, DBM/fibrin glue scaffolds have been investigated for osteoinductive capability with skin-derived mesenchymal stem cell–like cells (SDMSCs).[171] After 4 weeks of culture, osteogenic differentiation was confirmed by relative gene expression and flow cytometry.[171]

Overall, raw materials offer bioactive signaling potential that is unmatched by synthetic biomaterials. Optimization of raw material components and fabrication methods may alleviate the need to supplement tissue engineering scaffolds with immobilized or solubilized growth factors.

2.8 DISCUSSION

Integration of two components of the tissue engineering triad—scaffolds and signals—can be accomplished by utilizing raw material strategies in tissue engineering constructs. Raw materials can present physical, chemical, adhesive, and

mechanical cues to cells without the addition of immobilized or solubilized bioactive molecules. Moreover, collagen, GAGs, and bioceramics can be blended into composites using additional synthetic polymers and/or other raw materials based on the desired scaffold properties. Kruger *et al*.[34] characterized the ability of type I collagen to mineralize in comparison to PLGA when seeded with human mesenchymal stem cells (hMSCs) subjected to osteogenic media. Collagen scaffolds mineralized within 8 weeks of culture, while PLGA scaffolds displayed mineralization after 12 weeks.[34] Time differences were ultimately attributed to degradation of PLGA, which ultimately changed the matrix rigidity, porosity, scaffold architecture, and pH balance that can disrupt cell signaling in the local microenvironment. These results highlight an important distinction between bioresorbable and biodegradable tissue engineering constructs. Bioresorbable scaffold materials are generally raw materials that the body is able to recognize and incorporate into surrounding tissue. However, biodegradable scaffolds tend to break down in the body over time, creating alterations in the local microenvironment and microstructure of the scaffold that may adversely affect cell-biomaterial interactions. Arguably, the ability of a scaffold to integrate into surrounding tissue is one of the most crucial interactions that governs the success of the implanted construct.[172] While both synthetic polymers and the aforementioned raw materials possess distinct strengths and weaknesses, bioresorbability of scaffolds *in vivo* is certainly a crucial aspect of scaffold fabrication and development.

Additionally, selection of the most appropriate raw materials for the target tissue remains another important, yet controversial issue. While most researchers tend to utilize raw materials that are present in the native ECM, cartilage tissue engineering solutions tend to conflict between the selection of type I versus type II collagen (Table 3). Several raw material approaches utilize collagen type I to regenerate articular cartilage,[26, 28, 31, 33, 37, 45, 53, 56, 78] *despite the well known fact that the collagen of hyaline cartilage is predominately type II rather than type I*. Studies by Berendsen *et al*.[36] and Ng *et al*.[32] attempted to address this raw material debate. Berendesen *et al*.[36] found that chondrocyte-mediated contraction occurred only on collagen type I gels but not on collagen type II gels, allowing chondroctyes to maintain their phenotype on collagen type II gels, which confirmed by relative gene expression of matrix proteins and matrix metalloproteinases. Contraction seemed to be a contributing factor to the dedifferentiation of chondrocytes in the case of collagen type I gels.[36] The authors acknowledged that their results pointed toward collagen type II as the material of choice for cartilage tissue engineering, however, whether this outcome occurred because type II collagen presented a superior cell-biomaterial response or a catabolic response of cells to reorganize and produce their own collagen type II has yet to be determined.[36] However, raw material strategies using collagen type II to mimic the native ECM have been employed by other groups with similar success.[22, 53, 61] Contrasting data were obtained in a study by Ng *et al*.[32] where no difference was found between the effects of collagen type I and type II gels on mesenchymal stem cell proliferation and contraction. It is important to note, however, that differences in cell

type, seeding density, seeding technique, and crosslinking method could all contribute to the discrepancy between these studies. An additional study examining two-dimensional culture of chondrocytes on collagen type II versus aggrecan-coated polystyrene found that aggrecan coated surfaces best retained chondrogenic phenotype over four passages and collagen type II surfaces tended to induce loss of chondrogenic phenotype.[173] Logically, collagen type II would appear as the raw material of choice for articular cartilage scaffolds, but future studies examining both collagen type II and aggrecan will be necessary to confirm the most appropriate chondroinductive raw material for cartilage applications.

A similar debate exists in the bone tissue engineering community involving the choice between HAp and β-TCP as scaffold components. Rojbani *et al*.[174] examined the differences in osteoconductivity of HAp and β-TCP microparticles. The particles were loaded into calvarial defects in rats and supplemented with and without simvastatin. Results concluded that β-TCP proved to be a superior osteoconductive scaffold, resulting in greater bone formation compared to HAp, and the addition of simvastatin tended to increase bone regeneration in both of the bioceramic scaffolds. The authors attributed the success of β-TCP to faster degradation, which allows for a synchronized equilibrium between particle degradation and new bone formation.[174] No composite scaffolds incorporating either material were used in this study, however, investigation of hybrid materials containing both HAp and β-TCP would be needed to resolve the conflicting strategies of these materials, since these ceramics are not frequently used as sole scaffold components. In addition, exploration of each materials'

osteoconductive potential in a nanoparticle format would also be necessary. Ultimately, the ability of β-TCP to resorb much more quickly than HAp can provide an appealing solution for hastened bone in-growth.

Finally, trends in FDA approved tissue engineering scaffolds suggest that many areas of tissue engineering have failed to conquer the translational barrier from laboratory benches to clinical solutions. Healon® and Synvisc® are examples of HA formulations used clinically for ophthalmologic and orthopedic applications, respectively.[73] Human allograft DBM products, such as Allomatrix®,[170] DBX®,[170] Puros®,[175] and Grafton®[171] are also commercially available. Healos FX®, Collapat II®, and Biostite® are collagen type I-based medical products used clinically for various applications.[136] However, most attribute the failure of many other tissue engineering strategies to lie in the distinction between medical devices and combination products, respectively.[176, 177] Combination products often employ the use of biologics—cells, drugs, or growth factors—and must be proven in animal studies and a series of three clinical trials, likely spanning over 8 years before approval.[177-179] Medical devices do not contain biologics and can often be classified as a Class II device or under 510k approval (depending on application), alleviating the need for the three phases of clinical trials.[176, 178] Raw materials such as collagen, SIS, and human DBM contain endogenous growth factors and adhesive cues to aid in signaling, without the addition of biologics to the scaffold. Therefore, raw materials could provide a method for translating effective tissue engineering scaffolds to the clinic without all of the additional associated cost and time associated with combination products.

In summary, raw materials present a crucial subset of biomaterials for tissue engineering scaffolds. It is no coincidence that industry has already been using raw materials such as hyaluronic acid, collagen, and DBM in their regenerative medicine products. Quite simply, industry employs these materials because they produce results, although academia may be able to contribute more sophisticated and more effective designs by being more in tune to this classification of materials in our design strategies. Collagen, GAGs, and bioceramics can modulate cell-biomaterial interactions and provide building blocks to give tissues a jump start in the regeneration process. Many strategies have incorporated raw materials in constructs with exact ratios of these components in native tissue. However, a much larger subset of tissue engineering approaches rely on the tunability and predictability of synthetic polymer scaffolds. Studies suggest that composite materials may be the best method for combining both schools of thought. In the ongoing quest to find "perfect" tissue engineering scaffolds, it is essential that researchers look to the composition and structure of native tissue for material selection and design inspiration.

CHAPTER 3: Decellularized cartilage as a chondroinductive material for cartilage tissue engineering

CHAPTER PURPOSE:

The purpose of this chapter was to determine the feasibility of decellularized cartilage (DCC) as a raw material for cartilage regeneration applications. This consisted of 3 study phases:

- Decellularization
- Material Characterization
- Cell Response

At each phase, methods and results are detailed in the following sections. This chapter is written in the format of a manuscript for the possibility of future submission. A preliminary conclusion long with future recommendations are also given.

3.1 ABSTRACT

Components found within the extracellular matrix (ECM) have emerged as an essential subset of biomaterials for tissue engineering scaffolds. In the present study, decellularized cartilage (DCC) fragments were developed to be utilized as a raw material scaffold or scaffold component. Bovine articular cartilage was decellularized and examined for removal of cellular material and preservation of native glycosaminoglycans (GAGs). 99% of cells were removed, while over 87% of native GAGs remained following decellularization. Further processing to produce a fine

powder of DCC and characterize particle size and chemical composition were performed. Transmission electron microscopy (TEM) revealed a dry DCC particle size of 25-30 nm and only minor composition discrepancies existed between native and DCC specimens as confirmed by Fourier transform infrared spectroscopy (FTIR). A pellet culture study was used to examine gene expression in rat bone marrow stem cells (rBMSCs). Gene expression analysis of chondrogenic markers showed a two-fold increase in collagen type II expression and a three-fold increase in Sox9 expression in pellets supplemented with DCC powder versus control (blank) cell pellets. An encapsulation study revealed that supplementing rBMSC-encapsulated agarose hydrogels with 10 mg DCC/mL or 100 mg DCC/mL agarose helped hydrogels maintain similar DNA content to initial values (week 0) over a 3-week culture period, while gels containing no DCC lost over half of their initial DNA content over the same culture period. Overall, DCC may be a new chondroinductive material that can provide microenvironmental cues and signaling to promote stem cell differentiation in cartilage regeneration. This study is the first of its kind to assess stem cell gene expression in response to DCC, grind DCC into a nanopowder to allow incorporation of this raw material into many different scaffold formulations, and perform complete cell removal (>97%), while retaining close to native GAG concentrations.

3.2 INTRODUCTION

Osteoarthritis is expected to affect over 100 million adults in the United States by the year 2020 creating an economic burden of over $100 billion on the healthcare

system.[180] Cartilage defects, whether caused by osteoarthritis, joint trauma, or other disease, have provoked a wide variety of tissue engineering scaffold strategies in recent years. Traditionally, cartilage tissue engineering scaffolds have utilized synthetic polymer components to form hydrogels or other porous matrices. Poly(ethylene glycol) diacrylate (PEG-DA) and poly(D,L-lactic-co-glycolic acid) (PLGA) are some of the most common synthetic polymers used in cartilage regeneration scaffolds.[168] However, the main disadvantage of synthetic biomaterials for cartilage tissue engineering is their inherent lack of bioactive signaling molecules or microenvironmental cues to direct stem cell differentiation.

Collagen, glycosaminoglycans (GAGs), and other ECM-based matrices are key examples of ''raw materials'' that can be incorporated into scaffolds for a wide variety of applications. For cartilage tissue engineering, the most widely used raw material building blocks for scaffolds include collagen type I,[26, 28, 31-33, 36, 59, 67, 86] collagen type II,[22, 60-62] hyaluronic acid,[66, 76, 77, 90] and chondroitin sulfate.[50, 53, 54, 56, 97] Cartilage ECM is made up of approximately 70% water (w/w), 20% collagen (types II, IX and XI), and 10% aggrecan. [181] The ECM-rich nature of hyaline cartilage – volume ratio of 95% matrix to 5% chondrocytes – makes it an ideal candidate for decellularization.[182]

Utilizing decellularized cartilage as an ECM-based raw material, however, has scarcely been investigated in the literature. In theory, acellular hyaline cartilage would be expected to provide a scaffold rich in collagen type II, aggrecan, and endogenous growth factors following the decellularization process. Some groups have attempted to decellularize cartilage as an intact tissue block,[165, 166] while others have shattered or

cut thin slices of the tissue to allow for better penetration of solutions during the decellularization procedure.[167-169, 182] The majority of studies have investigated chondrocyte viability and gene expression in response to decellularized cartilage tissue with the goal of maintaining chondrogenic phenotype.[182, 183]

The goal of the present study was to investigate the development of decellularized bovine hyaline cartilage as a potential chondroinductive raw material, both alone and as a scaffold component. As mentioned previously, current work in the literature has only examined the use of DCC to help main chondrogenic phenotype of chondroctyes; however, this study aimed to examine preliminary chondroinductivity of DCC, i.e., the ability of DCC to provide a microenvironment that would induce chondrogenic differentiation of bone marrow stem cells. A previous study by Cheng *et al*.[184] examined the potential of porcine articular cartilage to induce chondrogenesis in adipose-derived stem cells using gene expression analysis. The distinction between this work and the present study lies in the decellularization process. This group minced cartilage into fragments and lyophilized immediately to form a porous scaffold – no decellularization techniques were performed and no confirmation of cellular removal was addressed.[184] Another study used human articular cartilage and examined chondrogenic induction of bone marrow stem cells.[168] However, this group used immunohistochemistry to stain for chondrogenic markers and did not quantitatively evaluate gene expression.[168] In addition, growth factors (transforming growth factor beta-1 (TGF-β1) and basic fibroblast growth factor (bFGF)) were used to influence chondrogenic differentiation in this study.[168]

In the present work, however, tissue decellularization was performed and confirmed prior to use as scaffolds. Bovine articular cartilage was harvested and decellularized and examined for DNA content, GAG content, chemical composition before/after decellularization, and particle size following grinding into a fine powder. DCC was then incorporated into agarose hydrogels along with rat bone marrow stem cells (rBMSCs) to examine cell proliferation. Then, the DCC tissue pieces were placed in pellet culture to determine their influence on rBMSC gene expression. No growth factors were used in culture medium this work to determine the influence of DCC alone as a chondroinductive material. To the best of our knowledge, this is the first time that stem cell response to DCC, in terms of gene expression response to decellularized hyaline cartilage tissue, has been characterized.

3.3 MATERIALS AND METHODS

3.3.1 Tissue preparation

Fresh bovine knee joints were obtained from Bichelmeyer Meats (Kansas City, MO) following sacrifice. Articular cartilage was excised, washed with phosphate buffered saline, processed into fragments using liquid nitrogen cryofracturing, and cryopreserved for storage until use. Cryopreservation was achieved by freezing the tissue in a cryoprotectant (RPMI with 10% FBS and 10% DMSO) at 1°C per minute using a controlled rate freezer (2100 Series, CustomBioGenics Systems, Romeo, MI) and stored at -180°C.

Previously cryopreserved cartilage fragments were thawed, packaged into dialysis tubing, and subjected to a reciprocating osmotic shock and multi-detergent and enzymatic washout protocol to remove cellular material as described previously.[185] Briefly, cartilage fragments were first treated in hypertonic salt solution (HSS) for two hours at 21°C under gentle shaking, followed by treatment with the non-ionic detergent Triton X 100 (TX100; 0.05% v/v; Sigma-Aldrich) in hypotonic, deionized, sterilized water for three hours. The tissue was rinsed with deionized water for ten minutes and subjected to a second round of treatment with HSS and TX100. The cartilage fragments were then treated in a Benzonase® solution (0.0625 KU ml^{-1}; Sigma-Aldrich) overnight at 37°C under constant shaking (220 rpm) to digest nuclear material. Following Benzonase® treatment, cartilage fragments were rinsed in deionized water and further treated with the anionic detergent sodium-lauroyl sarcosine (NaLS; 1.0% v/v; Sigma-Aldrich) for 24 hours at 21°C under constant shaking (220 rpm). At the conclusion of the NaLS treatment, cartilage fragments were rinsed and treated with ethanol (40% v/v, Sigma-Aldrich) under gentle agitation. Extraction of organic solvents was then performed using ion exchange resins (Amberlite, Sigma-Aldrich; Mixed Bead Resin TMD-8, Sigma-Aldrich; Dowex Monosphere, Supleco). The total duration of the decellularization process was 72 hours. Following decellularization, DCC was cryopreserved using the methods described previously and returned to cryostorage for later testing or processing.

In preparation for grinding, cryopreserved DCC fragments were thawed and subjected to five rinse cycles with deionized water to remove cryopreservation

medium. Tissue fragments were then lyophilized for 24 hours and then ground into a fine powder using a SPEX Freezer/Mill 6770 (SPEX Sample Prep, Metuchen, NJ) that was specifically designed for cryogenic grinding and pulverizing of tough and/or temperature sensitive samples, while immersed in liquid nitrogen. Conditions (impactor rate, grinding length, number of grinding cycles) were selected by preliminary studies to obtain a homogenous powder without burning the tissue by overgrinding. The resulting fine powder was removed and kept in the freezer at -20°C until further characterization or use. Prior to use for cell studies, DCC powder was sterilized by an Anprolene ethylene oxide sterilizer, using a 12-hour cycle (Andersen Products, Haw River, NC).

3.3.2 Biochemical analysis

Double stranded DNA (dsDNA) was quantified fluorimeterically using the Quanti-iT High Sensitivity dsDNA Assay Kit (Molecular Probes Inc., Eugene, OR) as described previously.[185] Briefly, fresh, cryopreserved, and decellularized tissue was digested using a ratio of 25 milligrams of tissue to 20 microliters of proteinase K. Complete digestion occurred after heating the tissue and solution at 56°C in a water bath overnight, with periodic vortexing. Total DNA purification was performed using the DNeasy Blood and Tissue Kit (DNeasy, Qiagen Inc., Valencia, CA) spin-column technique in accordance with the manufacturer's recommended protocol. Purified samples were treated with the Quanti-iT dsDNA Assay Kit according to the protocol recommended by the manufacturer to selectively bind dsDNA with a fluorescent dye.

Native and cryopreserved samples were prepared for the assay by making a diluted supernatant of 10 µl of sample with 190 µl of supplied buffer. Decellularized samples were not diluted. Solutions were analyzed in triplicate using a fluorescence microplate reader (ThermoFisher) at 486/528 nm excitation/emission wavelengths. This dsDNA analysis was performed for bovine cartilage tissue in fresh, cryopreserved, and decellularized states (n = 5). DNA content is reported as micrograms of DNA per milligram of hydrated tissue.

Sulfated GAG content was quantified colorimetrically using a commercially available sulfated GAG assay (Blyscan Sulfated Glycosaminoglycan Assay, Biocolor Ltd., UK). First, 50 milligrams of hydrated cartilage tissue was digested by adding 1.5 mL of papain solution comprising of 125 µg/mL papain (from papaya latex, Sigma), 5 mM N-acetyl cysteine, 5 mM ethylenediaminetetraacetic acid (EDTA), and 100 mM potassium phosphate buffer in ddH_2O (20mM monobasic potassium phosphate, 79 mM dibasic potassium phosphate in ddH_2O). The cartilage tissue was placed in microcentrifuge tubes containing papain digestion solution and left overnight at 60°C and then placed in the freezer at -20°C until future biochemical analysis. GAG content was then measured using a dimethylmethylene blue (DMMB) sulfated GAG assay as recommended by the vendor and was performed on fresh, cryopreserved, and decellularized cartilage specimens (n = 5). GAG content is reported in micrograms of GAG per milligram of hydrated tissue.

3.3.3 Transmission electron microscopy (TEM)

DCC dry powder samples (lyophilized for 24 hours prior) were prepared by suspending the cartilage powder in ethanol and agitating in an ultrasonic bath for 15 minutes. 10 mL of DCC sample in ethanol was placed onto copper mesh grid with lacey carbon film. The wet grids were allowed to air-dry for several minutes prior to being examined under TEM. The particle size and morphology were examined by bright-field and dark-TEM using an FEI Technai G2 transmission electron microscope at an electron acceleration voltage of 200 kV. High resolution images were captured using a standardized, normative electron dose and a constant defocus value from the carbon-coated surfaces.

3.3.4 Fourier transform infrared spectroscopy (FTIR)

Native and decellularized bovine cartilage were ground into fine powders as described previously. Separately, each of the powders was mixed with potassium bromide (KBr) and pressed into very thin tablets. Infrared spectra of each were obtained by using Perkin-Elmer Spectrum 400 Fourier transform infrared spectrophotometer (Perkin-Elmer, Waltham, MA) in transmission mode.

3.3.5 Swelling

Decellularized cartilage was dried for 24 hours by lyophilization following grinding as described previously. Dry DCC powder was weighed and placed into 2 mL microcentrifuge tubes and an initial volume measurement was taken. 1 mL of

phosphate buffered saline (PBS) was added to each tube and the powder was given 24 hours to swell. The volume and mass of the cartilage powder was then recorded and used to calculate an S-ratio and a V-ratio. The S-ratio was defined as the mass of the swelled tissue minus the mass of the dry tissue, divided by the mass of the dry tissue ($(M_{swell} - M_{dry})/M_{dry}$). The V-ratio was defined as the volume of the swelled tissue minus the volume of the dry tissue, divided by the volume of the dry tissue ($(V_{swell} – V_{dry})/V_{dry}$). Three trials were performed to quantify the swelling of the DCC dry powder.

3.3.6 Cell harvest, expansion, pellet culture, and encapsulation

Rat bone marrow stem cells (rBMSCs) were obtained from the femurs of male Sprague-Dawley rats (150-200 g, Charles River Laboratories) following a University of Kansas approved IACUC protocol (#175-08). Briefly, all rats were euthanized by exposure to CO_2 for five minutes with a death confirmation thoracotomy followed by removal of the leg bones. The femur was then separated from the tibia under sterile conditions and all excess muscle was removed. The marrow cavity was then flushed out of the femur using a syringe filled with 1% Antibiotic-Antifungal/PBS solution. All cells obtained were plated for expansion in monolayer and incubated at 37°C. The culture medium for rBMSCs was composed of Alpha MEM, 1% Penicillin-Streptomycin, 10% qualified fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA) and was changed every two days. At 80-90% confluence, cells were passaged and plated at 40,000 cells/cm^2. Cells were expanded to passage 4 (P4) to be used for pellet culture and encapsulation studies.

For formation into pellets, P4 cells were counted and placed into 15 mL centrifuge tubes at a density of 2 million cells/tube and centrifuged to create a cell pellet in the bottom of the tube for culture. Control samples were defined as an rBMSC pellet with no added DCC powder (-DCC), while the other experimental group was two million cells pelleted with 5 milligrams of DCC powder (+DCC). Each 15 mL tube was placed inside a 75 cm^2 flask to maintain air flow and sterile culture conditions in the incubator at 37°C. One mL of standard chondrogenic medium was placed in each tube. Medium consisted of high glucose DMEM, 1% Penicillin-Streptomyocin, 1X Insulin-Transferrin-Selenium (ITS)-premix, 100 µM sodium pyruvate, 1% non-essential amino acids (all listed previous from Invitrogen Life Technologies, Carlsbad, CA), 40 µg/mL L-proline (Sigma), 50 µg/mL L-ascorbic acid (Sigma), 100 µM dexamethasone (Sigma). Cell culture medium was changed on days three and six of the seven-day culture period.

Cell encapsulation in agarose hydrogels was performed as described in previous studies with slight modifications.[186, 187] First, a 3% w/v solution of agarose in PBS was prepared and autoclaved for 30 minutes. Meanwhile, rBMSCs were trypsinized to P4 and counted. After removal from autoclave and cooling in a sterile environment to 39°C, the cell suspension was added to the agarose solution in a 1:2 ratio to produce a 2% agarose solution with a seeding density of 10 million cells/mL. For the control group, no DCC powder was added to the agarose solution. For additional experimental groups, 10 mg DCC/mL agarose solution, 50 mg DCC/mL agarose solution, and 100 mg/mL agarose solution were added to make a total of four

experimental groups. For each group, agarose solution was pipetted into sterilized silicone rubber molds (~5mm diameter, ~2mm height, volume = 0.04 mL per gel), pressed between two glass slides, and cooled at 4°C for 10 minutes. The cell-encapsulated gels were then added to untreated 24-well plates (Becton Dickenson; Franklin Lakes, NJ), supplied with 2 mL standard chondrogenic medium (as described previously), and placed in a sterile 37°C incubator. Medium was changed every 48 hours for the 3-week duration of the study.

3.3.7 Quantitative polymerase chain reaction

In preparation for RT-PCR, pellet samples (n = 3) at day 1 and day 7 were first preserved in RNALater solution (Qiagen, Valencia, CA) and the RNA was isolated according to the manufacturer's guidelines using a RNeasy kit (Qiagen). Isolated RNA was converted to cDNA using a TaqMan High Capacity kit (Applied Biosystems, Foster City, CA) in an Eppendorf Realplex ThermoCycler. TaqMan Gene expression assays from Applied Biosystems for rat collagen type I (COL1A1, Rn01463848_m1), collagen type II (COL2A1, Rn01637087_m1), SRY-box 9 (Sox9, H Rn01751069_mH), aggrecan (Acan, Rn00573424_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Rn01775763_g1) were used in conjunction with an Eppendorf Realplex Real-time PCR System (Eppendorf, Happuage, NY). The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative level of expression for each gene as reported previously.[188] For quantification, one control sample without DCC (-DCC) at day 1 was designated as a calibrator, and GAPDH expression was used as an endogenous control.

3.3.8 Picogreen assay

Cell-encapsulated hydrogels from each group (n = 3) were harvested at week 0 (24 hours after encapsulation), week 1, and week 3. Hydrogels were homogenized and digested in a papain solution as described previously. DNA content was quantified using a Picogreen assay (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Based on previous studies, a conversion factor of 8.5 pg DNA/cell was used to calculate cell number for bone marrow stem cells.[189]

3.3.9 Statistical analyses

Statistical analyses were performed using a single factor analysis of variance (ANOVA) in IBM SPSS 22.0 software (SPSS, Inc., Chicago, IL), followed by a Tukey's Honestly Significant Difference *post hoc* test when significance was detected below the $p = 0.05$ value. All quantitative results (numerical values and figures) were expressed as the average ± standard deviation.

3.4 RESULTS

3.4.1 DNA content

DNA content (Fig. 2) was significantly reduced ($p < 0.005$) between fresh and decelled tissue (n = 5). The overall reduction of dsDNA content from fresh to decellularized was 98.9%.

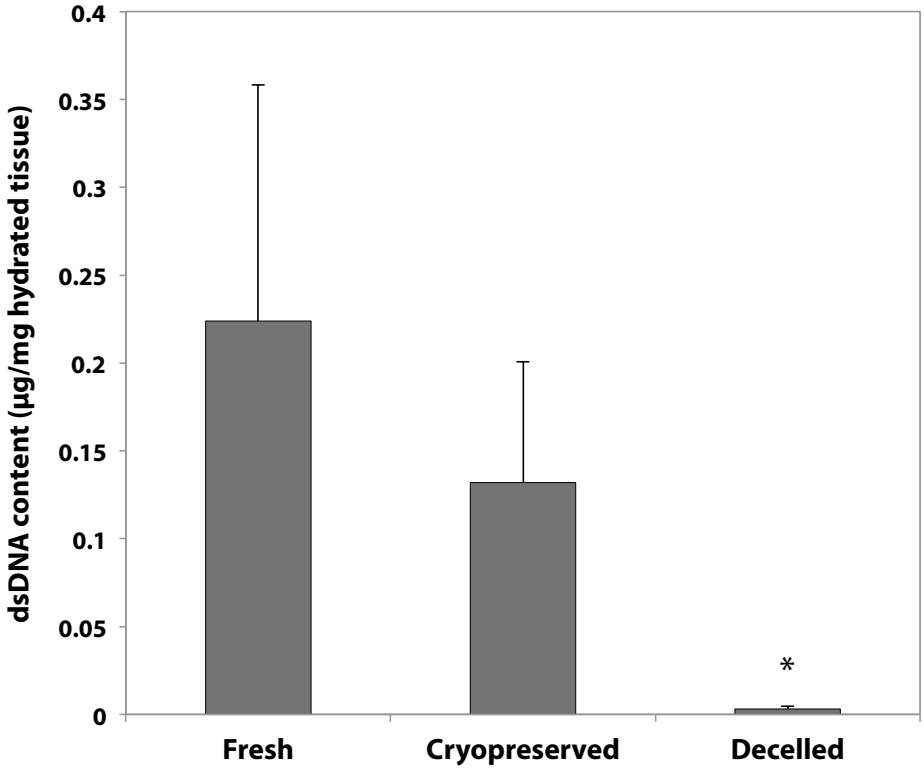

Figure 2. Double-stranded DNA (dsDNA) content in native, cryopreserved, and decellularized bovine cartilage tissue calculated as micrograms (µg) of dsDNA per milligram (mg) of hydrated tissue. All values are expressed as the average ± standard deviation (n = 5), $p < 0.05$, * = statistically significant difference from the control (fresh).

3.4.2 GAG content

GAG content (Fig. 3) slightly decreased in each stage of tissue processing (n=5) from fresh to cryopreserved to decellularized states. There was a significant reduction in GAG content from fresh to decellularized tissue ($p < 0.01$), although over 87% of native GAGs remained.

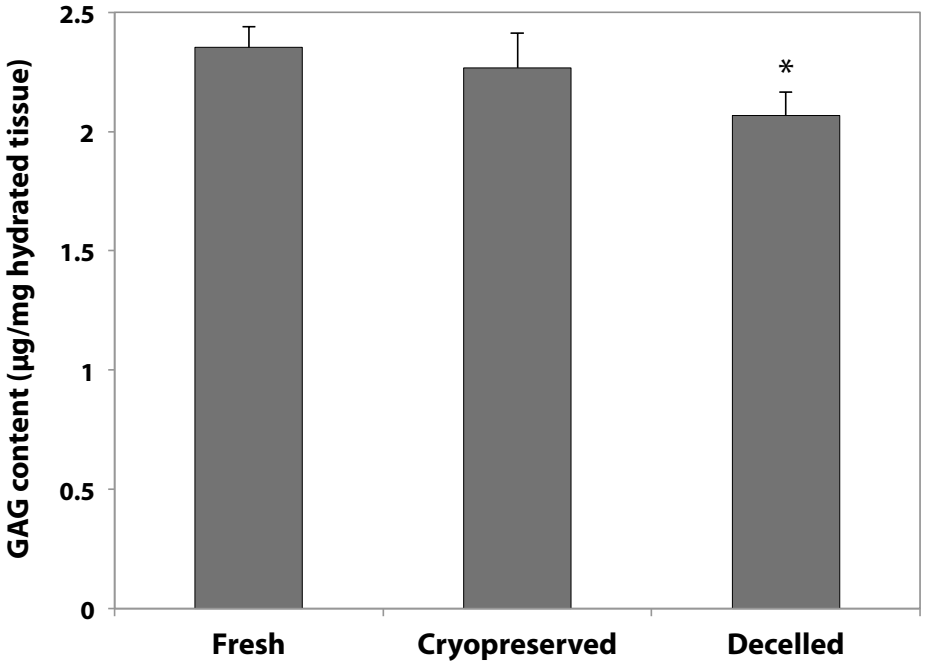

Figure 3. Glycosaminoglycan (GAG) content in native, cryopreserved, and decellularized bovine cartilage tissue calculated as micrograms (µg) of GAG per milligram (mg) of hydrated tissue. All values are expressed as the average ± standard deviation (n = 5), $p < 0.05$, * = statistically significant difference from the control (fresh).

3.4.3 Particle size

TEM revealed a fine and relatively monodisperse dry DCC powder with particle diameter ranging from 20-30 nm in size (Fig. 4 - A). Under higher magnification, DCC particles appear to have a hexagonal shape with slightly rounded edges (Fig. 4 – B).

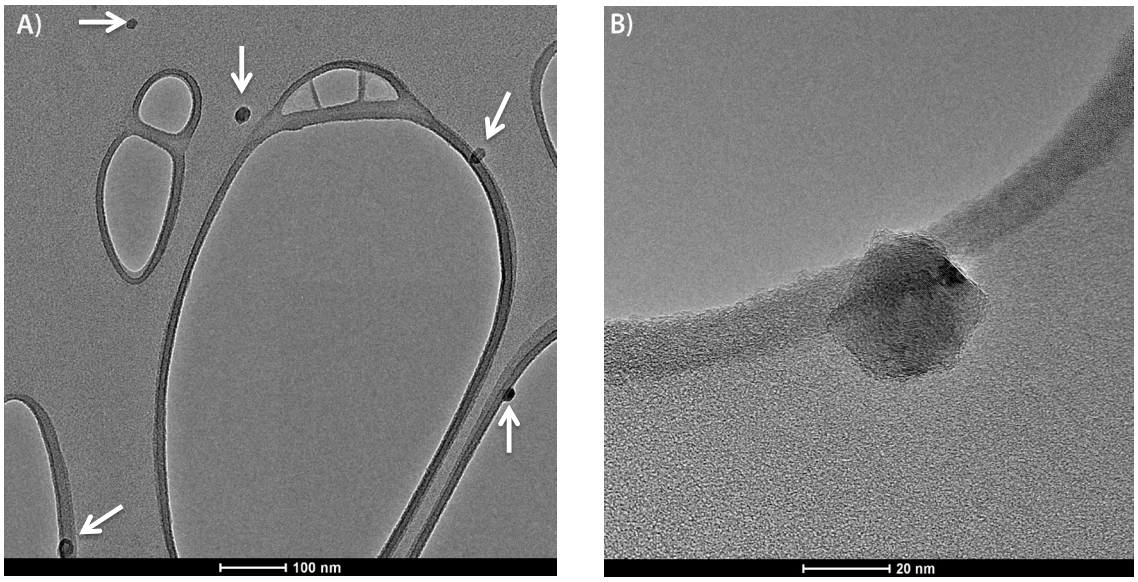

Figure 4. TEM images of DCC powder. White arrows distinguish DCC particles on a lacy carbon film. (A) shows a view of several particles of relatively equal diameters in the 20-30 nm range and (B) shows a zoomed in view of a single particle that is approximately 25 nm in diameter.

3.4.4 Chemical composition

FTIR spectra of native and DCC specimens (Fig. 5) showed nearly identical

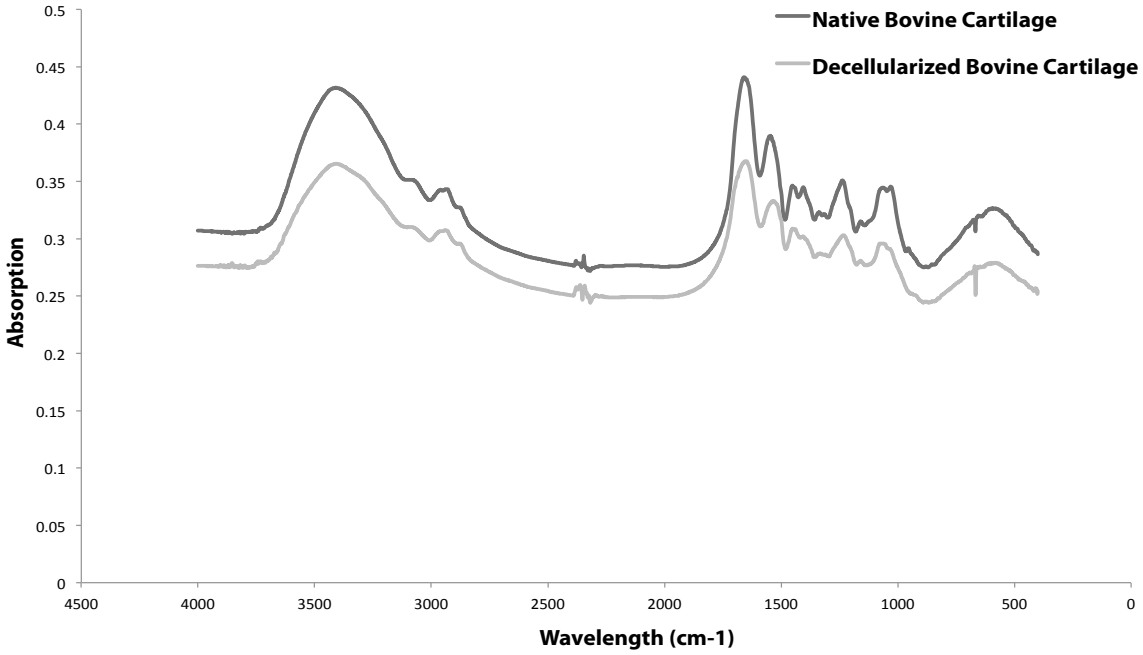

Figure 5. FTIR spectra of native and decellularized bovine cartilage. Both spectra appear to be relatively similar with only minor discrepancies.

signature peaks. Areas where the two samples may differ are noticed at 1405 cm^{-1} and 1029 cm^{-1} where peaks are missing from the DCC spectrum that are apparent in the native cartilage spectrum. Chemical composition appears relatively unchanged.

3.4.5 Swelling

From the swelling results of three trials with DCC dry powder, it is evident that the powder swells approximately 475% (Table 7). Therefore, in a hydrated state, it is reasonable to calculate that DCC particles would swell from 25-30 nm to approximately 150 nm.

Table 7. Swelling data for DCC dry powder.

Trial	Swell Density (mg/mL)	S-Ratio ((Mswell-Mdry)/Mdry))	V-Ratio ((Vswell-Vdry)/Vdry))
1	81.5	1178%	440%
2	79.5	1190%	478%
3	77.0	1253%	500%
Mean	79.3	1207%	473%
SD	2.2	40%	30%

3.4.6 Gene expression

Both the control (-DCC powder) and +DCC pellet groups expressed a small level of collagen type II at day 1 (Fig. 6). At day 7, however, collagen II expression increased significantly in both groups from day 1 and the +DCC pellet group expressed nearly two-fold significantly greater collagen II expression when compared to the control group ($p < 0.002$).

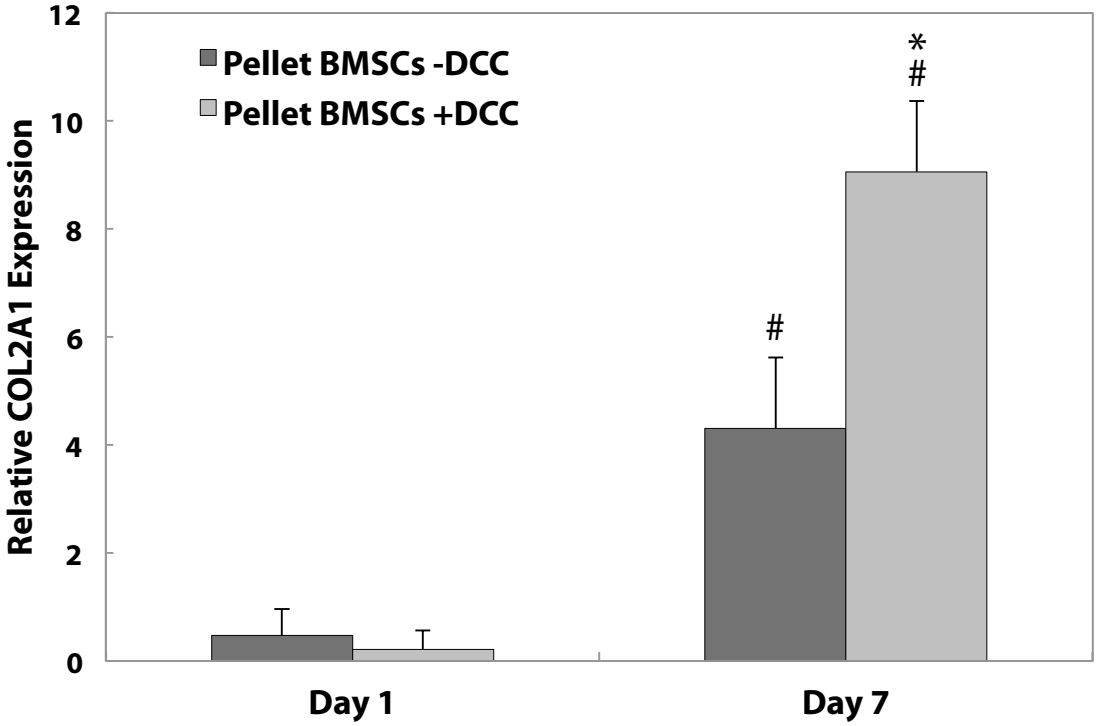

Figure 6. Relative gene expression of collagen type II (COL2A1) for pellet culture samples with (+DCC) and without DCC (-DCC) powder. All values are expressed as the average ± standard deviation (n = 3), $p < 0.05$, # = statistically significant difference from Day 1 value, * = statistically significant difference from the other group at that time point.

Sox9 expression (Fig. 7) was significantly greater in the control group (-DCC) when compared to the +DCC group at day 1 ($p < 0.014$). At day 7, Sox9 expression in the control group significantly decreased by over 66% in comparison to the day one value ($p < 0.008$). Sox9 expression was over three-fold greater in the DCC group when compared to the control at day 7, but this increased expression was not significant ($p = 0.256$).

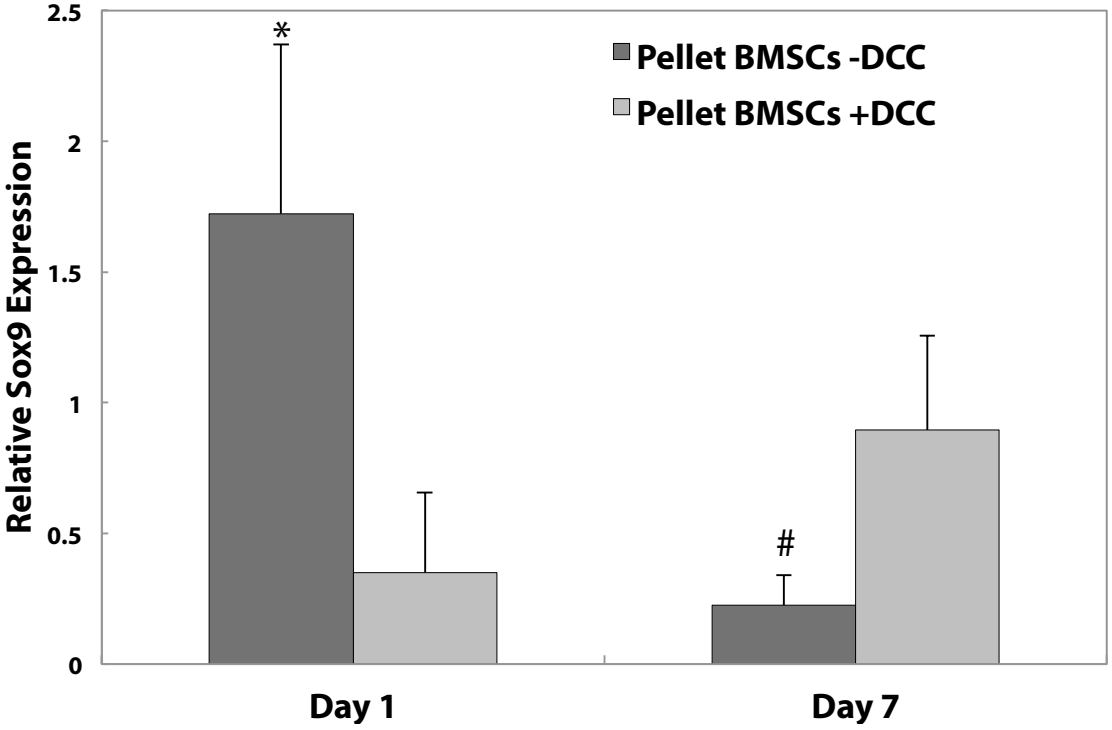

Figure 7. Relative gene expression of Sox9 for pellet culture samples with (+DCC) and without DCC (-DCC) powder. All values are expressed as the average ± standard deviation (n = 3), $p < 0.05$, @ = statistically significant difference from Day 1 value, * = statistically significant difference from the other group at that time point.

No significant differences were observed in collagen type I expression between groups or time points (Fig. 8). On day 1, the +DCC group seemed to have less collagen type I expression than the control ($p = 0.14$). Furthermore, collagen type I expression on day 7 in +DCC samples when compared to –DCC samples appeared slightly less as well ($p = 0.937$). Sizeable standard deviations in each group, however, did not allow for discernable differences between groups.

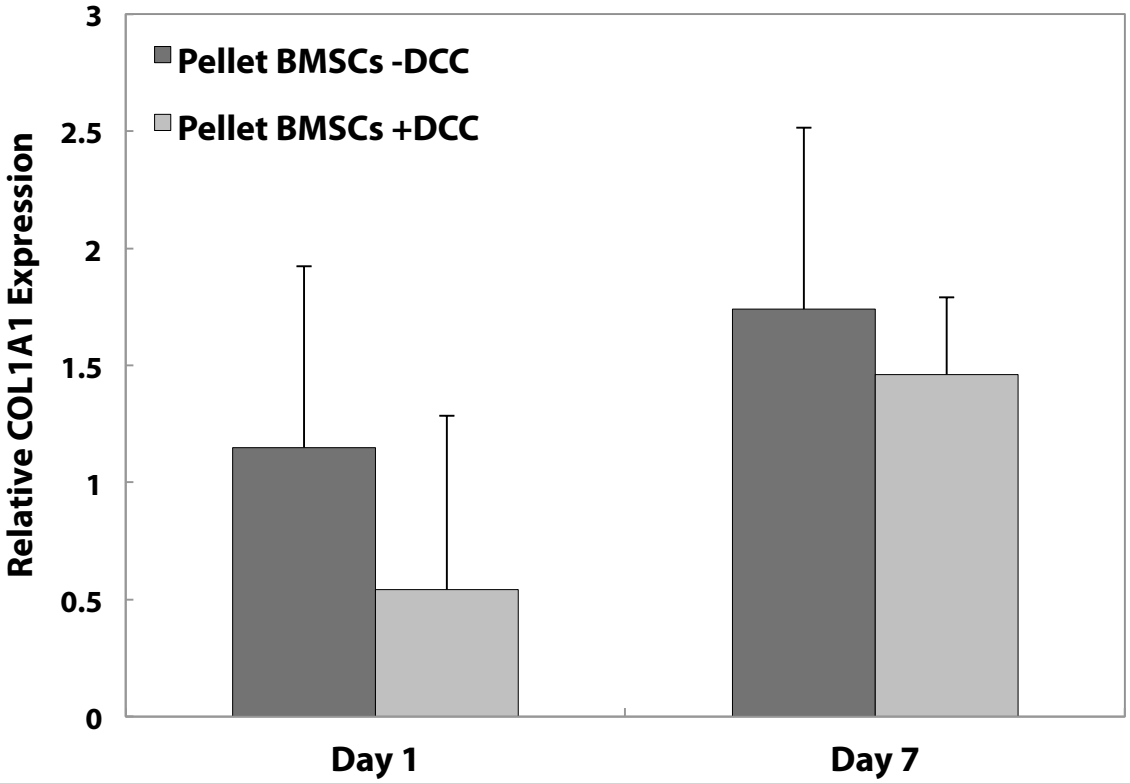

Figure 8. Relative gene expression of collagen type I (COL1A1) for pellet culture samples with (+DCC) and without DCC (-DCC) powder. All values are expressed as the average ± standard deviation (n = 3). There were no statistically significant differences in the expression of collagen type I between control BMSCs and BMSCs with DCC powder.

Like collagen type I, aggrecan expression (Acan) was not significant in either group or time point (Fig. 9). Aggrecan expression appeared be slightly greater in the +DCC group at both time points, however, the sizeable standard deviation each of these make it difficult to conclude with any certainty. The *p*-values comparing aggrecan expression of +DCC and –DCC groups were $p = 0.302$ and $p = 0.773$, for day 1 and day 7 respectively.

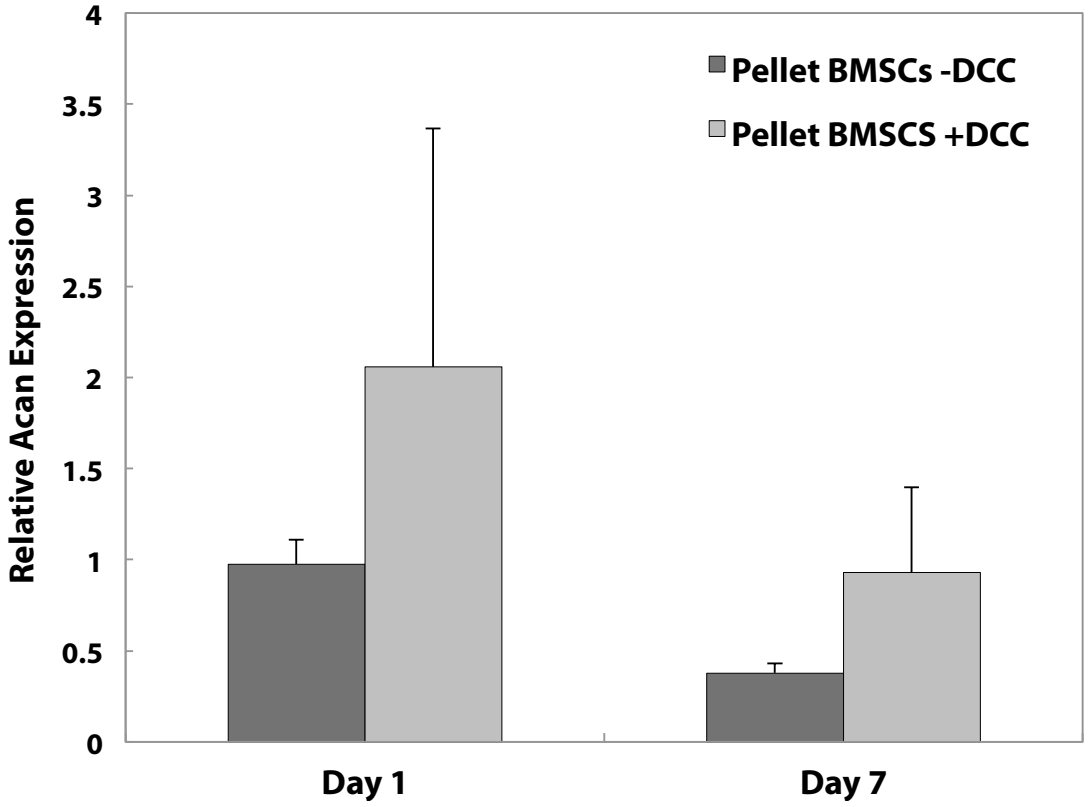

Figure 9. Relative gene expression of aggrecan (Acan) for pellet culture samples with (+DCC) and without DCC (-DCC) powder. All values are expressed as the average ± standard deviation (n = 3). There were no statistically significant differences in the expression of Acan between control BMSCs and BMSCs with DCC powder.

3.4.7 DNA content of cell-encapsulated hydrogels

At week 0 (24 hours following cell encapsulation), all groups had no significant differences in DNA content (Fig. 10). At week 1, all groups decreased in DNA content from week 0 but these changes were not significant. At week 3, the blank group (no DCC powder) significantly decreased in comparison to its week 0 value ($p < 0.025$). From week 1 to week 3, the 10 mg/mL DCC and 100 mg/mL DCC groups rebounded to near initial (week 0) values. The 50 mg/mL DCC group appeared to have degraded

more quickly then other groups and fell apart by week 3, which led to this group having the lowest DNA content at this timepoint.

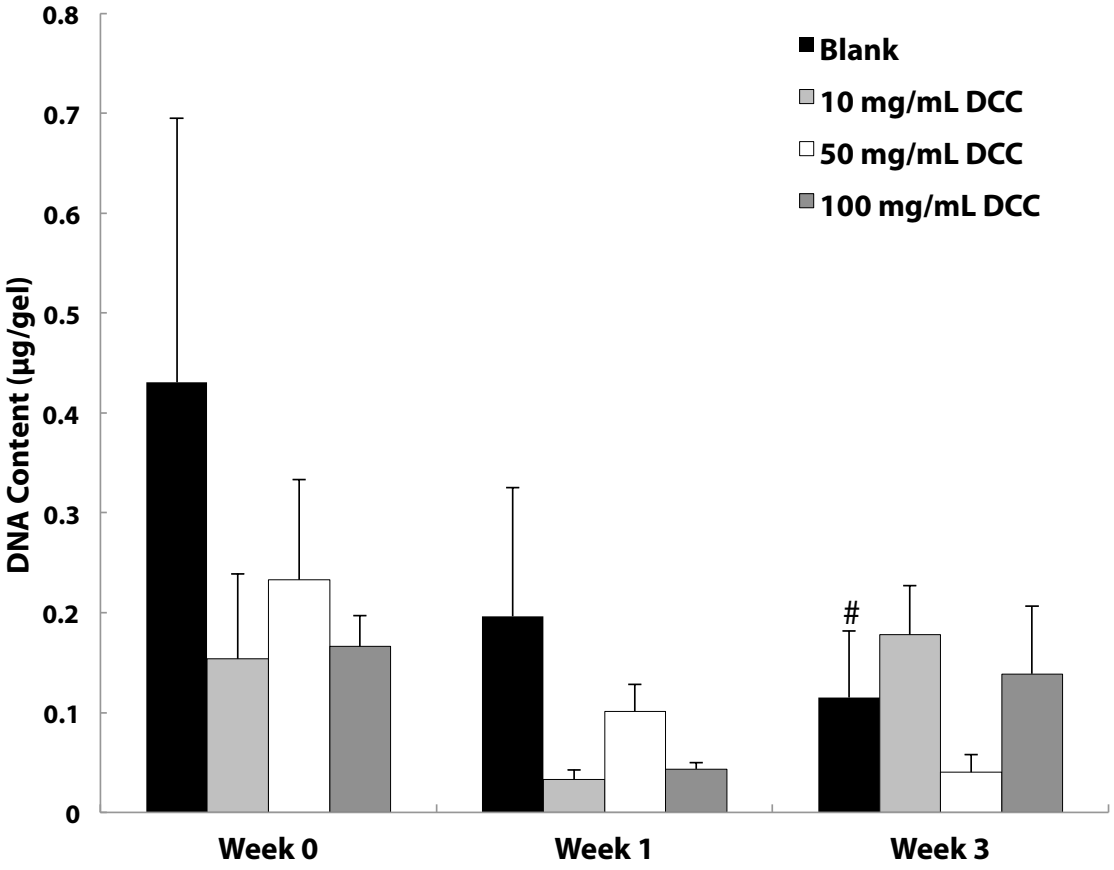

Figure 10. DNA content for groups at 0, 1, and 3 weeks. Values are reported as mean ± standard deviation (n = 3), $p < 0.05$, # = statistically significant change from week 0 value.

3.5 DISCUSSION

The primary objective of this study was to evaluate the potential of DCC as a raw material scaffold component for cartilage tissue engineering applications and to initially test its ability to induce chondrogenesis in bone marrow stem cells. To the best of our knowledge, this was the first effort to characterize stem cell response to

DCC through quantification of gene expression of chondrogenic markers (COL2A1, Sox9, Acan).

First, bovine articular cartilage was decellularized in tissue fragments and characterized for DNA content and GAG content in fresh, cryopreserved, and decellularized states. DNA content was reduced by 98.9% from fresh to decellularized states, while preserving over 87% of native GAG content. To the best of our knowledge, this is the greatest ratio of DNA reduction – GAG retention achieved by any decellularized cartilage study in the literature to date. The use of a highly-developed decellularization protocol utilizing advanced surfactants may be one reason for this high ratio. Additionally, tissue cryopreservation as close to euthanasia of the animal as possible may also contribute to the overall decellularization outcome.

A challenge in this study was finding a method for processing DCC fragments into a fine and more homogenous powder post-decellularization. The tough and gummy nature of cartilage tissue made it extremely difficult to find an apparatus for this procedure. After attempting several grinding options that ranged from homogenizers to dry ice grinders, the selection of a liquid nitrogen based mill and optimization of parameters for the SPEX Freezer/Mill produced a fine and homogenous powder that was confirmed to have a dry particle size in the range of 20-30 nm by TEM. Production of an ECM-based nanopowder is extremely rare in the literature; to the best of our knowledge, ECM-based nanopowder has never been reported with hyaline cartilage. The smallest particles of decellularized cartilage reported in the literature have been 2 μm “cartilage dust” by Ghanavi and

colleagues.[182]

Another factor that must be considered when examining particle size is swelling. Nearly all DCC studies in the literature have overlooked this parameter; however, the nature of cartilage tissue and its constituent highly negatively charged proteoglycans make DCC susceptible to sizeable swelling. In our experimental observations, DCC dry powder tends to swell about 4.75 times in volume in biological medium. This would translate to the DCC hydrated nanopowder being approximately 100 - 150 nm. Using similar logic, other DCC studies, such as the "cartilage dust" mentioned previously, would have demonstrated results with a hydrated particle size of about 10 μm. While it is not well understood which particle size may be more advantageous for cartilage tissue engineering scaffolds, it seems apparent that the nanopowder would be able to be incorporated into many different scaffold formulations – hydrogels, microparticles, colloidal systems – more readily than a micropowder.

One concern about this degree of tissue decellularization and tissue processing was the potential for chemical composition changes to occur. Transmission FTIR, however, confirmed only slight differences in the spectra of native and DCC tissue samples. The missing peaks in the DCC spectrum at 1405 cm^{-1} and 1029 cm^{-1} could represent crystallization in the tissue due to freeze-thaw cycles that are apparent in the steps from fresh cartilage tissue harvest to DCC nanopowder. It is not apparent that these wavelengths correspond directly to any well-known functional groups; however, higher resolution techniques may be helpful in making a more defined

determination.

Response of rBMSCs, quantified by gene expression, was a main focus of this study following the material development and characterization of DCC powder. While prior work in the literature has concentrated on maintenance of the phenotype of chondrocytes, this work centered on the ability of DCC to induce chondrogenesis by selecting chondrogenic markers (collagen type II, Sox9, and aggrecan) to quantify their expression in rBMSCs cultured in standard chondrogenic medium with and without DCC powder. It is important to note that no growth factors were added to this medium. Overall, the rBMSCs cultured with DCC powder appeared to have greater chondrogenic expression of collagen II and Sox9 markers by two-fold and three-fold, respectively. The gene expression results of aggrecan, the main proteoglycan in hyaline cartilage, appeared statistically inconclusive. Likewise, collagen type I expression would be expected to be downregulated during chondrogenesis due to its minute presence in hyaline cartilage tissue; however, those results were also statistically inconclusive. Larger sample sizes would be needed and should be a main goal for future work to determine the chondoinductivity of DCC.

Lastly, the study examined cell proliferation in response to DCC powder with rBMSCs encapsulated in an agarose hydrogel. Agarose was hypothesized to be a potential delivery vehicle for DCC in a three-dimensional scaffold. It was evident that gels containing 10 mg DCC/mL and 100 mg DCC/ mL retained DNA content over the 3-week culture period, while DNA content of blank hydrogels decreased significantly over the same period. The hydrogels containing 50mg DCC/mL appeared to degrade

substantially over the 3-week culture period suggesting that loading concentration may need to be examined more intensively in future work. While agarose was selected for its inert nature, it appears that rBMSCs may not fare as well encapsulated in this material as chondrocytes. Previous studies have shown that chondrocytes proliferate in agarose;[187, 190, 191] however, this study suggests that a different delivery method may be more suitable for rBMSC proliferation. Fibrin glue has been well studied and is used to deliver chondrocytes to chondral defects in the ACI procedure clinically.[192] Future studies with DCC should attempt to examine fibrin glue for the delivery of DCC in a thee-dimensional scaffold.

Lastly, we acknowledge that this work was not without limitation. Hyaline cartilage tissue properties and composition can vary greatly from batch to batch based on:

- Harvest time after euthanasia
- Age of the animal
- Species/breed
- Activity level of the animal

Therefore, the results obtained in this study regarding DNA content and GAG content could also vary. Repeatability should be addressed in future studies. Additionally, the assay used for GAG content examines only sulfated GAG content, which leaves out the only non-sulfated GAG, hyaluronic acid (HA). It is not well understood if HA is also preserved in the decellularization process. Sample sizes in this study for cellular response and proliferation were limited due to availability of tissue powder. Future

studies will need to occur with greater samples sizes. Finally, we acknowledge that the immunological response of cells to the DCC nanopowder was not characterized in this study. While skepticism about the biocompatibility of having not only an ECM-based material but also a nanomaterial may occur, future studies should examine this both *in vitro* and in animal models.

Overall, this study lays a foundation for future work developing and characterizing DCC as a chondroinductive material for cartilage tissue engineering. Preliminary gene expression results seem promising as well as the ability of our method to have an unmatched DNA reduction: GAG preservation ratio. In the search to find raw materials that provide both a biological scaffold and microenvironmental cues, DCC may help provide a solution to mitigate the growing problem associated with cartilage defects that exists throughout the world.

CHAPTER 4: Conclusion

Previous studies have attempted to decellularize hyaline cartilage and have examined the ability of this raw material to maintain phenotype of chondrocytes with success. The studies in this thesis made the first attempt to characterize stem cell gene expression in response to DCC powder. Our results examined the potential of DCC to induce chondrogenesis in bone marrow stem cells.

Bovine articular cartilage was harvested, cryopreserved, and decellularized. The tissue was evaluated for DNA and sulfated GAG content at fresh, cryopreserved, and decellularized states. The findings showed that this study was the first to demonstrate a high percentage of cell removal along with a high percentage of GAG retention. Additionally, this study was the first to process DCC into a nanomaterial for incorporation of this raw material into a wide variety of scaffold formulations. The chondroinductivity of DCC was tested in pellet culture with rBMSCs with and without DCC powder. Gene expression results yielded a two-fold upregulation of collagen type II and three-fold upregulation of Sox9 in pellets cultured with DCC. Aggrecan and collagen type I expression proved inconclusive. This may be attributed to a limited sample size or may suggest a partial differentiation of stem cells down a chondrogenic lineage.

Future work can address the limited sample sizes that were used for gene expression and cell proliferation in this study and examine repeatability. Use of a different delivery scaffold, such as fibrin glue, may be an advantageous choice for this project due to the use of fibrin glue in treating chondral defects clinically. Determining

non-sulfated GAG content in pre- and post-decellularized tissue should also be explored. Immunological response *in vitro* and in animal models would seem to be a high priority in order to take this work further. Determining proteoglycan concentration pre- and post-decellularization would be important to know and may be able to be quantified using ELISA assays. Finally, a comparison study between the ability of the DCC nanopowder developed in this study to retain phenotype of chondrocytes versus induce chondrogenesis of stem cells would seem to be of utmost importance to the field of cartilage tissue engineering. Currently, autologous chondrocytes are used clinically in the ACI procedure for treating cartilage defects; however, the potential ability of DCC to induce chondrogenesis of stem cells while also providing native cartilage building blocks may provide an alternative clinically in the future.

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