

DECELLULARIZED PLANT SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING

by

Melis Toker Bayraktar

B.S., in Chemical Engineering, Marmara University, 2017

M.S., in Biomedical Engineering, Boğaziçi University, 2019

Submitted to the Institute of Biomedical Engineering

in partial fulfillment of the requirements

for the degree of

Doctor

of

Philosophy

Boğaziçi University

2025

**DECELLULARIZED PLANT SCAFFOLDS FOR
CARTILAGE TISSUE ENGINEERING**

APPROVED BY:

Prof. Dr. Bora Garipcan
(Thesis Advisor)

Prof. Dr. Sedat Odabaş

Assoc. Prof. Dr. Banu İyisan

Prof. Dr. Lokman Uzun

Assist. Prof. Dr. Mustafa Kemal Ruhi

DATE OF APPROVAL: August 11, 2025

ACKNOWLEDGMENTS

I would like to express my utmost gratitude to my supervisor Prof. Dr. Bora Garipcan for his guidance throughout my PhD journey and his endless support during challenging times.

I also would like to thank Prof. Dr. Stephen Waldman from Toronto Metropolitan University for giving me the opportunity in his laboratory to collaborate. It's been an invaluable experience and I really appreciate all the support and contributions.

I am truly thankful for my laboratory colleagues and friends for their constant support and encouragement during times of hardship.

I am deeply grateful for my family for their unwavering support.

My special thanks to my husband for believing in me and being always there. I couldn't achieve this without you.

I dedicate this thesis to the loving memory of my father.

ACADEMIC ETHICS AND INTEGRITY STATEMENT

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ABSTRACT

DECELLULARIZED PLANT SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING

Cartilage is a load-bearing connective tissue with a limited self-healing capacity due to lack of blood vessels and innervation. Cartilage damage can occur from both trauma and degenerative joint diseases and current therapeutic options are mostly limited to symptom relief. Therefore, cartilage tissue engineering applications gain an importance to develop novel scaffolds for cartilage repair and regeneration. In this thesis, cellulose-based scaffolds from decellularized plants were investigated for cartilage tissue engineering applications. Lucky Bamboo (*Dracaena sanderiana*) was chosen since its pore structure and mechanical properties can mimic the native articular cartilage. First, the decellularization procedure was optimized for Lucky Bamboo samples. Second, material properties including pore structure, mechanical properties under compression and swelling behavior were characterized before and after decellularization to demonstrate the changes in scaffolds. Finally, decellularized Lucky Bamboo scaffolds were investigated for chondrocyte (cartilage-forming cells) culture up to 8 weeks. Cell adhesion and proliferation as well extracellular matrix (ECM) accumulation in decellularized Lucky Bamboo scaffolds were investigated with biochemical assays, histological and immunohistochemical methods. After 8 weeks of culture, a significant increase in cell proliferation throughout the scaffolds and significant production of collagen type II and aggrecan which are the most prevalent molecules of native cartilage tissue were observed. As a result, decellularized Lucky Bamboo scaffolds provided a three-dimensional (3D) microenvironment for the chondrocytes since the pore structure and mechanical properties can mimic the native cartilage tissue and promoted the production of ECM proteins and glycosaminoglycans from the cells.

Keywords: Plant Decellularization, Cellulose, Chondrocytes, Cartilage Tissue Engineering

ÖZET

KIKIRDAK DOKU MÜHENDİSLİĞİ İÇİN HÜCRESİZLEŞTİRİLMİŞ BİTKİ DOKU İSKELELERİ

Kıkırdak, yük taşıyan ve kan damarları ve sinir sistemi bulunmadığı için kendiliğinden iyileşme kapasitesi limitli olan bir bağ dokudur. Kıkırdak hasarları, travma veya dejeneratif eklem hastalıkları sebebiyle oluşabilir ve şu anki tedavi opsiyonları çoğunlukla semptom gidermekle sınırlıdır. Bu sebeple, kıkırdak doku mühendisliği uygulamaları, kıkırdak onarımı ve rejenerasyonu için yeni doku iskeleleri geliştirmek konusunda önem kazanmaktadır. Bu tez kapsamında, hücresizleştirilmiş bitkilerden elde edilen selüloz bazlı doku iskeleleri araştırılmıştır. Şans Bambusu (*Dracaena sande-riana*), gözenek yapısı ve mekanik özellikleri eklem kıkırdağını taklit edebileceği için seçilmiştir. İlk olarak, Şans Bambusu örnekleri hücresizleştirme prosedürü optimize edilmiştir. İkinci olarak, gözenek yapısı, sıkıştırma altındaki mekanik özellikleri ve şişme davranışı gibi malzeme özellikleri hücresizleştirme öncesi ve sonrası olarak karakterize edilmiştir. Son olarak, hücresizleştirilmiş Şans Bambusu örnekleri, 8 hafta boyunca kondrosit (kıkırdak oluşturan hücreler) kültürü için kullanılmıştır. Hücresizleştirilmiş Şans Bambusu içinde hücre tutunması, çoğalması ve ekstraselüler matris birikimi, biyokimyasal analizler, histolojik ve immünohistokimyasal metodlarla incelenmiştir. 8 haftalık kültür sonrasında, doku iskelesi boyunca hücre çoğalmasında belirgin bir artış ve önemli miktarda, eklem kıkırdağı ekstraselüler matrisinde en yaygın bulunan moleküller olan tip II kolajen ve agrekan, üretimi gözlemlenmiştir. Sonuç olarak, hücre-sizleştirilmiş Şans Bambusu doku iskeleleri, gözenek yapısı ve mekanik özellikleri eklem kıkırdağını taklit edebildiği ve hücrelerin ekstraselüler matris protein ve glikozaminoglikanlarını üretmesini desteklediği için kondrositler için üç boyutlu (3B) mikroortam sağlamıştır.

Anahtar Sözcükler: Bitki Hücresizleştirilmesi, Selüloz, Kondrositler, Kıkırdak Doku Mühendisliği

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LIST OF SYMBOLS

$(C_6H_{10}O_5)_n$	Cellulose
CO_2	Carbon dioxide
$NdFeB$	Neodymium iron boron
$CaCl_2$	Calcium chloride
$NaCl$	Sodium chloride
H_2O	Water
HCl	Hydrochloric acid
$NaOH$	Sodium hydroxide

LIST OF ABBREVIATIONS

3D	Three-dimensional
ADAMTs	A disintegrin and metalloproteinase with thrombospondin motifs
AMCA	Aminomethylcoumarin acetate
ANOVA	Analysis of variance
BC	Bacterial cellulose
BF	<i>Borassus flabellifer</i> (Linn.)
BMSC	Bone-marrow stem cells
BSA	Bovine serum albumin
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CH	Chitosan
COMP	Cartilage oligomeric matrix protein
CS	Chondroitin sulfate
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMMB	Dimethylmethylene blue
DTT	Dithiothreitol
ECM	Extracellular matrix
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
GAG	Glycosaminoglycans
Gel	Gelatin
GelMA	Methacrylated gelatin
GO	Graphene oxide
HA	Hyaluronic acid
H&E	Hematoxylin & Eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
hUCB-MSCs	Human umbilical cord blood-derived mesenchymal stem cells

HUVECs	Human umbilical vein endothelial cells
hWJMSCs	Human umbilical cord Wharton's jelly mesenchymal stem cells
IHC	Immunohistochemistry
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC	Nanoclay
OA	Osteoarthritis
PAMPS	Poly(2-acrylamido-2-methyl-1-propanesulfonic acid sodium salt)
PBS	Phosphate buffer solution
PCEC	Polycaprolactone-polyethylene
PCL	Polycaprolactone
PEG	Poly(ethylene glycol)
PFA	Paraformaldehyde
PGA	Poly(glycolic acid)
PLA	Polylactic acid
PLGA	Polylactic-co-glycolic acid
PNIPAM	Poly(N-isopropylacrylamide)
PRP	Platelet-rich plasma
PU	Polyurethane
PVA	Poly(vinyl alcohol)
RCF	Relative centrifugal force
RT	Room temperature
scCO ₂	Super critical carbon dioxide
SDS	Sodium dodecyl sulfate
TCP	Tissue culture plate
TGF β 1	Transforming growth factor beta 1
UV	Ultraviolet

1. INTRODUCTION

1.1 Motivation

Articular cartilage is a load-bearing connective tissue that functions to transfer mechanical loads to the underlying subchondral bone [1]. Lack of blood vessel innervation makes this tissue vulnerable to injuries, as it limits its self-healing capacity and leaves it susceptible to degenerative joint diseases. Both trauma and degenerative joint diseases, such as osteoarthritis, can cause cartilage damage that results in pain and disability [2]. Currently, most of the treatment options are limited primarily to symptom relief thereby necessitating the need for regenerative therapies, such as tissue engineering [3].

Cartilage tissue engineering investigates suitable scaffold materials to provide a microenvironment to support the growth of cells (chondrocytes or mesenchymal stem cells) resident within the scaffold [4]. Both natural (e.g. collagen, hyaluronic acid, chondroitin sulfate) and synthetic polymers (e.g. polycaprolactone, poly[glycolic acid], poly[ethylene glycol]) have been studied as possible scaffolds for cartilage tissue engineering, but not without limitations. Mostly, biocompatible natural materials do not have adequate mechanical properties to support the regeneration of cartilaginous tissue whereas synthetic materials don't have biological cues to support cellular attachment even though they can be synthesized with desired geometry and mechanical properties [5].

Cellulose, a linear homopolymer of $\beta(1-4)$ linked D-glucose units with the formula $(C_6H_{10}O_5)_n$ and the most widespread natural polymer, is a potential scaffold material for cartilage tissue engineering. Cellulose is biocompatible, has adjustable mechanical properties, and is easily processed [6,7]. Successful adhesion and proliferation of chondrocytes, as well as chondrogenic differentiation of stem cells on cellulose-based scaffolds have been previously reported [8,9]. Cellulose-based biomaterials can be syn-

thesized from different sources using different methods and plant decellularization is one of these methods [10]. The main advantage of plant decellularization is to retain the three-dimensional (3D) and porous cellulose architecture [11].

Plant decellularization is the removal of plant cells and proteins without disrupting the cell wall which is mainly composed of cellulose and provides a microenvironment for mammalian cell adhesion and proliferation [12]. The variety of plants can offer diverse structural and topographical properties in terms of mimicking human tissues. Plant decellularization has been investigated for different biomedical applications, including tissue engineering (bone, cardiac tissue, muscle, skin, and vascular structures) as well as drug delivery and lab-grown meat production [13]. However, this thesis is the first study to investigate the use of cellulose-based scaffolds derived from decellularized plants for chondrocyte (cartilage cell) culture.

In this thesis, Lucky Bamboo (*Dracaena sanderiana*) was chosen to mimic the cartilage tissue due to its pore structure and mechanical properties since these are the most important properties of cartilage scaffolds and generally inversely related [14]. Especially, total porosity, pore sizes, and the elastic modulus of Lucky Bamboo can provide a suitable microenvironment for chondrocytes [15, 16].

1.2 Objectives and Outline

The main aim of this thesis is to develop cellulose-based scaffolds for cartilage tissue engineering from decellularized plants. Since cartilage tissue has a very low self-healing ability and current treatment options are limited, obtaining such a scaffold can play a crucial role in cartilage tissue repair and regeneration. Lucky Bamboo (*Dracaena sanderiana*) is chosen to investigate this hypothesis, since its structural and mechanical properties can mimic cartilage tissue. The porous structure with appropriate pore sizes and Young's modulus similar to native cartilage tissue of decellularized Lucky Bamboo can promote chondrocyte proliferation and extracellular matrix (ECM) production.

The objectives of this thesis include the following.

- Optimization of Lucky Bamboo decellularization process by investigating different chemical methods.
- Evaluation the decellularization degree by observing the reduction in plant cells both quantitatively and qualitatively.
- Characterization and comparison of material properties including porosity, pore size, Young's modulus, compressive strength and swelling ratio of both native and decellularized Lucky Bamboo.
- Cell culture with primary chondrocytes obtained from bovine metacarpal-phalangeal joints for up to 8 weeks.
- Adhesion and proliferation of chondrocytes in decellularized Lucky Bamboo scaffolds.
- Investigation of ECM accumulation in decellularized Lucky Bamboo scaffolds with biochemical assays and histological/immunohistochemical analyses.

2. BACKGROUND

2.1 Cartilage Tissue

Cartilage tissue is a specialized type of connective tissue formed from chondrocytes that originate from mesenchymal stem cells and lacks the blood vessels, lymphatic system and nerves which limits its self-healing capacity. Cartilage has a dense extracellular matrix (ECM) composed of water, collagen and proteoglycans and these components provide the unique mechanical properties [17]. Three different types of cartilage are present in the body: (1) Hyaline cartilage is the most prevalent type and is found in joints, trachea, nose, sternum and ribs. It is primarily composed of type II collagen and proteoglycans and provides resistance to compressive forces by reducing friction due to its lubricant surface. (2) Elastic cartilage is found primarily in the eustachian tube, ear, epiglottis, and larynx to support the tissue against pressure and contribute to flexibility. (3) Fibrocartilage is composed mainly of collagen type I which makes it highly resistant to tension and compression and found in tendons, ligaments, intervertebral discs and some articular surfaces between bones [18].

2.1.1 Articular Cartilage: Structure and Properties

Articular cartilage is formed from 2 to 4 mm thick hyaline cartilage and its major function is the transfer of forces between articulating bones by reducing friction to facilitate joint movement [1]. Articular cartilage has a heterogeneous structure and is divided into four zones; Superficial, middle (transitional), deep and calcified zones. The thickness and the diameter of collagen fibrils are smaller in the superficial zone and increase towards the calcified zone. Also, the cell density is higher, cells are more flattened and collagen fibrils are parallel to surface at the superficial zone whereas the cell density decreases, cells are more spherical and collagen fibers become perpendicular in deeper zones. The intercellular matrix is composed of structural molecules and fluids

with a higher volume in the superficial (80%) zone and decreases volumetrically in the deep zone (65%). The remaining 20-35% of the matrix consist of the macromolecules that are synthesized by chondrocytes. As seen in in Figure 2.1, collagens form 50% of the extracellular matrix of chondrocytes whereas 20-35% consist proteoglycans and the remaining 15-20% glycoproteins. Type II collagen is the most prevalent collagen and makes 80-85% of total collagen in articular cartilage. Aggrecan is the most prevalent proteoglycan which is a large molecule consisting of a protein core with covalently attached glycosaminoglycan chains [1, 19].

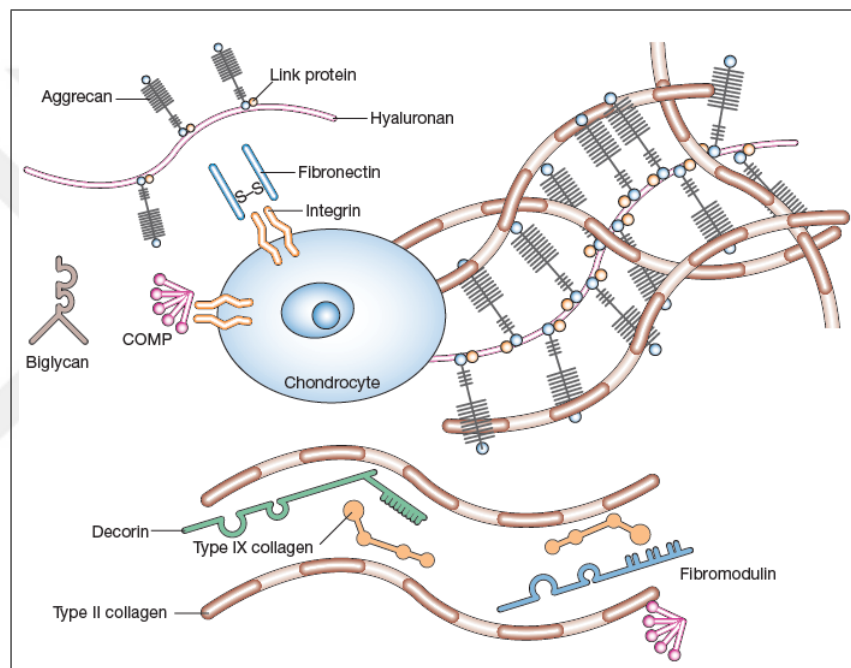


Figure 2.1 Extracellular matrix of cartilage consists of three classes of molecules: collagens (type II collagen is the most abundant); proteoglycans (aggrecan is the most abundant); other noncollagenous proteins (link protein, fibronectin, cartilage oligomeric matrix protein (COMP) and the smaller proteoglycans (biglycan, decorin and fibromodulin) [20].

2.1.2 Osteoarthritis

Osteoarthritis (OA), one of the most common musculoskeletal conditions and the leading cause of impaired mobility amongst the adult population, is a chronic disease that degenerates articular cartilage which is mostly diagnosed at knee joint but also affects the hips, shoulders, ankles and elbows [21]. In 2024, 528 million people worldwide, 10% of males and 18% of females 60 years of age or older, were reported to

have OA, suffering from chronic pain, reduced quality of life and high medical expenses. Hospitalization costs just in USA were reported as \$16.5 billion yearly [4, 22].

OA first starts with the loss of cartilage ECM proteins and the prevention of the proliferation of synovial cells which leads the gradual degradation of articular cartilage tissue (Figure 2.2). Eventually, it spreads to the underlying subchondral bone causing bone hardening (osteoclerosis) and formation of abnormal bony growths (osteophytes) and overall these pathologies can end up in a dysfunctional joint [23].

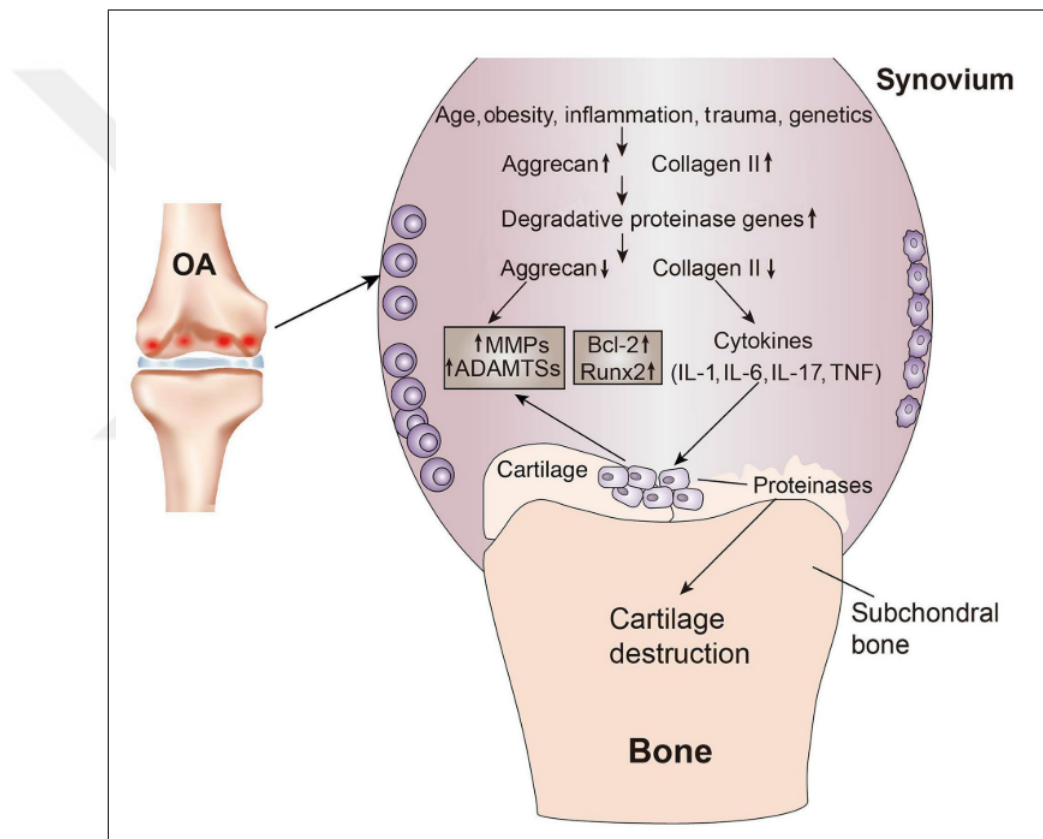


Figure 2.2 The changes in cartilage tissue with osteoarthritis (OA). With the progression of OA, degradation proteases expression increases whereas the expression of proteoglycans and type II collagen decreases which results in the production of numerous catabolic proteins such as MMPs (Matrix metalloproteinases) and ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs). The increase in proinflammatory cytokines (IL-1 β and TnF- α), apoptotic markers (Bcl-2 and Runx2 (runt-related transcription factor 2)) and transcription factors escalates the production of MMPs and ADAMTSs, ultimately leading to cartilage destruction. [23].

2.1.3 Current Treatment Options

Even though, ageing, genetics and obesity are the risk factors for the development of OA, the underlying pathogenesis is still unclear. Joint deformities including cartilage space narrowing and stiffness as well as chronic pain are the most common symptoms of OA. Current treatment options are mostly limited to symptom relief for reducing the pain and improving the quality of life [24]. Surgical methods that include microfracture surgery, osteochondral grafting and chondrocyte transplantation are limited to smaller defects and generally provide short-term relief of symptoms instead of complete healing. For example, after microfracture surgery, there is a high risk of clot formation and the regeneration of cartilage tissue mostly ends up with fibrocartilage formation instead of hyaline cartilage which has less durability [25]. Another option is cartilage grafting, however, availability of autografts is limited and finding allografts in suitable shape and size without causing any biomechanical imbalances is difficult [26]. Lastly, cell-based techniques have been used for cartilage repair but these techniques require harvesting the patient's cells and transplanting to the damaged tissue which results in more than one operation and increases the risks for patients [27]. Other than total joint replacement surgery, there is no effective therapy to prevent the progression of OA since it is an irreversible disease [24]. Total joint replacement is the removal of diseased joint and replacement with an artificial implant. This procedure has its own risks such as infection and the failure of the prosthetic by dislocation or fracture [28]. Therefore, tissue engineering approaches become important to find alternative methods for the repair of damaged cartilage.

2.2 Cartilage Tissue Engineering

Cartilage repair is still challenging due to its limited self-healing capacity and shortcomings of current treatment options. Therefore, tissue engineering approaches offer new therapeutic strategies for the regeneration of damaged cartilage by combining biomaterials with cell culture to mimic the native tissue [3]. Advancements in cartilage tissue engineering (Figure 2.3) have been made through both *in vitro* benchtop research

and *in vivo* studies with small animals (mice, rats and rabbits) by focusing on scaffold development, cell seeding and biochemical and biomechanical stimulation of obtained scaffolds [4].

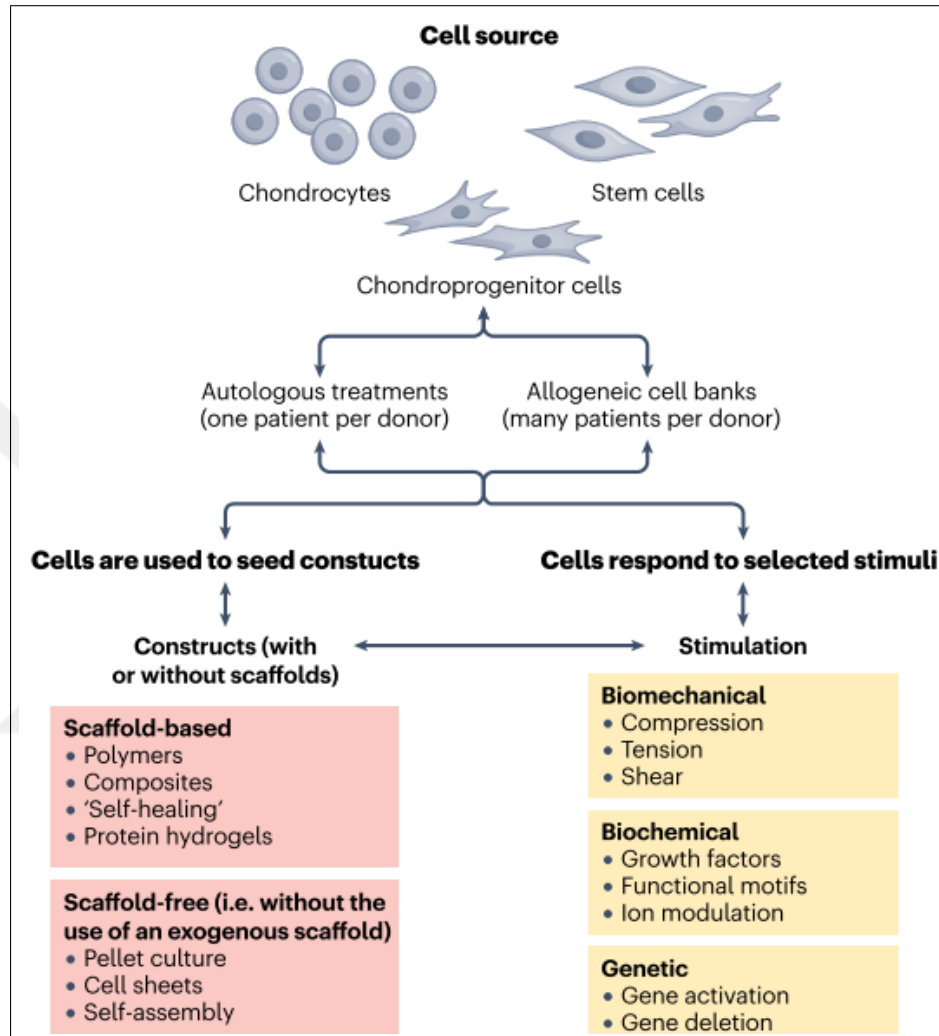


Figure 2.3 Articular cartilage tissue engineering strategies using the tissue engineering triad (cells, scaffold or scaffold-free approaches and biochemical or biomechanical stimulation). [4].

2.2.1 Cell Sources for Cartilage Tissue Engineering

In cartilage tissue engineering, different cell sources have been used to mimic chondrogenesis. Chondrocytes can be obtained from donors with a biopsy from articular cartilage, isolated and expanded in monolayers to further investigate. Autologous chondrocytes can be harvested from patient's own tissue but it causes further damage to healthy cartilage and increases the risk of multiple surgeries. On the other hand,

allogenic chondrocytes can be harvested from other donors but they might not be compatible with patient's needs and donor availability is very limited [4]. In addition, there is the risk of dedifferentiation of chondrocytes during culture. They can lose their round shape and become elongated fibroblast-like. Also, when they lose their phenotype, the synthesis of type II collagen and aggrecan decreases and they produce more type I collagen and versican [14].

Alternatively, stem cells have been used for cartilage tissue engineering as well. Mesenchymal stem cells (MSCs) are multipotent stem cells and they can be obtained from different tissues such as bone marrow, adipose tissue, umbilical cord and even from synovial tissue. MSCs have the ability of chondrogenic differentiation but their migration and attachment capabilities are limited [29]. The long-term disadvantage of using MSCs is their tendency to hypertrophic differentiation that results in endochondral ossification which ends up in bone formation instead of cartilage [4]. Other promising cell sources are chondroprogenitor cells that can differentiate into cartilage-forming cells (chondrocytes) without hypertrophic differentiation and can be derived from articular cartilage, synovium and adipose tissue [30]. Xue *et al.* compared the behavior of chondrocytes, chondroprogenitor cells and bone-marrow derived stem cells (BMSC) in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) scaffolds. They observed cartilage-like tissue formation with significant synthesis of collagen type II and glycosaminoglycans with chondrocyte and chondroprogenitor cell-seeded scaffolds, while the BMSC seeded scaffolds showed vascularization after 6 weeks of subcutaneous implantation. They concluded that in terms of chondrogenesis, cartilage progenitor cells were better than BMSCs but worse than chondrocytes [31].

Other studies reported that biomechanical and biochemical stimulation can improve functionality of cells and induce chondrogenesis. For instance, by applying tension and adding bioactive factors such as TGF β 1 (transforming growth factor beta 1), chondroitinase and lysyl oxidase-like to articular chondrocytes, more anisotropic constructs with higher Young's moduli were obtained [32]. In another study, fluid-induced shear stress and bioactive factors that were applied to self-assembled costal chondrocytes, improved the mechanical properties also increased collagen fiber density [33].

Different cell types can be used in scaffold-free approaches in cartilage tissue engineering with cell sheets, pellets or self-assembly cultures [4]. These approaches might be promising since cell-cell interaction can induce the tissue formation. However, heterogeneity in cell phenotype at different layers of cell aggregates is a major drawback. The superficial layers are composed of more fibroblast-like cells with higher collagen type I content whereas middle layers constitute more chondrocyte-like cells with collagen type II-rich ECM and the core layer mostly lacks the ECM with apoptotic cells [14]. Zhang *et al.* demonstrated that the micromass culture system (making self-assembly 3D mass by placing a high concentration of cells in small droplets) was more efficient in chondrogenic differentiation of MSC compared to the standard pellet culture systems where cells were aggregated into pellets by centrifuging. They also observed that, collagen type I and collagen type X expressions reduced in micromass culture systems, therefore, this approach is promising in terms of mimicking the hyaline cartilage [34].

Cells from different sources with different culture systems with or without chemical, mechanical or genetic stimulation can be used for cartilage tissue engineering. It is crucial to decide all these parameters based on specific application considering reproducibility and translation to clinical use.

2.2.2 Scaffold Materials for Cartilage Tissue Engineering

Scaffold-based approaches require biocompatible materials that can provide a 3D microenvironment to support cell attachment and proliferation and induce ECM synthesis. Also, a porous structure with desirable mechanical properties that can mimic native tissue is crucial for cartilage tissue engineering scaffolds [29]. The degradation rate is another important parameter since the scaffold should retain its structural integrity until the new tissue is formed which is at least 3 to 6 months. In addition, the by-products of the degradation should be non-toxic and not cause any adverse effects in body [22]. Such biomaterials can be obtained from synthetic or natural polymers as well as from their combinations as hybrid materials.

Synthetic polymers are advantageous due to adjustable structural and mechanical properties based on the specific application and stability for longer times. But, they don't have the biological cues to stimulate cell adhesion and proliferation [22]. Mostly used synthetic polymers for cartilage tissue engineering are poly(ethylene glycol) (PEG), polycaprolactone (PCL), polylactic acid (PLA), polyurethane (PU), poly(glycolic acid) (PGA) [5]. Fu *et al.* developed a porous scaffold from PCL and PEG by using solvent casting and particulate leaching methods. These scaffolds were both seeded with stem cells and implanted in rats with a cartilage defect. The results showed that PCL/PEG scaffolds supported both *in vitro* cell adhesion and migration as well as *in vivo* tissue regeneration after 8 weeks [35]. In another study, Tsai *et al.* developed PU scaffolds with dispersion method and seeded them with both chondrocytes and human MSCs. They observed that the proliferation of chondrocytes and glycosaminoglycans (GAG) synthesis were better in PU scaffolds compared to more conventional PLA scaffolds. Similarly, MSCs showed better chondrogenic differentiation in PU scaffolds [36].

On the other hand, natural materials are highly biocompatible and some of them even found in the human body and form the ECM of native cartilage tissue. Therefore, they have similar properties to native tissues and induce cell proliferation, differentiation and ECM production. However, the low stability under processing conditions and inadequate mechanical properties are the major drawbacks of natural polymers. Widely used natural materials in cartilage regeneration can be listed as collagen, hyaluronic acid (HA), chondroitin sulfate (CS), chitosan (CH), cellulose and fibrin [5]. Shi *et al.* obtained gelatin and silk fibroin scaffolds for cartilage regeneration with 3D printing technology and seeded them with bone-marrow stem cells (BMSC). The results of biochemical assays and histological analyses demonstrated that these scaffolds induced the proliferation, chondrogenic differentiation, and ECM synthesis of BMSCs [37]. In another study, Huang *et al.* developed gelatin/hydroxyapatite constructs by microextrusion 3D bioprinting and enzymatic cross-linking. They first observed the chondrogenic differentiation of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) *in vitro* on these scaffolds. Then, these cell-seeded scaffolds were implanted in pigs with cartilage defects and the results showed an efficient repair at

the damaged site after 24 weeks [38].

Hydrogels are also attractive sources as scaffold materials in cartilage tissue engineering since they have a 3D network of cross-linked hydrophilic natural and synthetic polymers with high swelling and fluid absorption abilities. These properties make hydrogels easy to mimic the native cartilage ECM. However, due to the gel-like form, they lack the high mechanical strength required for cartilage scaffolds [5]. Yang *et al.* developed a hybrid hydrogel from bacterial cellulose (BC) and poly(vinyl alcohol) (PVA)-poly(2-acrylamido-2-methyl-1-propanesulfonic acid sodium salt) (PAMPS). This hydrogel was designed with this composition since the cellulose can mimic the collagen in native cartilage ECM and provide tensile strength whereas PAMPS can mimic the other ECM components such as aggrecan-GAG structures [39]. Another example of hybrid hydrogel scaffold was obtained by Asadi *et al.* by combining synthetic and natural polymers as well as growth factors. Gelatin/polycaprolactone-polyethylene glycol (Gel/PCEC-TGF β 1) scaffolds were seeded with human adipose tissue derived mesenchymal stem cells and the results showed a high expression of type II collagen and aggrecan which proved the chondrogenic differentiation and ECM production [40].

2.2.3 Decellularized Cartilage ECM-Based Scaffolds

Another approach to obtain cartilage tissue engineering scaffolds is the decellularization of native cartilage tissue. Decellularization can be defined as the removal of cellular content from the cartilage, while preserving the ECM components that provide the structural and mechanical properties. Decellularization methods that were investigated for porcine, bovine and human cartilage tissues can be listed as chemical (generally sodium dodecyl sulfate treatment), physical (freeze-drying) and enzymatic (DNase, RNase, pepsin) [41].

Browe *et al.* compared the efficiency of decellularized cartilage scaffolds with microfracture technique which is the current standard of care for cartilage defects. They implanted solubilized decellularized articular cartilage scaffolds obtained from

porcine into damaged cartilage tissues in goats and. They observed that the cartilage regeneration was better with these scaffolds compared to microfracture technique [42]. Similar results were obtained in another study from Zhang *et al.* in which they implanted decellularized cartilage scaffolds seeded with human umbilical cord Wharton's jelly mesenchymal stem cells (hWJMSCs) into goats with cartilage defects. The results showed these scaffolds were better in terms of hyaline cartilage regeneration compared to microfracture technique [43].

Even though, some positive results with decellularized cartilage scaffolds were obtained, there are still some limitations. One of the challenges is to optimize the decellularization protocol since the cells should be completely removed from the tissue to not cause any immunogenicity but also the ECM should be kept intact, otherwise the structural properties of native tissue can be lost [44]. Another major challenge in obtaining porous scaffold materials without compromising the mechanical properties which was the problem also observed when scaffolds were obtained from the powder of decellularized articular cartilage. When the cartilage powder concentration was increased in scaffolds, the pore structure improved while decreasing the mechanical properties [45].

2.3 Plant-Derived Biomaterials and Scaffolds

2.3.1 Plants as Tissue Mimicking Scaffolds

Plants have been used by humankind since ancient civilizations in search of healing and curing diseases. Plants, such as ginseng, ephedra, juniper, coriander, myrtle, etc. discovered thousands of years ago, have been still used in modern pharmacology [46]. Moreover, plant-derived polysaccharides, proteins, and other extracts have been widely used in biomedical applications due to their biological and chemical properties. Nowadays, besides developing drugs, plants have also been investigated for tissue engineering applications due to their structural properties and surface topographies which enhance the ability of mimicking animal and human tissues. Tissue

engineering applications that aim to fabricate scaffolds for the repair and/or regeneration of damaged tissues or organs have achieved great success over the past couple of decades, but current approaches are not sufficient to design and produce perfectly suitable biomaterials [47–49]. Therefore, using plants as tissue scaffolds has drawn more attention [50].

The diversity of plants in terms of structural properties and surface topographies, abundant availability in a cost-efficient way, and simplicity of manufacturing and modification makes plants potential candidates as biomaterials for tissue engineering applications. In addition, the native vasculature, diverse pore structures, exceptional water uptake and transference ability, and wide range of mechanical characteristics of plants are favorable features for scaffold materials in terms of integration with native tissue and regaining the functionality of cells at the injured site when implanted. In particular, the inherent vasculature makes plants promising options because the major challenge with other polymeric biomaterials is neovascularization after the implantation of scaffolds. The vascular bundles are composed of xylem and phloem channels and they are responsible for water and nutrient transport in plants. Transpiration of water from leaves induces hydraulic conductance from roots to leaves. Therefore, vessels and interconnected porosity are also underlying features of water transport and retention ability. In addition, other structural properties of plants such as surface patterns and pore structure provide an appropriate microenvironment for cells to adhere and proliferate. Surface topography like microgrooves or channels can guide the elongation direction of cells. Moreover, aligned vascular channels found in plants like celery or asparagus were shown to guide the nerve or muscle cell alignment which is essential for them to gain their functionality [13, 16, 51–55].

The mechanical strength, durability, and elasticity of plants range on a large scale. For example, apple hypanthium tissue which has a Young's Modulus of 1 to 4 kPa can be utilized in soft tissues; whereas, palm fibers can have a Young's Modulus of up to 4 GPa, which is suitable for mimicking bone tissue [55]. However, this variability in mechanical properties can also be a disadvantage which is a crucial criterion for mimicking a target tissue. For example, bone-mimicking scaffolds need to be fa-

tigue resistant, or durability under cyclic loading is crucial for muscle tissue scaffolds. Therefore, these properties should be considered while designing a plant-based scaffold. Thus far, the data in the literature that investigated the mechanical characteristics of plant-based biomaterials are not sufficient to determine whether the strength, elasticity, or durability of plants are desirable for tissue engineering applications, specifically *in vivo* environments [13,52].

The degradation profile of plant-based biomaterials is still not well understood since the current data related to the degradation behavior of plants show alterations in the literature. The degradation rate of a scaffold material should be optimal; not too fast to allow the native tissue to integrate with the biomaterial and initiate the regeneration of damaged tissue and not too slow to prevent chronic inflammation [13]. Mahendiran *et al.* investigated the hydrolytic degradation in physiologic conditions of *Borassus flabellifer* (Linn.) and after 21 days 60% weight loss was observed [56]. However, Modulevsky *et al.* observed the degradation behavior of asparagus (*Asparagus officinalis*)-derived scaffolds *in vivo* and after 14 days they didn't observe any significant changes in scaffold sizes [54]. On the other hand, the result of *in vivo* degradation behavior of decellularized stems of pumpkin (*Cucurbita maxima* L.), papaya (*Carica papaya* L.) and castor (*Ricinus communis* L.) demonstrated that after 60 days, papaya and castor-based scaffolds disappeared completely and the weight loss of pumpkin scaffold reached 81.66% [57]. For controlling the degradation rate, surface modifications have been applied or enzymatic degradation (e.g., cellulase for cellulosic scaffolds) has been conducted. However, the by-products of surface coatings after degradation might be cytotoxic and can cause inflammation. Furthermore, the enzymes that accelerate the degradation such as cellulase are not found in mammals including humans. Therefore, only if the enzymes can be incorporated into scaffold materials, the results can also be relevant in *in vivo* studies [55,58]. Further research on the degradation behavior of plant-based scaffolds is necessary.

The diversity of plants offers a variety of structural, mechanical, and biochemical properties and enhances the chance of overlapping with the characteristics of human tissues and their mimicking ability. For example, decellularized spinach and chive

were investigated for kidney engineering and the results showed that their mechanical integrity and permeability which are crucial factors for functional kidneys were not appropriate for this application. Despite these drawbacks, spinach was studied by different researchers who reported that its vasculature might mimic the vessel structure of mammals and promote angiogenesis [59–62]. Therefore, the structural properties of plants should be considered when choosing the application area to find a good fit to mimic the related tissue. With all positive and negative aspects, using plants as tissue scaffolds is a promising research field and extensive investigations are required for more consistent results. Nevertheless, the diversity of plants offers a variety of physical structures, surface topographies, and chemical properties that can be appropriate for mimicking human tissues.

2.3.2 Decellularization of Plants

Decellularization is a leading technique for developing tissue scaffolds that involves removing native cells and nuclear material from organs and tissues while preserving the extracellular matrix (ECM). ECM maintains the structural support and provides biochemical and mechanical cues for cell adhesion and proliferation. Therefore, decellularized animal and human tissues create a more relevant cellular milieu compared to other polymeric biomaterials since they can mimic the native environment of cells [63].

Recently, decellularization of plant tissues has been investigated because they are more accessible and cost-efficient than animal products. The main principle of both animal and plant decellularization is to remove the genetic material from the native tissue. Animal ECM mainly contains proteins (collagen, fibronectin, etc.) and glycosaminoglycans, whereas plant ECM or the cell wall is predominantly composed of cellulose microfibrils that are crosslinked with polysaccharides such as pectin and glycans. There are also proteins and pigments found between this crosslinked network. Cell wall structure can differ based on the plant type but cellulose determines the framework and the content of hemicellulose and lignin that fill the cellulose microfibrils

can change between species [64–66].

Another difference is the removal of proteins in addition to genetic material in plants during the decellularization. The remaining cell wall is mostly composed of cellulose, therefore, plant decellularization can be considered as an alternative technique to obtain cellulosic scaffolds [59, 67, 68]. Cellulose, the most abundant natural polymer, is formed from a glucan chain with D-glucopyranose ring units linked by $\beta(1-4)$ glycosidic bonds and can be found in tunicates, algae, bacteria in addition to plant cell walls [69, 70]. Strong intramolecular hydrogen bonds provide strength and weak intermolecular forces contribute to the flexibility in cellulose. Therefore, due to the adjustable mechanical properties and biocompatibility, cellulosic materials have been widely used in tissue engineering applications including skin, bone, neural and vascular tissues [6, 16, 71–73].

The first study on plant decellularization was conducted by Modulevsky *et al.* in (2014). They chose apple (McIntosh red apple) due to its high porosity. They decellularized it by 0.5% Sodium Dodecyl Sulfate (SDS) treatment for 12 h, and modified the surface of decellularized apples with collagen and glutaraldehyde to enhance cell attachment. Their results showed that decellularized apple tissue can provide a 3D scaffold for the adhesion and proliferation of mammalian cells such as NIH3T3 fibroblasts, mouse C2C12 myoblasts, and human HeLa epithelial cells [74]. Following this study, different parts such as leaves, stems, and roots of diverse types of plants have been decellularized and investigated for various biomedical applications including tissue scaffolds, drug delivery applications and for laboratory-grown meat products [13, 55, 75]. For instance, leaf vasculature was used as a template for mimicking blood vessels, angiogenesis, and cardiac tissue engineering [59, 60]. As another example, microgroove-like surface topographies found in celery, green onion, asparagus, and grass blades were appropriate for the alignment of skeletal muscle cells [76, 77]. Therefore, it can be concluded that structural organization and surface patterns of plants are the most crucial factors to determine the application area.

2.3.3 Plant Decellularization Methods

Decellularization of plant and animal tissues utilize similar techniques but there are some fundamental differences between decellularized structures. The aim of decellularizing an animal tissue is to obtain ECM without any major damage to structural integrity and protein content. There are a few generally accepted criteria that need to be achieved in animal decellularization, which are: double-stranded DNA remaining in the scaffold should be lower than 50 ng per mg of dry ECM, remaining DNA fragments should be lower than 200 base pairs, and no nuclear content should be visible by 4',6-diamidino-2-phenylindole (DAPI) staining [54]. Although achieving these aforementioned criteria is necessary, depending on the application, other factors such as decellularized ECM structural integrity, mechanical properties, surface topography, and protein composition need to be maintained. Decellularization processes of tissues from different kingdoms of life (i.e., animal, plant, fungi, etc.) are largely the same but the intensity of applications is often different due to differences in structural robustness. Robustness of plants results in requirement of harsher decellularization processes than needed for animal tissues; however, these types of approaches for decellularizing plant tissues denature and/or remove proteins found in the ECM [48, 78]. As a result of harsher decellularization protocols, decellularized plants are often used as cellulose-based 3D scaffolds that have characteristic topographical properties. This results in the main difference between conventional animal tissue decellularization and plant tissue decellularization. Decellularized animal tissues can be classified as decellularized ECMs and decellularized plant tissues are often classified as 3D scaffolds since most of the protein content is being removed during the relatively harsh decellularization process. General decellularization methods can be categorized as chemical, physical, and enzymatic (Figure 2.4). Depending on a specific application, choosing a suitable approach for a plant requires careful investigation of structural properties and surface architecture.

2.3.3.1 Chemical Decellularization. Chemical-based plant decellularization is the most common among all possible alternatives due to its high decellularization

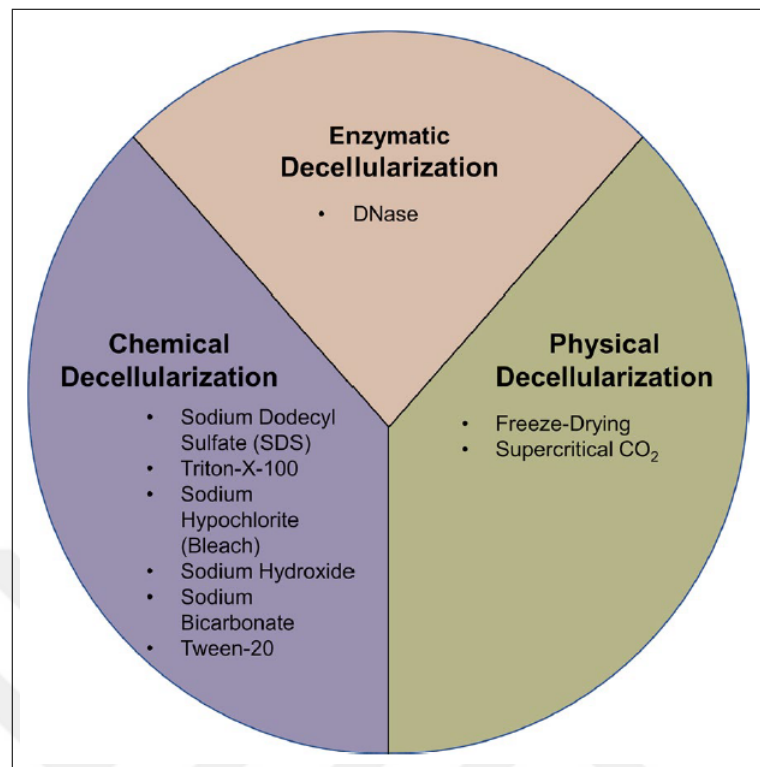


Figure 2.4 An overview of chemical, physical and enzymatic plant decellularization methods.

efficiency and its acceptance as a gold standard. Widely used chemical agents for chemical decellularization are detergent-based SDS (ionic) and Triton-X-100 (nonionic) solutions. Surfactants decellularize tissues by lysing the bonds between lipid molecules and lipid-protein interactions at the cell membranes [79,80]. Chemical decellularization can be applied to plants in two different ways: (1) Perfusion of the chemical through the plant vasculature or (2) Soaking the plant tissue in chemical solutions. For the perfusion decellularization, a pump module is used to deliver the chemicals from the stalk of a plant to the veins. This method is commonly used with leafy plants to investigate vascularization. Decellularization with soaking is more common and done by placing the whole plant tissue in a chemical solution for overall decellularization.

The golden standard and most used chemical in plant decellularization is an ionic surfactant, SDS. It decellularizes tissues through solubilizing cytoplasmic and nuclear membranes [81]. A vast majority of studies since plant decellularization started to gain attention in tissue engineering research, have utilized SDS as a main decellular-

izing agent. Depending on the plant and the application, concentrations between 0.1% and 10% SDS have been used for decellularization, and time frames of the applications have been reported starting from 12 hours to up to 5 days where a higher concentration results in a short treatment time [60–62, 68, 69, 82–86]. Triton X-100 is a nonionic surfactant that is widely used in general decellularization processes. Similar to SDS, Triton X-100 affects membranes but its mechanisms target lipid-lipid and lipid-protein bonds, specifically [81]. In plant decellularization, usage of Triton X-100 alone as a sole decellularizing agent is very rare and it is usually utilized together with SDS as a secondary chemical agent [87]. Triton X-100 is generally applied by using concentrations ranging between 0.1% and 0.5% for 1 to 2 days and it is often applied after the main SDS treatment [15, 16, 59–62, 77, 84, 88–94]. Apart from SDS and Triton X-100, there are some rare uses of other chemicals for plant decellularization. Sodium hypochlorite, commonly referred to as bleach, can be used alone rarely or in combination with other surfactants, mostly Triton X-100, for plant decellularization [95]. Generally being used in vasculature research, 0.1% Triton-X in 10% sodium hypochlorite is applied for removing color from the plants in order to obtain transparent platforms [62, 84]. Sodium hydroxide, sodium bicarbonate, and other surfactants such as Tween-20 have also been used rarely in the literature but their use is either alongside with other surfactants or in conjunction with high temperatures [77, 96, 97].

2.3.3.2 Physical decellularization. Usage of freeze-drying and supercritical CO₂ are considered physical plant decellularization approaches that are less common than their chemical counterparts. Using chemicals, especially detergents such as SDS and Triton X-100, may leave trace amounts of chemical residues even after careful washing. The main advantage of using physical decellularization methods is to keep 3D scaffolds surfactant-free so that the scaffolds would retain their biocompatibility. Freeze-drying is a dehydration process that involves freezing a sample and sublimating it under low pressure to remove its water content. Freeze-drying is rare in plant decellularization; nevertheless, it is reported that utilizing freeze-drying resulted in the removal of 90% of total DNA from a banana (*Musa balbisiana*) leaf without using post-freeze-drying chemical decellularization treatments [98]. Huang *et al.* successfully decellularized lo-

tus roots using freeze-drying and obtained scaffolds that promoted cell attachment and bone regeneration capabilities *in vivo* [99]. Lately, using supercritical carbon dioxide (scCO₂) has emerged as a strong alternative for general decellularization. Its non-toxicity, inertness, high diffusivity, and physiologically suitable critical point temperature (31.1 °C) make it viable to use in decellularization applications such as ram cardiac tissue, porcine dermal matrix, aorta, and cornea [100–103]. Only scCO₂ plant decellularization application in the literature has been successfully applied on baby spinach (*Spinacia oleracea*) leaves without any chemical treatment and obtained plant-based scaffolds were able to support fibroblast cell growth. Additionally, the total decellularization time using scCO₂ compared to chemical decellularization was 80% faster, making it extremely time-efficient [104].

2.3.3.3 Enzymatic Decellularization. Enzymatic methods remain comparatively untouched considering ever-improving implementations of chemical and physical methods. So far, only one *in vitro* study investigated the decellularization of transgenic hairy roots derived from tobacco (*N. tabacum*) with an enzyme as the main decellularization agent by combining it with freeze-drying, and DNase treatment. Postdecellularization nuclear content was significantly less compared to its controls [67]. Even though published results are promising, enzymatic approaches need to be investigated more in order to be compared against conventional methods.

2.3.4 Biotechnological Applications of Decellularized Plants

Biomedical applications of decellularized plants mainly center on creating 3D scaffolds for *in vitro* cell culture and *in vivo* implantation studies. Cellulose can act as a component to form composites with other materials and cellulosic surfaces can be functionalized; therefore, it is possible to tune its mechanical properties and surface chemistry [13, 105]. With the use of different biofunctionalization methods, such as surface coating, biomineralization, and cross-linking, cell adhesion can be facilitated in a variety of decellularized plant tissues. High water transport capacities and the

interconnected structure of plant tissues provide a microenvironment for cells to adhere and proliferate, in addition to the microstructure of plant frameworks leading to cellular alignment [16]. The initial approach of using decellularized plants as 3D platforms to culture different types of mammalian cells has begun with utilizing apple hypanthium. In the pioneer study, an apple hypanthium-derived cellulose-based scaffold was investigated to simulate physiological conditions for 3D mammalian cell culture [74]. Upon the success of this initial plant decellularization approach and its utilization as a 3D cell culturing scaffold, other types of plants were also starting to be tested. A wide range of different plants was later decellularized in order to assess their use in biomaterials research. Decellularized parsley, calathea (*Calathea zebryna*), bamboo (*Bambusoideae*), orchid (*Laelia anceps*), leek (*Allium porrum*), spinach (*Spinacia oleracea*), carrot (*Daucus carota*), celery (*Apium graveolens*), olive leaves and many others were shown to support adhesion and growth of various cells along with their ability for surface functionalization using various molecules and ECM proteins [13, 16, 60, 68, 76, 106, 107].

2.3.4.1 Tissue Engineering Applications Based on Plant Structures. Various plant species were decellularized mainly due to their unique surface architecture, topographies, and their potential to support specific cell types for specific culturing applications. Compared to conventional cell culturing, 3D scaffolds provide topographical cues to adherent cells and modulate cell behavior by triggering intracellular pathways [108]. These topographical cues can be in form of: elongated and interconnected channel-like patterns favoring cell adhesion and spreading [68, 109], porous structures to serve as bone tissue scaffolds [99, 110], a high aspect ratio of the surface conformation that guides cell alignment [76], linear microchannels which serve as vessels in scaffolds that have the potential to be utilized in both hard and soft tissue engineering [86] or in repeating microcavity patterns allowing stem cell differentiation [98].

Surface Topography: Micropatterns found on the surface of plant tissues including channel and groove-like shapes play an important role in guiding cell behavior which directly affects their functionality and differentiating abilities (Figure 2.5A).

Cheng *et al.* demonstrated that decellularized green onion promoted the alignment and differentiation of mouse and human muscle cells to form myotubes due to its microstructure (Figure 2.5B). The surface topography of green onion consisted of 20 μm wide and 10 μm deep microgrooves promoted cell growth and anisotropic alignment which made it a potential biomaterial for skeletal muscle tissue engineering [76]. Similarly, decellularized grass was investigated as a muscle tissue scaffold due to its long narrow blades composed of longitudinal and parallel striations on the surface. Allan *et al.* indicated that the topographical cues of grass guided muscle cells for self-alignment [77]. The effect of surface geometry was also studied with the outer skin of decellularized onion for stem cell proliferation and osteogenic differentiation. Imaging results demonstrated that cells expanded in parallel lines accordingly to the rectangular-shaped surface patterns [94]. Also, the decellularized leek was investigated due to its elongated channel-like surface patterns to observe the adhesion and proliferation of neuroblastoma cells [68]. Similarly, the effect of distinct microtopographies of cross-sectionally and longitudinally cut decellularized celeries on mesenchymal stem cell (MSC) behavior was studied. Cells aligned when they were cut longitudinally according to the directions of patterns and spread randomly when cross-sectionally cut due to lack of surface guidance [111].

Pore Structure: Interconnected porosity and pore size play a crucial role in cell adhesion and proliferation since the porous structure can help cell proliferation and migration, transfer of nutritional media, and gas exchange [73]. Therefore, various plants were investigated as tissue scaffolds due to their porosity (Figure 2.6). For instance, Contessi Negrini *et al.* studied apple, carrot, and celery since they kept their porosity within a size range of 70 to 420 μm after decellularization. According to pore sizes and mechanical properties, apple, carrot, and celery were suggested to be used as scaffolds for adipose, bone, and tendon tissues respectively, since they enabled the proliferation and differentiation of relevant cells [106]. Huang *et al.* suggested that decellularized lotus roots can be suitable scaffolds for bone tissue engineering due to their microstructure formed from pores with an average diameter of $\approx 100 \mu\text{m}$ [99]. *Borassus flabellifer* (Linn.) (BF) immature endosperm was investigated by Mahendiran *et al.* for bone tissue scaffolds due to its high porosity as well. Morphology

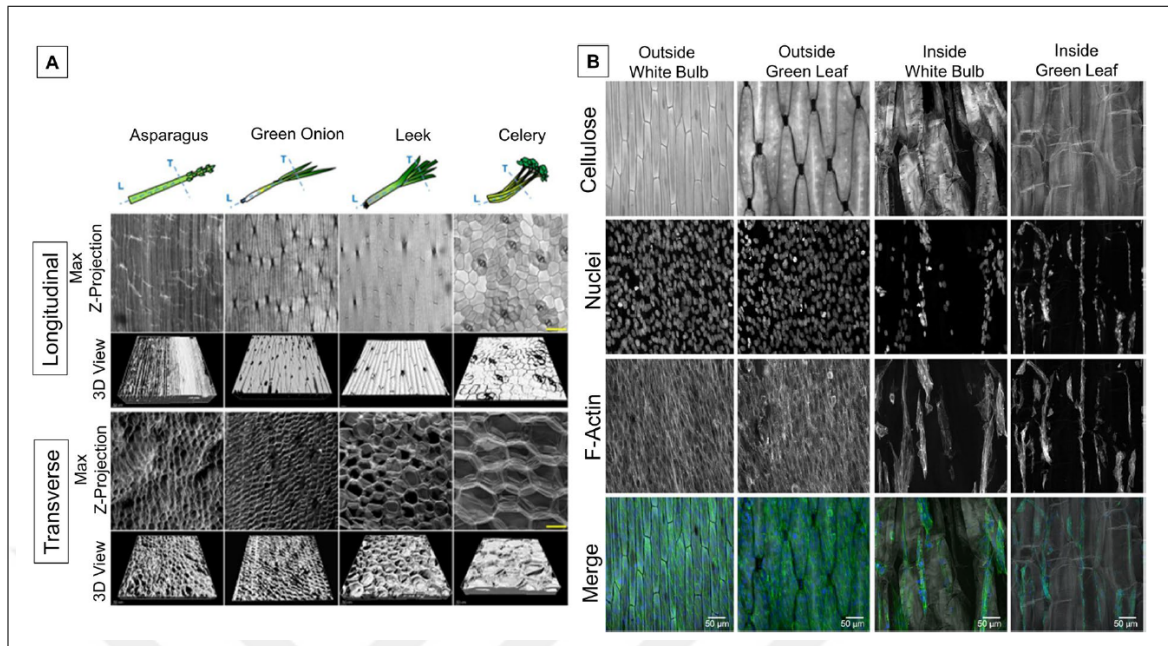


Figure 2.5 Different structures and surface topographies of cellulose scaffolds derived from decellularized plants. A) Anisotropic patterns were detected in asparagus, green onion, leek, and celery (scale bar = 100 μm) in both longitudinal and transverse directions (scale bar = 100 μm). 3D view of structures was shown below (scale bar = 50 μm). B) Surface microstructures of different sections of green onion. Both inner and outer sides of white bulb and green leaf were coated with fibronectin (50 $\mu\text{g}/\text{mL}$) seeded with C2C12 mouse myoblast cells. Images were obtained with confocal immunofluorescence analysis by staining decellularized plant with calcofluor white, gray for cell wall, NucGreen 488, blue for nuclei, Phalloidin, green for F-Actin to observe cell adhesion and proliferation [76].

studies showed that foam-like structure and interconnected pores of *Borassus flabellifer* (Linn.) (BF) immature endosperm were preserved after decellularization, oxidation, and surface modifications which provided a proper environment for cells to adhere and proliferate [112]. Similarly, the porous structure of decellularized celery stem made it a potential scaffold for osteogenesis. Pore size was measured as $323.15 \pm 78.93 \mu\text{m}$ and no significant changes were observed after scaffolds were coated with nano amyloids and biomineralized with nanohydroxyapatite. Li *et al.* concluded that decellularized and modified celery stem promoted osteogenesis both *in vitro* and *in vivo* [113]. Based on the studies from the literature, the interconnected porosity of various plants is advantageous especially for mimicking the bone substitutes.

Vascular Structure: The overall leaf structure of decellularized plants has been also utilized due to their venations, which provide both microstructures and vas-

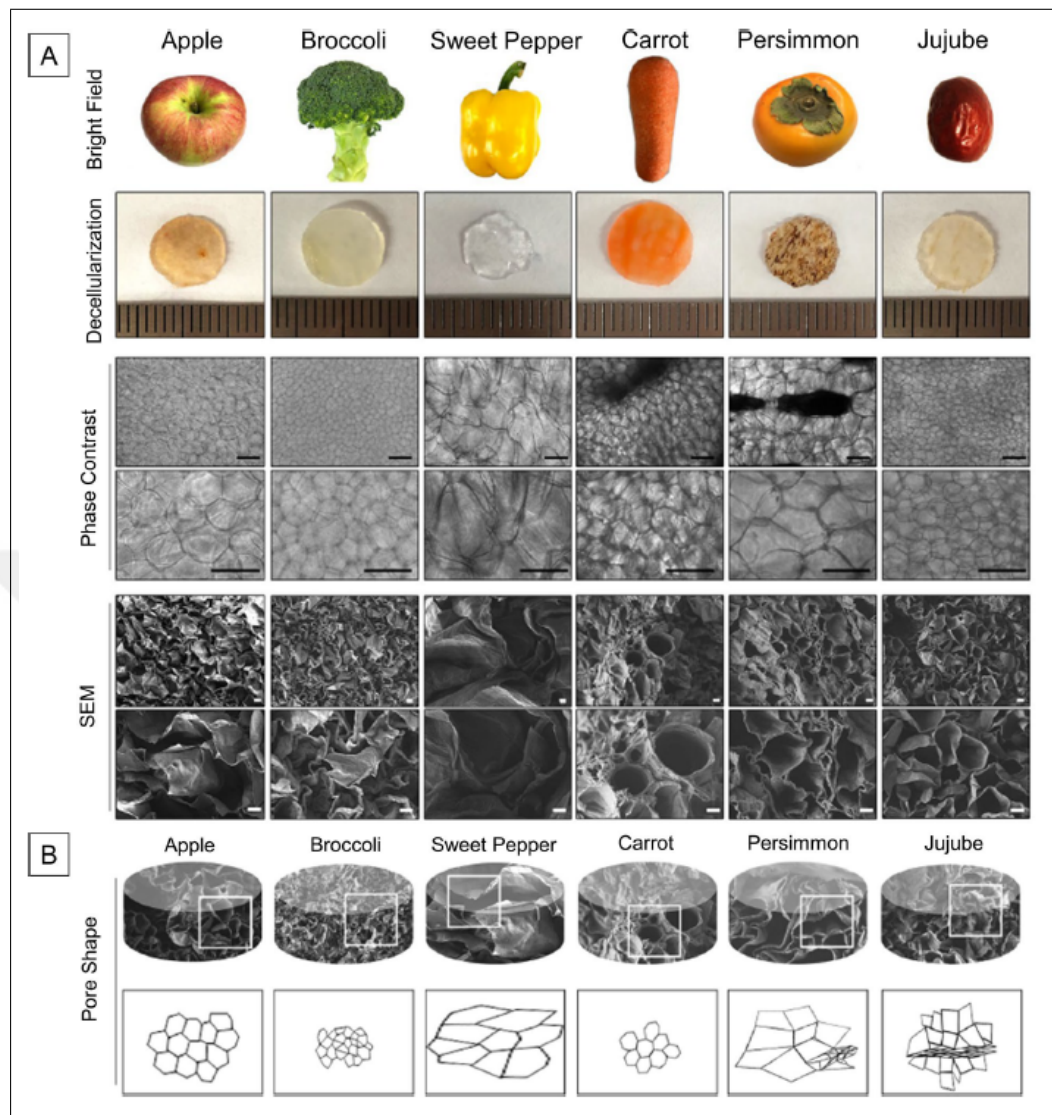


Figure 2.6 Plant-derived cellulose-based scaffolds have different pore structures with various sizes and shapes. A) Images of apple, broccoli, sweet pepper, carrot, persimmon, jujube before decellularization and after decellularization. Phase contrast images (magnifications: 100x and 200x) and scanning electron microscopic images of scaffolds obtained from decellularized plants (magnifications: 200x and 500x). B) Drawings of pore shapes and sizes of plant-derived scaffolds. [110]

cular networks. The first study that benefited from the similarities between plant and animal vessel structures was conducted by Gershlak *et al.* in 2017 (Figure 2.7). They demonstrated that stem cells differentiated on decellularized spinach leaves and became functional cardiomyocytes. The vascular structure of spinach and its perfusion ability provided a suitable platform for smooth muscle cells to proliferate and gain functionality which was the first step of neovascularization [59]. Dikici *et al.* also reported that decellularized baby spinach (*Spinacia oleracea*) leaves preparation was possible without

destroying their inherent vascular architecture and has shown the potential to stimulate neovascularization [60]. To develop a pre-vascularized scaffold, *Aptenia cordifolia* and spinach (*Spinacia oleracea*) leaves were recellularized by perfusion for vascular regeneration and the adhesion and growth of human red blood cells, human dermal microvascular endothelial cells, and human dermal fibroblasts were observed [60, 89]. Cabbage (*Brassica oleracea*) leaves also preserved their native vasculature after decellularization and supported the proliferation of endothelial cells which made them potential scaffolds for vascular grafting [88]. As another example, the microporous structure of chemically decellularized (by using SDS) *Ficus religiosa* and physically decellularized (by using scCO_2) spinach leaf skeletons encouraged attachment and proliferation of cells on the vascular surface, by proving that different approaches of decellularization can maintain structural integrity and adherent cell viability. Also, *Ficus religiosa* venation is reported to aid the transportation of oxygen and nutrients, ultimately resulting in a promising multi-layer 3D scaffold [97, 101]. Distribution of nutrients is still a fairly complicated challenge in tissue engineering, but recently, there have been substantial advancements and research in vascularized scaffold fabrication. The inner structure of plant leaf skeletons is proven to be a viable platform for vascularization-oriented cell culturing. Furthermore, Grilli *et al.* investigated the branched structure of plant leaves to observe whether they can provide a network of microchannels in a 3D scaffold. Wild fennel (*Foeniculum vulgare*), and dill (*Anethum graveolens*) were chosen due to the resemblance of their channel dimensions with human vessels and decellularized leaves were embedded in methacrylated gelatin (GelMA) for mimicking adipose tissue by integrating a vascular system in a 3D scaffold. Obtained platforms remained stable for 5 weeks and showed no cytotoxicity which were promising results in terms of using such scaffolds to simulate tissue microenvironment [114].

2.3.4.2 State-of-the-Art Biomaterials from Decellularized Plants. Cargo

Delivery Systems: Decellularized plants were further investigated as drug delivery platforms by Bai *et al.* Decellularized plant leaf and onion were loaded with polylactic-co-glycolic acid (PLGA)-based rapamycin nanoparticles and used in rat inferior vena cava patch venoplasty as an *in vivo* application model. Nanoparticle-perfused scaffolds

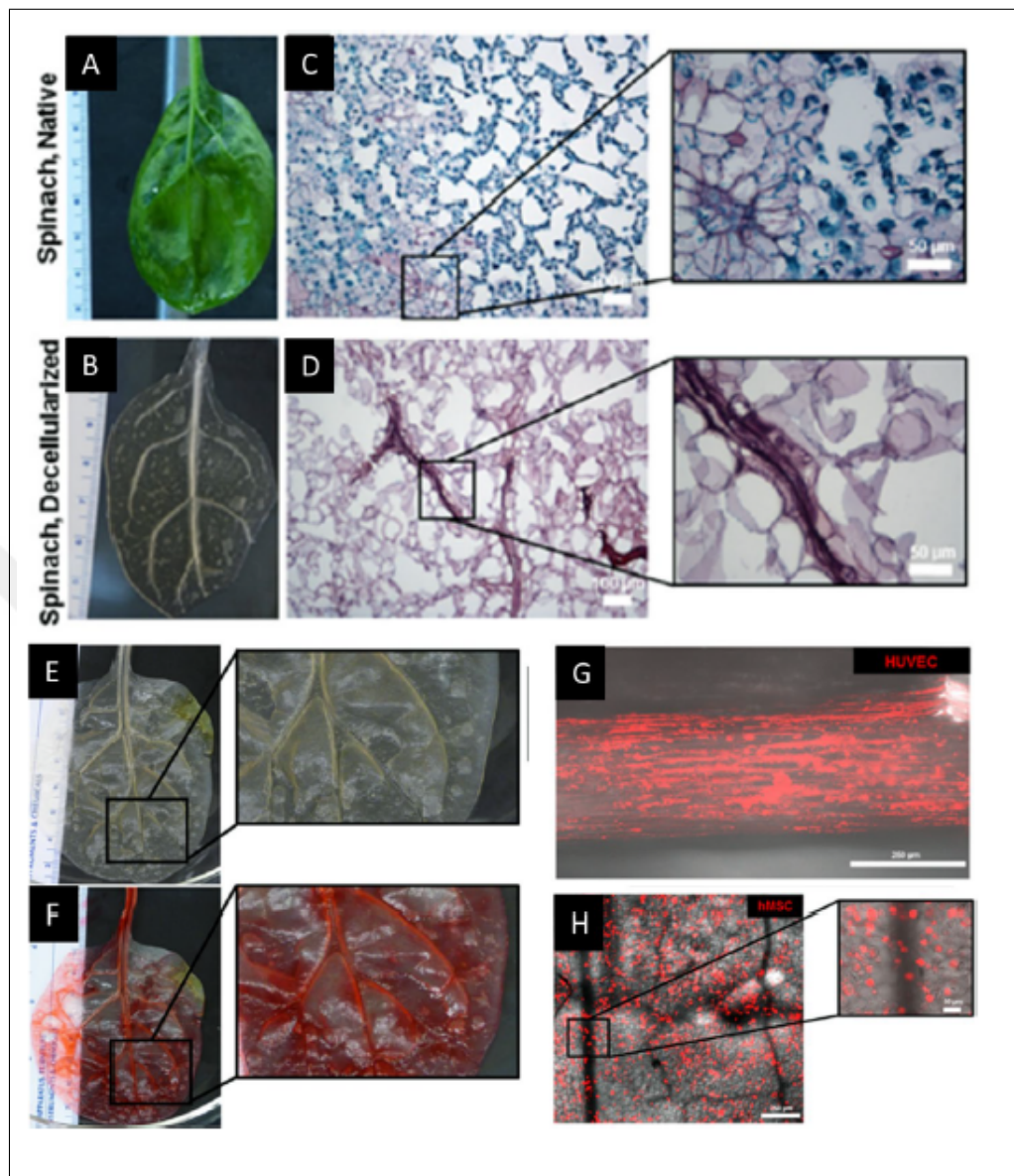


Figure 2.7 Appearances of spinach leaf (A) before and (B) after decellularization. Decellularized spinach leaves were stained with Safranin-O (red) and fast Green (bluish green). Blue dark spheres indicated chloroplasts, which are highly abundant in (C) native spinach leaves and mostly removed in (D) decellularized spinach leaves. Scale bars: 250 μm and 50 μm (insert). To demonstrate the perfusion capacity, Ponceau Red perfusion was applied to decellularized spinach leaves. Images of decellularized spinach (E) before and (F) after perfusion. Decellularized spinach leaves were seeded (G) human umbilical vein endothelial cells (HUVECs) by perfusion (scale bar: 250 μm) and (H) human mesenchymal stem cells (hMSCs) (scale bars: 250 μm for main image, and 50 μm for insert) [59].

decreased the neointima formation which can be described as scar tissue in tubular constructs such as blood vessels which proved the ability of nanoparticle loading and delivery of decellularized plants. Therefore, decellularized leaf and onion were suggested as a potential drug delivery platform and a vascular scaffold, respectively [115]. In another study, Predeina *et al.* developed a drug delivery and testing platform by us-

ing decellularized baby spinach leaves (*Spinacia oleracea*) due to their resemblance with human arterioles and capillaries (Figure 2.8A). They observed the behavior of loaded magnetic nanoparticles in complex vessel structure by using the spinach-derived model and suggested that this platform can enable targeted drug delivery. Furthermore, they concluded that such a model can also be used as an *in vitro* testing platform for monitoring the kinetics of pharmaceuticals [91]. Plant-derived cellulose scaffolds were also investigated as smart cargo delivery systems for growth factor release in a multitude of regenerative medicine by Mahendiran *et al.* After decellularization, platelet-rich plasma (PRP) was loaded in cellulose-based scaffolds and the effect of PRP was observed both *in vitro* and *in vivo*. Encapsulated PRP triggered the release of cytokines which promoted osteoblast growth and differentiation. Under *in vivo* conditions, PRP-loaded scaffolds showed enhanced collagen deposition and angiogenesis which proved the release of PRP [116]. Therefore, decellularized plants can also serve as microcarriers to encapsulate drugs and growth factors and for controlled release in the repair and regeneration of tissues.

Biosensors: Biosensors and soft actuators can be obtained by integrating decellularized plants with stimuli responsive materials. Zhao *et al.* conducted a proof-of-concept study (Figure 2.8B) by using decellularized spinach due to smooth surfaces that provide better contact area when patterning the surface of leaves with the temperature-responsive poly(N-isopropylacrylamide) (PNIPAM) polymer. Two experimental groups were used to control the change in morphology of materials when exposed to an external stimulus: 1) Nanoclay (NC)-PNIPAM, 2) Graphene oxide (GO)-NC-PNIPAM due to their temperature and light responsiveness, respectively. When ultraviolet (UV) light was applied and the temperature of the composite material was changed, twisting or bending occurred due to the strain mismatch between the swellable PNIPAM and non-swellable decellularized leaf. Biosensors can be designed by modifying decellularized plants with photothermal or thermal responsive hydrogels, utilizing the behavior of materials when subjected to environmental stimulus to control the alterations in their shapes and spatial organizations. Such biomaterials can be useful in tissue engineering and regenerative medicine as well as other biotechnological applications [117].

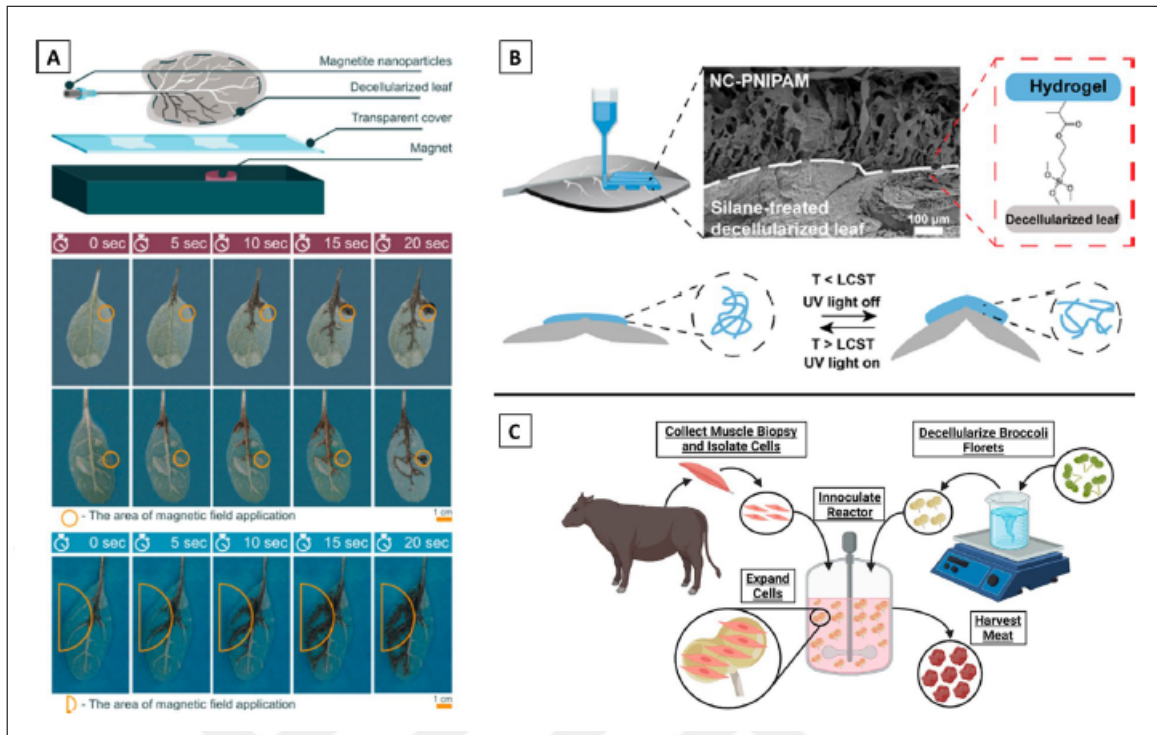


Figure 2.8 A) Drug delivery and testing platform developed by using decellularized baby spinach. Vascular bundles of decellularized leaves were filled with the 1.8% magnetite hydrosol. Experimental setup was prepared by placing magnetite hydrosol loaded leaves over neodymium magnet and behavior of magnetite hydrosol as a magnetic fluid was observed when a magnetic field applied. Magnetization hysteresis loop of the magnetite nanoparticles when 10 mm diameter round neodymium magnet (upper) and 47 mm diameter round NdFeB magnet (bottom) applied [91]. B) Proof-of-concept biosensor design by integrating decellularized spinach leaves with stimuli-responsive poly(N-isopropylacrylamide) (PNIPAM) polymer to control the shape of the material with external stimulus [117]. C) Main steps of lab-grown meat production. Isolation of muscle cells from animal, decellularization of broccoli florets, combining them in a reactor with dynamic conditions, growth of cells on decellularized broccoli to harvest meat [118].

3D habitats for nematodes: To study human diseases, model organisms such as nematodes (roundworms) have been used in laboratories due to genetic similarities. *Caenorhabditis elegans* (*C. elegans*) has been one of the commonly studied nematodes in standard laboratory conditions to observe the nictation behavior which can be described as moving the body in all directions while standing on the tail to locate food sources. The problem with studying *C. elegans* in a laboratory is that their habitat cannot be mimicked since they are cultured on agar plates and fed with controlled bacteria colonies. In nature, nematodes grow in rotting plants, especially in rotting fruits, and nourish the bacteria. Therefore, their physical traits and behaviors depend on the environment that they are found and to obtain more realistic results, they need to be cultured in a milieu similar to their native habitat. Inspired by mammalian cell culture

studies on decellularized plants, Guisnet *et al.* suggested that decellularized plants can provide 3D habitable scaffolds for the multi-generational culture of *C. elegans* in the laboratory. They chose Macintosh apples (*Malus domestica*) due to porosity, size, and structural integrity. Also, the transparency of decellularized apples made it easier to observe the behavior of *C. elegans* since they were also transparent. As a food source, they conducted bacteria culture with *Escherichia coli* (*E. coli*) on decellularized apples to obtain a controlled microbial environment for better mimicking the habitat of nematodes without causing significant contamination. Some critical parameters were determined during experiments including the type of apples since they should be rigid and colorless after decellularization, the storage conditions, sterilization steps and bacteria density were crucial for preventing contamination. The proof-of-concept studies demonstrated that decellularized plants can serve as habitat for nematodes however, further research is necessary [85,119].

Lab-grown meat: Lab-cultured meat has drawn more attention since it offers a cost-effective and eco-friendly approach to the unmet global food need especially dietary protein sources. The main steps of culturing artificial meat in a laboratory include the isolation of cells from animals such as adipocytes, myocytes, myosatellite, or embryonic stem cells, the growth, and differentiation of these cells on scaffold biomaterials and finally processing them as food products. Different manufacturing methods have been used for obtaining scaffolds for cultured meat including the decellularization of animal and plant tissues [120]. The first study where lab-cultured meat was obtained from decellularized spinach was conducted by Jones *et al.* Primary bovine satellite cells were cultured on decellularized spinach and 99% viability was achieved after 14 days. They also suggested that the native vasculature of spinach leaves provided a suitable platform to develop a meat product by promoting cell growth and differentiation [92]. Furthermore, in another study, broccoli florets were studied as microcarriers due to their resemblance in size and density with traditional microcarriers to obtain lab-grown meat for the first time by Thyden *et al.* (Figure 2.8C). Cell culture was conducted by inoculating primary bovine satellite cells into a reactor that contained decellularized broccoli florets and culture medium, and then dynamic conditions were created. Cell viability results showed that decellularized broccoli florets enhanced cell proliferation.

In addition, their nutritional content made them more advantageous compared to other microcarriers for meat products [118]. Furthermore, non-decellularized plants were also studied for developing lab-grown meat products. Twenty plant types including leek, spinach, iceberg lettuce, etc. were seeded with muscle stem cells obtained from different animal sources. The adhesion, alignment, and differentiation of muscle cells on these plant-based scaffolds were observed and these scaffolds were patented for culturing meat in a laboratory environment [121].

2.3.5 Non-Decellularized Plants as Scaffolds

Recently, plant-based scaffolds have been investigated without decellularization in order to demonstrate the differences and similarities between native and decellularized plant-based scaffolds. Only a few studies were conducted yet, however, the preliminary findings were promising in terms of the non-cytotoxicity of plants even though their genetic material remained. James *et al.* investigated palm fibers (*Maniharia Saccifera*) without decellularization and whether they can be used as hernia mesh or tendon scaffolds. Alkali treatment was applied to remove the lignin content for enhancing hydrophilicity. Then, mouse fibroblasts, human vascular smooth muscle cells, and human adipose-derived mesenchymal stem cells were seeded on the palm fibers. Cell viability and biocompatibility assays that were conducted on the cell-seeded palm fibers did not show any cytotoxic effect after 21 days of cell culture [122]. Bai *et al.* and Xie *et al.* investigated *Epipremnum aureum* leaves to obtain vascular patches for drug delivery. At first, leaves were decellularized, loaded with drugs, and implanted *in vivo* as vascular patches. After the implantation, a rapid scar tissue formation was observed. However, when the same experiments were repeated without decellularizing the leaves, results showed that the foreign body reaction was similar but the drug absorption ability of leaves increased with a homogenous distribution. The administration of the loaded drug significantly decreased scar tissue formation. Therefore, they obtained a more efficient drug delivery system with higher absorption capacity when they used fresh leaves as vascular patches [115, 123].

In addition, plant-based biomaterials have been widely used for tissue engineering due to their unique mechanical properties and biochemistry. For example, plant polysaccharides such as carrageenan, pectin, and ulvan can provide mechanical strength while plant extracts can be used as crosslinking agents to assemble the scaffold. Moreover, plant proteins can replace the ECM proteins and enhance cell attachment. Secondary metabolites that consist of alkaloids, glycosides, flavonoids, phenolics, etc. induce cell proliferation and differentiation, and essential oils extracted from flowers, leaves or roots show high antimicrobial properties [50]. Since all these components have specific properties that can be useful in scaffold design, using the whole plant without removing anything from the structure should also be promising and worth investigating.

The cytotoxic effects of plants can be different from each other and even can change between individuals just like food allergies. Therefore, more extended research is necessary for observing the biocompatibility and immune reactions of hosts when plant-based scaffolds are used without decellularization.

2.3.6 Future Perspectives

Plants have very desirable structural and chemical properties for biotechnological applications and the number of studies on decellularized plants has significantly increased and continues to draw more attention. In the meantime, the commercialization of decellularized plants became important with increasing patent applications and the founding of start-up companies that develop products by using plant-derived cellulose-based biomaterials. Decellularization protocols for a variety of plants, surface functionalization methods, culturing mammalian cells on decellularized plant scaffolds, and their use in tissue engineering and drug delivery were all patented [124–128]. On the other hand, Spiderwort was founded as a startup and developed two products from decellularized plants: CelluBridgeTM and CelluJuveTM which promote the regeneration of the spinal cord and heal dermal wounds. In pre-clinical trials, CelluBridgeTM was accepted by U.S. Food and Drug Administration as a Breakthrough Medical Device

due to its effectiveness in motor recovery in small animals [129].

Despite the developments in the use of plant-based scaffolds for biotechnological applications, there are still some considerations and unclarified points that should be further investigated. First of all, even though *in vitro* cell culture experiments on plant-based scaffolds were promising, the number of *in vivo* studies is very limited. And also, biocompatibility and biodegradation of scaffolds might differ *in vivo* environments. Thus far, researchers reported that decellularized plants didn't show any sign of inflammation or foreign body response and the scaffolds didn't degrade after *in vivo* implantation and preserved their original shapes. However, these studies should be extended; and the cytotoxic effect of plants, immune response even allergic reactions of hosts, and degradation behavior of scaffolds should be studied *in vivo*. The variations in immunogenicity and the mechanical stability among plant species make it difficult to classify them and assess their potential for biomedical use. Particularly important, after decellularization, mechanical strength of some plants decreases significantly, which is a major limitation for their use as tissue scaffolds.

Another topic that should be clarified is whether the decellularization of plants is necessary. Since decellularization is mostly achieved by chemical treatments with detergent-based solutions, the residues might be harmful to host tissues. Also, the mechanical strength and flexibility can be changed in an undesirable way after decellularization in some plants. Therefore, if plants do not cause any inflammation when implanted, the elimination of the decellularization process might be possible. However, more studies are needed to determine the biocompatibility of native plants. Because the data did not represent all types of plants.

Last but not the least, plants used in these studies were obtained from grocery stores, and the cultivating conditions were different from each other which result in batch-to-batch variations. The soil and water contents, climate, and chemicals (pesticides, insecticides) that were used to induce the growth of plants might affect the chemistry, physical properties such as the size, and morphology of both decellularized and native plants. Using different batches in each experiment can cause misleading

results. Increasing sample sizes during experiments can standardize the results. Another approach might be cultivating plants in a controlled laboratory environment. The standardization of plant samples is important in terms of obtaining more consistent results from structural, chemical, and physical analyses.



3. MATERIALS AND METHODS

3.1 Plant Decellularization

3.1.1 Optimization of Lucky Bamboo Decellularization

Lucky Bamboo (*Dracaena sanderiana*) was obtained from a local store, sliced into 1-2 mm thick discs (with a diameter of 11-12 mm) and washed with Milli-Q water. To optimize the decellularization process, different chemical methods were investigated. For the first trial, different concentrations of sodium hypochlorite (bleach) solutions were applied as follows: 0.2%, 1%, 2%, and 5% for 24, 48, and 72 hours [90]. For the second trial, to minimize the damage to the cellulose structure of Lucky Bamboo, 6 different methods with sodium dodecyl sulfate (SDS), Triton-X-100 and sodium hypochlorite were used (Table 3.1) [16], [15]. For further experiments, Lucky Bamboo samples were decellularized with 0.1 % SDS for 5 days under agitation at room temperature (Figure 3.1). The samples were then washed with Milli-Q water until no detergent remained and with Phosphate Buffer Solution (PBS) twice. For further chlorophyll removal samples were treated with absolute ethanol at 65 °C overnight. The samples were then washed again with PBS to remove the excess ethanol and kept in PBS at +4°C until further use.

Table 3.1
Different chemical methods for Lucky Bamboo decellularization.

	Decellularization Methods
1	0.1% SDS for 3 days
2	1% SDS for 3 days
3	0.1% SDS for 2 days followed by 10% bleach+0.1% Triton-X-100 for 2 days
4	0.1% SDS for 5 days
5	1% SDS for 5 days
6	1% bleach for 5 days

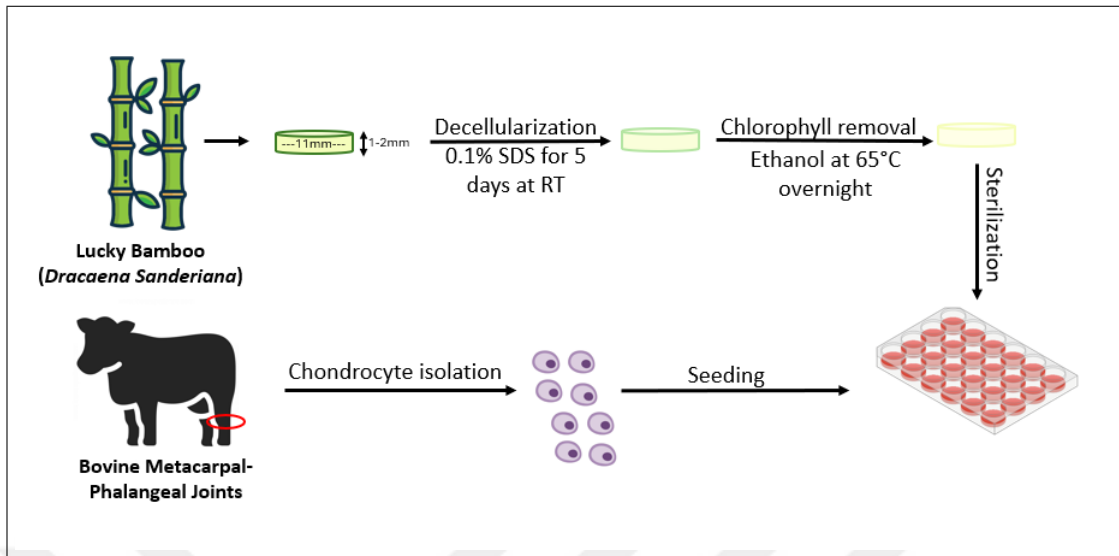


Figure 3.1 Schematic of Lucky Bamboo decellularization, chondrocyte isolation and seeding.

3.1.2 Evaluation of Decellularization Degree

3.1.2.1 DAPI staining. DAPI (4',6-diamidino-2-phenylindole) staining was applied to Lucky Bamboo samples before and after decellularization to demonstrate the plant cell reduction. Before staining, native and decellularized samples were fixed with a solution prepared with 4% paraformaldehyde (PFA), 0.1% Triton-X-100, 1% glutaraldehyde, 2 mM CaCl_2 , 1% sucrose. The samples were kept in the fixation solution overnight under agitation at room temperature. The samples were then rinsed with PBS three times to remove the remaining fixation solution. For the permeabilization step, samples were treated with 0.1% Triton-X-100 solution for 1 hour under stirring at room temperature. To prepare the DAPI staining solution, DAPI was dissolved in Milli-Q water with a concentration of 1 mg/mL and 10 μL of this stock solution was diluted in 10 ml of methanol. Samples were stained with the working solution for 5-15 minutes on a rocker in the dark, then excess dye was removed by washing with PBS. Zeiss Axio Observer 7 Fluorescence Microscope was used for imaging with the Aminomethylcoumarin Acetate (AMCA) filter (absorbance: 350 nm, emission: 450 nm). The images were taken at a magnification of 5X.

3.1.2.2 DNA quantification. Before DNA quantification, the samples were digested with enzyme solutions. Native and decellularized samples were cut into little pieces ($\sim 1/4$ of a disc), weighed, and placed in Eppendorf tubes. First, samples were digested with 30 μL of Proteinase K solution (0.5 mg/mL) in 720 μL of STE buffer (100 mM Tris (pH = 8.5), 5 mM Ethylenediaminetetraacetic acid (EDTA) and 200 mM NaCl) including 0.2% SDS at 60°C overnight [68]. For the second trial, the samples were first mechanically crushed with mortar and pestle and then Proteinase K solution was used as described in the first trial. Lastly, papain digestion was tried with three different concentrations, 80, 120, and 160 $\mu\text{g}/\text{mL}$. Samples were digested with 600 μL of papain in 20 mM ammonium acetate, 1 mM EDTA, and 2 mM dithiothreitol (DTT) (pH=6.2) for 72 hours at 65°C. For further digestion, the optimal method was determined as 80 $\mu\text{g}/\text{mL}$ papain for 72 hours at 65°C.

DNA quantification was then achieved with Hoechst dye 33258 assay [130]. Digested samples were diluted with a ratio of 1:6 in PBS and transferred to a dark 96-well plate. The dye solution was prepared by adding 2 μL of stock Hoechst dye (1mg/ml) to 20 ml of dye buffer (0.61g Tris, 0.19g EDTA in 500 ml of dH_2O , pH=7.4). 200 μL of dye solution was added to each well and the fluorescence intensity was measured at excitation/emission: 350/450 (nm) with a microplate reader (BioTek Synergy Neo2 Hybrid Multimode Reader). The DNA quantities were calculated based on the DNA standard curve (prepared from calf thymus DNA) and the values were normalized to the weight of the samples.

3.1.2.3 Nano-Computed Tomography (CT). Nano-CT was used to demonstrate the reduction in plant cells after decellularization. Native and decellularized samples were mounted with 1% agarose and scanned with phase contrast techniques (ZEISS Xradia 520, McGill University, Montréal, Canada). X-ray source voltage and power were 40 kV and 3 W. The exposure time was 30 seconds and the pixel size of the images were 1.97 μm and 1.53 μm for native and decellularized samples, respectively.

3.2 Material Characterization

3.2.1 Mechanical Testing

Compression tests were applied to measure the compressive strength, extensibility and Young's Modulus of native and decellularized Lucky Bamboo samples. A universal testing machine (United Systems STM-50kN) was used for measuring compressive strength and strain at failure (extensibility) and samples were compressed with a 5 kN load cell at a speed of 1mm/min. For measuring the Young's Modulus, a micromechanical tester (Mach-1) was used and samples were compressed with a 1.47N load cell at a speed of 0.3 mm/s.

3.2.2 Swelling Ratio

Native and decellularized Lucky Bamboo samples were freeze-dried overnight and the dry weights were measured. Samples were then placed in PBS at room temperature and the wet weights were measured periodically over a 24-hour period. The swelling ratio was calculated by using Eq. 3.1:

$$Swelling(\%) = (W_t - W_0) \times 100 / W_0 \quad (3.1)$$

where W_t is the weight of sample after time point t and W_0 is the weight of initial dry sample.

3.2.3 Pore Structure

The ethanol displacement method was used for determining the porosity (%). Native and decellularized bamboo samples were freeze-dried overnight and the dry weights were measured. Initial volumes of the samples were calculated by measuring the diameter and thickness of samples with a caliper. Samples were then placed in absolute ethanol for 24 hours at room temperature. The porosity (%) was calculated

by using Eq. 3.2:

$$Porosity(\%) = (W_f - W_i) \rho x 100 / V \quad (3.2)$$

where W_f is the final weight, W_i is initial dry weight, V is initial volume and ρ is the density of ethanol (0.785g/mL).

Pore sizes were determined by analyzing the Nano-CT images (described in Section 3.1.2.3.) of both native and decellularized Lucky Bamboo samples. For each group, three slices from top, middle and bottom of the sample were analyzed with Image J software. ~ 30 pores were measured for each slice and the average of all 90 measurements was reported as the sample pore size.

3.3 Cell Culture Studies

3.3.1 Chondrocyte Isolation

Chondrocytes were isolated from bovine metacarpal-phalangeal joints that were obtained fresh from a local slaughterhouse (Peel Meat Packers from Drayton, Ontario). Cartilage tissues were sliced from the condyle (Figure 3.2) and digested with 0.5% (w/v) protease in Ham's F12 media (Gibco) containing 25 mM HEPES (BioShop) for 1 hour followed by 0.15% (w/v) collagenase in Ham's F12 media containing 25 mM HEPES overnight. The digest was filtered with a cell strainer to remove the undigested particles or bone remnants. Cells were centrifuged (700 RCF for 7 minutes, 3 times), washed and then resuspended in F12 media with 25mM HEPES. Then, cells were cultured in F12+ 10% FBS media containing 1% antibiotics and 0.2% ascorbic acid [131].

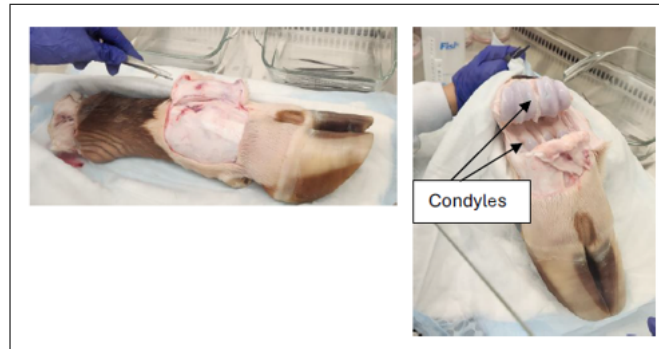


Figure 3.2 Bovine metacarpal-phalangeal joints where the chondrocytes were isolated.

3.3.2 Cell Seeding

Before cell seeding, decellularized Lucky Bamboo samples were sterilized with 70% ethanol followed by PBS wash twice. Then, ultraviolet light (UV; 254 nm) was applied to samples for 30 minutes. Viable chondrocytes were determined by trypan blue dye exclusion [132]. To optimize cell seeding, the following three methods were tried. First, a concentrated cell suspension was prepared (4×10^6 cells/ $60 \mu\text{L}$) and injected into the samples with a 20-gauge needle then the remaining media were added. Second, both sides of the samples were seeded with a concentrated cell suspension (2×10^6 cells/ $60 \mu\text{L}$). Cells were seeded on top of the samples and incubated for 3 hours, then, samples were flipped and the same number of cells were seeded on the other side of the samples. After incubating for another 3 hours the remaining media were added. Third, 4×10^6 cells were suspended in $400 \mu\text{L}$ and seeded regularly on samples. For further cell seeding, regular seeding (third) method was applied and samples were cultured at 37°C with 5% CO_2 and 95% relative humidity for up to 8 weeks with media exchanges every 2-3 days (1.5 mL).

3.3.3 Chondrocyte Adhesion and Proliferation on Decellularized Lucky Bamboo

For assessing the cell adhesion on decellularized bamboo, the effects of two conditions were investigated: 1) Collagen type II coating on samples to increase the

cell adhesion on bamboo, 2) Covering the well plate with agarose to prevent the cells from attaching to the plate [133]. Collagen type II coating was applied after sterilization of decellularized Lucky Bamboo samples. Samples were coated with 100 μL of collagen type II (Sigma) solution prepared in 0.1N acetic acid with a ratio of 1:3. Samples were left in the laminar flow hood overnight for acetic acid to evaporate and then washed with F12 media to remove the excess acetic acid. Well-plates were covered with 400 μL of 2.5% (w/v) agarose (Sigma) in F12 and then sterilized with UV light. The experimental groups are shown in Table 3.2.

Table 3.2
Experimental groups for measuring cell adhesion on decellularized Lucky Bamboo.

1	Tissue culture plate (TCP) (as control)
2	TCP+ Decellularized Lucky Bamboo
3	TCP+ Collagen Coated Decellularized Lucky Bamboo
4	Agarose (as control)
5	Agarose + Decellularized Lucky Bamboo
6	Agarose + Collagen Coated Decellularized Lucky Bamboo

3.3.3.1 Hoechst 33258 Assay. Cells were seeded on these samples with a concentration of 2.5×10^5 cells/mL (1×10^6 cells per sample). After 24 hours, samples were transferred to a new plate for further culture. The remaining media and agarose were digested with papain 80 $\mu\text{g/mL}$ papain and Hoechst dye 33258 assay was performed as described in section 3.1.2.2 to estimate the cell adhesion on decellularized Lucky Bamboo samples.

3.3.3.2 MTT Cell Proliferation Assay. Cell proliferation in decellularized Lucky Bamboo samples were assessed using the MTT kit (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Abcam). Samples were tested on days 1, 7, 14, 21, and 28 after cell seeding [134]. Culture media was removed and 50 μL MTT reagent with 50 μL of F12 were added to the samples. After incubating at 37°C for 3 hours, 150 μL MTT solvent was added and the plate was put on an orbital shaker for 15 minutes.

For the absorbance measurements, a clear 96-well plate containing the three replicates from each group was read at 590 nm with BioTek Synergy Neo2 Hybrid Multimode Reader.

3.3.3.3 Live/Dead Assay. Cell viability after 1,3,7 and 14 days of seeding was observed with Live/Dead Assay kit (Abcam). 3-4 μL of dye in 1mL F12 media were added to samples and incubated for 10-15 minutes at 37°C and 5% CO_2 . Confocal microscopy (Zeiss LSM 900 with Airyscan) was used for imaging.

3.3.4 Biochemical Analysis of Accumulated ECM

Chondrocyte-seeded decellularized Lucky Bamboo samples were harvested after either 4 or 8 weeks of culture, weighed and then digested with papain (as described in section 3.1.2.2). Aliquots of the digest were assayed for DNA, collagen and glycosaminoglycan (GAG) content.

3.3.4.1 DNA Quantification. Hoechst dye 33258 assay was performed to quantify the DNA accumulation on decellularized Lucky Bamboo samples as described in section 3.1.2.2.

3.3.4.2 Collagen Quantification. Hydroxyproline assay was performed to quantify the collagen amount in cell-seeded decellularized samples [135]. Digested samples were hydrolyzed by adding equal parts of 6N HCl and heating to 110°C for 18-24 hours. The hydrolysate was neutralized by adding equal parts of 5.7N NaOH and diluted with distilled water (Final dilution ratio was 1:6.). L-hydroxyproline in HCl was used as standard solution and diluted in distilled water to obtain the standard curve. 100 μL of samples and standards were transferred to a clear 96-well plate. 75 μL Chloramine T solution was added for 25 minutes at room temperature. Then, 75 μL Ehrlich's Reagent solution was added at 65°C for 20 minutes and at room temperature for another 20

minutes. The absorbance was read at 560 nm with BioTek Synergy Neo2 Hybrid Multimode Reader. Hydroxyproline amounts were calculated based on the standard curve and the values were normalized to the weight of the samples. The collagen content was calculated assuming that 10% of collagen mass is hydroxyproline [131].

3.3.4.3 Proteoglycan Quantification. Dimethylmethylen Blue (DMMB) dye assay was performed to quantify the sulphated-glycosaminoglycans (sGAG) that were produced by chondrocytes [136]. Before performing the assay, two different treatments were applied to samples to increase the detection ability of the DMMB dye assay. First, samples were digested with papain as described previously in section 3.1.2.2. and diluted with a ratio of 1:6 in 1% bovine serum albumin (BSA). Second, the GAG extraction was applied as follows: Samples were treated with 4M guanidine hydrochloride at 4°C overnight then the liquid parts were transferred to new tubes and samples were kept in absolute ethanol (3:1 ethanol: guanidine hydrochloride) at 4°C overnight. Samples were then centrifuged at 14000 rpm for 30 minutes at 4°C. After discarding the supernatant, samples were rinsed with 70% ethanol and centrifuged again at 14000 rpm for 10 minutes at 4°C. The second step was repeated three times and then samples were resuspended in 4M guanidine hydrochloride. Both digestion and extraction methods were tried with regular samples and also with freeze-dried (with liquid nitrogen) and crushed samples. Then, the following assay was performed: Chondroitin sulfate B sodium salt in PBS was used as the GAG standard solution. 10 μ L of standard solutions and digested or extracted samples were transferred to a clear 96-well plate. DMMB dye was dissolved in ethanol and 0.2% formic acid. 200 μ L DMMB dye was applied to each well and the absorbance was read at 525 nm with BioTek Synergy Neo2 Hybrid Multimode Reader. The sGAG amounts were calculated based on the standard curve and the values were normalized to the weight of the samples.

3.3.5 Histology & Immunohistochemistry

Harvested chondrocyte-seeded decellularized Lucky Bamboo samples were fixed with 4% PFA overnight and kept in 70% ethanol until tissue processing. Tissue processing was done with 70, 80, 95, and 100% ethanol treatments followed by xylene and paraffin treatments by using Leica TP1020 Automatic Tissue Processor. Then, samples embedded in paraffin were sliced with rotary microtome with a thickness of 10 μm .

For histological analysis, sections were stained with Hematoxylin & Eosin (H&E) (cell nuclei and ECM) or Safranin-O (proteoglycans) or Picro Sirius Red (collagens) or Masson's Trichrome (collagenous connective tissue) (Abcam) according to the manufacturer's instructions.

For immunohistochemistry, paraffin sections were rehydrated, and antigen retrieval was performed with 0.25 units/mL chondroitinase (Sigma) for 1 hour at 37°C followed by 0.25 units/mL keratinase (Sigma) for 30 minutes at 37°C. Sections were then blocked with 1% BSA (Sigma) in PBS for 30 minutes at room temperature. Slides were incubated with primary antibody; rabbit anti-bovine collagen type I (Abcam; ab138492, 1:500 or 0.875 mg/mL) or collagen type II (Abcam; ab34712, 1:250 or 1 mg/mL) or mouse aggrecan core protein (DSHB; 1-C-6, 0.5 $\mu\text{g/mL}$) at 4°C overnight. Slides were then incubated with the secondary antibody, goat anti-rabbit or goat anti-mouse HRP polymer (Abcam; ab214880 or ab214879) for 1 hour at room temperature. Slides were developed with the DAB substrate kit (Abcam, ab64238) according to the manufacturer's instructions, dehydrated, and mounted with Permount media.

3.3.6 Mechanical Testing After Cell Culture

The change in Young's modulus after cell culture due to cell and ECM accumulation and the effect of the culture period were assessed with compression testing as described in section 3.2.1. After culturing samples for 4 and 8 weeks, 10 samples for

each group were tested for Young's Modulus and the average values were calculated.

3.4 Statistical Analysis

All data was analyzed and visualized using Prism (GraphPad) and presented as mean $\pm$ standard deviation. Unpaired t-tests were used to compare two independent groups and one-way analysis of variance (ANOVA) with Tukey's post-hoc corrections was used for multiple comparisons.



4. RESULTS

4.1 Plant Decellularization

4.1.1 Optimization of Lucky Bamboo Decellularization

First, different bleach concentrations were tried for the decellularization and it was observed that bleach was too destructive for Lucky Bamboo samples. After treating the samples with 5%, 2%, and 1% of bleach, samples started to break down and eventually the cellulose structure disintegrated. Since the aim of decellularization is to remove nuclear material while keeping the structural properties intact, bleach treatment with these concentrations was found not suitable for Lucky Bamboo decellularization.

For the second trial, the methods indicated in Table 3.1 were applied. As seen in Figure 4.1, bleach-containing methods again caused structural disintegration in samples. On the other hand, SDS treatments were more efficient since the samples lost their color and the following ethanol treatment provided further chlorophyll removal which resulted in transparent samples without causing any disruptions to the cellulose structure.

For further experiments, 0.1% SDS treatment for 5 days followed by absolute ethanol treatment at 65°C overnight (for further chlorophyll) was applied as decellularization procedure (Figure 4.2) since an efficient reduction in plant cells was achieved with a lower concentration of detergent.

4.1.2 Evaluation of Decellularization Degree

4.1.2.1 DAPI Staining. DAPI staining was performed to observe the remaining nuclear material after the decellularization. Both native and decellularized Lucky Bam-

boo were stained with DAPI dye which binds to DNA of cells. As seen in Figure 4.3, the autofluorescence of Lucky Bamboo samples interfered with the results. In addition to plant cells, the pore structure was also stained and made impossible to detect the reduction in cellularity. Therefore, DAPI staining method was found unsuitable for measuring the DNA amounts of these samples.

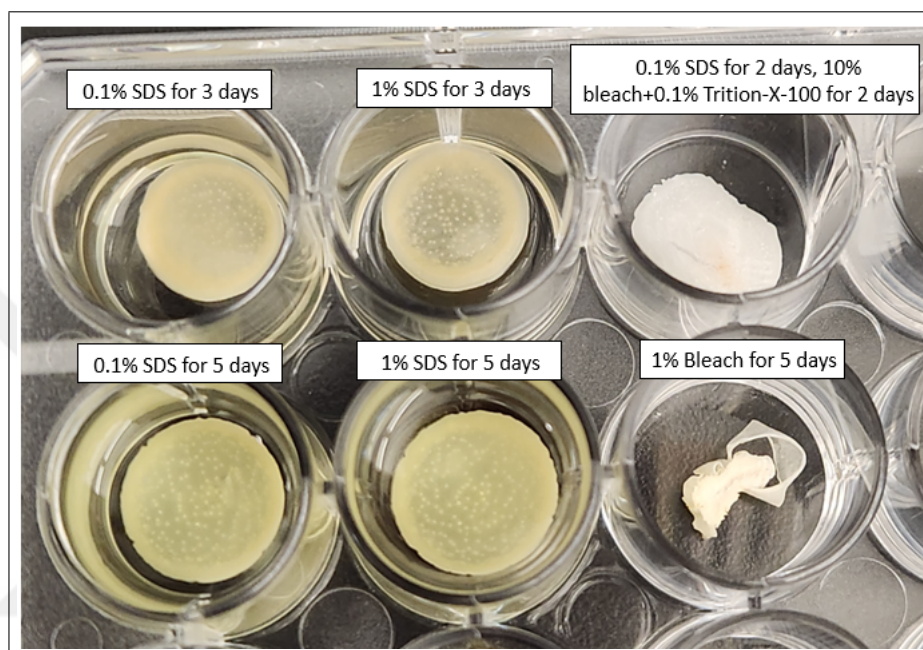


Figure 4.1 Visual assessment of Lucky Bamboo samples after different chemical treatments (After the decellularization, all samples were treated with absolute ethanol at 65°C.).



Figure 4.2 Visual changes in Lucky Bamboo before and after decellularization, A) native, B) after 0.1%SDS treatment, C) after absolute ethanol treatment.

4.1.2.2 DNA Quantification. DNA amounts were measured as 12.14 ± 2.82 , and 2.75 ± 1.46 ng DNA/mg sample for native and decellularized samples, respectively

(Figure 4.4a). DNA quantification confirmed the significant decrease in DNA amounts after 0.1% SDS treatment for 5 days followed by ethanol treatment overnight.

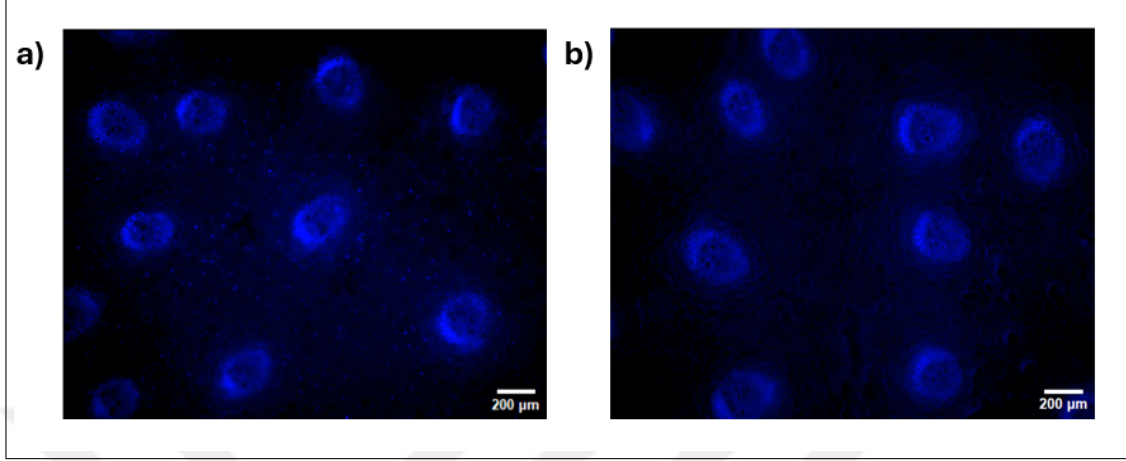


Figure 4.3 DAPI staining of a) native b) decellularized Lucky Bamboo samples demonstrating substantial autofluorescence. (Magnification: 5X).

4.1.2.3 Nano-Computed Tomography (CT). Nano-CT scanning (Figure 4.4b) also verified the significant reduction in cell amounts after applying the decellularization protocol. In addition, nano-CT images of native and decellularized bamboo samples demonstrated the three-dimensional (3D) structure that was composed of vascular bundles and remained intact after decellularization.

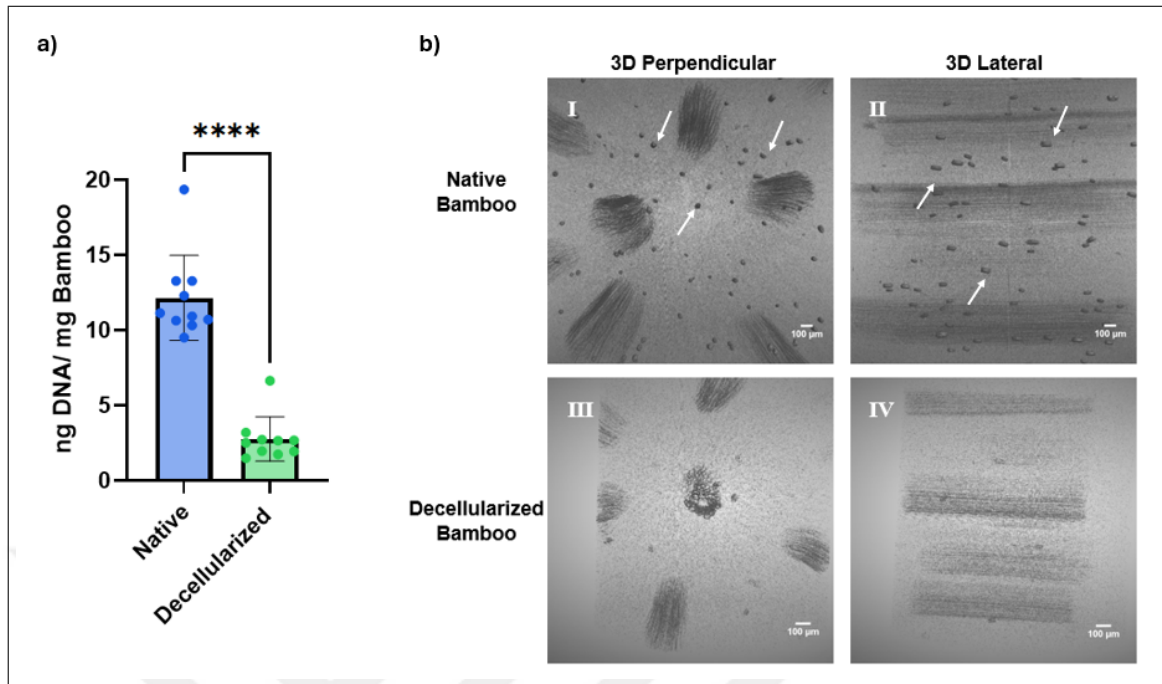


Figure 4.4 a) DNA Quantification with Hoechst assay for evaluating the decellularization degree of bamboo samples (**** $p < 0.0001$) b) Nano-CT scan images of native and decellularized bamboo samples (optical magnification: 4X).

4.2 Material Characterization

4.2.1 Mechanical Testing

To determine the effect of decellularization on mechanical properties, the Young's modulus, compressive strength and extensibility of native and decellularized Lucky Bamboo samples were measured. Young's moduli of native and decellularized samples (Figure 4.5a) were measured as 2.05 ± 0.55 and 0.89 ± 0.38 MPa, and the compressive strength (Figure 4.5b) were 11.34 ± 2.54 and 3.67 ± 2.98 MPa before and after decellularization, respectively. Strain at failure was measured as 0.71 ± 0.064 and 0.55 ± 0.214 , before and after decellularization (Figure 4.5c).

4.2.2 Swelling Ratio

The average swelling ratio of native and decellularized Lucky Bamboo samples was measured as 836.16% and 558.78% respectively, after keeping them in PBS for 24 hours (Figure 4.5d). The decellularization process with chemical and heating treatments caused a change in the swelling capacity of bamboo samples.

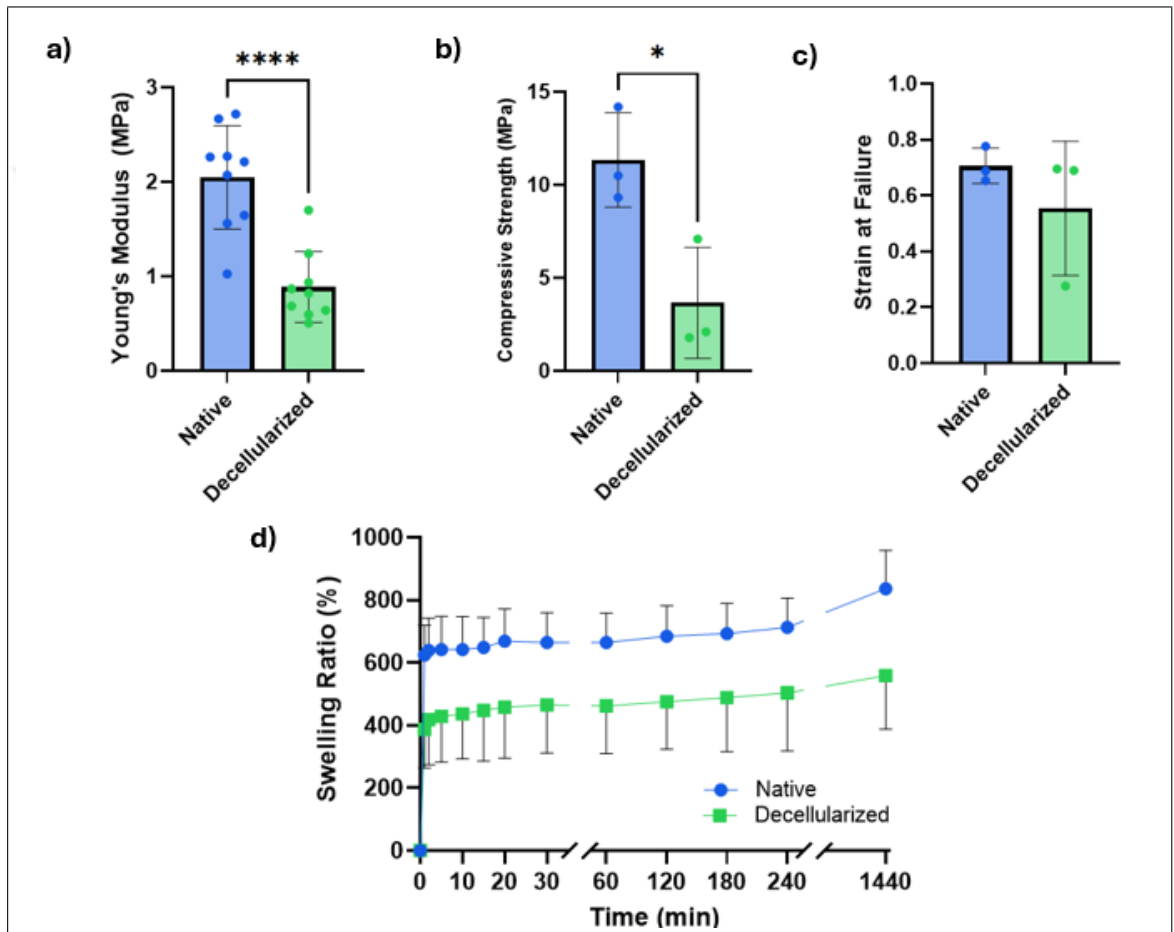


Figure 4.5 Mechanical test results; a) Young's modulus, b) compressive strength (* p<0.05, **** p<0.0001), c) strain at failure and d) swelling ratio (%) for native and decellularized bamboo samples.

4.2.3 Pore Structure

The pore diameters of native and decellularized bamboo samples were determined by analyzing the nano-CT images (Figure 4.6a-b). Pore size distribution in native and decellularized bamboo samples was demonstrated (Figure 4.6c-d) and the average pore diameters were calculated as $23.43 \pm 8.65 \mu\text{m}$ and $25.73 \pm 7.53 \mu\text{m}$, respectively (Table 4.1).

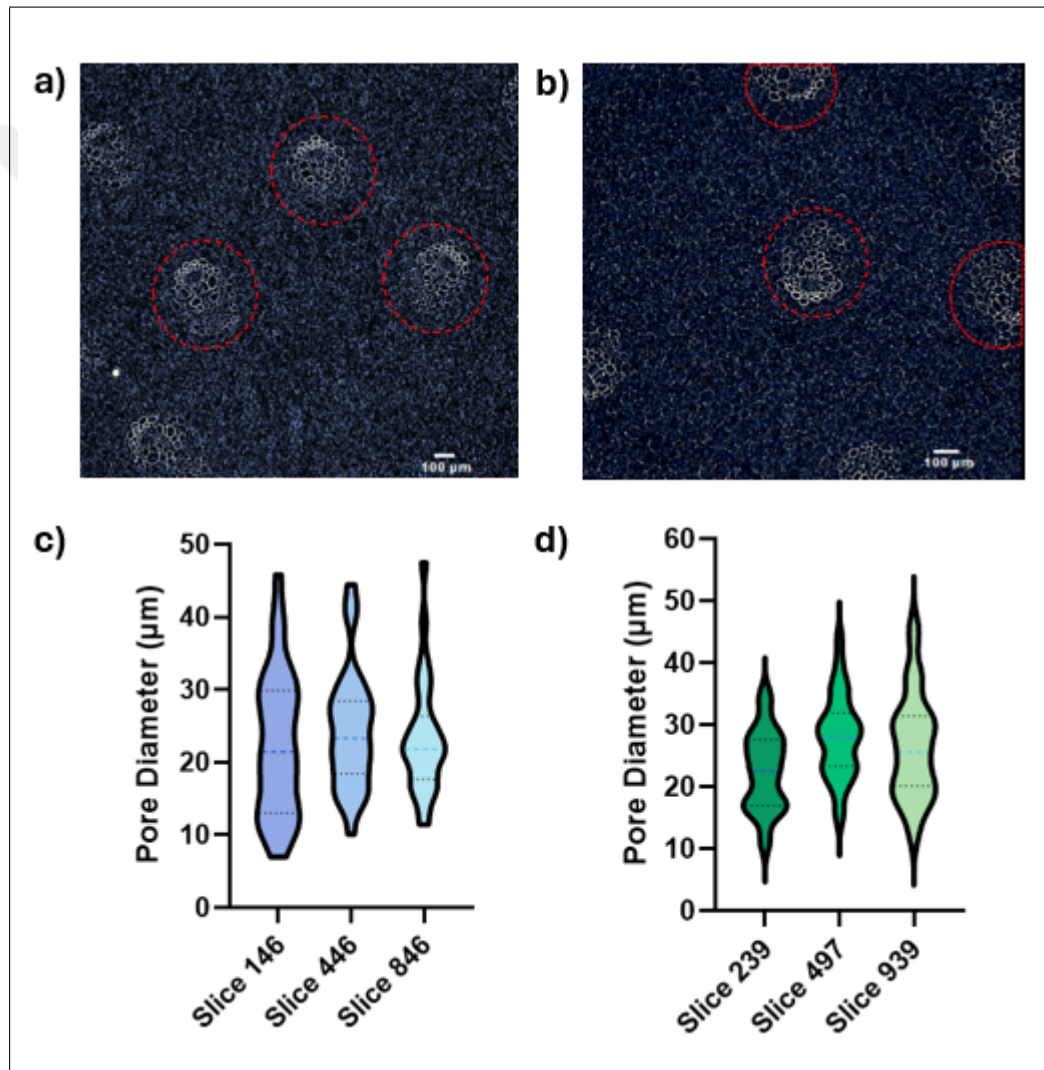


Figure 4.6 Cross-sectional slices from nano-CT images demonstrate the pores for a) native and b) decellularized bamboo samples (red dotted lines show the vascular bundles with larger pores), the distribution of pore sizes in three different slices belonging to c) native and d) decellularized bamboo samples.

The porosity values of native and decellularized Lucky Bamboo samples were measured as $79.86 \pm 13.22\%$ and $86.46 \pm 10.18\%$, respectively (Table 4.1). The difference

in overall porosity was not significant but the slight increase after decellularization can be explained by the removal of nuclear material and plant proteins after decellularization. Both the porosity percentage and pore diameters did not change significantly, which proved that the pore structure remained intact after decellularization.

Table 4.1

The total porosity (%) and average pore diameters for native and decellularized Lucky Bamboo samples.

Bamboo Sample	Porosity (%)	Average Pore Diameter (μm)
Native	79.86 ± 13.22	23.43 ± 8.65
Decellularized	86.46 ± 10.18	25.73 ± 7.53

4.3 Cell Culture Studies

4.3.1 Chondrocyte Adhesion and Proliferation on Decellularized Lucky Bamboo

Live/Dead assay was performed on Lucky Bamboo samples decellularized with 0.1% SDS treatment for 3 or 5 days, to observe the cell viability after 1, 3, 7, and 14 days of chondrocyte seeding as seen in Figure 4.7. However, the autofluorescence of bamboo interfered with Live/Dead assay results. Since the patterns of porous structure fluoresced too, it was difficult to determine whether they were chondrocytes or just the cellulose structure itself. However, the chondrocytes could be clearly seen inside the pores since there were voids before seeding cells. Even though it was shown that chondrocytes can attach on decellularized Lucky Bamboo samples, the results were not conclusive in terms of demonstrating the cell viability.

The chondrocyte adhesion on decellularized Lucky Bamboo samples was measured with DNA quantification (Figure 4.8a). 36.49 ± 15.24 % of seeded chondrocytes adhered on decellularized bamboo in 24 hours compared to TCP which was accepted as 100% cell adhesion. The effect of collagen type II coating on the cell adhesion was also

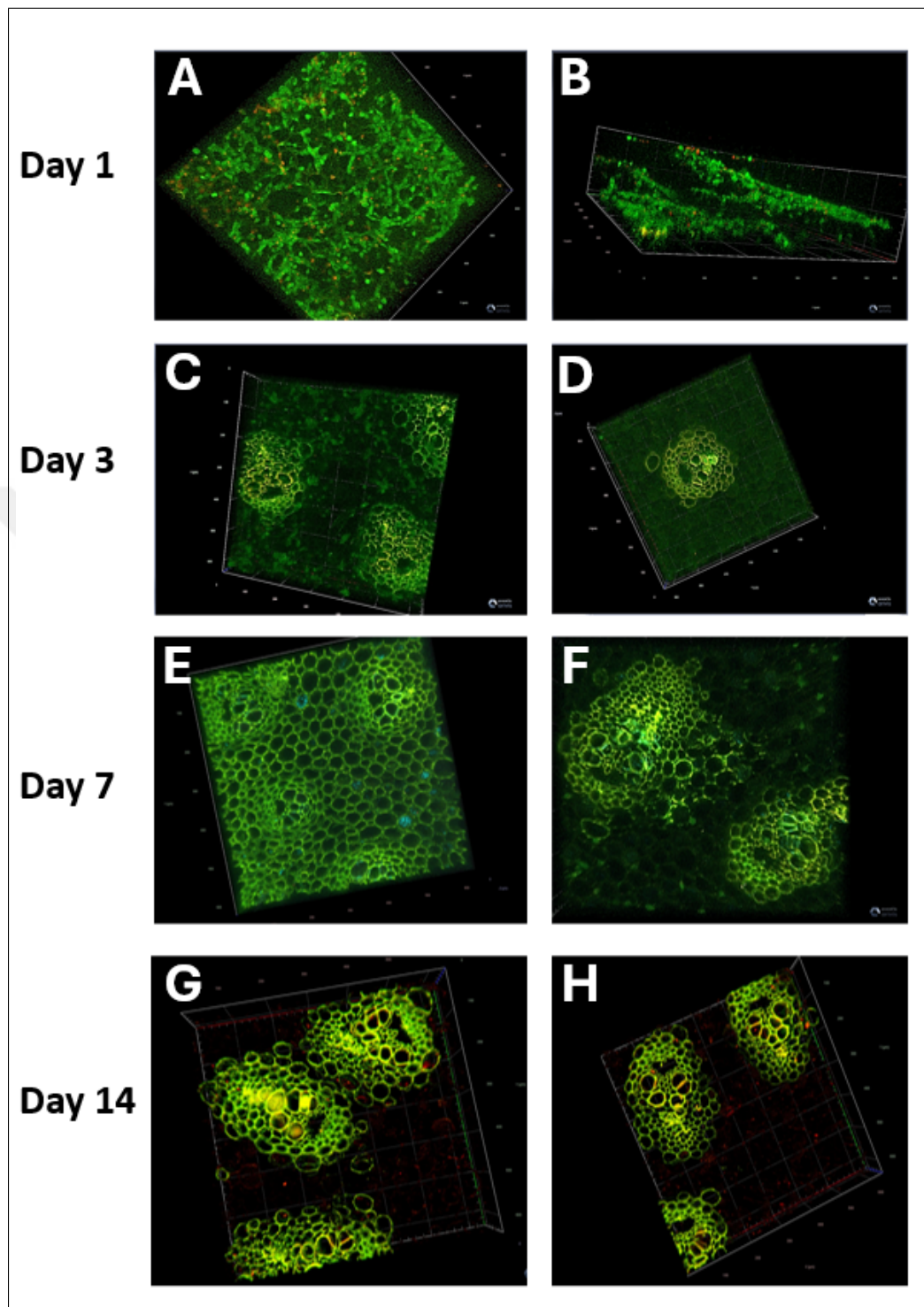


Figure 4.7 Live/Dead assay images for chondrocyte-seeded samples after 1,3,7 and 14 days. A, B, D, F, and H show the samples decellularized with 0.1% for 5 days. C, E, and G show the samples decellularized with 0.1% for 3 days.

assessed and cell adhesion was measured as $28.67 \pm 3.45\%$ which was lower compared to bare samples. In addition, the influence of covering the well plate with agarose

on cell adhesion was also investigated and the cell adhesion was slightly higher with $41.58 \pm 6.61\%$ but it wasn't a significant difference. Therefore, for further experiments, decellularized bamboo samples were seeded without any coatings.

The chondrocyte proliferation in decellularized Lucky Bamboo samples was investigated for 28 days (Figure 4.8b). Cell proliferation was stable for the first 21 days and significantly increased on day 28 which showed the cells can stay viable and expand in decellularized Lucky Bamboo scaffolds.

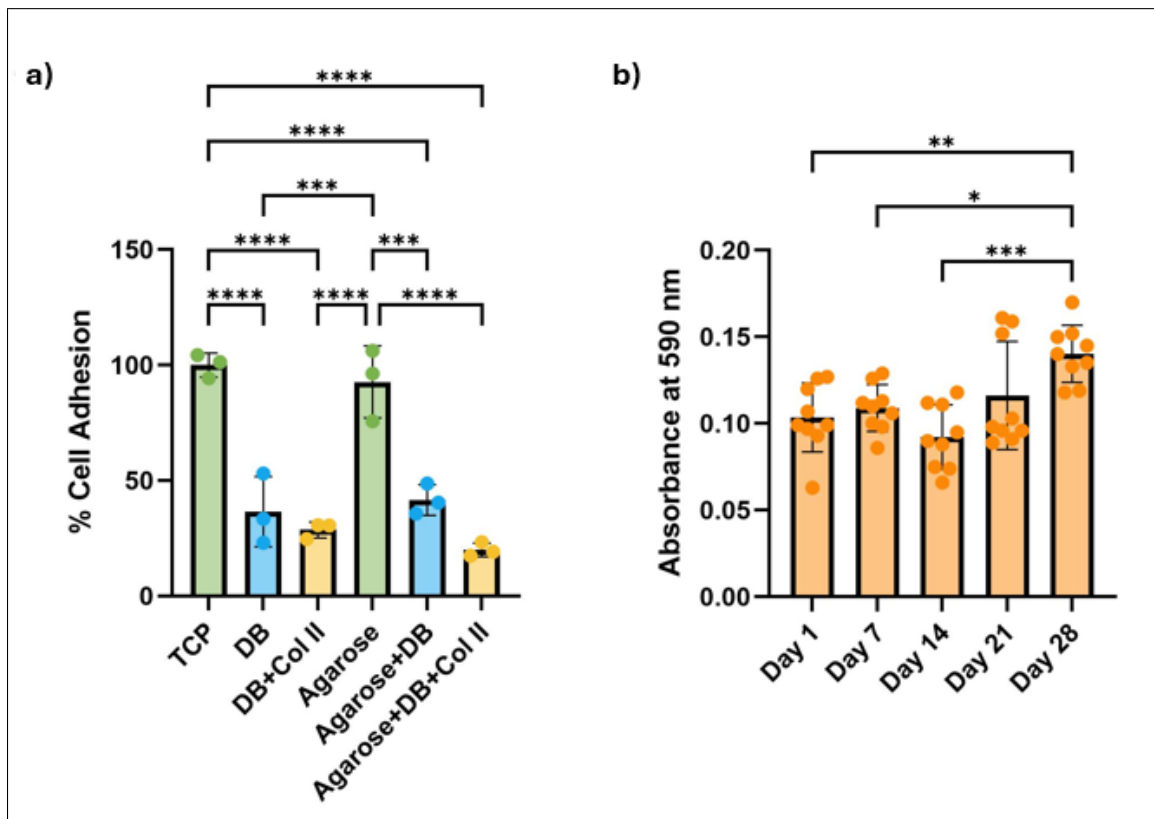


Figure 4.8 a) Chondrocyte adhesion on decellularized bamboo samples as well as the effect of collagen type II coating and covering the well plate with agarose on cell adhesion (*** $p < 0.0005$, **** $p < 0.0001$) (TCP: Tissue culture plate, DB: Decellularized bamboo, Col II: Type II collagen), b) MTT cell proliferation assay results for decellularized bamboo samples that were seeded with 10×10^6 cells/mL chondrocytes (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$).

4.3.2 Biochemical Analysis of Accumulated ECM

ECM accumulated within decellularized Lucky Bamboo scaffolds was analyzed biochemically to quantify DNA, collagen and sGAG contents after 4 or 8 weeks in

culture, compared to control decellularized scaffolds. DNA amounts accumulated in decellularized Lucky Bamboo after culturing cells for 4 and 8 weeks were measured as 41.93 ± 12.31 and 104.4 ± 21.93 ng DNA/mg sample, respectively (Figure 4.9a). There was a significant ($p < 0.0001$) increase (≈ 2.5 -fold) in DNA quantity considering the decellularized bamboo had 2.75 ± 1.46 ng DNA/mg sample before cell seeding.

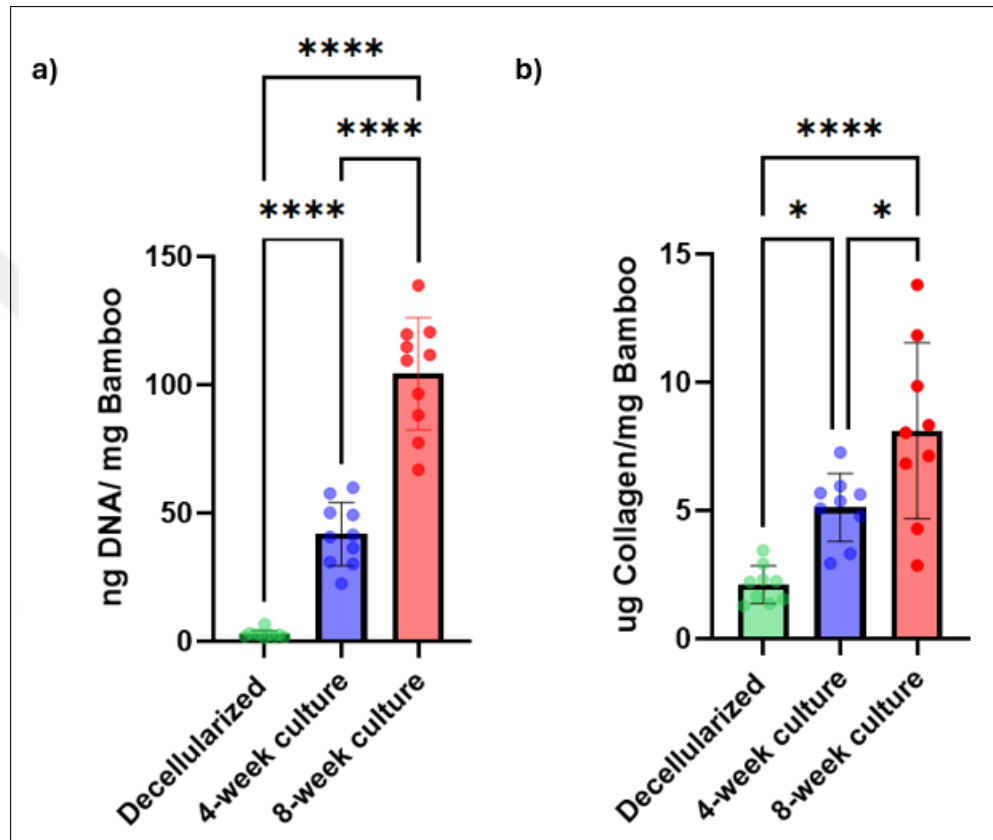


Figure 4.9 a) DNA quantification with Hoechst assay b) Collagen quantification with hydroxyproline assay results for decellularized and chondrocyte-cultured (4-8 weeks) decellularized bamboo samples (* $p < 0.05$, **** $p < 0.0001$).

Figure 4.9b demonstrates the total collagen amounts detected in decellularized and cell-seeded bamboo samples. The collagen amount detected in decellularized Lucky Bamboo (2.11 ± 0.73 μg collagen/mg bamboo) was negligible since collagen is not present in plant cell wall. On the other hand, there was a significant increase (≈ 1.6 -fold) in collagen amounts after 4 and 8 weeks of culture with 5.11 ± 1.33 and 8.11 ± 3.43 μg collagen/mg sample, respectively.

To measure the proteoglycan amount produced by cells during the culture pe-

riod, sulphated-GAG content was detected with the DMMB dye method on papain-digested samples. The average sGAG amounts were measured as 1.1 ± 0.72 , 0.81 ± 0.22 and $1.02 \pm 0.15 \mu\text{g GAG/mg bamboo}$ for decellularized, 4-week and 8-week cultured samples, respectively (Figure 4.10a). There wasn't a significant difference but sGAG amounts were incoherent between the groups. The values obtained from decellularized samples (control group) were in a wide range which showed that DMMB dye interfered with some compounds in the plant cell wall.

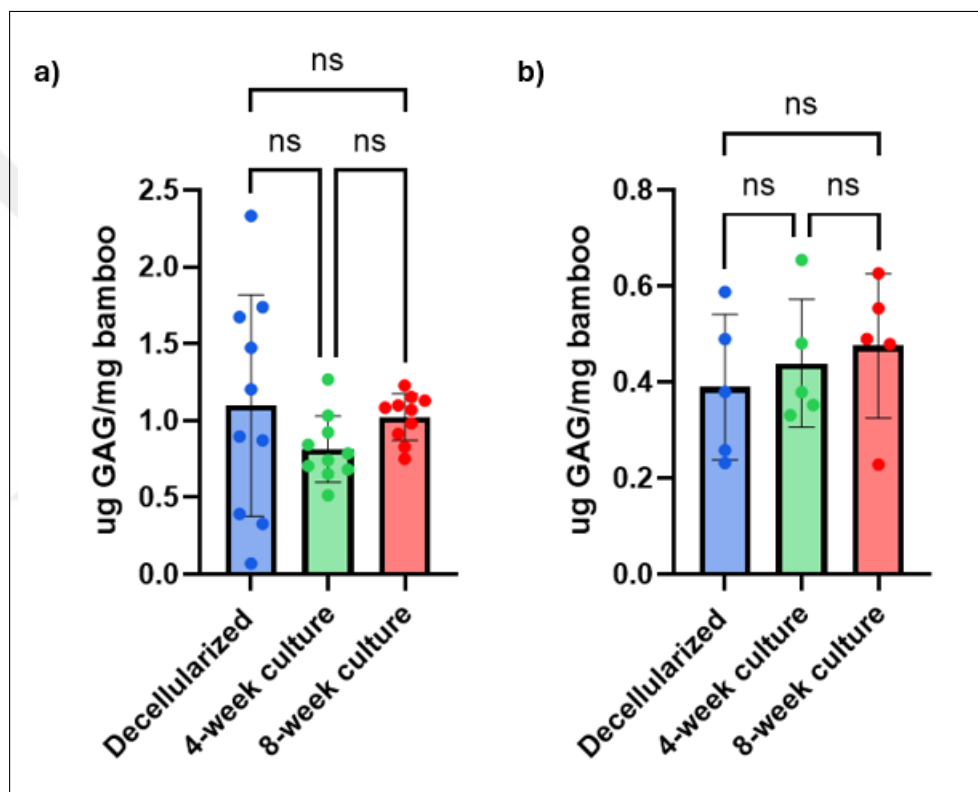


Figure 4.10 sGAG quantification with DMMB dye assay for decellularized and chondrocyte-cultured (4-8 weeks) decellularized Lucky Bamboo samples prepared by a) Papain digestion b) GAG extraction with guanidine hydrochloride (ns: non significant).

For the next trial, the GAG extraction method was tried with guanidine hydrochloride and then the DMMB method was applied to prevent the interaction of DMMB dye with the samples. As seen in Figure 4.10b, the average sGAG amounts were measured as 0.39 ± 0.15 , 0.44 ± 0.13 and $0.48 \pm 0.15 \mu\text{g GAG/mg bamboo}$ for decellularized, 4-week and 8-week cultured samples, respectively. There wasn't any significant difference between the groups but also the detection ability decreased with this method since the measured amounts were very low. Therefore, it was determined

that the GAG extraction method didn't work for these samples as well. It was concluded that the DMMB dye assay is not compatible with decellularized Lucky Bamboo samples.

4.3.3 Histology & Immunohistochemistry

4.3.3.1 Histology. Cell proliferation and ECM formation were shown with histological analysis as well. H&E staining images (Figure 4.11) showed that cells started to expand inside the decellularized bamboo samples after 4 weeks of culture but they were still concentrated at the seeding side of the sample. After 8 weeks, a homogenous cell distribution throughout the sample was achieved. Masson's trichrome staining results (Figure 4.11) also confirmed an increase in ECM formation (red: cell nuclei and proteoglycans, blue: collagen) after 8 weeks of culture.

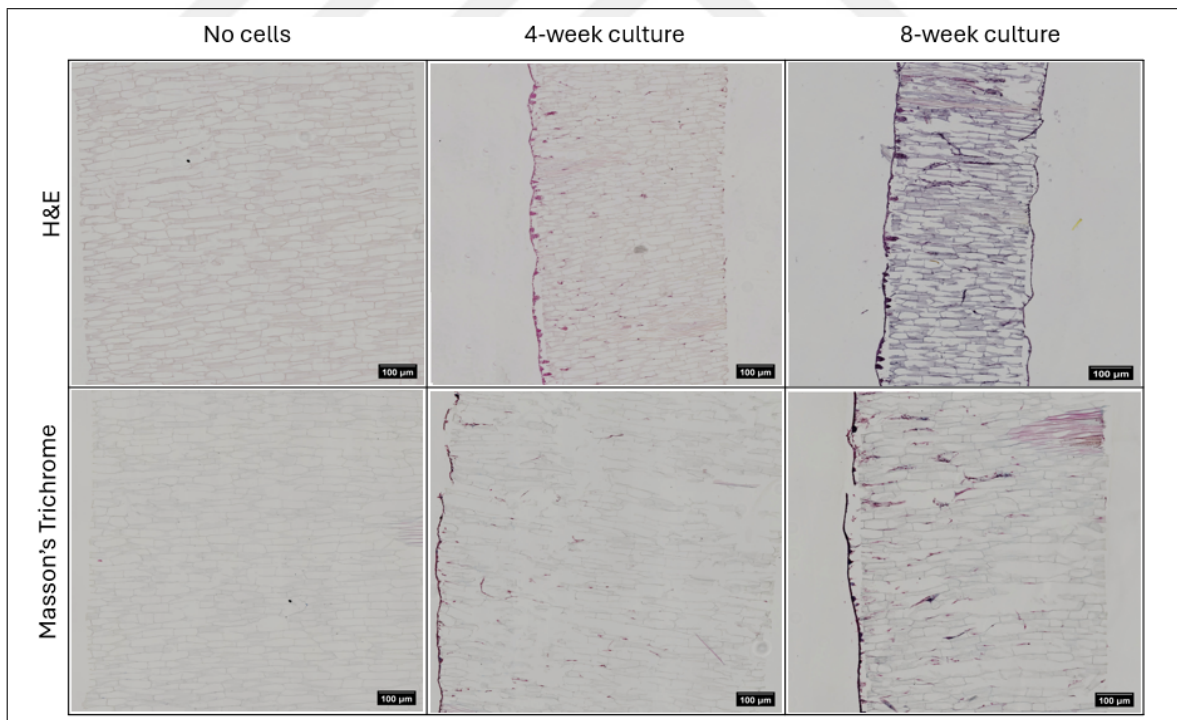


Figure 4.11 Connective tissue staining images; H&E (top) and Masson's trichrome (bottom) for decellularized, 4 and 8-week cultured bamboo samples (Magnification: 10X).

In addition, H&E staining of cell-seeded decellularized Lucky Bamboo after 8-

week culture (Figure 4.12; horizontal section parallel to the surface) demonstrated honeycomb-like patterns with resident chondrocytes, suggesting that surface topography played a role in cell proliferation.

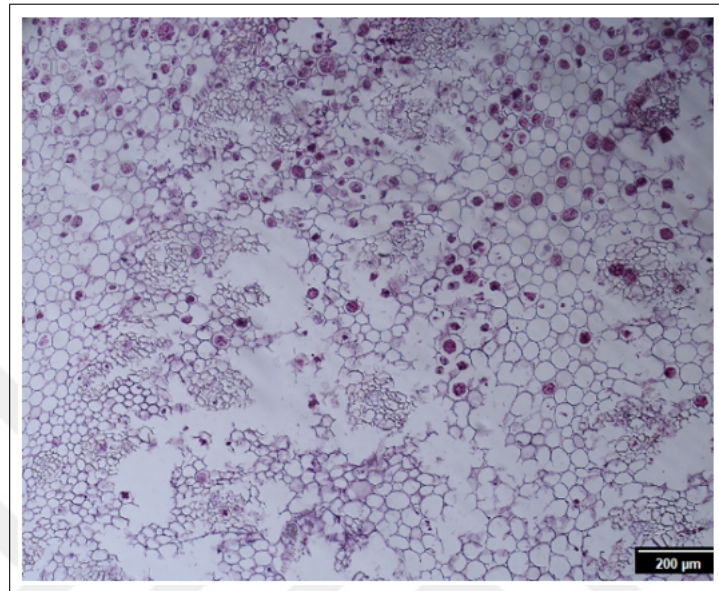


Figure 4.12 H&E staining results of cell-seeded, decellularized lucky bamboo after 8-week culture (Magnification: 10X).

Safranin-O (Figure 4.13a) and Picrosirius Red (Figure 4.13b) staining were performed to stain proteoglycan and collagen content produced by chondrocytes in decellularized Lucky Bamboo samples.

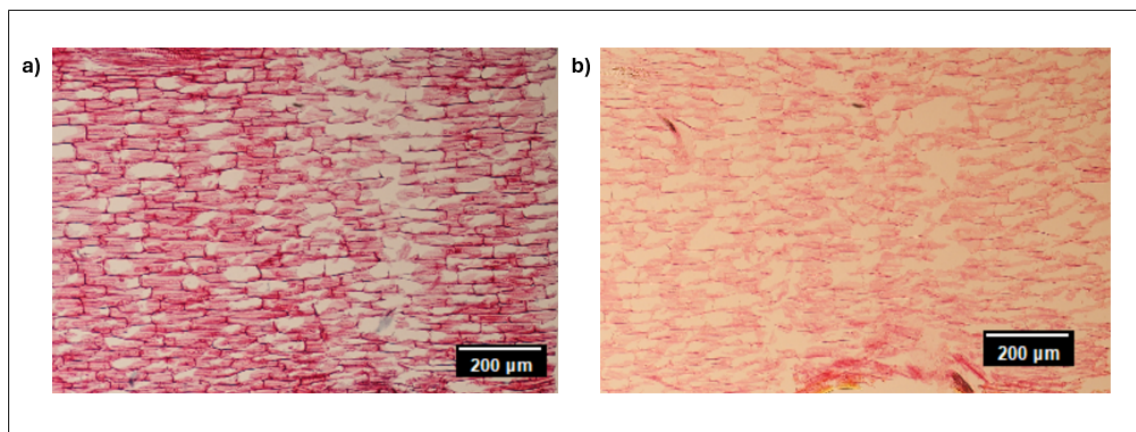


Figure 4.13 a) Safranin-O and b) Picrosirius red staining for decellularized Lucky Bamboo (control group without cell culture) (Magnification: 10X).

However, both dyes stained the negative control samples (not seeded with chon-

drocytes) which made it impossible to observe the proteoglycan and collagen formation since there was a significant background staining. Therefore, these methods were not appropriate for this study since the aim was to show the chondrocyte behavior on Lucky Bamboo samples.

4.3.3.2 Immunohistochemistry. For immunohistochemistry, the chemiluminescent method was used with HRP-polymer antibodies instead of the fluorescence method since the autofluorescence of bamboo interferes with fluorescent staining. First, the counterstaining with Hematoxylin was applied, and it was observed that even the negative control group was completely stained (Figure 4.14). Since the colors of HRP-polymer antibodies and hematoxylin were similar, it was hard to tell the difference. Therefore, for further immunohistochemistry, counterstaining wasn't applied.

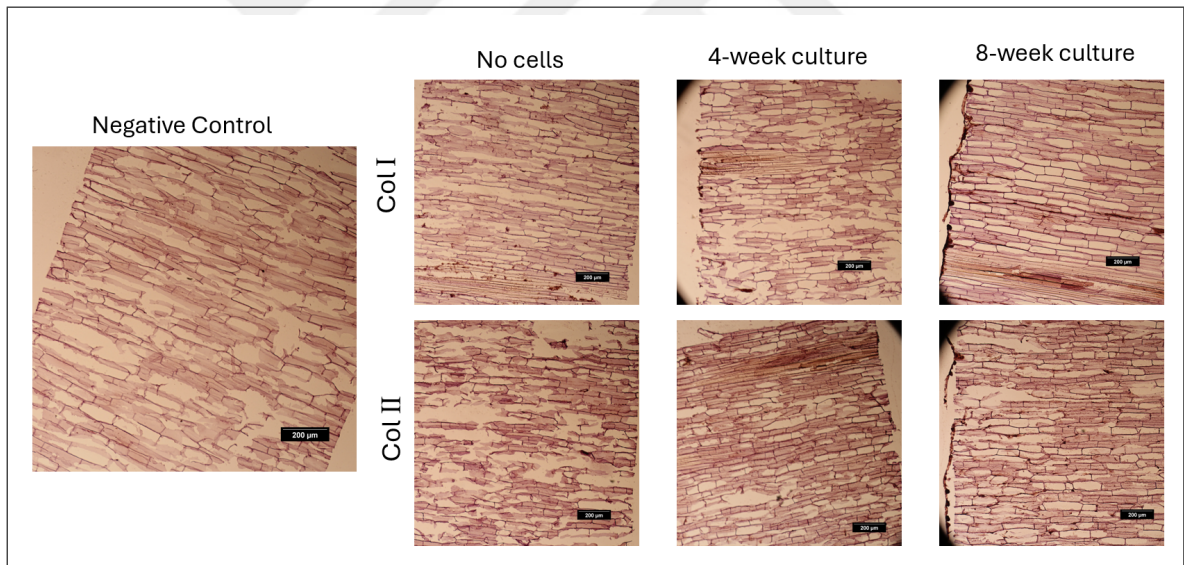


Figure 4.14 Type I and II collagen staining with Hematoxylin counterstaining of decellularized Lucky Bamboo seeded with chondrocytes (Cross-sectional images, magnification: 10X).

Immunohistochemical identification of collagen types (I and II) and aggrecan were similar to histology results (Figure 4.15). Scaffolds accumulated significant amounts of collagen type II and aggrecan which increased and was more homogenously distributed with time in culture. Collagen type I deposition was also observed with similar trends, but at significantly lower levels compared to collagen type II (Figure

4.15). Immunohistochemical staining indicated that the majority of cells remained as chondrocytes after 8 weeks of culture.

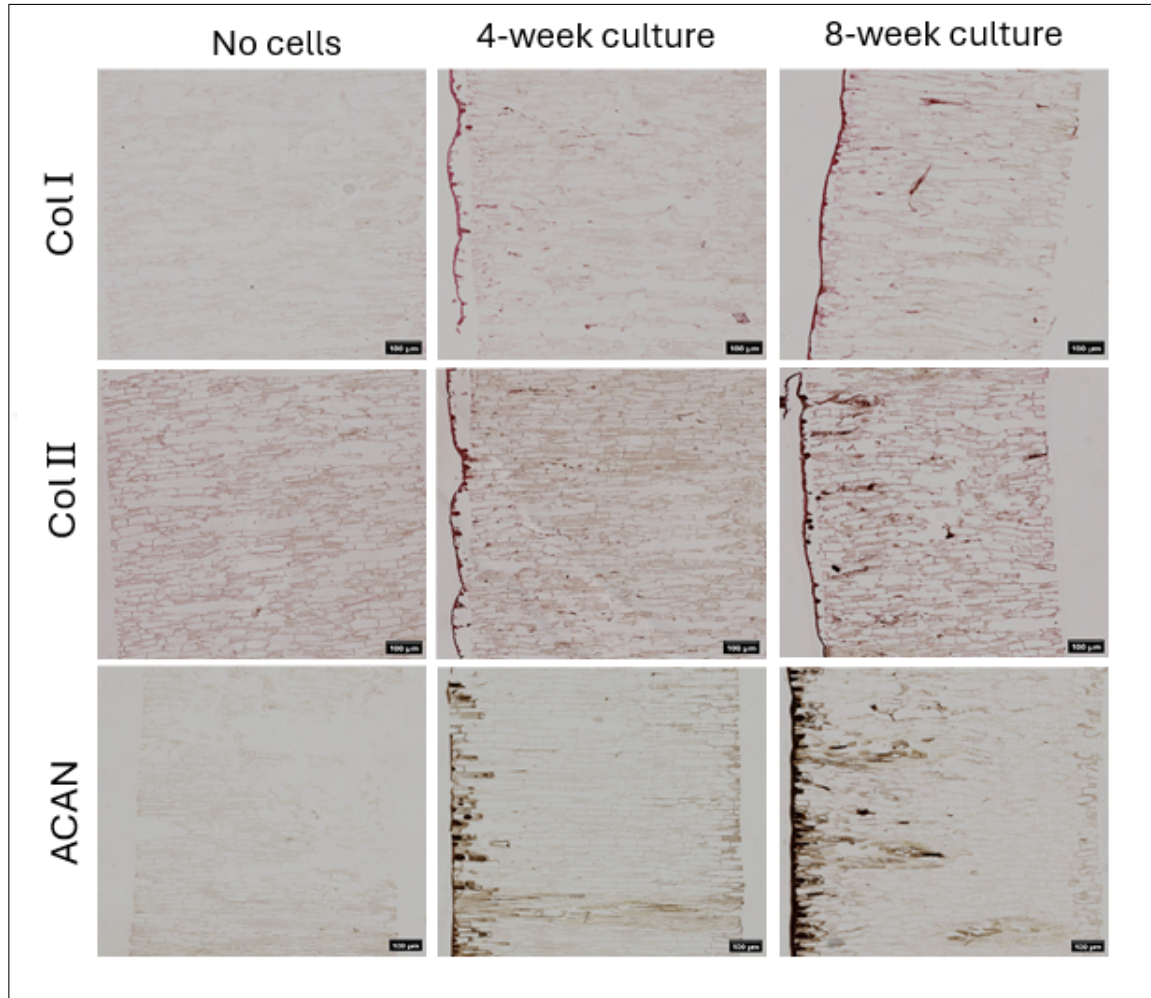


Figure 4.15 Immunohistochemical staining of chondrocyte-cultured decellularized bamboo samples for collagen type I (top), II (middle), aggrecan (ACAN) (bottom) Magnification: 10X.

4.3.4 Mechanical Testing After Cell Culture

Young's modulus measurements of decellularized Lucky Bamboo were repeated after cell culture (Figure 4.16). There wasn't a significant difference in Young's modulus after 4 and 8 weeks of culture. The Young's modulus of decellularized Lucky Bamboo (0.89 ± 0.38 MPa) was already in a similar range with native articular cartilage, and it remained at the same range after cell culture as well. However, the slight increase after 8 weeks (0.97 ± 0.28 MPa) compared to decellularized samples before cell seeding

can be explained with cell and ECM accumulation in the scaffolds since the Young's modulus approached even more to the Young's modulus of native articular cartilage.

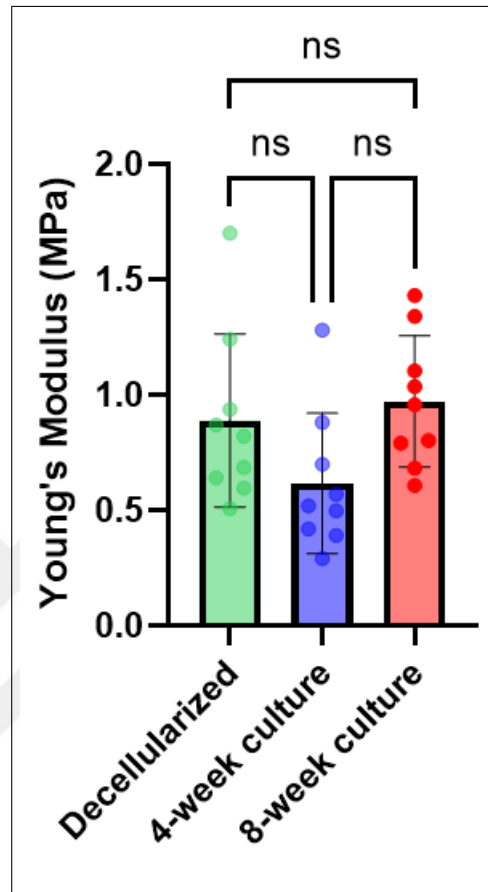


Figure 4.16 Young's moduli results of decellularized bamboo samples before and after cell culture (ns: not significant).

5. DISCUSSION

5.1 Decellularization of Lucky Bamboo

Decellularized plant-derived scaffolds have recently gained more attention and been investigated for various tissue engineering applications since they can provide diverse structural and mechanical properties based on tissue types [12]. In this thesis, Lucky Bamboo (*Dracaena sanderiana*) was chosen as a potential cartilage scaffold due to its pore structure and mechanical properties [15,16].

The decellularization procedure of Lucky Bamboo was optimized to obtain cellulose-based scaffolds with simple chemical treatments in a cost-efficient way. Different chemicals (bleach, Triton-X-100 and SDS) were tried while optimizing the decellularization procedure and it was observed that bleach and Triton-X-100 treatments caused disintegration in samples. On the other hand, SDS treatment significantly decreased the DNA amount and overall cell quantity without causing major disruption in the structure. Different concentrations of SDS (0.1% and 1%) were investigated and the effective lowest concentration (0.1%) was chosen to keep the damage to the structure at a minimum.

For animal tissue decellularization there are strict standards, such as DNA amounts should be less than 50 ng per mg dry ECM. However, this cannot be adapted to plant decellularization due to the diversity of plant structures [55]. As observed in this study, the DNA quantity of native lucky bamboo was measured as 12.14 ± 2.82 ng/mg tissue which was already less than 50 ng. 0.1% SDS treatment decreased the DNA amount by approximately 77% and nano-CT images confirmed the overall reduction in plant cells. Since keeping the structural properties of lucky bamboo intact was essential to obtaining a functional scaffold, the decellularization procedure was optimized with 0.1% SDS treatment followed by ethanol treatment for further chlorophyll removal.

5.2 Material Characterization

Material characteristics of Lucky Bamboo including swelling ratio, porosity and mechanical properties were analyzed to observe the changes after decellularization. The swelling ratio of bamboo samples decreased from 836.16% to 558.78% after decellularization. The chemical decellularization process and heating the samples caused a change in the swelling ratio since ionic strength, pH and temperature affect the swelling ratio [137]. However, the swelling ratio of decellularized Lucky Bamboo was in a similar range with the hydrogels used for chondrocyte culture in the literature [138].

In cartilage tissue engineering, one of the most challenging issues is to obtain cartilage scaffolds with the porous structure without compromising the mechanical properties and this has been a problem for both polymeric materials and decellularized cartilage tissues [14, 45]. However, decellularized Lucky Bamboo can provide the appropriate porosity with desired mechanical properties. Pore structure plays a significant role in chondrocyte adhesion and proliferation and the appropriate porosity range for cartilage scaffolds was reported as 70-98% [139, 140]. The average porosity of decellularized Lucky Bamboo wasn't significantly different from native one and measured as $86.46 \pm 10.18\%$ which perfectly fits the porosity range for chondrocyte culture. In addition, in the literature, the diameter of chondrocytes was reported as varying between $7\text{-}30\mu\text{m}$ depending on the anatomical layer [141]. The average pore diameter of Lucky Bamboo didn't significantly change after decellularization and was measured as $25.73 \pm 7.53\mu\text{m}$ which is in the same range with the diameter of chondrocytes that allows their proliferation inside the decellularized Lucky Bamboo scaffolds.

On the other hand, Young's modulus of native articular cartilage tissue was reported as $1.03 \pm 0.48\text{ MPa}$ whereas the compressive strength ranged between $0.8\text{-}15.5\text{ MPa}$ based on the tissue type [134, 138]. For Lucky Bamboo, Young's moduli were measured as 2.05 ± 0.55 and $0.89 \pm 0.38\text{ MPa}$ ($\approx 50\%$ reduction) for native and decellularized, respectively. After decellularization, the Young's modulus of Lucky Bamboo perfectly matched with the Young's modulus of native articular cartilage. Compressive strength values were measured as 11.34 ± 2.54 and $3.67 \pm 2.98\text{ MPa}$ ($\approx 67\%$

reduction) for native and decellularized Lucky Bamboo samples, respectively. They were also in the same range with native cartilage's compressive strength. Strain at failure of native cartilage changes between 30-50% and the strain at failure of Lucky Bamboo decreased from 71% to 55% after decellularization which is a similar range with native cartilage tissue [142]. Since the initial porosity of decellularized Lucky Bamboo scaffolds is high with $\approx 86\%$, it is expected that the mechanical properties of the scaffolds would approach closer that of native cartilage and become stiffer, stronger and less extensible with the increased cartilaginous ECM accumulation in time. This hypothesis should be confirmed in future studies. Overall, both the pore structure and mechanical properties of decellularized bamboo were suitable for chondrocyte culture.

5.3 Chondrocyte Adhesion and Proliferation in Decellularized Lucky Bamboo Scaffolds

To investigate the cell adhesion and proliferation on decellularized Lucky Bamboo, chondrocytes derived from the bovine metacarpal-phalangeal joint were seeded. Type II collagen coating was applied to samples before seeding to increase cell adhesion since it's the most prevalent ECM protein for articular chondrocytes [143]. However, the cell adhesion was measured as 28.67 ± 3.45 which was lower than the bare decellularized samples with the cell adhesion of $36.49 \pm 15.24\%$. The difference wasn't significant but collagen coating didn't improve the cell adhesion. Since the coating applied on the surface of the samples, the pores might be blocked preventing the cells from settling in the pores. On the other hand, agarose coating on well-plates was applied to increase the cell adhesion on decellularized bamboo since cells don't attach to agarose [133]. Decellularized Lucky Bamboo seeded in agarose-coated well plates showed a slight increase in cell adhesion with $41.58 \pm 6.61\%$ but it wasn't significant as well. Therefore, for further experiments, none of these coatings were applied because the main aim for using plants as scaffolds is their readily available structure that requires minimum modification.

MTT cell proliferation assay results demonstrated that cells remained viable and proliferated in the decellularized bamboo even after 28 days of culture. Accordingly, DNA assay after cell culture and connective tissue staining results (H&E and Masson's Trichrome) demonstrated that extending the culture period from 4 weeks to 8 weeks significantly increased the number of cells and ECM accumulation. The pore structure of the bamboo provided a 3D microenvironment for chondrocytes to grow. In addition, surface patterns may have affected cell proliferation as well. In the literature, it was reported that when chondrocytes were seeded on honeycomb-patterned polylactic acid (PLA) and poly(ϵ -caprolactone) films, they remained in spherical shape and produced abundant ECM compared to flat-surface films [144,145]. Similarly, honeycomb patterns of the Lucky Bamboo surface (Figure 4.12) supported chondrocyte proliferation since the cells accumulated inside the patterns and might contribute to the production of ECM contents as well.

5.4 ECM Accumulation in Decellularized Lucky Bamboo Scaffolds

ECM accumulation during the cell culture period was measured biochemically and histologically. A significant amount of collagen produced by cells after 8 weeks of culture was measured biochemically. However, DMMB dye assay results which was performed to quantify the sGAG accumulation (Figure 4.10) were not conclusive. Based on the literature, Lucky Bamboo could absorb sulfates from water and sulfated compounds accumulated in Lucky Bamboo [146]. Since SDS was used for decellularization, sulfates could have accumulated in samples and accumulated sulfate content might have interfered with the assay results since DMMB dye binds to sulfate groups [147].

Histologically, H&E and Masson's trichrome images demonstrated the cell proliferation and connective tissue formation in decellularized Lucky Bamboo. Similarly, chondrogenesis of mesenchymal stem cells (MSCs) and deposition of cartilaginous ECM on decellularized apple scaffolds were observed with histological analysis and gene ex-

pressions [148]. Furthermore, Picrosirius Red and Safranin-O staining were performed to demonstrate the collagen and proteoglycan amounts, respectively [149]. However, even though, collagen or proteoglycans are not present in the plant cell wall [150], the decellularized Lucky Bamboo samples that weren't seeded with chondrocytes also stained with both these dyes (Figure 4.13). According to the literature, these dyes also bind to lignified sections of the plant cell wall [151], therefore it was impossible to distinguish if the stained sections were related to chondrocytes or if they were the plant cell wall of the Lucky Bamboo. Therefore, it was determined that these methods are not compatible with decellularized Lucky Bamboo samples.

For further analysis, the types of collagens were determined with immunohistochemistry (IHC). The chemiluminescent method was used with HRP-polymer antibodies due to the autofluorescence of Lucky Bamboo samples. Autofluorescence was demonstrated with DAPI staining and Live/Dead images (Figure 4.3 and 4.7) as well as reported in the literature [152]. IHC results showed a significant amount of type II collagen production in the decellularized Lucky Bamboo after 8 weeks of culture. Collagen type II and aggrecan are hallmark macromolecules of the cartilaginous ECM [153,154] indicating that the seeded cells retained their phenotype which was proved that chondrocytes didn't dedifferentiate and remained functional. Similarly, type II collagen and aggrecan syntheses by chondrocytes on cellulosic scaffolds were observed in previous studies which verified that cellulose didn't induce the dedifferentiation of chondrocytes to fibroblasts [8,155,156]. In addition, some type I collagen synthesis was also observed which was expected since especially the chondrocytes at the flattened superficial zone of the cartilage tissue produce type I collagen to strengthen the ECM due to fluid flow at the superficial area compared to deeper zones [157]. Still, the majority of collagen content formed from type II which confirmed the accumulation of cartilage ECM.

The Young's modulus of decellularized Lucky Bamboo after 8 weeks of chondrocyte culture (0.97 ± 0.28 MPa) was still in the same range with the Young's modulus of native articular cartilage tissue [138]. Also, the slight increase compared to decellularized Lucky Bamboo (before cell seeding) (0.89 ± 0.38 MPa) could also verify the tissue formation, since previous studies showed the correlation between sGAG accumulation

and compressive properties of cartilage tissue [158].

5.5 Limitations

Even though results obtained in this thesis demonstrated that the structural and mechanical characteristics of decellularized Lucky Bamboo provided a suitable microenvironment for chondrocyte culture, there are also some limitations with biomaterials derived from decellularized plants as well as investigating them, that should be mentioned.

Plant samples were obtained from local stores therefore, high variability between the samples was observed. The cultivation conditions of each Lucky Bamboo might be different which changed its structural properties. High variation decreases repeatability of the experiments and increases standard deviations which made harder to get conclusive results.

Additionally, the chemical structure of plant cell wall made it more complex to adapt the experimental protocols that were developed for investigating cell behavior on other types of biomaterials. Most commonly used methods to investigate chondrocyte behavior didn't work for Lucky Bamboo samples. Some of the biochemical and histological assays couldn't be adapted for these samples since the chemicals interfered with the cell wall, especially the lignified regions.

6. CONCLUSION

The diversity of plant kingdoms and the simplicity of plant decellularization made it possible to obtain readily available tissue engineering scaffolds with desired material properties in a cost-efficient way. The promising results and increasing number of studies in this field for the last ten years can be the building stones for the application of decellularized plants in biomedical engineering and regenerative medicine in the future. In this thesis, the possible use of decellularized Lucky Bamboo as cartilage scaffolds was demonstrated. Lucky Bamboo could retain its cellulose porous structure after chemical decellularization and provide a suitable microenvironment for mammalian cells. The similar mechanical properties of decellularized Lucky Bamboo with native articular cartilage also played an important role in supporting cells to adhere and proliferate. After 8 week of culture, the biochemical and histological analyses confirmed that chondrocytes remained functional and produced ECM. More specifically, immunohistochemical analysis showed the accumulation of Collagen type II and aggrecan which are the most prevalent macromolecules found in chondrocyte ECM. Even though, further studies are necessary to produce these scaffolds in a larger scale with minimal variability, the results obtained in this thesis are very promising. As a conclusion, decellularized Lucky Bamboo can be a potential candidate for cartilage tissue engineering since it supported chondrocytes to proliferate and produce ECM due to its pore structure with appropriate pore sizes as well as desirable mechanical properties.

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