

# Plant Decellularization by Chemical and Physical Methods for Regenerative Medicine: A Review Article

## Abstract

Fabricating three-dimensional (3D) scaffolds is attractive due to various advantages for tissue engineering, such as cell migration, proliferation, and adhesion. Since cell growth depends on transmitting nutrients and cell residues, naturally vascularized scaffolds are superior for tissue engineering. Vascular passages help the inflow and outflow of liquids, nutrients, and waste disposal from the scaffold and cell growth. Porous scaffolds can be prepared by plant tissue decellularization which allows for the cultivation of various cell lines depending on the intended application. To this end, researchers decellularize plant tissues by specific chemical and physical methods. Researchers use plant parts depending on their needs, for example, decellularizing the leaves, stems, and fruits. Plant tissue scaffolds are advantageous for regenerative medicine, wound healing, and bioprinting. Studies have examined various plants such as vegetables and fruits such as orchid, parsley, spinach, celery, carrot, and apple using various materials and techniques such as sodium dodecyl sulfate, Triton X-100, peracetic acid, deoxyribonuclease, and ribonuclease with varying percentages, as well as mechanical and physical techniques like freeze-thaw cycles. The process of data selection, retrieval, and extraction in this review relied on scholarly journal publications and other relevant papers related to the subject of decellularization, with a specific emphasis on plant-based research. The obtained results indicate that, owing to the cellulosic structure and vascular nature of the decellularized plants and their favorable hydrophilic and biological properties, they have the potential to serve as biological materials and natural scaffolds for the development of 3D-printing inks and scaffolds for tissue engineering.

**Keywords:** *Plant leaves, plant tissue decellularization, scaffolds, three-dimensional bioprinting, tissue engineering*

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## Introduction

Biomedical engineering and tissue engineering are interdisciplinary fields that incorporate medical science and different fields of engineering to develop novel solutions for disease treatment. However, they are relatively new branches of science, and there are many challenges and opportunities in them. Important aspects of tissue engineering involve the treatment of diseases that require engineered tissue or whole-organ transplantation.<sup>[1,2]</sup>

The prevalence of some diseases and injuries causes the destruction of organs, and donors' shortages increase the need for organ transplantation. In the United States, the number of individuals awaiting organ transplants exceeds 100,000. Tissue

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engineering can solve this problem, and the organs can be produced. However, engineering tissue requires components such as cells, signaling molecules, and scaffolds that interact with each other. Specifically, signaling molecules and scaffolds provide biomechanical properties and regulate cell behavior. A variety of techniques, such as bioprinting, electrospinning, and decellularizing animal and plant tissues, can be applied to scaffold fabrication.<sup>[3]</sup>

Finding suitable scaffolds for cell culture in regenerative medicine is one of the main challenges of tissue engineering.<sup>[4]</sup> Several types of cell culture scaffolds include synthetic polymers and composites as well as natural ones derived from humans or animals. However, human and animal resource scaffolds are severely limited by cost, time, scarcity, and ethical issues.<sup>[5,6]</sup> Other natural alternatives are plant leaves

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or stems. Cellulose is one of the essential components of the plant cell wall, and cellulose scaffolds can support three-dimensional (3D) cell growth. According to previous researches, bacterial cellulose helps enhance the ability of mammalian cells to differentiate into target cells.<sup>[7]</sup> Cellulose biomaterials have many medical and tissue engineering applications owing to their extensive range of properties, including suitable physical, biochemical, and unique mechanical properties, as well as biocompatibility and bioactivity properties.<sup>[8]</sup>

Decellularization is a process used in tissue engineering to separate the extracellular matrix (ECM) from cells in order to obtain a cell-free, porous 3D natural scaffold that does not cause rejection by the body after cell culture and implantation. ECMs are made up of proteins and polysaccharides that provide biochemical and mechanical support to the surrounding cells.<sup>[9]</sup> The ECM is composed of proteins and polysaccharides, serving to provide adjacent cells with both mechanical and metabolic support.<sup>[10]</sup> Scaffold nanotopography is very important for cell growth and proliferation.<sup>[11]</sup>

This study employed original and review articles in the field of tissue decellularization, specifically on plant decellularization methods, highlighting the advantages offered for biological application by plants, such as their 3D architecture and vascularization for tissue engineering. The article focuses on the techniques employed in the fabrication of scaffolds derived from plants emphasizing their favorable attributes such as degradability, biocompatibility, mechanical properties, and interconnected porosity for nutrient fluid transport. The decellularized matrix derived from plants exhibits biocompatibility both in the laboratory and within the body.<sup>[12]</sup> It should be noted that there are various decellularization methods, such as detergent-based and physical approaches, which will be fully described.<sup>[13]</sup>

## Decellularization Methods

The process of decellularization encompasses the deliberate removal of cellular components and genetic material from tissue in order to generate a biologically derived scaffold. The aforementioned scaffold is employed in many tissue engineering applications, involving the generation and reconstruction of multiple tissue types.<sup>[3]</sup> In the realm of tissue decellularization, three primary methodologies were commonly implemented: chemical, physical, and enzymatic techniques and, in some cases, a combination of these procedures [Figure 1]. Each decellularization approach exhibits distinct characteristics and functions in a unique manner compared to other methods. For instance, chemical compounds alter the internal composition of samples or change the structure and destroy cells. In another way, mechanical methods disrupt cellular integrity by inducing unfavorable circumstances within the cell, leading to the rupture of the cell membrane and ultimately resulting

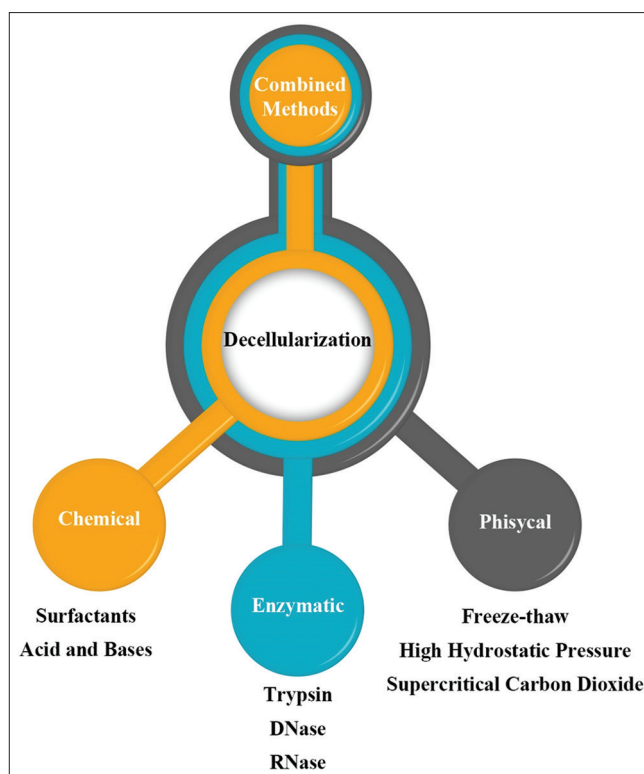


Figure 1: Main plant decellularization methods

in the acquisition of a cell-free 3D architecture.<sup>[14]</sup> In addition, a combined method of decellularization, such as a combination of chemical and mechanical methods, can be used in some decellularization processes and improve the results.<sup>[15]</sup> The decellularization time varies from 3 h to 3 weeks, depending on various factors such as plant type, chemical agent, and process. The decellularization process in the studies was performed at temperatures ranging from -80 to 90°C.

## Chemical methods

The process of plant chemical decellularization is a complex procedure that involves the use of various chemical agents, including surfactants, acids, and bases, to remove cellular components from plant tissues, resulting in the extraction of the ECM as the underlying scaffold. The complex process involves careful manipulation of chemical interactions within the plant structure in order to selectively dissolve cellular material while maintaining the essential architecture and composition of the ECM.<sup>[16]</sup> This technique employs the strategic application of specific chemicals to induce the disintegration of cell membranes and the disruption of cellular bonds. As a result, it enables the controlled extraction of cellular components while preserving the complex framework of the plant's ECM.<sup>[3,17]</sup> An alternative approach involves employing a balanced salt solution that incorporates a more intricate procedure and an extended duration of processing. The tissue or organ is washed using a balanced salt solution to eliminate any cellular component residuals and decellularization

agents, guaranteeing the absence of potentially harmful substances.<sup>[18]</sup>

### Surfactants

Surfactants are frequently employed for the purpose of disrupting cell membrane phospholipids through the disruption of membrane constituents. In general, surfactants used for decellularization include sodium dodecyl sulfate (SDS), Triton X-100, sodium deoxycholate, and 3-([3-cholamidopropyl] dimethylammonio)-1-propane sulfonate (CHAPS).<sup>[16]</sup>

SDS is widely recognized as an influential ionic surfactant because of its high ability to remove genetic material and other cells. It has the capacity to eliminate a minimum of 90% of DNA.<sup>[3]</sup> Perfusion is identified as one of the techniques employed for the utilization of surfactants in the process of decellularization. Perfusion is a physical methodology employed to facilitate the decellularization of tissue, wherein the cells are isolated and extracted from the ECM of a 3D structure.<sup>[19]</sup> In a research, it is reported that SDS had successfully been operated with perfusion to decellularize plant tissue, as is explained in decellularizing spinach leaves for seeding human endothelial cells.<sup>[20]</sup> In addition, surfactants are applied with immersion and agitation processes such as apple hypanthium tissue, which was employed for an *in vitro* 3D culture of NIH3T3 fibroblasts, mouse C2C12 muscle myoblasts, and human HeLa epithelial cells,<sup>[21]</sup> *Anthurium magnificum*, *Vanilla* stems, parsley stems, and *Orchid pseudobulbs*.<sup>[22]</sup> In a particular investigation, researchers decellularized celery stalks by soaking them in 0.1% SDS solution for 72 h.<sup>[23]</sup> Apple slices were decellularized in 0.1% SDS for 48 h. Parsley stems were first immersed in 1% SDS solution for 48 h and then dipped in 0.1% Triton X-100 solution with 10% sodium chlorite (NaClO<sub>2</sub>) as a bleaching agent in deionized water for 48 h.<sup>[24]</sup> In another study, plants including cucumber, carrot, broccoli, potato, green onion, asparagus, apple, leek, and celery were cut into small pieces of 1–2 mm in diameter and left in 1% SDS solution for 3 weeks.<sup>[25]</sup> In another research on apple fruit by Hickey *et al.*, samples of McIntosh red apples were stirred in a 0.1% SDS solution for 48 h at 180 RPM. This scaffold was used for C2C12 cell culture and was subcutaneously implanted.<sup>[26]</sup> Lee *et al.* decellularized the apple, sweet pepper, jujube, broccoli, carrot, and persimmon using 0.5% SDS.<sup>[27]</sup> In another study performed on plant green leaf and onion skin, 10% SDS and 10% NaClO<sub>2</sub> bleach were used for leaves, and 10% SDS was used for decellularization of onion skin due to its thinness and transparency. In addition, phosphate-buffered saline (PBS) was used for washing and removing detergents' excess.<sup>[28]</sup>

The other agent is Triton X-100; it serves as a nonionic decellularizing agent and effectively eliminates any remaining SDS residues. The combination of Triton X-100 and ammonium hydroxide has the capability to effectively

eliminate any residual traces of DNA.<sup>[29]</sup> In different studies, a combination of SDS and Triton X-100 has been successful. In addition to the aforementioned studies, other studies have been done on decellularization using these chemical compounds. In another study on spinach leaves by O'Donnell *et al.*, 10% SDS for 4 days and a mixture of 10% sodium hypochlorite and 0.1% Triton X-100 for 48 h were used for the decellularization process.<sup>[30]</sup> Walawalkar and Almelkar performed another study to find a decellularization method for cabbage and broccoli leaves with the potential to have the highest cellularity, the lowest cytotoxicity, and the best way to maintain the openness and integrity of the vascular network. PBS, 10% SDS, and finally, 1% Triton X-100 were perfused by injecting into the leaf vasculature to modify the decellularization.<sup>[31]</sup>

Wang *et al.* decellularized *Aptenia cordifolia* leaves using SDS, followed by a mixture of 0.1% Triton X-100 and 1% bleach.<sup>[29,32]</sup> Thippan *et al.* used a combination of 0.1% ethylenediaminetetraacetic acid (EDTA) and 0.1% Triton X-100 to decellularize *Piper betle*, *Sauropus androgynus*, *Basella alba*, *Centella asiatica*, and *Mentha spicata* leaves.<sup>[33]</sup> *Spinacia oleracea* leaves, *Solanum lycopersicum* plant, *Echinodorus grisebachii* plant, and lucky bamboo were decellularized by perfusion of 1% SDS for 48 h, followed by perfusion of a mixture of 10% bleach and 0.1% Triton X-100 for 48 h.<sup>[34]</sup> Herbs including *Calathea zebrina*, *Anthurium warocqueanum*, *A. magnificum*, *Solenostemon wasabi*, *Vanilla*, *Laelia anceps*, bamboo, parsley, and *Schoenoplectus tabernaemontani* became decellularized by soaking in 10% SDS solution for 5 days, followed by 0.1% Triton X-100 solution in 10% bleach solution for 48 h.<sup>[22,35]</sup>

In a study conducted by Jones *et al.*, decellularized spinach leaves were employed as a substrate for the cultivation of laboratory-grown meat with the intention of making it suitable for human consumption. The leaves were initially submerged in hexane for a duration of 3 min, followed by a subsequent immersion in a 1% SDS solution for a period of 5 days, thereby facilitating the creation of the scaffold. Subsequently, the leaves were subjected to 0.1% Triton X-100 solution containing 10% bleach for a duration of 48 h.<sup>[36]</sup> The research performed by Aswathy *et al.* involved the decellularization of bamboo in order to generate bone tissue. This was achieved through the utilization of a combination of various concentrations of SDS, Triton X-100, and bleach.<sup>[37]</sup>

### Acids and bases

Acids and bases such as peracetic acid (PAA), EDTA, and reversible alkaline swelling almost solubilize the membrane and genetic materials, causing tissue decellularization. The underlying cause of this behavior can be attributed to the electrical charge displayed by the acids and bases.<sup>[3]</sup> Harris *et al.* used PAA to decellularize spinach leaves. It should be noted that PAA is very corrosive and does not succeed in decellularization. For instance, the researchers

achieved successful decellularization of the spinach tissue with the utilization of PAA.<sup>[38]</sup> In their study on leaf decellularization, Varhama *et al.* developed a highly efficient method that, depending on the type of leaf, successfully achieved decellularization within a relatively short time of 180 min. The primary decellularization solution employed in the aforementioned method was sodium hydroxide. This procedure is carried out at high temperatures (90°C).<sup>[39]</sup>

### Enzymatic methods

Various enzymes were employed during the decellularization procedure in order to achieve substantial decellularization. Enzymes such as trypsin, deoxyribonuclease (DNase), and ribonuclease (RNase) have been included in this category. Note that trypsin is often used in combination with EDTA in decellularization processes. These enzymes are primarily used to break down cell–matrix bonds and disintegrate the fragments of genetic material. Phan *et al.* applied DNase to decellularize rice and investigated its effect on the cell nucleus by fluorescent imaging.<sup>[3,17]</sup> In summary, the enzymatic method represents a widely accepted and established technique to remove cells from tissues owing to its considerable efficacy and ability to maintain the integrity of the tissue structure.<sup>[18]</sup>

### Physical or mechanical approaches

Physical and mechanical methods are two decellularization techniques that can be used. These methods are more straightforward and more profitable than some chemical methods. In certain instances, they exhibit superior performance and necessitate a reduced time investment. Depending on the applied technique, they have different effects on the internal structures of the material.<sup>[40]</sup> Physical methods generally involve applying temperature and pressure. These methods break down cells and destroy adhesive proteins in the cell matrix. Physical treatments have three ways: Freeze–thaw, high hydrostatic pressure (HHP), and supercritical carbon dioxide (scCO<sub>2</sub>).

#### Freeze–thaw

One of the physical processes exploited is the freeze–thaw technique. Lowering the temperature to –80°C and then bringing it to the biological level (37°C) helps cells break down and decellularize.<sup>[3]</sup> *P. betle*, *S. androgynus*, *B. alba*, *Centella asiatica*, and *M. spicata* were transferred to the freezer and then thawed twice to decellularize.<sup>[33]</sup>

#### High hydrostatic pressure

HHP shows great potential as a method for the decellularization of tissues and organs. This technique enables the elimination of cellular components while preserving the ECM as a structural framework suitable for tissue engineering.<sup>[41]</sup> HHP is a technique that involves exposing tissue to increased pressures, typically ranging from 100 to 1000 MPa, for a designated period of time. HHP promotes the disruption of cellular membranes,

thereby promoting cell lysis and enabling the disintegration of cellular constituents. Furthermore, it maintains the structural and functional integrity of the ECM, thus preserving its native architecture to facilitate effective tissue regeneration. Nevertheless, HHP encounters obstacles such as the need for consistent decellularization methods and the optimization of pressure levels and exposure durations.<sup>[42,43]</sup> In contrast to alternative decellularization methods, the application of HHP in plant decellularization represents a relatively new field of study, necessitating further investigation to fully comprehend its capabilities and constraints.

#### Supercritical carbon dioxide

scCO<sub>2</sub> is used to decellularize plant tissues by applying CO<sub>2</sub> at a critical temperature of 31.1°C and a pressure above 7.40 MPa. These particular properties contribute to a significant infiltration of carbon dioxide into the tissue, resulting in the subsequent detachment of cells from the tissue.<sup>[3]</sup> Harris *et al.* have decellularized spinach leaves using scCO<sub>2</sub>; this method reduced the decellularization process to 4 h.<sup>[38]</sup> This method is recognized for its capacity to permeate porous materials, making it a promising candidate for decellularization procedures in diverse biological contexts, such as the decellularization of plant tissues.<sup>[44]</sup>

### Combined methods

Each of the aforementioned methods possesses certain advantages. Hence, combining these methods can lead to an optimal approach for decellularizing plant tissue. In addition, low-concentration surfactants can eliminate cellular debris. Therefore, the integration of both physical and chemical methodologies can enhance the overall efficacy of this particular process.<sup>[3]</sup> For example, in some investigations for decellularization of plant tissue, the tissue was immersed in a chemical solution with heat, and the solution was stirred. Therefore, this method will accelerate the decellularization process.<sup>[13]</sup> *In vitro* studies have provided evidence that the mechanical properties of tissues are compromised due to the multi-stage utilization of chemicals and enzymes. For example, some low-strength plant tissues are destroyed during these protocols. In one study, after soaking the samples of decellularized apples, carrots, and celery in a 0.1% SDS solution for 48 h, they were washed in a sonicator.<sup>[45]</sup> Harris *et al.* used scCO<sub>2</sub> with PAA in spinach leaf decellularization.<sup>[38]</sup> In a study of various methods of decellularizing *Ficus hispida* leaf, the researchers proposed a combined approach in which the samples were first placed in a solution containing 3% sodium bicarbonate and 5% bleach at 70°C.<sup>[13]</sup>

### Decellularized Tissues

Many researchers have worked on the decellularization of plant tissues, including the leaves, stems, and fruits of various plants such as apple hypanthium,<sup>[21]</sup>

carrot, cucumber, broccoli, green onion, potato,<sup>[25]</sup> *A. cordifolia*,<sup>[29]</sup> bamboo,<sup>[34]</sup> leek,<sup>[46]</sup> celery,<sup>[23]</sup> spinach,<sup>[20]</sup> and parsley.<sup>[24]</sup> Therefore, each researcher has used specific methods depending on the type of tissue chosen for decellularization, and they have used the obtained scaffold for tissue engineering applications. Table 1 provides a brief overview of the methodologies discussed in this review, along with their respective advantages and disadvantages.

## Plant Decellularization Assessment

A crucial factor in decellularization for tissue engineering applications is evaluating decellularized scaffolds and the number of cells removed from the native tissue. The assessment of these scaffolds will focus on eliminating cells, genetic remnants, and preserving structural proteins. To evaluate the success and effectiveness of the decellularization process and the quality of postdecellularization scaffolds, several factors were considered, including preserving mechanical properties and residual protein and DNA by different methods. Most protocols use the following tests to measure the effectiveness of decellularization.

### Mechanical properties

Preservation of plants' mechanical properties is one of the essential criteria in evaluating the decellularization process. These properties include elastic modulus, tensile strength, viscosity, and yield stress. Mechanical properties depend on the structural proteins of the ECM, such as collagen, laminin, fibronectin, and elastin.<sup>[47]</sup> For example, in the use of plant scaffolding as heart vessels, maintaining the leaf vasculature is very important because how cells are placed in the scaffold depends entirely on the shape of the scaffold. Changes in the plant's mechanical properties result from chemical and physical protocols. For instance, SDS alters the structure of the ECM by compressing the matrix collagen.<sup>[3]</sup> As a result, increasing the detergent concentration causes more changes in the mechanical properties of the ECM.

### Protein and DNA content

One way to evaluate decellularization performance is to measure the reduced immunogenicity of plant scaffolds. The scaffolds are not efficient and should be rejected if the immunogenicity level of the scaffold is high after implantation in the body.<sup>[47]</sup> Therefore, the residue of genetic material (DNA or RNA) is evaluated to measure the degree of immunogenicity. Previous research has suggested that there should be <50 ng of DNA per milligram of ECM, and the length of each piece of DNA should not exceed 200 base pairs. DNase and RNase break down nucleic acid and shorten its fragments.<sup>[3]</sup> Detergents such as SDS and Triton X-100 remove more than 90% of DNA, while SD and CHAPS are less successful. DNA and protein were measured in the study on leek decellularization by Toker *et al.*<sup>[46]</sup> using the DNA

isolation method and protein quantification assay from the mouse tail, respectively. Lacombe *et al.* quantified DNA and protein content.<sup>[34]</sup> DNA and protein contents were examined in spinach decellularization.<sup>[20,36]</sup> In bamboo tissue decellularization, in order to produce bone tissue, a PicoGreen assay was used to measure DNA content.<sup>[37]</sup>

## Histology

### Hematoxylin and eosin

Histology observations provide an additional route for promoting development and achievement in the field of decellularization. After staining, samples can be evaluated under a microscope.<sup>[47]</sup> H and E staining was used on celery (*Anethum graveolens*) stem scaffolds by C2C12 murine myoblast seeding<sup>[23]</sup> and parsley stems that were coated with human umbilical vein endothelial cells.<sup>[24]</sup> Lee *et al.* applied H and E staining to some fruits such as apple, carrot, broccoli, and sweet pepper to evaluate their histological properties.<sup>[27]</sup> The H and E staining technique was used for *S. androgynus*' scaffolds and for *A. cordifolia* leaves.<sup>[32,33]</sup> Apple hypanthium tissue was seeded with MC3T3-E1 preosteoblasts and stained.<sup>[48]</sup>

### Immunohistochemical analysis

Another staining-based process is immunofluorescence staining, which indicates the degree of decellularization. Toker *et al.* have used this method for evaluating leek decellularization.<sup>[46]</sup> In a study of the production of a heart patch from the decellularized leaf of spinach, the researchers used immunofluorescence staining to prove the adhesion of the collagen and fibronectin-coated cells to the scaffolds.<sup>[49]</sup> Santiago *et al.* evaluated the decellularization process using a test based on antibodies to assess the immunogenicity of the scaffold.<sup>[23]</sup> In the work on decellularized celery leaves for guided alignment of myoblast cells, they stained cellulose with 10% calcofluor (a fluorescence dye), and the samples were considered.<sup>[24]</sup> Cheng *et al.* performed immunoassays in their study using a mouse monoclonal alpha-myosin heavy chain antibody.<sup>[25]</sup> In the study of plant green leaves and onion skin, immunohistochemistry was used to count positive cell numbers, and immunofluorescence was used for staining and evaluating cellular nuclei.<sup>[28]</sup>

## Scanning electron microscopy

Scanning electron microscopy (SEM) facilitates the comprehension of the distinctions between decellularized and native tissues. Understanding of scaffold surface morphology could be advantageous. Lee *et al.* examined the apple, sweet pepper, jujube, broccoli, carrot, and persimmon with SEM by washing the samples with Dulbecco's phosphate-buffered saline followed by 2.5% glutaraldehyde as fixing a agent.<sup>[27]</sup> Santiago *et al.* used SEM to observe celery scaffolds.<sup>[23]</sup> In the study of spinach leaves by Gershlak *et al.*, SEM was used to study the surface structure.<sup>[20,50]</sup> Decellularized *S. oleracea*, *S. lycopersicum*, *E. grisebachii*, and *Kalanchoe fedtschenkoi*

**Table 1: A brief overview on advantages and disadvantages of plant decellularization methods**

Decellularization method	Materials and effects	Advantages	Disadvantages	Reference
Chemical methods				
Surfactants	SDS, Triton X-100, SD, and CHAPS (immersion and perfusion) Alter the membrane constituents	High elimination of genetic material ECM preservation Biocompatibility	Remaining residual SDS Immense washing process needed Depletion of bioactive substances Time-consuming process Structural alterations and changes in mechanical properties	[3,19,20,22,24-37]
Acids and bases	PAA, EDTA, and reversible alkaline swelling Solubilize membranes and genetic materials by their inherent electrical charge	Simple and accessible Tissue-specific optimization	Potential damage to extracellular matrix Limited control over selectivity Depletion of bioactive substances Time-consuming process	[3,38,39]
Enzymes	DNase RNase Trypsin Break down cell–matrix bonds and disintegrate fragments of genetic material	Specific cellular targeting and selectivity ECM preservation Less toxicity	Complexity and cost Time-consuming process Challenging control over enzyme activity	[3,17,18]
Physical methods				
Freeze–thaw	Significant change in temperature from –80°C to 37°C Help cells degrade	Cost-effective and simple Low chemical exposure Tissue architecture maintenance	Variable efficiency Limited control over selectivity Structural alterations Processing time	[3,33]
High hydrostatic pressure	Expose tissue to elevated pressure ranging 100–1000 MPa for specific duration Facilitate cell lysis and subsequent disintegration of cellular constituents	Biochemical composition and structure preservation Control and selectivity enhancement Reduced processing time	Equipment cost and complexity Structural alterations and possibility of ECM denaturation Optimization challenges Limited research	[41-43]
Supercritical carbon dioxide	Significant infiltration of CO <sub>2</sub> into the tissue at temperatures higher than 31.1°C and above 7.40 MPa Detachment of cells from tissue	Environmentally friendly Mild processing conditions Selective extraction Reduced procedure time Minimal use of harsh chemicals Ability to penetrate complex structures No significant damage to the structural integrity	Equipment complexity and cost Optimization challenges Limited research	[3,38,44]
Combined methods	Combination of two or more methods	Enhanced efficiency Tailored optimization Comprehensive removal of residual chemicals Controlled degradation of cellular components	Increased complexity and cost Optimization challenges	[3,13,38,45]

SDS – Sodium dodecyl sulfate; PAA – Peracetic acid; ECM – Extracellular matrix; EDTA – Ethylenediaminetetraacetic acid; SD – Sodium deoxycholate; CHAPS – 3-([3-cholamidopropyl] dimethylammonio)-1-propane sulfonate; DNase – Deoxyribonuclease; RNase – Ribonuclease

*variegata* were imaged by SEM after *in vitro* cell culture to assess cells' adhesion to the natural scaffolds.<sup>[34]</sup> The researchers also used SEM to evaluate the decellularization of the rat spinal cord for tissue regeneration applications.<sup>[51]</sup> Decellularized plants exhibit a diverse array of porous structures. Taking advantage of SEM enables researchers to effectively examine the microstructure and porosity of samples, facilitating the evaluation of pore size and area characteristics.

## Application of the Decellularized Plant Tissue

### Tissue engineering

The utilization of decellularized plant tissue offers prospects for the development of tissue engineering scaffolds. As previously stated, the application of these scaffolds offers several benefits, including favorable mechanical characteristics and minimal cell toxicity. Another advantage of these scaffolds is that they are vascularized, which can simulate tissue capillaries. For example, researchers decellularized spinach leaves in one study to produce mouse heart capillaries using cell culture in leaf veins.<sup>[48]</sup> Similarly, in another study, Jansen *et al.* conducted the decellularization of spinach and chive plant materials, subsequently cultivating them with renal cells in order to generate kidney tubules.<sup>[52]</sup> Researchers produced edible meat by culturing bovine primary satellite cells on decellularized spinach tissue.<sup>[36]</sup> In another study, scientists cultured human-induced pluripotent stem cell-derived cardiomyocytes (hiPS-CM) on a decellularized spinach scaffold and succeeded in producing a cardiac patch; they used collagen and fibronectin coating on the scaffold.<sup>[49]</sup> By decellularizing the apple hypanthium, researchers could regenerate the bone tissue.<sup>[48]</sup> Contessi Negrini *et al.* reported in their research that apple-derived decellularized scaffolds are suitable for adipose tissue engineering due to their porous structure and mechanical properties, carrot-derived scaffolds are ideal for bone tissue engineering,<sup>[45]</sup> and celery-derived scaffolds are suitable for tendon tissue engineering. Starch/cellulose nanofiber composites are used in cartilage tissue engineering owing to their excellent porosity, mechanical strength, and biodegradability. These scaffolds are somewhat biodegradable after about 20 weeks.<sup>[10]</sup> Modulevsky *et al.*, in another study on apple-derived scaffolds implanted in mice, examined the biocompatibility of plant cell-derived scaffolds. The square-shaped scaffolds implanted in the backs of the mice were examined for up to 8 weeks. According to reports, the scaffolds showed excellent biocompatibility and, in addition, almost retained their shapes. After 8 weeks, fibroblast migration in the scaffold, complete acceptance by the host, and vascularization in the scaffold were observed.<sup>[53]</sup> In the field of vascular tissue engineering, Dikici *et al.* studied spinach baby leaves, which were suitable for prevascularized tissue engineering constructs using a combination of two methods of *in vitro*

cell migration and tube formation on decellularized leaves.<sup>[54]</sup> The utilization of a solution containing 10% SDS in conjunction with Triton X-100 for decellularization revealed findings that suggest the viability of employing biodegradable scaffolds derived from plant stems. The scaffolds exhibited favorable mechanical properties and demonstrated biocompatibility, as evidenced by their ability to degrade gradually over time without inducing notable inflammation or toxicity. These scaffolds have the potential to serve as temporary grafts in tissue engineering applications.<sup>[55]</sup> The application of decellularized scaffolds in conjunction with other natural polymeric materials, such as chitosan, has been observed in the field of tissue engineering.<sup>[56]</sup> In another investigation, decellularized leaf structures were chosen in conjunction with gelatin methacrylate, which exhibited a branched architecture similar to a vascular network. Adequate channel dimensions and mechanical properties were observed in the 3D models, which make them suitable for replicating the microenvironment of adipose tissue. The cells exhibited metabolic adaptations, enabling their growth, proliferation, colonization of the entire microenvironment, and subsequent differentiation. The results obtained in this study demonstrate the potential of the proposed innovative approach, which employs natural plants for the vascular network. This approach effectively simulates the tumoral microenvironment in 3D scaffold-based models.<sup>[57]</sup>

### Wound healing

The decellularized plant tissue has the advantage of having vasculature and can be used in wound dressing.<sup>[58]</sup> Residues from the decellularized matrix of the plant play an important role in wound healing. Decellularized *Asparagus* was used to regenerate the rat spinal cord.<sup>[51]</sup>

### Bioprinting

In recent investigations, researchers have used a combination of decellularized scaffolds and bioprinting technology in tissue engineering. Natural polymers such as collagen, alginate, or ECM were used separately or together in some works.<sup>[59]</sup> The decellularized ECM is a mixture of natural polymers obtained from various plant and animal tissues. In this regard, the decellularized porcine liver in a thriving culture of hepatocytes was studied.<sup>[60]</sup> These materials can be used as bioink (a mixture of materials and cells in 3D bioprinting). Decellularized scaffolds can be used as a biological coating; the presence of these coatings helps regenerate the tissue and causes homeostasis.<sup>[61]</sup> Choi *et al.* used porcine skeletal muscle to regenerate the damaged muscle tissue.<sup>[62]</sup> Decellularized cellulosic biological materials can be used in composition with hydrogel inverse molding techniques with different biochemical cues (e.g., matrix proteins, growth factors, small molecules, etc.) to invade cells to attract or elevate the growth of specific cell types *in vitro*.<sup>[26]</sup> In one study, Pomerleau *et al.* used the decellularized plant tissue to

produce a 3D vascular structure for bioprinting; they applied the decellularized leaves of spinach to benefit from their vasculatures for water and nutrient transfer to the cells, resulting in better cell growth and adhesion.<sup>[24]</sup> In several studies, this technique has been employed to generate skin constructs so that a cell-free matrix can be utilized to prepare bioinks for tissue bioprinting.<sup>[63]</sup> The scientists could print the heart tissue on the decellularized spinach scaffold and the ear tissue on the apple scaffold using plant scaffolds.<sup>[64]</sup> As mentioned, cellulosic biomaterials can be used as bioink and biopaper for bioprinting applications.<sup>[14]</sup> In addition, Toker-Bayraktar *et al.* discovered that decellularized plant tissues can be pulverized following the decellularization process using a mortar or grinder. This powdered form can then be utilized as a constituent of a blended scaffold and combined with other materials, such as bioinks, for printing.<sup>[65]</sup>

### Plant-based scaffolds in the future

Based on the applications of plant scaffolds, it is expected that these technologies will be used in more extensive investigations in the future because of the inherent advantages associated with these scaffolds. For this purpose, several problems and shortcomings must be resolved, such as the high time for decellularization in chemical methods. Another drawback is the nonabsorbency of cellulose-based scaffolds, which makes their use in tissue engineering difficult. These scaffolds can be used more widely in tissue engineering by solving this problem. Following this method, multi-vascularized tissues with the specified diameters can be produced. Since these scaffolds, with their particular components such as cellulose, play an essential role in wound healing, they can be used as wound dressings.

Plant tissue vessels are expected to help with drug delivery to the wound site. Bai *et al.* showed that onion and leaf cellulose could be used as natural drug delivery systems and vascular patches with nanoparticle delivery abilities.<sup>[28]</sup> Thyden *et al.* focused on the versatility and edibility of laboratory-grown meat production by utilizing broccoli as a plant-derived cell carrier. The study emphasizes how plant-based carriers can help address environmental and ethical issues related to the production of meat. However, issues with technology, society, politics, and regulations must be resolved before broad adoption.<sup>[66]</sup>

### Conclusion

Decellularization is a pivotal technique in tissue engineering, involving the removal of cellular components from biological tissues while maintaining the integrity of the ECM. Decellularized scaffolds can then be repopulated with new cells. Several methodologies have been extensively studied in the process of decellularizing plants, each offering distinct advantages and challenges when applied to tissue engineering endeavors. In general, by

examining different sources, it can be concluded that most plant tissues can be decellularized by chemical methods, especially with varying concentrations of substances such as SDS, Triton X-100, and the bleaching agent. However, it ought to be noted that these techniques have the capacity to alter the biochemical composition of the ECM and introduce cytotoxic residues. This has the potential to compromise the functionality and biocompatibility of the resulting scaffold. Physical and mechanical approaches such as  $scCO_2$  or HHP are not successful in this process and can cause tissue disintegration and need to be optimized to ensure cell removal without further damage; however, the freeze-thaw method was used as a nondestructive method.<sup>[17]</sup> A combination of physical and chemical methods is observed in some protocols, for example, decellularization with a bleach solution at high temperatures and combining chemical decellularization with sonication.<sup>[13,45]</sup> However, the heating method changed the mechanical properties of the leaves. In another scenario, the researchers injected chemicals into the tissue and decellularized the plant leaf as a direct approach.<sup>[31]</sup> In all the aforementioned methods, the desired tissue was decellularized. However, choosing the proper protocol depends on the type and size of tissue and its intended use.<sup>[3]</sup> Therefore, selecting a suitable method can save time and reduce the number of chemicals entering the tissue. In this way, toxicity caused by chemicals can be modified.<sup>[16]</sup> The utilization of plant-based decellularization techniques has exhibited encouraging outcomes in the production of biocompatible scaffolds that induce minimal immune responses. Furthermore, the substantial availability, capacity for decomposition, and wide range of structures exhibited by plant-derived substances present a compelling substitute for animal-derived ECMs, effectively addressing ethical considerations and regulatory constraints associated with biomaterials sourced from animals. The techniques for plant decellularization have potential uses in medicine and a variety of tissue engineering applications. Despite plant decellularization advancements, there are several aspects that require further investigation involving the development of decellularization protocols to effectively maintain the biomechanical characteristics of the ECM, improve biocompatibility, and facilitate cellular repopulation and tissue regeneration processes. Furthermore, it is imperative to emphasize the importance of executing standard procedures and quality control measures in order to effectively enable the translation of plant-based decellularization techniques for use in clinical applications.

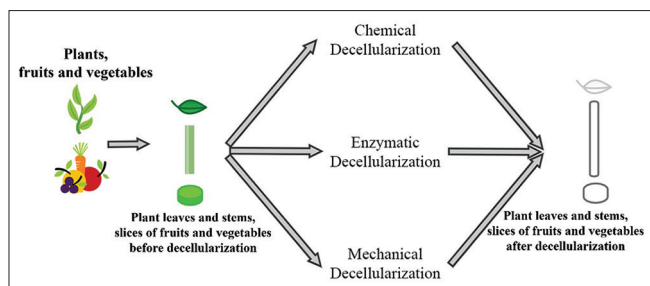
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### Graphical abstract

A brief explanation of decellularization of plants, vegetables, and fruits and obtaining 3D scaffolds to use in tissue engineering.





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### Conflicts of interest

There are no conflicts of interest.

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