MethodsX 11 (2023) 102385

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/methodsx

A typical method for decellularization of plants as biomaterials

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ARTICLE INFO

Method name:
Plant tissue decellularization with chemical methods

Keywords:
Biomaterials
Decellularization
Plant tissues
Tissue engineering

ABSTRACT

Decellularization is a process by which cells are removed from tissues or organs, leaving behind the extracellular matrix (ECM) structure. This process has gained interest in the fields of tissue engineering and regenerative medicine as a way to prepare suitable scaffolds for tissue reconstruction. Although the initial efforts come with the animal tissues, this technique can also be applied to various plant tissues with simple modifications, as plant-derived biomaterials have the benefit of being biocompatible and serving as a safe, all-natural substitute for synthetic or animal originated materials. Additionally, plant-derived biomaterials may help cells grow and differentiate, creating a three-dimensional environment for tissue regeneration and repair. Here we demonstrate a general method for plant tissue decellularization, including already experienced approaches and techniques.

- Exhibit the basic steps for plant decellularization, which may be applied to several other plant tissues.
- The proposed approach may be optimized considering various intended uses.
- Gives basic information for the determination of decellularization efficiency.

Specifications table

Subject Area:	Materials Science
More specific subject area:	Tissue Engineering
Method name:	Plant tissue decellularization with chemical methods
Name and reference of original method:	Toker, M., Rostami, S., Kesici, M. et al. Decellularization and characterization of leek: a potential cellulose-based biomaterial. *Cellulose* 27, 7331–7348 (2020). https://doi.org/10.1007/s10570–020–03278–4
Resource availability:	Standard Laboratory Equipment and Analytical Methods

Introduction

For decades, tissue engineering has shown promise for tissue repair and regeneration, which requires cells, growth factors, and most importantly, an appropriate scaffold that must support new tissue development, cell proliferation, and growth. These biological scaffolds can be designed from different biomaterials such as collagen, cellulose, hyaluronic acid, or fibrin of natural or synthetic origin [1]. Biological scaffolds derived from decellularized tissues and organs can be used directly from the tissue itself as a biomaterial in which tissues are decellularized by chemical, physical, and enzymatic processes, and the extracellular matrix (ECM) is preserved.

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https://doi.org/10.1016/j.mex.2023.102385
Received 25 December 2022; Accepted 16 September 2023
Available online 17 September 2023

Decellularization removes cellular material from a tissue or organ, leaving behind a cell-free scaffold consisting of an extracellular matrix (ECM), whose composition is attached to the tissue or organ from which it originates while leaving an intact vascular network [2]. The extracellular matrix (ECM) component remaining from the decellularized tissue/organ is unique to each tissue or organ. This can naturally result in an inconsistency between tissues or organs obtained from different patients or decellularized by different methods, due to variables such as age, organism, and the characteristics of the decellularization protocol used [5,6]. This can naturally result in an inconsistency between tissues or organs obtained from different patients or decellularized by different methods, due to variables such as age, organism, and the characteristics of the decellularization protocol used [3,4]. In addition, a significant amount of additional research is required before whole decellularized organs can be considered a clinically practical option [5].

Recently, researchers have turned to plant tissues due to the cheap and abundant resources and simple application of the method, as well as studies beginning with the decellularization of various animal tissues and showing its effectiveness in tissue regeneration or similar applications. Decellularized plant tissues have great potential due to their ability to mimic plant and animal vascular network structures as well as animal tissues [6].

Plant tissues, such as leaves, stems, and roots, have ECM structures that are primarily composed of structural polysaccharides, such as cellulose and xyloglucans. Plant ECM structures are generally less complex and organized than animal tissues. However, the polysaccharides in the structure are organized into fibers that provide mechanical support and strength to the tissue. The ECM of plant tissues also contains other types of molecules, such as cellulose and pectin, which can contribute to the mechanical properties of the tissue [7].

The most abundant component of plants is cellulose, which forms the main structural component of the cell wall and provides support and protection for the cell. It also plays a role in maintaining the shape of the cell and helps to prevent the cell from collapsing due to osmotic pressure. Cellulose and other plant polysaccharides such as pectin and hemicellulose have been well studied to be used as biomaterials in clinical practice, tissue engineering, drug delivery systems, and as biocompatible and wound-healing materials [8–10]. Here, we describe a typical, easy-to-apply decellularization method for the use of plants as biomaterials.

Materials and method

Plant decellularization

Equipment

- Micropipette
- Erlenmeyer flask
- Beaker
- Magnetic stirrer (C-MAG HP-7, IKA)
- Hot plate (C-MAG HP-7, IKA)
- Vacuum pump (Rocker 410, Isolab)

Reagents

- Sodium Dodecyl Sulfate (SDS) (Sigma, Cat no: 8,170,341,000)
- Phosphate-buffered saline (PBS), pH 7.4 (Gibco, Cat no: 10,010,023)
- Absolute ethanol (Merck, Cat no: 1,070,172,511)

Methods

1. Supply fresh leeks from the grocery store and store at −20 ˚C prior to use.
   Tips and tricks: One may also use other plant tissue parts: such as succulent leaves, cabbage leaves, spinach leaves, rooted plants like carrot, celery, and turmeric, or other parts such as apple and flower stems.
2. Slice the samples layer by layer and cut into 2.5 cm x 6 cm pieces.
3. Wash with deionized water (dH2O) under vacuum for 3 h and keep at −80 ˚C overnight.
4. Place the samples in a beaker containing 1.0% Sodium Dodecyl Sulfate (SDS) solution (250 mL) and keep the beaker on a magnetic stirrer hot plate at 37 ˚C for 5 days.
   Tips and tricks: Based on plant type, SDS solution can be used within a concentration range of 0.1–10.0% and in combination with Triton-X-100 or other detergent solutions.
5. Wash the samples with dH2O under vacuum until foam disappears and twice with Phosphate Buffer Solution (PBS, pH 7.4).
6. Place samples in absolute ethanol at 65 ˚C overnight for the removal of chlorophyll.
7. Wash decellularized samples with PBS twice and keep them in PBS at 37 ˚C overnight to remove excess ethanol.
8. Store decellularized samples at 4 ˚C until further use.
   Optionally, samples may also be subjected to at least 24 h of lyophilization to get dried samples. Samples should be kept at −80 ˚C overnight prior to lyophilization.

Assessment of the decellularization efficacy

Genomic DNA quantification

Equipment

- Hot plate (C-MAG HP-7, IKA)
- Vortex (IKA)
- Centrifuge (320R, Hettich)
- Spectrophotometer (Nanodrop 2000c, Thermo Scientific)

Reagents

- Sodium Chloride-Tris-EDTA (STE) buffer (100 mM Tris (pH=8.5), 5 mM EDTA, 0.2% SDS, 200 mM NaCl) (Merck, Cat no: 85,810)
- Proteinase K solution (Sigma, Cat no: P2308)
- 70% Ethanol (Sigma, Cat no: 51,976)

Methods

1. Weigh and transfer the decellularized samples into a 2.0 mL microtube, add 720 µL STE buffer and 30µL Proteinase K solution (to a final concentration of 10 mg/mL stock solution) onto the decellularized samples, and keep in a water bath at 55 ˚C for 3 h. Mix the contents with a vortex at one-hour intervals at top speed for 10 s.
2. Keep the solution of decellularized samples at 70 ˚C for 5 min, then on ice for 5 min to inactivate Proteinase K.
3. Transfer the solution to a tube and centrifuge it for 10 min at 14.000 rpm, then transfer it to a new 2.0 mL microtube containing 720 µL of isopropanol by decantation. Centrifuge the tube for 5 min at 14.000 rpm again, and after removing the supernatant, wash the pellet with 70% ethanol. Repeat this step twice, then let the DNA dry for 1–2 min, then resuspend it at 55 ˚C for 1 hour.
4. Obtain quantitative results by reading the DNA content (µg/mL) from the spectrophotometer.

Tips and tricks: One can basically calculate the dsDNA amount using the equation given below, using the absorbance of the sample at 260 nm.

Equation: 50µg/mL x OD_{260} x dilution factor

Nuclear staining

Equipment

- Beaker
- Petri dishes
- Micropipette
- Magnetic stirrer (C-MAG HP-7, IKA)
- Orbital shaker (KS 4000, IKA)
- Fluorescence microscope (Leica DFC 295)

Reagents

- Formaldehyde (Sigma, Cat no: 47,083-U)
- Sodium hydroxide (NaOH) (Sigma, cat no: SZBF0910V)
- Triton-X-100 (Merck, cat no: K47973303 846)
- Glutaraldehyde (Loba Chemie, Cat no: LM0193A1103)
- Calcium chloride (CaCl2) (Merck, Cat no: C4901)
- Sucrose (Sigma, Cat no: S9378)
- PBS (Gibco, cat no: 10,010,023)
- DAPI (4,6-diamidino-2-phenylindole) stain (Invitrogen, Cat. no: D1306)
- Methanol (Sigma, Cat no: 179,337)

Methods

Fixation steps:

1. Prepare the fixation solution with 4.0% formaldehyde in PBS and adjust the pH to 7.0 with 1 N NaOH. Add Triton-X-100 (0.1%), glutaraldehyde (1.0%), 2 mM CaCl2 and 1.0% (w/v) sucrose to the fixation solution and keep it on ice prior to use.
2. Place the decellularized samples in the fixation solution overnight.
3. Wash the samples with PBS three times to remove formaldehyde from the samples.
4. Place the samples in 0.1% Triton-X-100 solution for one hour at room temperature.

Staining steps:

5. Aliquot DAPI dye according to the manufacturer's guidelines by dissolving the dye in methanol, and then diluting it with 2.1 μL of the methanolic stock solution in 100 μL PBS. Dilute the intermediate solution 1:1000 in PBS to obtain the staining solution.
6. Add the staining solution to the decellularized leek samples and keep on a shaker gently for three minutes.
7. Rinse samples with PBS twice.
8. Image the stained samples with a fluorescence microscope under violet filter.

Method validation

The goal of decellularization is to preserve the extracellular matrix while removing all cellular parts, whatever the tissue origin is. Decellularization is often used to create scaffolds for regenerative medicine, in which damaged or diseased tissue is replaced with healthy, functional tissue. Animal tissues have extracellular components similar to human tissues; however, the extracellular matrix of plants is primarily composed of cellulose, hemicellulose, lignin, and pectin. Therefore, the aim of plant decellularization is mostly to maintain the structural integrity needed to be used as a candidate biomaterial for tissue engineering and regenerative medicine. Within this plant decellularization approach, one can obtain a lamellar structure as in a leek, cabbage, or spinach or a porous structure as in a turmeric, carrot, or apple (Fig. 1).

The use of decellularized plant tissues as scaffolds for tissue engineering has various benefits. The ECM provides a natural framework for cells to develop on and is both plentiful and accessible. Additionally, plant tissues may be acceptable for use in allogeneic graft applications since they do not cause an immune response in individuals [6,11]. The wide range of features offered by different plants provides a unique opportunity for the development of tissue-specific scaffolds based on the morphological, physical, and mechanical properties of each plant. In plant decellularization, the decision to decellularize a plant as a whole or to slice it before decellularization depends mainly on the intended use in the application and also depends on several other factors, such as preserving structural integrity and mechanical properties, or providing proper porosity last but not least, considering the standardization of the sample size. The ability to design the shape and size of the chosen plant, according to the desired scaffold type for tissue regeneration has a significant advantage. For instance, carrot-derived scaffolds with a heterogeneous structure and sizes ranging from approximately 70–130 μm can be utilized as bone fillers in non-load-bearing conditions [12], comparable to the porosity of scaffolds designed for bone tissue regeneration such as collagen/hydroxyapatite scaffolds with a range of 50–100 μm [13]. Another example is celery-derived scaffolds, which can be obtained by slicing and hollowing the stem pith to create longitudinally oriented structures with aligned pores, suitable for mimicking the anisotropic connective tissue structure of tendons, due to their orientation that facilitates cell alignment [13].

Pore sizes may affect animal cell attachment and growth. The pore sizes of the plant tissues may vary. The important issue with the pore sizes is that the decellularization process may alter the pore sizes. The difference in pore sizes before and after decellularization may be minimal in hard plant tissues like bamboo, while the pore sizes may be increased in soft plant tissues like leaves after decellularization due to long oscillation and washing steps [14]. Moreover, plant tissues have analogous network structures that can

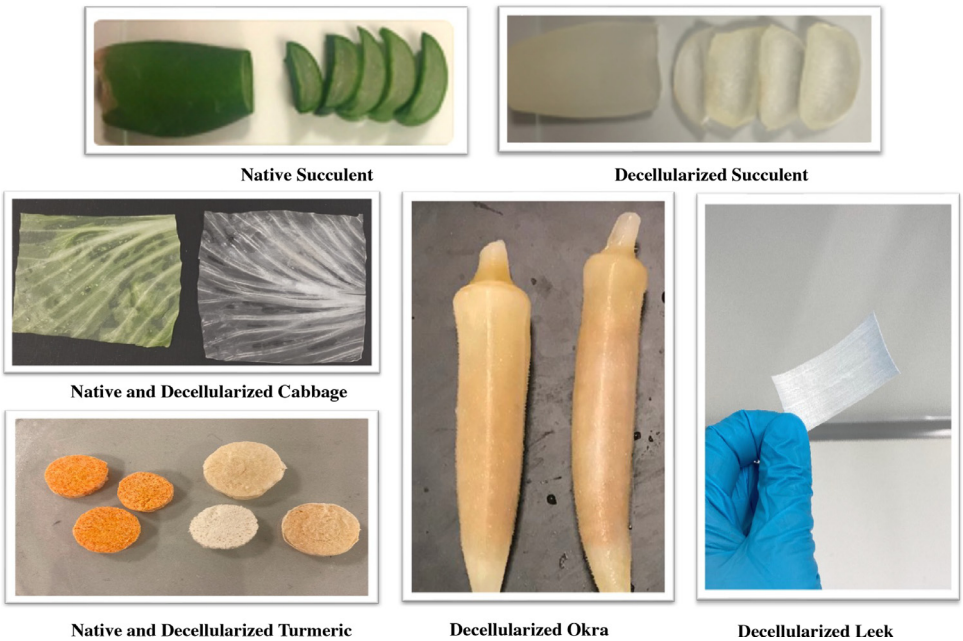

Fig. 1. Various examples for plant decellularization performed by our Group.

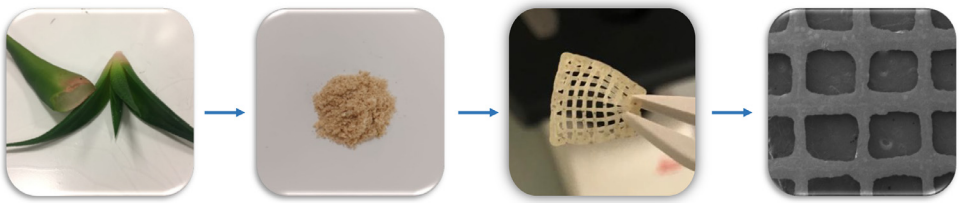

Fig. 2. An example for the use of decellularized plants.

be used to mimic animal vascular or neural networks. Previously, we reported leek decellularization and showed its potential to be used as a biomaterial due to its interconnected and elongated channel-like structure [15].

Surfactant such as SDS is used for the decellularization of plants; however, it should be noted that smaller tissues / tissue slices may require a low concentration of SDS and may need less time for the decellularization, while larger plant parts and whole plants may need a higher concentration of SDS and may need more time for an effective decellularization [13].

The decellularization efficacy should be evaluated by various assessments, whatever form is used. The main assessment is the quantification of genomic DNA content after decellularization, either by spectroscopically or by nuclear staining. The efficacy of decellularization can be determined by comparing of the ratio of DNA content before and after decellularization. Although there is still debate about the limit of DNA content after decellularization, <50 ng dsDNA per mg extracellular matrix (ECM) dry weight; <200 bp DNA fragment length, and a lack of visible nuclear material in tissue sections stained with DAPI or Hematoxylin and eosin staining are widely accepted criteria [16]. The one crucial method for whole plant tissue decellularization is to check the structural integrity and morphological characteristics of the final form by Scanning Electron Microscopy (SEM).

Decellularized plant tissues can also be powdered after decellularization by using a mortar or grinder to be used as a kind of component of a blended scaffold in their powder form. These scaffolds may be prepared via conventional scaffold preparation methods such as molding and casting or trending techniques such as 3D printing (Fig. 2). Finally, although recellularization of structures using appropriate cells and maintaining structural and mechanical properties remains challenging, decellularized-based plant tissues have the potential to be used as scaffolds for *in vitro* and *in vivo* tissue repair and regeneration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Melis Toker-Bayraktar: Methodology, Data curation, Visualization, Writing – original draft. **Melek İpek Ertugrul:** Methodology, Data curation, Writing – original draft. **Sedat Odabas:** Conceptualization, Methodology, Writing – review & editing, Writing – original draft. **Bora Garipcan:** Conceptualization, Methodology, Writing – review & editing, Writing – original draft.

Data availability

Data will be made available on request.

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