Video Article

# Two Methods for Decellularization of Plant Tissues for Tissue Engineering Applications

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## Abstract

The autologous, synthetic, and animal-derived grafts currently used as scaffolds for tissue replacement have limitations due to low availability, poor biocompatibility, and cost. Plant tissues have favorable characteristics that make them uniquely suited for use as scaffolds, such as high surface area, excellent water transport and retention, interconnected porosity, preexisting vascular networks, and a wide range of mechanical properties. Two successful methods of plant decellularization for tissue engineering applications are described here. The first method is based on detergent baths to remove cellular matter, which is similar to previously established methods used to clear mammalian tissues. The second is a detergent-free method adapted from a protocol that isolates leaf vasculature and involves the use of a heated bleach and salt bath to clear the leaves and stems. Both methods yield scaffolds with comparable mechanical properties and low cellular metabolic impact, thus allowing the user to select the protocol which better suits their intended application.

## Video Link

The video component of this article can be found at https://www.jove.com/video/57586/

## Introduction

Tissue engineering emerged in the 1980s to create living tissue substitutes, and potentially address significant organ and tissue shortages[1]. One strategy has used scaffolds to stimulate and guide the body to regenerate missing tissues or organs. Although advanced manufacturing approaches such as 3-D printing have produced scaffolds with unique physical properties, the ability to manufacture scaffolds with a diverse range of achievable physical and biological properties remains a challenge[2,3]. Moreover, due to a lack of a functional vascular network, these techniques have been limited in regenerating 3-dimensional tissues. The use of decellularized animal and human tissues as scaffolds has aided in circumventing this problem[4,5,6,7]. However, high cost, batch-to-batch variability, and limited availability may limit widespread use of decellularized animal scaffolds[8]. There are also concerns about potential disease transmission to patients and immunologic reaction to some decellularized mammalian tissues[9].

Cellulose, derived from plant and bacterial sources, has been extensively used to generate biomaterials for a wide range of applications in regenerative medicine. Some examples include: bone[10,11], cartilage[12,13,14] and wound healing[15]. Scaffolds that are comprised of cellulose have an added benefit in that they are durable and resistant to being broken down by mammalian cells. This is due to the fact that mammalian cells do not produce the enzymes necessary to break down cellulose molecules. In comparison, scaffolds produced using macromolecules from the extracellular matrix, such as collagen, are readily broken down[16] and may not be well suited to long-term applications. Collagen scaffolds can be stabilized by chemical cross-linking. However, there is a trade-off due to the inherent toxicity of the cross-linkers that affect the biocompatibility of the scaffolds[17]. Conversely, cellulose has the potential to remain present at the site of implantation for prolonged periods of time because it is impervious to enzymatic degradation by mammalian cells[18,19,20]. This can be altered by tuning the rate of degradation through hydrolysis pretreatment and co-delivery of the scaffolds with cellulases[21]. The biocompatibility of decellularized plant-derived cellulose scaffolds *in vivo* has also been demonstrated in a study done on mice[22].

Through hundreds of millions of years of evolution, plants have refined their structure and composition to increase the efficiency of fluid transport and retention. Plant vascular vessels minimize hydraulic resistance by branching into smaller vessels, similar to the mammalian vasculature according to Murray's law[23]. After decellularization, the plant's complex network of vessels and interconnected pores is maintained. Considering the vast number of distinct plant species readily available, plant-derived scaffolds have the potential to overcome design limitations currently affecting scaffolds in tissue engineering[24,25]. For instance, Modulevsky *et al.* demonstrated that angiogenesis and cell migration occurred when decellularized apple tissue was implanted subcutaneously on the back of a mouse[22]. Similarly, Gershlak *et al.* showed that endothelial cells could

be grown within the vasculature of decellularized leaves[24]. In a separate experiment, Gershlak *et al.* were also able to show that cardiomyocytes could be grown on the surface of leaves and were able to contract[24].

Plants also include complex organization from the cellular to the macroscopic scale, which is difficult to achieve even with the most advanced manufacturing techniques developed to date. The complex hierarchical design of plant tissues makes them stronger than the sum of their constituents[26]. Plants possess a plethora of different mechanical properties ranging from rigid and tough components such as stems, to much more flexible and pliable ones such as leaves[27]. Leaves vary depending on species in terms of size, shape, break strength, the degree of vascularization, and can carry different degrees of hydrophilicity. Overall, these plant properties suggest that decellularized plants can serve as unique and highly functional medical devices, including as tissue engineering scaffolds.

This protocol focuses on two methods to decellularize plant tissues, such as leaves and stems, for use as scaffolds in tissue engineering. The first method is a detergent-based technique that uses a series of baths to remove DNA and cellular matter, which has been adapted from a widely used technique to decellularize mammalian and plant tissues[6,22,25,28,29,30]. The second method is detergent-free and is adapted from a "skeletonization" protocol generally used to remove the soft tissues of leaves[31]. Prior work showed that simmering leaves in a bleach and sodium bicarbonate solution facilitated separation of the vasculature from the surrounding soft tissue[31]. This technique can be cited back to experiments carried out in the 17$^{th}$ and 18$^{th}$ centuries, such as the work of Albertus Seba[32] and Edward Parrish[33]. These experiments centered around leaving plant matter, such as leaves and fruit, submerged in water for extended periods of time (weeks to months) and allowing the softer tissues to decay away naturally. Here the "skeletonization" approach is adapted to use milder conditions, such as longer incubation times at lower temperatures, to remove cellular residues and to avoid significantly disrupting the soft tissue structure. For the experiments detailed herein, three plant types were used: *Ficus hispida*, *Pachira aquatica* and a species of *Garcinia*. Results of DNA quantification, mechanical tests, and impact on cellular metabolic activity from both methods are described.

## Protocol

# 1. Decellularization of Plant Tissue Using the Detergent-based Approach

1. **Use fresh or frozen *F. hispida*, leaf samples. Freeze unused fresh samples in a -20 °C freezer and store for future use (up to a year).**
   NOTE: Use stem or leaf tissue of nearly any desired plant. Extended storage times can cause damage to the tissues.
   1. Determine the size and shape of samples to be processed on the basis of the sample's intended use (*i.e.* samples cut into strips are well suited for mechanical testing applications, meanwhile 8 mm disc samples are useful in multi-well culturing applications). Cut the leaf into 8 mm discs with a sharp, clean biopsy punch while submerged under room temperature (20-25 °C) deionized $H_2O$.
      Note: This protocol can be used on whole leaves and stems. However, smaller samples will decellularize faster.
   2. Incubate the samples for 5-10 min at room temperature (20-25 °C) deionized $H_2O$ on a shake plate set to a low speed setting to wash and/or thaw them. Use enough deionized $H_2O$ to ensure all samples are thoroughly wetted.

2. **Prepare a solution of 10% (w/v) sodium dodecyl sulfate (SDS) in deionized $H_2O$. Place the samples in a suitable container (a glass or plastic dish is ideal) and add SDS solution to completely cover the samples. Incubate samples for 5 days at room temperature (20-25 °C) on a shake plate set to a low speed to prevent damage to the samples.**
   NOTE: Do not overcrowd the container as this can slow down the decellularization process and lead to uneven treatment by the SDS. During this step, samples should acquire a brown hue.
   1. After 5 days, replace the SDS solution with deionized $H_2O$. Incubate the samples for an additional 10-15 min on the shake plate to thoroughly rinse off any residual SDS solution.

3. Prepare a 1% (v/v) non-ionic surfactant in 10% (v/v) bleach solution. To make a 500 mL solution, mix 5 mL of the non-ionic surfactant with 50 mL of bleach then add 445 mL of deionized $H_2O$. Submerge samples in the freshly prepared solution.
   NOTE: The non-ionic surfactant/bleach solution does not have a long shelf life, therefore, should be used within 48 h of preparation.
4. **Replace the non-ionic surfactant/bleach solution every 24 h until the samples are completely cleared (refer to Figure 1A for visual comparison). Then incubate the sample in deionized water on a shake plate for 2 min to rinse off the excess non-ionic surfactant/bleach solution. Set the shake plate to a low setting to prevent damaging the samples.**
   NOTE: The time required to clear the samples used varies, depending on the type and species of the plant. The complete discoloring of the samples is indicative of full decellularization (perform DNA quantification to ensure thorough clearing).
   1. Lyophilize the samples to store them up to a year (flash freezing using liquid nitrogen is preferred, freezing samples in -80 °C is also acceptable) and store them at room temperature (20-25 °C) in low humidity.
   2. Reconstitute samples in Tris-HCl (10 mM, pH 8.5) using enough to coat samples. Rinse samples 2-3 times in serum-free media gently using a micropipette before use (*i.e.* use 150-300 µL per rinse, in a standard 24 well plate).
      NOTE: Tris-HCl buffer and serum-free media (*i.e.* DMEM, but any basic cell culture media can be substituted) are more effective in lowering the impact to cell viability of SDS treated samples than those treated with deionized $H_2O$ alone.

# 2. Preparation of Samples Using the Detergent-free Decellularization Approach

NOTE: The initial steps of this procedure coincide with steps 1.1-1.1.2 (see above).

1. Prepare a 5% (v/v) bleach (NaClO) and 3% (w/v) sodium bicarbonate ($NaHCO_3$) solution. Warm the solution in a fume hood to 60-70 °C for *F. hispida* leaf samples cut into 8 mm discs while stirring on a hot plate.
   NOTE: The sodium bicarbonate can be substituted with sodium carbonate ($Na_2CO_3$), or sodium hydroxide (NaOH). The desired temperature varies greatly (from room temperature to 90 °C) and should be adjusted according to the properties of the samples used.

2. Once the solution reaches the desired temperature range, submerge the samples and reduce the stirring speed to avoid damaging them. After the samples are visibly cleared (refer to **Figure 1B** for visual comparison), remove from the bath carefully. Incubate samples once in deionized $H_2O$ for 1-2 min to remove excess bleach solution.
   NOTE: The time required to clear the samples can vary widely. For example, cut parsley can be cleared in 10-15 min, while thicker and/or larger samples such as whole leaves or stems can take hours in high-temperature baths to clear completely.
3. Lyophilize the samples and store them at room temperature (20-25 °C) in low humidity.

## Representative Results

Both methods yielded scaffolds that were suitable for cell culture and tissue engineering applications. **Figure 1** shows the general workflow of the decellularization process using an intact leaf for the detergent-based method and cut samples (8 mm diameter) for the detergent-free method. Successful decellularization of *Ficus hispida* tissues following both methods yielded clear and intact samples (**Figure 1A** and **1B**). It was possible to decellularize whole plant tissues (**Figure 1A**); however, smaller samples appeared to clear faster and more effectively. A potential issue observed with the detergent-based approach involved heterogeneous decellularization as a result of premature removal from the non-ionic surfactant bath (**Figure 1C**). A drawback of the detergent-free method is that damage can occur due to prolonged incubation in the heated bath (**Figure 1D**).

The mechanical properties of decellularized scaffolds prepared using both methods were investigated using uniaxial tensile testing as was done in other studies[24,34]. This testing yielded the maximum tangent modulus (MTM) (**Figure 2A**), strain at failure (SAF) (**Figure 2B**), and ultimate tensile strength (UTS) (**Figure 2C**) for *F. hispida* and *Pachira aquatica* samples. *P. aquatica* samples prepared using both decellularization protocols displayed similar mechanical properties across all three of the parameters measured. The *F. hispida* samples showed a similar trend in all cases except the UTS testing. In particular, samples prepared using the detergent-based (SDS) protocol had higher average UTS results than those prepared with the detergent-free (Bleach) approach. These results show that samples collected from different plant species may produce distinct decellularized scaffold properties when prepared via the two decellularization protocols.

To measure DNA removal, samples were cleared using either method and their genomic DNA was isolated using a previously established protocol[35]. Both decellularization methods (detergent-based and detergent-free) significantly decreased the genomic DNA content of samples to 2.47 ng of DNA/mg (n = 3, s = 0.18) and 2.71 ng of DNA/mg of tissue (n = 3, s = 1.60) respectively (**Figure 2D**). Non-decellularized samples had 104.67 ng of DNA/mg of tissue (n = 3, s = 26.21) (**Figure 2D**). The substantial decrease in DNA content after decellularization indicated an effective removal of plant cell matter.

The impact on cell viability of the two decellularization methods was assessed by measuring the metabolic activity of Human dermal fibroblasts (hDF) cultured for 2 days in the presence of decellularized *P. aquatica* or *Garcinia* scaffolds. hDFs had higher metabolic activity when cultured in the presence of scaffolds decellularized via the detergent-free method versus those prepared via the detergent-based method (**Figure 3A**). To ensure thorough removal of the reagents used during the decellularization, an additional set of experiments was performed in which the prepared scaffolds were washed extensively prior to cell culture. Two separate washing methods were tested on samples prepared using the detergent-based approach: a wash in Tris-HCl buffer (10 mM, pH 8.5) followed by a wash in Serum Free Media *vs.* 2 washes in deionized $H_2O$. Both approaches used final washing steps with 1x PBS. The impact to the mammalian cells subsequently exposed to the decellularized plant scaffolds was measured using the same metabolic activity assay as above. Tris-HCl buffer (10 mM, pH 8.5) followed by Serum Free Media helped reduce the impact on cellular metabolic activity compared with deionized $H_2O$, making samples treated with the detergent-based approach comparable to samples from the detergent-free method (**Figure 3B**).

It was previously shown that mammalian cells could grow on scaffolds prepared using the detergent-based approach[25]; therefore, it was necessary to demonstrate this on samples prepared by the previously untested detergent-free method. Mesenchymal stem cells (MSCs) were seeded on decellularized *F. hispida* leaf samples (8 mm discs) at 20,000 cells per scaffold and allowed to incubate for 24 h. These scaffolds were functionalized prior to cell introduction with an RGD-Dopamine conjugate to facilitate adhering. **Figure 4** illustrates images collected from a typical sample. A bright field image was collected to show the overall structure of a section of the sample used (**Figure 4A**). The MSCs were stained using calcein and imaged with a fluorescence microscope (**Figure 4B**). The images were then overlaid (**Figure 4C**) to display the location of the growing cells on the surface of the leaf.

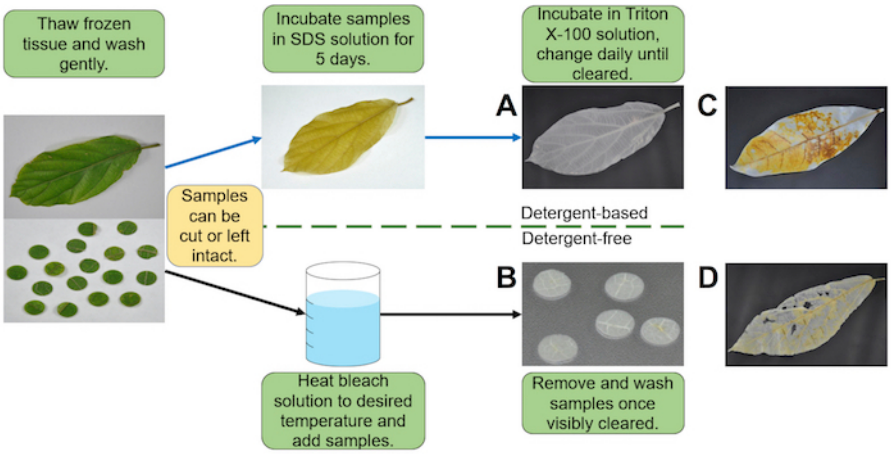


**Figure 1. The general workflow for decellularization of plant tissues.** Typical workflow for the decellularization of plant tissues (washing and preparatory steps are universal); (**A**) top path is for the detergent-based method (**B**) bottom for the detergent-free method. (**C**) An incomplete/ failed decellularization of a *P. aquatica* leaf using the detergent-based method due to premature removal from the non-ionic surfactant/bleach bath (**D**) A damaged decellularization of a *F. hispida* leaf due to prolonged incubation in the heated bleach solution in the detergent-free method. Please click here to view a larger version of this figure.

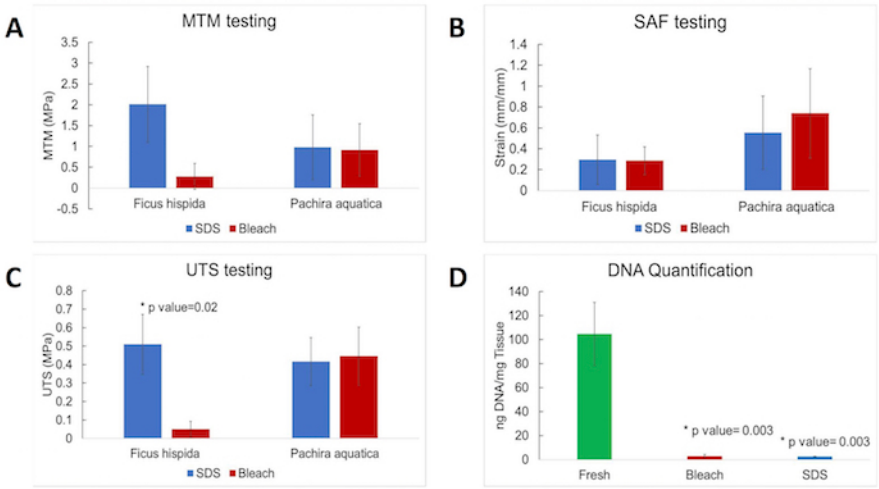


**Figure 2. Comparison of mechanical properties and effectiveness of decellularization between methods.** Mean ± SD; statistical analysis was done using the two-sample t-test and ***p*** values listed where statistically significant (less than or equal to 0.05). Comparison of mechanical testing results between samples of *F. hispida* and *P. aquatica* (blue bars used for detergent-based approach, red for the detergent-free, and green for untreated samples) (*F. hispida* detergent-based n = 4, detergent-free n = 2; *P. aquatica* detergent-base n = 3, detergent-free n = 4) (**A**) Maximum Tangent Modulus (MTM) (**B**) Strain at Failure (SAF) (**C**) and Ultimate Tensile Strength (UTS). (**D**) A dsDNA quantification assay was run in triplicate on three samples from each group: untreated (Fresh, n = 3), the detergent-free method (Bleach, n = 3) and the detergent-based method (SDS, n = 3), *p* values are for decellularization method group compared to untreated sample group. Please click here to view a larger version of this figure.

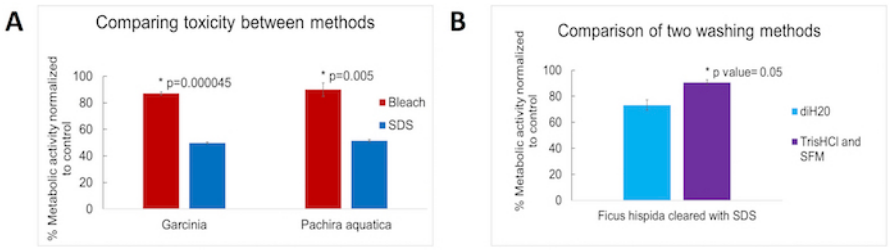


**Figure 3. Metabolic activity assay results comparing impact between the two decellularization methods.** Mean ± SD; statistical analysis was done using the two-sample t-test and *p* values listed where statistically significant (less than or equal to 0.05). Cellular metabolic activity was measured in the presence of tissue samples of both *P. aquatica* and a species of *Garcinia* (normalized to control wells with no sample added) to display a (**A**) Comparison of the metabolic impact of the two decellularization methods (n = 3 for each group) and (**B**) Comparison of the impact to metabolic activity using two washing methods in detergent-based protocol: Tris-HCl buffer (10 mM, pH 8.5) combined with Serum Free Media (n = 4) *vs.* deionized $H_2O$ (n = 4). Please click here to view a larger version of this figure.

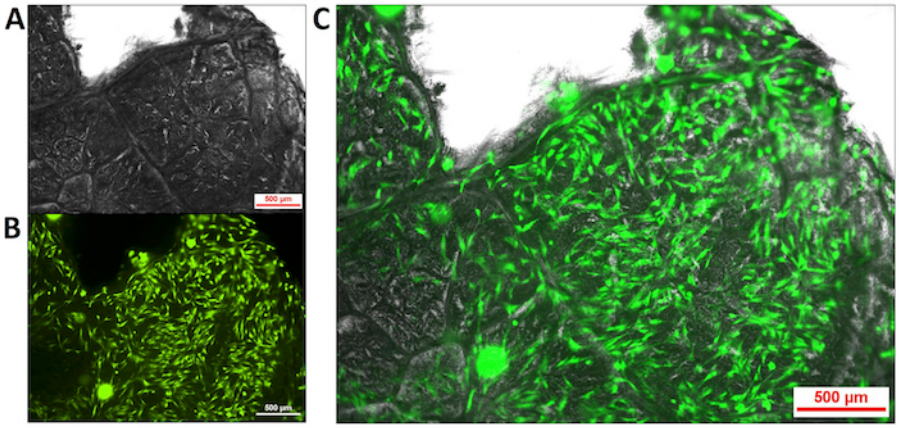


**Figure 4. Mesenchymal stem cells (MSCs) growing on the surface of decellularized *Ficus hispida* leaf, functionalized with a RGD-Dopamine conjugate.** MSCs were grown on *F. hispida* leaves, decellularized by the detergent-free method and functionalized with an RGD-Dopamine conjugate (500 µm scale bars were added for reference) (**A**) Bright field image of a section of decellularized leaf (**B**) Fluorescence image of calcein stained MSCs growing on leaf surface (**C**) Merged bright field and fluorescence image. Please click here to view a larger version of this figure.

## Discussion

Herein, two methods to decellularize plant tissues are described. The results presented here, coupled with the results of prior studies[25], suggest that the protocols put forth are likely applicable to a wide spectrum of plant species and can be performed on both stems and leaves. These procedures are simple and do not require specialized equipment, so plant decellularization can be carried out in most laboratories. It is noteworthy that after decellularization, the scaffolds must be functionalized to facilitate mammalian cell adhesion. Different functionalization techniques to enable mammalian cell adhesion and growth on plant tissues have been described elsewhere[25] and are not the topic of the current manuscript.

The final step in both decellularization protocols presented here (**Figure 1**) was critical to their success. When performing either method do not overcrowd the bath as this will result in an uneven clearing. These methods can be applied to whole leaves and stems; however, this will increase the length of time required to clear them. Make sure that the samples are submerged entirely to keep the clearing consistent throughout the entire sample. Ideally, decellularization should clear plant tissues of most cellular matter while minimizing structural damage. It is likely that the detergent-free method can be modified as needed to work with desired specimens. Higher bath temperatures may result in faster clearing times but may also result in more damage to the samples if they are over-incubated. Lower temperatures may require longer incubation times and would be appropriate for more fragile samples. The concentration of bleach in the solution can also be changed to accelerate or decelerate the decellularization process. When testing new samples for use with the detergent-free method, it is recommended to start with a bath temperature of 50-60 °C with frequent checks on the progress of the decellularization (ideally every 15-20 minutes) until they are adequately cleared. This temperature range was well tolerated by most of the plant species that were tested during optimization of the technique. Upon completion, the samples can be inspected visually and manually to ensure they have not been over-incubated and damaged irreparably as would be indicated by tearing or disintegration upon removal from the bath. Samples prepared using this approach show a lessened impact on the cell growth (refer to **Figure 3A**) than the detergent-based approach with only washing in deionized $H_2O$. However, it is recommended to wash additionally with Tris-HCl (10 mM, pH 8.5) and serum-free media, especially in sensitive applications.

The detergent-based (SDS) method is likely applicable to most plant species and is especially useful for clearing whole leaf and delicate samples because it requires minimal physical agitation. As mentioned above, the final step is critical for the decellularization of samples. Samples with distinct physical properties can be cleared by adjusting the incubation time in the non-ionic surfactant baths. A significant drawback of this process is that decellularized tissues may contain traces of the detergents used, which can subsequently influence the cellular metabolic activity

of mammalian cells. Therefore, thorough wash steps are recommended before use. Here it was found that samples washed with Tris-HCl buffer (10 mM, pH 8.5) followed by serum-free media had higher cell viability than those washed with deionized $H_2O$ before their use **(Figure 3B)**.

When storing untreated samples for future use in either method, it is important to monitor them for damage to ensure that the resulting scaffolds will not be impacted. Damage can appear as discoloration of the plant tissues as well as cracking or excessive brittleness when handled. It is recommended to monitor the samples on a weekly basis so as to utilize them before they are no longer useful and need to be disposed of. The onset of damage varies between specimen types, with thicker more robust samples lasting longer.

In general, plants hold much promise as a potential source of biomaterials. This is bolstered by their naturally developed intricate structures and characteristics that help them grow in their native environments. Successful utilization of plant tissues as tissue engineering scaffolds proposes a potentially cheap alternative to costly, and often difficult to design, biomaterials. Plant tissue's surface topography, which inherently varies from one species to another, can also be harnessed to direct spatially oriented cellular growth[25]. For example, a previous study has shown that human cells respond to topographical cues present on decellularized plants[25], thus these scaffolds can be used to culture cells in highly organized patterns[25]. Another feature that is desirable in tissue engineering is *de novo* perfusability. Plant tissues have evolved over hundreds of millions of years to be extremely efficient at fluid transport, and their vascular networks can be efficiently perfused[24]. This property can be potentially used to guide cellular migration and angiogenesis with the ultimate goal of creating replacement tissue for clinical applications. This is bolstered by the findings of previous studies that demonstrated that biomaterials with the high surface area and interconnected porosity are conducive to cell proliferation and when implanted *in vivo*, enable inward migration of the host cells into the implant[36,37,38,39]. The high surface area and the interconnected porosity are also the hallmark of plant tissues[40,41,42], thus plant-derived scaffolds have the potential to integrate with the surrounding tissues *in vivo*. Moreover, recent studies found that decellularized leaves can be successfully perfused with cells[24] and that when human cells were seeded on decellularized plant tissues, they conformed to their topographical cues[25]. When taken all together, plant-derived scaffolds possess the necessary properties to be successfully applied towards tissue engineering applications.

## Disclosures

The authors have nothing to disclose.

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