

Review

Decellularization of tissues and organs

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Abstract

Decellularized tissues and organs have been successfully used in a variety of tissue engineering/regenerative medicine applications, and the decellularization methods used vary as widely as the tissues and organs of interest. The efficiency of cell removal from a tissue is dependent on the origin of the tissue and the specific physical, chemical, and enzymatic methods that are used. Each of these treatments affect the biochemical composition, tissue ultrastructure, and mechanical behavior of the remaining extracellular matrix (ECM) scaffold, which in turn, affect the host response to the material. Herein, the most commonly used decellularization methods are described, and consideration given to the effects of these methods upon the biologic scaffold material.

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1. Introduction

Biologic scaffolds derived from decellularized tissues and organs have been successfully used in both pre-clinical animal studies and in human clinical applications [1–8]. Removal of cells from a tissue or an organ leaves the complex mixture of structural and functional proteins that constitute the extracellular matrix (ECM). The tissues from which the ECM is harvested, the species of origin, the decellularization methods and the methods of terminal sterilization for these biologic scaffolds vary widely. Each of these variables affects the composition and ultrastructure of the ECM and accordingly, affects the host tissue response to the ECM scaffold following implantation. The objective of this manuscript is to provide an overview of the various methods that have been used to decellularize tissues, and the potential effects of the various decellularization protocols on the biochemical composition, ultrastructure, and mechanical behavior of the ECM scaffold materials.

2. Rationale for decellularization of ECM

Xenogeneic and allogeneic cellular antigens are, by definition, recognized as foreign by the host and therefore induce an inflammatory response or an immune-mediated rejection of the tissue. However, components of the ECM are generally conserved among species and are tolerated well even by xenogeneic recipients [9–12]. ECM from a variety of tissues, including heart valves [13–19], blood vessels [20–23], skin [24], nerves [25,26], skeletal muscle [27], tendons [28], ligaments [29], small intestinal submucosa (SIS) [30–32], urinary bladder [2,33,34], and liver [35] have been studied for tissue engineering and regenerative medicine applications. The goal of a decellularization protocol is to efficiently remove all cellular and nuclear material while minimizing any adverse effect on the composition, biological activity, and mechanical integrity of the remaining ECM.

Any processing step intended to remove cells will alter the native three-dimensional architecture of the ECM. The most commonly utilized methods for decellularization of tissues involve a combination of physical and chemical treatments. The physical treatments can include agitation or sonication, mechanical massage or pressure, or freezing and thawing. These methods disrupt the cell membrane, release cell contents, and facilitate subsequent rinsing and removal of the cell contents from the ECM. These physical treatments are generally insufficient to achieve complete decellularization and must be combined with a chemical treatment. Enzymatic treatments, such as trypsin, and chemical treatment, such as ionic solutions and detergents, disrupt cell membranes and the bonds responsible for intercellular and extracellular connections. Tissues are composed of both cellular material and ECM arranged in variable degrees of compactness depending on the source of the tissue. The ECM must be adequately disrupted

during the decellularization process to allow for adequate exposure of all cells to the chaotropic agents and to provide a path for cellular material to be removed from the tissue. The intent of most decellularization processes is to minimize the disruption and thus retain native mechanical properties and biologic properties.

3. Description of decellularization protocols

The most robust and effective decellularization protocols include a combination of physical, chemical, and enzymatic approaches. A decellularization protocol generally begins with lysis of the cell membrane using physical treatments or ionic solutions, followed by separation of cellular components from the ECM using enzymatic treatments, solubilization of cytoplasmic and nuclear cellular components using detergents, and finally removal of cellular debris from the tissue. These steps can be coupled with mechanical agitation to increase their effectiveness. Following decellularization, all residual chemicals must be removed to avoid an adverse host tissue response to the chemical. The efficiency of decellularization and preservation of the ECM can be assessed by several methods. The mechanisms of physical, enzymatic, and chemical decellularization for a variety of tissues are reviewed in the following sections and in Table 1.

3.1. Physical methods

Physical methods that can be used to facilitate decellularization of tissues include freezing, direct pressure, sonication, and agitation. Snap freezing has been used frequently for decellularization of tendinous and ligamentous tissue [36–41] and nerve tissue [42]. By rapidly freezing a tissue, intracellular ice crystals form that disrupt cellular membranes and cause cell lysis. The rate of temperature change must be carefully controlled to prevent the ice formation from disrupting the ECM as well. While freezing can be an effective method of cell lysis, it must be followed by processes to remove the cellular material from the tissue.

Cells can be lysed by applying direct pressure to tissue, but this method is only effective for tissues or organs that are not characterized by densely organized ECM (e.g., liver, lung). Mechanical force has also been used to delaminate layers of tissue from organs that are characterized by natural planes of dissection such as the small intestine and the urinary bladder. These methods are effective, and cause minimal disruption to the three-dimensional architecture of the ECM within these tissues.

Mechanical agitation and sonication have been utilized simultaneously with chemical treatment to assist in cell lysis and removal of cellular debris. Mechanical agitation can be applied by using a magnetic stir plate, an orbital shaker, or a low profile roller. There have been no studies performed to determine the optimal magnitude or frequency of sonication for disruption of cells, but a standard ultrasonic cleaner appears to be as effective at

Table 1
Commonly used decellularization methods and chaotropic agents

Method	Mode of action	Effects on ECM	References
Physical			
Snap freezing	Intracellular ice crystals disrupt cell membrane	ECM can be disrupted or fractured during rapid freezing	[36–42]
Mechanical force	Pressure can burst cells and tissue removal eliminates cells	Mechanical force can cause damage to ECM	[33,35]
Mechanical agitation	Can cause cell lysis, but more commonly used to facilitate chemical exposure and cellular material removal	Aggressive agitation or sonication can disrupt ECM as the cellular material is removed	[19,21,33,35]
Chemical			
Alkaline; acid	Solubilizes cytoplasmic components of cells; disrupts nucleic acids	Removes GAGs	[33,43–46]
Non-ionic detergents			
Triton X-100	Disrupts lipid–lipid and lipid–protein interactions, while leaving protein–protein interactions intact	Mixed results; efficiency dependent on tissue, removes GAGs	[2,21,28,29,35,43,58]
Ionic detergents			
Sodium dodecyl sulfate (SDS)	Solubilize cytoplasmic and nuclear cellular membranes; tend to denature proteins	Removes nuclear remnants and cytoplasmic proteins; tends to disrupt native tissue structure, remove GAGs and damage collagen	[18,24,25,29,35,59,60]
Sodium deoxycholate		More disruptive to tissue structure than SDS	[18,24,25,29,35,59,60]
Triton X-200		Yielded efficient cell removal when used with zwitterionic detergents	[18,24,25,29,35,59,60]
Zwitterionic detergents			
CHAPS	Exhibit properties of non-ionic and ionic detergents	Efficient cell removal with ECM disruption similar to that of Triton X-100	[21]
Sulfobetaine-10 and -16 (SB-10, SB-16)		Yielded cell removal and mild ECM disruption with Triton X-200	[18,24,25,29,35,59,60]
Tri(<i>n</i> -butyl)phosphate	Organic solvent that disrupts protein–protein interactions	Variable cell removal; loss of collagen content, although effect on mechanical properties was minimal	[28,29]
Hypotonic and hypertonic solutions	Cell lysis by osmotic shock	Efficient for cell lysis, but does not effectively remove the cellular remnants	[21,29,61–63]
EDTA, EGTA	Chelating agents that bind divalent metallic ions, thereby disrupting cell adhesion to ECM	No isolated exposure, typically used with enzymatic methods (e.g., trypsin)	[13,68–70]
Enzymatic			
Trypsin	Cleaves peptide bonds on the C-side of Arg and Lys	Prolonged exposure can disrupt ECM structure, removes laminin, fibronectin, elastin, and GAGs	[13,68–70]
Endonucleases	Catalyze the hydrolysis of the interior bonds of ribonucleotide and deoxyribonucleotide chains	Difficult to remove from the tissue and could invoke an immune response	[18,21,29,82]
Exonucleases	Catalyze the hydrolysis of the terminal bonds of ribonucleotide and deoxyribonucleotide chains		

removing cellular material as placing the tissue on an orbital shaker. In all of these procedures, the optimal speed, volume of reagent, and length of mechanical agitation is dependent on the composition, volume, and density of the tissue.

3.2. Chemical methods

3.2.1. Alkaline and acid treatments

Alkaline and acid treatments are used in decellularization protocols to solubilize the cytoplasmic component of

the cells as well as remove nucleic acids such as RNA and DNA. For example, acetic acid, peracetic acid (PAA), hydrochloric acid, sulfuric acid, and ammonium hydroxide (NH₄OH) can effectively disrupt cell membranes and intracellular organelles [33,43–46]. However, these chemicals also dissociate important molecules such as GAGs from collagenous tissues.

A variety of porcine tissues including SIS and layers of the urinary bladder [e.g., submucosal layer (UBS) and the basement membrane plus tunica propria (UBM)] have been decellularized using PAA at concentrations of

approximately 0.10–0.15% (w/v). This treatment is highly efficient at removing cellular material from these thin ECM structures, and simultaneously disinfects the material by entering microorganisms and oxidizing microbial enzymes [47,48]. The effects of PAA treatment on the ECM components have been studied extensively. Several types of collagen including types I, III, IV, V, VI, and VII have been identified in SIS and/or UBM following treatment with PAA [31,49], however, the microstructure of the collagen fibers has not been closely examined following such treatment. The ECM retains many of the native GAGs including hyaluronic acid, heparin, heparin sulfate, chondroitin sulfate A, and dermatan sulfate following PAA treatment [50]. It has also been shown that laminin and fibronectin are present in the ECM scaffolds following exposure to PAA [49,51]. PAA treatment preserves the structure and function of many of the growth factors that are resident in the ECM, including transforming growth factor- β , basic fibroblast growth factor, and vascular endothelial growth factor [52,53]. PAA does not appear to have any adverse effect on the mechanical behavior of the biologic scaffold [33]. Both SIS and UBM have been shown repeatedly to serve as excellent substrates for in vitro cell culture [51,54–56], and have been successfully used for many tissue-engineering applications in vivo following decellularization and disinfection with PAA.

3.2.2. Non-ionic detergents

Non-ionic detergents have been used extensively in decellularization protocols because of their relatively mild effects upon tissue structure. Non-ionic detergents disrupt lipid–lipid and lipid–protein interactions, but leave protein–protein interactions intact so that proteins within a tissue or organ following non-ionic detergent treatment should be left in a functional conformation [57].

Triton X-100 is the most widely studied non-ionic detergent for decellularization protocols. Exposure of tissue to Triton X-100 for periods ranging from several hours to 14 days [2,21,28,29,35,43,58]. Decellularization of tissues with Triton X-100 has shown mixed results. When Triton X-100 was used to decellularize a heart valve, complete removal of nuclear material was observed with maintenance of the valvular structure after 24 h. However, cellular material was found in the adjacent myocardium and aortic wall [15]. With regard to the ECM components, Triton X-100 led to a nearly a complete loss of GAGs and decreases in the laminin and fibronectin content of the valve tissue [15]. Other studies showed that Triton X-100 was not effective at completely removing cellular material from a blood vessel, tendon, and ligament after exposure for up to 4 days [21,28,29]. Nuclear material was observed by histological staining in all of the tissues and immunohistochemical staining showed the presence of the cytoskeletal protein vimentin in the anterior cruciate ligament (ACL) after treatment with Triton X-100. It was found that treatment with Triton X-100 severely altered the tendon with respect to the tensile strength of collagen fibers

isolated from the tendon. Conversely, treatment with Triton showed no effect on the collagen content in the ACL. Treatment with Triton X-100 also showed mixed results with regards to GAG content. All GAGs were removed from a heart valve after treatment with Triton X-100 for 24 h, while there was no difference in the sulfated GAG content in the ACL after treatment with Triton X-100 for 4 days. Although Triton X-100 can be an effective decellularization method, its efficacy is dependent upon the tissue being decellularized and the other methods with which it is combined in a given decellularization protocol.

3.2.3. Ionic detergents

Ionic detergents are effective for solubilizing both cytoplasmic and nuclear cellular membranes, but tend to denature proteins by disrupting protein–protein interactions [57]. The most commonly used ionic detergents are sodium dodecyl sulfate (SDS) and sodium deoxycholate and Triton X-200 [18,24,25,29,35,59,60].

SDS is very effective for removal of cellular components from tissue. Compared to other detergents, SDS yields more complete removal of nuclear remnants and cytoplasmic proteins, such as vimentin [29]. SDS tends to disrupt the native tissue structure, and causes a decrease in the GAG concentration and a loss of collagen integrity. However, it does not appear that SDS removes collagen from the tissue.

Sodium deoxycholate is also very effective for removing cellular remnants, but tends to cause greater disruption to the native tissue architecture when compared to SDS. There are no reports of tissue decellularization using sodium deoxycholate alone, so it is difficult to isolate its effect on the remaining ECM of a tissue. Sodium deoxycholate was also combined with several zwitterionic detergents to decellularize nerve tissue, however, it was found that only a combination of Triton X-200 with the zwitterionic detergents yielded a completely decellularized nerve ECM [25,60].

3.2.4. Zwitterionic detergents

Zwitterionic detergents exhibit the properties of both non-ionic and ionic detergents. Zwitterionic detergents have a greater tendency to denature proteins than non-ionic detergents. Examples of zwitterionic detergents include 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), which has been studied for decellularization of blood vessels [21], and sulfobetaine-10 (SB-10) and -16 (SB-16), which have been used for decellularization of nerves [25,60]. CHAPS treated artery tissue has histologically normal collagen and elastin morphology and the collagen content appears to remain similar to that of the native artery. CHAPS treatment results in a significant decrease in the burst pressure and maximum stress of arterial tissue, but the decrease is comparable to the burst pressure of arteries subjected to treatment with Triton X-100 and hypotonic/hypertonic solutions [21]. Peripheral nerves have been decellularized

with SB-10 and SB-16 in combination with Triton X-200, an ionic detergent. The combined treatments had a less detrimental effect on the structure of the nerve ECM than combined treatments with Triton X-100 and sodium deoxycholate [25,60].

3.2.5. *Tri(n-butyl)phosphate*

Tri(*n*-butyl)phosphate (TBP) is an organic solvent that is used to inactivate viruses in blood without compromising the coagulation factor activity. Recently, TBP has been used as a chaotropic agent for decellularization of tendon and ligament grafts. Treatment with TBP yielded complete removal of nuclear remnants from rat tail tendon and the midsubstance of an ACL graft, although the removal of cellular material was incomplete at the insertion of the ligament to bone. TBP treatment did not have an effect on the tensile strength of collagen fibers isolated from the rat tail tendon as compared to native control, but for the ACL, treatment with TBP led to a decrease in collagen content [28,29]. TBP appears to be a promising chaotropic agent for decellularization that has minimal effect on the mechanical behavior of the ECM and is worthy of further study.

3.2.6. *Hypotonic and hypertonic treatments*

Osmotic shock with a hypotonic or hypertonic solution such as deionized water or low ionic strength solution is used to lyse the cells within tissues and organs [21,29,61–63]. A treatment in a hypotonic solution (10 mM Trizma HCl, 5 mM EDTA) for 11 h followed by an 11 h treatment in a hypertonic solution (50 mM Trizma HCl, 1 M NaCl, 10 mM EDTA) can cause cell lysis, but does not generally remove the resultant cellular remnants from the tissue [21]. Additional enzymatic or chemical treatments are typically necessary to facilitate removal of cellular debris. Removal of DNA remnants from the tissue can be particularly difficult due to the “sticky” nature of DNA and its tendency to adhere to ECM proteins.

3.2.7. *Chelating agents*

Chelating agents, such as EDTA and EGTA, form a ring-shaped molecular complex that firmly binds and isolates a central metal ion. It has been shown that divalent cations, such as Ca^{2+} and Mg^{2+} , are necessary for cell attachment to collagen and fibronectin at the Arg–Gly–Asp receptor [64–67]. By binding the divalent cations that are present at the cell adhesions to the ECM, these agents facilitate removal of the cellular material from the tissue. EDTA is typically used in combination with trypsin.

3.3. *Enzymatic methods*

Enzymatic techniques of decellularization include the use of protease digestion, calcium chelating agents, and nucleases [13,68–70]. Trypsin is one of the most commonly used proteolytic enzymes in decellularization protocols. Trypsin is a highly specific enzyme that cleaves the peptide

bonds on the carbon side of arginine and lysine if the next residue is not proline [71]. The maximal enzymatic activity of trypsin occurs at 37 °C and at a pH of 8. Nucleases such as endonucleases catalyze the hydrolysis of the interior bonds of the ribonucleotide or deoxyribonucleotide chains whereas exonucleases catalyze the hydrolysis of the terminal bonds of deoxyribonucleotide or ribonucleotide ultimately leading to the degradation of RNA or DNA [72].

The efficacy of enzymatic treatments for removal or separation of the cellular material from the ECM has been studied for a variety of tissues. Some studies show efficient removal of cellular material from porcine pulmonary valves after treatment with 0.05% trypsin/0.02% EDTA for 24 h with agitation [19], while other studies show less efficient decellularization. For example, porcine aortic valves subjected to treatment with 0.5% trypsin, 0.05% EDTA, 0.02% Gentamicin, 0.02 mg/ml DNase and 20 µg/ml RNase-A in Milli-Q water for up to 17 h at 37 °C with agitation rendered the cells non-viable, but did not lead to removal of cellular material from the tissue [15].

Enzymatic methods of decellularization are not without an adverse effect upon the extracellular components of tissues and organs. Prolonged treatment with trypsin/EDTA causes disruption of the normal pulmonary valve ECM structure, but does not affect the amount of collagen in the tissue [19]. Trypsin/EDTA does, however, substantially reduced the laminin and fibronectin content of the ECM. Prolonged exposure to trypsin/EDTA greatly decreases the elastin content and GAGs over time, with *o*-sulfated GAGs (chondroitin sulfates, keratin sulfates, and dermatan sulfates) showing the greatest decrease. Such treatments can contribute to a decrease in tensile strength of up to 50%. The remaining ECM after such enzymatic decellularization protocols still supports endothelial cell growth in vitro despite the removal of ECM components [15,19]. It is desirable to limit the duration of exposure to trypsin/EDTA treatments to minimize the disruptive effects upon the ultrastructure and composition of the ECM.

3.4. *Protease inhibitors*

During the decellularization protocols, a number of proteases can be released from the disrupted cells. For long duration chemical treatments, the presence of proteases can cause damage to the native ECM ultrastructure. For this reason, it may be desirable to include protease inhibitors such as phenylmethylsulfonylfluoride (PMSF), aprotinin, and leupeptin to the solutions in which the tissue is immersed. A buffered solution of pH 7–8 further inhibits many proteases, and the control of temperature and time of exposure to the lysis solutions can also limit the activity of the proteases.

3.5. *Antibiotics*

One concern for long duration chemical decellularization methods is the presence of bacteria, which can contaminate

the remaining ECM material. A number of protocols have therefore included antibiotic solutions such as penicillin, streptomycin, or amphotericin B [29,59,73–75]. However, if antibiotics or antibiotic residues remain in the scaffold material after the decellularization protocol, regulatory agencies may consider the material a drug rather than a medical device, which increases the complexity of regulatory approval.

4. Effects of tissue variability upon decellularization

The efficiency of a given decellularization method or protocol is dependent upon the tissue of interest. Despite identical times of exposure and a greater concentration of trypsin (0.5% vs. 0.05%), Grauss et al. [15] found that a trypsin-based decellularization protocol was ineffective at removing the cellular material from the rat aortic heart valve, while Schenke-Layland et al. [19] showed complete removal of cells from a porcine pulmonary valve. It is likely that subtle nuances in the application of the protocols had an effect on the efficiency of removal that are not entirely clear, such as the application of three-dimensional agitation by Schenke-Layland et al. [19]. Studies by Cartmell [28] and Dunn and Woods and Gratzer [29], which studied decellularization of rat tail tendon and porcine ACL, respectively, showed differences in the decellularization results that cannot be easily explained by differences in the protocols studied. These studies show that the effectiveness of decellularization and the alterations to the ECM vary depending on the source of the tissue, the composition of the tissue, the tissue density, and other factors. For each tissue that is studied, it will be necessary to optimize the decellularization protocol to obtain acceptable cell removal.

5. Verification of cell removal

There are a number of methods available to determine the efficiency of the removal of cellular material from tissues. Standard histological staining with Hematoxylin and Eosin can serve as a first line of inspection to determine if nuclear structures can be observed. Alternative histological stains such as Masson's Trichrome, Movat's Pentachrome, or Safran O can be used to examine tissues for the presence of various cytoplasmic and extracellular molecules. Immunohistochemical methods can also be utilized for specific intracellular proteins, such as actin and vimentin [29].

Inspection for the presence of DNA can be performed by staining the specimen with DAPI or Hoechst, which are both fluorescent molecules that bind to the AT clusters in the minor groove of DNA [76–79]. In addition, assays using propidium iodide and PicoGreen have been developed to provide quantitative data regarding the presence of DNA within a specimen [80]. DNA probe techniques have been utilized to track the fate of DNA from allografts after implantation, and could also be utilized to determine

whether any DNA is present in the decellularized tissue [81]. Polymerase chain reaction (PCR) or electron microscopic methods are possible but not typically used to examine for the presence of remnant nuclear material or cytoplasmic debris due to the technical complexity and expense of these procedures for routine work.

In addition, to determining what has been removed, it is also necessary to confirm that desirable components of the ECM are retained, such as adhesion proteins like fibronectin and laminin, GAGs, growth factors, elastic fibers, and collagens which will be required for infiltration of the matrix by cells of choice in vitro or in vivo. Mechanical testing of the ECM after treatment provides insight into presence and integrity of the structural proteins within the scaffold.

Although the above methods provide important information regarding the effectiveness of the decellularization methods, the biologic consequences of small amounts of nuclear material or cytoplasmic debris within the remaining scaffold materials is unclear. There are no reports showing a direct cause–effect relationship between such cellular remnants and an adverse host response to date.

6. Removal of residual chemicals

The decellularization methods described above include a wide variety of chemicals, which are used because of their inherent abilities to damage cells. If the chemicals remain within the tissue in high concentrations after treatment, then it is likely that they will be toxic to host cells when the scaffold is implanted in vivo. There is a need for development of assays to quantify the presence of residual chemicals in the decellularized scaffold material. Similarly, some of the processes that have been described above include enzymes commonly derived from bovine sources (i.e., DNase, RNase, trypsin). These enzymes can potentially invoke an adverse immune response by the host.

7. Conclusion

Complete decellularization of most tissues will require a combination of physical, enzymatic, and chemical treatments, and the protocol will be dependent on the tissue of interest. It is generally desirable to use the mildest protocol possible that yields an acellular material without disruption of the structural and functional component of the ECM. A typical progressive approach would be to start with treatment in a hypotonic or hypertonic solution followed by a mild non-ionic or zwitterionic detergent. If necessary, an enzymatic treatment of trypsin/EDTA can be added prior to the detergent treatment to assist in breaking the bonds between the cell membranes and the ECM. Finally, if these treatments are still inadequate to remove the cellular material, an ionic detergent such as SDS, deoxycholate, or Triton X-200 can be added to the decellularization protocol.

It is clear that physical, enzymatic, and chemical treatments can have substantial effects on the composition, mechanical behavior, and host response to biologic scaffolds derived from the decellularization of native tissue and organs, and could have important implications for subsequent use for in vitro and in vivo applications. The removal of adhesive proteins and GAGs from the scaffold could slow cell migration onto the scaffold and the bioactivity of the scaffold itself. Disruption of the collagen network can change the mechanical behavior and collagen fiber kinematics of the scaffold, which can have an effect on the load bearing capacity of the scaffold and alter the mechanical environment to which the cells are exposed. Degradation is another important factor that relates to the mechanical behavior and bioactivity of the scaffold that could be affected by decellularization treatments. The chemical treatments could compromise the ECM scaffold in a way that makes it more susceptible to enzymatic degradation in vivo, which would lead to a rapid decrease in the strength of the scaffold.

It is unlikely that any combination of methods will remove 100% of all cell components from a tissue or organ. However, it seems apparent that methods which remove most or all of the visible cellular material result in biologic scaffold materials that are safe for implantation. A number of naturally occurring ECM devices and related decellularization protocols have received regulatory approval for use in human patients, including human dermis (Alloderm[®], LifeCell, Corp.), porcine SIS (SurgiSIS[®], Cook Biotech, Inc.; Restore[®], DePuy Orthopaedics, Inc.), porcine urinary bladder (ACell, Inc.), and porcine heart valves (Synergraft[®], CryoLife, Inc.). The growing list of biologic scaffolds used for tissue engineering/regenerative medicine applications makes the continued development of decellularization protocols a clinically relevant and important effort.

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