Review

Decellularization for the retention of tissue niches

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Deana Moffat[1], Kaiming Ye[1,2] and Sha Jin[1,2]

Abstract

Decellularization of natural tissues to produce extracellular matrix is a promising method for three-dimensional scaffolding and for understanding microenvironment of the tissue of interest. Due to the lack of a universal standard protocol for tissue decellularization, recent investigations seek to develop novel methods for whole or partial organ decellularization capable of supporting cell differentiation and implantation towards appropriate tissue regeneration. This review provides a comprehensive and updated perspective on the most recent advances in decellularization strategies for a variety of organs and tissues, highlighting techniques of chemical, physical, biological, enzymatic, or combinative-based methods to remove cellular contents from tissues. In addition, the review presents modernized approaches for improving standard decellularization protocols for numerous organ types.

Keywords

Decellularization, extracellular matrix, tissue and organ, chemical method, physical method, biological method, enzymes

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Introduction

To circumvent the disparity between the number of organ donors and transplant recipients, studies have been focused on using decellularized tissue scaffolds to help regenerate organs i*n vivo* or *in vitro*. The decellularized tissues provide proper cues and anatomical structures for cells to proliferate, differentiate, and organize into adequately functioning tissues. Of all scaffolds used in regenerative medical research, those generated directly from or designed to mimic the qualities of the extracellular matrix (ECM) are considered the gold standard for making proper graft to repair damaged tissues or organs. The ECM contains physiochemical cues needed to regulate accurately cell phenotype and function, as well as to provide a foundation for the desired organ's natural structure. Native ECM is ideal for regenerative medicine and tissue engineering approaches, as it retains an original tissue anatomical structure. Therefore, retention of native protein niches and physiochemical properties is the primary goal of decellularization. Often, a decellularization protocol must be customized in accordance with the origin of the tissue, as there is no singular ideal approach that can fit all. In addition, proteomic studies have revealed that the proteomic content of the matrisome varies according to the tissue from which the ECM is sourced.[1] Hu et al.[2] has implied that that age and sex may also play a lesser, but still notable role in the proteomic makeup of the ECM. This corroborates recent findings by Ozcebe et al.[3] which indicate recellularization potential of ECM varies by donor age. To generate an ECM-derived scaffold, a plethora of decellularization protocols have been generated over the past decades, each with their own unique advantages and disadvantages. Some of these protocols often contradict to each other, due

[1]Department of Biomedical Engineering, Binghamton University, State University of New York (SUNY), Binghamton, NY, USA
[2]Center of Biomanufacturing for Regenerative Medicine, Binghamton University, State University of New York (SUNY), Binghamton, NY, USA

Corresponding author:
Sha Jin, Department of Biomedical Engineering, Thomas J. Watson College of Engineering and Applied Sciences, State University of New York, Biotechnology Building BI 2612, Binghamton, NY 13902, USA.
Email: sjin@binghamton.edu

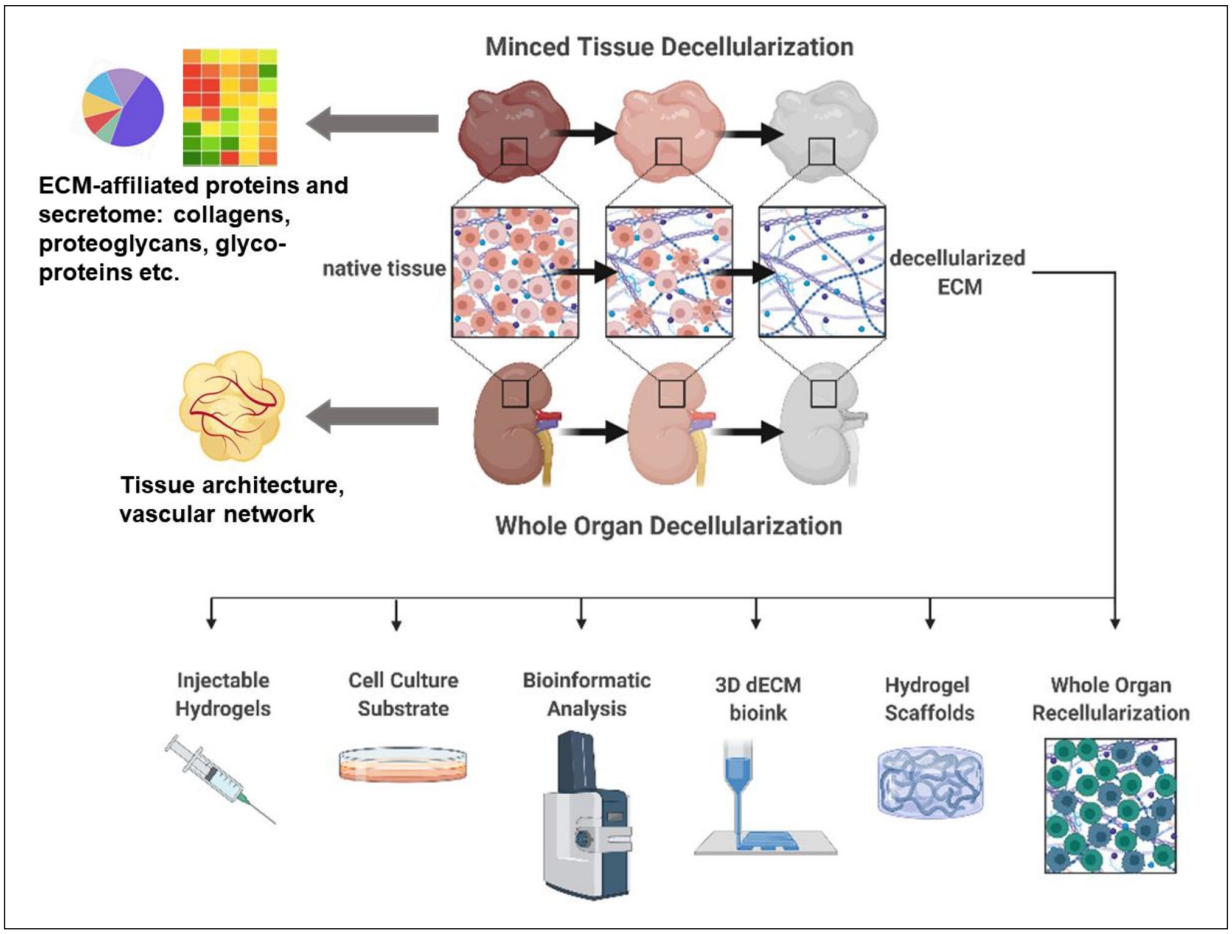

Figure 1. Decellularized ECM procurement and tissue engineering applications. Decelluarized ECM contains ECM proteins and secretome that facilitate cell proliferation and differentiation. Tissue structure and vascular network retained after whole organ decellularization provide alternative tissue graft materials.

to diverse experimental conditions including different tissue types, temperatures, flow applications, pH, etc. As a result, it is difficult to directly compare the efficiency of each individual component utilized. This review aims to highlight a variety of decellularization approaches, compare the methods used as a basis for decellularization of tissue to procure a well-decellularized ECM with minimal damage to the biomechanical structure and proteomic makeup. We provide a comprehensive analytical interpretation on the most recent advances in decellularization strategies for a variety of organs and tissues, highlighting techniques of chemical, physical, biological, enzymatic, or combinative-based methods to remove cellular content from tissues. In addition, we collectively present modernized approaches for improving standard decellularization protocols for various types of tissues and organs.

Decellularization for ECM retrieval

The ECM is a naturally occurring, dynamically active macromolecular network arranged in a highly organized tissue-specific manner. It is predominantly composed of collagens, proteoglycans, elastin, glycoproteins, and secreted factors based on tissue type (Figure 1). These components establish the ECM as a mechanically stable basement membrane that serves as a structural support for anchored cells. The bioactive molecules and growth factors secreted into the ECM by the cells allow for conformational changes in its structure, which induce chemical signals that regulate cell proliferation, differentiation, adhesion, migration, polarity, and apoptosis. ECM has been shown to preferentially promote the differentiation of cells from the same tissue origin. Stem cells or stem cell-derived cells seeded into the ECM have their cell morphology influenced toward the cell lineage of the ECM origin tissue, regardless of the origin of the cells being seeded.[4,5] Interestingly, it has been shown that ECM from various species can support human stem cell proliferation and differentiation if the ECM constructs are sourced from the same tissue type as the organ of interest.[6,7] Although the properties of ECM make it an ideal model for implantable scaffolds for tissue engineering purposes, the ECM's

intricate biomechanical and biochemical composition make it difficult to replicate its properties from inorganic materials.[8] As such, it is of the utmost importance to develop methods to obtain naturally occurring ECM for tissue engineering. Decellularization, therefore, has been extensively studied in the past two decades.

Decellularized ECM has shown success as scaffolds for generation of a variety of tissue and organ types.[9–15] Typically, decellularization results in the retention of larger macromolecules such as polysaccharides and constructive collagens due to their size and degree of crosslinking, while smaller ECM-associated components like growth factors, chemokines, and signaling molecules are more easily washed away.[16] The loss of these molecules cripples the performance ability of the resulting tissue microenvironment to act as a tissue specific platform for recellularization. Different decellularization techniques vary in the rates of lost protein niches. Crap et al.[17] has stated that successful decellularization should be determined on the basis of producing (I) ECM which does not contain more than 50 ng of DNA per dry weight, (II) residual DNA fragments no longer than 200 bp, and (III) no visible nuclear components. Several strategies have since been able to successfully decellularize tissues to retrieve the ECM by this definition. Figure 1 illustrates two major decellularization processes, that is, minced tissue and whole organ decellularization, for ECM procurement and potential applications in tissue engineering and regenerative medicine. Minced or sectioned decellularized tissues can be reconstituted and used as cell culture substrates or as injectable hydrogels to improve cell proliferation, differentiation, and engraftment.[7,18–22] Decellularized tissue ECM proteomics and subsequent bioinformatics analyses allow distinct applications, including but not limited to the understanding of tissue microenvironments associated diseases such as tumor metastasis and signaling molecules crucial to in vitro tissue generation (Figure 1).[18,23–26] Hydrogels prepared from decellularized tissues can also be used as a bioink for 3D bioprinting of tissue or as a disease model.[27–32] Decellularized animal whole organs retain their original 3D structure and vasculature network and thus are applied to repopulate human cells for organ regeneration.[33–35]

Decellularization techniques

Common decellularization techniques utilize chemical, physical, biologic, or enzymatic methods to remove cellular content from the tissue in order to retrieve naturally occurring ECM. To overcome the deleterious effects associated with a single particular decellularizing method or agent, techniques are often used in combination. The specific tissue type being decellularized should also be taken into consideration, as the same decellularizing protocol can produce different results in different tissues.[36] We discuss the decellularization techniques in details in the following.

Chemical treatment-based decellularization

The efficiency of chemical treatment-based decellularization varies according to the size, density, cellularity, thickness, and lipid content of the starting tissue. Chemical agents are a favorite among decellularization strategies, as there exists a plethora of evidence to validate their ability to quickly and efficiently rid tissue of native cells. Despite this, chemical agents usually are incapable of completely stripping cellular components and can leave trace amounts of dsDNA, mitochondrial DNA, mitochondria, and membranous phospholipids, the presence of which can jeopardize subsequent recellularization attempts and invoke immune responses.[17] Moreover, chemical agents are typically cytotoxic and must be followed with additional washing steps to rid the decellularized construct of remnant chemical residues. Chemical agents can be compounded together, used in tandem, or combined with other decellularizing strategies to expedite the decellularization processes. In protocols where multiple decellularizing agents are used, the order by which a tissue sample is exposed to different chemical agents plays a critical role in the proteomic and biomechanical features of the resulting ECM.[37] Chemical decellularizing agents are categorized as being either ionic detergents, non-ionic detergents, zwitterionic detergents, solvents, acids, bases, or hypertonic and hypotonic solutions.[38]

Detergents, also known as surfactants, are the most commonly used chemical means for decellularization. Owing to their popularity, they are generally cheap, quick, and efficient decellularizing agents. The most common detergents for decellularization are the nonionic detergent Triton X-100 and the ionic detergent Sodium Dodecyl Sulfate (SDS). Triton X-100 removes cellular content by disrupting lipid-lipid and protein-lipid interactions without affecting protein-protein interactions, whereas SDS disrupts protein-protein interactions and solubilizes cell membranes. Ionic detergents like SDS have the benefit of being able to effectively remove nuclear materials in shorter time frames compared to other chemical treatments.[39–42] This, however, is at the expense of greater damage to the ECM matrisome, as treatment of tissue with SDS can result in an altered microstructure that diminishes the biomechanical integrity of the ECM.[43] The concentration of SDS and tissue exposure time must therefore be optimized for any SDS-based protocol, as increased exposure is directly linked to decreased ECM biomechanical properties,[44] Also easily lost in SDS decellularizations are fibronectins, glycosaminoglycans (GAGs), proteoglycans, and ECM regulators and secreted factors.[45] Thus, SDS is associated with removal of significant amounts of biochemical cues contained in a native ECM. Most successful

SDS-based decellularizations use a concentration between 0.1% and 1% SDS.[46–49] However, decellularization at SDS concentrations as low as 0.01% in ovine small intestine and as high as 2% in articular cartilage have been performed to varying degrees of success.[44,50]

Remnant SDS within a decellularized matrix is difficult to remove, which is detrimental to recellularization due to its high cytotoxicity, potentially due to the exposure of the collagen C and N termini which reveal antigenic sites.[51] As such, SDS treatment is commonly followed by Triton X-100. Alternatively, decellularizations using nonionic detergents like Triton X-100 are considered to be less harsh on tissue than ionic detergents, and are therefore useful for decellularizations where preservation of the ECM proteome is important.[39] Although milder than ionic detergents, Triton X-100 is still itself a strong detergent and its use can lead to proteomic complications, especially at high concentrations. Normally decellularization protocols do not use concentrations of Triton X-100 in excess of 1%, though some protocols have found success with concentrations as high as 3% so long as exposure length was reduced accordingly.[43,47,52] Like all other detergents, the adverse effects of Triton X-100 include, but are not limited to, significantly increased stiffness of the resulting matrix, lowered recellularization potential due to cytotoxic chemical remnants, and loss of bioactive molecules such as GAGs and proteoglycans.[39]

In recent years, several new detergents have been discovered for decellularization protocols. The ionic detergents sodium deoxycholate (SDC) and sodium lauryl ether sulfate (SLES) have gained popularity upon discovery that they contain decellularizing abilities on par with or superior to SDS-based protocols. Both SDC and SLES have been shown to better preserve collagen and GAG content than SDS while showcasing superior biocompatibility following recellularization.[39,53–56] SDC can successfully decellularize tissues at concentrations up to 4%,[57,58] although increased concentration of SDC does not contribute to higher nuclear removal rates and results in increased damage to structural integrity, despite its inability to degrade collagens.[57,59] Additionally, SDC decellularizations must be followed with agents such as Deoxyribonuclease (DNase) to reduce induction of DNA agglutination at the tissue surface.[57] To date, the requirement of DNase for successful SDC decellularization has only been overcome in cardiac tissue, as described by Methe et al.[56] Regardless, SDC has proven to be successful for decellularization of several tissue types, including rat peripheral nerve,[57] auricular cartilage,[58] murine ovary,[53] and porcine heart valve,[39] while SLES has successfully decellularized of heart, kidney, ovary, and bone tissue,[16,60,61] demonstrating the versatility of both ionic surfactants. SLES is particularly intriguing, as Emami et al.[16] found that SLES may be superior to not only SDS, but also other decellularizing agents, including Triton X-100 and enzymatic Trypsin/EDTA for decellularization of histologically dense bone tissue. Li et al.[62] advised that the addition of dextrose perfusion pretreatment step prior to SLES exposure assists in further protecting collagens from degradation by the detergent. Potassium Laurate (PL), a naturally occurring ionic detergent, is also of interest. In 2019, Obata et al.[63] was the first to describe its potential for decellularization of rat lung. They reported that PL was capable of sufficient cellular and DNA removal while significantly reducing ECM damage associated with SDS. Most ostensibly, these results indicate the potential for the development of PL based protocols to permit effective detergent based decellularization of easily damaged thin tissues. As such, further investigations into the potential of PL to act as a decellularizing agent are necessary. The only nonionic detergents other than Triton X-100 to be investigated for use in decellularization are Tween 20 and Tween 80, both of which consistently produce inadequate decellularizations apart from a study by Chaschin et al.[67] who found that inclusion of Tween 80 in decellularization of human aorta by supercritical carbon dioxide (scCO2) was beneficial.[64–66]

Zwitterionic detergents share properties with both ionic and nonionic detergents. They have been shown to preserve the ECM ultrastructure but tend to be limited in their ability to completely remove cellular content.[39,68] For this reason, they are typically followed by treatment with enzymatic techniques to complete the decellularization. They are known to target and break protein-protein bonds in a similar fashion to ionic detergents but are far less aggressive. 3-[(3-cholamidopropyl) (dimethylammonio]-1-propane sulfonate (CHAPS) decellularizes tissues by disrupting lipid-protein and lipid-lipid interactions.[68] Its reduced permeating qualities limit CHAPS' ability to remove nuclear DNA, thereby making CHAPS more applicable for decellularization of thin tissues.[68–70] CHAPS also highly retains the biomechanical properties of native tissue, making it ideal for tissues, which must be capable of contracting with ease such as heart or lung.[70] Other zwitterionic detergents Sulfobetaine 10 and Sulfobetaine 16 (SB10 and SB16, respectively) induce cell apoptosis, resulting in improved cell removal, which eliminates the need for vigorous washing steps.[71] Physical agitation is used to gently assist in coaxing apoptotic cells from the matrix.[71,72] SB-10 and SB-16 have been shown to result in better retention of ECM basement membrane integrity and higher rates of cell removal when compared to ionic detergents.[73] Both detergents are typically followed by enzymatic treatments, such as DNase, to promote DNA fragmentation and reduce immunogenicity of the ECM construct.[39,71] In the case of CHAPS treatment, immunogenicity can also be reduced by induction of a physiologically accurate pH during decellularization to reduce inflammation upon later implantation.[74,75] Song et al.[71] describes that cellular apoptosis is more quickly induced

when SB-10 and SB-16 are combined with another apoptosis-inducing agent, camptothecin, while McCrary et al.[57] found that SB-10 and SB-16 treatment could improve SDC decellularizations.

Solvents, another category of chemically-based decellularizing techniques, are generally inadequate for decellularization. They can be used as an initiating step for decellularization to remove lipids and reduce fat content of the tissue.[76–78] Exercising caution when using solvent-based techniques is encouraged, as the use of solvents often results in damage to the 3D microstructure and reduces the likelihood of successful recellularization.[17] Solvents for decellularization include alcohols, acetone, tri-n-butyl phosphate (TNBP), dimethyl ether (DME), and urea. Alcohols dehydrate native cells to lyse them and have been shown to delipidate tissues. The use of alcohols is effective for the removal of fat from thick, dense muscle tissue. However, they can crosslink and precipitate collagens, altering the structural integrity of the 3D proteomic network.[17] Alcohols such as isopropanol,[77,79] glycerol,[80] ethanol,[81–83] and methanol[21] have all proven to be effective for removal of lipid content, though some reports indicate that isopropanol may be a superior agent for this purpose.[84] Like alcohols, acetone removes cellular content from ECM by acting on lipids. It is typically used in conjunction with ethanol, albeit these ethanol-acetone solutions dehydrate the ECM and result in significant increases in stiffness.[85,86] Because acetone has a dehydrating effect on ECM, some studies use it to chemically dry and sterilize collagen matrices rather than to decellularize them.[87] Due to its sterilizing properties, it has reduced immunogenicity compared to detergent-based techniques.[81] Typically, acetone results in extensive adverse impacts on the biomechanics of ECM and is not recommended for use in tissues which are contracting or load bearing.[87,88]

TNBP, which does not share the same dehydrating effect as alcohols or acetone, has been shown to be capable of successful removal of DNA content from the ECM membrane when used in tandem with multiple enzymatic and chemical decellularizing agents. Recently, TNBP has been utilized to assist in physical scCO2 decellularization strategies, though these processes can be lengthy.[39,89,90] Unlike alcohols and acetone, TNBP is not useful for decellularization of dense muscle tissues, as increased concentrations or exposure times do not improve nuclear removal rates, but also do not further damage the matrisome of the ECM.[91,92] Unlike alcohols and acetone, TNBP may promote collagen crosslinking as opposed to degrading them.[90,91] It can also act as a principal decellularizing agent, and has been effective in protocols for decellularization of porcine diaphragm,[91] rabbit tendon,[89] porcine trabeculae,[90] and human vein.[93]

Dimethyl ether (DME) and urea are decellularizing solvents which are not yet extensively studied, and it is therefore difficult to infer their effect on the ECM. Kanda et al. demonstrated that DME under subcritical temperatures, followed by DNA fragmentation with DNase, can effectively replace SDS in decellularization protocols for porcine aorta and ostrich carotid artery.[94,95] In addition to reducing structural damage to the ECM, DME may also reduce the immunogenic response properties. Urea is a powerful solubilizing agent with a high affinity for antigen removal. Addition of urea to decellularization protocols showed severe alterations to the histoarchitecture, elastin, and GAG content of the ECM in bovine bone and pericardium.[96,97] These findings indicate that urea may not be an ideal agent for use in future decellularization attempts.

Protocols which utilize acids and bases for decellularization have also been developed. Peracetic acid (PAA) can be used as both a decellularizing and disinfecting agent. In this way, it is much like alcohols and acetone, though it may result in fewer significant impacts to the biomechanical structure of the ECM.[98] Similar to but stronger than PAA is acetic acid (AA), which is more likely to damage the structural integrity of the ECM by destroying or removing collagens, although it has a negligible effect on smaller bioactive molecules such as GAGs.[99] For tendon, acetic acid or hydrochloric acid can be utilized to successfully strip calcium from the matrix prior to decellularization by detergent.[100] Ammonium-hydroxide, a mildly basic compound, disrupts the cell membrane and breaks down the cell wall while disrupting hydrogen bonds to induce cell lysis. Typically, following ammonium-hydroxide treatment, an ionic or nonionic detergent solubilizes the cell membrane and separates the proteins from the matrix. Ammonium-hydroxide has been reported to have been utilized in protocols to successfully decellularize liver,[101–104] urological tissue,[34] and mesenchymal stem cell derived ECM.[105] It is considered to work best in tandem with Triton X-100 under perfusion or static conditions.[101,106,107] Treatment with enzymes like DNase following decellularization with ammonium hydroxide and Triton X-100 has been recorded to result in up to 100% DNA removal in thin cell sheets.[106]

Unlike all other types of chemical decellularizations, hypertonic and hypotonic solution-based decellularizations are consistently described as having little to no negative impact on the proteome of the ECM along with powerful removal of cellular DNA.[7,18] Hypertonic solution rinses followed by hypotonic solution rinses induce cell lysis by an osmotic shock to decellularize tissue. This technique seeks to capitalize on the initial incubation in the hypotonic water step used in many decellularization strategies and limit exposure to harsh chemicals known to strip ECM of native proteins. Previously, most attempts to utilize this technique resulted in subpar immunogenic conditions for implantation into a host biosystem.[108,109] However, recent advancements in the technique have increased removal rates of cellular content, thereby making the resulting constructs more biocompatible. Recent advances

by our group have shown that NaCl and distilled water changes under agitation can produce porcine pancreatic ECM, which meets criteria for successful decellularization. Interestingly, this protocol resulted in DNA removal rates similar to or exceeding those of an SDS and Triton X-100 based techniques.[2,7,18] Similar studies which use hypertonic NaCl solution to decellularize cornea have indicated a downside with this technique, in which the tissue grafts produced by these decellularized corneal grafts remained transparent in rabbit eyes for 6 months,[110,111] which is approximately half the amount of time that grafts produced by SDS-containing isotonic buffer decellularized corneal grafts.[112] Due to the lack of decellularizing strength associated with this technique, only minced or sectioned tissue samples have been successful. Thus, no hypertonic/hypotonic solution change protocol has been developed to date for whole organ decellularization. Table 1 summarizes numerous chemical methods that have been developed and utilized to decellularize tissues based on the classification of the chemicals with comparison of their advantages and disadvantages.

Physical/Mechanical decellularization

Physical methods of removing cellular content from tissue work by disrupting cell membranes and creating unfavorable cellular environments that can induce apoptosis. Physical decellularization has the advantage of producing a uniform effect throughout tissue. Moreover, their effect is more predictable than chemical or enzymatic decellularizing agents. Physical treatment alone is often insufficient for decellularization. While they can induce cell lysis, they are ineffective for complete removal of cell or nuclear remnants. Nonetheless, they can be used in conjunction with chemical, biological, or enzymatic decellularizing agents to reduce exposure times and aid in the retention of ECM proteomic content. Vacuums, high hydrostatic pressure (HHP), freeze-thaw cycles, scCO2, and sonication are all physical methods commonly used for decellularization.

Vacuum-assisted decellularizations refer to any decellularization technique that is aided or accelerated by the usage of a negative pressure. Though the use of negative pressure systems is ineffective for decellularization on its own, it has been found to be effective when used in combination with other physical methods as well as chemical or enzymatic methods.[122,123] These systems can significantly reduce the decellularization time without sacrificing additional proteins, allowing for more efficient processes and reduced exposure to potentially damaging agents.[122]

HHP bursts cells with minimal risk of the type of protein denaturation associated with nonphysical strategies. Pressures over 150 MPa are required to achieve adequate cell death, though pressures over 500 MPa can potentially result in ECM protein denaturation.[124–126] Supercooling pretreatment of tissue before HHP decellularization can also assist in reducing the likelihood of protein denaturation.[126] HHP has the disadvantage of not retaining the biomechanical properties of the original tissue as well as other physical methods like freeze-thaw cycles. However, it has been found to produce immunologically superior decellularized matrices that increase likelihood of achieving recellularization.[127]

Freeze-thaw cycles result in thermal shock-induced cell death upon immersing tissue in liquid nitrogen. Freeze-thaw is used as a precursor step to decellularization and cannot effectively decellularize any kind of tissue alone.[128,129] Therefore, freeze-thaw cycles have been followed by washes with detergents such as Triton X-100,[130–132] solvents like isopropanol,[133] and enzymes such as trypsin.[58] This method assists in retaining a majority of the ECM's 3D structural integrity and allowing for reduced exposure required for adequate cell removal by chemical or enzymatic agents.[134] Freeze-thaw cycles are particularly applicable for use in tissues when treat with highly damaging agents known to harm ECM components, such as SDS and SDC. However, it should be carried out with caution, as they can also cause main components of the ECM to rupture and make recellularization difficult.[135]

scCO2 is notable for its ability to decellularize tissues in a fraction of the time that it takes for most chemical agents. The carbon dioxide used in this technique is an ideal gas for decellularization, as it is nontoxic, inflammable, relatively inert, and cost effective. The exact mechanism by which scCO2 removes cells and cellular content from a tissue has been widely disputed, although previous beliefs that it is the result of high pressure induced cell bursting have been disproven.[136–138] Recently, attempts to identify the main mechanism by which it decellularized tissue has brought claims that it may induce hypoxia, though this is still uncertain. Previous studies found scCO2 to be inadequate for developing viable scaffolds, as the final matrices were often too dehydrated for reseeding. Pre-saturating scCO2 with water overcomes tissue dehydration.[139] In addition, combination of scCO2 with 2% PAA can successfully decellularize tissues without damaging the vasculature or proteome of their ECM. PAA was found to be superior to all other solvents when paired with scCO2.[140] Combination of scCO2 with ethanol also results in the production of successfully decellularized and immunologically inert acellular matrix from pig esophagus, albeit, to a lesser extent than detergent-based approaches.[141]

Sonication is a technique that allows decellularizing agents to better permeate a tissue. Sonication has been used to successfully assist in decellularization of aorta,[142] artery,[143] larynx,[144] and cartilage.[145] The cavitation intensity during the course of sonication is influenced by pH, temperature, viscosity, diffusion rate of dissolved oxygen and vapor pressure, and solubility of gas in liquid. These conditions are heavily influenced by the concentration of

Table 1. The advantages and disadvantages using different chemicals for tissue decellularization.

Classification	Decellularizing agent	Mechanism	Advantages	Disadvantages	References
Ionic Detergents	Sodium Dodecyl Sulfate (SDS)	Breaks non-covalent bonds	Consistently removes over 90% of cellular content	Requires vigorous rinsing Damages biomechanical integrity Removes collagens, fibronectin, and small bioactive molecules	Alizadeh et al.[40], Xu et al.[43], Elder et al.[44], Xing et al.[45], Schmitt et al.[46], Wang et al.[49], Chakraborty et al.[51], Schmid et al.[113], Dal Sasso et al.[114], Fernandez-Perez and Ahearne[115]
	Sodium Deoxycholate (SDC)	Disrupts cell membrane	Higher retention of GAGs and collagen than SDS Higher biocompatibility than SDS Incapable of degrading collagens	Induces immune response Induces DNA agglutination Removes GAGs and growth factors	Simsa et al.[39], Alshaikh et al.[53], McCrary et al.[57], Rahman et al.[58], Hwang et al.[59]
	Sodium Lauryl Ester Sulfate (SLES) & Sodium Lauryl Sulfate (SLS)	Disrupts cell membrane	Higher retention of GAGs and collagen than SDS Higher recellularization potential than SDS Preserves biomechanical integrity and microarchitecture	Removes collagen and GAGs	Emami et al.[16], Ma et al.[54], Keshvari et al.[55], Kawasaki et al.[61]
	Potassium Laurate (PL)	Solubilizes membrane proteins	Better retention of GAGs, elastin, and collagen than SDS Higher recellularization potential than SDS Preserves biomechanical integrity and architecture Reduced inflammation compared to SDS treated tissue	Not extensively studied	Obata et al.[63]
Nonlonic Detergents	Triton X-100	Disrupts lipid-lipid and lipid-protein interactions	Higher recellularization potential than SDS and SDC Does not require extensive rinsing	Increases stiffness Removes GAGs and proteoglycans	Simsa et al.[39], Xu et al.[43], Luo et al.[47], Liao et al.[116]
	Tween 20 & Tween 80	Induce cell lysis	Protects proteins from denaturation Compatible with several decellularizing agents	Insufficient for decellularization alone Minimally impactful Reduces biomechanical integrity	Aeberhard et al.[64], Heidarzadeh et al.[65], O'Neill et al.[66]
Zwitterionic Detergents	CHAPS	Disrupts lipid-lipid and lipid-protein interactions	Retains biomechanical integrity	Insufficient for decellularization alone Unable to permeate tissue pH dependent	Mendibil et al.[68], Marin-Tapia et al.[69], Qiu et al.[70], Tsuchiya et al.[74], Zvarova et al.[75]
	SB10 & SB16	Induces apoptosis	Retains small bioactive molecules Retains biomechanical integrity Does not require vigorous rinsing	Not extensively studied	Song et al.[71], Hudson et al.[73]

(Continued)

Table 1. (Continued)

Classification	Decellularizing agent	Mechanism	Advantages	Disadvantages	References
Solvents	Alcohols	Dehydrate and lyse cells	Effective for removing fat from thick tissues	Crosslinks and precipitates collagens Increases stiffness	Crapo et al.[17], Lumpkins et al.[85], Kabirian and Mozafari[86]
	Acetone	Acts on lipids	Sterilizes ECM Reduces immunogenicity	Increases stiffness	Van de Walle et al.[81], Lumpkins et al.[85], Gorschewsky et al.[87], Gorschewsky et al.[88]
	Tri(n)butyl Phosphate (TNBP)	Disrupts protein-protein interactions	Less structurally damaging than alcohols and acetone Promotes collagen crosslinking Increases protein retention and recellularization potential Compatible with detergent-based methods and physical methods	Insufficient for decellularization alone	Simsa et al.[39], Xing et al.[89], Duarte et al.[90], Deeken et al.[91], Yang et al.[117]
	Urea	Disrupts non-covalent bonds	Reduces immunogenicity Removes cytotoxic detergents	Primarily used as an antigen removal agent as opposed to a decellularization agent Disrupts biomechanical integrity Disrupts collagen organization	Wong et al.[96], Wong et al.[97]
	dimethyl ether (DME)	Acts on lipids	May reduce immunogenicity Retains biomechanical integrity	Insufficient for decellularization alone Not extensively studied	Kanda et al.[94], Kanda et al.[95]
Acids and Bases	Acids	Solubilizes cell membrane and disrupts nucleic acids	Negligible reduction of small bioactive molecules such as GAGs Can strip calcium from bone tissue	Damages collagen	Syed et al.[98], Abaci and Guvendiren[99], Zhao et al.[100], Dong et al.[118], Datta et al.[119]
	Bases	Denature chromosomal and plasmid DNA	Can achieve 100% DNA removal in thin tissue samples	Removes growth factors Reduces biomechanical integrity	Kajbafzadeh et al.[101], Farag et al.[106], Poornejad et al.[120]
Hypotonic & Hypertonic Solutions		Induce cell lysis by osmotic shock	Gentler removal of cells than detergent-based methods High retention of ECM components	Difficult to achieve acceptable cellular removal Ineffective for whole organ decellularization Can result in ECM swelling	Hu et al.[2], Dahl et al.[108], Woods and Gratzer[109], Lee et al.[121]

the decellularizing agent. Sonication protocols use low concentrations of SDS, as this agent can aggressively solubilize cellular content.[145] However, sonication is itself a physically aggressive process, which may or may not cause structural damage to the ECM.[146] Ultrasonic baths are better able to allow smooth penetration of chemical agents into tissue than sonicators while also causing less damage to the ECM ultrastructure. This, however, is at the cost of longer protocols.[147] Table 2 highlighted decellularizing methods with advantages and disadvantages of each physical method.

Biological treatment-based decellularization

Biological treatment-based decellularization aims to induce apoptosis of cells. These apoptosis-inducing agents are looked upon favorably for decellularization protocols because they result in contained cell debris that can more easily be washed away. Few studies have investigated the role of apoptosis-inducing agents, but those which indicate that they likely involve complicated mechanisms, leaving the field open for further exploration. Cytotoxic drugs, hydrogen peroxide, and hypoxia are means of inducing apoptosis in tissue to facilitate the removal of cells from the tissue.

To date, cytotoxic drugs are generally utilized only to improve the efficiency of zwitterionic detergent decellularizations and are not effective decellularizing agents alone.[150,151] Camptothecin, a cytotoxic drug, is employed in decellularization as it inhibits DNA topoisomerase I. It has been utilized in several studies and has shown great promise for decellularization of sciatic nerve, as it preserves anatomical architecture while retaining small bioactive molecules.[71,151] Cornelison et al.[151] reported that camptothecin was necessary for cellular removal through hypertonic/hypotonic buffers. Further treatment with DNase over hypotonic buffers led to effective DNA removal for nerve tissue.

Rotenone, another cytotoxic compound and a strong mitochondrial class I inhibitor, mediates apoptosis by inducing oxidative stress.[150,152,153] Treatment of tissues with rotenone for up to 24 h prior to detergent based decellularization of cell sheets has shown no significant benefit in terms of DNA removal, although this could be due to the already efficient strategy to decellularize cell sheets.[150] The effect of rotenone in decellularization of tissues thicker than cell sheets has not yet been reported and is worth investigation. The cytotoxic drug Latrunculin B induces changes in cell shape and actin organization. It has been identified as an efficient DNA removal agent, and has even been found to produce greater reduction of DNA in skeletal muscle than standard Triton X-100 and SDS/Triton X-100 methods. However, this was at the cost of notable reduction in structural integrity.[154–156] Other apoptotic inducing compounds for decellularization, including analogs of those previously listed, have not yet been extensively tested.

Apart from cytotoxic drugs, hydrogen peroxide or hypoxia can also be used to induce apoptosis for decellularization purposes. In addition to being a strong induction of apoptosis, hydrogen peroxide (H_2O_2) also shows promise as a sterilizing agent, which may make it useful for recellularization or implantation purposes. H_2O_2 cannot act as the major component of a decellularization protocol, as it has been found to be largely unhelpful when tested in combination with PAA/ethanol and scCO2 mediated decellularization strategies.[157,158] Isolation of tissue under hypoxic conditions can be used to assist in the removal of cellular content from tissue. N_2 is particularly useful as a hypoxic agent for decellularization. It has been utilized with semi-successful results in porcine cornea but has not been tested in any other tissue.[159,160] Table 3 showed advantage and disadvantages of the biological decellularization techniques.

Enzymatic decellularization

Enzymatic digestion can improve decellularization by digesting tissues with one or a combination of several enzymes to allow for decellularizing agents to diffuse through the tissues more easily. This makes enzymatic treatments particularly alluring for use in dense tissues that are difficult to decellularize. Despite the benefits associated with enzymatic digestion, these approaches are difficult to reproduce. They also magnify the risk of significantly altering the structural and proteomic composition of the ECM.

Trypsin is an aggressive enzyme that specifically cleaves at the C-terminus of lysine and arginine to disrupt the tissue microstructure, allowing for accelerated solubilization by detergents or enzymatic chelating agents.[163,164] As these bonds help crosslink collagens and elastin, trypsin is rarely used as a principal agent for decellularization.[114] Use of trypsin (0.05%–0.2%) typically is restricted to an initial pretreatment step before decellularization with chemical agents.[114,164,165] Higher concentrations or lengthy exposure to trypsin can result in detrimental damage to biomechanics of ECM. Attempts to utilize trypsin as a principal decellularizing agent have resulted in inadequate DNA removal and severely damaged biomechanical properties of ECM.[166] Ethylenediamine tetra acetic acid (EDTA) is a chelating agent that decellularizes ECM by targeting the Ca^{2+} and Mg^{2+} ions that maintain bonds between the ECM and native cells. Most protocols that use EDTA also include trypsin to help cleave the bonds between cells.[37] Though most protocols pair EDTA with trypsin, its versatility allows it to also be paired with ionic detergents and non-ionic detergents.[37,167] Miranda et al.[37] coupled EDTA with Tris buffer and used it for murine skeletal muscle decellularization by induction of an osmotic

Table 2. Advantages and disadvantages of physical decellularizing methods.

Decellularizing agent	Mechanism	Advantages	Disadvantages	References
High Hydrostatic Pressure	Induces necrosis	Reduced likelihood of protein denaturation	Proteins can denature at pressures above 600MPa Reduces biomechanical properties	Le et al.[124], Frey et al.[125], Zemmyo et al.[126], Watanabe et al.[127]
scCO2	N/A	Decellularizes tissue quickly	Dehydrates ECM Increases stiffness	White et al.[136], Dillow et al.[137], Isenschmid et al.[138], Sawada et al.[148]
Freeze-Thaw Cycles	Induces necrosis by thermal shock	Leaves majority of ECM components intact Reduces decellularization time	Insufficient for decellularization alone Can cause ECM components to rupture	Levorson et al.[128], Thibault et al.[129], Li et al.[134], Liu et al.[135]
Vacuums	Negative pressure system aids decellularization	Reduces decellularization time Facilitates uniform exposure to decellularizing agents	Insufficient for decellularization alone	Butler et al.[122], Wang et al.[123]
Sonication	Rupture cell membrane	Helps decellularizing agents permeate tissue Reduces decellularization time	Can impact microarchitecture and biomechanics of ECM	Azhim et al.[142], Yusof et al.[146], Rabbani et al.[147], Manalastas et al.[149]

shock. This approach, while successful, was improved with additional treatment by ionic and nonionic detergents but was still less effective than standard ionic or nonionic protocols.[37,167]

Nucleases such as DNase, RNase, and benzonase are generally used as post-treatment steps in chemical, physical, or biological decellularization. These agents fragment nuclear contents to achieve considerable removal of cellular materials from the matrix. DNase has been useful for decellularization of peripheral nerve,[57,168] kidney,[169] artery,[170] and trachea tissues,[171] among several others. Similar to DNase, ribonuclease (RNase) can selectively break down the nucleic acids that make up RNA. DNase and RNase are particularly useful for removing nucleotides from ECM following treatment with lysis-inducing decellularization agents. Studies indicate that the addition of DNase treatment steps can improve the retention of biomechanical properties and GAGs in several chemical, enzymatic, and physically based decellularization strategies.[57,123,172] Despite this, it does not fully remove nuclear materials and can still result in incomplete decellularization when coupled with chemical and enzymatic methods.[39] Benzonase, while also unable to act as a principal decellularizing agent, is compatible for use with several detergent-free decellularization protocols.[173–175] DNase can be used as a replacement for TNBP, which similarly works to reduce negative impacts caused by chemical agents, as DNase achieves similar results with shorter protocols and higher mechanical stability and GAG content.[39] ECM treated with DNase or RNase must be put through numerous rinse cycles, as they are immunogenic compounds that can hinder recellularization attempts.[176]

Enzymatic digestion can also be carried out by proteases and esterases, such as dispase, collagenase, phospholipase A2, and chondroitinase ABC. Dispase is a neutral protease that dissociates cells quickly but gently from a tissue by selectively cleaving at fibronectin and collagen IV. This makes it ideal for decellularizing the basement membrane of tissues, which are predominantly composed of collagen IV and laminin. Dispase is also good for preventing cell aggregation.[177] It is generally used to dissociate cells from thinner tissue membranes such as lung or cornea, but it can be used in succession with other chemical or enzymatic agents to decellularize denser tissues.[110,178,179] Collagenase cleaves at collagen II in cartilage and collagens I and III in all other tissues. Collagenase treatment can be used to selectively metabolize collagens from the ECM, permitting better proteomic analysis of other components of the ECM matrisome using mass spectrometry. Kuljanin et al.[180] found that the use of collagenase depleted the relative abundance of collagen in the ECM of bone and adipose tissue from 90% to less than 10%. Phospholipase A2 is an esterase which hydrolyses the phospholipids in cells but does not disrupt collagen and proteoglycan content, allowing for insignificant structural damage to the ECM but noticeable reduction of GAG content.[181] Phospholipase A2 can assist in the removal of lipid content but is inadequate for removal of cellular content from a tissue. Thus, it is commonly used in combination with chemical detergent or non-detergent methods to produce adequate decellularized ECM.[181–185] Chondroitinase ABC can aggressively digest proteoglycans, making it useful for the decellularization of dense cartilage tissue.[186] As such, it can be utilized to enhance the removal of cellular remains from the cartilage ECM. Chondroitinase ABC is directly associated

Table 3. Advantages and disadvantages of biological strategies for tissue decellularization.

Agent	Mechanism	Advantages	Disadvantages	References
Cytotoxic Drugs	Induce apoptosis	Retains small bioactive molecules	Insufficient decellularization Can cause damage to structural integrity Increases immunogenicity	Song et al.[71], Novoseletskaya et al.[150], Cornelison et al.[151], Giordano et al.[153], Reyna et al.[154], Fishman et al.[155], Desouza et al.[156]
Hydrogen Peroxide	Induce apoptosis	Sterilizes ECM Decreases immunogenicity	Inefficient as a principal decellularizing agent	Gosztyla et al.[157], Hennessy et al.[158]
Hypoxia	Induce apoptosis	Can be used to assist in recellularization following decellularization	Inefficient as a principal decellularizing agent Only somewhat successful in assisting decellularization	Amano et al.[159], Isidan et al.[160], Han and Flynn[161], Colombo et al.[162]

with extreme reduction of GAG content, which changes the mechanical properties of the ECM and increases stiffness.[186,187] However, for cartilage decellularization, this may be ideal, as the reduction of GAG content can improve later recellularization efforts and subsequently be restored.[186–188] Apart from cartilage, Chondroitinase ABC has also been utilized for decellularization of tissues associated with the peripheral nervous system, such as sciatic nerve and peripheral nerve. It is able to digest the chondroitin sulfate proteoglycans which inhibit neuronal repair after injury, thereby permitting axonal growth.[189–191] Chondroitinase ABC treated ECM scaffolds appear to permit adequate recellularization, which indicates great potential for use in tissue engineering.[186–188,192,193] Table 4 outlined types of enzymes widely used in decellularization, their mechanism, advantages, and disadvantages.

Recent novel approaches to decellularization by tissue type

Figure 2 summarizes a variety of novel approaches that have been recently developed and tailored for decellularization of various tissue types.

Bone

Organic bone ECM is a complex heterogeneous composite material composed predominantly of collagen types I, III, and V as well as low levels of proteoglycans, glycoproteins, and small signaling molecules.[201] Decellularization of bone has a variety of uses, as it contains the ideal properties to be developed into 3D bioinks and hydrogel scaffolds for tissue engineering, and surgical meshes. In clinical settings, decellularized bone allografts have demonstrated excellent bone regeneration capability comparable to that of autologous bone grafts, which signifies the importance of the ECM in naturally occurring bone reconstruction.[202] For these reasons, optimizing decellularization protocols for bone tissue is highly appealing. During decellularization, pretreatment of bone tissue with chelating agents or acids can be performed to first demineralize the bone. As these agents can break down several key ECM components that contribute to the biomechanics of bone, the concentration and exposure time must be taken into consideration.[203] Many decellularization protocols use combinative chemical and biological decellularizing approaches. Multiple chemical reagents are effective for decellularizing bone tissue, but the most commonly utilized are SDS and Triton X-100. Addition of acids to these decellularization processes is also possible, although they tend to result in lower success rates.[204] While these agents have been able to successfully decellularize bone tissue, they all can destruct essential ECM components such as collagens, GAGs, and growth factors. If not washed away properly, they can also cause increased immunogenicity due to the retention of cellular waste.[16]

Emami et al.[16] recently investigated the effectiveness of multiple detergents for bone decellularization. They found 0.5% SLES to outperform typical SDS and trypsin/EDTA protocols in regard to DNA removal rates, retention of critical ECM proteins, and recellularization potential. Rasch et al.[205] investigated sonication as a potential new means of bone decellularization. They determined sonication to be a superior means of removing DNA from bone tissue when compared to SDS treatment. They also found the final ECM product to have good recellularization potential and biocompatibility. These characteristics led to the conclusion that sonication produces a matrix comparable to commercially available products. However, the effectiveness of retaining the native architecture and biomechanical properties was not evaluated and is worth further evaluation.[205] Hashimoto et al.[206] was the first to report that a high hydrostatic pressure (HHP) of 980 MPa followed by treatment with nucleases could produce decellularized ECM from bone (Figure 2). However, Nakamura et al.[207] later found HHP to be inferior for overall cell removal compared to SDS, but superior for retaining the ECM microenvironment.

Cartilage

Naturally occurring cartilage has highly limited healing capabilities, making the generation of scaffolds for tissue

engineering by decellularization alluring.[208] Different types of cartilage have different structural composition and permeabilities, and thus require individualized decellularization protocols.[209] Cartilage is a highly dense tissue, making decellularization and subsequent recellularization highly difficult. Despite this, several successful decellularization strategies have been developed for various cartilaginous structures.[210–212] Generally, pretreatment with physical methods such as freeze thawing, snap-freezing, or tissue smash can be performed to assist in the decellularization, as the ice crystals they produce can create more pores in the tissue, thereby reducing the exposure time to damaging reagents. Other pretreatments that have been explored to optimize decellularization of hyaline cartilage include pulverization of the tissue, which increases the surface area and promotes slightly better permeation of chemical agents into the tissue.[213,214] These steps are commonly followed by combinative chemical-enzymatic treatments. The chemical detergents used most in cartilage decellularization are SDS and Triton X-100, although some studies have utilized SLES. These detergents are then quickly followed by treatment with enzymes such as trypsin-EDTA, DNase, or RNase to completely remove cellular DNA and prevent nucleic waste from sticking to the matrix.[215,216] Due to the detrimental nature of these compounds on the ECM matrisome, the exposure time to these agents must be highly controlled. Since some types of cartilage are denser than others and therefore require longer subjection to decellularizing agents, the specific tissue type must be considered when determining the necessary exposure time. Hyaline cartilage, which is present between most joint surfaces, is the most extensively studied cartilage for decellularization. The ECM of hyaline cartilage consists of predominantly collagens, particularly collagen II. It is also extensively composed of GAGs and laminin. Auricular cartilage, present within the outermost part of the ear, is highly elastic and flexible as a result of more elastic fibers in its ECM composition compared to hyaline cartilage. Fibrous cartilage makes up the intervertebral disks and menisci in ligaments and tendon. Its ECM composition is unlike any other cartilage in that fibrous cartilage contains collagen I only, as college II was not detected.[217]

Decellularization of cartilage tissue should aim to retain as much of the original GAG content as possible, as it is predominantly the GAG content which grants cartilage its unique mechanical properties.[218,219] Unfortunately, exposure to common decellularizing agents tend to result in the destruction of GAGs, causing an increase in matrix stiffness. Despite this setback, reduction of GAG content has been theorized to assist in the recellularization potential of the ECM, as GAG depletion reduces density and increases the porosity of the final matrix. Depleted GAGs could potentially return to the structure following successful recellularization.[186] As such, several cartilage decellularizations aim to use agents which reduce GAG content, as may assist in the successful recellularization of the final construct. In the case of cartilage tissue, further study into the importance of GAG retention should be performed in order to determine what properties constitute an ideal scaffold. Regardless, only three recent investigations at least somewhat successfully detail means for overcoming GAG reduction. Rahman et al.[58] attempted to optimize standard decellularization protocols to retain GAG content and found that detergent decellularizations involving treatment with trypsin are able to retain GAGs slightly better than treatment with other enzymatic agents, although GAG content overall was still significantly reduced.[58] This was likely due to the aggressive means by which trypsin cleaves at amino acid bonds permitting a more rapid decellularization and reducing overall exposure time to all reagents. Most auspicious to the field, however, were the progress made by Vas et al.[220] They established a vacuum-assisted osmotic shock method capable of retaining up to 85% of GAGs during decellularization of porcine costal cartilage, while Shen et al.[221] developed a means of decellularizing cartilage using freeze-thaw, followed by treatment in water and sonication to preserve GAG content (Figure 2).[220] Clearly, further investigation into the effectiveness of sonication and vacuum-assisted decellularization of cartilage must be conducted.

Adipose

Adipose tissue is important for cushioning and supporting the internal organs and is made up of adipocytes, which are dependent upon the basement membrane to provide mechanical support as well as facilitate adipogenesis. This basement membrane is predominantly made up of collagen types I and IV, laminin, and proteoglycans.[222] Mechanical disruption or dilapidation steps should be performed prior to cell removal in order to permit proper invasion by decellularizing agents. While decellularization of adipose is not as extensively studied as many other tissue types, it has been shown that adipose tissue can be decellularized using detergent, enzymatic, or solvent-based techniques. Thomas-Porch et al.[223] sought to compare several previously described combinative chemical and enzymatic protocols for adipose decellularization and found that while all were capable of decellularizing adipose tissue, the agents used influenced the proteome of the resulting decellularized matrix. This echoed several years of similar findings by studies, which analyzed the effects of detergents and enzymes on other tissue types as well. As such, investigation into alternative strategies for decellularization of adipose is encouraged.

A detergent-free decellularization protocol for adipose tissue, described by Flynn et al. involved freeze-thaw cycles followed by isopropanol treatment, which was then followed by enzymatic digestion with trypsin-EDTA, DNase, RNase, and lipase to remove cellular content and

Table 4. Summary of varied enzymatic techniques applied to tissue decellularization.

Classification	Agent	Mechanism	Advantages	Disadvantages	References
Nucleases	DNase	Cleaves nucleotide bonds	Compatible with several decellularizing agents Removes remnant nuclear content Does not impact proteomic content of ECM	Insufficient for decellularization alone Induces immune response	Simsa et al.[39], McCrary et al.[57], Wang et al.[123], Ramm et al.[172], Oliveri et al.[176]
	RNase	Cleaves nucleotide bonds	Compatible with several decellularizing agents Removes remnant nuclear content Does not impact proteomic content of ECM	Insufficient for decellularization alone	Sart et al.[194], Wallis et al.[195], Rademacher et al.[196]
	Benzonase	Cleaves nucleotide bonds	Improves retention of small bioactive molecules and biomechanical properties Compatible with several decellularization agents	Insufficient for decellularization alone	Simsa et al.[39], Liu et al.[173], Godehardt et al.[174], Dong et al.[175]
Proteases	Trypsin	Cleaves lysine and arginine	Compatible with several decellularizing agents	Insufficient for decellularization alone Damages biomechanical integrity	Dal Sasso et al.[114], Olsen et al.[163], Sajith[164], Grauss et al.[165], Zou and Zhang[166]
	Dispase	Cleaves fibronectin and collagen IV	Assists cell removal from thick tissues Prevents cell aggregation	Removes ECM structural components Damages microstructure	Asadi et al.[177], Spurr and Gipson[197], Gonzalez-Andrades et al.[198]
	Collagenase	N/A	Permits better detection and identification of ECM proteins	Removes collagen Damages microstructure	Kuljanin et al.[180], Palka and Phang[199]
	Phospholipase	Hydrolyze ester bonds	Reduces immunogenicity Compatible with several decellularizing agents	Insufficient for complete removal of lipids	Wu et al.[181], Chen et al.[182], Gessner et al.[183], Huang et al.[184], Li et al.[185]
Esterases	Chondroitinase ABC	Removes chondroitin sulfate and dermatan sulfate side chains from GAGs	Assists in decellularizing dense tissues Removes CSPGs in neural tissue	Aggressively reduces GAG content Increases ECM stiffness	Bautista et al.[186], Natoli et al.[187], Neubauer et al.[189], Boyer et al.[190], Bradbury et al.[191], Shaya et al.[200]
Chelating Agents	EDTA	Targets Ca2+ and Mg2+ ions to cleave cell-ECM bonds	Compatible with several decellularizing agents	Less effective cell removal than detergents	Miranda et al.[37], Xu et al.[43], Haupt et al.[167]

remnant lipid content.[84] This protocol retained adequate levels of collagen IV and laminin to support adipocytes in the future. Since then, few studies have been published which claim to have developed adequate decellularization by alternative means. For this reason, most recent advancements in adipose decellularization have focused on optimizing or comparing existing approaches.[224–227] Despite these attempts, detergents and acids of varying concentrations and exposure times have consistently proven to be too harsh on adipose tissue, resulting in full decellularization being accompanied by a reduction or complete removal of structurally supportive laminin.[226–229] One successful attempt to develop a detergent-free method of decellularizing adipose tissue has been reported recently. This protocol, developed by Wang et al,[230] utilized scCO2 to adequately decellularize adipose tissue with ethanol as a modifying agent (Figure 2).

Cornea

The ECM of the cornea is composed of water, inorganic salts, proteoglycans, glycoproteins, and several types of

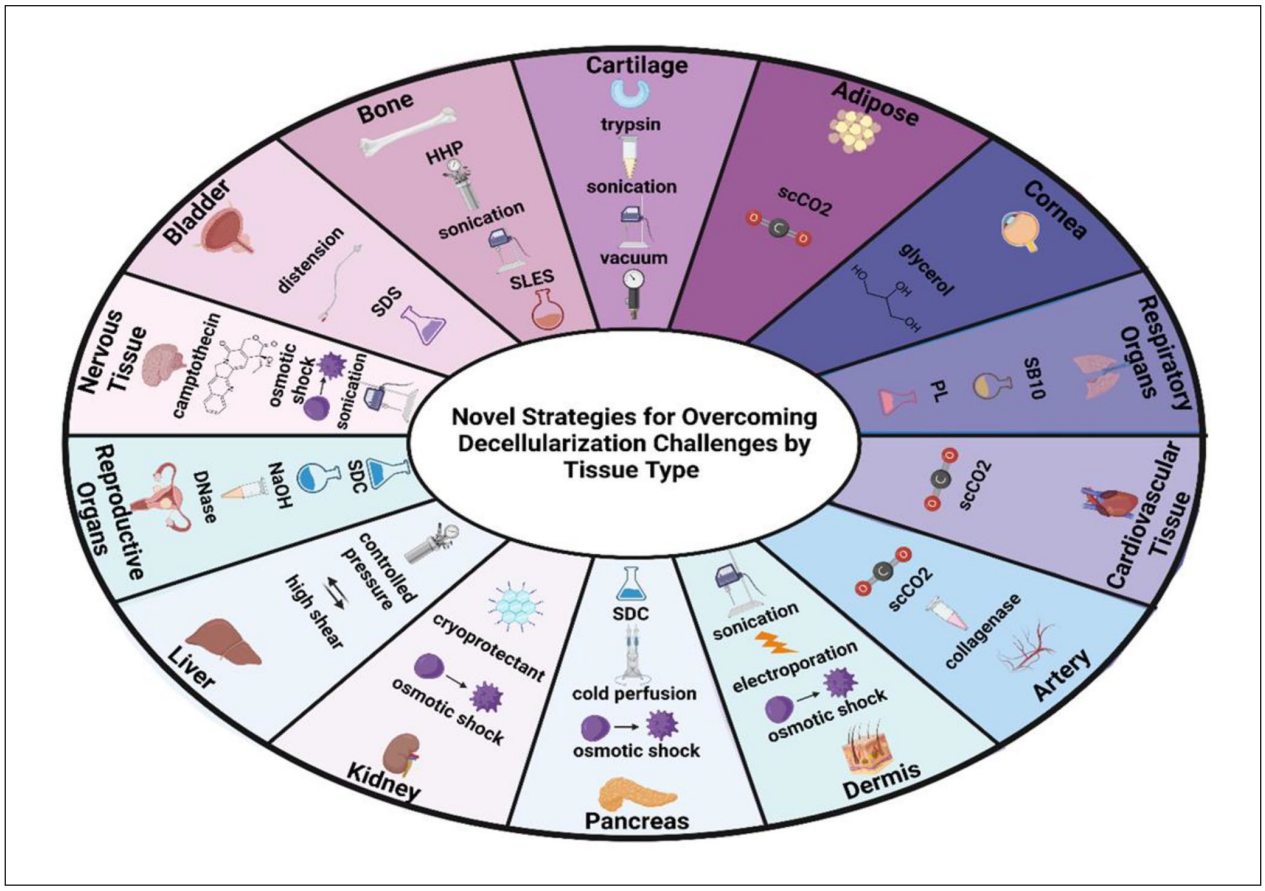

Figure 2. Novel strategies to improve standard decellularization protocols organized by tissue type.

collagens.[231] Similar to other tissues, a successful decellularization should aim to preserve as much of the native ECM components as possible. Unlike other tissues, decellularization of cornea must aim to produce a fully transparent matrix. Several conventional chemical and physical decellularization methods have been applied to corneal tissue, although most cause some undesirable results such as reduced transparency, damage to the ECM microstructure, dehydration, and/or edema.[160,232,233] As such, several studies focus on uncovering newer unconventional decellularizing techniques. For instance, some studies indicate that phospholipase A2 or human serum with electrophoresis are capable of maintaining ECM structural proteins and transparency, albeit no protocol has been optimized to attain adequate cell removal. Perhaps the most promising recent advancement, described by Lin et al.,[80] indicates that decellularization of cornea can be performed using glycerol with chemical crosslinking (Figure 2). The application of glycerol to the tissue through a pressure based osmotic system followed by gamma-ray irradiation preserved the fibrous collagen morphology and GAG content. Moreover, it provided a fully transparent and non-immunogenic decellularized graft with long-term stability.[80]

Respiratory organs

Due to the importance of the lungs to be able to expand and collapse, it is critical that a lung decellularization protocol be optimized to retain as many of the native organ's mechanical properties as possible. In particular, the ultrathin "air-blood barrier" between the alveoli and capillaries are of the utmost importance to preserve during decellularizations, as this permits the exchange of gases between the blood and the lungs.[234] The thinness of the lungs allows them to be decellularized quickly by a variety of agents. As such, several protocols have been published which indicate that the lungs can be decellularized by chemical, enzymatic, physical, and combinative methods.[235,236] Most commonly, lung tissue is decellularized by perfusion through the airways, vasculature, or both, with combinations of detergents such as SDC and Triton X-100, or individually by low concentrations of SDS or CHAPS. Protocols have utilized different concentrations for each reagent, exposure times to reagents, routes for perfusion, and order of reagent administration, each with differing levels of success. Tebyanian et al.[236] found that a detergent-based approach using 2 mM CHAPS and 0.1% SDS for 24 h maintained the microarchitecture of rat lung better than 2 mM CHAPS and 0.5% Triton X-100 for 96 h. It

is important to note that a donor tissue source must be taken into consideration when choosing a means of introducing decellularizing agents into the tissue, as lung from species of differing sizes cannot be decellularized under the same conditions. For instance, pressure-based perfusion of decellularizing agents into the tissue should be avoided for lungs sourced from larger donors, such as human or pig, but are acceptable for lungs sourced from smaller donors such as mouse or rat. Palma et al.[235] overcame this barrier by developing a constant pressure-based perfusion system to introduce SDS into horse lung that was able to maintain most of the native collagen, elastin, fibronectin, and GAG content, although the procedure resulted in the collagen area being significantly reduced and increased stiffness of the ECM compared to native tissues. Obata et al.[63] found that decellularization of rat lung by the naturally occurring detergent, potassium laurate, could significantly reduce the damage to the ECM microstructure often caused by commonly used ionic and non-ionic detergent-based protocols. Interestingly, the use of potassium laurate for decellularization resulted in an increased recellularization potential and a significantly reduced immune response upon implantation compared to lung decellularized by SDS.[63] Song et al.[71] found that the zwitterionic detergent, SB10 following treatment with the cytotoxic drug, camptothecin, was able to successfully decellularize rat lung while significantly reducing the damage to the ECM collagen structure when compared to a commonly utilized Triton X-100 and SDS treatment.[71] The potential of using potassium laurate and SB10 for detergent-based decellularization of thin tissues is worth further investigation (Figure 2).

Cardiovascular tissue

ECM sourced from the heart is composed mostly of collagens, fibronectin, and elastin. These components permit the heart to be durable, strong, and flexible.[237] Cardiovascular tissue remains to date one of the most extensively studied organs for decellularization, which has allowed for the development of several protocols capable of adequate cell removal as well as retention of biomechanical properties. While cardiovascular tissue can be decellularized using several combinative detergent-based approaches, protocols have been developed with the express purpose of enhancing the effectiveness of individual detergents alone. The most common detergents used are SDS, SDC, and Triton X-100. Several studies claim to have discovered an optimized protocol for whole organ, valve, and sectioned cardiovascular tissue decellularizations across several species.[238–240] These protocols typically utilize either physical methods, such as osmotic shock, or enzymatic methods, such as treatment with trypsin-EDTA, prior to decellularization with detergents in order to improve results. Post-decellularization, treatment with low concentrations of enzymes is used to wash away remnant nuclear waste from the matrix. Sokol et al.[241] tested multiple standardized detergent-based pericardium decellularization protocols and found that while multiple detergent-based protocols adequately removed cellular content, protocols which use single step detergent or enzyme decellularization were most likely to negatively impact the collagen structure of the resulting matrix. Single step decellularization also led to higher levels of trace nucleic acid in the matrix than combinative approaches.[241] SDS and Trypsin enzyme combinative approaches in particular are of interest, as they were shown to result in the most optimal proteomic and biomechanical results.[241]

Despite the heart being a relatively durable organ and the adequate decellularizations performed by combinative detergent-based approaches, the potential for detergents to damage the mechanical properties of the ECM, particularly GAG content that provides much of the heart's mechanical strength, remains indisputable. Thus, recent attempts to do away with detergents for cardiovascular decellularization are becoming increasingly popular. Decellularization by scCO2 treatment has been studied as a potential means for avoiding detergent induced damage to ECM components, however, this method can dehydrate the ECM.[242] Cesur et al.[243] found that a combinative Triton X-100 and scCO2 method could overcome the weaknesses associated with both decellularizing techniques.

Artery

Arteries contain three layers: the intima, media, and adventitia, each with different ECM composition. The tunica intima consists mainly of laminin and collagen IV, the media consists mostly of collagen II, elastin, glycoproteins, and GAGs, and the tunica adventitia is predominantly collagen I, elastin, and proteoglycans.[244] Because this tissue is so thin, elastic, and structurally complex, it is considered difficult to decellularize while maintaining its ECM components. To attain the best results, it has been suggested that prior to decellularization, an additional step may be taken to begin cell lysis, such as washing in water under physical agitation or introducing freeze-thaw cycles.[130,245] Similar to other tissues, artery is most commonly decellularized using detergents, including SDS, EDTA, SDC, CHAPS or Triton X-100. These detergents can be used alone or in combination and produce satisfactory results. Additionally, some protocols have shown that use of either trypsin or hypo/hypertonic solutions in tandem with these detergents can produce greater success rates.[246,247] While many studies have reported successful decellularization of blood vessels by detergent-based methods, they have been applied under varying experimental conditions, making it difficult to assess the usefulness of each individual detergent. Simsa et al.[39] sought to overcome these discrepancies in experimental conditions to determine the efficacy of individual agents in commonly utilized detergent-based protocols. Although protocols which use Triton

X-100, SDS, SDC, and CHAPS as the main decellularizing reagent all produced sufficiently decellularized matrices with acceptable biodegradability and mechanical properties, Triton X-100 based protocols were shown to result in the greatest recellularization potential. However, it was noted that it is essential for Triton X-100 decellularization be followed up with enzymatic treatment with DNase to ensure removal of nuclear remnants.[39] Most recently, attempts to overcome the need for detergents using scCO2 as a decellularizing agent have been described (Figure 2). Gil-Ramirez et al.[248] indicated that scCO2-ethanol mediated decellularizations followed by enzymatic treatment with benzonase may be a potential means for detergent-free arterial decellularization. scCO2 was shown to successfully decellularize arterial tissue, although it resulted in some external damage to the tissue. This is to be expected, as scCO2 is known to dehydrate the ECM, and thus it may not be ideal for procedures that require an intact final matrix. Another issue with arterial decellularizations is the fact that many arterial walls are protected by a watertight lining that spans the length of the arterial wall. Tuan-Mu et al.[249] recently discovered that pretreatment of umbilical cord with collagenase permitted better decellularization of the arterial walls within. Collagenase treatments were able to remove the watertight lining of the abluminal surface, in turn allowing for improved perfusion of 1% SDS detergent and reducing the overall decellularization process to less than 24 h.[249]

Decellularization of thinner tissues, such as artery, may be improved by the development of a camptothecin and SB10 approach. Blood vessels are known to be best decellularized using combinative detergent-based and enzymatic techniques, but the thinness of these tissues can result in overexposure to harmful agents, resulting in unwanted removal of protein niches. Attempts to utilize non-detergent based methods are difficult as well, as several of these non-detergent agents can dehydrate the ECM, reducing elasticity and damaging the matrix. To date, no studies have been published showing the utilization of SB10 or SB16 as the main decellularizing agents for thin elastic tissues such as artery. Camptothecin, which gently mediates apoptosis, followed by rinses with SB10, a zwitterionic detergent shown to effectively remove cellular content with a low risk of resulting in unwanted protein removal, may prove successful. However, this likely would still require further enzymatic or chemical treatment to adequately remove cellular content, which may decrease the mechanical strength of arterial ECM.[71,151,250] It is also necessary for satisfactory cellular removal with commonly used detergents such as Triton X-100.[39] For this reason, thin tissue decellularized with SB10 or SB16 could potentially require shorter posttreatment exposure times to highly damaging enzymes for adequate cell removal.

Dermis

Dermal tissue, like arterial tissue, contains multiple layers in order to perform a multitude of functions. These layers make decellularization by gentle decellularizing agents difficult, as the density of dermal tissue makes infiltration of non-surfactant agents nearly impossible. As such, decellularization of dermal tissue generally requires treatment with aggressive decellularizing agents such as surfactants and enzymes. Dermis must first be delipidated before decellularization that is most commonly carried out using combinative detergent and enzymatic treatments. While surfactant-enzymatic methods are adequate for decellularization, recently, multiple protocols have demonstrated that dermal ECM can be procured by using detergents in combination with hypo/hypertonic solution changes to induce osmotic shock, as opposed to enzymatic treatment.[251–253] Apart from osmotic shock-based methods, other attempts to remove or reduce the need for enzymatic exposure step from dermal tissue decellularization protocols have not necessarily proven to be more effective than standard chemical and enzymatic treatment protocols.[254,255] Because surfactant-enzymatic methods have been thus far the most practical and common decellularizing protocols for dermal decellularization, recent advancements by Koo et al sought to reduce exposure time to these harsh chemicals by altering standard chemical-enzymatic treatment to include hypo/hypertonic pretreatments along with physical sonication and electroporation methods.[256] The results of the study show promise in the premise of combining standard detergent-enzymatic decellularizations with physical decellularization methods, and further investigation into combinative physical/chemical/enzymatic treatment is worth exploring.

Pancreas

Pancreatic decellularization is typically carried out by Triton X-100, SDS, or enzymatic agents. Attempts to define the best detergent reagents for decellularization consistently indicate that Triton X-100 is superior to other detergents in that it typically results in far less structural damage to the ECM components of the matrix.[257] This may be due to the ability of Triton X-100 to interrupt lipid-lipid and lipid-protein bonds. Reagents used for pancreatic decellularization must be capable of breaking the protein-protein and protein-lipid bonds within the tissue, though this aspect of pancreatic decellularization is not widely reported on. Still, Triton X-100, as well as several other detergent-based protocols are well documented for the proteomic makeup of pancreatic ECM.[257,258] Recent advancements in pancreatic decellularization tend to focus on either developing protocols to optimize maintenance of the pancreatic ECM proteome or overcoming lipid barriers to successfully infuse decellularizing agents homogeneously throughout the tissue. For whole organ decellularization, Sackett et al.[259] developed a protocol that used a novel homogenization step followed by SDC treatment under agitation to successfully delipidate and decellularize human pancreas samples that contained up to 70% lipid content by weight. The subsequent matrix was determined to be non-immunogenic and had excellently

retained collagen and laminin content, but only partially retained GAG content.[259] A study performed by Panjota et al.[260] echoed this conclusion that 4% SDC could better preserve structural pancreatic ECM components than Triton X-100 or SDS in canines. SDC is known to be incapable of degrading collagens, which may explain its ability to better retain the fibrous structural components of pancreas. Elebring et al.[261] indicated that detergent-based techniques could be optimized by adjusting the temperature at which detergent-based decellularization occurs. They reported that the use of "cold-perfusion," in which a 4% SDC and 6% Triton X-100 reagent mixture was perfused through pancreas at a constant temperature of 4°C, assisted in the maintenance of pancreatic ECM ultrastructure.[261] This may have been due to the fact that the low temperature inhibits the activation of the enzymatic proteases native to the pancreas which can be triggered by exposure to detergents (Figure 2). Bi et al.[7] and Hu et al.[2] demonstrated that effective rat and porcine pancreatic decellularization with maintenance of key ECM proteins could be achieved through nondetergent-based methods. Their studies indicated that hyper/hypotonic solution changes under agitation were able to sufficiently remove up to 99% and 98% DNA from rat and porcine, respectively, while maintaining collagen, laminin, and GAG content, though investigation into potential impact on the biomechanics and ultrastructure were not reported.[2,7,18]

Kidney

Recent studies have demonstrated progress in optimizing kidney decellularization to better maintain an intact vascular tree by means of physical, chemical, and biological methods.[169,262] Both Yang et al.[262] and Feng et al.[169] investigated the usefulness of several different cryoprotectants to reduce the damage caused by the freezing process to the kidney's vascular network. While kidneys have been successfully decellularized by a variety of detergents, they are most commonly decellularized by Triton X-100, SDS, or combinative SDS and Triton X-100 protocols.[263–265] Multiple studies have indicated that the addition of a freeze-thaw step to kidney decellularization by Triton X-100 allows for relatively short treatment time with the chemical agent.[266,267] However, freeze-thaw is also known to cause damage to the overall structure of the ECM as well as the vascular network by expanding blood vessels by nearly 200% of their original size.[267] Several cryoprotectant agents could be used to optimize the freeze-thaw decellularization process. The study results indicated that conditions such as crystallization temperature, freeze-thaw temperature, rate of cooling, and concentration of each cryoprotecting agent must be considered before choosing which cryoprotectant is best suited for use in a freeze-thaw decellularization protocol (Figure 2).[169,262] Regarding recent advancements in renal decellularization by chemical methods, Poornejad et al.[268] optimized a whole organ SDS detergent protocol by inducing osmotic shock on porcine kidney. By cycling between perfusion of SDS-containing NaCl solution at gradually increasing flow rates, they were able to significantly reduce chemical treatment time and increase preservation of collagens, and GAGs.[268] Despite being relatively successful, there remains the potential for kidney decellularization for better retention of ECM ultrastructure. More specifically, establishing a protocol that better preserves the vasculature of native kidney is of the utmost importance.

Liver

The liver is mainly composed of laminin, elastin, fibronectin, and collagen types I, III, and IV, as well as sulfated GAGs.[269,270] Decellularization of liver has been extensively studied. Livers that have been decellularized by the techniques discussed above maintain their structural and proteomic components, promote cell proliferation, attachment, and migration, and show acceptable biocompatibility. Typically, for liver, whole organ decellularization is performed using perfusion methods, but can also be achieved using immersion-based methods at the cost of losing the 3D architecture and vascular network. Whole liver decellularization usually use various combinations of chemical, physical, and biological methods such as detergents like Triton X-100 or SDS, hypo/hypertonic solution changes involving NaCl, and enzymatic treatment with DNase. Currently, the most vital obstacle to overcome for liver decellularization is inefficient diffusion of decellularizing reagents through the native whole organ. Several recent advances have been made in liver decellularization—many of which have determined that the addition of external pressure or forces acting on the liver during decellularization are beneficial. Struecker et al.[271] was the first to report that pressure-based decellularizations could produce optimized liver scaffolds. They reported that the application of controlled oscillating pressures from 0 to 35 mbar during perfusion of detergents into porcine liver assisted in decreasing the amount of time required for decellularization to be achieved.[271] Similarly, Willemse et al.[272] found that decellularization carried out at constant pressure of 120 mmHg can assist in reducing the required exposure time to Triton X-100, thereby minimizing the damage incurred on essential ECM components. Mazza et al.[273] applied high shear stress during decellularization to produce ECM scaffolds from human liver (Figure 2). They found that agitating the liver at 45× *g* significantly decreased the time required to achieve successful decellularization of sectioned tissue.[273] These results indicate that further exploration into the application of external forces on tissue during decellularization are necessary.

Reproductive organs

SDS appears to be the commonly utilized reagent for decellularization of ovary and uterus, but is well known to be

cytotoxic if inadequately cleared from the final ECM product.[11,274] As a result, some attempts have been made to find alternative detergents for ovarian decellularization. Alshaikh determined that decellularization of murine ovary by 2% SDC better preserved GAGs, collagen, and elastin fibers than 0.5% SDS treatment at the cost of retaining slightly higher DNA content. The inclusion of a DNase enzymatic treatment step further improved DNA removal for all decellularization protocols in this study except for Triton X-100, which was determined to be entirely ineffective for adequate cell removal from ovary, a result shared by a recent study performed on ovine uterus, despite previous studies indicating its potential in rat uterus.[275–279] Similarly, Padma et al. found ovine uterus treated with 2% SDC to be slightly superior to uterus treated with 0.5% SDS.[280] Pennarossa et al.[281] sought to improve upon standard SDS decellularization protocols by developing a combinative physical and multichemical approach. They subjected porcine ovaries to freeze thaw cycles prior to immersion in 0.5% SDS, 1% Triton X-100, and 2% SDC. This protocol functionally decellularized the matrix and maintained an intact collagen fiber network. The protocol also preserved the elastin and GAGs while displaying high affinity for recellularization. The study did not examine the significance of each agent throughout the decellularization, and thus could not affirm or deny the claims made by Alshaikh et al. surrounding the impact of Triton X-100 on ovarian decellularizations. An attempt to remove surfactants from porcine ovarian decellularization entirely was described by Eivazkhani et al.,[282] which employed sodium hydroxide, a decellularizing reagent rarely used due to the high risk of tissue denaturation. While this protocol did lead to more structural damage than SDS, it produced a matrix with greater potential for successful recellularization later.[282]

Nervous tissue

ECM sourced from nervous tissue is mainly composed of collagen types II and IV, laminin, and fibronectin.[283,284] As is the case with most tissues, chemical methods, namely detergents, are the favored decellularizing agents for nerve. Traditionally, the most widely utilized protocol for decellularization was pioneered by Sondell et al,[285] which has demonstrated sufficient myelin and Schwann cell removal as well as adequate neural regeneration with optimal biocompatibility. This protocol used a combinative detergent effort using Triton X-100 SDC to effectively remove cellular content. Later, the decellularization protocol was optimized by Hudson et al.[73] who removed SDC entirely in favor of using the less aggressive zwitterionic detergents SB10 and SB16, replaced Triton X-100 with the now discontinued Triton X-200, and added multiple shorter wash steps. Several adaptations have since been made to this protocol and multiple studies have sought to find entirely new means of nervous tissue decellularization. Nieto-Nicolau et al.[286] developed a successful combinative multi-chemical and enzymatic approach that preserved collagen IV and laminin content, known to make up the basal lamina that is essential for axon growth. This technique, which used SB 10 and 16, Triton X-100, hypertonic NaCl, and DNase treatment steps left no cytotoxic remnants and removed the MHC II receptor, known to be the antigen most responsible for inducing immune response and thereby demonstrating the biocompatibility of the matrix.[286] Suss et al.[287] found that sonication during chemical decellularization does not help to remove DNA content, but can assist in removing cellular debris and myelin-sheaths. Cornelison et al.[151] developed a novel apoptosis-inducing decellularization protocol using camptothecin and hyper/hypotonic solution treatment, indicating the potential to overcome structural damage and immunogenicity concerns associated with detergents (Figure 2).

Bladder

Urinary bladder regeneration requires a scaffold that can produce tissue with the biomechanical properties to easily expand and contract. An engineered bladder must also contain a muscular wall and urothelium which responds to dynamic changes in sensory and autonomic provocation.[288] As it is an extensively studied organ for decellularization, several protocols have been developed and found to be effective. Distension of the native bladder prior to beginning decellularization can assist the process due to the reduced thickness of the tissue wall.[289–291] Decellularizations are most commonly carried out by PAA and ethanol or SDS and Triton X-100, although trypsin-EDTA and SDC protocols have been developed.[292–297] Recent updates in the field indicate that PAA and ethanol may not be ideal for decellularization of bladder.[48] Only Kao et al.[298] has recently sought to overcome this obstacle by optimizing SDS protocols (Figure 2). To enhance the efficacy of the detergent, SDS was prepared in a buffer to ameliorate its degradative characteristics. Subsequently, 1% SDS treated bladder-derived hydrogel scaffolds surpassed the decellularization standards set forth by PAA protocols within the realm of cytotoxicity, immunogenicity, and cell removal.[298] In the future, it may be beneficial to decellularize urinary bladder with other ionic surfactants under organ distension. For instance, SLES has consistently shown to remove cellular and nuclear material on par with SDS while also better maintaining the biomechanics of the ECM. Alternatively, since the issue associated with PAA is its inability to fully remove cellular content, perhaps the stronger analog, acetic acid may prove useful for correcting this issue, though exposure time would likely need to be optimized to discourage dehydration of the ECM. Figure 2 highlights recently developed approaches for improving decellularization efficacy specific to a tissue type. Table 5 exhibited some examples of novel step-by-step protocols for tissue

Table 5. Step-by-step protocols for decellularizing different types of tissues.

Organ	Donor species	Protocol	Comments	Reference
Bone	Homo sapien	2 h 750 uL PBS and agitate [30 m wash in deionized water 15 m centrifuge at 1850x*g*] (x3) 10 m 20 kHz sonication in 1 mL 3% hydrogen peroxide 10 m 20 kHz sonication in 1 mL 70% ethanol 10 m deionized water 15 m centrifuge at 1850x*g* 30 m wash in deionized water	Acceptable biocompatibility Comparable to commercially available bone ECM products	Rasch et al.[205]
Bone	Bovine	Wash in PBS 48 h 0.5N HCl wash in PBS 24 h 0.5% SLES wash in PBS 24 h 1% Triton X-100 24 h wash in PBS	Greater preservation of ECM proteins compared to other surfactants	Emami et al.[16]
Heart	Murine	soak in 2 mL ethanol 6 h 35 MPa $scCO_2$ wash 5 days in PBS & DNase	Retains significantly more collagens and GAGs compared to SDS and Triton X-100	Seo et al.[141]
Heart	Caprine	2 h 0.1% Triton X-100 Wash with PBS 1 h $scCO_2$	Does not dehydrate the ECM Avoids structural damage caused by long exposure to Triton X-100	Cesur and Laçin[243]
Dermis	Porcine	6 h deionized water 12 h hypertonic solution (1 M NaCl, 10 nM EDTA, 50 mM Tris-HCl) 8 h wash buffer 12 h hypotonic solution (5 mM EDTA, 10 mM Tris-HCl) 8 h wash buffer	Avoids enzymatic digestion Preserves more GAGs, collagen, and elastin than several other strategies Maintains similar biomechanical properties to native tissue	Greco et al.[253]
Dermis	Porcine	Rinse with deionized water x3 6 h 0.25% trypsin-EDTA Rinse with deionized water x3 6 h 0.1% SDS in 70% isopropanol Rinse with deionized water x3 12 h 1% Triton X-100 in 70% isopropanol Rinse with deionized water x3 12 h 100% isopropanol Rinse with deionized water x3	Effectively decellularizes skin while maintaining ECM proteomic content Sufficiently biocompatible Combinative surfactant-alcohol approach increases ECM protein retention compared to surfactant alone	Ventura et al.[77]
Dermis	Homo sapien	Rinse with deionized water 1 M NaCl solution 0.05% trypsin-EDTA 2% SDS 1% Triton X-100 Rinse again 2 h 40 kHz sonication Electric stimulation Sonication	Addition of hypertonic solution changes, sonication, and electroporation reduce exposure time to decellularizing agents Maintained structural integrity better than standard surfactant-enzymatic dermal decellularizations	Koo et al.[256]
Lung	Murine	0.0035% Triton X-100 Rinse in PBS and 1 M NaCl 0.15% PL & 0.5% Triton X-100 Rinse in PBS	Preserved microstructure Decreased immunogenicity compared to SDS based approaches	Obata et al.[63]

(Continued)

Table 5. (Continued)

Organ	Donor species	Protocol	Comments	Reference
Ovary	Murine	16 h 2% SDC wash in deionized water 30 m 40 U/mL DNase 24 h wash in deionized water 30 m 0.1% PAA 24 h wash in PBS	Preserved collagen fiber networks, GAGs, and elastin	Alshaikh et al.[275]
Nerve	Murine	1 d 5uM camptothecin 24 h hypertonic 4X PBS 30 m wash in 2X PBS 30 m wash in 1X PBS (x2) 36 h 75 U/mL DNase 30 m wash in 1X PBS (x2)	Cells in early stages of apoptosis more easily washed away than cells in secondary stages Decellularized tissue architecture nearly identical to native tissue Retained important ECM proteins	Cornelison et al.[151]
Cornea	Porcine	Wash in PBS 4 h immerse in % glycerol Wash in preservation solution 2 h glycerol buffer solution Wash in preservation solution Irradiate at 25KGy	Produced high transparency graft Maintained mechanical properties Showed long-term stability, low immunogenicity, and good biocompatibility	Lin et al.[80]

decellularization. The outcomes of these protocols were highlighted as well.

Future directions

Even though the ECM matrisome of various tissues are similar, the varied abundance of the ECM proteins maintains the uniqueness of the tissue type and plays a substantial role in directing cell fate.[299] The proteome of the ECM can be difficult to analyze due to the complexity of large, insoluble, crosslinked, and glycosylated proteins. Thus, it is difficult to determine whether decellularization results in the retention of the entire proteome. Decellularized ECM must be characterized following decellularization to confirm that the properties of the resultant scaffold construct will be able to promote re-seeded cell proliferation and not invoke an immune response upon implantation into a host biosystem. Residual DNA, cytotoxic chemicals, or disruption of the native mechanical structure could all result in unfavorable outcomes upon recellularization or implantation of the final decellularized construct.

Currently, no singular decellularizing agent can be used to retrieve a decellularized ECM scaffold with perfectly identical proteome to that of native ECM, even though tremendous strategies for tissue- and organ-specific decellularization have been developed. For this reason, decellularization protocols must cautiously determine which agents are best for use with a particular tissue of interest. Many decellularization protocols can therefore unintentionally increase the risk of inefficient nuclear removal rates, produce unintentionally cytotoxic constructs, or induce strong immune responses from the host system upon implantation. Therefore, further studies must be performed to determine the extent to which these obstacles can be overcome. For instance, recent investigations have sought to introduce drugs, such as Rosiglitazone and Honokiol to decellularized ECM to reduce immunogenicity.[300,301] Raptinal has been identified as a possible apoptosis-inducing decellularizing agent.[151] Additionally, while many techniques described throughout this review have been shown to reduce GAG content, some studies have suggested that partial loss of GAGs may be beneficial for recellularization.[302] Investigation into the level of acceptable GAG removal should be performed to better characterize the usefulness of individual decellularizing agents. While the importance of using tissues from an organ of interest has been stressed, recent findings have indicated that decellularized plant could potentially be used as an alternative to some types of decellularized animal tissues for scaffolding. Current research progresses on plant decellularization and their potential applications of plant-derived scaffolding have been introduced and summarized elsewhere.[303] Taken together, we provide a comprehensive and up to date review focusing on a variety of decellularization techniques that have been extensively investigated lately. Further advancements in decellularization strategies would facilitate the clinical applications of the decellularized biomaterials for tissue repair and disease treatment.

Author contributions

DM wrote the manuscript. SJ and KY revised the manuscript. All authors read and approved the final manuscript.

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ORCID iD

Sha Jin https://orcid.org/0000-0002-8033-8110

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