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Advances in abiotic tissue-based biomaterials: A focus on decellularization and devitalization techniques

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ABSTRACT

This Review explores the growing and diversifying field of tissue-derived abiotic constructs for tissue engineering applications, with main focus on decellularization and devitalization techniques and principles. Acellular fractions derived from biological tissues, such as the extracellular matrix (ECM), have long been considered a valuable approach for the generation of numerous scaffolds and more complex constructs. The removal of the cellular content has been considered essential to prevent the development of adverse immunological reactions. Nevertheless, the discovery of promising features of certain cellular components has sparked interest in the use of inactivated or devitalized cellular fractions for several applications, particularly in regenerative medicine and inflammation control. Devitalization has been described for several clinical applications, but remains poorly explored in terms of in vitro constructs compared to decellularization methods currently available. In this review, we present and critically evaluate a spectrum of approaches for the decellularization of whole-organs and in vitro constructs, and the most prevalent devitalization techniques, with a discussion on their implications on scaffolds composition, structure, and potentially therapeutic properties. Processing methodologies to achieve optimal cell-based abiotic materials and approaches for their effective characterization are described and discussed. The application of these materials in healthcare, with most focus on regenerative approaches and including examples of commercially available products, is also addressed.

1. Introduction

Mammalian tissues and organs are appealing sources of structural (bio)materials for healthcare due to their resemblance to human tissues. In fact, the preservation of proteins and other domains in mammals has been widely studied, suggesting that evolutionary processes have converged into high biochemical and functional conservation, probably related to the common structures and functions of these macromolecules between species. Although the field of living materials, comprising highly responsive and adaptive living materials including cell types of different origins - including bacteria, algae and mammalian cells - have been steeply growing over the last few years, this Review will focus exclusively on abiotic products amenable to be processed from mammalian tissue sources [1,2]. While living materials are of utmost importance and hold great promise in fields such as tissue engineering [3], regeneration [4], and immunotherapies [5], their passage to clinical application may be slower due to still poorly studied aspects such as long-term functional predictability and overall safety concerns (e.g., cell migration, immune rejection, control of cell death mechanisms) [6,7]. Overall, the design and fabrication of abiotic materials from whole tissues and organs relies on the assumption that abiotic tissue byproducts, as well as their fractions, maintain physiological mimicry and partial biologic potential. For tissue regeneration purposes, these matrices are expected to provide biochemical and/or structural support for tissue ingrowth or cellular modelling through the provision of cell adhesion sites, induction of cell proliferation, and directing of differentiation pathways [8,9].

A literature analysis of the last decades shows a clear predominance of decellularization techniques associated to the processing of matrices of mammalian origins to be used as biomaterials. On the one hand, this is a logic path considering that xenogeneic cellular cues are well-reported triggers of immune rejection, and most extracellular matrix-based materials, even in clinics, are from animal origin (e.g., porcine, and bovine origins) [10,11]. It is worth noting that, only very recently, gene editing tools have enabled removing genes associated to xenogeneic rejection, with promising yet short-term success results in clinical trials focused on

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organ transplantation [12,13]. Decellularized ECMs (dECM) from human origin are not frequently used to process products for widespread use. Additionally, the allogeneic character between donors and hosts may hamper their immediate application as off-the-shelf products if cell membrane molecules responsible for immune recognition would be kept [10,11], although this question is open for cell types with low immunogenicity, including mesenchymal stem cells [14] and perinatal tissues [15]. A last aspect widely associated with the need for the decellularization of biological matrices is related to the immunogenic role of DNA and other fragments associated to common cell death pathways [16]. Overall, these three aspects – control over xenogeneic response, prevention of allogeneic rejection, and protection of the host from toxic or immunogenic fragments – can be identified as the main driving forces for the need or high convenience in decellularizing tissues or organs prior to their implantation and widespread use as mammalian tissue derivatives [10,16,17].

Although ECMs have mostly been isolated directly from animals, the retrieval of ECMs from in vitro tissues are a growing trend [10]. In this case, the direct use of human cells is facilitated, as well as the selection of autologous cells, or even the study of poorly immunogenic cell types capable of differentiating into several tissue types, such as mesenchymal stem/stromal cells (MSCs) [10,18]. The use of such matrices, decellularized or simply devitalized by different means, have opened recent avenues to better understand and identify applications that strictly require decellularization procedures [19,20].

From a clinical perspective, the use of devitalized tissues, in opposition to carefully decellularized matrices, is not a new subject *per se*. In fact, devitalization has been used in several clinical fields, including the devitalization of infected or damaged tooth [21,22], of cancerous tissues for posterior implantation in situ [23,24], as grafts for defect-filling and reconstruction and to promote regeneration, including muscular [25,26] and bone autografts [27], as well as allografts [28] and xenografts from several tissues [29], such as corneal transplants [30], and as some commercial available membranes [31,32]. The technological exploration of devitalized in vitro tissues, however, is a new field still in its infancy, with seminal works focusing on the ability of devitalized cellular aggregates to promote cell adhesion and mediate the immune response [33,34], and to release retained factors capable of directing osteogenic differentiation in vivo [33,35]. One important aspect of devitalized matrices is not only their more rapid processing comparing to time-consuming decellularization processes, depending on the methodologies applied, but also the possible interest in keeping cellular cues in the processed biomaterials. While this approach apparently contradicts the established rules for safety associated to decellularized matrices, the exploration of preserved cell surface proteins may equip these cellular materials with versatile features, including precise and targeted immunomodulation ability [20,36,37]. It is worth noting that cell death mechanisms seem to be of high importance in the generation of immunologically overall safe devitalized materials [16].

The preservation of the biochemical and cell signalling cues of proteins and other components has been much valued, mostly in the processes to obtain extrudable solutions, which can be later crosslinked by physical or chemical methods to fulfil requirements associated with specific applications, such as defect filling [8]. The maintenance of structural features has been mostly valued in the decellularization of full organs, mostly by taking advantage of accessible vascular or lymphatic networks to perfuse decellularization reagents throughout the full volume of the organs, without structural and architectonic losses. In general, the preservation of architectonic features of organs and tissues requires gentler decellularization methods, when compared to the ones that give rise to extrudable liquid or liquid-like solutions [8,38]. The preservation of the intricate composition and ultrastructure of macromolecules that compose decellularized matrices has also gained momentum in the last years, as well as the preservation of previously overlooked components, currently known for their importance in cell signalling, including proteoglycans and lipids [36,39].

In this Review, we address the use of tissue-derived abiotic constructs – starting from classical fully decellularized matrices to more recently explored in vitro devitalized constructs -, considering different sources (e.g., whole organs and tissues retrieved from animal sources, or in vitro cell assemblies) and processing methodologies to achieve functional biomaterials for healthcare applications. We review the main principles of decellularization, including the diverse methodologies for the preparation and compositional characterization of decellularized matrices; a critical discussion about their implications in the biochemical and structural features of original tissues or organs is provided. A similar analysis is provided for devitalized matrices, with a more critical discussion about what is still to study in the field. Fig. 1 outlines the workflow for the production and characterization of both decellularized and devitalized scaffolds. For all types of cell-derived materials, focus is given to tissue-based materials approved for clinical use, as well as to clinical trials currently addressing the topic. A critical discussion on the specific adequacy of different types of tissue-derived materials is provided, while still poorly understood mechanisms are also highlighted as inspiring topics for future research.

2. Decellularization to obtain cell-free extracellular matrices (ECMs)

The extracellular matrix is a highly organized 3D network that surrounds and interacts with tissue-resident cells by exchange of biochemical signals, which influences processes such as proliferation, differentiation, adhesion, organization and migration [17,40]. The ECM is composed of extracellular proteins secreted by tissue-resident cells, such as collagen, elastin, fibronectin, laminin, as well as glycosaminoglycans (GAGs) and water [8,38]. It can be classified in two main types that differ in terms of biochemical composition and structure: the interstitial matrix that surrounds cells in conjunctive tissues, and pericellular matrices that directly involve cells [41,42]. The interaction between cells and the ECM is mediated by cell surface receptors, such as proteoglycans, integrins and the hyaluronan receptor CD44 [41]. The ECM serves not only as a structural scaffold but also as a reservoir for a wide range of bioactive molecules, including growth factors, cytokines, and chemokines. These molecules are stored within the ECM and can be released upon specific biochemical cues [41,42]. Moreover, the ECM is capable of undergoing dynamic remodeling, where components such as collagen, elastin, and proteoglycans are degraded or synthesized in response to both mechanical and biochemical signals, thus allowing tissues to adapt to altered physiological conditions or injury. This ability to modulate ECM composition and architecture plays a crucial role in development, wound healing, fibrosis, and tumor progression [41,42]. Whole-organs, tissues, microtissues or cell cultures can be decellularized to generate decellularized ECM scaffolds [40]. This process ideally involves the removal of cellular material often associated with the development of adverse immune reactions, with preservation of most molecular components [43]. ECM scaffolds are associated with the development of a constructive remodeling response, which is thought to be necessary to produce functional tissue after implantation. This response is dictated by the innate immune system and the adaptive immunity and is associated with an anti-inflammatory and wound-healing environment [10,44]. On the other hand, ineffective decellularization is thought to trigger an adverse inflammatory response that could affect the constructive remodeling process and result in an adverse immune reaction and/or transplant rejection [10,44].

The first report of a decellularization technique was made by William E. Poel in 1948 generating an acellular homogenate from muscle tissue samples [45]. Later, in the late 1970s, extracellular matrices were isolated from biopsies using chemical and physical decellularization approaches [45]. But it was the promising results of a decellularized porcine small intestinal submucosa (SIS) graft for the regeneration of Achilles tendon in 1995 [29,45]that raised interest in the use of decellularized ECMs as scaffolds for regenerative medicine [45]. In the

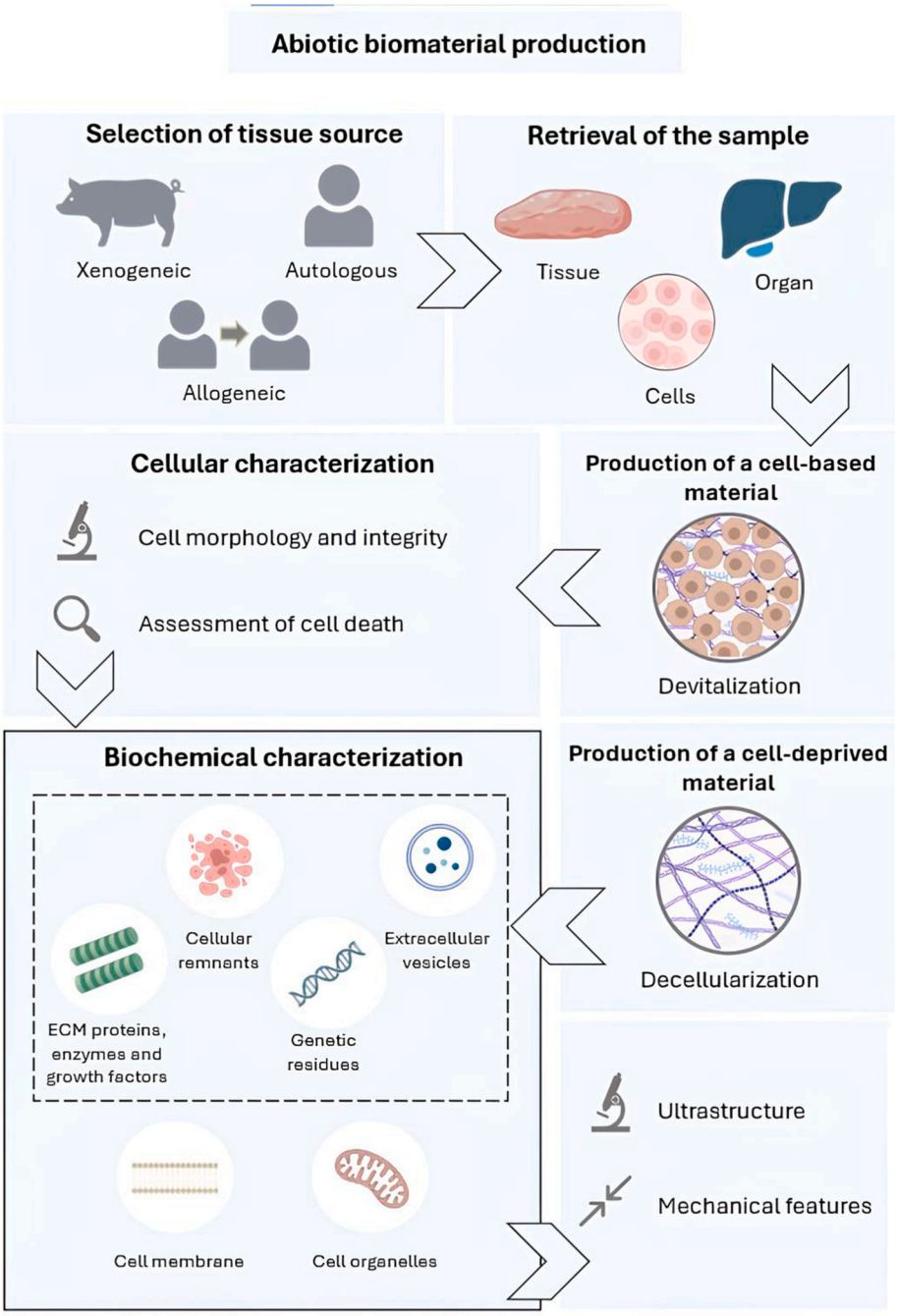

Fig. 1. Schematic representation of the pre and post devitalization and decellularization process, including the selection of tissue source and sample, in addition to characterization and evaluation of key parameters of the resulting scaffold. Created with Biorender.com.

subsequent years, substantial research efforts were dedicated to this field, culminating in a significant milestone in 2008, when scientists successfully achieved the decellularization and subsequent recellularization of a complete mouse heart [45,46]. In the following year, techniques for decellularizing the trachea based on enzymes and detergents were developed [44,47]. Since then, several organs have been decellularized to create acellular scaffolds. It has been demonstrated that these scaffolds, once transplanted, can perform physiological functions [48–51]. This process appears to rely on the recruitment and differentiation of stem cells [10,44]. Another alternative is the recellularization of scaffolds with patient cells and subsequent implantation to promote tissue regeneration or replace a damaged or missing organ [38]. dECM scaffolds have been used to aid in the reconstruction and remodeling of musculoskeletal, nervous, gastrointestinal, and cardiovascular tissue. They have also been employed as valuable models for studying numerous diseases and pathological states, such as tumors, and for investigating drug metabolism [8,9]. dECM scaffolds have been processed and modified to form hydrogels, bioinks for 3D bioprinting, and other processing techniques such as electrospinning [50,52]. More recently, a bioreactor was developed to support the recellularization of a lung dECM scaffold [53]. Researchers are now focused on carrying out proteomic and bioinformatic studies of the dECM to map it and to better characterize the matrisome, thus allowing the development of scaffolds that better mimic the structure and composition of the native ECM [38,44]. Decellularization techniques are classified into three main classes: physical, chemical, and enzymatic. Fig. 2 synthesizes the decellularization workflow, the evaluation and assessment of the scaffolds' properties and its posterior processing and modifications. Combined

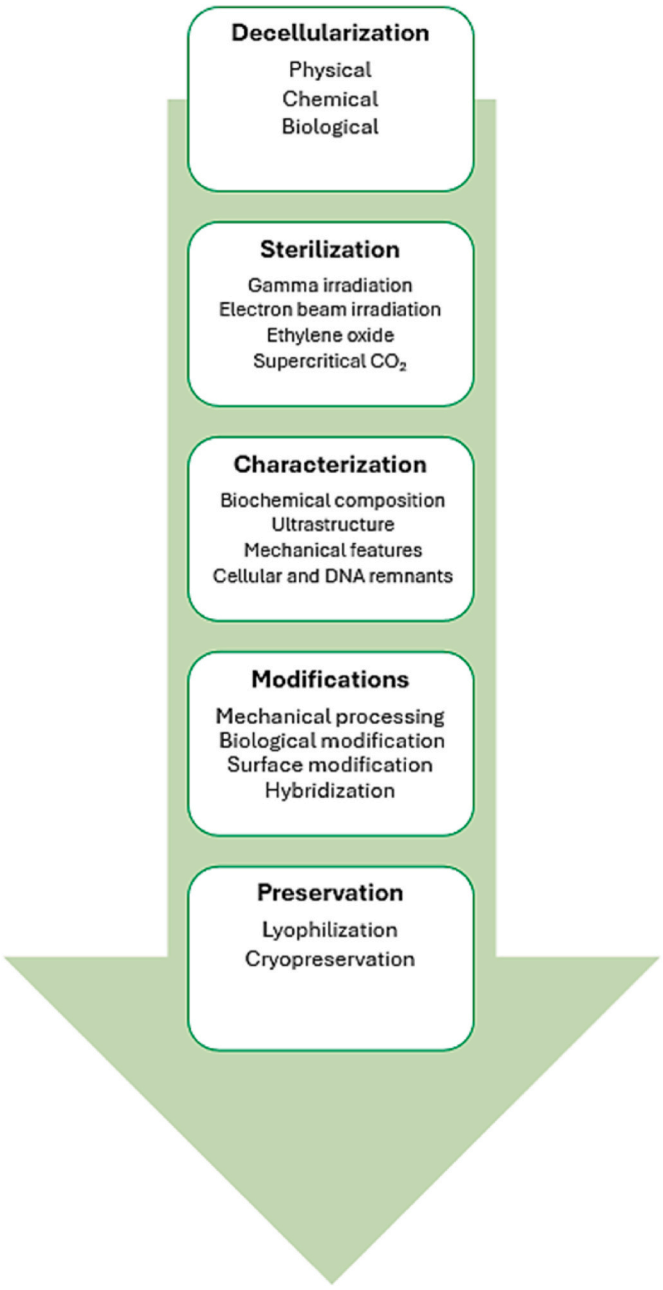

Fig. 2. – Decellularization workflow, from decellularization methods to further characterization and modification of the decellularized matrix.

strategies are often employed to achieve a higher level of decellularization, and the most effective protocols in the literature mostly incorporate a combination of all (Fig. 3) [54]. A description of the primary methods within each class and their advantages and disadvantages are described in the next section.

Decellularization methods differ considering the original tissues to be processed, and the route employed for its delivery [55]. The composition of the ECM is also affected by donor age and differs among specific tissues and organs [10]. Each tissue has unique mechanical properties and distinct ultrastructures, finely tuned to specific tissue functions. Consequently, decellularization methods must be tailored to a particular tissue [10]. The choice of method will depend on several tissue-specific factors, such as cellular and lipid content, cell density, thickness, and shape [10,17]. It is important to note that all decellularization methods will alter the ultrastructure and composition of the original ECM to some degree, and that complete removal of all cellular components is not possible [9]. Age, gender, and species are typically not considered when designing a decellularization protocol. However, it is known that the ECM of tissues from older donors may have lower levels of collagen and other characteristic ECM proteins, which can ultimately reduce the elasticity and mechanical properties of the matrix [56]. Taken this in consideration, for tissues from older donors, it may be necessary to select a protocol that is not overly aggressive, ensuring that it does not significantly alter the mechanical properties and composition of the ECM, and enables preserving tridimensional structure when necessary. Some studies have shown that different protocols should be adopted for tissues of different ages [57,58]. For example, fetal organ-derived tissues require lower detergent concentrations compared to the same organs in adult tissues. A study by Hu et al. conducted a proteomic analysis of decellularized pancreatic matrices [59]. It was demonstrated that the ECM from younger donors contained approximately 70 % more ECM proteins than that of adult donors. Additionally, it was shown that some ECM proteins are gender-specific, being found exclusively in male or female donors. Regarding interspecies differences, one study demonstrated that a protocol used for porcine hearts was ineffective for human hearts [58]. This discrepancy could be attributed to the fact that the human hearts were cadaveric, whereas the porcine hearts were fresh. It was previously demostrated that age increases fibrosis and stifness in cardiac tissue due to ECM crosslinking and changes in ECM composition [58]. Nevertheless, the influence of age, gender, and species on ECM composition and its impact on the decellularization process are not yet fully understood, but it is important to take these aspects into consideration during the formulation of a decellularization protocol and in the posterior evaluation of the resultant scaffold. For a more extensive characterization of ECM composition, the database MatrisomeDB includes a collection of ECM proteomic data from 15 healty tissues, 6 cancer types and other pathological conditions [60]. Additionally, another mass spectrometry-based study analyzed ECM from 25 mouse organs [61].

2.1. *Physical methods*

Physical methods act by disrupting the cell membrane and destroying the cell-matrix adhesive proteins [54,62]. As most physical methods fail to remove all cellular and genetic content, they are often used in combination with enzymatic and chemical treatments [54]. The lysing of the cellular membrane allows the release of cellular debris and facilitates the subsequent integration of chemical and enzymatic reagents in the cell [55]. Afterward, a washing step is required to remove all cellular and genetic material. Efficient DNA removal is difficult to achieve since the DNA can adhere and bind to the ECM fibers [54]. Some forms of residual DNA have been associated with the development of an immune response, and the quantification of DNA removal is used as an indicator of the efficiency of the decellularization [9]. The previous methods can have deleterious effects on the composition and structure of the ECM, and can present some level of toxicity. Due to these reasons, physical methods with greater effectiveness are under investigation, such as vacuum-assisted decellularization [55,62,63]. Physical approaches include the temperature, pressure, mechanical force and mechanical agitation, electroporation, immersion, and supercritical fluids (Table 1) [54,55].

2.1.1. *Temperature*

Freeze-thaw cycles involve submitting the samples to freezing temperatures (usually lower than −80 °C) and biological temperatures (37 °C) [62]. Rapid temperature shifts can form intracellular ice crystals and induce cell lysis. The number of freeze–thaw cycles performed and the temperature variation should be adapted to the type of tissue being decellularized and the degree of ECM preservation required since this process can cause some level of disruption to the ECM network [8,54]. Nevertheless, the mechanical properties appear not to be affected by the freeze-thaw process on robust tissues [17]. Studies have reported that

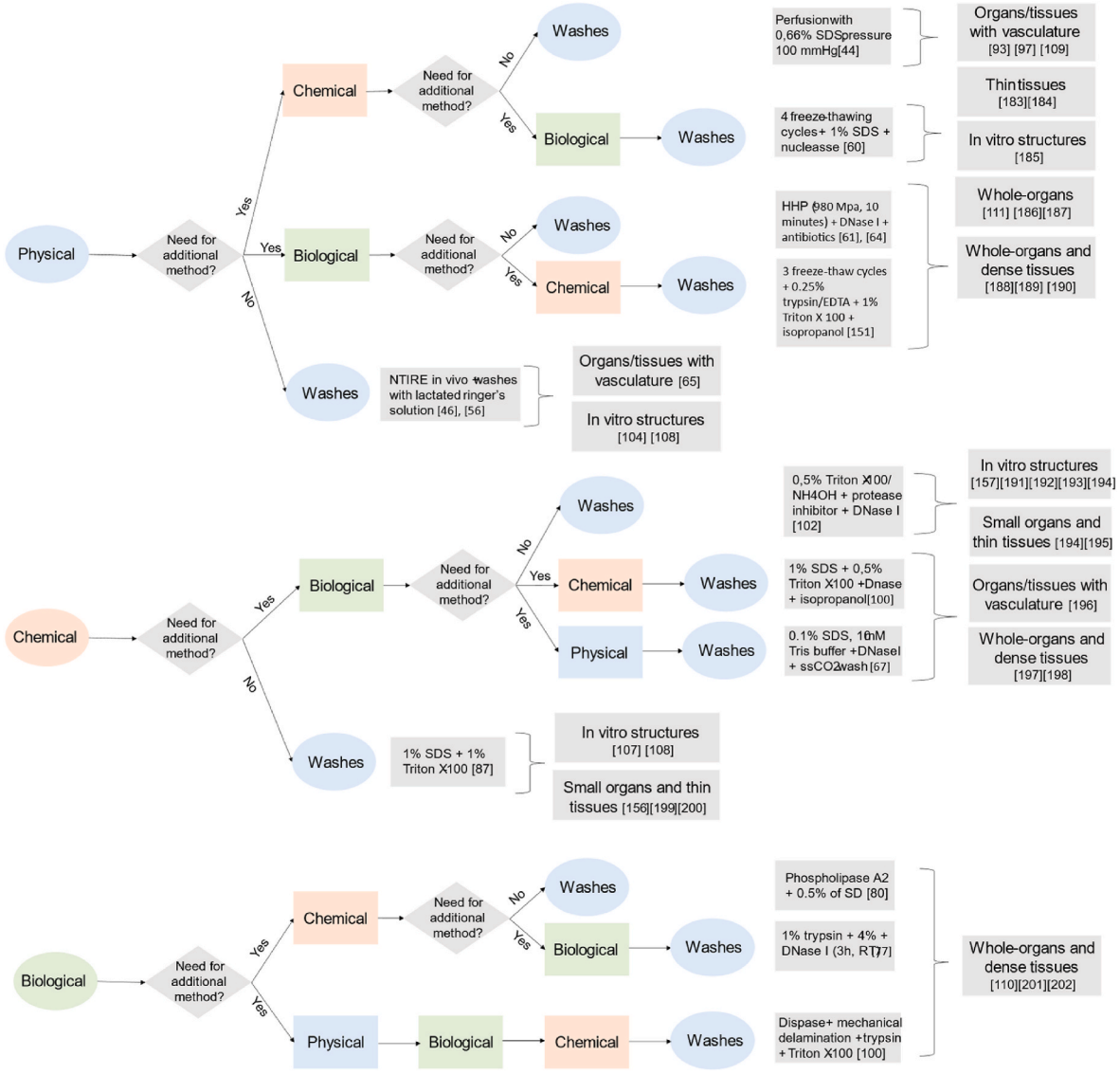

Fig. 3. – Flowchart of the most common combined decellularization methods and examples of reported protocols.

the use of a cryoprotectant, such as DMSO, can minimize structural damage to the tissue without affecting cell lysis [9]. Following the cycles, cellular debris must be removed using detergents and enzymes [54]. Burk et al. performed a freeze-thaw-based decellularization method in large equine tendons [64]. Freeze-thaw consisted of 5 cycles of freezing in liquid nitrogen for 2 min and thawing in phosphate-buffered saline (PBS) for 10 min at 37 °C. Following freeze-thawing, the tissue was treated with 1 % Triton X-100 for 48 h or with 1 % SDS for 48 h. Results showed no morphological alterations, and the scaffolds were biocompatible. However, residual nuclei and residual DNA were found [64]. Ning et al. used a similar freeze-thaw procedure for the decellularization of Achilles' tendons from beagle dogs, but incubated the tendon tissue in a nuclease solution of DNase and RNase [65]. The results demonstrated the almost complete removal of all cellular and genetic material and preservation of the ECM ultrastructure, as well as preservation of mechanical strength and specific growth factors and proteoglycans.

2.1.2. Pressure

Pressure gradients are useful for hollow tissues such as veins and for thicker tissues such as tendons, as they can facilitate the delivery of decellularization reagents into the tissue and eliminate cellular remnants [8,66]. Pressure gradients are considered an effective decellularization method and have minor effects on the mechanical properties and structure of the ECM [8,9]. Another benefit of pressure gradients is that they shorten processing times and dispense the use of harsh chemical agents to achieve an effective degree of decellularization [66]. High hydrostatic pressures (HHP) also eliminate the need for aggressive chemicals. This method consists of directly applying high pressures greater than 600 MPa homogeneously throughout the tissue to disrupt the cell membrane [12,67]. HHP is effective as a decellularization method on its own and leads to higher removal of cell residues in comparison to detergent-based methods [9,62]. Furthermore, this process is relatively fast and destroys viruses and bacteria, thus sterilizing the tissue [17,68]. Nevertheless, it has some disadvantages that need to be taken into account. Some studies reported some level of DNA remnants in the samples after the application of this method and the denaturation of ECM proteins, such as collagen and elastin, affecting their mechanical properties [68]. Additionally, the baric formation of intracellular ice crystals can partially destroy the ECM structure and extra washing and rinsing steps are required to completely remove cellular components [17,68]. Several studies have reported that applying HHP at a pressure of 980 MPa could effectively decellularize soft and hard tissues such as blood vessels [69], cornea [70], and bone

Table 1
Summary of main physical mehtods for decellularization reported in the literature, usually applied in combined strategies, including the principle of cellular removal, main advantages and disadvantages.

Physical Method	Principle	Advantages	Disadvantages	Ref
Temperature	Rapid temperature oscillations form intracellular crystals and induce cell lysis	Dispenses the use of chemical treatment	Structural damage and alteration of mechanical properties; Requires additional treatments to remove cellular and genetic residues	[8,9,17, 64,72]
Pressure	HHP consists in directly applying high pressures homogeneously through the tissue, disrupting the cell membrane	Minor effects in mechanical and structural properties. Effective in cellular content removal for high pressures.	Higher pressures can lead to protein denaturation and tissue fragmentation; possible formation of intracellular baric ice crystals; some studies report insufficient removal of cellular components	[8,9,17, 72, 79–81]
Electroporation	Application of microsecond electric pulses in the tissue, leading to the formation of micropores in the cellular membrane	No structural damage or reduction of protein levels	Size restriction; NTIRE is often performed in situ, but is considered insufficient and can cause inflammation in vivo	[17,38, 79,82]
Mechanical force	Direct application of mechanical force by scarping in order to remove surface cell layers	Used in thick and dense tissues to remove cell layers. Usually dispenses the use of chemical treatment	Can cause damage to the ECM and the basement membrane, affecting structural and mechanical features	[9,17,38, 64,79,83]
Agitation and immersion	Immersion of the tissue with a decellularization solution under dynamic conditions, which facilitates cell removal	Easily performed; can be applied for many tissue types	Need for optimization for different tissue types; Possible ECM structural changes	[8,9,38, 64,81]
Sonication	Generation of acoustic cavitation bubbles disrupts the cell membrane	Facilitates the integration of decellularization solutions in the cell and aids in the removal of cellular residues	Cavitation may affect the tissues' structure and mechanical features	[8,79,81, 84]
Supercritical fluids	Removes cellular residuals after treatment with a chemical agent	Do not alter ECM composition, network and mechanical properties; Easily removed from the tissue; Sterilizing effect	Usually needs to be used in combination with other methods	[9,17,38, 79,81,85]
Perfusion	Perfusion of decellularization solutions through the vasculature of the tissue/ organ, facilitating cell removal	Effective cell removal when combined with decellularization solutions; Can reach deeper areas of the organ/tissue	Can not be applied to tissues without a vasculature network; Needs to be used in combination with other, usually chemical, methods; Can cause disruption of capillaries and ECM structure	[9,38,79, 84–86]

[71]. For porcine corneas and porcine blood vessels, the 980 MPa pressure was applied for 10 min at a temperature of 10 or 30 °C and then the tissue was washed with DNase I solution and antibiotics. It was verified that there was no structural damage, and the mechanical properties of the tissue were not affected. After transplantation in vivo, it was demonstrated that the scaffold was functional [69,72].

2.1.3. Electroporation

Nonthermal irreversible electroporation (NTIRE) involves the application of microsecond electric pulses across a tissue, inducing the disruption of the cell membrane's electrical potential. This leads to the formation of micropores in the cellular membrane, followed by loss of homeostasis and cell death [17,38]. The mechanisms involved in the removal of cellular debris are not precisely understood, but the ECM components appear to be intact following treatment with NTIRE [17, 73]. This method has some limitations, including size restriction for the tissue being decellularized and the need to be performed in vivo in order to avoid immune rejection, since the mechanism of cellular removal appears to be mediated by the immune system [17,66].

2.1.4. Mechanical force

The application of mechanical force to cell layers on the surface of an organ with planes of dissection such as the skin, the SIS and the urinary bladder, in combination with enzymes, a hypertonic solution or a saline solution, can effectively remove the cells from the basement membrane [17,38,54]. The physical removal of the surface layers can be achieved by scraping with a sharp tool. Nevertheless, the direct application of mechanical force can cause damage to the ECM and the basement membrane, affecting their ultrastructure, so the force applied must be carefully controlled [9,17,66]. Two types of direct mechanical force can be applied: mechanical force using a sharp object (e.g., forceps) to scrape and remove tissue layers, as seen in skin or amniotic membrane decellularization [74]; and mechanical scraping/stress using a sharp object to remove superficial cellular components or induce cell lysis [75]. The amount of applied force is relative and depends on the type of tissue being decellularized. Denser tissues require greater force to remove layers or thick basal membranes [9]. The optimal force applied should be tailored to the tissue type and the desired level of preservation of the final scaffold, depending on the protocol and its optimizations. After applying mechanical scraping, a structural and morphological analysis of the ECM should be performed to assess whether the applied force during the decellularization protocol was excessive — potentially damaging the ECM — or insufficient to remove the necessary cellular content and layers [9,38,76].

2.1.5. Agitation and immersion

This method consists of immersing a tissue in a solution with a decellularization agent while providing mechanical agitation with a magnetic plate, an orbital shaker, or a rotating chamber [38,54,66]. The combination of immersion and agitation facilitates cell lysis and cell removal from the basement membrane. These methods are often used in conjunction with chemical treatments and enzymes in order to obtain more effective decellularization [8]. Agitation and immersion are mostly used for thinner and avascular tissue sections such as the basement membrane from the urinary bladder and the small intestine, but decellularization protocols have been described for various types of tissues/organs [9,38,68]. Thinner tissues require less exposure time, while thicker tissues require longer exposure to a combination of decellularization agents [9]. Parameters such as agitation length and speed, decellularization agent concentration and volume, and extent of the procedure are dependent on the dimension, thickness, and composition of the tissue [38,54,68]. This method can be easily performed when compared with perfusion decellularization, but agitation can also deteriorate the ECM structure [8]. Sonication is considered a subtype of mechanical agitation in which ultrasonic waves at frequencies above 20 kHz are applied to a tissue or organ immersed in a bath [8,66]. The generation of acoustic cavitation bubbles disrupts the cell membrane and facilitates the penetration of the decellularization agent into the scaffold, leading to the rupture of the cell membrane and removal of the cellular components [66,68,77]. However, the phenomenon of cavitation must be controlled in order to avoid unwanted damage to the tissue's structure and mechanical properties [66,77].

2.1.6. Supercritical fluids

Supercritical fluids such as supercritical carbon dioxide ($scCO_2$) can burst through the tissue to remove cellular remnants after treatment with a chemical agent, such as ethanol, to achieve complete decellularization [17,77]. The gas-like low viscosity and high diffusivity permits a rapid and efficient mass-transfer within the matrix. Carbon dioxide has the advantage of being an inert substance, as it does not alter the ECM mechanical properties [9]. Nevertheless, the pressure required for the supercritical phase can disrupt the ECM. This method eliminates the need for lyophilization required for ECM processing and storage, and the need for a sterilization step because supercritical carbon dioxide has a bactericidal effect and inactive viruses [17,77]. Additionally, as carbon dioxide is highly permeable, it will be swiftly removed from the tissue and further washing steps won't be required [66]. This decellularization method is considered simple and fast to perform [77]. Casali et al. developed a hybrid method that combines SDS treatment with presaturated $scCO_2$, performed in porcine aorta. The tissue was exposed to 0.1 % SDS, 10 mM Tris buffer, and 0.2 mg/mL DNase I for 48 h, followed by a wash with $scCO_2$ for 1 h. SDS enhanced cell lysis and facilitated the penetration of $scCO_2$ into the cell to further remove cellular remnants. The results showed complete decellularization of the tissue with preservation of the hydration state, matrix ultrastructure, and mechanical properties [78].

2.1.7. Perfusion

Antegrade and retrograde perfusion are mostly used to decellularize whole organs or big sections of tissue. In this method, the chemical decellularization agents are perfused through the organ's native vasculature, and the cellular components are removed [66,77]. This method allows for more effective decellularization, as cells are in close proximity to the vasculature and can reach deeper areas of the organ/-tissue [9,79]. Different perfusion protocols have been developed for numerous organs such as the heart, liver, kidney, lung, and small intestine [66]. However, this method cannot be applied to organs/tissues without vasculature [38]. Additionally, perfusion pressure can cause disruption of capillaries and ECM structure if the flow rate isn't precisely controlled [17,66]. Other parameters such as the perfusion route, the solution being perfused and the density and dimension of the organ/-tissue may affect the outcome of the decellularization process [77]. Another disadvantage of this method is the complexity of the perfusion devices [38]. Schmitt et al. developed an optimized protocol for the decellularization of whole organs, which was tested in rat kidneys. This protocol used SDS in combination with perfusion. To minimize damage to the ECM, 0.66 % SDS was used for 1 h at a pressure of 100 mmHg. The results showed complete decellularization of the tissue with preservation of the matrix architecture and basal membrane. After recellularization, the cells demonstrated a high survival rate [80].

2.2. Chemical methods

Chemical treatments are the most widely used decellularization methods and act by solubilizing the cell membranes, breaking protein-lipid bonds, and disrupting the DNA and RNA molecules [47,55]. These methods include acids/bases, hypotonic/hypertonic solutions, detergents, solvents, chelating agents, and toxins (Table 2) [38]. The majority of chemical treatments effectively remove most cellular and genetic material, but can also decrease or damage the ECM proteins and present some degree of toxicity [55,62]. In order to reduce these issues, chemical methods may be used in combination with enzymatic approaches and all chemical residuals must be carefully washed out from the final scaffold [81,87]. For thicker tissues, protocols include a combination of different chemical agents [88]. As previously stated in this review, chemical treatments can also be used in conjunction with perfusion and agitation techniques.

Table 2
Summary of chemical techniques for decellularization, usually applied in combined strategies, most widely available in the literature, including the principle of cellular removal, main advantages and disadvantages.

Chemical Method	Principle	Advantages	Disadvantages	Ref
Ionic detergents	Solubilization of cellular membranes and nucleic acids	Highly efficient in cellular content removal	Protein denaturation, loss of GAGs and growth factors; Difficult to remove from the ECM, multiple washes are required	[8, 9, 17, 72, 94]
Non-ionic detergents	Solubilization of cellular and nuclear membranes	Ideal to disrupt lipid-lipid and lipid-protein bonds; Usually do not affect protein-protein interactions and collagen integrity	Incomplete removal of cellular components Can cause the reduction of certain proteins and affect ECM ultrastructure	[8, 9, 17, 38, 64]
Zwitterionic detergents	Solubilization of cellular and nuclear membranes	Effective removal of cellular debris	May disrupt the basement membrane and mild disruption to the ECM structure	[8, 9, 64, 65]
Acids/Bases	Solubilize cytoplasmatic components and cellular membranes; disruption of nuclear material	Effective in removing cellular components, specially in thicker tissues and whole-organs; Some acids can act as sterilizing agents	Acids can cause protein denaturation, depletion of GAGs and growth factors and can affect ECM architecture; Bases can degrade and disrupt collagen crosslinks, reduce GAGs and growth factors, and affect the structure and mechanical features of the ECM	[8, 9, 17, 94, 95]
Hypertonic/ Hypotonic solutions	Induce osmotic shock, leading to cell lysis, dehydration and disruption of DNA-protein interactions	Minimal alterations to ECM composition and structure;	Incomplete removal of cellular content and DNA remnants	[9, 17, 38, 64]
Organic solvents	Alcohols and acetone act by causing cell dehydration and cell lysis. Can also remove nuclear components; TnBP disrupts protein-protein interactions	Alcohols and acetone are effective in removing lipidic fractions and nuclear content, usually after a prior step of cell membrane disruption; TnBP highly effective in removing cellular content without significant biochemical and mechanical alterations	Alcohols and acetone can cause denaturation and precipitation of proteins, affecting ECM composition and structural and mechanical properties; may lead to the fixation on cells (see devitalization techniques); TnBP can	[9, 17, 64, 96]

(*continued on next page*)

Table 2 (*continued*)

Chemical Method	Principle	Advantages	Disadvantages	Ref
			potentially reduce collagen	
Chelating agents	Bind divalent metals at cell-matrix adhesions, facilitating the dissociation of cells from the ECM	Effective in the removal of the cellular fraction when combined with trypsin	Low efficacy in cellular content removal; Denaturation of proteins and disruption of protein-protein interactions	[8, 9, 17, 38]
Toxins	Latrunculin B disrupts the actin cytoskeleton	Useful for the decellularization of skeletal muscle; Eliminates genetic remnants with preservation of GAGs	Limited to muscle tissue	[8, 9, 17]

2.2.1. Detergents

Ionic detergents such as sodium dodecyl sulfate (SDS), sodium deoxycholate (SD), and Triton X-200 act by solubilizing cellular membranes and nucleic acids [8]. However, the high ionic strength of these detergents usually leads to protein denaturation by disrupting protein bonds, and loss of GAGs and growth factors from the ECM [54,88]. SDS is one of the most used decellularization reagents due to its high efficiency in eliminating cellular components. Several studies in different tissues/organs have shown that SDS treatment in concentrations between 0.1 and 1 % removes at least 90 % of all genetic material [68]. Nevertheless, it also depletes GAGs and signaling proteins and affects collagen integrity [54,62]. Additionally, SDS is difficult to remove from the ECM due to its polarity and can affect cytocompatibility [8]. To minimize these effects, one approach is to employ SDS in multiple washing steps with lower concentrations and shorter exposure times, while also reducing the temperature used [9]. SD is another example of an ionic detergent. Compared to SDS, SD is less effective at removing genetic material, but better preserves the ECM [8]. One drawback of SD is its potential to induce DNA clumping, an issue that can be mitigated by using the surfactant in conjunction with DNase I [62].

Non-ionic detergents also act by solubilizing cellular and nuclear membranes, but in contrary to ionic detergents, the protein-protein interactions and the collagen integrity and orientation remain intact [38]. Although the ECM ultrastructure and growth factors are not affected, the cellular components are not completely removed. To resolve this issue, non-ionic detergents could be used in combination with other agents or physical treatments [8,38]. These detergents, such as Triton X-100, are ideal for breaking lipid-lipid and lipid-protein bonds and therefore are mostly employed in tissue delipidation. It has been shown that Triton X-100 also removes GAGs from the ECM [9,17]. Ionic and non-ionic detergents, such as Triton X-100 and SDS, are often combined to minimize the detrimental effects on the ECM and to increase the removal of cellular debris [38,54]. Moreover, the addition of a physical method can improve the decellularization rate. Xu et al. developed a protocol based on the two detergents in conjunction with HHP for the decellularization of porcine Achilles tendon entheses [89]. Different conditions were tested, but the optimal results for decellularization and matrix preservation were obtained when washing the tissue with 1 % Triton X-100 and 0.5 % SDS for 48 h under a hydrostatic pressure of 200 mmHg. Results showed that ECM structure, biochemical features, and mechanical properties were preserved [89].

Zwitterionic detergents include 3-[(3-cholamidopropyl) dimethylammonio]-1-proppane-sulfonate (CHAPS), sulfobetaine-10 (SB-10) and sulfobetaine-16 (SB-16). These detergents have properties of both ionic and non-ionic detergents [55]. Tsuchiya et al. demonstrated that there is greater collagen retention in lung scaffolds decellularized with CHAPS compared to SDS-based decellularization, resulting in higher mechanical integrity [90]. Another study showed that the zwitterionic detergents SB-10 and SB-16 were more effective in removing cellular residues compared to ionic detergents [91]. Those detergents seem to consistently remove cellular components better than non-ionic detergents and cause less damage to the ECM components and architecture than ionic detergents [54]. CHAPS is more suitable for thinner tissues such as blood vessels or the lung, retains collagen and GAGs, and removes at least 95 % of genetic material [9]. However, it may disrupt the basement membrane and cause mild disruption to the ECM structure [8].

2.2.2. Acids/bases

Acids and bases solubilize cytoplasmatic components and the cell membrane, disrupt nuclear material, and induce the hydrolytic degradation of molecules [38]. However, most acids can denature proteins, such as collagen, as well as GAGs and growth factors, and affect the ECM architecture [8,9]. The most commonly used acids are peracetic acid (PAA), hydrochloric acid, acetic acid, and deoxycholic acid [9,38]. PAA has been applied in low concentrations (0.1 %) for the decellularization of thin tissues sections such as SIS and urinary bladder matrix (UBM) [9, 88]. Studies have shown that this concentration minimizes damage to the ECM structure and its constituents [92]. Adjusting the concentration and exposure time to these compounds in decellularization protocols is important for reducing the damage caused by acids [9]. Additionally, PAA has the advantage of eliminating bacteria and viruses, acting as a sterilizing agent [38]. Acetic acid is another acid frequently used for decellularization and it does not remove GAGs. However, it has been shown to reduce the collagen content, and affects the resulting scaffold strength [9,17]. Similar to acids, bases act by solubilizing cellular and nuclear materials [38]. Examples of bases frequently employed as decellularization methods include sodium hydroxide, ammonium hydroxide, and calcium hydroxide [9]. These compounds are commonly used to decellularize thicker tissues such as the dermis, and whole-organs [8,9]. Bases with a pH higher than 11 are particularly effective at removing cellular components since they cause the DNA to denature [38]. However, the high pH affects the structure and mechanical properties of the ECM by degrading and disrupting collagen crosslinks and also reduces GAGs and growth factors from the tissue [9, 17]. Both acids and bases are often used in combination with other decellularization methods.

2.2.3. Hypotonic/hypertonic solutions

Hypertonic and hypotonic solutions induce osmotic shock, leading to cell lysis or cell dehydration and disruption of DNA-protein interactions [9,17]. Hypertonic solutions such as sodium chloride (NaCl) typically remove proteins, while hypotonic solutions such as Tris-HCl have shown more efficacy in eliminating nuclear material without negatively affecting the ECM composition [38]. To combine the effects of both solution types, those are often employed alternately in multiple decellularization cycles and can be easily removed from the tissues, reducing their potential toxicity [9,17]. Although hypertonic and hypotonic solutions facilitate cell lysis, they do not completely remove cellular debris, such as DNA remnants. For this reason, these solutions are usually combined with other chemical agents [54].

2.2.4. Organic solvents

Alcohols and acetone can be used for the decellularization of dense tissues following a physical method and/or permeabilization with detergents by dehydrating and lysing cells [17]. Ethanol, methanol, and isopropanol effectively solubilize and remove lipids from adipose tissue and the liver [9]. Ethanol and methanol also remove nuclear components and inactivate pyrogens [9,17]. Acetones may be used for delipidating tissues, but due to the significant damage to the ECM, they are mostly used for fixation and sterilization. Similar to acetone, treatment with ethanol and methanol also dehydrates and fixates tissues and can denature and precipitate proteins [9,17].

Tri(n-butyl)phosphate (TnBP) is an organic solvent that has been used for the inactivation of blood viruses while preserving the activity of blood coagulation factors [9,54]. TnBP disrupts protein-protein interactions and have shown to be more effective than SDS and Triton X-100 in the decellularization of tissues such as tendons and ligament grafts [17,54]. Treatment with TnBP does not significantly affect biochemical and mechanical properties. However, it can potentially reduce collagen and only partially remove cellular proteins [9,17]. Bottagisio et al. tested different concentrations of TnBP in combination with PAA for the decellularization of equine tendons [82]. The results showed that exposing the tissue to 1 % TnBP for 24 h and to 3 % PAA for 4 h, both under agitation, yielded an acellular scaffold with good mechanical and biochemical properties. Furthermore, reseeding with fibroblasts showed that the resulting scaffolds were not cytotoxic and were biocompatible [82].

2.2.5. Chelating agents and toxins

Chelating agents such as ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA) bind divalent metals at cell-matrix adhesions, which cause the dissociation of the cell from the ECM [9]. These compounds are often combined with other agents due to their low efficacy in cell removal. Many decellularization protocols use EDTA with trypsin or chemical detergents [8,17]. K. Gupta et al. used 0.25 % trypsin containing 0.02 % EDTA for the decellularization of goat lungs. Following the treatment with trypsin-EDTA, the tissue was exposed to 0.1 % SDS in PBS for 6–8h and then to RNase (20 μg/ml) and DNase (0.2 mg/ml) for 24 h. The ECM matrix retained collagen, elastin, and glycoproteins [93]. Nevertheless, some studies have reported that chelating agents can disrupt protein-protein interactions and denature proteins [38]. Latrunculin B is a toxin that can be used for the decellularization of skeletal muscle since it disrupts the actin cytoskeleton [9]. This approach successfully eliminates cellular DNA while preserving GAGs [17]. In comparison to detergents and enzymatic methods, the use of this toxin in combination with DNase treatment and hypertonic/hypotonic solutions has resulted in greater decellularization of the skeletal muscle in terms of DNA removal [8].

2.3. Enzymatic methods

Enzymes can be useful as decellularization agents due to their high specificity for the biological substrate, targeting and removing unwanted cellular and genetic remnants [9,62]. Some enzymes act by disrupting the cell membrane and breaking bonds within the cells and between the cells and the extracellular matrix [54,55]. Proteases (e.g. trypsin, collagenase, dispases), nucleases (e.g. DNase, RNase), and esterases (phospholipase A2, lipase) are examples of enzymatic agents that usually supplement other chemical and physical treatments (Table 3) [9,17,38]. Enzymatic methods present some disadvantages, namely the detrimental effects on the ECM structure and the possibility of enzymatic residuals causing an adverse immune response [17,38].

2.3.1. Nucleases

Nucleases such as DNase and RNase catalyze the hydrolysis of phosphodiester bonds of nucleotides, cleaving deoxyribonucleotide and ribonucleotide chains, respectively [38]. Nucleases are commonly used alongside detergents to destroy residual DNA/RNA fragments in the scaffold and to remove detergent leftovers [9,38]. Nevertheless, these enzymes can alter the ECM structure and affect mechanical properties, as well as reduce GAGs, collagen, and laminin [9]. Additionally, nucleases are difficult to wash out from the scaffold and enzymatic remnants can trigger an adverse immune response, impeding recellularization and transplantation [9,38].

2.3.2. Proteases

Trypsin is a serine protease that specifically cleaves peptide bonds on the carbon side of lysine and arginine, disrupting cell-matrix adhesions [17,38,54]. Trypsin is frequently used as a decellularization agent since it can effectively remove cellular and nuclear components and can facilitate the integration of further decellularization agents [17]. Trypsin removal of ECM components is dependent on time, and longer incubation times are often required for complete decellularization. However, the tissue's exposure time to this enzyme should be minimized since it disrupts elastin and specifically targets collagen, compromising the mechanical properties of the scaffold [17,88]. The concentration of trypsin used should also be taken into account [38]. For the decellularization of porcine trachea, Giraldo-Gomez et al. added 1 % trypsin (3 h at 4 °C) to 4 % SD (4 h, RT) and DNase I (3 h, RT) in a five-cycle protocol [97]. Results showed complete removal of cellular components and preservation of mechanical properties. It was demonstrated that trypsin promoted fast decellularization compared to the protocols in which trypsin was not added, but the action of this enzyme may have resulted in the removal of chondrocytes from the tracheal cartilage [97]. Dispase is a bacterial protease that has been used in the decellularization of thicker tissues such as skin and cornea [8,9,17]. This enzyme cleaves fibronectin and collagen IV in the basement membrane, is useful for isolating epithelial sheets and can prevent cell aggregation [9,83]. Since dispase cannot remove cells within the tissues, it is often used in combination with physical methods, such as mechanical abrasion [83]. A study reported that, when compared to trypsin, dispase showed a greater level of cellular removal. However, it caused substantial damage to the ECM structure [84]. Collagenases cleave peptide bonds within collagen, and can be used for decellularization at low concentrations or when the

Table 3
Summary of commonly applied enzymatic methods for decellularization, usually applied in combined strategies, including the principle of cellular removal, main advantages and disadvantages.

Biological Method		Principle	Advantages	Disadvantages	Ref
Nucleases	DNase/RNase	Catalyze the hydrolysis of phosphodiester bonds of nucleotides	Highly effective in removing genetic remnants	Can alter ECM structure, affect mechanical properties and reduce GAGs, collagen and laminin; Difficult to wash out from the scaffold and can trigger an immune response after transplantation	[9,38]
Proteases	Trypsin	Disruption of cell-matrix adhesions	Effective in removing cellular content, specifically in thicker tissues/organs with multiple layers	Long exposure times can disrupt elastin and collagen, affecting mechanical features; Most be combined with other decellularization agents	[17, 38,64, 83]
	Dispase	Cleaves collagen IV and fibronectin in the basement membrane	Effective in decellularizing thicker tissues and isolating epithelial sheets	Not effective in deeper areas, must be combined with other methods; Can cause substantial damage to ECM structure	[8,9, 17,83]
Esterases	Phospholipase A2	Hydrolyzes phospholipidic components within the cell membrane	Preservation of collagen and proteoglycans	Usually combined with other decellularization agents; Can reduce the levels of GAGs	[8,9, 38]
	Lipase	Catalyze the hydrolysis of ester bonds in triglycerides	Effective for tissue delipidation and dermal tissues	Only useful in combination with other decellularization agents [8,9,38]	[17, 38,83]

preservation of collagen and ECM structure is not required for the intended application [17,55,88].

2.3.3. Esterases

Phospholipase A2 is an esterase that hydrolyzes phospholipidic components within the cellular membrane [38]. Since this enzyme does not affect collagen and proteoglycans, the ECM structure is mostly preserved, but it can reduce the levels of GAGs in the scaffold [8,9]. Phospholipase A2 is usually combined with detergent treatments, and Wu et al. used 200 U/ml of phospholipase A2 and 0.5 % of SD to decellularize porcine corneas. The results showed adequate decellularization and preservation of the ECM ultrastructure and mechanical properties [85,98]. Lipase is another esterase that catalyzes the hydrolysis of ester bonds in triglycerides and can be used to aid in tissue delipidation [17,38]. Lipase is only useful when used in combination with other solvents, such as alcohols, and has been applied for the decellularization of dermal tissues [17,83,88].

2.4. Decellularization strategies – a classification based on tissue origin

The dECM scaffolds can be categorized into three primary groups based on the source of the ECM: organ-derived ECM, tissue-derived ECM, and cell culture-derived ECM [38] (Fig. 4). Scaffolds derived from organs and tissues have the same composition, ultrastructure, biological activity, and microenvironment as the native tissue/organ compared to cell culture-derived ECM [38,86]. Biological factors and cues stored within the ECM are tissue-specific and can promote cell survival and differentiation. A study has reported that factors bond to the dECM could promote specification of uncommitted fetal stage precursor cells in a tissue-specific manner [99]. Another study has described that dECM from lung matrices promoted lineage commitment of human lung progenitor cells [96]. The preservation of the composition, biological ratios, and organization of the collagen fibers, as well as mechanical properties, regulate several processes, including proliferation, cellular adhesion and migration, and differentiation [38,86]. Additionally, these scaffolds preserve the original vasculature of the native tissue [40]. However, the organ/tissue-derived scaffolds present some disadvantages. First, the limited reproducibility due to variability between the scaffolds from different donors, meaning that the specific properties of the ECM, such as stiffness, degradability, and porosity, are difficult to modify and customize to a particular application or be patient-specific [38,86]. The use of this approach is also dependent on the availability of biological material. Autologous tissue faces the issue of limited accessibility and surgical complications. On the other hand, allogeneic and xenogeneic tissue can cause donor site morbidity, immunological rejection, and disease transmission [38,94]. Furthermore, some tissues, such as stem cell niches, are difficult to obtain since they cannot be isolated [94]. Organ/tissue-derived dECM are commonly used to create ECM models for research, to generate artificial organs and tissues, and in many clinical applications, such as breast reconstruction and wound dressing [40]. Cell-derived scaffolds are produced by cells in 2D or 3D cultures that secrete ECM proteins. These ECM scaffolds are relatively easy to achieve and the characteristics and properties can be customized and modified [40,100]. Additionally, they present minimal batch-to-batch variations, and the risk of disease transmission and immunological rejection is very low [38,94]. However, these scaffolds are difficult to produce at a large scale and have fewer similarities with the native tissue in terms of composition, structure, and mechanical features [40]. Cell-derived scaffolds have been used as cell culture substrates and as models for basic research and drug testing. Several cellular types, including stem cells and cancer cells, have been employed in cell culture and have demonstrated specific and unique cellular functions [40]. The generation of cell culture-derived scaffolds will be discussed in more detail later in this review.

2.4.1. Whole organ-derived ECM

Whole-organ decellularization consists of producing an acellular 3D scaffold from a complete organ. Whole organ-derived ECM holds significant potential in tissue engineering and regenerative medicine due to its preserved native architecture, biochemical composition, and bioactive properties [111]. Furthermore, whole organ-derived ECM is being explored for the development of scaffolds in whole-organ bioengineering, where it acts as a decellularized matrix for recellularization with autologous or allogeneic cells, offering promising prospects for functional organ transplantation [9,111,112]. Additionally, whole organ-derived ECM can be processed into hydrogels, bio-inks, or electrospun fibers, which are widely used in 3D bioprinting, in vitro cell culture models, and drug screening platforms [111]. These materials provide versatile platforms for creating complex tissue structures and conducting high-throughput testing for drug efficacy and toxicity. The ECM's ability to retain essential extracellular proteins also makes it an ideal candidate for disease modeling, enabling researchers to study organ-specific pathophysiology within a more physiologically relevant context [8,38,111]. Moreover, whole organ-derived ECM has potential applications in cancer research, as it can be used to create tumor microenvironment models that mimic the extracellular matrix's role in cancer progression [113]. The most commonly used technique is perfusing a decellularization agent through the native vasculature of the organ [44]. Antegrade or retrograde perfusion allows for a uniform and effective removal of cellular components without disrupting the organ's structure and can be applied to any solid organ, ideally for large organs with an extensive vasculature network [55,100,114]. The application of this method requires specialized and complex bioreactor systems and, eventually, the ECM microstructure can suffer substantial damage if the perfusion pressure and the reagent concentrations are not adequately properly controlled [115]. Immersion and agitation is an alternative method used for the decellularization of thin organs or without a large vasculature system, such as the trachea and dermis [17,115]. A dynamic environment allows for a more homogeneous exposure to detergents and other decellularization agents, and this method is simple to perform in comparison to perfusion techniques [38]. When the preservation of the organ's native structure is not required for the desired purpose, the organ can be sliced to increase the surface area and shorten the decellularization time. As mentioned earlier, agitation can cause some degree of disruption to the ECM structure [115]. The choice of the decellularization agent depends on the characteristics of the organ, including cell density, thickness, size, shape, lipid content, and vasculature, as well as the features of the decellularization agent and the intended application of the dECM scaffold [17,100]. Most protocols use a combination of several agents, including detergents such as SDS, SD, Triton X-100, and CHAPS, nucleases, and trypsin, and many protocols include a freeze-thawing step [100,114]. For whole vital organs, including the liver, lungs, and kidney, perfusion and freeze-thaw are the most frequently used techniques [9]. Osmotic solutions can be employed for the decellularization of composite organs, such as the trachea, uterus, and testes, in combination with enzymatic and other chemical treatments. Amorphous organs with high lipid content, such as the brain and pancreas, have been decellularized using freeze-thaw, acids and bases, and mechanical force and usually require treatment with a solvent/alcohol [9,17,116,117].

2.4.1.1. Heart. For whole-heart decellularization, antegrade or retrograde perfusion on the aorta or coronary vessels with a decellularization agent is a commonly used technique [114]. The decellularizing agents are usually detergents, such as SDS, SDC, Triton X-100, and polyethylene glycol (PEG), and enzymes with chelating agents, such as trypsin-EDTA. The use of hypotonic/hypertonic solutions and acids has also been described [62,114]. Perfusion of 1 % SDS and 1 % Triton X-100 is an effective and frequently used approach [100,118]. A protocol for porcine heart decellularization with retrograde aortic perfusion

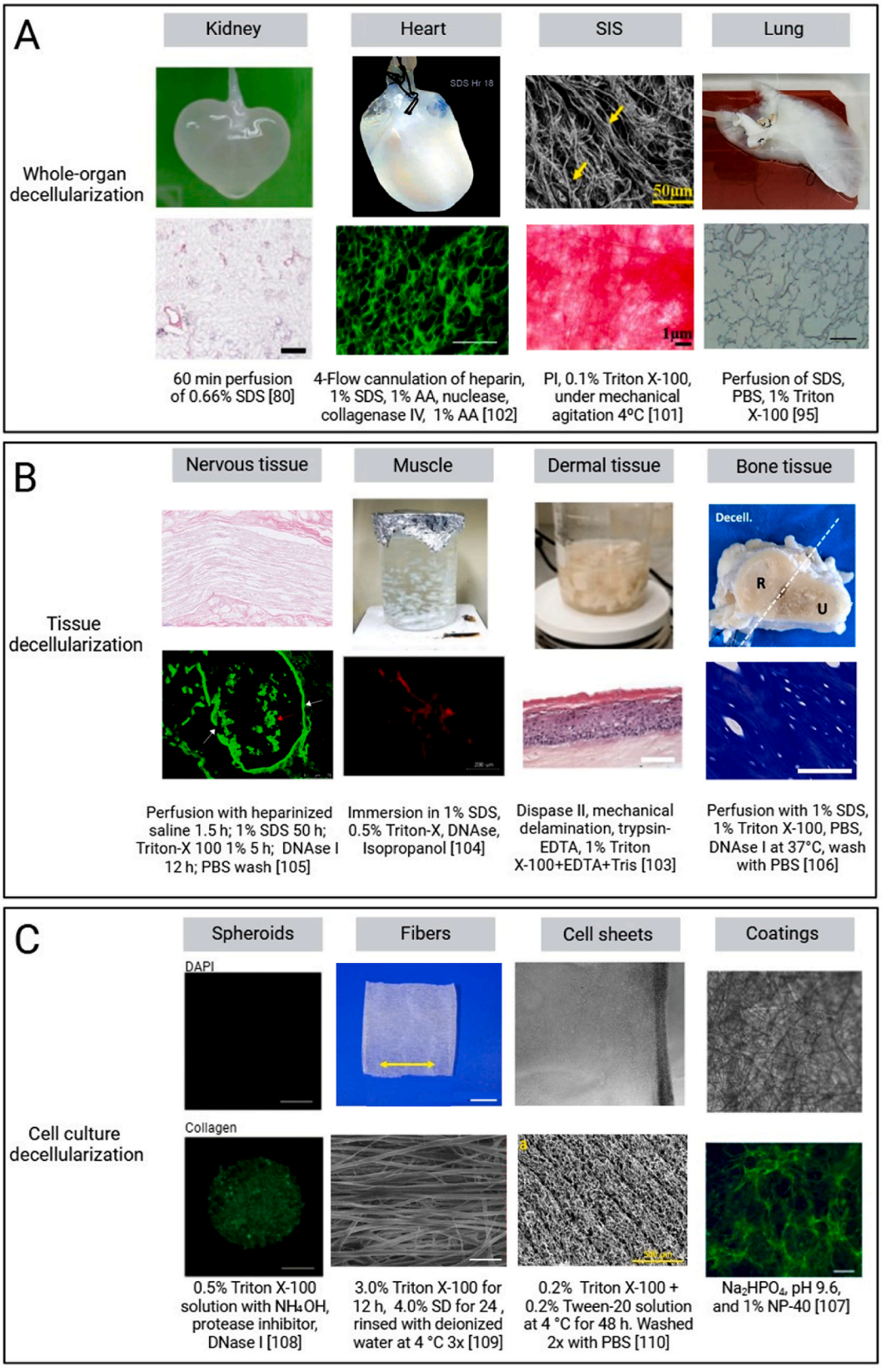

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Fig. 4. – Decellularization strategies. A) Examples of protocols for whole organ decellularization, namely kidney, heart, SIS and lung. Kidney: Representative macroscopic pictures of decellularized rat kidneys and HE-Staining; Heart: Representative image of decellularized rat heart and Collagen I staining of collagen network; SIS: FESEM image of surface morphology and H&E staining of decellularized goat gut; Lung: Decellularized porcine lung by perfusion through the pulmonar artery and staining. Ref. from Refs. [80,95,101,102]. B) Methods for tissue decellularization, such as nervous tissue, muscle, dermal and bone tissue. Nervous tissue: Stainings for H&E, collagen IV/DAPI for decellularized porcine vascularized nerve grafts; Muscle: Decellularization process of porcine skeletal muscle tissue and immunohistochemistry of laminin (red) and nuclei (blue) of dECM; Dermal tissue: Method for decellularization of porcine skin and HE-staining of resulting dECM; Bone tissue: Images of decellularized forelimb and Masson's trichrome staining of decellularized cortical bone. Images reproduced from Refs. [103–106], after copyright permission granted from publishers; C) Decellularization of in vitro small 3D structures, including spheroids, fibers, cell sheets and dECM coatings. Spheroids: DAPI and collagen staining of dECM derived from MSC spheroids; Fibers: Representative images of core-shell electrospun fiber; SEM micrographs of aligned electrospun fibers; Cell sheets: Representative images of ECM sheets post-decellularization and SEM image of SEM of decellularized sheets; Coatings: Bright field image the decellularized synthetic fibers and fluorescence image of staining with anti-fibronectin antibodies (green). Images reproduced from Refs. [107–110], after copyright permission granted from publishers. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of Triton X-100, trypsin-EDTA, sodium azide (NaN_3), and deoxycholic acid showed high preservation of collagen I and elastin, as well as basement membranes. The DNA was almost completely removed and the mechanical features were maintained [119].

2.4.1.2. Lung. In the case of lung decellularization, perfusion is applied through the vasculature and/or airways of the lung, usually within the pulmonary artery or the right ventricle and the trachea, and involves the use of fewer detergents [62,114]. Most protocols for mouse, rat, and porcine lung decellularization apply only one or two detergents, such as SDS, Triton X-100 and SDC, and CHAPS. Additionally, hypertonic solutions such as NaCl and DNase/RNase treatment could be added [8, 100]. Ott et al. used a method to decellularize rat lungs based on antegrade perfusion of 0.1 % SDS through the pulmonary artery. The results showed the generation of whole-lung scaffolds with preserved vasculature, airways, and ECM content, and no cellular or genetic remnants [48].

2.4.1.3. Digestive system. For liver decellularization, perfusion through the portal vein or the inferior vena cava with detergents has been described for many animal models, such as rats, mice, rabbits, and pigs [62,114]. Commonly used combinations of detergents include 1 % SDS with 1 % Triton X-100, different concentrations of Triton X-100 with 0.1 % SDS or 0.1 % ammonium hydroxide, and the combination of 0.25 % and 0.5 % SDS [100,120]. Intestine-derived ECM is one of the most used scaffolds in the field of regenerative medicine. Decellularization of SIS scaffolds involves an initial step of mechanical abrasion of the intestine tissue to separate different layers. Further cell removal is usually performed using agitation and immersion or perfusion with decellularizing agents. SD, SDS and Triton X-100, hypotonic solutions, peracetic acid, and DNase are commonly used substances [8,118].

2.4.1.4. Kidney. Kidney decellularization strategies include perfusion through the renal artery with a series of chemical and enzymatic solutions. Similar to lung decellularization, 1 % SDS with 1 % Triton X-100, and different concentrations of Triton X-100 with 0.1 % ammonium hydroxide have also been described for rat and pig kidney decellularization, respectively [8,62]. SDS in different concentrations (0.5 %, 1 %) with DNase, Triton X-100 with SDS and DNase, and cycles of 0.5M NaCl with 0.5 % SDS were also reported for porcine kidney decellularization. For human samples, SDS (0.5 % or 1 %) with Triton X-100 or DNase or a conjunction of the three can be applied [100,121].

2.4.1.5. Decellularization devices. Several personalized devices and systems have been used to perform whole-organ decellularization, including customized bioreactors, tanks, small chambers and flasks, and beakers on rotary shakers [38,115]. The lack of commercially available devices nowadays does not allow for the production of decellularized ECM scaffolds in large quantities and is limited to research purposes applications. Currently, there are a few commercially available options for decellularization devices in the market, including the Eber tubular chamber, Harvard Apparatus: HPC-3, and Harvard Apparatus: ORCA [115]. There is a need to develop new automated equipment with standardized parameters to enable the production of dECMs scaffolds on an industrial scale and facilitate its clinical application [38,115].

2.4.2. Tissue-derived ECM

Tissue-derived ECM scaffolds are obtained from the decellularization of different types of tissues, including tendons, adipose tissue, heart valves and blood vessels, nerves, and tissue laminates from the urinary bladder, intestine, and dermis [9,62]. Agitation and immersion with chemical agents is the most employed decellularization method for tissue sections, although the methods and reagents choice depends on the tissue's unique features [44]. Mechanical delamination is another method usually applied. For laminates of thin tissues such as the urinary bladder, pericardium, and intestine, mechanical force is commonly used for the removal of layers and the exposure to detergents and acids is minimal in order to avoid damaging the tissue [9,17]. In contrast, thicker and dense tissue layers such as the dermis, require longer exposure times to decellularization agents as well as longer rinsing times [9,116,117].

2.4.2.1. Vascular tissues. Luminal perfusion with decellularization agents is ideal for hollow tissues, such as umbilical veins [44,122], but the use of immersion and agitation has also been reported [123]. Vascular tissue decellularization is based on detergents, such as SDS, SDC, Triton X-100, CHAPS, and EDTA. Additional treatment with trypsin, hypotonic and hypertonic solutions, and nucleases have also been reported [118]. $SsCO_2$ and NTIRE have also been described for the decellularization of blood vessels, heart, skin, and neural tissues [44]. Omid et al. decellularized ovine coronary arteries by immersion on a hypertonic solution (1.2 % NaCl for 2 h), followed by a wash with distilled water (2 h) and incubation with trypsin (0.025 %) for 24 h under mild agitation [123]. A final treatment with Triton X-100 (1 %, 48 h) was applied, followed by washes in distilled water and PBS and sterilization with Pen-Strep (10 %). All the steps were performed at 4 °C, and the authors referred that the low temperature may lower the kinetic rate of the reactions and minimizes structural damage, what was confirmed by similar mechanical strength and swelling ratio as the native tissue [123]. Montoya et al. compared two decellularization protocols for human umbilical veins, one based on rotary agitation and other based on a connective flow within perfusion bioreactors [122]. In both methods, a decellularization solution based on chemical agents (20 % acetone, 20 % water and 60 % ethanol) was used. Overall, it was verified that the veins submitted to connective flow decellularization had greater removal of cellular components, phospholipids and total protein, and showed better preservation of mechanical and structural features of the ECM [122].

2.4.2.2. Adipose tissue. Similar to other amorphous organs, adipose tissue can be decellularized using freeze-thaw and washes in osmotic solutions as an initial lysis step, followed by polar extraction with isopropanol or lipase for lipid extraction, and a detergent or enzymatic treatment. DNases and RNases nucleases are also applied if the scaffold

is intended for in vivo implantation [9,124]. Dunne et al. decellularized human adipose tissue using three freeze-thawing cycles (frozen at −80 °C and thawed at RT), and further treatment with 0.5 M and 1 M NaCl, 0.25 % trypsin/EDTA, 1 % Triton X-100, and isopropanol [125]. Later, the matrices were lyophilized and sterilized with 70 % ethanol. SEM analysis confirmed the removal of cells and generation of a porous fibrous network. Cancer breast cells were later cultured in these scaffolds, resembling the growth profiles observed in xenografts in comparison to two-dimensional cell cultures. These matrices displayed potential for being used as models for cell culture and drug testing [125]. Anderson et al. decellularized human subcutaneous adipose tissue with a protocol that combined physical and chemical approaches [126]. First, the tissue samples were mechanically processed and rinsed. Then, the tissue was incubated with 3 % peracetic acid for 3 h and a final treatment with 1 % Triton X-100 overnight was applied. The resulting scaffold was snap frozen and milled to create an injectable off-the-shelf matrix. The decellularization protocol produced a matrix composed of collagen and other ECM proteins derived from the adipose tissue. In preclinical studies, this material demonstrated biocompatibility and pro-regenerative properties. Additionally, it was well tolerated in early clinical trials, and cellular and immune infiltration was observed in the adipose tissue implants [126].

2.4.2.3. Neural tissue. For neural tissues, most decellularization protocols are based on surfactants in low concentrations, including Triton X-200, SB-10, and SB-16 [91,118]. Additionally, osmotic methods are commonly used as an initial lysis step, and nucleases can be applied to ensure proper DNA removal [118]. Arash et al. decellularized nerve segments for repair of sciatic nerve injury in rats [127]. Bovine nerve tissue was decellularized by immersion in SB-10 (125 mM) for 12 h, followed by SB-16 (0.6 mM) and Triton X-100 (0.14 %) for 12 h, under agitation. Later, the tissue was incubated again in the previous solutions, SB-10 for 7 h and SB-16/Triton X-100 for 12 h. Then, the samples were washed with ddH_2 for 15 min and immersed in a DNase/RNase solution (1 U/mL; 50 U/mL) for 6 h. Further analysis revealed the removal of cells, myelin and axons, with maintenance of structure in the nerve xenografts. After transplantation in rats with sciatic nerve injury, regenerated myelinated axons and Schwann cells were present in the injury site, suggesting that these xenografts can be used for peripheral nerve repair [127]. Kong et al. combined decellularized nerve tissue with a chitosan solution to generate a composite scaffold with antibacterial activity [128]. The sciatic nerves were decellularized by immersion in 4 % Triton X-100 for 6 h, followed by incubation with 4 % SD for 12 h at 4 °C. The scaffold was later sterilized, lyophilized and ground into a powder. The composite scaffold demonstrated high biocompatibility and could promote the proliferation and migration of Schwann cells [128].

2.4.2.4. Skin. The production of dECM from skin tissue involves de-epithelialization, delipidation, and the removal of cellular components [62]. The protocol developed by Sarmin et al. for the decellularization of porcine skin consisted in freeze-drying the skin, followed by dissociation from the epidermis by dispase II (50 U/L, 12–16 h) and mechanical delamination to remove the epidermis from the dermis [103]. Treatment with trypsin (0.25 %/0.1 % EDTA, 1 h, 40 °C) and Triton X-100 (1 %, 6 h, RT) was applied to eliminate the cellular components. It was demonstrated that the resulting scaffold resembled the native skin in terms of collagen fibers and matrissomal proteins, and could be used as in vitro models that replicate the characteristics and functions of native skin [103]. Kamalvand et al. tested several decellularization protocols in fish skin for skin injury repair [129]. The most promising protocol in terms of biocompatibility, cell adhesion and preservation of structural and mechanical features included a combination of physical and chemical approaches. The initial step involved freeze-thawing the fish skin, followed by incubation in a hypertonic solution for 24 h and exposure to 0.25 % Triton X-100 for 24 h. Later, the tissue was incubated in a hypotonic solution for 24 h and freeze-dried. The results demonstrated low-cytotoxicity and removal of genetic fragments [129].

2.4.2.5. Bone tissue. For the decellularization of bone tissues, protocols using physical, chemical, and enzymatic methods have been described [62,130]. Chemical methods are commonly used, such as chelating agents with trypsin, chelating agents with detergents such as Triton X-100 and SDS, or a combination of detergents, along with acids and bases. Physical methods including HHP, freeze-thawing, and $scCO_2$ have been frequently used due to the effective removal of cellular components and preservation of the ECM structure [118,131]. Jeon et al. decellularized a culture layer of osteoblasts using a hypertonic solution (2.0 M KCl) and a detergent (0.2 % Triton X-100). After the decellularization procedure, no nuclei were observed. The ECM was further treated with osteogenic differentiation medium. It was demonstrated that treatment for 4 weeks could promote osteogenic differentiation when re-seeded with osteoblasts. In order to improve the osteogenic activity of decellularized bone matrices, Cui et al. incorporated peptides derived from the epitope of bone morphogenetic protein-2 into hydrogels [132]. These hydrogels were further injected in porous decellularized bone tissue. The decellularization of cancellous bone of pig femur was performed by immersing the tissue in chloroform/methanol (1:1) for 1 h under agitation, and an ultrasonic cell breaker was used to remove the lipidic content. Later, the samples were incubated in a Tris-HCL buffer with 0.5 % dispase enzyme and 1 % Triton X-100 for 3 h. Additionally, an ultrasonic treatment was applied to enhance the removal of cellular debris. The functionalized decellularized scaffold was biocompatible, preserved mechanical properties and promoted the proliferation of bone marrow mesenchymal stem cells. Additionally, in vivo studies demonstrated improved repair of critical radial defects in rabbits [132]. Duarte et al. combined $scCO_2$ with TnBP to decellularized trabecular bone [133]. The first step included six freeze-thawing cycles of freezing in liquid nitrogen for 2 min and thawing at RT. Different decellularization protocols including the two techniques alone and combined were tested. The combination of these methods resulted in a scaffold with increased removal of genetic material and minimal alterations to the ECM structure [133].

2.4.2.6. Cartilage. Cartilage decellularization involves similar methods, usually an initial treatment with freeze–thawing or osmotic solutions, followed by trypsin–EDTA, Triton X-100 or SDS, and nucleases [118]. Zeng et al. decellularized cartilage from pig knee joints to produce decellularized cartilage-derived hydrogels [134]. First, the cartilage tissue was lyophilized for 24 h and milled to form cartilage powder. The powder was later incubated with a solution of Tris-HCL and Triton X-100 (1 %) for 24 h at 4 °C under agitation. Then, the samples were treated with a solution of DNase (50 U/mL) and RNase (20 μg/mL), followed by incubation with trypsin-EDTA for 12 h at 4 °C. Further analysis indicated that collagen was mostly preserved, some DNA remnants were retained and GAGs were partially removed. The injectable hydrogels demonstrated chondrogenic and immunomodulatory capacity in vitro and in vivo [134]. A study from Das et al. compared the efficacy of two chemical decellularization methods for cartilage xenogeneic tissue [135]. The decellularization was performed by incubation with a hypotonic solution (10 mM Tris–HCl, 5 mM EDTA, 1 μM PMSF) for 8 h at 37 °C, followed by a treatment with a hypertonic solution (50 mM Tris–HCl, 1 M NaCl, 10 mM EDTA, 1 μM PMSF) for 8 h at 37 °C. This protocol was repeated three times. The second decellularization method consisted in treatment with 1 % Triton X-100 for 48 h at 37 °C. Further analysis demonstrated that both protocols resulted in removal of most genetic material (85 % and 90 %) and preservation of mechanical and structural features. A three-month study in vivo indicated that the decellularized scaffolds could promote osteochondral regeneration in rabbits' knee joints [135].

2.4.2.7. Skeletal muscle. For skeletal muscle and tendons, gentler methods are usually employed in order to not disrupt the native ultrastructure and modify the ECM content. Milder detergents, weak acids, salt solutions, and a final nuclease treatment are frequently used, mostly in low concentrations and short exposure times [118]. A study from Urciuolo et al. compared three methods for the decellularization of rat lower limbs [136]. All protocols relied on perfusion of decellularization agents with a peristaltic pump (flow rate of 1 ml/min). The first protocol used latrunculin B (50 nM, 2 h, 37 °C), followed by washes in salt solutions and a final treatment with DNase in 1 M NaCl for 2 h. The second method consisted in perfusion with 4 % SD at RT for 4 h, followed by a DNase solution in 1 M NaCl for 2 h, RT. In the last protocol, the limbs were treated with 0.25 % SDS for 72 h. Histological analysis confirmed the absence of cellular nuclei in all decellularized samples, removal of collagen and GAGs and loss of specific sarcolemma proteins, specially in muscle tissue treated with SD. However, all acellular scaffolds could promote cell migration and differentiation in a volumetric muscle loss model and preservation of ECM proteins appears to be correlated with improved myofiber three-dimensional organization and cell homing [136].

The decellularization of tissues is more complex than the decellularization of whole organs, mainly due to the absence of an organized vascular system that allows for the uniform distribution of decellularization reagents [54,88]. The use of combinations of methods can be beneficial for both organs and tissues, as it enables more thorough decellularization at a deeper level. These methods often need to be adapted to the specific characteristics of the organ/tissue, including the presence or absence of vasculature, porosity, multiple tissue layers, density, and morphology [9,54,88].

2.4.3. Cell culture-derived ECM

The first step in creating cell culture-derived matrices is choosing the type of cells to culture. Common sources include fibroblasts, MSCs, chondrocytes, and osteoblasts [73,94]. Fibroblasts have been widely used due to their capacity to generate an ECM highly abundant in collagen. On the other hand, MSCs can be cultured with specific conditions in order to produce ECM that mimics a desired tissue [94,107]. The cell types used can be primary cells harvested from tissues/organs or immortalized cell lines. ECM from primary cells better resemble the native structure and microenvironment of the original tissue, but to obtain a sufficient number of cells, they often need to be cultured and expanded in vitro [94,137,138]. Furthermore, the number of passages and culturing conditions has to be tuned carefully to not significantly alter the original phenotype. In contrast, immortalized cell lines can be generated in large amounts with the same phenotype [94,138]. The disadvantage of using immortalized cells is the significant biochemical differences from the original tissue since these cells are usually derived from cancerous cells [137,139]. The deposition of ECM in vitro is often slow and yields low quantities, leading to the use of strategies to produce larger amounts. Some of these techniques include the introduction of supplements and/or macromolecules and the optimization of culture conditions [94,108]. Macromolecular crowding (MMC) involves culturing cells in a confined space and introducing supplements to enhance interactions between ECM components, simulating in vivo tissue conditions [140]. This technique promotes ECM proteins' supramolecular interactions, structure, folding, and stability, influencing ECM crosslinking and remodeling [108,140]. MMC also influences enzymatic activity, interactions with smaller factors/molecules, and improves the reactions' thermodynamics [94,140]. Crowding agents are concentrated polymer solutions that facilitate protein-protein interactions, including poly(ethylene glycol), Ficoll, dextran, and carrageenan [94,140]. Additionally, other techniques can enhance the production of proteins or specific ECM factors and characteristics. For example, hypoxia can promote the production of collagen and angiogenic factors, while supplementing the culture medium with ascorbate leads to a more robust production of proteins, particularly collagen [94].

There are several strategies to generate cells in culture based on 2D or 3D cultures. For 2D cultures, the cells can grow in an adherent monolayer, within parallel groves to generate an aligned ECM or as a coating on the surface of a scaffold [94,137]. For 3D cultures, the cells could be cultivated within a degradable 3D scaffold, in the format of cellular aggregates, such as spheroids, or as cell sheets. Each strategy has its advantages and drawbacks. Cells in monolayers can be easily removed by decellularization methods, can be generated in specific and customized patterns, as an aligned ECM or as a more intricated network, and can also be integrated with other materials to create hybrid structures [94,138]. The retention of ECM on the surface of scaffolds after decellularization allows for better biocompatibility. 3D cultures better resemble the native structure of the ECM, especially cellular aggregates, and can be produced in more complex formats using degradable or non-degradable scaffolds. Cellular aggregates dispense the use of a scaffold, but individual aggregates result in lower quantities of ECM with decreased thickness when compared to larger constructs based on scaffolds [94,137,139]. In comparison with ECM derived from 2D cultures, ECM structures derived from 3D cultures show better stability and similarity to native cellular niches [94]. Cell culture-derived ECM has the advantage of allowing control over the design, geometry, and intrinsic characteristics of ECM components. Since these characteristics are predetermined, it is possible to choose designs that facilitate the decellularization process, such as structures with interconnected porosity [94,138].

Cell culture-derived ECM is decellularized using similar methods used for tissue and organs, but they are usually gentler methods to avoid damaging the structure or activity of the ECM, especially in the case of 2D cultures. Most protocols use enzymes, detergents, mechanical approaches, or a combination of various methods, depending on the cell culture format and the desired result and application [40,94].

2.4.3.1. Adherent monolayers. For adherent monolayers, Triton X-100 and alkaline solutions, such as ammonium hydroxide, have been used. Further DNase treatment could also be applied to improve the degree of decellularization. In the case of scaffold coatings, freeze–thaw cycles are usually used for the decellularization of the surface cell layer [137]. Goyal et al. used ECM derived from an NIH/3T3 culture as a coating for synthetic fibers [107]. The hybrid biomaterial was decellularized using a lysis buffer consisting of 8 mM Na_2HPO_4, pH 9.6, and 1 % NP-40, a non-ionic surfactant. The material was first incubated at 37 °C for 15 min and incubated a second time with fresh lysis buffer for 90 min. The decellularization protocol did not adversely affect the ECM structure and fiber integrity. The resultant hybrid biomaterial promoted cell adhesion and new ECM assembly, showing better biocompatibility and functionality compared to the material without the ECM coating [107,141].

2.4.3.2. Fibers. ECM fibers can be created using degradable scaffolds, as described by Roberts et al. [142]. NIH/3T3 fibroblasts were cultured in a degradable hollow fiber membrane for three weeks. After the formation of the fibers, they were decellularized using chemical and enzymatic agents. First, the samples were rinsed with 10 mM Tris-HCl with 1 % EDTA and 10 KU/ml aprotinin. Then, the fibers were immersed in 0.1 % SDS for 24 h. In the last step, a treatment with a reaction buffer containing 50 U/mL DNase I and 1U/mL RNase for 3 h was applied. The characterization of the fibers showed the retention of proteins, proteoglycans, and matrix factors. Additionally, the fibers were able to support the cultivation of fibroblasts in vitro and showed no adverse response in the host after transplantation [142].

2.4.3.3. Spheroids. For cellular aggregates, the application of Triton X-100 with DNase and lyophilization has been reported. Chiang et al. successfully decellularized 3D spheroids seeded with 5000 cells using Triton X-100 with preservation of collagen type I and laminin, as well as

the native protein network [108]. Treatment with this detergent also removed 98 % of double-stranded DNA (dsDNA). They tried other approaches using freeze-thaw cycling with DNase I, but results showed that only 73 % of genetic material was extracted. Furthermore, they verified that the decellularization of spheroids with higher cell density (10 000 and 12 000) was less successful, with a higher retention of DNA content (85.7 % and 84.6 %) [108].

2.4.3.4. Cell sheets. Xing et al. tested three methods for the decellularization of fibroblast cell sheets, including 0.5 % SDS, 0.05 % SDS, and freeze-thawing cycles [143]. The SDS solutions were applied after the previous treatment with 1 M NaCl, 10 mM Tris, and 5 mM EDTA. High-concentration SDS removed 90 % of genetic material, but failed to preserve the ECM content and affected its mechanical strength. On the other hand, freeze-thaw cycling only removed 12 % of DNA but did not compromise the ECM structure and mechanical strength. Low-concentration SDS showed mixed results [143].

3. ECM-rich non-decellularized biomaterials: Devitalized materials

Devitalization involves the inactivation of cells within a biological tissue. Therefore, in contrast with decellularization, it does not require the use of detergents for the complete removal of cells and its fragments from the tissue [144]. On the other hand, while the degree of ECM preservation may be greater in devitalization, cellular content retained within the scaffold have been hypothesized to lead to immune adverse reactions and immunological rejection in vivo [10]. Commonly used techniques for devitalization include the use of alcohols, acetone, heat, HCl, freeze-thawing, sonication, pasteurization, radiation, and HHP treatment [145,146]. Another approach is the induction of apoptosis using chemical substances, such as caspase inductors, to inactivate all viable cells without altering the tissue's microenvironment [147]. Scaffolds produced using only physical methods are often considered and referred to as devitalized materials since these methods retain most cellular components [148]. Table 4 summarizes approaches reported in the literature used for tissue devitalization. There is some ambiguity around the term devitalization, and some authors consider it the initial step of a decellularization process. Furthermore, certain devitalization protocols incorporate washing steps to remove cellular remnants, with some even employing DNase or chemical reagents, making the process more akin to a decellularization procedure, and suggesting that some authors may use the terms interchangeably [149]. Here, we will address devitalization as a process in which cellular remnants are deliberately kept in the structure of the final biomaterial.

Fig. 5 synthesizes the devitalization workflow, including sterilization methods, assessment of the scaffolds' composition and general properties, and preservation mehtods. These processes will be further discussed in section 4. The main techniques currently employed to devitalize biological scaffolds and their main advantages and disavantages will be discussed in this section.

3.1. Induction of apoptosis

The induction of apoptosis with chemical substances or genetic modifications is a valuable approach for the inactivation of viable cells with high preservation of the ECM structure and composition [16,38,150]. Cytotoxic drugs include camptothecin and rotenone, which induce apoptosis through the inhibition of DNA topoisomerase I and the induction of oxidative stress, respectively [16,38]. Apoptosis occurs during tissue and organ development and is associated with tissue regeneration and remodeling by promoting the proliferation of progenitor cells and the release of signaling molecules [16]. Pigeot et al. developed a devitalization technique for hypertrophic cartilage based on cells retrovirally transduced with an apoptotic system that induces the dimerization of caspase 9 [147]. The tissues were rinsed once with PBS prior to live/dead viability staining and/or implantation in vivo to remove cellular residues. The results showed a high percentage of dead cells (95 %) with preservation of the osteoinductive capacity of the ECM. The human-derived cartilage was implanted in immunocompetent rabbits and the results showed formation of new bone and mineralization. No adverse immunological reaction was observed, and the authors speculated that the formation of apoptotic bodies, instead of cellular debris released upon necrosis, may have decreased the immunological response [147]. While most decellularization methods are associated with the development of a necrotic response and the release of immunological cytokines and factors that result in a pro-inflammatory response, techniques that induce apoptosis are associated with the

Table 4
Summary of techniques for devitalization, including the principle of cell inactivation, main advantages and disadvantages.

Devitalization Method		Principle	Advantages	Disadvantages	Ref
Induction of apoptosis		Induction of apoptosis with chemical substances or genetic modifications	Effective cell removal without the release of immunogenic components; Specific degradation of genetic residues; High preservation ECM composition and structure	Complex process that requires precise control	[16,38,146,150,151]
Physical	Freeze-thawing	Rapid temperature oscillations form intracellular crystals and induce cell lysis	Preservation of ECM proteins and factors	Leakage of cellular content through the compromised membrane, including immunogenic debris; Limited performance after transplantation	[16,146,151]
	High hydrostatic pressure (HHP)	Direct application of high pressures homogeneously through the tissue, resulting in the inactivation of cells	Homogeneous devitalization of cells in composite tissues; Preservation of ECM biochemical and structural features	Elevated pressure can result in necrotic response, denaturation of proteins and formation of baric ice crystals	[33,67,145,152]
Thermal treatment		High temperatures cause the thermic inactivation of cells	Inexpensive and easy to perform; Heat treatment bellow 80 °C can preserve biological activity	High temperatures (above 80 °C) can cause protein denaturation and loss of biological activity	[118,153,155–159]
Dehydration	Organic solvents	Dehydration and cell lysis	Easy to perform; preservation of original structure of the scaffold	Can cause protein denaturation and precipitation, affecting ECM composition and structural and mechanical properties	[8,9,20,38]
	Air-drying	Air-drying caused dehydration and inactivation of cells	Simple and inexpensive method; Maintenance of biomechanical feature;	Not widely used; limited tissue sources explored	[19]
	Lyophilization	Removal of all water from cells inactivated cells	Simple to perform; Can create porous scaffolds	Simple to perform; Can create porous scaffolds	[33,161,163]

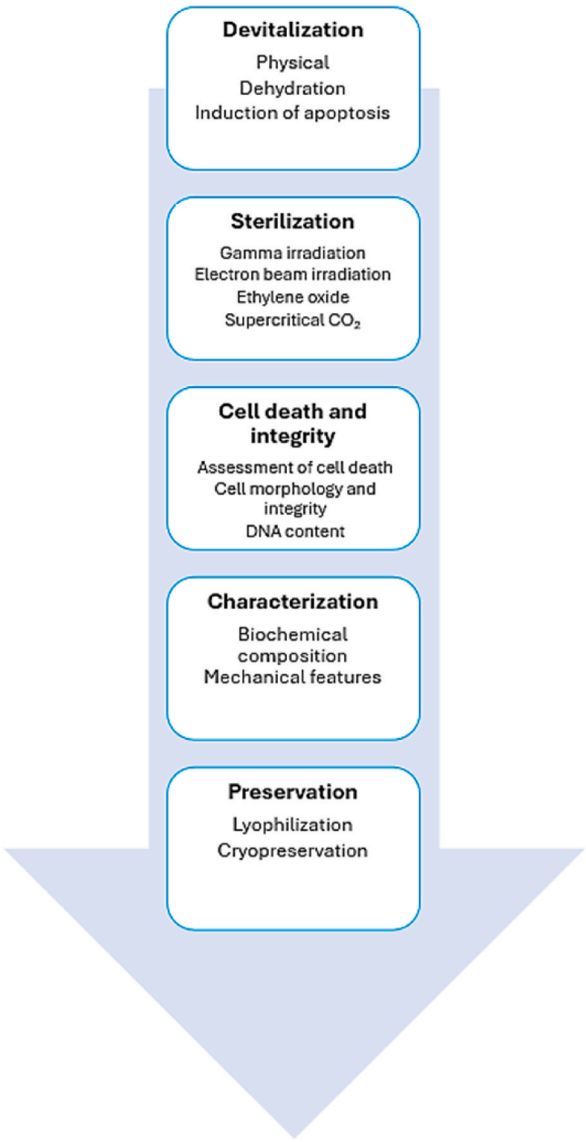

Fig. 5. – Devitalization workflow, including the main devitalization methods and posterior assessment and characterization.

preservation of most cellular remnants within apoptotic vesicles, a decreased release of immunogenic organelles and components and the development of a pro-regenerative response [151]. In this system, the DNA is specifically degraded, which prevents its release to the intercellular space. The dsDNA breaks in 180 base pair length fragments, which is consistent with the threshold defined for ECM scaffolds (DNA fragment length inferior to 200 base pairs) [16,17].

3.2. Physical methods

3.2.1. Freeze-thawing

Freeze-thawing is considered an initial step of decellularization, but some protocols described the use of this technique to devitalize biological constructs. Freeze-thawing is a classic devitalization method associated with disruption of cell membrane and leakage of cellular content, and induces cell death primarily by necrosis [16,151]. It has been reported that cell inactivation by the induction of apoptosis is preferred to avoid the development of an immunological response [16,151]. Consistent with this observation, devitalization protocols based on freeze-thawing often demonstrate limited regenerative performance, despite superior preservation of ECM and retention of ECM factors in comparison to decellularization methods [16,146,151]. Garg et al. devitalized autologous minced muscle grafts for muscle loss repair in rats [25]. The protocol used for devitalization included a freezing step with liquid nitrogen, thawing, and heating at 65 °C for 10 min. The authors refer that a temperature of 65 °C is not expected to alter the ECM structure, since the collagen denatures at a temperature range of 70–85 °C; nonetheless, effects on other proteins may be observed. The devitalized grafts were analyzed and results showed lysed cellular debris and no cell proliferation. The ECM proteins, namely collagen and laminin, were maintained, and the ECM structure was mainly preserved. However, when implanted in vivo, the grafts showed limited regenerative capacity, resulting in a fibrotic mass [25]. Beck et al. compared the chondroinduction capacity of devitalized porcine cartilage with decellularized porcine cartilage encapsulated in hydrogels [35]. The devitalization was performed with freeze-thawing cycles, while the decellularization was achieved through osmotic solutions, detergents, and enzymes. Both matrixes were freezer-milled to form a fine powder and lyophilized. Results showed that the devitalized cartilage had superior rheological properties and was superior at promoting chondrogenesis in rat bone marrow mesenchymal stem cells (BMSC). The authors stated that the architecture and biological activity of the ECM was superior in devitalized cartilage, and would be preferred over decellularized cartilage in certain applications [35].

3.2.2. High hydrostatic pressure

HHP treatment has been recently described for devitalization and has been shown to preserve the biochemical features of the native ECM. HHP can either trigger an apoptotic or necrotic response, depending on the pressure levels applied [67]. A necrotic response is associated with a proinflammatory environment that could lead to immunogenic reactions. Waletzko et al. studied the effect of different pressure ranges on the initiation of apoptotic and necrotic pathways in human osteoblasts and chondrocytes [152]. It was demonstrated that a pressure range of 250–300 MPa leads to metabolic inactivation of cells with both apoptotic and necrotic responses [33,152]. Another study has also described that pressures of 200 MPa resulted in an apoptotic response in mammalian cells, while pressures higher than 300 MPa triggered mainly necrotic pathways [67]. This technique is cost-effective, dispenses the use of toxic and aggressive chemicals, and can homogenously devitalize cells even in composite tissues [145].

3.3. Thermal treatment

Autoclavation has been used to inactivate autologous grafts. This method maintains biological compatibility, provides an exact defect-filling graft, and avoids the complications associated with the use of scaffolds from allogeneic sources. However, several studies have reported that the high temperatures used in the autoclavation process (above 100 °C) can denature proteins and result in a loss of biological activity [153]. Asmara et al. demonstrated that autoclave treatment (134 °C) for 15 min resulted in loss of osteoblast number, reduced levels of BMP-2, and lower osteoinductive properties in bone autografts from mice [154]. On the other hand, it was demonstrated that heat treatment below 80 °C can preserve biological activity. Sugiura et al. evaluated the long-term effects of moderate heat treatment (70 °C) for 15 min in autologous bone grafts. Results showed no severe complications, gradual remodeling, new bone formation, and adequate functionality [154]. Other studies have demonstrated that pasteurized grafts can have high complication rates [155]. Sugiura et al. evaluated the long-term effects of pasteurization (70 °C for 15 min) on autografts. Inlay grafts showed fewer complications when compared with intercalary and composite grafts, which led to major complications. The study also demonstrated that the combination of these grafts with vascularized fibular grafts could reduce major complications and have more beneficial outcomes [156]. Furthermore, pasteurization has the advantages of being relatively inexpensive, non-invasive, and simple to perform [157,

158]. Additionally, cell devitalization by low temperatures has also been described [138]. Bourgine et al. referred that, in contrast to freeze-thawing methods, small temperature variations, ideally between 10 and 45 °C, result in cell apoptosis instead of cell necrosis [118,158,159].

3.4. Dehydration

3.4.1. Organic solvents

Cell devitalization by dehydration can be induced by solvents and alcohols, air-drying the tissue sample, or by lyophilization. Alcohols are mostly used as fixating agents by dehydrating cells and causing cell lysis [8,38]. Established protocols to devitalize cells for live/dead assays use 70 % ethanol [145]. Alcohols, such as ethanol, methanol, and acetone, have been widely used since are considered safe when completely removed, effective, inexpensive, and easy to perform. Zhang et al. devitalized bone cancerous tissue with 99 % ethanol for 30 min in vitro, which was further replanted in situ [24]. Results demonstrated that clinical outcomes were similar in comparison with patients with prostheses. Furthermore, Sousa et al. used methanol for 24 h to devitalize human adipose-derived stem cells spheroids [20]. Results showed that all cells within the constructs were not viable and the main structure of the spheroid was preserved. Additionally, it was demonstrated that the fixed aggregates had an anti-inflammatory effect on macrophages and could decrease the levels of inflammation and promote the expression of tissue reparative markers in mouse skin wounds [20].

3.4.2. Air-drying

Air-drying is a simple and inexpensive method that could be used to devitalize tissue samples or ECM assembled in vitro [19]. The protocol described by Magnan et al. for the devitalization of cell-assembled ECM consisted of rinsing the tissue in distilled water and drying at room temperature in sterile conditions, which was further stored at −80 °C [19]. The authors considered that devitalization was preferred then decellularization since the tissue originated from allogenic fibroblasts that have low immunogenicity. The scaffolds were further rehydrated in water, and it was verified that the devitalization process did not significantly affect the tissue's thickness and strength [19]. In a clinical study, the implantation of these allogenic grafts did not trigger an adverse immune response [151]. Similar outcomes were observed in commercial products based on allogenic fibroblasts [160–162].

3.4.3. Lyophilization

Lyophilization is mostly used as a long-term preservation method, but some studies have described the use of this technique to create porous devitalized scaffolds. Cheng et al. described a protocol to devitalize porcine cartilage and create spongeous scaffolds [163]. The tissue was first homogenized, then centrifugated, and resuspended in distilled water. Later, the samples were frozen at −80 °C and lyophilized for 24 h. The resulting porous scaffold was capable of inducing chondrogenic differentiation of ASCs seeded within the scaffold [163]. Longoni et al. used a mild devitalization procedure that includes a lyophilization step to devitalize soft callus-mimetic cartilaginous spheroids for endochondral bone regeneration [33]. The authors have previously demonstrated that vital allogenic cartilage did not trigger an adverse immune response, and it was confirmed that the devitalized spheroids could promote the formation of new bone without evoking a detrimental response in an immunocompetent rat model, although it was verified the presence of residual DNA [33]. Cartilage is considered to be immune-privileged since it is avascular and the cartilage ECM prevents the contact between chondrocytes and immune cells [161]. Allogeneic cartilage tissue has been used in clinical practices without major detrimental immune reactions, and an allograft with living chondrocytes (DeNovo Natural Tissue) has shown promising results in clinical studies [164–166].

The choice between devitalized or decellularized depends on the intended application and the desired format and properties of the final scaffold. Decellularization is preferred when the priority is to avoid the development of an adverse immunological reaction, while devitalization is ideal for tissues from autologous sources or low immunogenic sources [19,35,146]. A compilation of the most used devitalization methods and respective examples are represented in Fig. 6.

4. Considerations on general post-processing steps for abiotic cell-derived materials

4.1. Sterilization and preservation

Devitalized and dECM scaffolds need to be efficiently sterilized and disinfected before their application or implantation in vitro and/or in vivo [8]. Sterilization removes all organisms, including bacteria and viruses, and possibly toxic residues [17,38]. In contrast with sterilization, disinfection cannot remove bacterial spores [8]. The sterilization process should remove all microorganisms without altering the chemical, physical, and mechanical properties of the scaffold and not induce toxicity. However, most methods cause some degree of modification to the ECM, such as collagen cross-linking [38]. Besides the deleterious effects on the scaffold, the choice of method also depends on the characteristics of the scaffold, the intended application, and the time interval until use [8]. Irradiation is a physical technique that includes gamma irradiation, Ultraviolet (UV) irradiation, and electron beam irradiation. Irradiation strongly penetrates microorganisms and destroys their nucleic acids, proteins, and enzymes. Gamma irradiation has been used to sterilize scaffolds from different tissues and has been tested in several doses [8,9]. Even low doses of gamma radiation cause structural disruption, since it leads to the denaturation of collagens and other ECM proteins. Higher doses can alter the mechanical properties, promote enzymatic degradation of the ECM, and affect biological compatibility [9,38]. UV radiation is ideally used for the disinfection of thin scaffolds and surfaces. Large tissues and organs cannot be completely disinfected with this method [17]. Ethylene oxide is an alkylating agent that disrupts DNA and proteins and inactivates macromolecules, affecting cellular metabolism [38,55]. However, it can significantly affect the mechanical features and leave behind toxic residues that can cause adverse immune responses in the host after implantation [9,62]. Peracetic acid is used for disinfection since it can destroy the enzymes and cell walls of bacteria and do not produce any toxic residues. Nevertheless, the oxidation of PAA can cause ECM crosslinking and alter the ECM properties [62]. Alcohols have been used as disinfectants for many tissues since they can disrupt the microorganism's proteins without causing significant damage to the scaffold's structure. However, similar to other disinfectants, they do not remove bacterial spores [8,168]. Antibiotics can also be applied to destroy bacterial components without affecting the structure of the ECM scaffold, but do not affect viruses and spores [62]. As mentioned earlier in this review, $scCO_2$ can have a sterilizing effect. Recently, it has been reported that it can destroy bacterial and viral components, produces minimal alterations to the structure and mechanical properties, and has reduced toxicity [17,168].

Since devitalized and dECM scaffolds have short availability, preservation and long-term storage are necessary for the clinical translation and commercialization as on-the-shelf products [43,55]. Cryopreservation at low temperatures involves the use of DMSO as a protective agent and could be performed with snap freezing in liquid nitrogen or with slow-rate freezing [55]. Lyophilization is another preservation method commonly applied since it removes the water from the scaffolds and increases its stability [43]. Compared to cryopreservation, lyophilization does not form large ice crystals and dispenses the use of toxic cryopreservation agents. Alternatively, scaffolds in PBS with antibiotics and antimycotics at − 20 °C or −80 °C could be used for longer-term preservation, and at 4 °C in the same conditions for short-term preservation. However, most methods lead to an alteration and/or degradation of biochemical and mechanical properties with time [55].

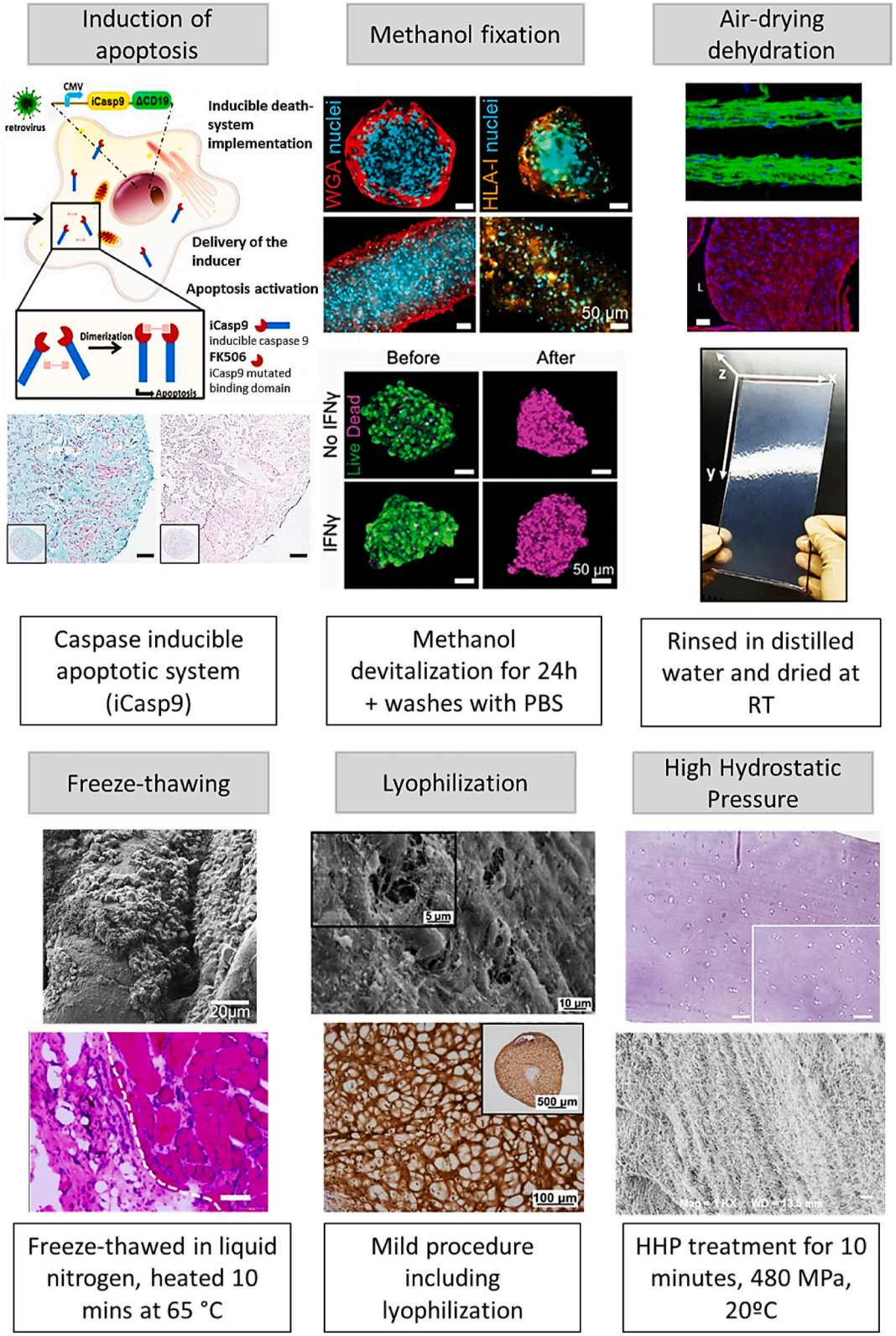

Fig. 6. – Devitalization methods. Induction of apoptosis: Mechanism of devitalizathion by inducible caspase 9 (iCaspase). Safranin-O and Collagen II stainings [150]; Methanol fixation: HLA/DAPI, WGA/DAPI, Live/dead stainings of devitalized spheroids and fibers [20]; Air-drying dehydration: Staning for collagen I, fibronectin. Air-dried living membrane composed of fibroblasts and ECM [19]; Freeze-thawing: SEM images of devitalized minced muscle graft; Hematoxylin and eosin staining of defect area transplanted with devitalized muscle graft [25]; Lyophilization: SEM images of surface porosity of lyophilized spheroids. Collagen type II staining [33]; High hydrostatic pressure: H&E staining of devitalized cartilage. FESEM images of ECM structure of hyaline cartilage [167]. All images are reproduced after copyright permission granted from publishers.

4.2. Compositional and mechanical characterization

4.2.1. Biochemical composition

Following decellularization or devitalization, it is essential to perform a comprehensive characterization of the resulting materials, which includes assessing their biochemical composition, ultrastructure, and mechanical properties. For decellularized matrices, it is particularly important to identify and quantify DNA residues and assess the presence of other fragmented cellular components [8,9,38]. Criteria to verify effective decellularization have focused on quantifying the genetic material, including the number of visible nuclei, the quantification of dsDNA in the scaffolds, and the size of DNA fragments [9]. The focus on the genetic material comes from the fact that DNA remnants are associated with an adverse immune response in the host [17]. To verify the presence of nuclei, histologic and immunofluorescent techniques, such as hematoxylin and eosin and DAPI stains, can be applied, as well as electron microscopy. DNA quantification is performed using dsDNA intercalators such as PicoGreen® and propidium iodide (PI) and, possibly, polymerase chain reaction (PCR). Gel electrophoresis is useful to measure the length of the remaining fragments [9,17,55]. Additionally, it is necessary to evaluate other cellular components, such as membrane-associated molecules and mitochondria, using histological stainings of thin tissue sections, SEM and TEM. Fluorescence and confocal microscopy are useful to visualize specific ECM and cellular components. These techniques are often combined or overlaid with TEM imaging to identify and visualize a higher number of structures and molecules, resulting in superior resolution [169]. The same ultramicroscopy techniques can be employed for devitalized materials, enabling the evaluation of membrane integrity, organelles, vesicles, and scaffold ultrastructure. Furthermore, cell devitalization/death must be evaluated using assays such as live/dead staining, tetrazolium reduction, resazurin reduction, protease activity assays, and ATP assays [170]. The threshold for both cellular and genetic components is difficult to establish since unfavorable remodeling and immune responses vary between tissues, the scaffold's source, and the anatomic location where it is implanted [9,17].

In addition to assessing the genetic fraction and identifying cellular organelles, the biochemical characterization of decellularized materials focuses on identifying and quantifying ECM components through histology, immunohistochemistry, and quantitative assays [8,169]. These macromolecules include ECM constitutive proteins, such as the different types of collagen fibers, elastin, laminin, fibronectin, as well as GAGs. For devitalized materials, besides quantifying protein and GAG content, the lipid component and other cellular fractions may also be assessed depending on the intended application [8,9].

4.2.2. Conservation of mechanical and structural properties

The scaffolds' structure and architecture can be analyzed through microscopic techniques. Transmission electron microscopy (TEM) provides detailed information about the ECM network, such as the orientation of collagen fibers, its diameter and position [8,168]. In regard to devitalized materials, TEM can also provide detailed imaging of cell membranes integrity and cellular organelles [169]. On the other hand, a scanning electron microscope (SEM) is useful to analyze the scaffold's tridimensional structure, surface, relative position and morphology with high resolution [169]. Additionally, polarized light microscopy observation of histological stainings could reveal any structural alterations within the collagen fibers [168]. Furthermore, second harmonic generation (SHG) microscopy allows the visualization of ECM components from several types of samples in unstained and unfixed tissues [171]. For analysis of isolated macromolecules, the components must be separated and homogenized. Negative staining of collagen fibrils with phosphotungstic acid (PTA) is a useful and simple method to evaluated the macromolecular structure. Rotary evaporation is a technique that consists in evaporating platinum-carbon whereas the sample rotates, separating the macromolecules with high contrast [169].

Other important aspects to evaluate in decellularized and devitalized scaffolds are the mechanical properties, including elastic modulus, viscous modulus, tensile strength, and when applicable, anisotropic or isotropic features [68]. Most commonly, abiotic tissues are tested using uniaxial mechanical testing, and parameters including elastic modulus and ultimate tensile/compressive strenght are reported, as summaries in Table 5. Rheological and dynamic mechanical analysis also enable the characterization of the viscoelastic properties of the materials under distinct test modes (e.g. tensile, compression, shear etc.) [8,168]. Atomic force microscopy has also been used to evaluate the mechanical strength of the processed scaffold and compare it with the native tissue [8]. Overall, it is believed that the mechanical features of decellularized materials are mostly defined by the ECM structural proteins, such as collagen, fibronectin, laminin, and elastin [60], and mechanical testing can be used to indirectly evaluate the preservation of the ECM components after decellularization and assure the maintenance of the tissue's function [168,172].

Most studies focusing on the mechanical assessment of abiotic cellular matrices employed uniaxial and biaxial testing, primarily assessing the elastic modulus and ultimate tensile stress (UTS). The mechanical properties seem to be mainly affected by the use of detergents or enzymatic methods but also seem to depend on exposure time, the type of tissue being decellularized, and the species of origin. The most common observed alterations in mechanical properties include a decrease in stiffness and elasticity of the decellularized ECM, often ascribed to the degradation of its protein structure and of key proteins contributing to mechanical strength (e.g., collagen) and elasticity (e.g., elastin). Interestingly, some approaches appear to enhance the preservation of mechanical strength, including the use of zwitterionic detergent CHAPS, PAA, and certain physical methods (freeze-thaw cycles, supercritical CO_2, perfusion). Owing to the complexity and diversity of methods applied for decellularization, the full elucidation of the effects of different decellularization agents on ECM mechanical properties would require a more extensive revision, and would be probably dependent on the existence of data acquired with controlled and comparable settings.

4.2.3. Processing and modifications

After decellularization, the ECM scaffold can be used in the original format or can be further processed and undergo modifications in order to improve functionality and biocompatibility [8] (Fig. 7). Mechanical alterations include solubilization, fragmentation, or turning the dECM into powder [94]. ECM powder or solubilized ECM is used to form hydrogels or bio-inks that can be employed in many technologies, such as electrospinning or 3D bioprinting. In electrospinning, the dECM solution is used to generate fibrous scaffolds with tunable characteristics, such as architecture, porosity, and mechanical features. Moreover, using bioinks, 3D bioprinting enables the production of 3D constructs that resemble the native tissue structure with controllable shape, composition, and pore size [8,44]. Biochemical alterations include the addition of functional groups, drugs, growth factors, or genetic modifications [8,94]. Cross-linking is a method commonly used to combine dECM scaffolds with biological and/or chemical materials to increase ECM stiffness and biocompatibility. Surface modification or coating is also used to improve mechanical and angiogenic properties, cytocompatibility and to repair possible ECM damage caused during the decellularization process [8]. Modification of the matrices with photocrosslinkable domains allows to obtain hydrogels with tunable mechanical properties under the action of light [15]. Modification of the matrices also permits control over the rheological properties and injectability of the materials, that can further be integrated as bioinks in injectable systems. Hybridization of dECM with synthetic materials is another strategy to refine mechanical features, provide a structure more similar to the native tissue, and facilitate implantation and integration [8,62,94].

Devitalized scaffolds generally undergo minimal processing or modifications, being primarily used in their original format, preserving

Table 5
Effect of decellularization protocols on the mechanical properties of typical dECM scaffolds.

Method	Summarized Protocol	Tissue	Measured parameter (before and after (→) decellularization)	Ref
Physical + Biological	HHP (980 MPa; 10 or 30 °C)	Porcine aortic blood vessels	*Elastin region elasticity (10^5 x Pa)* 10 °C: 2.2 → 8.2; 30 °C: 2.2 → 2.4 *Collagen region modulus of elasticity (10^5 x Pa)* 10 °C: 26.7–9.6; 30 °C: 26.7 → 16.7 *Ultimate tensile strength (UTS, 10^5 x Pa)* 10 °C: 8.0 → 3.6; 30 °C: 8.0 → 5.9 *Failure strain (%)* 10 °C: 67.7 → 63.2; 30 °C: 67.7 → 60.5	[69]
Chemical + Biological + Physical	0.1 % SDS 48 h under agitation in $ssCO_2$; DNase I; RNase	Porcine aorta	*Uniaxial ring test stress (kPa)* 32.7 → 21.9	[78]
Physical + Chemical	200 mmHg hydrostatic pressure - 0.5 % SDS + 1 % Triton X-100 for 48 h	Porcine entheses	*Uniaxial test system:* *Maximum force (N):* 31.9 → 51.4 *Young's modulus (N):* 26.5 → 39.8 *Maximum elongation (mm):* 0.26 → 0.20	[89]
Physical + Chemical + Biological	Freeze-thawing; 1 % TBP for 24 h RT under agitation; 0,0025 % DNase RT 4 h; 3 % PAA	Equine tendons	*Stress-relaxation tests:* *Elastic modulus (MPa):* ≈ 270 → 270 *Failure strain (%):* ≈ 16 → 17 *Failure stress (MPa):* ≈ 18 → 15	[82]
Biological + Chemical + Biological	1 % Trypsin 3 h, 4 °C; 4 % SDS 4 h RT; DNase I 3 h RT;	Porcine tracheas	*Uniaxial tension test:* *Young's modulus (MPa): 0.25* → 0.26	[97]
Physical + Biological/ Chemical + Chemical	Freeze-thawing; 0.02 % trypsin 3 h; 3 % Triton X-100 2 h RT + 4 % deoxycholic acid solution 2 h RT; 0.1 % PAA/4 % ethanol;	Porcine hearts	*Biaxial burst strength:* *Average maximum force (N):* 130.77 → 113.99 (left ventricle); 132.49 → 125.00 (right ventricle) *Average extension at maximum force (mm): 30.26* → 26.30 (left ventricle); 34.72 → 26.10 (right ventricle)	[119]
Chemical, Physical	3 protocols: 1 0.5 % SDS 0.5 h h RT 2 0.05 % SDS 0.5 h RT 3 Freeze-thawing (3x)	Human fibroblast cell sheet	*Mechanical strength:* *Elastic modulus (Pa)* 1 ≈ 470 → 100 2 ≈ 470 → 260 3 ≈ 470 → 360 *Viscous modulus (Pa)* 1 ≈ 670 → 240 2 ≈ 670 → 490 3 ≈ 670 → 710	[143]
Chemical + Biological + Chemical	Bicarbonate-mixed salt solution with PLA2 (200 U/ml); 0.5 % SD, 6 h 37 °C; same without SD 2 h 37 °C;	Porcine corneal stroma	*Stress–strain curves: Areal modulus, corneal curvature.* No significant difference in curvature variation and areal modulus between groups	[98]
Chemical	0,2 % Triton X-100 + 0,2 % Tween-20, 48 h 4 °C	Skeletal muscle cells	*Biaxial Test System:* *Stiffness (mN/mm):* ≈ 39 → 22	[173]
Chemical	2 protocols: 1 Hypotonic solution (10 mM Tris–HCl) 8 h 37 °C; hypertonic solution (50 mM Tris–HCl, 1 M NaCl) 8 h 37 °C (3x) 2 1 % Triton X-100 48 h 37 °C.	Goat cartilage	*Elastic modulus (GPa):* 1 *1.47 → 3.05* 2 *1.47 → 2.31* *Hardness (GPa):* 1 *0.06 → 0.08* 2 *0.06 → 0.10* *Stress relaxation indentation (μN s−1)* 1 *2.68 → 4.28* 2 *2.68 → 3.58*	[135]
Physical + Chemical	4 protocols: freeze-thawing (6 cycles) + $ssCO_2$ + TnBP (1 % or 0.1 %) freeze-thawing (6 cycles) + 1 % TnBP freeze-thawing (6 cycles) + $ssCO_2$	Pig trabecular bone	*Uniaxial compression testing:* *Young's modulus (MPa)* *$scCO_2$ 1 h: 47.61 → 66.24* *TnBP 48 h: 47.61 → 57.31* *$scCO_2$-1 % TnBP 3 h: 47.61 → 50.24* *Ultimate Strength (MPa)* *$scCO_2$ 1 h: 4.00 → 7.81* *TnBP 48 h: 4.00 → 7.01* *$scCO_2$-1 % TnBP 3 h: 4.00 → 7.53* *Ɛ at Yield (%)* *$scCO_2$ 1 h: 20* → 25 *TnBP 48 h: 20* → 27 *$scCO_2$-1 % TnBP 3 h: 20* → 24 *Ɛ at Break (%)* *$scCO_2$ 1 h: 68* → 62 *TnBP 48 h: 68* → 68 *$scCO_2$-1 % TnBP 3 h: 68* → 84	[133]
Physical	Mechanical force + $ssCO_2$ (ethanol co-solvent)	Human skin	*Young's Modulus (ratio of native)* 1.0 → 1.0 *Critical strength (ratio of native)* 1.0 → 1.0	[174]

(*continued on next page*)

Table 5 (*continued*)

Method	Summarized Protocol	Tissue	Measured parameter (before and after (→) decellularization)	Ref
Physical + Chemical	Freeze-thawing, hypertonic solution 24 h, 0.25 % Triton X-100 24 h, hypotonic solution 24 h	Fish skin	*Uniaxial tensile tests:* *Tensile stress (MPa)* 32.77 → 21.25 *Young's modulus (MPa)* 284.14 → 192.15	[129]
Physical, Chemical, Enzymatic, Mixed	First step of freeze-thawing (−20 °C/21 °C) 1 0.1 % SDS 2 3 % Triton X-100 3 0.5 % Trypsin 4 2.4 U/mL dispase 5 Liquid nitrogen −196 °C for 4 h 6 Enzymatic procedure, and then chemical procedure 7/8–15 % and 85 % glycerol	Skin tissue	*Uniaxial tensile testing for enzymatic procedure (before sterilization):* *Young's modulus (MPa)* *Dispase: 66.6* *Trypsin: 33.9* *Strain (mm/mm)* *Dispase: 0.20* *Trypsin: 0.16* *Stress (MPa)* *Dispase: 11.2* *Trypsin: 5.6*	[175]
Chemical	4 % $NaClO_4$ (1–4 months)	Bone tissue	*Young's modulus (GPa):* Radius: 7 → 7 Ulna: 6 → 9 *Yield strength (MPa):* Radius: 168 → 113 Ulna: 120 → 124 *Compressive strength (MPa):* Radius: 173 → 116 Ulna: 125 → 129	[176]
Chemical + Physical, Chemical + Physical + Enzymatic	4 protocols: 1 0.05 % SDS, 0.1 % Triton X-100 2 Degrease treatment, Trypsin, 0.05 % SDS 3 1 % SDS, 1 % Triton X-100 4 Hypertonic, 0.1 % Triton X-100 All under mechanical agitation	Caprine goat SIS	*Tensile tests:* *UTS (MPa):* 1 10.10 → 4.03 2 10.10 → 5.12 3 10.10 → 3.68 4 10.10 → 5.90 *E-modulus (MPa)* 1 56.05 → 24.67 2 56.05 → 32.67 3 56.05 → 28.34 4 56.05 → 38.22	[101]
Chemical, Enzymatic	1 % SDS for 5 d, 0,5 % Triton X-100 for 24 h, 50 U/mL DNase for 12 h, 0,1 % PAA for 4 h	Porcine tibialis anterior muscle	*UTS (kpa):* ≈0.2 → 1.9 *Elastic modulus (kPa):* ≈3.6 → 9.5	[104]
Physical + Chemical + Enzymatic	Perfusion decellularization 12 ml/min - 1 % SDS, 1 % Triton X-100, DNase I	Porcine forelimbs	*Hardness values (HV, Vickers unit)* Radius: ≈4 → 10 Ulna: ≈3 → 6 *3-point bending tests (N):* Radius: ≈102.3 N/3.3 mm → 76.2 N/6.1 mm Ulna: ≈137.4 N/5.4 mm → 193.6 N/4.5 mm	[177]
Physical + Chemical	0.5 % SDS, 0.1 % NH_4OH; under agitation RT	Rat calvaria	*3-point bending tests (N):* 33.86 → 28.61	[178]
Chemical	3 protocols; 1 2 h 1.8 mM SDS 2 2 h 8 mM CHAPS 3 3 % Tween-20 2 h, 4 % SDS 2 h, 0.1 % PAA	Human/Porcine lungs	*Uniaxial stress-strain curves: tangential modulus and peak stress.* Tangential modulus and peak stress higher for human than for porcine tissue. CHAPS decellularization - highest tangential modulus and peak stress for human and porcine tissue. Most compliant tissue – 3 step and SDS methods in human and porcine tissue.	[179]

the structure and characteristics of the native tissue. These materials are often applied as grafts for implantation and to promote tissue regeneration. Preservation of the original structure maintains cellular and membrane integrity, preventing leakage of intracellular content. Tissues with low-immunogenicity, such as cartilage, are commonly used in clinical practices and in some commercially available products, both in their native format and processed forms. As referred in previous sections, some studies have reported materials derived from cartilage that have been freeze-milled, lyophilized, and encapsulated in hydrogels [35]; or homogenized, centrifuged, resuspended and lyophilized to form porous scaffolds [163].

4.3. Immunogenicity

Immunogenicity is one of the most crucial parameters to evaluate in the decellularized and devitalized scaffold, as it directly influences the clinical application of these materials and their entrance into the market. Immunogenicity can be measured by performing ELISAs, multiplex and antibody arrays assays, macrophage polarization assays, mixed lymphocyte reaction (MLR), cytotoxicity assays, flow cytometry and quantitative polymerase chain reaction (qPCR) [18,180,181]. Immunogenic responses following host implantation are mainly caused by allogeneic and xenogeneic domains and retained cellular components within the matrix, particularly genetic elements such as DNA and RNA [16,172]. Genetic remnants and other cellular components act as damage-associated molecular patterns (DAMPs) that are recognized by pattern recognition receptors (PPRs) in innate immune cells, and promote the transcription of pro-inflammatory genes. The production of pro-inflammatory cytokines and chemokines activate and polarize macrophages to the M1 phenotype and recruit other immune cells. DAMPs are also associated with an activation of complement cascades and a response from T helper 1 (Th1) cells [16]. ECM damage and/or

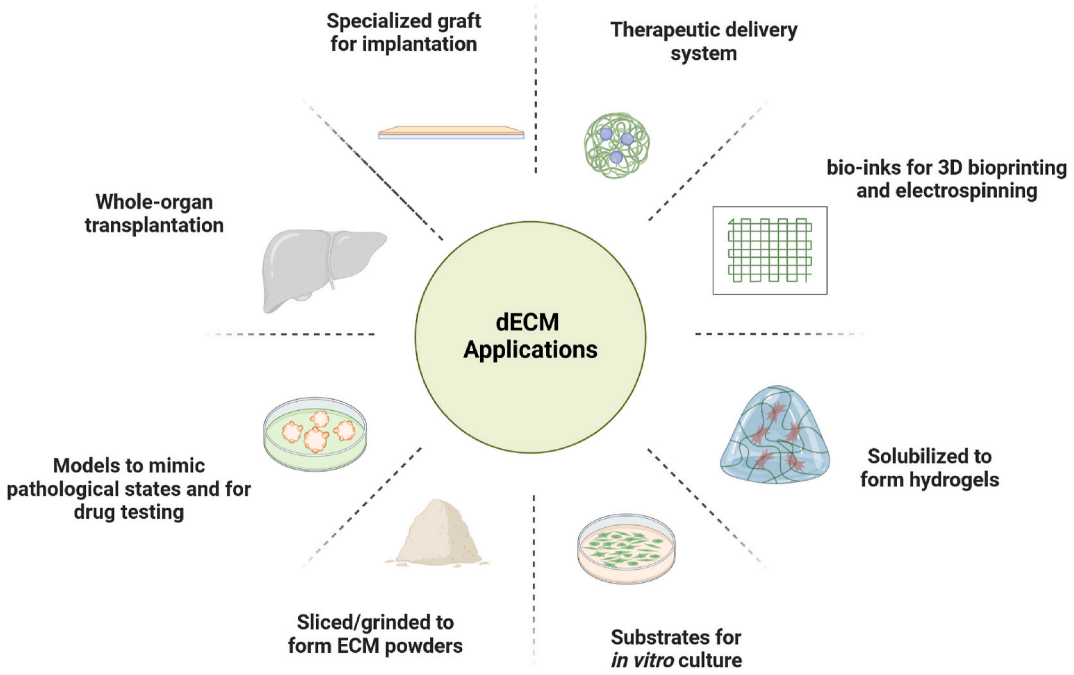

Fig. 7. Decellularized ECM modifications and main applications. Created with Biorender.com.

degradation during the decellularization procedure, such as alterations in protein structure and fragmentation, is also associated with the exposure of new antigen sites and the release of immunogenic small particles that attract and activate immune cells [16]. The α-Gal epitope and major histocompatibility complexes (MHC) are the main triggering antigens responsible for immunorejection in vivo [62]. The α-Gal epitope is absent in humans, but is found in non-primate mammals and can trigger a hyperacute rejection in scaffolds derived from xenogeneic tissues, such as transplants from pigs. MHC class I and MHC class II are located in the cellular membrane and can trigger responses from T cells and natural killer cells in both allografts and xenografts. Besides MHCs, other minor histocompatibility antigens present in these grafts could drive an immunological response against the dECM or devitalized scaffold [16]. Additionally, some ECM components such as collagen IV can also lead to immunogenic reactions [62,172].

Strategies to prevent a host immune rejection include the recellularization of the scaffold with autologous cells or genetically engineered hypoimmunogenic cells, modulatory coatings on the surface of the scaffold, and immune cloaking [8]. Moreover, residual agents from the decellularization and sterilization can be retained within the scaffold and cause adverse immune reactions and cytotoxicity, therefore affecting further recellularization. The quantification of toxic chemical remnants, such as SDS, can be performed using visible-light spectroscopy, gas chromatography, and methylene blue binding assays [8,168].

As previously discussed, the response to devitalized materials varies significantly depending on the method of devitalization and the tissue source. Apoptosis and fixation with organic solvents preserves cellular residues, genetic material, proteins, and immunogenic particles within the cells or apoptotic vesicles [20,150,151]. More aggressive devitalization methods lead to the release of these components, which can activate PRRs and attract immune cells, resulting in unfavorable responses such as inflammation, allo-antibody production, and scaffold/-transplant rejection [16,151]. The immune response to biomaterials is also dependent on the immunogenicity of the donor tissue, the age of the donor, its biocompatibility with the host and the cultivation conditions [10,18]. Taking the example of MSCs, these cells are considered hypoimmunogenic due to low expression of MHC class I and II molecules and due to their capacity to promote immunosuppressive responses [18]. Therefore, these cells are widely used as the cell source for the production of various pro-regenerative materials, and more recently, their immunomodulatory potential has been explored, which will be discussed in more detail in another section [182].

4.4. Recellularization

In order to produce a functional tissue or organ, it's necessary to repopulate the decellularized ECM scaffold [68]. Recellularization with autologous cells is ideal since they do not trigger an immunological reaction and transplant rejection. Stem cells are the most commonly used cell type for recellularization due to their high proliferation capacity and the ability to differentiate into several cell types, with MSCs and hematopoietic stem cells (HSCs) being the most used stem cells [8,38]. MSCs have been widely employed in tissue regenerative medicine due to their tissue healing capacity. Moreover, induced pluripotent stem cells (iPSCs) are genetically reprogrammed cells, which can be derived from the patient's body. Although iPSCs show greater biocompatibility, the generation of these cells is time-consuming and they can possibly originate tumors [8]. The chosen cell type and method for recellularization mainly depend on the characteristics of the dECM scaffold. Cell culture-derived matrices, such as cell sheets, are seeded with just one or two cell types by covering the material with the cell suspension. In the case of more complex structures with three dimensions, such as spheroids or successive cell sheets, a cell suspension is applied between the cell sheets or the constructs [68]. For thin tissues with single layers, injection of the cells is frequently used. For more complex tissues with multiple layers, the cells are injected within each tissue layer to facilitate cell seeding. In the case of thicker tissues and organs, injection of the cells or perfusion through the vasculature are the preferred methods [8,68]. Due to this reason, it is indispensable that the native microvasculature of the organ remains intact. To evaluate its state, micro-computed tomography, angiography, fluoroscopy, fluorescent dyes, and contrast agents can be employed [100,183]. Additionally, the effective recellularization of the vascular lumens with endothelial cells is important to avoid thrombosis and hemorrhages after transplantation to the host [100,114]. Recellularization of a whole organ is assisted by a bioreactor system that simulates its original physiological conditions, including temperature, pH, oxygenation, flow rate, and pressure [44,183]. Ideally, the organ will be colonized by cells, serving as a scaffold for cell

attachment, proliferation, and differentiation [88]. It is expected that the scaffold acquires the organ's basic functionality, and several studies in vitro and in animal models have reported limited function of recellularized dECM scaffolds, such as contraction and electrical conductivity in heart, filtration, secretion and reabsorption in kidney, albumin secretion and urea synthesis in liver and gas exchange in lung [38,68]. However, the clinical application of these scaffolds still faces many challenges [44].

4.5. *In vitro and in vivo applications – focus on commercial products*

Decellularized ECM scaffolds have been employed in several in vitro and in vivo applications with promising results in preclinical and clinical studies. In vitro applications include 3D models that mimic the organ or tissue structure, composition, and mechanical features to recapitulate a certain disease or pathological state, such as tumoral microenvironments, and to serve as a platform to test drugs and to perform drug screening [8,184]. In vivo applications include grafts for tissue regeneration, organ/tissue transplantation, and as a support for cell transplantation. Additionally, dECM scaffolds have been explored to assist therapeutic delivery systems due to their low immunogenicity and biocompatibility, increasing drug stability [184]. Tables 6 and 7 summarize some in vitro and in vivo applications of these matrices. Furthermore, there are some commercially available materials derived from decellularized matrices (Table 8), including grafts and injectable materials from many tissue types.

As previously discussed, devitalized or fixed products are less employed in therapeutic fields, but historically have been mainly used as grafts for defect-filing and tissue reconstruction [25,26], mostly in cases of severe skin wounds, such as ulcers and burns, and for bone [27], cartilage [147] and corneal transplants [30] from autografts, allografts and xenografts [28,29]. Later, it was applied for tooth devitalization in order to remove the infected or damaged dental pulp [21,22], in cancerous tissues to kill malignant cells for posterior re-implantation [23,24] and in the form of immunotherapy in which devitalized cancerous cells and tissues are injected to stimulate an immune response in the host [185]. Currently, there are some commercial devitalized products, including Epifix® and WoundPlus™ Membrane [31,32]. These grafts are derived from dehydrated and devitalized human amniotic membranes and can be used for acute and chronic wounds. Allografts based on human amniotic membranes have low immunogenicity since the epithelial cells do not express HLA-A, B, or C and contain immunoregulatory components [15].

5. Challenges and future perspectives

Decellularized ECM scaffolds can be generated from different sources, including whole-organs, tissues, and cell cultures, and their high versatility allows for their application in several formats, including cell sheets, hydrogels, powders, fibers, and whole-organs with preserved structure and vasculature [55]. Devitalized materials, especially in vitro-engineered scaffolds, may hold great potential by preserving surface markers and cues, and culminating in denser, easier to handle and faster to process materials [20]. The safety of these biomaterials seems to be dependent on the devitalization procedure and the tissue of origin, among other factors intrinsic to the host and implantation [10,20,208].

Table 6
Examples of in vitro applications of dECM matrices in tissue engineering.

In vitro	Source	Species	Structure preservation	Application	Decellularization method	Results	Ref
Humanized rat hearts	Complete hearts	Rat	Yes	Drug testing models	"4-Flow" perfusion 1 % SDS, heparin, 1 % antimycotic and antibiotic, endonuclease, collagenase IV solution	Functional vessels and valves post-decellularization. After recellularization, the humanized heart mimic the vasculature and mechanical compliance of the whole organ	[102]
Human adipose tissue-derived extracellular matrix (hDAM)	Adipose tissue samples	Human	Yes	Model for breast cancer and drug testing	3 freeze-thaw cycles NaCl solution (0.5 M and 1 M), 0.25 % trypsin/EDTA, 1 % Triton X-100, and isopropanol	3D porous nanofibrous networks retained the inherent properties of the native ECM and could mimic the microenvironment of breast tissues	[125]
Pancreatic hydrogel	Non-diabetic pancreata	Human	No	Scaffold or substrate for in vitro cell culture	Freeze-thawing, homogenization and decellularization with deoxycholate. Lyophilization, digestion in pepsin/HCl and neutralization to form a fibrous 3D gel	Maintenance of ECM components, do not show toxicity to the growth and differentiation of several types of cells	[186]
Decellularized kidneys	Kidneys	Porcine	Yes	Model system for Idiopathic calcium oxalate stones	Intact kidney perfused through the arterial cannula with 3 % Triton X-100, DNase, 3 % Triton X-100, 4 % SDS	The tissues showed similarities with the native plaques, presenting mineral spherules, collagen with intrafibrillar mineral and similar morphological features	[187]
dECM bio-ink for integration in 3D liver-on-a-chip	Liver tissue	Porcine	No	Model for drug testing	Samples were sliced, treated with 0.5 % Triton X-100 in 1 M NaCl + 1 % SDS	The dECM bioink promoted stem cell differentiation and increased the function of HepG2 cells. The 3D chip demonstrated superior functionality in comparison to 2D and 3D cultures, and effective drug response	[188, 189]
Hydrogel for 3D in vitro model	Brain tissue	Human	No	Patient specific model for glioblastoma multiforme tumor	Decellularizing solution of 1 % Triton X-100, 0.1 % NH_4OH	The tumor model successfully mimicked the specific ECM microenvironment found in a glioblastoma patient and helped to understand how the ECM microenvironment influenced cell invasion	[190]
Hydrogel with methacryloyl domains	Amniotic membrane	Human	No	Building blocks for bottom-up approaches, cell culture assays	1 % SDS overnight + 1 % Triton X-100 30 min + washes with DPBS + DNase/RNase (in 5 mM Tris-HCl + 10 mM $MgCl_2$) for 3 h at 37 °C	AMMA hydrogels could support 3D stem cell culture for up to 7 days, resulting in proliferative cells	[15]

Table 7
Examples of in vivo applications of dECM matrices in tissue engineering.

In vivo	Source	Species	Structure preservation	Application	Decellularization method	Results	Ref
Decellularized BMSCs sheets	BMSCs	Rabbit	Yes	Cell sheets for osteochondral reconstruction	0.5 % SDS 1 % Triton X-100 DNase enzyme	The application of cell sheets was found to enhance the recruitment of BMSCs and promoted regeneration of osteochondral defects.	[191]
Pancreatic hydrogel	Non-diabetic pancreata	Human	No	Allograf tissue engineering	Freeze-thawing, homogenization and decellularization with deoxycholate. Lyophilization, digestion in pepsin/HCl and neutralization to form a fibrous 3D gel	No adverse immune response in vivo in a humanized mouse model after transplantation	[186]
Whole-liver grafts	Liver	Human	Yes	Transplantable whole-liver grafts	Perfusion through the portal vein and hepatic artery with 4 % Triton X-100 + 1 % ammonium hydroxide, and 0.9 % NaCl + DNAse-I solution + 0.9 % NaCl	Absence of allo-reactivity. The grafts were compatible with cells following reseeding with MSCs or umbilical vein endothelial cells	[192]
Hydrogels derived from adipose tissue extracellular matrix (AdECM)	Adipose tissue samples	Human	No	Hydrogel delivery system	Mechanical processing, alcohol and salt solution. Disinfection with low concentrate peracetic acid	In vitro studies with ASCs showed low cytotoxicity and cell survival within the hydrogel delivery system. Cell culture studies also showed that this system can influence adipocyte formation, influencing adipose tissue formation	[193]
dECM powder-based bio-ink	Liver	Porcine	No	Bio-ink for 3D bioprinting	Livers mechanically chopped into pieces, immersed in a detergent solution of 1 % Triton X-100 and 0.1 % ammonia	Great mechanical properties and 3D printability. In vitro studies demonstrated cytocompatibility with endothelial cells and primary mouse hepatocytes	[194]
Electrospun dECM scaffolds	Skeletal muscle tissue	Rabbit	No	Skeletal muscle reconstruction	Incubated in 0.025 % and 0.05 % EDTA, 1 % Triton X-100 with 1 % antibiotic under agitation. Agitated in 10 mM tris-HCl and 50 mM tris-HCl +1.5 M NaCl	Studies demonstrated that bioactive electrospun dECM scaffolds could influence cell proliferation and differentiation and control the development of myotubes	[195]

Table 8
Commercially available dECM and applications.

Source	Format	Commercial name	Application	Results	Clinical trial iD
Human dermis	Graft implant	AlloDerm SELECT™ RTM	Skin regeneration and breast reconstruction	Some clinical trials have demonstrated that the use of AlloDerm in breast reconstruction can lead to minor and/or major complications, but can provide psychological and aesthetic benefits [196,197].	–
Bovine pericardium	Graft implant	Lyoplant® Onlay	Dura mater repair	Clinical results showed that Lyoplant is safe and efficient for the repair of dura mater defects [198,199]	NCT02678156
Porcine peritoneum	Surgical mesh	Meso BioMatrix®	Breast reconstruction	In clinical data, histologic analysis revealed cellular infiltration and product resorption, but some patients suffered at least one complication [200,201]	NCT01823107
Porcine SIS	Graft implant	OASIS® Wound Matrix	Acute and chronic wounds repair	Clinical trial demonstrated that oasis wound matrix causes reduction in wound size [202]	NCT00570141
Porcine aortic root	Bioprosthesis	Prima™ Plus	Heart valve replacement	Results of a clinical trial showed that the valves enabled high hemodynamics and presented low postoperative morbidity and mortality [203,204]	NCT00377871
Human dermis	Graft implant	Graftjacket Now™	Tendon and ligamentous tissue regeneration	Clinical studies demonstrated complete integration of the patches in tendon tissue. Patients reported improvements in pain, function and other clinical outcomes [205,206]	–
In vitro human BMSCs	Matrix with preserved 3D structure	CELLvo™ Matrix	Substrate for MSCs expansion	Results showed that this matrix enables efficient isolation of stem cells from primary cells and expansion of stem cells without loss of potency [207]	–

In recent years, commercially available dECMs have emerged to support the regeneration of various types of tissues. Nevertheless, regardless of the high potential of decellularized matrices to be applied in tissue regenerative medicine, there are still many drawbacks and challenges that limit its translation and broad application in clinical practices [38,139]. In order to produce scalable and off-the-shelf products, standardized protocols for decellularization with minimized deleterious effects through the ECM and reduced toxicity are in demand; It is necessary to establish widely accepted criteria to assess the removal of cellular and genetical components, degree of matrix preservation, and quantification of toxic remnants [38,68,168]. There is also a need for more efficient and less detrimental sterilization and disinfection methods that could be applied in a systematized manner [8,43]. The tissue source is another cause of variability that needs to be taken into account when producing commercial dECM since it can lead to differences in structure and composition between scaffolds [43]. Another challenge is the possible immune adverse reactions triggered by matrices derived from xenogeneic and allogeneic sources and that affect its clinical translation [10,11]. With more efficient decellularization strategies and a more cautious evaluation of the resulting scaffolds, allied with a better understanding of these immune reactions, these drawbacks can be overcome in the future. The constant progress in this

field and the development of new techniques for the refinement and modification of these materials will soon enable their broad application in regenerative medicine [38,139,168].

As ideas for the future, a promising approach that has not yet been extensively explored is the production of recombinant ECM [209,210]. Currently, most companies only offer the possibility of producing individual recombinant proteins from the ECM in large quantities from cell lines and bacteria. Although it is difficult to replicate the intricate and complex ECM microenvironment and structure, this approach is not dependent on animal or human sources, decreasing the risk of disease/pathogen transmission and immunological rejection. Additionally, these in vitro expression systems have the advantages of being relatively inexpensive and highly reproducible [209,210]. AMSBIO has developed several commercially available recombinant ECM proteins, including an animal free recombinant protein coating (MAPTrix™) genetically incorporated with bioactive peptides that mimic the ECM biological activity [211]. Alternatively, the genetic modification of cells and animals allows for the production of an ECM with the desired properties and functions. A cell line was genetically engineered by retroviral transduction to overexpress vascular endothelial growth factor alpha (VEGF) in order to improve vascularization in the implantation site [210]. Another example is the overexpression of heparan sulfate proteoglycan 2 (HSPG2) with CRISPR/Cas9 to improve the function of neural stem cells [209]. Furthermore, many animal models with gene deletions or mutations have been used to study the processes that control ECM production and organization, and to mimic certain diseases and pathological states [40,210].

Recently, a study demonstrated that extracellular vesicles (EVs) released by cells and retained in the ECM can characterize the extracellular microenvironment, particularly the tumor microenvironment [212]. To this end, surface markers of EVs from the dECM of tumors at different stages were analyzed. Differences were found in epithelial cell and platelet surface markers, as well as in a regulatory T cell activation markers. These markers were predominantly identified in more advanced colorectal tumors, suggesting their potential use as a prognostic method [212]. Indeed, it is well known that EVs mediate interactions between cells and the ECM, playing a crucial role in ECM remodeling by altering both its structure and composition [213]. Therefore, the understanding and characterization of EVs as important fractions of abiotic structural materials derived from tissues may be a promising field enabling the preparation of more effective and targeted biomaterials.

CRediT authorship contribution statement

Diana F. Tavares: Writing – original draft, Methodology, Formal analysis, Data curation. **João F. Mano:** Writing – review & editing, Validation, Conceptualization. **Mariana B. Oliveira:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mariana Oliveira reports financial support was provided by LEO Foundation. Mariana Oliveira reports financial support was provided by Foundation for Science and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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