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Extracellular matrix decellularization approaches for 3D tissue printing

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Abstract: 3D bioprinting holds transformative potential for the field of regenerative medicine, offering unprecedented opportunities for the fabrication of complex, living tissues. Central to this technological innovation is the development of suitable bioinks that can accurately replicate the native cellular environment. Decellularized extracellular matrix (dECM) has emerged as a promising candidate due to its inherent biocompatibility, bioactivity, and structural resemblance to native tissues. Decellularization is a crucial process in tissue engineering that involves the removal of cellular components from the extracellular matrix (ECM) to create scaffolds suitable for tissue regeneration. This article provides a review of some decellularization methods, categorizing them into physical, chemical, and biological approaches. The article discusses the advantages and limitations of each method, highlighting the need to balance effective decellularization with the preservation of the ECM's functional properties. Understanding these methods is critical for developing optimized scaffolds for various tissue engineering applications.

1 Introduction

The field of tissue engineering and regenerative medicine is witnessing a revolution with the advent of 3D bioprinting technology, which allows for the precise construction of complex tissue structures layer by layer. A critical component of this technology is the bioink, a substance composed of living cells and biomaterials that can be printed into tissue-like structures. Among various bioink options, decellularized extracellular matrix (dECM) is emerging as a highly promising candidate due to its unique biochemical and mechanical properties. Derived from natural tissues, dECM retains the intricate composition of proteins, growth factors, and structural molecules, providing an optimal environment for cell growth and differentiation. This article explores the potential of dECM as a bioink in 3D printing, examining its advantages, challenges, and the latest advancements in its application for fabricating functional living tissues. By leveraging the inherent biological cues of dECM, researchers aim to create more physiologically relevant tissue constructs, advancing the frontier of personalized medicine and organ transplantation [1,2]. However, the process of decellularization, which involves removing cellular components from the ECM while preserving its structure and function, is a delicate and complex task. Different decellularization techniques, physical, chemical, and biological, offer unique advantages and challenges that influence the quality and efficacy of the resulting dECM. The choice of method can significantly impact the mechanical properties, biocompatibility, and bioactivity of the dECM, thereby affecting the success of the bioprinted tissue [3,4].

2 Extracellular matrix

There is no tissue in the body that consist of homogenous compositions of its extracellular matrix (ECM), cell phenotype, and mechanical characteristics. Extracellular matrix and its constituents influence the survival, self-renewal, and proliferation of the cells. The mechanical properties and the composition of ECM play critical roles in fate of the stem cell [1]. The ECM comprises a diverse array of macromolecules whose specific composition and structural organization vary among different tissues. The primary components of ECMs include fibrous-forming proteins such as collagens, elastin, fibronectin (FN), laminins, glycoproteins, proteoglycans (PGs), and glycosaminoglycans (GAGs), the latter of which are characterized by their high acidity and hydration levels. In most tissues, the predominant constituents of ECMs are fibril-forming collagen type I, while in cartilage, collagen type II is most prevalent. These collagen types are associated with other collagen forms, ECM proteins, and

PGs, forming extensive fibrillar structures. These multimolecular assemblies are interlinked with ECM molecules, which further interact with one another, thereby constructing the complex three-dimensional matrix network (Figure 1) [2].
Mammalian tissue is comprised of more than 300 ECM proteins and multiple ECM-modifying enzymes, ECM-binding growth factors, and other ECM-associated proteins that mediate structural, mechanical, biophysical, and biochemical cues to cells (Table 1) [3].

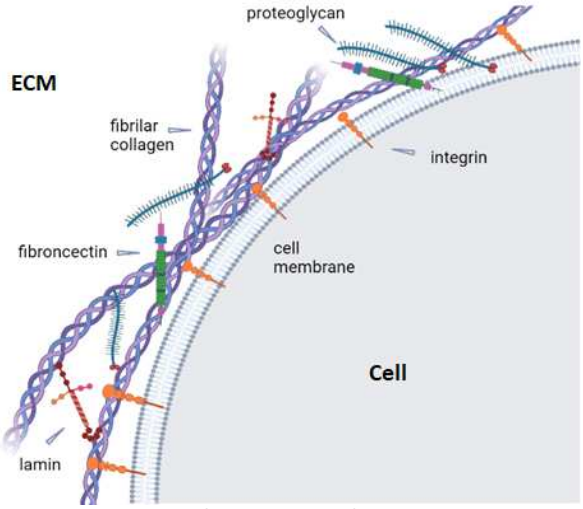

Figure 1 Illustration of composition of extracellular matrix (ECM), showing cellular engagement with biomolecules and primary components of ECM (created with Biorender.com)

The integrins are a family of α, β heterodimeric receptors that mediate dynamic linkages between extracellular adhesion molecules and the intracellular actin cytoskeleton [4]. Proteoglycans are complex glycoconjugates that contain one or more glycosaminoglycan chains, such as chondroitin sulfate, heparan sulfate, or keratan sulfate, covalently bound to a protein. Fibronectin mediates a wide variety of cellular interactions with the extracellular matrix (ECM) and plays important roles in cell adhesion, migration, growth, and differentiation. The lamins are the major architectural proteins of the animal cell nucleus and ensure mechanical stability of the nuclear membrane [5].

One of the significant roles of ECM is the provision of 3D structural support as a substrate in cellular migration and as a transmitter of biomechanical forces [6]. The mechanisms by which ECM offers these activities are varied. The transmission of mechanical cues is provided by: (a) signalling through direct cellular binding; (b) through the sequestration and regulation of soluble growth factors and cytokines. Therefore, ECM can be considered a highly specialized substrate for both mechanical support and functional substrate for cell growth and signalling. The components of ECM also provide spatial separation between specialized sections of tissue, such as the basement membrane separating the mucosal lining of the intestine from the submucosal tissue. The extracellular matrix also mediates the stress processes and regulates cell proliferation and phenotype expression based on the current state of the cell and tissue [11]. The key to the cell signalling is an interaction between ECM proteins and integrins, which are heterodimeric transmembrane receptors. Integrins bind to the ECM protein as their ligands, therefore they can respond to any mechanical or biochemical change in ECM [6] [7]

Table 1 Components of extracellular matrix (ECM) with brief description of their functions [3,8,9,10]

ECM	Activity	
Collagen	dictates the tissue architecture, shape, and organization	
Proteoglycans	Decorin	regulate collagen fibril assembly
	Lumican	
	Aggrecan	causes elasticity and high biomechanical resistance
		binds hyaluronan – facilitating of *chondrocyte-chondrocyte* and *chondrocyte-matrix* interactions
		arranges the extracellular areas between neurons in the brain
	Versican	modulates cell adhesion, migration, proliferation
	Neurocan	modulates cell adhesion and migration
	Brevican	may play a role in maintaining the extracellular environment of mature brain
Lamins	create network between cells and receptors on cell surface	
	essential for early embryonic development and organogenesis	
Fibronectin	provides attachment and migration of cells, growth, differentiation	
Elastin	provides elasticity to the tissue	

3 Decellularised extracellular matrix

As there is no singular ideal approach for decellularization, the protocol must be customized according to the source of the tissue. Same decellularization process can bring different results in different tissues [11]. For the successful preparation of dECM without any adverse host reaction, it is necessary to remove cells from tissues while preserving the native ECM components and structure (Figure 2). Any residual cell material is responsible for the induction of an inflammatory response and subsequently an immune reaction [12]. The immune response is tightly connected with any DNA residues, as the host recognizes any unfamiliar genetic material and evaluates it as a threat, therefore, starting the processes of the immune system to eliminate it [13]. However, at the moment, it is not possible to reliably

remove all the genetic information from the ECM in the process of decellularization. Therefore, there are three criteria established to describe dECM: 1) maximum of 50 µg residual double-stranded DNA per mg of ECM dry weight, 2) DNA fragments smaller than 200 base pair, and 3) 4', 6-diamidino-2-phenylindole (DAPI) or hematoxylin and eosin (H&E) staining proving lack of visible nuclear material [1,9,14].

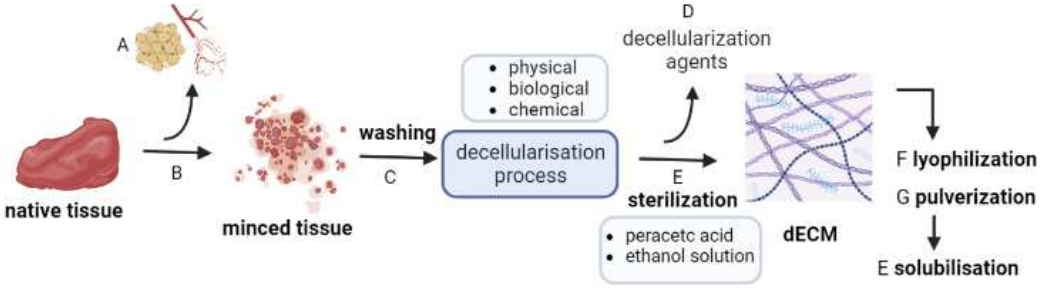

Figure 2 Process of preparation of dECM (Created with Biorender.com)

There are various techniques for decellularization, such as physical, enzymatic, chemical, or their combination (Table 2). The decellularization can alter the composition of ECM therefore it is essential to select a fitting mechanism to maintain the wanted structure It is crucial to mention that any of the chosen methods disrupt the composition of ECM on some level therefore the goal is to minimize the harmful effects [15]. It is also important to note, that each tissue requires a specific approach to achieve successful decellularization with ECM properties preserved [16].

3.1 Physical decellularization

Physical ways of removing cellular material from tissues work by breaking cell membranes and producing unfavourable cellular conditions that can induce apoptosis [13]. Lack of chemicals used in physical processing and the benefit of being consistent throughout tissue makes physical decellularization a common method between researchers. Furthermore, effects of the process are more predictable than those of chemical or enzymatic decellularizing agents. However, physical treatment alone is frequently insufficient for decellularization [15]. While it can cause cell lysis, it is unsuccessful at totally eliminating cell or nuclear remains. Nonetheless, it can be utilized in tandem with chemical, biological, or enzymatic decellularizing agents to reduce exposure times and enhance ECM proteomic content retention [17].

One of the earliest physical processes to achieve decellularization is cyclic freeze-thaw method. It is cost-effective process to prepare dECM. However, recent studies show, that extensive cyclic freeze-thaw decellularization can cause and immune response and impact the biochemical abilities of dECM, as the microstructure of a collagen at molecular level could be gradually altered by ice creation several cycles in [19]. Pu Luo et al. prepared dECM through repeated freeze-thaw cycles with combination with other methods to design an efficient decellularization method. Extracellular matrix was obtained from porcine peritoneum, underwent n = 0 (control) 3 and 7 freeze thaw cycles and then the decellularization continued with alkaline and acids, organic solvents, and hypotonic/hypertonic solutions.

Table 2 Summary of methods used in decellularization of extracellular matrix with their advantages and disadvantages

Method	Procedure	Pros	Cons
Physical	Freeze-thaw cycle	tissue integrity	relatively high DNA content left
		preserved elastic modulus	alteration of collagen fibrils
	Ultrasonic waves	whole cell removal	uncontrolled cavitation
			destructive effects on retinal tissue
	Hydrostatic pressure method	collagen well preserved	addition of chemical agents to remove DNA residues
	Electroporat-ion	preservation of ECM - neighboring tissue	limited size of the decellularized tissue
Chemical	Acids and bases	sterilization capabilities	insufficient cell removal
		separation of cellular components	alters mechanical properties
	Triton X-100	removal of cellular residues from thin tissue	elastin decrease
		myosin and actin preserved	effectivity varies between tissues
	SDSc	effective in dense tissues	damage of collagen
			removing growth factors
Biological	Trypsin	does not affect the amount of collagen	prolonged exposure removes disrupts ECM
		effective cell removal	removes laminin, fibronectin elastin
	Endo/Exo-nucleases	hydrolysis of the terminal bonds of RNA and DNA	difficult to remove from the tissue
			invokes an immune response

The resulted mass was subsequently lyophilised and sterilised as per standard procedure. As typical temperatures for freeze-thaw cycle are - 20° C and - 80° C, it takes a lot of time to remove the cell material thoroughly, however in this study, the liquid nitrogen was used to freeze the tissue for 5 minutes and then thawed at the room temperature. Subsequent analysis with histological staining confirmed no visible nuclei. Surface morphology and pore size of the dECM structure were characterized to analyse the impact of freeze-thaw cycles (FTC). SEM images showed a decrease in average pore size from 10 mm to 5 mm after 7 FTC and collagen diameter was half the size of that in the control group. Mercury intrusion porosimetry (MIP) and atomic force microscopy (AFM) confirmed the theory that the pore size of the structure decreased with an increasing number of FTC [18] .

3.2 Chemical decellularization

Depending on the initial tissue's size, density, cellularity, thickness, and lipid content, chemical treatment-based decellularization's efficacy varies. To speed up the decellularization processes, chemical agents can be mixed with one another, applied simultaneously, or used in conjunction with other decellularizing techniques. The proteomic and biomechanical characteristics of the produced ECM are significantly influenced by the order in which tissue samples are subjected to various chemical agents in protocols that employ several decellularizing agents. The nonionic detergent Triton X-100 and the ionic detergent Sodium Dodecyl Sulfate (SDS) are the most often used detergents for decellularization [19,20]. SDS breaks protein-protein interactions and disintegrates cell membranes, whereas Triton X-100 eliminates cellular content by impairing lipid-lipid and protein-lipid connections without impacting protein-protein interactions. Any SDS-based approach must consequently optimize SDS concentration and tissue exposure period, as higher exposure is directly related to decreasing ECM biomechanical characteristics. Ionic detergents, such as SDS, have the advantage of being able to successfully remove nuclear materials in less time than conventional chemical treatments. This comes at the expense of more damage to the ECM matrisome, as SDS treatment may result in a modified microstructure that reduces the biomechanical integrity of the ECM [17]. Normally, decellularization treatments do not use Triton X-100 concentrations more than 1%, while some techniques have demonstrated effectiveness with concentrations that are as high as 3% as long as exposure time is adjusted proportionately. Despite its inability to break down collagens, SDC can successfully decellularize tissues at concentrations of up to 4%. However, larger concentrations of SDC don't lead to higher nuclear elimination rates and result in significant structural integrity damage. Furthermore, SDC decellularizations must be followed by agents such as Deoxyribonuclease (DNase) to minimize DNA agglutination at the tissue surface [14]. The Hudson method is a decellularization technique for peripheral nerves that relies on the use of the detergent Triton X-200 to remove cellular components while preserving the extracellular matrix [21]. McCrary et al. aimed to optimize a new chemical decellularization method for peripheral nerves using sodium deoxycholate (SDC) and other reagents to match the effectiveness of the Hudson method. The optimized process, involving 3% SDC and a 3-hour DNase incubation, successfully preserved extracellular matrix components and removed cellular debris more effectively than the Hudson method. Results showed that the novel method maintained a similar proteomic profile to the Hudson method, reduced cellular protein counts, and did not leave cytotoxic residues, making it a viable alternative for peripheral nerve repair [22].

3.3 Biological decellularization

Some studies have shown that use of detergents like SDS or Triton-X in decellularization process disturbs the collagen and glycosaminoglycan (GAG) in ECM, which significantly decreases its mechanical strength and viscoelasticity [20]. To preserve native content of dECM, enzymes and chelating agents can be used. Shanto et. al used a combination of trypsin and EDTA in decellularization process in cartilage tissue. Distilled water was used to rinse the lyophilized bone fragments and then decellularized in 0.05 % trypsin and 0.02 % EDTA solution with continuous stirring at 37 °C for 24 hours. The process continued with subsequent steps in order to remove the cellular materials. The remaining GAGs and collagen components were analysed using kits the Blycan™ sulphated glycosaminoglycans assay kit, and collagen assay kit. The analysis showed gradual increase in collagen and GAG contents after 3 weeks of in vitro culture with rat bone marrow-derived mesenchymal stem cells (rBMSCs) [23]. DNase, RNase, and benzenes are all frequently used as subsequent processing steps in chemical, physical, or biological decellularization. According to research, the addition of DNase treatment steps can improve the retention of biomechanical characteristics and GAGs in a variety of chemical, enzymatic, and physically based decellularization methods [1].

4 Discussion

Decellularization techniques have made significant strides, enabled the removal of cellular components while preserved the ECM's structural and biochemical integrity. This preservation is crucial for maintaining the bioactivity and mechanical properties of the scaffolds, which are essential for successful tissue engineering applications [24,25]. The integration of decellularized ECM in 3D printing holds the promise of creating scaffolds that closely mimic the native tissue environment, thereby enhancing cell adhesion, proliferation, and differentiation. This bioactivity is vital for the formation of functional tissues and their integration with host tissues post-implantation

[26]. Various decellularization methods, including chemical, enzymatic, and physical approaches, have been explored, each offering distinct advantages and limitations. Physical decellularization methods, while effective at removing cellular material, often compromise the structural integrity of the ECM, making them less ideal for applications requiring high fidelity in matrix architecture. Chemical methods, although efficient at thorough cell removal, can leave behind toxic residues that may interfere with cell viability and function post-bioprinting. Biological decellularization, with its enzymatic specificity, provides a more controlled approach, preserving the biochemical composition and structural properties of the ECM, but may require optimization to ensure complete decellularization [20,21,24]. Ultimately, the choice of decellularization method must strike a balance between the efficiency of cellular removal and the preservation of the ECM's natural properties. This balance is crucial to producing a dECM scaffold that supports cell adhesion, proliferation, and differentiation. Continued research and refinement of these techniques will be essential to advancing the field of bioprinting and enhancing the functionality of engineered tissues [25,26].

5 Conclusion

In conclusion, the advancements in decellularization techniques have significantly impacted the field of tissue engineering, particularly in the context of 3D bioprinting. These techniques have successfully enabled the extraction of cellular components from extracellular matrix (ECM) while retaining its essential structural and biochemical characteristics [22,24,25], which are vital for developing effective tissue scaffolds. The discussion calls for further research to optimize decellularization processes, explore the combination of ECM with other biomaterials to enhance scaffold properties, and conduct comprehensive in vivo studies to validate the clinical efficacy of these bioprinted tissues. The selection of an appropriate decellularization technique is pivotal in the creation of decellularized extracellular matrix for bioprinting applications. Physical, chemical, and biological decellularization methods each offer distinct advantages and drawbacks that must be carefully considered depending on the specific requirements of the tissue engineering application.

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