



## ***A Systematic Review of Tissue Engineering of a Total Artificial Heart: Focus on Perfusion Decellularization***

Mansoor Gullabzada<sup>1,\*</sup>, Uzma Gullabzada<sup>2</sup>, Fatima Zahid<sup>3</sup>, Md Badsha<sup>4</sup> and S. Rangrej<sup>5,\*</sup>

<sup>1</sup>BSc Spec Honors & MEng Biomedical Engineering, Ryerson University

<sup>2</sup>BSc Honors Kinesiology, McMaster University

<sup>3</sup>Bachelor of Health Studies, York University

<sup>4</sup>BSc Biochemistry & Molecular Biology, University of Dhaka

<sup>5</sup>MD, Assistant Professor & Chair of Research Committee, Saint James School of Medicine

### Article info

Received 02 March 2022

Revised 01 April 2022

Available Online 10 April 2022

\*Corresponding author: Mansoor Gullabzada and S. Rangrej, Saint James School of Medicine, HRDS Inc. 1480 Renaissance Drive, Suite 300 Park Ridge, IL 60068 USA

### Abstract

**Introduction:** A leading cause of death worldwide is heart disease, and in the United States alone, 6.2 million individuals live with heart failure. The recent ability to recreate the vascular network of cardiac Extracellular Matrix (ECM) suggests the feasibility of engineering whole heart constructs. Tissue engineering method such as perfusion decellularization allows for effective removal of nearly all cellular composition of a heart while maintaining the native mechanical integrity of the scaffold. This scaffold can be reseeded with stem cells in the hopes of generating a functional heart. The goal of this systematic review was to assess the various perfusion decellularization methods and its effects upon the biologic scaffold.

**Methodology:** A comprehensive systematic literature review search was carried out through Pubmed, MEDLINE and Google Scholar using the search terms “whole heart decellularization”, “whole organ decellularization” and “perfusion decellularization”. The inclusion criteria for this research were as follows: scholarly or peer-reviewed studies, published within the last 20 years in the English language, and primary studies in which whole cardiac organ decellularization was performed. Chosen articles were those examining the various decellularization methods and its effects upon the biologic scaffold. Excluded were studies regarding heart valve decellularization, tissue decellularization, cardiac patch, tissue recellularization, articles in foreign languages, articles dated prior to 2001, literature reviews, systematic reviews, and duplicate articles.

**Results:** Chemical agents such as acid and bases cause hydrolytic degradation of biomolecules which could reduce ECM strength and eliminate growth factors from the matrix. Compared to other detergents such as Triton X-100, sodium dodecyl sulfate yields a more complete removal of nuclear material. A combination of these various approaches has shown an increased efficacy of the decellularization process. The decellularization of a large solid organ such as the heart requires several sophisticated steps. Perfusion decellularization is achieved via anterograde or retrograde perfusion of the intrinsic vascular network of the heart by utilizing decellularizing agents (i.e., chemicals and/or enzymes). Hodgson et al., 2017 demonstrates a combined strategy in which the decellularization of porcine hearts is accomplished in 24 hours and results in 98% DNA removal with only 6 hours of detergent exposure.

**Conclusion:** *There have been significant advances to address the heart disease epidemic. A promising approach is perfusion decellularization which allows for complete DNA content removal while maintaining the ECM scaffold integrity. This scaffold is then re-cellularized with stem cells in the hopes of creating an artificial heart. There are several challenges that need to be surpassed before bio-artificial hearts can be used to replace in vivo function. Future research is directed towards optimizing types of cells and cell sources used to repopulate decellularized hearts, seeding strategies and bioreactor systems to provide in vitro conditions required for organ maturation.*

**Keywords:** *Decellularization; Whole organ perfusion; Heart decellularization; Perfusion decellularization; Total artificial heart*

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## Introduction

Congestive heart failure occurs when the heart muscle is unable to pump sufficiently to maintain blood flow to meet the body's needs [1]. Heart failure is caused by numerous conditions such as cardiomyopathy, congenital heart defect, and coronary heart disease [2]. In the United States alone, 6.2 million individuals live with heart failure. On a broader perspective, 17.9 million people die each year from heart failure [3]. The best treatment available for end-stage heart failure is cardiac transplantation. However, the demand for transplantation greatly exceeds the number of available donor hearts. Moreover, patients receiving cardiac transplant are faced with the risk of rejection and the chance of developing health conditions associated with immunotherapies. These immunotherapies lead to immune suppression which subsequently increases the susceptibility to infection and cancer [4]. The creation of an artificial heart can potentially solve this dilemma. There have been numerous advances in medicine for the functional replacement of the heart organ including assistive devices, bio-prosthetic total artificial hearts and bio-artificial hearts.

The very first total artificial hearts were Liotta-Heart and Jarvik-7 which are pneumatic pulsatile devices [5]. The SynCardia (Jarvik-7) is an artificial heart that has been implanted in more than 11,000 patients with the longest duration of support being 3.75 years [6]. Another promising total artificial heart is CARMAT. To reduce the risk of thrombosis, the membrane of CARMAT is made up of bovine pericardial tissue [7].

Due to the biocompatibility and device limitations of the bioprosthetic artificial hearts, a new promising tissue engineering approach for functional replacement of

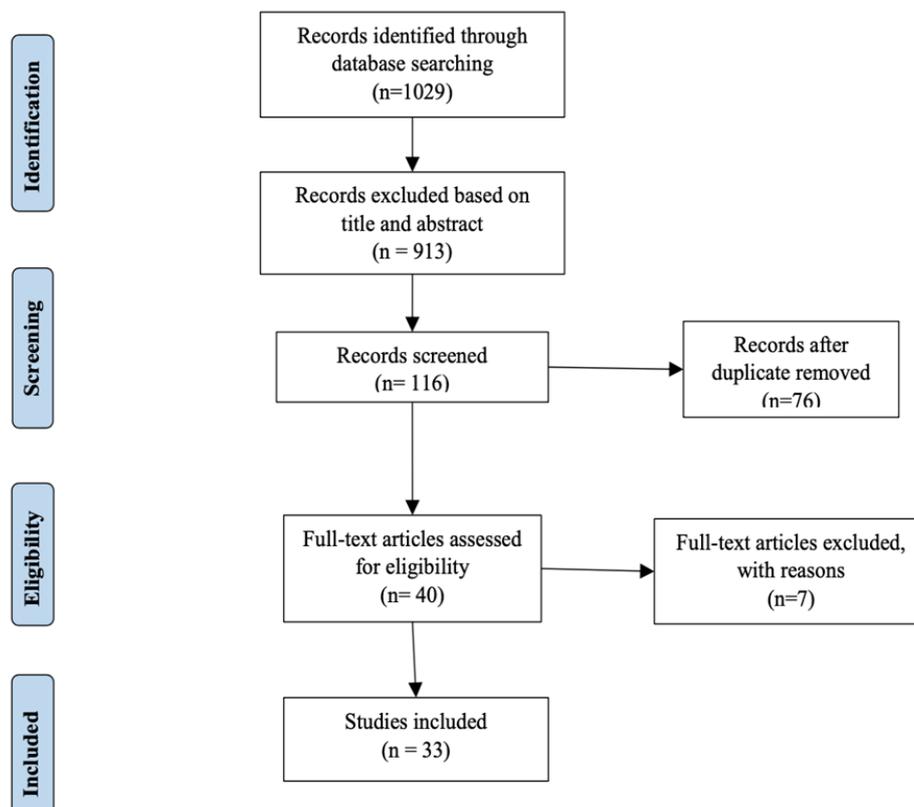
heart organs has been developed, known as decellularization and recellularization. The engineering whole heart had been viewed as a hypothetical construct in tissue engineering because of the complex vascular network needed to support the high rate of cardiomyocyte oxygen consumption. The recent ability to recreate the vascular network of cardiac ECM suggests the feasibility of engineering whole heart constructs through tissue engineering. The ECM is in constant communication with the resident cell population, therefore the structure and composition of the ECM changes in response to the metabolic activity of the resident cell and mechanical demands of tissue. This vital mutualism between the ECM and resident cells is a great asset for the use of ECM material over synthetic material [8]. Decellularization is a process by which the removal of cells and its nuclear material takes place resulting in a biologic scaffold composed of ECM. The ECM is a naturally occurring scaffold material made up of resident cells of each tissue and organ. ECM components are dictated by tissue type. Particularly, the cardiac ECM consists of glycoproteins, proteoglycans and glycosaminoglycans [9]. The ECM has bio-inductive properties, and these properties are attributed to its fibroblast growth factor and vascular endothelial growth factor components. During the degradation of the ECM scaffold, fibroblast growth factor and vascular endothelial growth factor are responsible for remodeling of the ECM by way of angiogenesis, mitogenesis and deposition & organization of the new host [10]. There are various decellularization methods including physical, chemical, enzymatic processes, or a combination of these. Each method affects the biochemical composition, tissue ultrastructure and

mechanical behavior of the ECM scaffold as well as affects the associated host response that the scaffold will prompt when used as a template for organ reconstruction [11]. Perfusion decellularization is furthermore achieved through anterograde or retrograde perfusion of decellularizing agents through the use of the intrinsic vascular network of the organ [12]. The goal of decellularization is to effectively remove all cellular composition, while maintaining the native mechanical integrity of the scaffold. Therefore, extra-caution should be taken in choosing the method of decellularization of the heart. The goal of this systematic review is to assess the various decellularization methods and its effects upon the biologic scaffold.

## Methods

The objective of this research was to perform a comprehensive systematic literature review search through a variety of resources including: Pubmed, MEDLINE and Google Scholar. Search terms “whole heart decellularization”, “whole organ decellularization”, and “perfusion [AND]

decellularization” were used to identify relevant studies discussing. Boolean operators such as “[AND]” were used when needed. Citations were screened and assessed for quality via Rayyan, a web application. If an article met the below listed exclusion criteria, it was excluded from the analysis. The inclusion criteria for this research were as follows: scholarly or peer-reviewed studies, relevant articles published within the last 20 years, articles published in the English language, and primary studies in which whole cardiac organ decellularization was performed. Chosen articles were those examining the various decellularization methods and its effects upon the biologic scaffold of the heart. Excluded were studies pertaining solely to heart valve decellularization, tissue decellularization, cardiac patch decellularization, tissue recellularization, articles in foreign languages, articles dated prior to 2001, literature reviews, systematic reviews, and duplicate articles. The primary outcome was the successful removal of all native cellular components of a heart organ, i.e., cardiomyocytes and its intracellular material, while maintaining the native mechanical integrity of the whole organ ECM scaffold.



**Figure 1:** Flow-chart for the selection.

## Results

**Table 1:** Summary of primary outcome findings.

First author	Decellularization method	Effects on ECM	References
Wainwright et al., 2010	Retrograde Aortic Perfusion: Trypsin, EDTA, NaN <sub>3</sub> , Triton X-100, and deoxycholic acid	No cell nuclei or dsDNA were visible based on H&E and DAPI stain. Movat's Pentachrome stain of the C-ECM revealed the absence of muscle cells. The presence of collagen type I & II was detected by Movat's and Herovici's stain. Anti-human collagen IV antibodies showed presence of collagen IV. DNA quantification revealed great reduction in DNA (0.66 ng DNA/mg sample vs. 8.48 ng DNA/mg sample [p=0.014]). This equates to a 92% reduction in DNA.	[13]
Hulsmann et al., 2015	Perfusion Decellularization: Detergent solution S1 (0.5% SDS +0.5% Sodium Deoxycholate), S2 (Aqua bides) & S3 (PBS)	The dry weight of the heart was reduced from 176±10.5 mg to 23±1.9 mg. The total content of DNA of native rat hearts decreased from 685±80 µg on average per heart to approximately 325±23 µg.	[14]
Hodgson et al., 2017	Perfusion of PBS alternating with distilled water, then heart is perfused with alternating cycles of SDS and distilled water. Lastly, the heart is perfused with Triton X-100, another lysing agent, and water	This perfusion method successfully removed 98% of DNA content with 6 hours of detergent exposure.	[15]
Garry et al., 2016	Langendorff Perfusion [12 ml PBS, 60 ml of 1% SDS in dH <sub>2</sub> O for 14 hours, 10 ml 1% Triton X-100 (diluted in distilled water), followed by 10 ml dH <sub>2</sub> O, followed by 60 ml PBS containing 1x penicillin streptomycin]	H&E showed no nuclei. DNA content of the decellularized heart reduced from 68.08 ± 2.25 µg to 4.73 ± 2.27 µg. Immuno-staining identified presence of collagen IV. This equates to 93% reduction in DNA.	[16]
Taylor et al., 2018	Decellularized under constant pressure of 120 mmHg measured at the aortic root. 4 h of hypertonic solution (500 mM NaCl), 2 h of hypotonic solution (20 mM NaCl), 120 h of 1% SDS, and a final wash with 120 L of 1X PBS. During the final wash with 10 L of 1X	Angiogram showed the preservation of coronary vasculature. Less than 10% DNA content of the cadaveric heart was seen in all regions of the heart. Nonlinear optical imaging of myocardium layers from thick areas (i.e., RV, LV, and septum) in a decellularized human heart confirmed cell removal.	[17]

	PBS, add 500 mL of sterile neutralized 2.1% peracetic acid solution, neutralized with 10N NaOH, leading to a 0.1% peracetic acid solution (v/v) in PBS		
Ozlu et al., 2019	Perfusion decellularization through two cycles, first cycle perfused with deionized water, PBS, trypsin, 1% SDS, 3% Triton X-100 solution, deoxycholic acid, peracetic acid, PBS, deionized water, PBS, and deionized water perfusion. Second cycle perfused with deionized water, PBS, trypsin, 1% SDS solution, 3% Triton X-100 solution, deoxycholic acid, peracetic acid, PBS, and deionized water perfusion.	There were no visible cell nuclei based on Crossman's modified triple staining. Blue color verified the presence of connective tissue. The cell density was found to be $284 \pm 411$ cells/mm <sup>2</sup> in the decellularized heart compared to native heart which had $3718 \pm 399$ cells per mm <sup>2</sup> . This equates to 7.6% residual nuclear material in the decellularized heart.	[18]
Meşinã et al., 2019	Large Hearts (pig & human): Cannulation of the ascending aorta. Perfusion with a solution of 30g of NaCl and 2L of demineralized water for 20 minutes. Wash with 2L dH <sub>2</sub> O, repeat this 3x. Perfusion with solution of 6g of Bromelain and 2L of dH <sub>2</sub> O to perfuse the heart at 37°C for about 1 hour. Alternation of 2L demineralized water + 30g NaCl/2L dH <sub>2</sub> O for 20 minutes each. Then perfusion with sodium lauryl sulfate (SLS) (10g SLS in 2L of dH <sub>2</sub> O) for 24 hours. After completion of decellularization, NaCl + demineralized water/demineralized water alternation. Lastly the heart was perfused with hydrogen peroxide 3% and acetic acid solution in a 7/1 ratio (1.4 L hydrogen peroxide + 200 ml acetic acid) for 2 hours.	Histological examination showed that all hearts (human, pig, and rat) lost intracellular components. Furthermore, it showed the maintenance of collagen, proteoglycan and elastin. Quantitative DNA analysis showed a significant reduction in DNA in decellularized hearts.	[19]

	<p>Small Hearts (Rat): 3 alternate perfusions of demineralized water + NaCl/ demineralized water in the same concentrations using the dialysis pump to administer the substances through retrograde coronary perfusion as with the pig hearts. Then perfusion with SLS (10g SLS in 2L of demineralized water) for 24h.</p>		
<p>Dal Sasso et al., 2020</p>	<p>Retrograde Perfusion: Prior to the actual decellularization process, to remove the blood residues, perfusion of the heart with a solution of PBS, pH 7.4, added to 5 UI/mL enoxaparin sodium.</p> <p>Perfusion with protease activity inhibition (30 min, pump speed 1.5 mL/min, room temperature). Antioxidation (30 min, 1.5 mL/min, RT). SDS-based perfusion (5.5 h, 10 mL/min, RT). Wash in deionized water (30 min, 1.5 mL/min, RT). Triton X100-based perfusion (1 h, 1.5 mL/min, RT). Wash in PBS (overnight, 1.5 mL/min, RT). Benzoylase-based treatment (1500 U/cm<sup>2</sup>; 2 × 24 h incubation, mild agitation, 37 °C). 2,3-butanedione monoxime (20 mM) was added at each step starting from the first PBS washout until SDS-based treatment to induce muscle relaxation and optimize whole organ perfusion. Maintain the decellularized heart in PBS supplemented with 3% penicillin-streptomycin and</p>	<p>Histological analysis of the heart valve, blood vessels and ventricle showed absence of cell. Moreover, absence of cell elements was noted in coronary vessels. Maintenance of collagen/elastin was seen. Autofluorescence of cardiomyocytes and elastin was observed in native hearts by TPM but mostly lost after decellularization</p>	<p>[20]</p>

	0.25% amphotericin B and store it at +4 °C		
Weymann et al., 2011	Retrograde Perfusion: hearts were perfused at a constant pressure of 100mmHg by Langendorff mode delivering 4% SDS in PBS at 2.0 L/min for 12 h. During the process, the hearts were washed with PBS for 15 min every 3 h to remove residual substances. The final washing step after SDS treatment included perfusion with PBS for another 24h to remove remnant detergents and cell debris at 1.5 L/min.	The native porcine hearts appeared to be almost acellular based on histological examination. Light microscopy of decellularized hearts stained with Movat's pentachrome stain, Herovici's stain and Masson's trichrome stain showed preservation of fiber orientation, composition of ECM collagen and elastic fibers. The total DNA content decreased significantly compared to controls (84.32±3.99 ng DNA/mg tissue in the decellularized group vs. 470.13±18.77ng DNA/mg tissue in the control group (P<0.05)	[21]
Ott et al., 2008	Antegrade coronary perfusion with 10uM adenosine for 15 mins, 1% SDS for 12 hours, 1% Triton X-100 for 30 mins, PBS with antibiotics for 124 hours.	Histological evaluation showed no nuclei or contractile elements. Collagen type 2 & 3, laminin and fibronectin remained within the matrix. Perfusion with red Mercox resin showed larger cardiac vessels and the smaller third- and fourth-level branches were patent.	[22]
Merna et al., 2013	Whole heart perfusion with four different detergent solutions made up with Trypsin/EDTA (0.05%)/NaN <sub>3</sub> (0.05%) and/or Triton/ EDTA (0.05%)/NaN <sub>3</sub> (0.05%). Varied amount of Trypsin and Triton were used in different experiments to examine the effect of both Trypsin and Triton, Trypsin-only, and Triton-only on ECM	DNA content: Treatment with detergent solutions containing both Trypsin and Triton showed almost 90% removal of DNA, whereas Trypsin-only and Triton-only eliminated approximately 59% and 40% of DNA, respectively.  Decellularized tissues retained compressive modulus less than 20% of non-decellularized tissues.  Except Triton-only detergent, all other detergent solutions reduced the amount of collagen from ECM.  Collagen microstructure: Trypsin-only treatment preserved collagen organization closer to that of native ECM, whereas Triton-only produced disheveled collagen fibers.  Only Triton-only detergent solutions-maintained integrity of Elastin fiber to a great extent.	[23]

		Elastin microstructure was largely retained in Triton only decellularization conditions where others disrupted the fine fiber architecture.	
Akhyari et al., 2010	Software-controlled automatic coronary perfusion-decellularization using detergent solutions: heparinized PBS, 10 mM adenosine, 1% SDS, 1% DCA, 0.05% NaN <sub>3</sub> , 20% glycerol, and 25 mM EDTA in 0.9% NaCl solution; 1% Saponin	A nuclear material free (H&E staining) and cellular content free (PSG/FG staining and $\alpha$ -actin immunostaining) homogenous ECM with abundant collagen type IV fiber was achieved	[24]
Akhyari et al., 2018	Pressure-controlled perfusion-based decellularization with detergent solutions (SDS, 0.75% and DCA, 0.25%)	Almost no cytosolic proteins were detected in western blot analysis. Presence of ample collagen fibers were confirmed by Semi-blue staining with osmium tetroxide. Compared to the native hearts, the DNA content in decellularized hearts was significantly low ( $121.69 \pm 9.7$ ng and $67.36 \pm 9.15$ ng per mg dry mass, respectively ( $p=0.0286$ ))	[25]
Sarig et al., 2012	Trypsin-Triton based three steps perfusion decellularization method involved, 1) alternating hyper/hypo-tonic solutions (1.1% and 0.7% (w/v) NaCl in double distilled water; respectively); 2) treatment with trypsin (trypsin supplemented with 0.02% (w/v) EDTA); and 3) washing off detergent with 1% (v/v) Triton-X-100 in PBS supplemented with 1% (v/v) ammonium hydroxide	Scanning electron microscopy and multiphoton microscopy analyses revealed that the resulting trypsin-perfused yielded a non-immunogenic ECM, removed cells and residual proteins from the organ more effectively, and preserved ultra-structure of collagen fibers (types I, II, III, and V) compared to other decellularization procedures, such as stirring, sonication, or SDS/Triton-based procedures.	[26]
Nguyen et al., 2018	Four-flow cannulations method: Ascending aorta, superior vena cava, pulmonary artery and vein were cannulated to perfuse detergent (heparin, PBS solution, and 1% SDS)	This method preserved the overall 3D shape of the heart which was closer to the native heart. Total DNA content was <50 ng/mg. Multiphoton imaging displayed the original organization of collagen and elastin fibers.	[27]

Cohen et al., 2021	Automated perfusion decellularization: machine-controlled flow rates of detergent (0.01-0.1% isotonic SDS) at 4 °C and perfusion pressure of up to 120 mmHg	Organs free of vascular-tree endothelial cells were attained	[28]
Park et al., 2018	Pulsatile pattern flow of detergent (1% SDS) was performed during the decellularization process.	Reduced amount of genomic DNA was detected in ECM compared to that with non-pulsatile pattern perfusion	[29]
Methe et al., 2014	5 porcine hearts were washed with phosphate buffered saline (PBS) containing 1% penicillin, 1% streptomycin, and 1% amphotericin B. After being frozen and subsequently thawed, the hearts were washed with Distilled Water (D/W). 8 cycles of perfusion treatment with 4% sodium deoxycholate (SDC), 1% Triton X-100 and thorough washing with (D/W) was completed in order to remove any cellular material which was verified with histological analysis. The hearts then underwent perfusion with 0.1% peracetic acid in PBS for 3 h at room temperature, after which they were kept in PBS containing antibiotics and an antifungal solution.	DNA quantification showed no remaining cardiomyocyte nuclei or nuclear material in all 4 heart chambers. The quantification study also demonstrated that the number of ECM components including hydroxyproline, collagen, elastin, and GAGs was not significantly decreased after perfusion of both atria and ventricles. Although the number of GAGs was found to be significantly reduced in the left ventricle due to the decellularization process, which was confirmed with Masson's trichrome staining for collagen. Immunohistochemical staining for elastin, fibronectin, heparan sulfate and hyaluronic acid revealed the arrangement of ECM components was not altered after the decellularization process. Furthermore, there was no significant reduction in the quantity of growth factors remaining including leptin, FGF-1, IL-8, VEGF-C, and VEGF-A after the decellularization process.	[30]
Guyette et al., 2016	Human hearts were obtained either after donation by cardiac death (DCD) or brain death (DBD). 40 hearts were applied with aseptic pressure-controlled antegrade coronary flow in a custom organ chamber at 60 mmHg. The hearts were then decellularized via perfusion using 1% SDS for 168 hours, deionized H <sub>2</sub> O for 24 hours, 1% Triton-X100 for 24 hours, and lastly PBS washes for 168 hours. The	A 99.05% reduction in double-stranded DNA (3.27±3.12 ng/mg wet tissue; P<0.05) was achieved with endonuclease perfusion of decellularized hearts.  All decellularized hearts revealed maintenance of ECM components such as insoluble collagen, a fair decrease in sulfated glycosaminoglycans, and reduced quantity of $\alpha$ -elastin and soluble collagen via biochemical analysis. An analysis of proteomics demonstrated an 89.14% decrease in the cardiac proteome (consisting of 967 proteins)	[31]

	<p>decellularized hearts retained 346.17±27.92 ng/mg of residual double-stranded DNA, which was then treated with endonuclease to yield a more complete removal of DNA content.</p>	<p>as only 105 unique proteins were identified in the decellularized heart scaffolds. Collagens (I, III, and IV), fibronectin and laminin were maintained in the ECM scaffolds as evidenced by histological analysis. Moreover, the cardiac extracellular matrix architecture was retained with visible spacing between myofibers and the disappearance of nuclear material as well as the myosin heavy chain (MHC) myocyte marker. Lipid molecules which are insoluble were found to remain on the epicardium after the decellularization process, but additional analysis verified the absence of cells and preservation of the ECM scaffold. Pentachrome staining revealed the decellularized vasculature maintained its key structural features, such as the intima, media, adventitia, and elastic laminae.</p>	
Kitahara et al., 2016	<p>9 porcine hearts were collected and stored at -80°C for 24 h. The hearts were perfused at a constant pressure of 100 mmHg with 1% sodium dodecyl sulphate and 1% Triton X-100, after which it was maintained at a temperature of 37°C. The remaining decellularized whole-heart scaffolds were then sterilized with gamma irradiation.</p>	<p>H&amp;E staining demonstrated a lack of nuclei in the decellularized hearts, but the extracellular matrix was found to be unaffected when compared with a normal heart. Furthermore, DAPI staining revealed no nuclei remaining in the decellularized scaffold. ECM components such as collagen types I and IV and laminin were found to be preserved through immunohistochemical analysis. Meanwhile, intracellular cardiac troponin T had completely been removed after the procedure. The results indicate successful removal of cellular material while preserving the extracellular matrix scaffold.</p>	[32]
Lee et al., 2016	<p>6 porcine hearts were subjected to a decellularization process consisting of a 4-hour hypertonic (500 mM NaCl) perfusion, a 2-hour hypotonic (20 mM NaCl) perfusion, 60-hour 1% SDS perfusion, and a final 40-liter 1X PBS wash. A PID (Potential-Integral-Derivative) controller was utilized at the aortic root to maintain a constant perfusion pressure of 60 mmHg. The standard upright heart retrograde perfusion method was applied along with two</p>	<p>It was revealed the inverted method of retrograde decellularization perfusion was superior to the vented method, which was demonstrated by a greater coronary perfusion efficiency, greater collagen and elastin content inside the aortic valve, greater cell debris outflow, decreased DNA content, and lastly, better retention of the whole heart shape after the procedure. The quantification of DNA and SDS were significantly less in the inverted heart than in the upright or vented hearts. There was no significant difference in the amount of GAG content remaining in most areas of the heart when comparing the 3 heart preparation methods. In all, the initial GAG content</p>	[33]

	new retrograde decellularization processes, which consisted of inverting the heart and venting the apex to decrease inflow rate.	recovered was 50% and 10-30% of the original DNA was retained.	
Sanchez et al., 2015	39 human hearts were decellularized via antegrade coronary perfusion through the ascending aorta using a 1% sodium dodecyl sulfate (SDS) detergent in deionized water for 4 to 8 days, which was followed by rinsing with 20L of penicillin-streptomycin-containing phosphate-buffered saline (PBS). The hearts were then analyzed for degree of cell removal and retention of ECM using methods such as angiography, endoscopy, staining and much more. 13 cadaveric hearts were used as controls.	The results of the endoscopy revealed integrity of all 4 chambers, the valves, the papillary muscles, the chordae tendineae and the valve leaflets. Moreover, H&E stain demonstrated no visible nuclei as less than 5% of the original DNA content of the decellularized heart was remaining in the scaffold. Silver staining, Masson's trichrome staining and immunostaining of the ECM scaffold revealed that both the structural and basement membrane components of the extracellular matrix (collagen, fibronectin, and laminin) were all preserved. The vascularity of the scaffold was also retained according to angiography and echocardiography tests.	[34]
Park et al., 2012	Heart was cannulated, rinsed with heparinized phosphate buffered saline (PBS). Then 1% sodium dodecyl sulfate (SDS) in distilled water was circulated at approximately 100 mL/min using a peristaltic pump for 48 hours. This procedure included multiple rinses with distilled water to prevent clogging of the small vessels due to cellular debris. The process of decellularization continued until the organs showed an entirely white appearance. As a final step, PBS solution was perfused for 24 hours to remove chemicals.	The heart showed a white appearance with well-preserved morphology. X-ray angiography & CT scan showed intact vasculature of the scaffold. Double stranded DNA content was decreased by 94.2%. H&E staining revealed no evidence of nuclear or cytoplasmic residues.	[35]

Kawasaki et al., 2015	Sodium lauryl ester sulfate heart perfused with detergent for 12 h and deionized water for 15 mins. Followed by 1 % TritonX-100 for 30 min and 500 U/ml of DNase in phosphate buffered saline for 24 h. Each perfusion was rinsed with deionized water (three cycles).	Type 1 and IV collagen, fibronectin, and laminin demonstrated preservation of ECM structure and components. A basement membrane was also maintained.	[36]
Oberwallner et al., 2014	The human left ventricular myocardial tissue method of decellularization included incubation in 0.5% (wt/vol) sodium dodecyl sulphate (SDS) for 9 h, 5% (vol/vol) triton x-100 for 48 h, and 4% (wt/vol) sodium deoxycholate for 40h. PBS solution was then used to dissolve and samples were then incubated in reagents with orbital shaking taking place at 4°C. Followed by 3 washing cycles in PBS and overnight rinse in PBS along with penicillin/streptomycin and nystatin.	H&E and picosirius red staining were performed following fixation with 4% buffered paraformaldehyde, paraffin embedding and microtome sectioning. decellularized samples were positive for laminin and fibronectin by immunohistology. All ECM components were least partially preserved. ECM fibers within a myocardial fiber were largely maintained after removal. H&E and Sirius red staining also showed collagen network of fibrotic ECM to be denser than the non-infarcted tissue after decellularization	[37]
Fernig et al., 2017	27 Adult porcine hearts were obtained. 21 hearts treated with sodium dodecyl sulfate (SDS)/ Triton X-100, 3 hearts with OGP, and 3 hearts treated with 3-cholamidopropyl dimethylammonio-1-propanesulfonate (CHAPS) through retrograde aortic perfusion via aortic cannulation of a whole porcine heart.	<p>&lt;50 ng DNA per mg ECM dry weight from each of the 4 heart chambers and lacked visible nuclear material tissue sections stained with H&amp;E. The final DNA content in the endocardium and epicardium of the right ventricle was greater than that of the left ventricle.</p> <p>The collagen content of the hearts decellularized with SDS was similar to the collagen content found in the native heart. OGP and CHAPS have significantly less collagen content than the native heart.</p>	[38]

Delgado et al., 2021	Porcine heart aorta cannulated and washed in 0.1% phosphate buffered saline, deionized water, 4% sodium dodecyl sulfate (SDS), and 1% Triton X-100 and PBS	Limited DNA remnants. ECM components such as collagen type 1, elastin, and glycosaminoglycans were successfully maintained	[39]
Hulsmann et al., 2018	Coronary perfusion is customized in detergent based decellularization semi-automatic pressure controlled. Ovine and rodent hearts decellularized via coronary perfusion with sodium dodecyl sulfate (SDS), desoxycholic acid (DCA), double distilled water and phosphate buffered saline (PBS).	DNA remnants could still be identified within the dense collagenous matrix. DNA analysis of decellularized hearts showed $88 \pm 26$ % reduction of DNA ( $P < 0.0001$ ) as compared to non-decellularized native controls.  A large cell free ECM with preserved matrix microarchitecture, including patent, myocardial vessels compared to non-decellularized native controls. Western blot analysis indicated an almost full removal of cardiac actin and only marginal detection of desmin.	[40]
Weymann et al., 2014	Antegrade coronary perfusion using a modified Langendorff decellularization model in 32 porcine hearts. Perfusion pressure of 100 mmHg in combination with 4% sodium dodecyl sulfate (SDS) in phosphate buffered solution (PBS) with antibiotic containing PBS penicillin-streptomycin.	Decellularized hearts lacked intracellular components but retained specific collagen fibers, proteoglycan, elastin and mechanical integrity; quantitative DNA analysis demonstrated a significant reduction of DNA compared to controls (82.663.2 ng DNA/mg tissue vs. 473.2613.4 ng DNA/mg tissue, $p < 0.05$ ).	[41]
Tao et al., 2015	Hearts obtained from female Sprague Dawley rats. The decellularization process included: PBS, 80% glycerol + 0.9% NaCl + 0.05% NaN <sub>3</sub> + 25mM ethylenediaminetetraacetic acid (EDTA); S2, 4.2% sodium deoxycholate + 0.05% NaN <sub>3</sub> . S3, 1% sodium dodecyl sulfate (SDS) + 0.05% NaN <sub>3</sub> S4, 3% Triton X-100 + 0.05% NaN <sub>3</sub> and S5, 0.05% NaN <sub>3</sub>	The colour of the rat's heart changed to a light brown and whitish color. Decellularized heart stained with Missons trichrome and examined with immunofluorescent staining of actin, cTnl, collagen type 1, and nuclei. There is no indication of cardiomyocyte specific protein nor nuclei. Collagen type 1 is positive in the whole heart section which indicates that the natural heart framework remains intact.	[42]

Remlinger et al., 2012	The decellularization of the porcine heart through the method of coronary retrograde perfusion. Protocol utilizes: Trypsin solution, Triton X-100, 2X Phosphate Buffered Saline (PBS), and sodium deoxycholate solution (DSD)	Histological staining for cell nuclei showed less than 50 ng of DNA was present per mg of dry weight of the tissue, and that any DNA was less than 200 bp in size.	[43]
Taylor et al., 2021	A catheter was placed into the carotid through the femoral arteries to the aortic arch in adult and neonatal rats. A solution of 1% sodium dodecyl sulfate (SDS) was placed in deionized water at 80mm hg. Therefore, obtaining the translucent organs tissues that were washed with deionized water and phosphate saline buffer (PBS).	The decellularization process both in neonatal and adult rats successfully decellularized by whole body systemic perfusion.  ECM fiber orientation is preserved along with the epicardial wall in heart. Decellularized vessel lumens and basement membranes were visible. Low levels of DNA preserved	[44]
Akbarzadeh et al., 2019	Twenty adult sheep ovine hearts via Antegrade perfusion. Distilled water, 1% SDS solution, 1% Triton X-100 in distilled water and PBS containing penicillin-streptomycin.	DAPI staining after decellularization indicated absence of cell nuclei of the left ventricle comparing the native control.  Approximate 95% reduction ( $p < 0.05$ ) in the DNA content of the decellularized scaffold compared with that of the native organ this indicates effective removal of cells and cellular DNA from the scaffold.	[45]

## Discussion

The main goal of decellularization is the removal of all cellular material without losing the composition & mechanical integrity of the ECM. There are various decellularization methods such as physical, chemical, enzymatic processes, or a combination of these. Each method affects the biochemical composition, tissue ultrastructure and mechanical behavior of the ECM scaffold. They furthermore affect the associated host response that the scaffold will prompt when used as a template for organ reconstruction. In our review, it was evident that chemical agents removed cardiomyocytes and any intracellular content with a high degree of efficacy. For example, Sanchez et al. (2015) used a

chemical detergent (1% sodium dodecyl sulfate detergent) via antegrade coronary perfusion to wash out intracellular content and the results demonstrated that the integrity of valves, all 4 chambers, the papillary muscles, and even fine structures such as the chordae tendineae and valve leaflets were maintained. As well, H&E stain showed no nuclear material and the residual content of the decellularized heart was less than 5%, which means there was a 95% intracellular content removal. Masson's trichrome, silver staining, and immunostaining of the scaffold showed that both the structural and basement membrane components of the ECM (collagen, fibronectin, and laminin) were retained [34]. Many of the studies included similar results, with the highest degree of DNA content removal being 99%

[31]. With regards to contrasting different chemical detergents available to decellularize whole hearts, it was found that sodium dodecyl sulfate (SDS) yielded a more complete removal of nuclear material when compared to other detergents such as Triton X-100 [47]. On the downside, chemical agents such as acids and bases cause hydrolytic degradation of biomolecules which could reduce ECM strength and eliminate growth factors from the matrix [46].

Moreover, there are decellularization methods that utilize enzymes such as trypsin & endonucleases to remove protein and DNA content, respectively. Of many included studies in this review, a combination of both chemical and enzymatic decellularization approaches were used by five studies. Specifically, in a study by Merna et al. (2013), they yielded a 59% decrease in DNA content when a trypsin solution was solely perfused through the coronary vessels of the heart. However, when combined with a chemical agent, Triton, a 90% decrease in DNA content was produced [23]. This finding demonstrates that using enzymes as the primary decellularization method does not successfully remove all intracellular material, and to maximize the efficacy of decellularization, a combination of enzymatic and chemical agents should be used. Furthermore, previous research has shown that prolonged exposure to enzymes disrupt the ECM structure and removes laminin, fibronectin, and GAGs. In addition, these enzymes are difficult to wash out afterwards from the remaining scaffold. Lastly, there are physical agents to decellularize tissues and whole organs, including a snap freezing method in which intracellular ice crystals disrupt the cell membrane and a mechanical force method in which high pressure burst cells and tissue. However, it has been found that these methods damage the ECM greatly. In our review, no studies utilizing physical agents were included due to the inclusion criteria [8].

Perfusion decellularization is achieved by anterograde or retrograde perfusion of decellularizing agents through the intrinsic vascular network of the heart. In this approach, the vascular network offers a route of detergent delivery, a process of removal of cellular debris to be excreted through the venous system and lastly, a route of delivery for nutrients and oxygen to the cells during the recellularization process. The use of perfusion decellularization has been successfully used to clear an organ of its cellular material. However, the

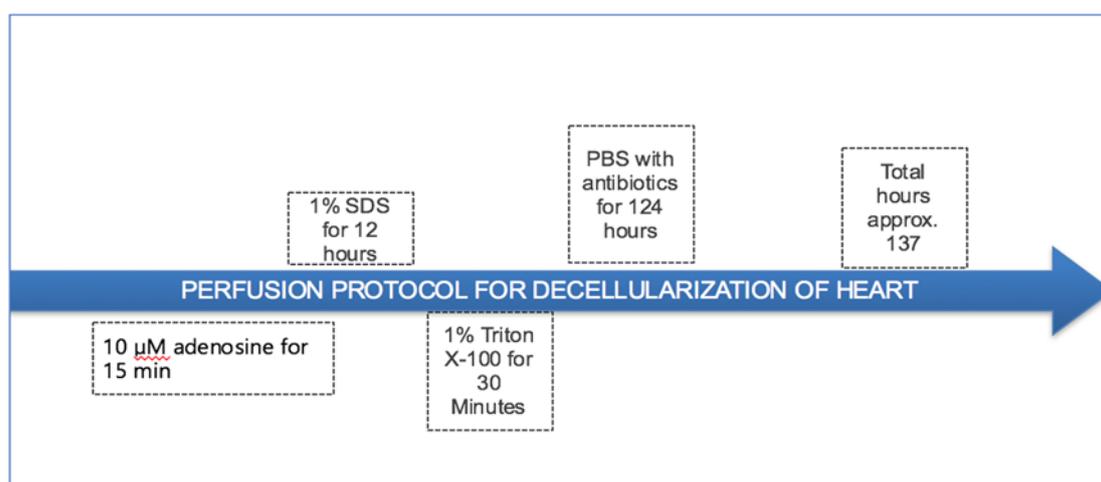
degree of success varies depending on the concentration of solution and amount of exposure time. As demonstrated by Garry et al. (2016), perfusion with 12 ml PBS, 60 ml of 1% SDS in distilled water (dH<sub>2</sub>O) for 14 hours, 10 ml 1% Triton X-100 (diluted in distilled water), followed by 10 ml dH<sub>2</sub>O and thereafter 60 ml of PBS containing 1x penicillin streptomycin (Pen-Strep) yielded a 93% decrease in DNA content. Hodgson et al. (2017), furthermore used a phosphate-buffered saline (PBS) alternating with distilled water, where the heart is perfused with alternating cycles of SDS and distilled water, followed by perfusion with Triton X-100, another lysing agent, and water. This process was accomplished in 24 hours and resulted in 98% DNA removal with only 6 hours of exposure to detergent.

Another example of perfusion decellularization technique of the heart organ is shown in *Figure 1* [22]. In this approach, antegrade coronary perfusion of rat hearts on a modified Langendorff apparatus was carried out. The protocol included use of 10 $\mu$ M adenosine for 15 mins, 1% SDS for 12 hours, 1% Triton X-100 for 30 mins and PBS with antibiotics for 124 hours. Histological evaluation showed no presence of nuclei or cardiac contractile elements. Extracellular materials such as collagen type 2 & 3, laminin and fibronectin remained within the matrix.

The goal of decellularization is to have a biocompatible ECM fit for reseeding with stem cells in the hopes of generating a functional heart. Recellularization involves rebuilding autologous vasculature and cardiac muscle. The heart is composed of various cells: atrial, ventricular and pacemaker myocardial cells; valvular, endocardial, and epicardial cells; arteries, veins, and capillaries; lymphatics; parasympathetic neurons, fibroblasts and myofibroblasts. The challenge with recellularization of a scaffold is its repopulation with an appropriate ratio of these cells and the placement of the cells to their appropriate location in the scaffold. The source of cells for whole organ recellularization include embryonic stem cells, fetal cells, induced pluripotent stem cells, adult derived stem/progenitor cells, umbilical cord blood cells, primary tissue or organ-derived cells, and organ-derived progenitors. The creation of induced pluripotent stem cells changed the field of stem-cell research. Inducible pluripotent stem cells can be expanded to large numbers and give rise to complex tissue cell types making them a reasonable cell source for tissue engineering applications. The number

of cells required for seeding the decellularized heart matrix is dependent on the biomechanical duties of the heart. As the heart is required to be fully functional at the time of implantation, a very high initial seeding percentage is needed. The average heart possesses approximately  $10^8$  cardiomyocytes/cm<sup>3</sup> [48]. Seeding strategies in recellularization of the heart include intramural injections or infusions of cells into the vasculature followed by perfusion. The optimal method has yet to be determined. As mentioned previously, Ott et. al (2008) decellularized rat hearts by coronary perfusion using SDS and Triton X-100 detergents, and subsequently reseeded the constructs with cardiac and endothelial cells. By day four,

macroscopic contractions were observed. After eight days of maturation the construct under physiological load and electrical stimulation could generate a fetal heart pump like function with a force equivalent to 2% of an adult's heart or 25% of the force of a heart belonging to a 16-week-old. This tissue engineering and regenerative medical approach is still in its infancy. There are several challenges that need to be surpassed before bio-artificial hearts can be used to replace in-vivo function. Future research is directed towards optimizing types of cells and cell sources used to repopulate decellularized hearts, seeding strategies and bioreactor systems to provide in vitro conditions required for organ maturation.



**Figure 2:** Chemical approach for decellularization of heart organs. Abbreviation: SDS, sodium dodecyl sulfate.

## Conclusion

The heart is a vital muscular organ that pumps blood through blood vessels to the rest of the body. Heart disease is the leading cause of death. There have been significant advances to address the heart disease epidemic by utilizing assistive devices, bio-prosthetic total artificial hearts and bio-artificial hearts. Due to the biocompatibility and device limitations of the bio-prosthetic total artificial heart, a new promising tissue engineering approach for functional replacement of heart organ has been developed via decellularization. Decellularization is a process by which the removal of cells and its nuclear material takes place, resulting in a biologic scaffold composed of an ECM which remains to be re-cellularized and developed into a whole heart organ. There are various decellularization methods, but those of a large, solid organ require several sophisticated steps compared to the decellularization of

small organs and/or sections of tissues. Thus, perfusion decellularization allows for nearly complete DNA content removal while maintaining the integrity of the ECM scaffold. The scaffold is then re-cellularized with stem cells in the hopes of creating an artificial heart. Future research is directed towards optimizing the types of cells and cell sources used to re-seed decellularized hearts, specific seeding strategies and bioreactor systems to provide the most suitable in vitro conditions needed for organ development.

## Conflict of interest statement

All authors declare that they have no conflicts of interest.

## Data access statement

All research data supporting this publication is provided in full in the results section of this paper.

### Ethics statement

The research committee board at Saint James School of Medicine has approved this research.

### Funding statement

The author(s) received no financial support for the research, authorship and publication of this paper.

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**Citation:** Gullabzada M, Gullabzada U, Zahid F, et al. A Systematic Review of Tissue Engineering of a Total Artificial Heart: Focus on Perfusion Decellularization. *J Protoc Biol Methods* 2022; 2: 102. doi: [10.31531/edwiser.jpbm.1000102](https://doi.org/10.31531/edwiser.jpbm.1000102)

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