

# ON TISSUE ENGINEERING OF PIG, HUMAN, AND NON-HUMAN PRIMATE TISSUES

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2020

**Front cover:** Immunofluorescence staining of  $\alpha$ -smooth muscle actin (red) and tropomyosin (green) in the normal porcine esophagus.

On tissue engineering of pig, human, and non-human primate tissues  
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*To my mother and brother*  
*To my wife and our children*

In the memory of my father



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## ABSTRACT

### BACKGROUND

Demand for donor organs for transplantation has been increasing every year more than the actual supply of suitable donor organs. One of the major problems associated with allogeneic transplantation includes lifelong immunosuppression. Tissue engineering and regenerative medicine is a growing field that uses knowledge of stem cell biology, developmental biology, immunology, and bioengineering to replace diseased and damaged tissues or organs. Tissue engineered (TE) hollow organs and tissues derived from natural extracellular matrix (ECM) have been used in several preclinical and clinical studies. More complex three-dimensional organs such as heart, liver, lungs, and kidney have been studied extensively both in-vitro and in-vivo in preclinical settings, but clinical experience is lacking. There is an increasing demand for understanding the composition of ECM, cell-ECM interaction in-vitro and in-vivo, and how tissue engineered organs behave immunologically after implantation. The current thesis focuses on investigation of decellularization methods for heart (porcine), esophagus (porcine, baboon, and human) and larynx (porcine); and recellularization of esophagus (porcine and human). It was also investigated during various time-points of recellularization if stem cells were able to synthesize ECM proteins, tissue specific proteins and growth factors, and if stem cells were able to differentiate into tissue-specific cells.

## METHODS

In Paper I, a detergent based decellularization method was developed to create acellular whole porcine hearts. The cardiac ECM was then characterized for its structural and mechanical properties. In Paper III, physical and chemical methods were developed to decellularize porcine larynx. Decellularized larynx was analyzed microscopically for its ultrastructural changes and presence of cells. In Paper II, decellularization and recellularization (with human amniotic mesenchymal stem cells and epithelial cells) of porcine esophagus was carried out. In Paper IV, decellularization of pig, baboon, and human esophagus was performed as per the method described in Paper II. Paper IV studied the cell-ECM interaction during recellularization of human esophagus with human amniotic mesenchymal stem cells by using the stable isotope labeling with amino acids in cell culture (SILAC) technique.

## RESULTS

Decellularization of heart, larynx, and esophagus was achieved successfully, with loss of cell nuclei, preservation of major ECM proteins such as collagen and elastin, preservation of growth factors, and maintaining three-dimensional structures of the tissues and organs. Decellularized esophagus was characterized by preservation of matrisome and non-matrisome proteins in the ECM using proteomics-bioinformatics analyses. Recellularization of pig and human esophagus was evidenced by stem cell proliferation, differentiation, and tissue specific protein synthesis by seeded stem cells. SILAC assay showed synthesis of newly produced proteins in the recellularized esophagus by seeded stem cells including ECM (collagens and fibronectin), cell-ECM signaling molecules (integrins), ECM regulators, secreted factors, skeletal muscle proteins, and proteins required for contraction of striated muscle.

## CONCLUSIONS

The decellularization protocol for heart, larynx, and esophagus was effective in removing cells while preserving ECM. Recellularization of esophagus showed the potential of human amniotic-derived stem cells for different tissue engineering applications. The SILAC based proteomics method can replace use of conventional proteomics in TE field to differentiate between cell and ECM proteins

**Keywords:** Decellularization, esophagus, heart, larynx, proteomics, recellularization, SILAC, stem cells, tissue engineering

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# LIST OF PUBLICATIONS

The thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. Methe K, Bäckdahl H, Johansson B R, **Nayakawde N**, Dellgren G, Sumitran-Holgersson S, 2014, An Alternative Approach to Decellularize Whole Porcine Heart. *BioResearch Open Access*, 3(6), 327-338.
- II. **Nayakawde NB**, Methe K, Banerjee D, Berg M, Premaratne GU, and Olausson M, 2020, *In Vitro* Regeneration of Decellularized Pig Esophagus Using Human Amniotic Stem Cells. *BioResearch Open Access*, 9.1, 22-36.
- III. **Nayakawde NB**, Methe K, Premaratne GU, Banerjee D, and Olausson M. Combined Use of Detergent and Ultrasonication for Generation of an Acellular Pig Larynx. (Submitted)
- IV. **Nayakawde NB**, Sihlbom C, Thorsell A, Banerjee D, Premaratne GU, Ul Haq U, Rivas Wagner K, Berg M, and Olausson M. Investigation of Extracellular Matrix Proteins in Decellularized Pig, Human, and Baboon Esophagus by Proteomics. (in manuscript)



# SUMMARY IN SWEDISH

## BAKGRUND

Efterfrågan på donerade organ för transplantation är mycket större än tillgången. Ett av de största problemen i samband med organ-transplantationer är de biverkningar som immundämpande läkemedel förorsakar. Genom ökad kunskap inom regenerativ medicin och de försök att tillverka kroppsegen vävnad som nu utvecklas, försöker man återskapa organ och vävnader från ett ramverk som kan bestå av kroppens stödjevvnader utan levande celler – ett så kallat bio-matrix/bio-scaffold eller extracellulär matrix (ECM). Regenerativ medicin är ett växande område som använder kunskap om stamcellsbiologi, utvecklingsbiologi, immunologi och bioteknisk tillämpning för att ersätta sjuka och skadade vävnader eller organ. ECM från ihåliga organ och vävnader tillverkat med hjälp av de och recellulariseringstekniker har använts i många prekliniska och kliniska studier. Mer komplexa tredimensionella organ som hjärta, lever, lungor och njurar har studerats omfattande i experimentella miljöer, men de har för närvarande ännu inte nått klinisk användning. Det finns ett ökande behov av att förstå sammansättningen av ECM, cell-ECM-interaktion in-vitro och in-vivo och hur organ tillverkade med vävnadsteknik beter sig immunologiskt efter implantation. Med lyckade resultat skulle nackdelar med transplantation och immundämpande läkemedel då kunna undvikas. Den aktuella avhandlingen fokuserar på undersökning av decellulariseringsmetoder för hjärta (svin), matstrupe (svin, babian och människa) och larynx (svin); och recellularisering av matstrupen (svin

och människa). Den undersöker också vid olika tidpunkter efter recellularisering om stamceller kan syntetisera ECM-proteiner, vävnadsspecifika proteiner och tillväxtfaktorer och differentiering av stamceller till vävnadsspecifika celler.

## **METODER**

I Studie I utvecklades en decellulariseringsmetod för att skapa hela acellulära hjärtan, baserat på tvättmedelsliknande kemikalier. Hjärt-ECM undersöktes sedan för dess strukturella och mekaniska egenskaper. I Studie III vidareutvecklades sedan decellulariseringstekniken med en fysikalisk och kemisk metod för att decellularisera luftstrupe på gris. Decellulariserad luftstrupe analyserades såväl elektronmikroskopiskt, för dess ultrastrukturella förändringar, som ljusmikroskopiskt för att bedöma närvaron av celler. I Studier II och IV utfördes decellularisering och recellularisering (med humant amnion härledda mesenkymala stamceller och epitelceller) av matstrupe från gris. I Studie IV utfördes decellularisering av svin, babian och mänsklig matstrupe, enligt metoden som beskrivs i Studie II, och cell-ECM-interaktionen under recellularisering av den mänskliga matstrupen med humana amnion-mesenkymala stamceller genom stabil isotopmärkning med en aminosyra i cellkultur (SILAC) teknik, studerades. I avhandlingen användes olika metoder som histologi, immunohistokemi, immunofluorescens, Luminex, proteomics, SILAC (med nLC-MS / MS) för att karakterisera decellulariserade och recellulariserade vävnader och organ.

## **RESULTAT**

Decellularisering av hjärta, struphuvud och matstrupe uppnåddes framgångsrikt, med förlust av cellkärnor, konservering av viktiga ECM-proteiner såsom kollagen och elastin, samtidigt med bevarande av tillväxtfaktorer och upprätthållande av tredimensionell struktur i vävnader och organ. Decellulariserad matstrupe visade bevarade matrisome och icke-matrisome proteiner i ECM genom proteomik-bioinformatik analyser. Recellularisering av svin och mänsklig matstrupe visade stamcellsproliferation, differentiering och vävnadsspecifik proteinsyntes av utsådda stamceller. SILAC-analys visade syntesen av nyproducerade proteiner i den recellulariserade matstrupen av utsådda stamceller innefattande ECM (kollagener och fibronektin), cell-ECM-signalmolekyler (Integriner), ECM-regulatorer, utsöndrade faktorer och skelettmuskel proteiner och proteiner som krävs för kontraktion av tvärstrimmiga muskler.

## **SLUTSATSER**

Decellulariserings protokoll för hjärta, struphuvud och matstrupe var

effektiva för att ta bort celler med till synes intakt ECM. Recellularisering av matstrupen visade på potentialen hos humana amnion-härledda stamceller för olika vävnadstekniska tillämpningar. Den SILAC-baserade proteomics metoden resulterade i ny information om cell-ECM-interaktion, ej tidigare funnen med standard proteomics undersökning.



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Heart decellularization and recellularization

In-vivo decellularization

In-vivo maturation of partially recellularized organs

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# ABBREVIATIONS

ADSCs	Adipose derived stem cells
AMSCs	Amniotic mesenchymal stem cells
bFGF	Basic fibroblast growth factor
BMSCs	Bone marrow stromal cells
COL12A1	Collagen alpha-1(XII)
COL6A1	Collagen alpha-1(XI)
COL6A2	Collagen alpha-2(XI)
COL6A3	Collagen alpha-3(XI)
DAPI	4', 6-diamidino-2-phenylindole
DC	Decellularized
DNase-I	Deoxyribonuclease-I
dsDNA	Double stranded deoxyribonucleic acid
ECM	Extracellular matrix
EpCAM	Epithelial cell adhesion molecule
FN1	Fibronectin-1
GAGs	Glycosaminoglycans
H&E	Hematoxylin and eosin
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HSPG2	Heparan sulphate proteoglycan 2
HUVECs	Human umbilical vein endothelial cells
IHC	Immunohistochemistry
iPSCs	Induced pluripotent stem cells
ITGB1	Integrin beta-1
MI	Myocardial infraction
MP	Movat's pentachrome
MSCs	Mesenchymal stem cells
MT	Masson's trichrome
nLC-MS/MS	Nano-scale liquid chromatographic tandem mass spectrometry
PDGF	Platelet-derived growth factor
PLGA	poly(lactic-co-glycolic acid)
PRELP	Prolargin
RC	Recellularized
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SILAC	Stable isotope labeling with amino acid in cell culture
SIS	Small intestine submucosa
TE	Tissue engineering
TEM	Transmission electron microscopy
TnBP	Tri(n-butyl) phosphate
UB	Urinary bladder
VCAN	Versican
VEGF	Vascular endothelial growth factor



# DEFINITIONS

**Bioreactor** – Can guide stem cells for appropriate cell differentiation and tissue development by providing the necessary biochemical and physical regulatory signals.

**Decellularization (DC)** – Decellularization is a process by which cells are removed from human or animal organs utilizing physical, chemical, or enzymatic treatment leaving acellular ECM.

**Extracellular matrix (ECM)** – Complex three-dimensional structure of extracellular proteins of structural, adhesive, and proteoglycans providing structural and biochemical support to the surrounding cells.

**Mass spectrometry** – Analytical technique that measures mass-to-charge ratio of ions produced by ionization technique.

**Proteomics** – Large scale experimental analyzes of proteins and proteome by means of protein purification and mass spectrometry.

**Recellularization** – Recellularization is a process of growing seeded cells on decellularized scaffold or synthetic scaffold with the aim of creating a functional organ that could replace or repair damaged tissue or an organ in-vivo.

**Stem cell** – cells with potential to self-renew and capacity to differentiate.

**Tissue engineering (TE)** – Tissue engineering field involves knowledge of engineering and life science to create laboratory-grown tissues and organs to restore, maintain, and improve the function of damaged tissues or organs.



# INTRODUCTION

## **Tissue engineering**

A shortage of donor organs remains the primary obstacle in the field of transplantation worldwide. According to the American Transplant Foundation, 20 persons die every day in the USA due to the lack of available organs for transplantation. In recent years, tissue-engineered (TE) organs have been suggested as a promising alternative source of organs for transplantation. The tissue engineering field involves knowledge of engineering and life science to create laboratory-grown tissues and organs to restore, maintain, and improve the function of damaged tissues or organs<sup>1</sup>. The field of tissue engineering was first introduced by Langer et al. in 1993<sup>1</sup>. But the first development of the field was as early as in 1963 when Becker et al. observed the differentiation of hematopoietic stem cells in the murine spleen<sup>2</sup>. The first scaffold seeded with cells was implanted in a rabbit knee joint. An allograft of decalcified bone was seeded with chondrocytes from rabbit articular cartilage which led to repairing the cartilage defect of the knee joint<sup>3</sup>. Embryonic stem cell research in mice and humans has led to the development of stem cell transplant therapy for cancer patients<sup>4,5</sup>. Many in-vitro studies on the use of biological/synthetic scaffolds, with or without cells, were developed in the laboratory setting. The purpose was to correct organ defects in various animal models which in turn founded the use of TE grafts/organs in clinical studies<sup>6</sup>.

**Table 1:** Notable advances in the tissue engineering and regenerative medicine field over the years

Year	Milestone	Reference
1963	Observed differentiation of hematopoietic stem cells in the spleen	Becker A.J. et al. <sup>2</sup>
1975	First decellularization of various organs (basement membrane isolation)	Meezan E. et al. <sup>7</sup>
1977	Chondrocytes were grown in scaffold and implanted in mice	Green W.T. et l. <sup>3</sup>
1981	Identification of embryonic stem cells	Martin G.R. et al. <sup>4</sup>
1985	Cell transplantation	Russell P.S. <sup>8</sup>
1993	Tissue engineering concept	Langer R. et al.
1998	Identification of embryonic stem cells in humans	Thomson J.A. et al. <sup>5</sup>
2001	Transplantation of TE pulmonary artery in a 4-year-old girl	Shin'oka T. et al. <sup>9</sup>
2006	Mouse with growing human ear cartilage on the back	Vacanti C.A. et al. <sup>10</sup>
2006 & 2011	Transplantation of bladder and urethra (synthetic scaffold, autologous cells)	Atala A. et al. <sup>11</sup> Raya-Rivera A. et al. <sup>12</sup>
2008	Recellularized beating rat heart	Ott H.C. et al. <sup>13</sup>
2011	Use of TE organs for in-vitro drug discovery	Elliott N.T. et al. <sup>14</sup>
2014	Transplantation of bone marrow stem cells to repair cartilage	Yamasaki S. et al. <sup>15</sup>

### *TE- Tissue engineering*

#### **Clinical application of TE construct**

The clinical use of TE organs started in the early 21<sup>st</sup> century. In 2001, a TE vessel graft was created for a 4-year-old girl. The scaffold was made from polycaprolactone–polylactic acid copolymer with woven polyglycolic acid. This scaffold was then seeded with the girl's autologous peripheral vein cells. Subsequently, the graft was transplanted into the child in order to replace an occluded pulmonary artery<sup>9</sup>. Seven months after the transplant, the patient was doing well without any occlusion of the transplanted TE arterial graft. Atala et al. succeeded in the first clinical application of an autologous TE bladder substitute in seven patients who were in need of a cystoplasty<sup>11</sup>. The patients were either transplanted with a graft consisting of collagen alone (decellularized bladder submucosa) or collagen or polyglycolic acid seeded with autologous urothelium and muscle cells. These cells had been isolated from the patient's bladder biopsy. Post-transplant follow-up after 22-61 months showed an improvement of the bladder function of the TE

**TABLE 2.** Clinical use of tissue-engineered esophagus

Year	Scaffold	Number of patients	Study
2011	Decellularized porcine SIS (Surgisis)	5	Badylak et al. <sup>17</sup>
2012	Autologous epithelial cell sheet	9	Ohki et al. <sup>18</sup>
2012	Decellularized porcine SIS (Surgisis)	3	Hoppo et al. <sup>19</sup>
2014	Porcine UB (MatriStem)	4	Nieponice et al. <sup>20</sup>
2016	Human skin (AlloDerm)	1	Dua et al. <sup>21</sup>

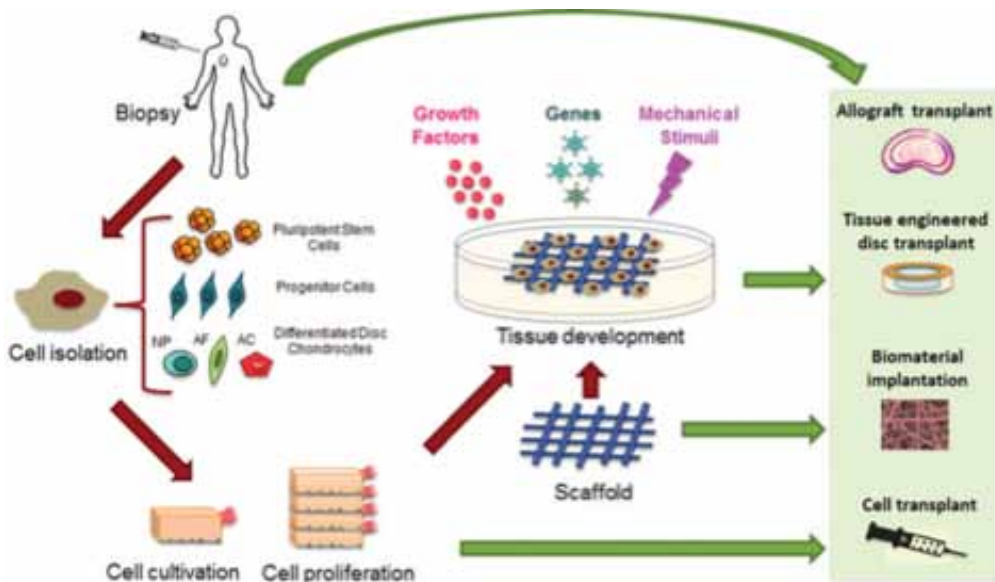
*SIS-small intestine submucosa, UB-urinary bladder*

bladder constructs with minimal postoperative complications. Although many TE organs have been successful in clinical practice, they are currently limited to simple hollow organs such as the esophagus (Table 2), blood vessels, trachea, urethra, and bladder<sup>9, 11, 12, 16-22</sup>. Currently, more complicated TE organs like the heart, kidney, liver, and lungs have not made their way to clinical trials.

### Why do tissue engineering?

The demand for donor organs greatly outpaces the supply of donated organs. Many people die waiting for a suitable donor organ, and this number is increasing yearly. While there is an upward trend in the annual number of deceased and live donors, it never covers the actual demand for transplantation<sup>23</sup>. There is an increasing demand to study other alternatives to fill the organ shortage.

Although conventional transplantation can save the lives of patients in addition to improving their quality of life, it is associated with the risk of allograft rejection. Many types of immunological barrier exist between donor and recipient, namely, ABO blood group, HLA typing, and cross-matching. Hyperacute rejection tends to occur within hours after transplantation due to the presence of anti-donor antibodies in the host which facilitates coagulation and destruction of the graft. Acute rejection occurs from late in the first week to months after transplantation. This is primarily governed by the recipient's T-cell response to the donor allograft and innate immunity. Chronic rejection occurs after months to years after allograft transplantation. This is mediated by both anti-donor antibody and innate immune cell activation, which in turn cause allograft rejection<sup>24</sup>. Transplanted patients are put on lifelong immunosuppressive drugs to avoid the risk of allograft rejection. Unfortunately, due to this immunosuppressive therapy, they are at increased risk of various opportunistic fungal and viral infectious diseases. Common opportunistic pathogens include cytomegalovirus,



**FIGURE 1.** Tissue engineering and regenerative medicine concept. Adapted from Moriguchi Y. et al.<sup>26</sup>

herpes simplex virus, tuberculosis, aspergillus, candida, and pneumocystis. They are also at increased risk of common bacterial infections leading to urinary tract infections, cholangitis, and life-threatening conditions like pneumonia. Other medical conditions associated with the long-term use of immunosuppressants include skin cancer, solid organ and hematological malignancy, bone marrow suppression, and an increased risk (50% deaths in renal transplant patients) of cardiovascular disease<sup>25</sup>.

TE organs have the potential to address issues of organ shortage and to avoid the immunosuppressive drugs required after conventional organ transplantation. TE organs can be made from a biological source (ECM) or a synthetic scaffold, with or without autologous cells, thereby avoiding immunosuppressive drugs (Fig.1).

### Types of scaffold

The basic backbone of a TE organ is a 3D scaffold, cells, and growth factors necessary for the generation of the tissue or organ for in vivo transplantation or implantation<sup>1, 27</sup>. The scaffolds used in TE are generally derived from a natural

source (ECM) or synthetically made in the laboratory. This 3D scaffold is essentially the structure of the organ without cells in it. The scaffold plays a vital role in creating an optimal microenvironment lending support to the seeded cells *in-vitro* or incoming cells *in-vivo* (in the host body). It mimics the host's tissue or organ. Synthetic scaffolds should resemble the quality of natural ECM. They do this by providing not only mechanical support to cells, but also by supporting cell proliferation, migration, and differentiation<sup>28</sup>. The design and composition of a synthetic scaffold is based mainly on the tissue/organ type and place of implantation. The criteria for an ideal synthetic scaffold include non-toxicity, sterility, biocompatibility, desired degradation rate, as well as appropriate surface and mechanical properties. Apart from this, the synthetic scaffold must not cause an immune reaction or foreign body reaction upon *in-vivo* implantation.

Natural ECM is derived from biological organs and tissues by the process of decellularization. Decellularization is a process by which cells are removed from human or animal organs utilizing physical, chemical, or enzymatic procedures<sup>29</sup>. Decellularized organs are complex 3D structures consisting of proteins and growth factors left over after the decellularization process. The major advantage of using natural ECM over a synthetic scaffold is that the natural ECM comes from the human or animal body. Thus, they retain the structure and growth factors necessary for cell proliferation and survival. However, the quality of natural ECM is dependent on limiting damage to the native organ or tissue during the decellularization process to preserve the architecture, proteins, and growth factors in the resulting product. Natural ECM should also be immunologically inert, thereby not causing an immune reaction or risk of rejection upon *in-vivo* implantation. Ideally, natural ECM should be mechanically stable, non-toxic, sterile (free from bacteria and viruses), and with no donor cells (DNA).

## **Synthetic scaffold**

### **Natural polymer-based ECM**

Both synthetic and natural origin polymers are used to create synthetic scaffolds. Natural polymeric scaffold materials include collagen, hyaluronic acid, alginate, chitosan, elastin, fibrinogen, and silk<sup>30</sup>. These natural polymeric ECMs are superior to synthetic ones with regard to biocompatibility, biodegradability, and cell adhesion properties<sup>31,32</sup>. However, they lack the batch consistency, lower degradation rate, and good mechanical strength of synthetic ECMs. Since natural polymers are usually derived from plant or animal sources, they are also more likely to cause an immune reaction after implantation.

## **Synthetic polymer-based ECM**

Synthetic polymeric scaffolds have greater uniformity in terms of mechanical strength, degradation rate, and sterility. Batch variability is less of a concern. However, there are some drawbacks associated with such scaffolds, such as lack of key growth factors and protein structure for cell attachment and growth. Another problem associated with such scaffolds is that the degradation of the polymers *in-vivo* can lead to an inflammatory reaction<sup>33</sup>. The most common synthetic scaffolds are made from Poly (lactic acid), Poly (glycolic acid), Poly (lactic acid-co-glycolic acid), Poly (caprolactone), and Polydioxanone. Such scaffolds have been in use for different tissue engineering applications such as skin, cartilage, bone, ligament, tendon, vessel, nerve, bladder, and liver<sup>34</sup>. The production of synthetic scaffolds is generally carried out by electrospinning, phase separation, leaching techniques (creation of porous scaffold by use of paraffin, sugar, and gelatin in polymer solution and later dissolved by immersion), and computer-aided design technique (3D printing)<sup>34</sup>.

## **3D porous scaffold**

This type of scaffold is created by using natural (marine resources) or synthetic materials. Such scaffolds have a major advantage over other synthetic scaffolds because they do not tend to cause an inflammatory reaction upon *in-vivo* degradation.

## **Hydrogel based scaffold**

In recent years, hydrogel-based 3D scaffolds have become very popular in the TE field due to their hydrophilic nature which is similar to that of natural ECM. The hydrated structure of hydrogel creates a complex 3D biocompatible matrix which can be coupled with living cells by bioprinting technology. Such techniques are already used in the process of tissues such as skin, blood vessels, bone, and cartilage<sup>35</sup>.

## **Injectable hydrogel**

These soft hydrogels are an attractive source for the delivery of cell-based scaffold to correct defects between soft and hard tissues. A natural polymer such as silk fibroin hydrogel has been suggested as a corrective gel filler for cartilage and bone repairs<sup>36</sup>.

## **Natural scaffold**

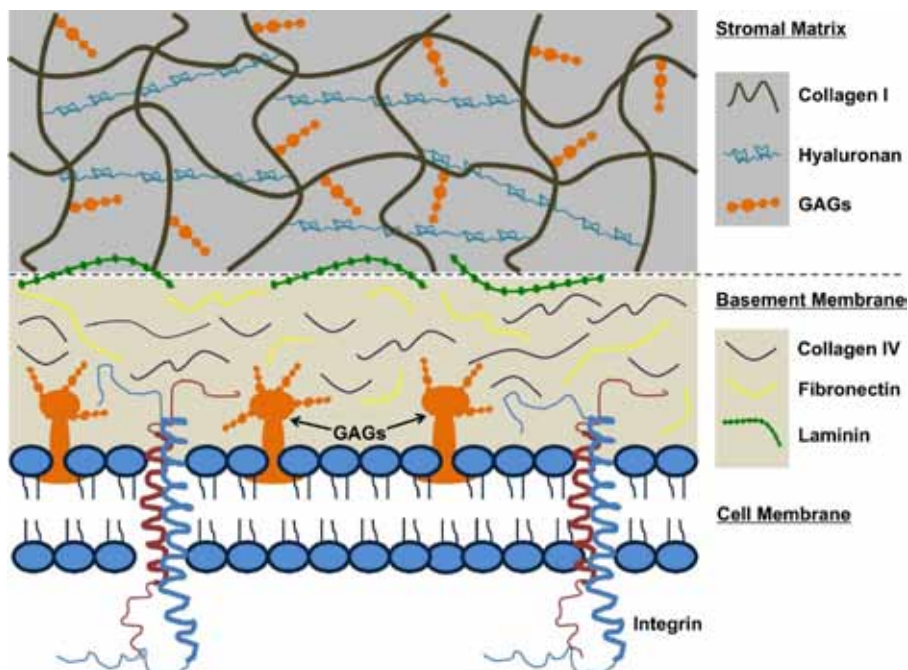
Natural scaffolds are generally derived from animal or human tissues after

**TABLE 3.** Commercial natural ECM products prepared with decellularization methods.

Product (Manufacturer)	Tissue Source	Application Focus
AlloDerm® (Lifecell Corp.)	Human dermis	Soft tissue
AlloPatch HD™, FlexHD® (Musculoskeletal Transplant Foundation)	Human dermis	Tendon, breast
NeoForm™ (Mentor Worldwide LLC)	Human dermis	Breast
GraftJacket® (Wright Medical Technology Inc.)	Human dermis	Soft tissue, chronic wounds
Strattice™ (Lifecell Corp.)	Porcine dermis	Soft tissue
Zimmer Collagen Repair Patch™ (Zimmer Inc.)	Porcine dermis	Soft tissue
TissueMend® (Stryker Corp.)	Bovine dermis	Soft tissue
MatriStem®, Acell Vet (Acell Inc.)	Porcine urinary bladder	Soft tissue
Oasis®, Surgisis® (Cook Biotech Inc.)	Porcine small intestine	Soft tissue
Restore™ (DePuy Orthopaedics)	Porcine small intestine	Soft tissue
FortaFlex® (Organogenesis Inc.)	Porcine small intestine	Soft tissue
CorMatrix ECM™ (CorMatrix® Cardiovascular Inc.)	Porcine small intestine	Pericardium, cardiac tissue
Meso BioMatrix™ (Kensey Nash Corp.)	Porcine mesothelium	Soft tissue
IOPatch™ (IOP Inc.)	Human pericardium	Ophthalmology
OrthoAdapt®, Unite® (Synovis Orthopedic and Woundcare Inc.)	Equine pericardium	Soft tissue, chronic wounds
CopiOs® (Zimmer Inc.)	Bovine pericardium	Dentistry
Lyoplast® (B. Braun Melsungen AG)	Bovine pericardium	Dura mater
Perimount® (Edwards Lifesciences LLC)	Bovine pericardium	Valve replacement
Hancock® II, Mosaic®, Freestyle® (Medtronic Inc.)	Porcine heart valve	Valve replacement
Prima™ Plus (Edwards Lifesciences LLC)	Porcine heart valve	Valve replacement
Epic™, SJM Biocor® (St. Jude Medical Inc.)	Porcine heart valve	Valve replacement

*Adapted from Crapo P. et al<sup>29</sup>.*

the decellularization process. Natural ECM is a 3D complex structure of proteins, growth factors, and signaling molecules without donor cells. ECM is composed of different structural and functional proteoglycans, cell adhesion proteins, and extracellular vesicles that all give support to the cells. This natural ECM is evolutionarily conserved and created inside the living body by the resident cells. Moreover, natural ECMs have proved to be much better in supporting cell



**FIGURE 2.** Natural ECM proteins, their arrangements, and attachment sites to the cell. GAGs- Glycosaminoglycans. Adapted from Kuraitis, D. et al.<sup>39</sup>

differentiation, mitogenesis, and chemotaxis than natural polymer-based ECMs<sup>37</sup>.

The concept of decellularization was first attempted in 1975, but the application of this technique for TE and regenerative medicine started in the late 20<sup>th</sup> century<sup>7</sup>. Recently, allogeneic and xenogeneic ECM prepared by decellularization techniques have been used in clinical transplantation of bladder, vein, and esophagus<sup>11, 16, 17, 19, 21</sup>. Commercial, off-the-shelf products, consisting of allogeneic and xenogeneic ECM prepared by decellularization processes, are available for clinical use (Table 3)<sup>29</sup>. Theoretically, the risk of rejection for allogeneic and xenogeneic decellularized ECM implants is lower since the natural ECM lacks immunogenic donor cells. However, the in-vivo effect of donor ECM needs further investigation. Another drawback associated with natural ECM is the risk of bacterial and viral infection during the decellularization process. Sterilization of such scaffolds after the decellularization process with acids/radiation/alcohol is therefore recommended.

## **Composition of natural ECM**

### **Collagen**

ECM is mainly made up of collagen (~85%) protein which gives structure, shape, and strength to the natural scaffold. In total, 28 different types of collagen have been identified, and their type differs depending on the tissue and organ in question<sup>38</sup>. Collagen type I found abundantly in the human body, predominantly in tissues such as skin, tendon, and bone. Collagen type II is mainly found in cartilage and collagen type IV (Fig.2) is mostly present in the basement membranes. Other proteins found in the ECM which contain a collagen-like triple helix structure are EMILIN-1 and 2, adiponectin, and complement C-1.

### **Elastin**

Elastin is a particularly important protein of the ECM for maintaining the mechanical load-bearing properties, elasticity, and contractibility in organs such as skin, lungs, artery, and elastic cartilage. Elastin is derived from tropoelastin polymer and helps make up elastic fibers in the tissues. Elastin is responsible for mechanical integrity, structural organization, and biological signaling in the ECM<sup>40</sup>. It is essential to preserve elastin in natural ECM during decellularization because elastin plays a major role in cell adhesion, proliferation, and differentiation by activating cellular receptors such as integrins and GAGs. Elastic fibers are co-existing with other structural fibers, e.g. collagen.

### **Fibronectin**

Fibronectin is the second most abundant protein found in the ECM after collagen. It is found both as a soluble form in blood and in fibrillar form in the ECM. Fibronectin is a dimeric molecule and has a binding site for ECM molecules including collagen, fibrin, and heparan sulfate. Different fibronectin variants are also responsible for cell binding through cell signaling receptor integrin and differentiation<sup>41</sup>. It is widely used as a coating material for cell adhesion and growth in cell culture plates.

### **Laminin**

Laminin is a large molecular-weight glycoprotein, mostly found at the basement membrane of the ECM. In humans, there are 11 distinct laminin chains found as alpha, beta, and gamma subunits<sup>42</sup>. It has high-affinity binding sites for collagen IV and heparan in the ECM. Laminin alpha chain of C-terminus of the

long arm laminin is believed to be responsible for attachment of cell signaling molecules of integrin which is essential for cell adhesion, migration, and differentiation<sup>43</sup>. Laminin is also expressed in the blood vessel basement membrane and is important for cell adhesion. Other essential adhesive proteins found in the ECM include vitronectin, thrombospondin, and tenascin.

### **Glycosaminoglycans (GAGs)**

GAGs are negatively charged complex carbohydrates consisting of mucopolysaccharides. They are hydrophilic and viscous molecules present on cells and in the ECM. Because of their viscosity, they tend to store growth factors and bind to chemokine, cytokine, and adhesion molecules<sup>44</sup>. GAGs are classified as sulfated and non-sulfated. Non-sulfated GAGs contains hyaluronic acid. Hyaluronic acid acts as a protective lubricant in joints and cartilage. Sulfated GAGs include heparan sulfate, chondroitin sulfate, keratin sulfate, and dermatan sulfate. Chondroitin sulfate gives mechanical strength to cartilaginous tissues. Heparin is another example of a potent anti-coagulant used clinically. Other types of extracellular GAG's containing proteoglycans are versican, biglycan, decorin, fibromodulin, and testican<sup>45</sup>.

### **Growth factors**

Growth factors are essential for cell survival, proliferation, and differentiation. Many growth factors are bound and stored in the ECM through binding sites of ECM proteins such as fibronectin, heparan sulfate, and heparin<sup>46</sup>. Growth factors such as vascular endothelial growth factors (VEGF), hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGF) bind to fibronectin in the ECM<sup>47, 48</sup>. It is also believed that growth factors are released by the degradation of ECM proteins.

### **Matrisome proteins**

In mammals, the core matrisome is comprised of ~300 ECM proteins<sup>47</sup>. Matrisome proteins are classified as core matrisome proteins and matrisome associated proteins. Studies on matrisome proteins in the ECM has shown beneficial in the field of cancer research to identify abnormal tumor matrix proteins<sup>49-51</sup>. Matrisome proteins are also crucial in cell-cell interaction and cell-matrix interaction.

### **Decellularization**

Decellularization is achieved by the use of physical, chemical, or enzymatic

methods on allogeneic or xenogeneic tissue or organs in order to remove cellular material. It can be achieved by employing perfusion of the organ through its vasculature or the lumen of the hollow organs such as blood vessels, trachea, esophagus, and intestine. Each method of decellularization has its advantages and disadvantages. Any decellularization method causes some degree of damage to the ECM proteins and the mechanical structure of the ECM. So the choice of decellularization method, exposure time, and temperature mostly depends on the type of tissue, its cellular density, mechanical strength, and thickness of tissue<sup>29</sup>. For effective decellularization in tissues, especially when dense, a combination of physical, chemical, and enzymatic decellularization methods are used.

### **Physical methods**

Physical methods for decellularizing tissues and organs include freeze-thawing, application of force (compression), pressure, electroporation, and sonication.

In the freeze-thaw process, ice crystal formation causes a breakdown of the cell membrane in the tissue. Dead cells trapped in the tissue can be removed by a washing step combined with other decellularization methods such as chemical decellularization<sup>52, 53</sup>. Like every other method, the freeze-thaw method also adversely affects the resultant scaffold architecture, but it does not cause a major change in the mechanical properties of the decellularized tissue<sup>29</sup>.

Force and pressure aided decellularization is an effective method, but it can cause damage to the ECM. Decellularization of blood vessels can be achieved through the combination of pressure methods and washing with mild detergents<sup>54</sup>.

Non-thermal irreversible electroporation involves high pulsing energy selectively damaging the cell membrane without producing heat. This method is useful in limiting ECM destruction to a minimum level. Electroporation has been used to decellularize porcine livers effectively<sup>55</sup>.

### **Whole organ perfusion**

Perfusion-decellularization with detergents or chemicals work by applying transmural flow pressure across the vessel wall of the 3D organ, causing uniform cell lysis when compared to agitation with chemicals<sup>56</sup>. Vascularized organs such as the heart, lungs, kidneys, and liver offer an advantage in that the existing vascular network can be used to deliver decellularizing agents effectively and uniformly without disturbing the 3D architecture of the scaffold. Hollow organs, such as blood vessels, esophagus, and intestines, can be perfused through the

luminal side of the organ for effective decellularization<sup>57</sup>.

### **Agitation decellularization**

Agitation with detergents or solvents can be used to decellularize thick and thin tissues. It acts by constant contact and diffusion of the chemical into the tissue, thereby causing cell death and removal of the cells. This technique is useful for non-vascular tissues like the cartilaginous tissues of the trachea and larynx which require more rapid agitation rates for a longer period of time. It is also useful for thin tissues like skin, ligaments, small intestinal submucosa, and bladder.

### **Ultrasonication**

Sonication can be useful for decellularization of dense tissue where diffusion of chemicals is of great concern. Sonication works by converting electrical signals into physical vibration of the tissue. It acts by mechanically loosening the collagenous matrix of the dense tissues to dislodge cells from the ECM. Sonication creates heat during the application. To avoid ECM damage caused by heat, tissue samples are generally immersed in a cold solvent during the process. Sonication can be used for the decellularization of cartilage, ligaments, and skin.

### **Chemical methods**

Acids and alkalis work by solubilizing cytoplasmic components of cells and thereby destroying nucleic acid and causing cell lysis. But this method has a destructive effect on ECM proteins such as collagen, GAGs, and growth factors<sup>29</sup>. Frequently used acids include peracetic acid and acetic acid whereas bases commonly used include sodium hydroxide and sodium sulfites. Acid treatment combined with detergent treatment has been used to decellularize porcine dermal matrix<sup>58</sup>.

Hypotonic and hypertonic solutions can be used for decellularization either alone or in combination with detergents for organs such as blood vessels and bladder<sup>59,60</sup>. It acts by providing an osmotic shock to the cells and disrupts DNA-protein bonding.

### **Detergents**

The surface acting agents are a popular method for decellularization of various 3D organs and flat sheet tissues<sup>29</sup>. They are classified as non-ionic, ionic, and zwitterionic (a type of surfactant having one positive and one negative charge) detergents. Most detergents act by solubilizing cell membrane and dissolving nuclear DNA from protein in the tissue.

Ionic detergents such as sodium deoxycholate (SDC) and sodium dodecyl sulfate (SDS) are examples of popular choices for decellularization. However, they tend to act on protein-protein interaction of the ECM which causes loss of ECM proteins during the decellularization process. Although they are very efficient in cell removal, GAGs are usually significantly reduced when using ionic detergents. On the other hand, elastin is well preserved in the tissue after this type of decellularization process<sup>29, 57, 61</sup>.

Non-ionic detergents such as Triton X-100 act on the DNA-protein interaction to remove cells from even denser tissue. It also acts on lipid-to-lipid and lipid-to-protein bonds in the tissue, but it is somewhat milder in action than SDC and SDS.

CHAPS and sulfobetaine-10 and 16 are zwitterionic detergents that act the same way as ionic and non-ionic detergents, but with greater efficiency and less destruction of the ECM proteins. CHAPS provides effective cell removal potential in thin tissue when used alone, but it fails to remove the cells from thick tissues completely<sup>58</sup>.

## Enzymes

Enzymes such as nuclease (DNase-I and benzonase) are highly specific for the removal of DNA from the tissue, but they are difficult to wash away from the tissue. They are usually preferred in combination with ionic detergents in detergent-enzymatic protocols. These protocols can be used for various tissues ranging from hollow organs, such as esophagus to cartilaginous trachea tissues<sup>57, 61, 62</sup>.

The use of enzymes such as trypsin, dispase, and collagenase tend to disrupt ECM proteins like collagen and may have the unfortunate consequence of affecting the mechanical properties of the tissue. Xenogeneic tissues are generally treated with enzyme  $\alpha$ -galactosidase to remove Gal epitopes from the tissues. Gal epitopes can trigger hyperacute rejection in humans and monkeys when receiving xenogeneic organ transplants.

## Other solvents

Alcohols such as ethanol and acetone act by dehydration of cellular material as well as acting on lipids in the ECM. These solvents are very effective in decellularization of dense tissue structures, but at the same time, they tend to crosslink with the ECM proteins rendering them chemically ineffective.

Tributyl phosphate (TnBP) works on protein-to-protein interaction in the ECM and tends to destroy collagen tissue.

## Characterization of DC tissues

### Sterility

Sterility of the decellularized scaffold is one of the vital parameters considering its *in-vivo* application or use for *in-vitro* studies. Unsterile or infected implantable grafts can cause infection upon in-vivo implantation. Peracetic acid in low concentrations can be used to sterilize decellularized scaffolds. Other sterilization methods for ECM include UV radiation, gamma radiation, and ethylene oxide. Xenogeneic ECM always poses a risk of viral infection, so using virucide agents is mandatory.

### DNA

It is a well-known fact that residual DNA in the decellularized ECM can cause an immune reaction upon in-vivo implantation<sup>63</sup>. Although cell debris in the ECM is not alive, it will be enough to cause an inflammatory reaction and even rejection in the host. Double-stranded DNA can be quantified by using DNA isolation and measurement with a spectrophotometer. The minimal acceptable criterion mentioned and generally accepted by the TE field is <50 ng/mg of dry weight<sup>29</sup>. The length of DNA fragments present in the ECM should also be below 200 bp. Finally, nuclear staining with 4', 6-diamidino-2-phenylindole (DAPI) and H&E should indicate that no positive (blue) colored cell nuclei should be visible in the decellularized ECM.

### Gal-epitopes

Gal epitopes on the xenogeneic decellularized ECM can cause an inflammatory reaction and hyperacute reaction upon *in-vivo* implantation. Immunohistochemistry (IHC) can be useful to detect alpha-Gal epitopes in xenogeneic ECM.

#### Mechanical test

It is necessary to investigate the effect of the decellularization method on the mechanical properties of the decellularized tissue. Biomechanical testing of decellularized tissue such as cartilage, bone, and ligament is crucial prior to in-vivo study to increase the chance of success. It is also important in in-vitro recellularization where cells modify their shape and size according to the 3D environment. There are several ways to determine the mechanical properties of decellularized organs, and the choice of method is mainly based on the type of organ or tissue. For soft tissues, elastic modulus, burst pressure, and stress-strain relationship are commonly used biomechanical tests.

## **ECM proteins and growth factors analyses**

Decellularized organs are mainly composed of proteins and growth factors after removing the cellular material from the tissue or organ. Investigation of different cellular, cytoplasmic, nuclear, and ECM proteins is possible with the help of IHC, biochemical assays, Luminex detection, and proteomics analyses. Most researchers have only focused on the detection of the immunogenic component of nuclear proteins, assuming that other cell-membrane and cytoplasmic proteins do not cause an immune reaction. However, there is a knowledge gap in the understanding of ECM-detergent protein complex and non-nuclear donor cell protein in decellularized tissue or organ and how it will behave in the *in-vitro* and *in-vivo* settings.

Proteomics is a powerful and sensitive method to identify proteins and peptides in the tissue sample. From a very small sample size, thousands of proteins can be identified. With the available bioinformatics tools, the prediction of different signaling and molecular pathways is now possible by referring to a list of identified proteins. In addition, quantitative proteomics is able to detect and quantify the amount of protein in the analyzed sample. Similarly, Luminex technology can detect as little as 1 picogram of growth factors in the ECM.

## **Recellularization**

Recellularization is a process of growing seeded cells on the decellularized scaffold or synthetic scaffold with the aim of creating a functional organ that can replace or repair a damaged tissue or organ *in-vivo*. Three basic components required for recellularization are a decellularized scaffold, a cell source, and media containing growth factors. In order to support 3D scaffold culture and provide an *in-vivo* like environment, a bioreactor is pivotal.

## **Cell source**

Stem cells have the ability of self-renewal, and they are able to differentiate into multiple lineages without losing their characteristics<sup>64</sup>. They should also possess qualities such as availability in large quantities, be easy to harvest, have great differentiation capacity, and be safe for patient use (should not form teratoma).

## **Adult cells**

Adult stem cells are not totipotent but pluripotent. Attempts have been made to recellularize porcine and human organs with tissue-specific primary adult cells. However, bearing clinical applications in mind, adult primary cells are limited in terms of their use because their proliferation and expansion capacities are

insufficient to generate human-size recellularized organs.

### **Mesenchymal stem cells (MSCs)**

#### **Bone marrow stem cells (BMSCs)**

Recent advances have shown that BMSCs have been used effectively in the clinic due to their capacity to differentiate and patient safety due to their inherent autologous nature<sup>65</sup>. However, collection of bone marrow from the patient is an uncomfortable and painful procedure which may lead to complications in the form of harvest site infection<sup>66</sup>. Older patients have also been shown to have a smaller population of bone marrow stem cells which are unable to generate sufficient cell numbers for recellularization. Consequently, better sources of stem cells are needed for recellularization.

#### **Adipose-derived stem cells (ADSCs)**

ADSCs have become popular due to the fact that they can be isolated easily from subcutaneous fat by liposuction without major surgery. Like BMSCs, ADSCs have the capacity to differentiate into multiple lineages<sup>67</sup>. Also, in-vitro studies on ADSCs have shown a reduction in the inflammatory and T-cell response<sup>68</sup>.

#### **Human amniotic derived stem cells**

Human amniotic epithelial cells are ectodermal derived epithelial cells and found in contact with amniotic fluid in the amniotic sack. Amniotic mesenchymal stem cells (AMSCs), on the other hand, are found in the layer beneath the epithelial layer (basement membrane). Both epithelial and mesenchymal stem cells have immunomodulatory properties, and they can be isolated from the discarded amniotic membrane in billions. AMSCs show positive staining for mesenchymal stem cell markers of CD90, CD166, and CD105<sup>69</sup>. Amniotic membrane has been used routinely in clinics to treat burn patients without immunosuppressants<sup>70</sup>,<sup>71</sup>. Amniotic stem cells can be used for the recellularization of natural ECM, and can, therefore, be suitable for transplantation without any need for immunosuppressants.

#### **Induced pluripotent stem cells (iPSCs)**

iPSCs are reprogrammed somatic cells with specific pluripotent genes to generate embryonic-like stem cells. These types of cells have an advantage in that they do not cause an immune reaction. They are pluripotent, so the expansion and proliferation are good. Billions of cells can be grown and used for the recellularization of decellularized organs. Recently, iPSCs have been used to create

recellularized human and rat lungs<sup>72</sup>.

### **Bioreactor**

A bioreactor in TE is used to culture the 3D decellularized tissues or organs. It maintains sterility, provides space for the 3D structure of ECM and cells, and delivers growth factors and culture media during the recellularization. Static culture conditions would never be able to give stimulus for the generation of a recellularized 3D organ with homogenous cell distribution. Further, in static culture, if the organ is thicker, necrosis occurs in the middle of the organ due to insufficient nutrient delivery, and waste removal is limited since, in this setting, the nutrient exchange happens only by diffusion<sup>73</sup>. Bioreactors used in the TE field are generally custom made for different types of organs. Vascularized organs require proper settings such as perfusion inlet and outlet for arteries and veins, monitoring of pressure and resistance in the blood vessels, temperature, pH, glucose, oxygen level, and lactate. Whereas avascular tissues like cartilage, ligament, and bone require mechanical stimulus in a chamber bioreactor (spinner flask bioreactor).

The currently used bioreactors have only focused on some of the fundamental requirements, such as culture media circulation, pressure, and oxygen delivery, etc. The demand for assessing cellular health, metabolism, physiological conditions of acid and salt balance, and osmolarity in the bioreactor is growing. The ex-vivo recellularization of organs required advanced engineering skills to develop non-invasive techniques for analyses of cell coverage, mechanical properties, and mechanical stimulus<sup>74</sup>.

### **Cell-ECM interaction**

It is crucial to understand how cells-ECM interaction takes place during recellularization. It is known that cells have specific binding sites for the ECM receptors that act in a lock-and-key pattern for cell-ECM communication<sup>37</sup>.

### **Non-integrin**

Syndecan is one of the cell surface receptors that binds to ECM collagen, fibronectin, thrombospondin, and basic fibroblast growth factor (bFGF). CD 44 is another cell surface protein, which binds to collagen types I and IV and hyaluronan in the ECM. This binding is vital for cell adhesion and migration.

### **Integrin**

Integrins are cell surface receptors responsible for cell adhesion, proliferation,

survival, and differentiation by connecting the extracellular domains to cytoplasm<sup>75</sup>. Integrins have alpha and beta subunits. Cell-ECM binding is mostly governed by integrin signaling. Different types of integrin bind to a number of ECM ligands through focal adhesion. For the homogenous recellularization of 3D organs, it is crucial to study integrin binding to ECM.

### **Characterization of recellularized tissues or organs**

Seeded cells can be visualized by DAPI and H&E staining. The growth of cells can be analyzed with histological staining. Quantitative cell measurement can be performed by counting cells on a histologically stained section of recellularized tissue. This will give an idea of whether cells have grown homogeneously in the recellularized tissue or not. Electron microscopy can help to understand cell-ECM interaction at the ultrastructural level.

DNA quantification of the recellularized tissue sample gives a quantitative measurement of cellular growth in the tissue.

Biomechanical properties of the recellularized graft are known to decide the fate of the graft upon *in-vivo* implantation. Mechanical testing of recellularized tissues can be performed to test the effect of *ex-vivo* culture conditions at 37°C and has an impact on the strength of the recellularized tissue. IHC can be helpful to understand if the seeded stem cells have differentiated into tissue-specific cells.

Proteomics can be helpful in understanding the effect of seeded stem cells on the matrisome proteins of decellularized tissue. This is also important in understanding if the seeded cells have produced new proteins in the ECM. Stable isotope labeling with amino acids in cell culture (SILAC) is extremely useful in understanding the nature of newly synthesized proteins from seeded stem cells in the recellularized tissue. In this assay, culture media containing heavy isotopically labeled amino acids can be used to grow stem cells during recellularization. Proteomics analyses of recellularized samples shows both an ECM proteins peak (light) and a stem cell (heavy) labeled protein peak which can differentiate newly produced proteins from native ECM proteins. This technique can also be used in *in-vitro* settings to investigate if stem cells are able to produce ECM proteins and replace existing ECM proteins.

Confirmation of proteomics data can be performed using Western blot and IHC. Gene expression analyses of recellularized tissue can be done with polymerase chain reaction (PCR).

TABLE 4. In-vitro recellularization of the esophagus

Species (if natural scaffold)/Synthetic	Scaffold	DC	RC	Cells	Year	Author
Dog	Porcine urinary bladders	Yes	No	Dog Tx model	2006	Nieponice et al. <sup>76</sup>
Pig	DC esophagus	Yes	Yes	Canine BMSCs	2013	Bo tan et al. <sup>77</sup>
Mouse	Porcine urinary bladders	Yes	No	Mouse Tx model	2013	Nieponice et al. <sup>78</sup>
Synthetic	Poly(ester urethane) scaffolds with Silk fibroin	No	Yes	Primary esophageal smooth muscle cell	2016	Hou et al. <sup>79</sup>
Pig	Electrospun synthetic nanofibre matrices	No	Yes	Pig mucosal esophageal cells	2017	Barron et al. <sup>80</sup>
Pig	DC esophagus	Yes	Yes	ADSCs	2018	Luc et al. <sup>81</sup>
Rat	DC esophagus	Yes	Yes	Human mesoangioblasts and mouse fibroblasts	2018	Urbani et al. <sup>82</sup>
Synthetic	Synthetic polyurethane electro-spun grafts	No	Yes	Adipose-derived mesenchymal stem cells	2018	La Francesca et al. <sup>83</sup>
Pig	DC esophagus	Yes	Yes	Human aortic smooth muscle cells or human ADSCs	2018	Wang et al. <sup>84</sup>
Pig	Small intestine submucosa (SIS) with poly(lactic-co-glycolic acid (PLGA)	Yes	Yes	Human esophageal smooth muscle cells	2019	Syed et al. <sup>85</sup>
Human	3D printed cell mass	No	No	Normal human dermal fibroblasts, human esophageal smooth muscle cells, human BMSCs, and human umbilical vein endothelial cells	2019	Takeoka et al. <sup>86</sup>
Pig	DC esophagus	Yes	Yes	Human amniotic MSCs and epithelial cells	2020	Nayakawde et al. <sup>87</sup>

*Tx- Transplantation, DC- Decellularized, BMSCs- Bone marrow stem cells, ADSCs- Adipose-derived stem cells, MSCs- Mesenchymal stem cells.*

**TABLE 5.** In-vitro and in-vivo tissue engineering of the heart

Milestone	In vitro/in vivo	Reference	Year
Pioneering work DC and RC of rat heart	In vitro	Ott et al. <sup>13</sup>	2008
Decellularization of whole porcine hearts	In vitro	Wainwright et al. <sup>88</sup>	2010
RC of DC cardiac patches and their implantation for MI.	In vivo	Godier-Furnemont et al. <sup>89</sup>	2011
Use of DC cardiac patch in RVOT	In vivo	Wainwright et al. <sup>90</sup>	2012
First recellularization of whole heart with iPSCs	In vitro	Lu et al. <sup>91</sup>	2013
DC and RC of Rat hearts	In vitro	Yasui et al. <sup>92</sup>	2014
DC and RC of Rat hearts	In vitro and in vivo	Robertson et al. <sup>93</sup>	2014
Porcine cardiac ECM + HUVEC and murine neonatal cardiac cells	In vitro	Weymann et al. <sup>94</sup>	2014
Decellularization of human heart + recellularization with different cell types	In vitro	Sanchez et al. <sup>95</sup>	2015
DC pericardium for MI	In vivo	Manton et al. <sup>96</sup>	2015
DC porcine cardiac patches + adipose derived cells implanted into the pig	In vitro and In vivo	Perea Gill et al. <sup>97</sup>	2016
DC cardiac patches + hiPSC for MI in rat	In vivo	Wang et al. <sup>98</sup>	2016
Porcine DC cardiac patches for MI in rat	In vivo	Sarig et al. <sup>99</sup>	2016
DC cardiac patch from pig and DC pericardial patch from human + ATMSCs implanted onto MI site in pigs and retrieved at 40 days	In vivo	Perea Gill et al. <sup>100</sup>	2018
DC porcine cardiac patches + ASCs from rat and pigs and implanted into the rats MI site	In vivo	Shah et al. <sup>101</sup>	2018

*DC- Decellularized, RC- Recellularized, MI- Myocardial infraction, iPSCs- induced pluripotent stem cells, ECM- Extracellular matrix, HUVEC- Human umbilical vein endothelial cells, RVOT-Right Ventricular Outflow Tract, hiPSC- Human induced pluripotent stem cells, ATMSCs- adipose tissue mesenchymal stem cells, ASCs- Adipose derived stem cells*





# AIM OF THE THESIS

The specific aims of this thesis were:

- To investigate an effective decellularization protocol for the heart.
- To investigate an effective decellularization protocol for the esophagus and study the fate of human amniotic stem cells when seeded onto chemically decellularized esophagus scaffold.
- To investigate an effective decellularization protocol for the larynx with minimum damage to the ECM.
- To test if a SDC/DNase-I decellularization protocol is effective for baboon and human esophagus. To identify if stem cells produce proteins and ECM proteins in the recellularized human esophagus.

## **Hypothesis**

We hypothesized the following:

- Human size whole hearts can be decellularized with intact ECM
- Human size esophagus can be decellularized with intact ECM
- Human size larynx can be decellularized with intact ECM
- Human size esophagus can be recellularized with human amniotic stem cells

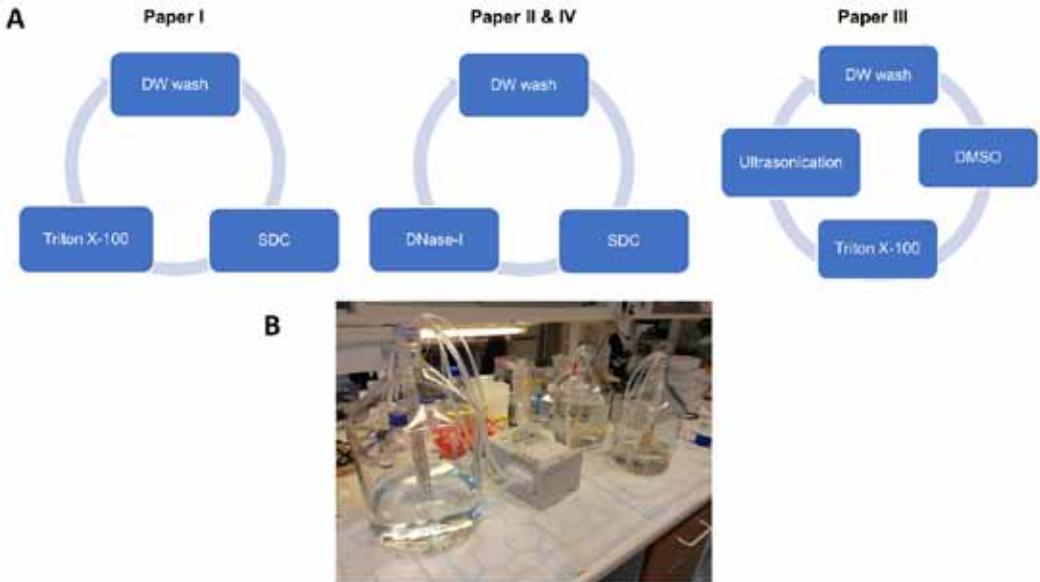


# MATERIAL AND METHODS

Methods used in Paper I-IV are described in this section. The detailed description of each experiment is found in the Materials and Methods part of papers I-IV.

## **Organ procurement**

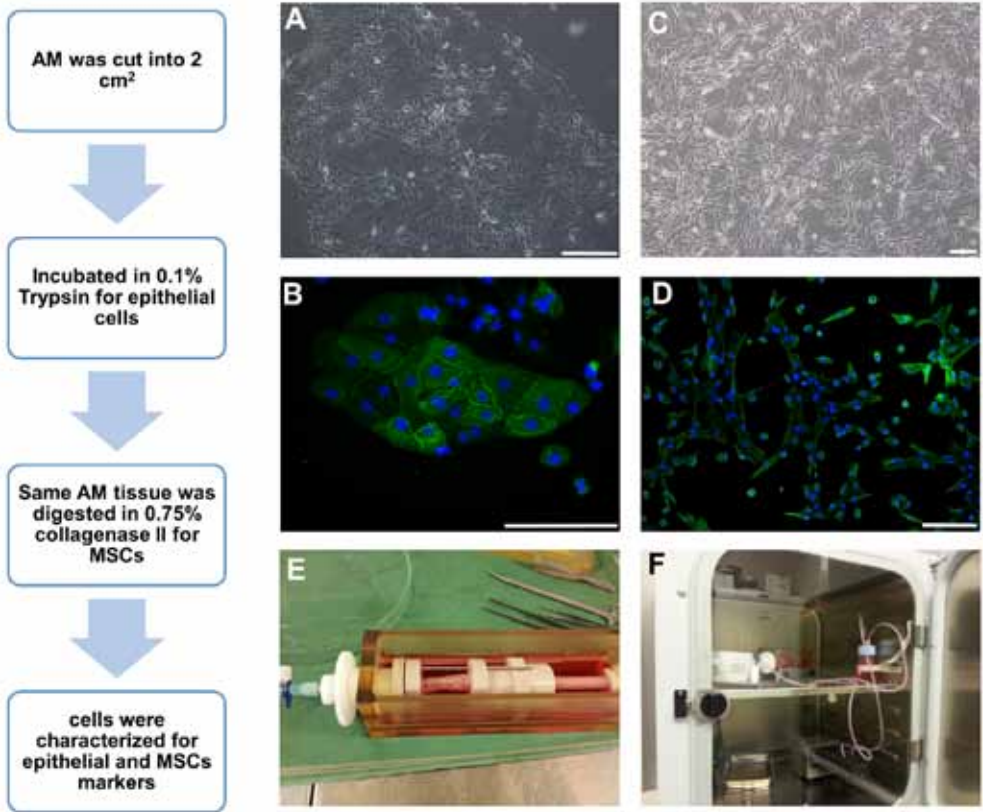
Pig heart (Paper I), esophagus (Paper II & IV), and larynx (Paper III) were collected from deceased pigs, weighing 45-50 kg and 1-4 hrs after death. These were procured from The Experimental Biomedicine Centre, Gothenburg University from pigs, either terminated for unrelated studies, after surgical training or discarded under refinement, reduction, and replacement (3R's). Human esophagus (Paper IV) was obtained from deceased donors (medical research) at Sahlgrenska University Hospital, Gothenburg. The local ethical committee approved the permit for use of human esophagus in this study (DNR-161-11 and T491-14). With regard to baboon (*Papio hamadryas*) esophagus (Paper IV), these tissues were retrieved from euthanized baboon at Mannheimer Foundation (IACUC), Florida, and imported to Gothenburg Sweden under permit from Jordbruksverket (Dnr 4.10.18-11077/17, Nr51136-17). Human amniotic membranes were collected from discarded placenta and membranes at Sahlgrenska University Hospital/Östra, Gothenburg.



**FIGURE 3.** Representation of how detergents were used for decellularization of heart, esophagus, and larynx in Paper I-IV (**A**). Overview of decellularization setup used in Paper I-IV (**B**).

## Decellularization

Decellularization protocols involve the use of physical, chemical, or enzymatic treatment of native tissue and organs with an aim to remove the native cell material. Donor DNA is known to cause an inflammatory reaction upon in-vivo implantation. Decellularized grafts are usually devoid of harmful native DNA, but preserve extracellular matrix proteins in the scaffold. The decellularization protocol that was used for heart, esophagus, and larynx was based on tissue type, its density, complexity, and cellularity of the organ. For each organ, we used customized methods of (physical+chemical or physical+chemical+enzymatic) decellularization. In most protocols, non-ionic detergent Triton X-100 and ionic detergent SDC were used. DNase-I is an endonuclease that is effective in removing nuclear debris from the ECM. We used DNase-I in Paper II and Paper IV. The agitation method of decellularization primarily works by passive diffusion, and it is mainly limited by tissue density/thickness. In contrast, perfusion decellularization effectively penetrates the thick tissues where vascularization remains intact.



**FIGURE 4.** Stem cell isolation, expansion, characterization, and recellularization setup. Flow chart (Left panel) for isolation of human amniotic mesenchymal and epithelial cells. **(A)** Human amniotic epithelial cells in culture, and **(B)** strong expression of epithelial cell adhesion molecule (EpcAM; green), epithelial cell marker in these cells at passage three. **(C)** Cultured human amniotic derived mesenchymal stem cells (MSCs) and its positive staining for the mesenchymal stem cell marker vimentin (green) **(D)** in immunofluorescence microscope at passage five. Nuclei (blue) were counterstained with DAPI. **(E)** A perfusion-rotation Harvard prototype bioreactor showing organ holding chamber with the recellularized esophagus and **(F)** media reservoir connected to the main chamber connected through a silicone tubing. Scale bars: 200  $\mu\text{m}$  (A-D). AM- Amniotic membrane, MSCs- Mesenchymal stem cells, Adapted and modified from *Nayakawde et al.*<sup>67</sup>.

The perfusion method is based on the creation of pressure difference in the lumen of vessels, thereby causing cell lysis. The fresh detergent solution helps clear the dead cell debris and some soluble ECM components out of the ECM.

Pig heart (Paper I) is a dense, complex 3D structure. Hence we used perfusion-agitation with a combination of non-ionic detergent Triton X-100 and

ionic detergent sodium deoxycholate (SDC) (Fig. 3A). Chemicals were perfused through cardiac vessels followed by agitation, and lastly, washing with distilled water (DW).

In Paper II, we compared three decellularization methods, where perfusion-decellularization of protocol 1 (SDC/DNase-I) was based on the detergent-enzymatic method. Protocol 2 was based on Triton X-100/Tri-n-butyl phosphate (TnBP)/DNase-I and protocol 3 used a non-detergent (sonication/DNase-I) decellularization method.

Pig larynx (Paper III) is a dense cartilaginous organ composed of different structures such as muscle, cartilage, vessels, nerves, and esophagus. We used perfusion-agitation-sonication with a combination of detergent (DMSO and Triton X-100). Ultrasonication helped in providing mechanical damage to the cell membrane and collagen structures, and perfusion and agitation helped in washing away dead cells from the ECM of the larynx.

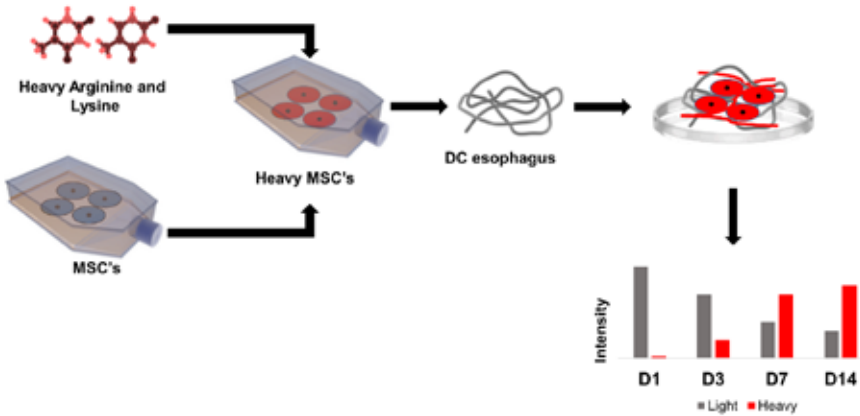
In Paper IV, the chosen method was detergent-enzymatic application (protocol 1 from paper II) combined with perfusion-decellularization for pig, baboon, and human esophagus. Fig. 3B shows the perfusion setup used for decellularization of the pig, baboon and human esophagus with SDC/DNase-I method.

## **Recellularization**

Recellularization is the attachment and growth of seeded cells onto acellular tissue. This growth of cells largely depends on the types of cells used and the quality of ECM in the decellularized organ. In Paper II, human amniotic mesenchymal and epithelial stem cells from amniotic membrane were isolated. Amniotic stem cells have immunomodulatory properties, and they have the potential to differentiate into multiple cell lineages such as muscle cells and epithelial cells. The human amniotic membrane is a generally discarded tissue after delivery in obstetric clinics. Cells derived from amniotic membranes are an excellent alternative to adult stem cells and bone marrow stem cells.

## **Amniotic stem cells isolation and characterization**

Amniotic stem cells were isolated in the manner mentioned in the flow chart of Figure 4. In brief, cells were isolated by stepwise enzymatic treatment of Trypsin and Collagenase type II. MSCs and epithelial cells were then centrifuged several times before plating in order to remove enzymes in culture plates. Before seeding the expanded cells onto a scaffold, cells were characterized for EpCAM and vimentin (Fig. 4 B&D).



**FIGURE 5.** Schematic diagram of the SILAC experiment (paper IV). Amniotic MSCs were grown in media containing isotopic labeled amino acid of heavy  $^{13}\text{C}_6$   $^{15}\text{N}_4$  L-arginine HCl and  $^{13}\text{C}_6$   $^{15}\text{N}_2$  L-lysine-2HCl for 5-6 passages. The human esophagus was decellularized by SDC/ DNase-I protocol. Heavy labeled cells were seeded on the rings of esophagus and cultured in 6 well plates for 14 days. Proteomics analyses of the recellularized esophagus on days 1, 3, 7, and 14 showed heavy labeled red peak and light proteins (from DC esophagus ECM) in grey peak. DC- decellularized, MSCs- Mesenchymal stem cells.

## Bioreactor

The bioreactor stimulates cells resulting in their adhesion and growth on an acellular scaffold. We used a perfusion-rotation Harvard prototype bioreactor (Fig. 4E) for the recellularization of pig esophagus (Paper II) with some custom modification of the reservoir for epithelial media (lumen). The bioreactor was custom made for tubular organs such as esophagus, aorta, and trachea. The bioreactor was also autoclave proof and easy to assemble in laminar airflow. Stem cells were seeded inside and outside of the acellular esophagus attached to the bioreactor and later maintained in epithelial media for luminal perfusion and mesenchymal stem cell media outside the reservoir. Tissue samples during recellularization were analyzed on days 4, 7, and 14 for histological investigation.

## SILAC assay

Stable isotope labeling with amino acids in cell culture (SILAC) was used to detect newly produced proteins in the recellularized human esophagus (Paper IV). With SILAC, one can know exactly which proteins are produced by seeded cells and which are derived from the acellular scaffold. The use of conventional proteomics in tissue engineering applications, such as in recellularization studies, does not allow for such a detailed and definitive interpretation of results as can be

obtained using SILAC. Using the SILAC method, cells are generally grown (4-5 passages) in a special medium with minimal growth factors and dialyzed serum instead of normal serum. This SILAC medium contains isotopically labeled heavy arginine and lysine which are taken up by cells and start producing proteins. These newly synthesized proteins can be identified using MS and are characterized by heavy and light peaks (Fig. 5).

To detect newly produced proteins in the recellularized esophagus, the SILAC method was used for the recellularization of the human esophagus. Prior to recellularization, DC human esophagus was cut into rings and primed by incubating tissue in SILAC heavy media for 24 hrs in the incubator. Human amniotic MSCs were grown in SILAC heavy media in media containing isotopic labeled heavy amino acid of  $^{13}\text{C}_6$   $^{15}\text{N}_4$  L-arginine HCl and  $^{13}\text{C}_6$   $^{15}\text{N}_2$  L-lysine-2HCl for 5-6 passage. Expression of CD166 marker for mesenchymal stem cells was observed on the heavy cells before seeding onto the DC scaffold. Heavy labeled cells were allowed to grow in heavy media on the rings of DC human esophagus for 14 days in a 6 well plate. At days 1, 3, 7, and 14 time-points, tissue samples were further analyzed by proteomics (nLC-MS/MS) to determine newly produced proteins by these stem cells.

## Characterization of de and recellularized organs

### Histology

Histologically, confirmation of cell removal from decellularized heart, esophagus, and larynx was performed with DAPI (Paper I & III) and H&E (Paper II & IV). MT (Paper I-IV) and MP (Paper II) were performed to better understand the levels of collagen, mucin, and elastin in native and decellularized organs.

IHC and immunofluorescence staining were performed on native and decellularized tissue sections to investigate ECM proteins such as elastin (Paper I-III), laminin (Paper I-III), fibronectin (Paper I-III), heparan sulphate (Paper I & III), hyaluronic acid (Paper I & III), chondroitin sulphate (Paper III), Integrin beta 1 (Paper IV), and collagen IV (Paper IV).

In the recellularized esophagus, H&E (Paper II) and MT (Paper IV) were used to analyze the presence of cells in the tissue. IHC was used in paper IV to investigate the expression of ITGB1 and collagen IV. MT staining was used (Paper IV) to calculate collagen expression during recellularization on days 1, 3, 7, and 14.

### DNA

The absence of nuclear material in the decellularized tissues was analyzed by

DNA quantification and compared with the DNA level in native tissue. Evaluation of decellularization and recellularization was performed by dsDNA quantification (Paper I-IV).

### **Biomechanics**

Tensile strength is particularly important for an organ like the heart due to its function of constant pumping of the blood throughout the body. It also helps in maintaining the 3D structure of the organ and plays an important role in cell migration and differentiation. Mechanical tests were performed on native and decellularized heart to check for the tensile strength of the bioscaffold (Paper I).

### **ECM quantification**

Quantification of structural proteins and proteoglycans were performed in Paper I and II by a biochemical method. Lyophilized tissue samples of native and decellularized tissues were used to detect collagen, elastin, and GAGs using the Sircol assay kit.

### **Luminex**

Angiogenic growth factors play an important role in transplantation studies. We studied 17 angiogenic growth factors in native and decellularized tissue samples of heart and larynx (Paper I and III) by Luminex technology.

### **Proteomics**

Proteomics is a powerful tool to identify thousands of proteins from a tiny biological sample. Decellularized pig, human, and baboon esophagus (Paper IV) tissue samples were analyzed to investigate the dominant protein groups in the three tissues. PANTHER database was used to generate bioinformatics pathways of identified proteins in the proteomics analyses.

### **SEM and TEM**

Ultrastructural changes (which are difficult to identify by light microscopy) in the decellularized heart (Paper I) and larynx (Paper III) were identified with SEM and TEM analyses. The same analyses were also used to analyze damage to the collagen and elastin fibers arrangement due to ultrasonication during decellularization in Paper III.

### **Proliferation and Apoptosis**

To investigate if the seeded stem cells undergo proliferation or apoptosis

(paper II) in the recellularized esophagus, we used immunostaining for ki67 (594-red) and Click-iT plus 488 kit. Positive cells were counted by taking ten random images (200X) at each recellularization time point. Apoptotic cells and proliferative cells were counted using CellProfiler software (version 2.2.0).

### **Statistical methods**

Detailed statistics are described in each paper. In brief, the data were checked for normal distribution using the Shapiro–Wilk test (Paper I-IV). For normally distributed data, paired t-test (Paper I and IV) and ordinary one-way ANOVA followed by Tukey’s multiple comparison post hoc test (Paper IV) were used. We also used non-parametric tests when data did not pass normality distribution. The Mann Whitney U test (Paper I, II, and IV) and Kruskal-Wallis test, followed by Dunn’s multiple comparison post hoc test (Paper II, III, and IV), were used to check the statistical difference between the groups. The statistical analyzes was carried out by Graph Pad Prism 8.2 software.





# RESULTS

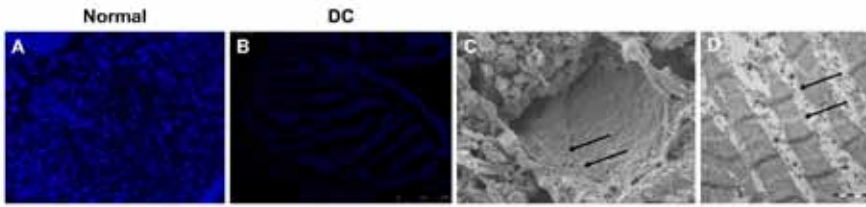
## **Paper I – An alternative approach to decellularize the whole porcine heart**

Decellularization of porcine hearts was achieved successfully after eight decellularization cycles of SDC and Triton X-100. DAPI staining showed prominent blue nuclei in the native heart, but absence of nuclei in the DC heart tissue sections (Fig. 6). DNA quantification confirmed a loss of DNA in the DC heart compared with the native heart. The lowest amount of DNA was found after DC cycle 8.

DC heart tissue stained for collagen, elastin, fibronectin, heparan sulfate, and hyaluronic acid showed preservation of ECM proteins and structural arrangement of ECM of heart comparable to native heart tissue. Quantitatively, levels of hydroxyproline, collagen, and elastin in DC heart were not significantly reduced compared to the respective levels in native heart tissue. However, levels of GAGs in DC hearts were significantly reduced compared to native heart tissue. With respect to effects on the chambers of the heart, there were lower levels of GAGs in the left ventricle suggesting more decellularization in this chamber.

Even after detergent treatment of the heart during decellularization, angiogenic growth factors were present in DC heart irrespective of the heart chamber (auricles and ventricles). Growth factors such as leptin, FGF-1, and VEGF-A were present in DC heart at levels almost equal to those in native heart tissue.

The absence of native cells in endocardium, pericardium, veins, and arteries indicates effective decellularization of the heart by the perfusion-agitation



**FIGURE 6.** Histological and SEM evaluation of DC heart. DAPI positive cells (blue) in the normal heart (A). Whereas, in the decellularized heart, no cells were observed (B). SEM micrograph showing the luminal surface of the probable vessel (arrow) were observed in the decellularized heart (C). TEM micrograph showing the actin-myosin band in the decellularized heart (D). DC- Decellularized. *Adapted and modified from Methe et al<sup>62</sup>.*

method. Perfusion of detergents through the vasculature of the heart allowed access to deep tissue areas for better cell removal. Cardiomyocytes (cell nuclei) were removed from the ECM, but contractile actin-myosin bundles were present in the DC heart.

Biomechanics testing of DC heart revealed that the right ventricle wall had higher elasticity than the left ventricle. These results support the ECM quantification of the heart where elastin was higher in the right ventricle than in the left ventricle. The right ventricle also withstood higher stress than DC left ventricle and native heart walls.

## **Paper II- *In vitro* regeneration of decellularized pig esophagus using human amniotic stem cells**

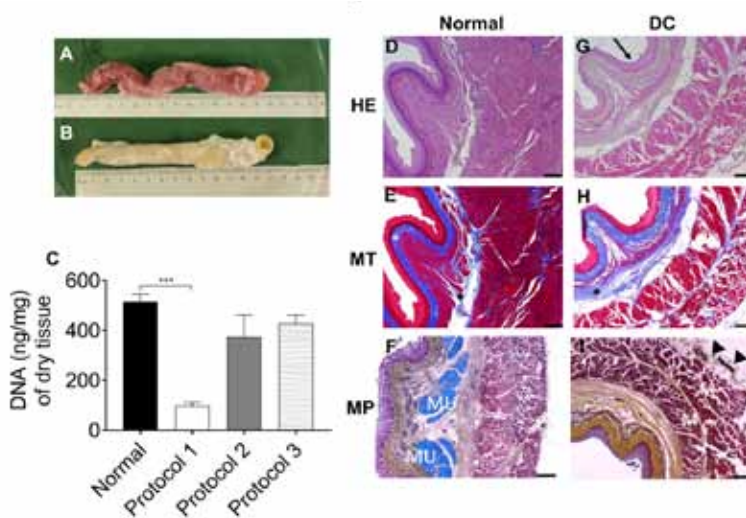
Three decellularization protocols were compared to achieve DC esophagus. Protocol 1 (SDC/DNase-I) was better at removing cell nuclei than both protocol 2 (TnBP/Triton X-100/DNase-I) and protocol 3 (sonication/DNase-I) as confirmed by H&E staining and DNA quantification (Fig. 7). The histological evaluation confirmed the preservation of tissue architecture, epithelial basement membrane, collagen, and elastin in protocol 1 derived DC esophagus compared with native esophagus tissue (Fig.7D-I). Blue colored mucus glands in the esophagus were also removed after the decellularization process (Fig.7I).

A total of 12 hours SDC treatment during DC did not result in accumulation of detergents in the acellular esophagus as confirmed by spectrophotometric analyses. Immunofluorescence staining for ECM proteins revealed that collagen IV, fibronectin, and elastin were abundantly present in the DC esophagus, whereas laminin expression was localized around blood vessel structures and near footprints of smooth muscle fibers. However, collagen types I, III, and V were not detected in normal and DC esophagus tissues.

Quantitative estimation of ECM proteins such as elastin, GAGs, soluble and insoluble collagen in the normal and DC esophagus was investigated by the biochemical method. No significant loss of elastin and insoluble collagen was observed in the DC esophagus compared with the native esophagus. But GAGs and soluble collagen were most affected in the decellularization process. In the decellularized esophagus, we unexpectedly found smooth muscle actin without cell nuclei but no skeletal muscle protein expression, and only weak expression of xeno-antigen, alpha-gal expression compared with native esophagus, using immunostaining.

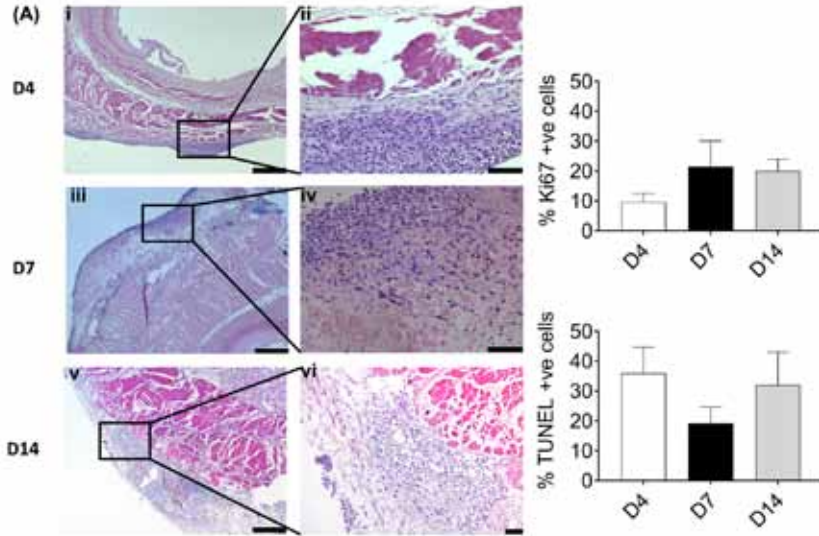
Histologically, cells were observed on day 4 with rounded nuclei near mucosal, submucosal, and adventitia tissue (Fig. 8A). On day 7, more cells were seen infiltrating from adventitia to the muscularis externa. The majority of cells were rounded, but some were also elongated. On day 14, multiple layered cells were observed around the adventitia, but some were also seen near the basement membrane of the epithelial lining. Overall, a relatively large number of cells were observed in the adventitia, muscularis externa, and submucosa, but only a small number of cells were seen near the epithelial lining of the acellular esophagus. Cell quantification showed that significant growth of cells occurred from days 4 to 14.

Immunostaining of recellularized esophagus on day 14 revealed differentiation



**FIGURE 7.** Macroscopic and microscopic analyses of protocol 1 decellularized esophagus; and DNA quantification. Native esophagus (**A**) coloured pink was similar in size to DC esophagus (**B**) colored white. DNA quantification of the normal and decellularized esophagus (**C**) showed 82% of DNA is removed in Protocol 1 (n=7), but no significant difference was found in Protocol 2 and 3 compared to normal tissue. Microscopically, decellularized esophagus (**G-I**) shows the preservation of the tissue architecture, with a prominent epithelial network without a cell nucleus (arrow) compared to the normal esophagus (**D-F**). Masson's trichrome (MT) stain shows the presence of blue collagen (asterisk) in the decellularized tissue compared to the native tissue (E, H). Movat's pentachrome (MP) staining showing the presence of black elastic fibers (arrowhead), red muscle (M) tissue but absence of blue colored mucin (MU) in decellularized tissue compared to the normal tissue (**F, I**). (n=9); Scale bars: 500  $\mu$ m (**D-I**). Error bars represent standard error of the mean (SEM); normal and Protocol 1 (n=7), Protocol 2 and 3 (n=5). Kruskal-Wallis test followed by Dunn's multiple comparison post hoc test was used to calculate p-value; \*\*\* represents  $p=0.0008$ . DC-decellularized, HE- Hematoxylin & Eosin, MT- Masson's trichrome, MP- Movat's pentachrome, MU- Mucin. Adapted and modified from *Nayakawde et al.*<sup>87</sup>

of seeded stem cells into a few endothelial and smooth muscle cells. Moreover, some cells were still positive for MSCs marker vimentin on recellularization day 14. Cell proliferation and apoptosis were studied in the recellularized esophagus by Ki67 marker and TUNEL immunofluorescence staining, respectively (Fig. 8 right panel). There was a numerical increase in proliferating Ki67 cells on days 7 and 14 compared with day 4, but there were no statistically significant differences between the three time-points. The highest number of apoptotic cells (TUNEL) was observed on day 4. On day 7, the least number of cells were observed. But on day 14, a higher percentage of apoptotic cells was observed compared with day 7. However, there was no significant difference in the percentage of apoptotic cells in recellularized esophagus between the three time-points.



**FIGURE 8.** Microscopic characterization and quantification of cell proliferation and apoptosis in the recellularized esophagus. **(A)** H&E staining showing the growth of mesenchymal cells (blue nuclei) in the esophageal tissue on days 4, 7, and 14 at low (**i, iii and v**) and high (**ii, iv, vi**) magnification. The percentage of positive Ki67 and TUNEL cells (**right panel**) in the tissues were counted by CellProfiler software. No significant difference was seen in Ki67 and TUNEL marker between days 4, 7, and 14. The results represent mean  $\pm$  SEM, where  $p < 0.05$  was considered as statistically significant using the Kruskal-Wallis test followed by Dunn's multiple comparison post-hoc test. Scale bars: 750  $\mu$ m **A (i, iii, and v)**, 100  $\mu$ m **A (ii, iv, and vi)**. Adapted and modified from *Nayakawde et al*<sup>87</sup>.

### **Paper III- Combined use of detergent and ultrasonication for generation of an acellular pig larynx**

Seventeen decellularization cycles of perfusion-agitation-sonication were applied to obtain DC larynx. The resultant acellular larynx appeared whitish while vessels, muscles, and cartilaginous structures were preserved. H&E staining of native larynx structures showed a well-arranged ECM with prominent blue colored nuclei. In the DC larynx, however, epiglottis, trachea, vocal cord, and esophagus were devoid of cell nuclei but with similar tissue architecture as compared to native larynx structures.

SEM and TEM analyses showed decellularization affected the ECM structure of the acellular larynx. Elastic cartilage of the epiglottis contained more elastic fibers, but thyroid, cricoid, and trachea dominated in their collagen fiber content. The dsDNA in DC larynx structures of epiglottis, muscles, trachea, thyroid, and cricoid cartilage were below 50 ng/mg.

IHC of the ECM structures of DC larynx showed the expression of some important proteins including structural, adhesion, and proteoglycans. Immunohistochemical characterization of cartilaginous structures mostly revealed expression of laminin, fibronectin, heparan sulfate, hyaluronic acid, and chondroitin sulfate. Elastin was found in the acellular epiglottis, but it was absent in other structures of the DC larynx. Blue colored collagen was expressed in all structures of DC larynx compared to the native larynx, as investigated by Masson's trichrome stain.

Luminex technology was used to detect angiogenic growth factors in the native and DC larynx. In total, 17 growth factors were studied. DC thyroid muscle retained more growth factors than epiglottis and trachea. Among all 17 growth factors, proteins strongly detected across all structures were G-CSF, leptin, FGF-1, follistatin, HGF, and VEGF-A. Generally speaking, most of the growth factors were decreased in the DC larynx structures compared with native larynx.

## **Paper IV- Investigation of extracellular matrix proteins in decellularized pig, human, and baboon esophagus by proteomics**

Pig, baboon, and human esophagus were decellularized using the SDC/DNase-I protocol, which was based on our previously published protocol for decellularization of porcine esophagus<sup>87</sup>. Pig esophagus required shorter exposure to SDC/DNase-I (3 DC cycles) than baboon and human esophagus (4-5 DC cycles) to generate completely acellularized esophageal matrices. After decellularization, gross morphological pictures of the esophagus appeared whitish in color with similar size and shape to the native organ.

Histological evaluation showed clear signs of blue-colored nuclei in the native esophagus of the pig, baboon, and human, but the cells were absent in the respective DC esophagus. DC baboon esophagus showed better ECM preservation compared to native tissue than DC pig and human esophagi.

Immunohistochemical analyses showed that the expression of HLA types I and II were absent in DC human esophagus compared with the native esophagus.

DNA quantification of DC baboon and human esophagus showed significant loss of dsDNA compared with respective native organs. There was a slight increase in the dsDNA in recellularized human esophagus compared to DC esophagus, but there was no significant difference between DC and RC human esophagus irrespective of which time-points were studied.

Investigation of ECM proteins in the DC pig, baboon, and human esophagus was performed by nLC-MS/MS based proteomics analyses. In total, 773, 2,166, and 4,557 proteins were identified, respectively. For data analyses, proteins were further filtered with at least two peptides for a protein.

Bioinformatics analyses of pig, baboon, and human esophagus was performed by using protein accession ID's of the selected protein list. Among other detected pathways, the integrin signaling pathway was the most prominent one for all three species. Other important pathways that were detected in all three species included Wnt signaling pathway, PDGF signaling pathway, EGF receptor signaling pathway, and angiogenesis.

For matrisome analyses of the pig, baboon, and human esophagus, proteomics data were mapped onto the human matrisome database (<http://matrisomeproject.mit.edu/>). Protein list (accession ID's) were used to achieve a list of core matrisome and matrisome associated proteins. ECM glycoproteins and collagens were dominant core matrisome protein groups in DC human and baboon, whereas proteoglycans were found to be present to a lesser extent compared to other core

matrisome proteins. Matrisome-associated proteins were also detected in the DC human and baboon esophagus. In DC pig esophagus, both core matrisome and matrisome-associated proteins were detected, but in a low number of proteins compared with DC human and baboon esophagus. Matrisome-associated protein such as secreted factors was not detected at all in the DC pig esophagus. Nine matrisome proteins that were common in all three species of DC esophagus were EMILIN1, FN1, COL12A1, COL6A1, COL6A2, COL6A3, HSPG2, PRELP, and VCAN (versican).

Proteomics results were further validated for the presence of collagen fibers in the native and DC baboon and human esophagus by Masson's trichrome staining. Blue colored collagen fibers were quantified by measuring the % positive area of the stain. In both human and baboon esophagus, the decellularization process resulted in a significant increase in collagen in the DC esophagus compared to the native esophagus.

Recellularization of DC human esophagus with SILAC labeled cells was performed to study the difference of native proteins of the esophagus and newly produced heavy labeled proteins by the seeded cells. Proteomics analyses were carried out with nLC-MS/MS. The proteomics analyses showed one peak for unlabeled protein and another peak for heavy labeled protein (Fig. 5). For calculations, the summed abundance of light peptides (unlabeled) was divided by the heavy peptides (newly produced proteins) for each protein and sample. A low ratio indicates newly synthesized proteins. To be able to extract interesting proteins, the criteria for quantified proteins were at least two unique peptides, and quantification values had to be present in at least three samples in a group. The threshold for D14/D1 ratio for median and average values was set to  $\leq 0.5$ . In total, 735 proteins were detected by mass spectrometry. After further calculation of peptides and fold change, a total of 60 newly produced proteins were analyzed by bioinformatics.

Investigation of newly synthesized proteins in the RC human esophagus was evaluated by analyzing intensity of mass spectrometry. Skeletal muscle development and contraction proteins myosin-7 and myosin-14 were found in newly synthesized heavy proteins in RC esophagus samples. Important structural protein collagen alpha and collagen type IV were also found in newly produced proteins by stem cells.

Protein accession ID's of unique heavy peptides were used to generate matrisome classification. ECM glycoproteins and ECM regulatory proteins were found to a greater extent than other matrisome proteins. A gradual decrease of light

peptides from day 1 to day 14 was observed in all proteins of core matrisome and matrisome-associated proteins.

Bioinformatics analyses of unique heavy proteins for biological processes showed that upregulated proteins mainly belonged to the following pathways: extracellular matrix organization, cell adhesion, cellular protein metabolic process, proteolysis, and cell differentiation. Extracellular matrix organization and metabolic process were the two most represented pathways. Cellular component pathway analyses showed most newly produced proteins can be ascribed to membrane, extracellular, cytoplasm, organelle lumen, cytosol, and nuclear protein categories. The highest percentage of proteins belonged to the extracellular matrix and cellular processes.

Validation of proteomics results of the SILAC experiment was performed by immunofluorescence staining for collagen IV and ITGB1. Matrisome analyses of newly produced proteins in the RC esophagus revealed that different types of collagen and integrin proteins were present in the samples. Two proteins (collagen IV and ITGB1) were selected based on pathway analyses and matrisome analyses for verification by immunofluorescence staining. Strong expression of collagen IV was observed in the recellularized esophagus at all time-points. Staining for ITGB1 was mostly present near seeded stem cells and around muscularis externa of the RC esophagus at all time points. These results support the value of proteomics and bioinformatics analyses of the RC esophagus.



## DISCUSSION

In the first paper our results showed that decellularization was successful with preserved cardiac ECM proteins, growth factors, and biomechanical properties while removing cellular material from the ECM. Previous studies on decellularization of whole heart from rat and pig showed the possibility of cardiac tissue engineering while preserving ECM<sup>13, 88, 102-106</sup>. But most of these studies used harsh detergents or enzymes like SDS or trypsin for decellularization. Such harsh chemicals and enzymes resulted in a negative impact on the decellularized ECM such as loss of ECM proteins and loss of mechanical properties of the tissue<sup>107</sup>. Therefore, we evaluated the possibility of using mild detergent Triton X-100 in combination with SDC for perfusion-agitation decellularization of whole porcine hearts.

The effectiveness of the decellularization of thick organs is determined by choice of detergents used for the decellularization process. A combination of two or more detergents instead of a single detergent together with a physical/chemical method of decellularization accelerates the decellularization time<sup>29</sup>. However, all decellularization methods have inherent disadvantages regarding their impact on acellular tissue, making them less suitable for recellularization or *in-vivo* implantation. Triton X-100 has been used in combination with other detergents, and it tends to be gentler on the tissue, preventing biomechanical damage to the tissue during decellularization<sup>108</sup>. SDC, on the other hand, proved to be effective and less destructive than SDS. Decellularization of myocardial tissue with an SDS containing protocol resulted in much stiffer tissue<sup>109</sup>. Use of a SDC/Triton X-100 protocol for decellularization of anterior cruciate ligament (ACL) proved

to be more effective than trypsin/EDTA or SDS treatment<sup>110</sup>. Acellular ACL, however, lost a significant number of GAGs compared to native tissue by all decellularization protocols. This supports our finding of better decellularization with SDC/Triton X-100 but loss of GAGs from the ECM. Another study on decellularization of porcine aortic valve leaflets compared SDC/Triton X-100 and detergent-enzymatic protocols where they found that both protocols were effective in removing cells, preventing destruction of collagen and elastin fibers, and maintaining ECM structure integrity<sup>111</sup>.

Given the vascularity of the heart, perfusion of the vasculature proved to be effective in the present study, and agitation provided better exposure of the heart to chemicals, thereby shortening the decellularization time. Minimal exposure time of detergents during decellularization is known to avoid detergent accumulation in the tissue. We did not use DNase-I in this study because DNase-I is difficult to remove from the tissue despite extensive washing steps during decellularization. It is also known to cause an inflammatory reaction when DNase treated tissue is implanted into the body.

We observed cytoplasmic remnants of actin and myosin in the decellularized heart. The decellularization method was unable to remove these cytoplasmic materials. Other studies have also reported cytoplasmic proteins remnants after decellularization<sup>112,113</sup>. However, the positive or negative effects of residual cytoplasmic actin and myosin are unknown.

Recently, several other groups have also performed human-sized whole heart using the detergent decellularization technique<sup>114-119</sup>. Most of these studies used a perfusion-decellularization technique with application of a pressurized system to enhance the decellularization process. Momtahan et al. used a bioreactor with pressure controlled decellularization with SDS/Triton X-100 which resulted in a complete acellular heart with decreased exposure to detergents than conventional perfusion-decellularization technique<sup>116</sup>. Hulsmann et al. investigated effects of perfusion-decellularization with detergents (SDC/SDS) on rodent and human-size ovine heart and protein loss during decellularization process<sup>114</sup>. Current method of analyzing quality of DC ECM is at the end of the process, but according to Hulsmann et al. it is necessary to check for the quality of ECM during the decellularization process to maintain the quality control in the process of decellularization<sup>114</sup>. Akhyari et al. performed ovine heart decellularization with SDS/SDC with pressure-controlled perfusion decellularization and reduced the time of detergent exposure to just three cycles of decellularization<sup>119</sup>. Taylor et al. showed that the whole human heart can be DC by placing it in an inverted

position, in a pressurized pouch and perfusing it with hypotonic/hypertonic/SDS treatment thereby preserving intact coronary vasculature. All recently published papers on decellularization of whole hearts showed a reduction in detergent exposure and time for decellularization than our method of decellularization.

Heterotopic transplantation of decellularized and recellularized pig heart was first tried in pigs with limited success<sup>120</sup>. In the present transplantation study, decellularized hearts were recellularized with porcine MSCs and were transplanted in pigs. After 3 days, in both DC and recellularized transplanted heart, although there were no signs of thrombus in the main chambers of the heart, there was thrombus formation in the coronary artery and inflammatory cell invasion in the heart tissue. This suggests the importance of endothelial cell coverage of the vascular organ. This study was unable to differentiate between MSCs and endothelial cells in the heart that led to thrombus formation, despite the fact that pigs were given anticoagulants until hearts were removed from the body for analyses. Recellularization and transplantation studies of decellularized heart have had limited success in rats but human-sized heart recellularization and transplantation is still a challenge. This is due to the size difference which requires a large number of cells for recellularization of pig/ovine/baboon/human heart compared to the rat or mice heart.

In paper II we investigated three protocols and showed that perfusion with the SDC/DNase-I protocol successfully decellularized whole porcine esophagus with minimal damage to the native ECM. DC esophagus accepted seeding with amniotic membrane-derived MSCs and epithelial cells with subsequent cell growth and proliferation.

Decellularization of rat esophagus was reported by Ozeki et al. in 2006<sup>121</sup>. In this study, SDC and Triton X-100 were used separately to perform agitation-decellularization. SDC treated esophagus was found to be superior to Triton X-100 by preserving ECM proteins and removing DNA. In another study, rodent esophagus was decellularized with SDS/EDTA using the agitation method<sup>122</sup>. It was not until 2012 that human-sized esophagus (porcine and ovine) was decellularized with SDS or with SDC/DNase-I by agitation and perfusion<sup>57, 123, 124</sup>. Studies using perfusion-decellularization of porcine esophagus resulted in uniformly decellularized tissue with intact ECM<sup>57, 81, 84</sup>. However, tissue specific differentiation of cells during recellularization of acellular esophagus has not been performed to-date. We evaluated three protocols of perfusion-decellularization of porcine esophagus with SDC/DNase-I, TnBP/Triton X-100/DNase-I, and

non-detergent decellularization with ultrasonication/DNase-I.

Perfusion-decellularization of the esophagus preserved ECM architecture, structural, adhesive, and basement membrane proteins. Only GAGs were depleted in the decellularized esophagus. GAGs are important for cell adhesion and growth factor storage. Reduction of GAGs after decellularization with the SDC based protocol was also supported by other studies which used SDC/SDS based protocols for decellularization of pig and rat esophagus<sup>57, 81, 125, 126</sup>. However, Wang et al. managed to preserve GAGs after decellularization of pig esophagus with a combination of latrunculin B + potassium chloride + potassium iodide + DNase-I<sup>84</sup>. But latrunculin B and potassium chloride can be toxic following *in-vivo* implantation. Another study which used SDS/EDTA/DNase-I based decellularization of pig esophagus showed preservation of GAGs such as heparan sulfate and dermatan sulfate but loss of chondroitin sulfate by immunostaining<sup>127</sup>. However, this research group used qualitative measurement (IHC) to analyze GAGs and not quantitative techniques. Only one study has shown that SDC/DNase-I perfusion decellularization of rat esophagus preserved GAGs<sup>82</sup>. This could be due to the decellularization carried out at 4°C. This could have minimized the loss of GAGs compared to other studies and compared to our study where we decellularized esophagus at room temperature and at 37°C. A modified protocol able to preserve GAGs or replace damaged GAGs in DC esophagus would be advantageous.

Gal epitopes in xenogeneic scaffolds can cause hyperacute rejection upon implantation into humans<sup>128-130</sup>. In our study, the decellularization method successfully removed Gal epitopes from the cell surface, but very weak staining for Gal epitopes was observed on the decellularized esophagus. Previous studies on decellularization of xenogeneic esophagus lack details of the remaining Gal epitopes on the acellular esophagus<sup>57, 81, 122-124</sup>. We speculate that the use of short treatment with  $\alpha$ -galactosidase would be able to remove Gal epitopes even from the ECM surface.

Several *in-vivo* studies have been reported describing transplantation of recellularized esophagus<sup>131</sup>. Some studies focused on using synthetic scaffold with organ-specific cells like smooth muscle cells and epithelial cells for recellularization<sup>85, 86</sup>. Other studies suggest using ADSCs or BMSCs<sup>81, 83, 86</sup>. However, adult stem cells such as ADSCs and BMSCs have limitations such as cell expansion. We used human amniotic MSCs and epithelial cells. In the recellularized esophagus, most cells were initially found around the adventitia. Cells started to infiltrate muscle and submucosa from day 7 to day 14. Tissue-specific stem cell differentiation was limited to endothelial and smooth muscle cells. Recellularization with

tissue-specific smooth muscle, skeletal muscle, endothelial, epithelial, and mucin in all areas of the human-sized esophagus has not been reported in any in-vitro recellularization studies<sup>81, 84, 127</sup>. We speculate that the loss of GAGs during decellularization could have caused a negative impact on the recellularization process. Recently, Urbani et al. described a two-step method for recellularization of rat esophagus. Human mesoangioblast, fibroblast, and neural cells were added as a first step and epithelial cells as a second step (after in-vivo maturation in the rat omentum) under both static culture conditions and dynamic recellularization in the bioreactor<sup>82</sup>. Recellularized esophagus in this study showed cell distribution in all areas of the esophagus with proliferation of smooth muscle cells and epithelial cells. Decellularized rat esophagus also preserved GAGs during the decellularization process. So, preservation of GAGs could have resulted in a favourable recellularization in this study. Nevertheless, the complexity and size of rat esophagus and pig esophagus is significant. Rat esophagus is quite small in size and shape and requires small numbers of cells for repopulation compared to pig or human esophagus. Also, scale-up of rat esophagus regeneration settings to human-size organ is usually associated with problems such as huge numbers of cells, a bioreactor, and technology transfer required for recellularization.

Recently, partial or full-thickness esophageal defects have been surgically replaced with decellularized xenogeneic and allogeneic ECM material in the clinic<sup>17, 19-21</sup>. It is widely accepted in the field of TE that even the recellularized scaffold, when implanted or transplanted *in-vivo*, would start to be degraded by host cells. Cells in the recellularized graft would gradually be replaced by the host's own cells. Even though the esophagus is a fairly complex organ, *in-vivo* regeneration of tubular organs is possible after transplantation. We postulate that even partially recellularized esophagus can be transplanted into a large animal model such as pig. Recellularized esophagus can be allowed to mature in a subcutaneous space, it could later be transplanted in the same recipient pig to correct full-thickness esophageal defects.

In paper III we demonstrated that larynges can be decellularized using a combination of detergent and sonication methods without using DNase-I. We also showed the methodological approach to be successful in preserving some of the vital proteins and growth factors necessary for regeneration of larynx. Several studies have focused on larynx tissue engineering but very few studies showed effective decellularization of whole human-sized larynx<sup>132-136</sup>. Earlier studies were more focused on removing cells from soft tissue and not chondrocytes from tough cartilage tissue<sup>132-134</sup>. However, recent studies on canine larynx decellularization

used perfusion decellularization with SDS/Triton X-100 in a pressurized system<sup>136</sup>. Another study on pig larynx decellularization used a latrunculin B/potassium chloride/potassium iodide/DNase-I and SDC/Triton X-100/DNase-I immersion technique<sup>135</sup>. Both protocols resulted in loss of nuclei and preservation of ECM proteins. However, in a canine decellularization study, DNA levels in DC larynx structures were higher than the acceptable limit (>50 ng/mg) stated in the literature<sup>29</sup>.

The larynx is a complicated organ consisting of elastic cartilage, hyaline cartilage, skeletal muscle, arteries, veins, and thyroid glands. The challenge was to decellularize the entire 3D structure with minimum damage to soft tissues of the larynx. We used perfusion decellularization to reach the deep-seated vascular area of thyroid muscle and other soft tissues. The carotid artery was used for perfusion of detergents since the thyroid artery proved too small to cannulate. Cartilage is avascular tissue composed mainly of tightly packed collagen fibers where nutrient supply to the innermost area of cartilage is maintained through diffusion. Agitation is proven to be a suitable method for decellularization of various cartilaginous structures<sup>137, 138</sup>. To decellularize cartilaginous structures, we used ultrasonication treatment to loosen fibers of collagen and to allow detergents to reach cartilage structures. Sonication is proven to be an effective method for decellularization of organs such as blood vessels, trachea, bone, meniscus, and small intestine<sup>139-144</sup>. Sonication decellularization of porcine trachea was achieved in 4 cycles of SDC/DNase-I together with sonication (in an ultrasonic bath) that resulted in loss of DNA (<50 ng/mg) in decellularized trachea compared to native. However, this study failed to completely remove chondrocytes from the cartilage matrix<sup>141</sup>. This study<sup>141</sup> does not support our findings that our method successfully removed chondrocytes from most parts of porcine larynx. The difference could be that Tchoukalova et al. used a sonication bath and we used an ultrasonicator (probe sonicator)<sup>141</sup>. Ultrasonication tends to impact more energy on the sample than a bath sonicator. We speculate that more impact of such high energy could have a positive effect on removal of chondrocytes from cartilaginous larynx. A study of sonicated assisted decellularization of porcine aorta showed complete decellularization of aortas and upon subcutaneous implantation into rats for 5 weeks, minimal inflammatory reaction compared to native tissue<sup>140</sup>. However, xenogeneic implantation of native tissue, at least theoretically, tends to cause a severe inflammatory reaction compared to DC aortas.

In our paper, harsh treatment of sonication was coupled with mild detergent of Triton X-100 and organic solvent DMSO for decellularization of larynges. We believe that use of harsh chemicals such as SDS or SDC with Triton X-100

coupled with sonication-agitation could have caused significant damage to the laryngeal ECM. Triton X-100 is an effective decellularization agent when used in combination with other chemicals such as SDC or SDS but when it is used alone or with DNase-I to decellularize rabbit tracheas, it fails to remove chondrocytes from the cartilage<sup>145</sup>. DMSO is widely used in cell freezing media for its ability of penetration. One recent study showed that when SDS was used together with DMSO, it behaved as an enhancer for decellularization of porcine aortas. This protocol was also effective in cell removal and preserves ECM<sup>146</sup>. In another study on decellularization of rat uterus, a Triton X-100/DMSO decellularization protocol was effective in preserving GAGs but elastin was reduced compared to a SDC based protocol<sup>112</sup>. However, DNA quantification of decellularized uterus revealed that higher levels of DNA were found with a Triton X-100/DMSO protocol. There was also a mechanically weaker scaffold with compromised ECM organization compared to results achieved with an SDC based protocol<sup>112</sup>. These results on rat uterus decellularization are quite different to our findings in terms of DNA remnants and ECM organization. However, in addition to Triton X-100/DMSO treatment, we also used sonication so effective removal of DNA in our protocol could have been due to the combined effect of detergents and sonication. But another study, published by the same group, achieved recellularization (with BMSCs) of patches of acellular uterus tissue subjected to Triton X-100/DMSO and SDC based protocols<sup>147</sup>. After recellularization in this study, cell coverage was significantly more present using the Triton X-100/DMSO DC uterus than SDC DC protocol. When these recellularized grafts were transplanted in rats with defect uteri, Triton X-100/DMSO generated uterus patches supported pregnancy in rats. The findings obtained in uterus tissue engineering recellularization studies further support our use of a Triton X-100/DMSO based decellularization protocol.

Our decellularization method was sufficient to reduce the double-stranded DNA level below the widely accepted minimum DNA level (50 ng/mg) in TE field<sup>29</sup>. This study showed the presence of chondroitin sulfate, hyaluronic acid, and collagen in most areas of the decellularized larynx. This proved that GAGs are retained in most parts of the acellular larynx. Ultrasonication tends to do more damage to the ECM than detergent treatments. In our studies, we were able to preserve collagen in the decellularized larynx. We also found angiogenic growth factors such as VEGF, BMPs, leptin, HGF, and angiopoietin-2 still present after decellularization. These growth factors are important in chondrocyte differentiation, cartilage development, chondrogenesis, and osteogenesis<sup>148</sup>. We speculate that these preserved growth factors in the decellularized larynx can be

beneficial in future recellularization or *in-vivo* implantation in large animal models.

This study shows an alternative decellularization protocol of combination of harsh treatment (sonication) and mild detergents to preserve laryngeal ECM but to remove cells from all areas of the larynx structure.

In paper IV we investigated whether our earlier pig decellularization protocol (Paper II) could be used for decellularization of baboon and human esophagus. We demonstrated successful decellularization with minimal ECM destruction, using histological and DNA quantification assays. Furthermore, matrisome analyses of recellularized human esophagus proteins revealed increased production of secreted proteins, ECM regulators, ECM glycoproteins, and proteins related to striated muscle regeneration and contraction. This suggests that our recellularized esophagus scaffold did not show fibrotic regeneration but it showed tissue specific protein synthesis.

Decellularization of baboon esophagus is not reported in the literature. Research on NHP would overcome problems associated with genetically variability, and scale up issues associated with small animal research to human research. However, due to ethical issues research on primates is reserved as a last option before human trials. Notably, de/recellularization of human esophagus was reported by Green et al<sup>149</sup>. However, in this study, esophagus was first cut into pieces (5 cm<sup>2</sup>) and then decellularized by removing the epithelial layer.

Proteomics of decellularized pig, baboon, and human esophagus is another area not yet reported. We carried out proteomics analyses of decellularized pig, baboon, and human esophagus using nLC-MS/MS to determine the status of ECM protein after decellularization. The greatest number of proteins was identified in baboon esophagus whereas pig esophagus contained the least number of proteins. The bioinformatics analyses proved to be an invaluable tool for identifying important signaling pathways such as integrin, Wnt, PDGF, EGF, and angiogenesis signaling pathways.

Previous studies on characterization of decellularized ECM were limited to structural, adhesive, and functional proteins. However, current knowledge on ECM proteins tells us that around 1056 matrisome proteins are present in the matrisome of human ECM<sup>150</sup>. The complexity of huge numbers of ECM proteins and signaling molecules that may interact with cell-ECM (if subjected to recellularization or *in-vivo* implantation) requires investigational tools like proteomics and bioinformatics. Matrisome analyses provided information on how ECM proteins were distributed between pig, baboon, and human esophagus. It also

provided valuable information for core matrisome proteins and the matrisome associated protein list present in the ECM. Integrin and different types of collagen were predominantly present in the matrisome analyses. Integrin subtypes have ligands directed towards the core matrisome proteins collagen, laminin, and fibronectin. Matrisome proteins shared in all three species of the decellularized esophagus included EMILIN1, FN1, COL12A1, COL6A1, COL6A3, HSPG2, PRELP, and VCAN.

The initial use of SILAC in a tissue engineering mechanistic study by Muller et al. studied expression of ECM proteins from fibroblasts during cell-artificial ECM recellularization<sup>151</sup>. Use of SILAC in mechanistic approaches to understand ECM protein turnover during recellularization has also been studied in vocal cord and human lung tissue<sup>152-154</sup>. Li et al. showed a relationship between decellularized vocal fold ECM and seeded cells during recellularization<sup>152</sup>. Rendin et al. showed that ECM proteins in decellularized diseased lungs directs fibroblasts to deposit pro-fibrotic proteins in the ECM whereas, decellularized lungs from healthy donors showed different responses during recellularization with human lung fibroblasts<sup>154</sup>. In our study, SILAC assay demonstrated 735 newly produced proteins in the recellularized human esophagus. Myosin-7 and Myosin-14 were examples of newly produced proteins from stem cells. Myosin-14 tends to be involved in intracellular signaling and morphogenesis<sup>155</sup>. Bioinformatics of newly produced proteins in recellularized esophagus showed that stem cells produce proteins of extracellular matrix, cell adhesion, cell metabolic process, and cell differentiation. We postulate that stem cells were not only differentiating in the scaffold, but they were also producing ECM proteins and other proteins necessary for ECM remodeling. This finding is important for future applications since cells that attach to decellularized esophagus simultaneously start to independently synthesize new ECM proteins.

Our study contributes new knowledge to the evolving study of proteomics, bioinformatics, and matrisome analyses of decellularized pig, baboon, and human esophagus. Moreover, these results demonstrate the clinical potential of using SILAC in tissue engineering of esophagus to study protein turnover during *in-vitro* recellularization with amniotic stem cells.



# CONCLUSIONS

- The porcine heart can be decellularized with a perfusion-agitation (with Triton X-100/SDC) method, but cytoplasmic actin-myosin was difficult to remove.
- The SDC/DNase-I decellularization protocol effectively decellularized pig esophagus, and partial recellularization of the esophagus was achieved with human amniotic stem cells.
- Decellularization with perfusion-agitation-sonication with non-ionic detergents and DMSO can be an effective and alternative way to decellularize larynx and other hard tissues like cartilage, and skin.
- The SDC/DNase-I decellularization protocol is not species-specific for the esophagus. SILAC labeled stem cells in recellularized human esophagus synthesized cellular and ECM proteins. SILAC is an effective method to characterize the precise nature of proteins synthesized by stem cells.



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# REFLECTIVE STATEMENTS

- Decellularization methods for preservation of ECM of heart, larynx, and esophagus successfully remove nuclear material and preserve important ECM proteins which have a beneficial role in organ regeneration according to the literature. However, heart and larynx decellularization protocols used in this thesis exposed these organs to detergents for a longer duration of time. According to the literature, long-term exposure to detergents is harmful to ECM. So, it could be beneficial to modify the current protocols to expose the organs to detergents for shorter times. Ultimately, it is necessary to ensure the biocompatibility and cellular toxicity of decellularized heart and larynx are conducive to recellularization and implantation studies in animals. For larynx studies, it would be advisable to carry out biomechanical testing to test mechanical properties after decellularization.
- Recellularization of the esophagus showed partial growth of human amniotic stem cells on both xenogeneic and allogeneic (pig and human) esophagus. Amniotic stem cells showed endothelial and smooth muscle differentiation in the recellularized pig esophagus. Procedures that employ human-sized organ recellularization, covering all areas of 3D organs, have still yet to be performed. In recellularized esophagus studies, more functional analyses of cells are necessary. There is opportunity to improve decellularization strategies. In particular, proteins such as GAGs should be preserved for complete recellularization in all layers of the esophagus with organ-specific differentiation of seeded stem cells.
- In Paper IV, the identification of newly synthesized proteins in the recellularized human esophagus by SILAC and proteomics provided an exciting and novel approach to investigate recellularized scaffold. This technique is useful in understanding stem cell-ECM interactions at the protein level. SILAC-based proteomics techniques should prove to be beneficial in future *in-vitro* TE studies in understanding exactly which proteins derive from stem cells and ECM structures. More confirmatory experiments for protein and gene levels are necessary in this area of research.
- Clinical application of de/recellularized organs has had limited success to date. Regulatory hurdles, challenges in recellularization, insufficient knowledge of immunogenicity, risk of bacterial and viral infection, and the risk of cancer development remain some of the key reasons to be overcome.



# FUTURE PERSPECTIVES

## **Heart decellularization and recellularization**

We plan to modify our decellularization protocol for the heart. Accordingly, we will just use perfusion with a high concentration of detergents for a shorter time. We plan to characterize the resultant ECM histologically and by proteomics analyses to check the status of the proteins and growth factors level. Damaged or missing proteins in the decellularized heart will be either injected in the heart or coated with synthetic protein. This heart will then be recellularized with human amniotic stem cells or fetal cardiac cells.

## **In-vivo decellularization**

Decellularization of organs with any method has potential drawbacks which can hamper *in-vitro* recellularization and further *in-vivo* application. We plan to develop a protocol for the decellularization of hollow organs inside the animal body itself. Donor blood vessels or esophagus will be treated outside the recipient animal with much milder chemicals. Grafts can then be transplanted into an animal for a specified time to achieve acellularity. This technique will be more clinically relevant than currently used de/recellularization *ex-vivo* techniques.

## **In-vivo maturation of partially recellularized organs**

Chemically decellularized organs and natural decellularized (in-vivo decellularized) organs can be seeded with amniotic stem cells for one week in the incubator. This partially recellularized organ can be implanted into an animal for a specified time with or without blood vessel anastomosis for *in-vivo* recellularization. After in-vivo maturation, the organ will be characterized for recipient cell infiltration and tissue-specific differentiation, the functionality of the specific organ, mechanical tests, and proteomics analyses.



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