POTENTIAL THERAPEUTIC APPLICATIONS OF NOVEL BIOENGINEERED TISSUES AND ORGANS USING METHODS OF DECELLULARIZATION AND RECELLULARIZATION

TISSUE ENGINEERING OF ORGANS

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Confidence and hard work is the best
medicine to kill the disease called failure.
It will make you a successful person.

Dr. A.P.J. Abdul Kalam

Potential therapeutic applications of novel bioengineered tissues and organs using methods of decellularization and recellularization

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Dedicated to my parents for their
unconditional support in making this thesis possible

ABSTRACT

The transplantation of personalized organs or tissues will benefit patients with various diseases and disorders. Decellularization is a method to generate an acellular, non-immunogenic natural scaffold. The personalized tissue can be generated after recellularization with recipient stem cells and it can be transplanted to recipient without the need for immune suppression. The current thesis focuses on developing decellularization and recellularization strategies for simple tissue (human saphenous veins), complex tissue (porcine skin) and organs (porcine pancreas and kidneys). In Paper I, decellularization of human saphenous veins is demonstrated followed by recellularization with peripheral blood and endothelial media perfusion in a bioreactor to show cell attachment at the lumen of the vein. In Paper II, the application of acellular porcine skin as a gel mixed with peripheral blood mononuclear cells (PBMC) in mice with skin wounds revealed a faster healing rate, complete wound closure, increased collagen deposition and improved angiogenesis. Papers III and IV demonstrate that porcine pancreas and kidneys decellularized in 4°C and room temperatures respectively resulted in loss of nuclei and the preservation of extracellular matrix proteins. The recellularization of pieces of acellular pancreas and kidney with human fetal pancreatic or kidney progenitor cells showed the attachment, infiltration and proliferation of human cells. The recellularized pancreas pieces expressed the characteristic exocrine (α-amylase) and endocrine (c-peptide, glucagon) markers. The recellularized kidney pieces also showed cell growth over the acellular matrix and the increased expression of important transcription factors involved in kidney development. Taken together, protocols for the decellularization of saphenous veins, skin, pancreas and kidneys were established. The recellularization of veins with peripheral blood and the application of porcine skin gel with PBMC may benefit patients with vascular diseases and burns respectively. The recellularization of the acellular pancreas and kidney with human fetal stem cells demonstrates the potential of fetal cells in further functional studies and may be in whole-organ recellularization experiments. The technique of decellularization and recellularization to bioengineer tissues and organs may thus have important implications in the field of regenerative medicine and ultimately organ transplantation.

Keywords: Tissue engineering, Decellularization, Recellularization, Saphenous vein, Bioreactor, Wound healing, Skin gel, Pancreas, Kidneys, Ephrins and Human fetal stem cells

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SAMMANFATTNING PÅ SVENSKA

Transplantation av individanpassade organ eller vävnader kan komma att gynna patienter med flera olika sjukdomar. Decellularisering är en metod där celler avlägsnas från en vävnad för att generera en naturlig skaffold utan celler som på vilken immunsystemet inte kommer att reagera och stöta bort. Den individanpassade vävnaden kan genereras genom recellularisering med mottagarens stamceller och kan därefter transplanteras utan att mottagaren behöver behandlas med immundämpande läkemedel. Denna avhandling fokuserar på att utveckla decellulariserings- och recellulariseringsstrategier för enkel vävnad (ven från människa), komplex vävnad (hud från gris) och intakta organ (bukspottskörtel och njure från gris). I Studie I redovisas en detaljerad metod för att decellularisera mänskliga vener samt att perfusion av skaffold av vener med perifert blod följt av cellodlingsmedium i en bioreaktor resulterade i infästning av celler till venens innerväggar. I Studie II resulterade decellularisering i ett fullständigt avlägsnande av cellkärnor från grishud. Appliceringen av skaffold av grishud i gelform tillsammans med mononukleära celler från perifert blod till hudsår hos möss visade en snabbare läkningshastighet, fullständig sårförslutning, ökad kollagenproduktion samt förbättrad nybildning av blodkärl. Studie III och IV visar att bukspottskörtel och njure från gris som decellulariserats i kyla respektive rumstemperaturer resulterade i avsaknad av cellkärnor och bevarande av extracellulära matrixproteiner. Recellulariseringen av delar av skaffold från bukspottskörtel och njure med humana fetala stamceller från bukspottskörtel eller njure visade infästning, infiltrering och tillväxt av humana celler. De recellulariserade delarna av bukspottskörtel uttryckte också karaktäristiska exokrina (α-amylas) och endokrina (c-peptid, glukagon) markörer. De recellulariserade delarna av njure visade ett ökat uttryck av viktiga transkriptionsfaktorer involverade i njurutveckling. Sammanfattningsvis har protokoll för decellularisering av mänskliga vener, samt hud, bukspottkörtel och njure från gris fastställts. Recellularisering av vener med perifert blod och applicering acellulär grishud i gelform tillsammans med mononukleära celler från perifert blod kan gynna patienter med kärlsjukdomar eller brännskador. Recellulariseringen av skaffod från bukspottkörtel och njure med humana fetala stamceller visar potentialen hos fosterceller för ytterligare studier samt för fortsatt recellularisering av intakta organ. Decellularisering och recellularisering kan således ha en viktig framtida betydelse för regenerationsbiologi och slutligen för organtransplantation.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Vijay Kumar Kuna,** Bo Xu and Suchitra Sumitran-Holgersson. Decellularization and Recellularization Methodology for Human Saphenous Veins.
Journal of Visualized Experiments. 2018; 137, e57803.

II. **Vijay Kumar Kuna*,** Arvind Manikantan Padma*, Joakim Håkansson, Jan Nygren, Robert Sjöback, Sarunas Petronis and Suchitra Sumitran-Holgersson. Significantly Accelerated Wound Healing of Full-Thickness Skin Using a Novel Composite Gel of Porcine Acellular Dermal Matrix and Human Peripheral Blood Cells.
Cell Transplantation. 2017; 26(2): 293-307.

III. Erik Elebring, **Vijay Kumar Kuna,** Niclas Kvarnström and Suchitra Sumitran-Holgersson. Cold-perfusion decellularization of whole-organ porcine pancreas supports human fetal pancreatic cell attachment and expression of endocrine and exocrine markers.
Journal of Tissue Engineering. 2017; 8: 2041731417738145.

IV. **Vijay Kumar Kuna*,** Sanchari Paul*, Bo Xu, Robert Sjöback and Suchitra Sumitran-Holgersson. Human fetal kidney progenitor cells regenerate acellular porcine kidneys via upregulation of key transcription factors involved in kidney development.
Manuscript.
* – Equal author contribution

CONTENTS

ABBREVIATIONS

CD	Cluster of differentiation
CK	Cytokeratin
DAPI	4', 6-diamidino-2-phenylindole
DLK-1	Delta-like 1 homolog
DNase	Deoxyribonuclease
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
EPC	Endothelial progenitor cells
Eph	Ephrin
FGF	Fibroblast growth factor
GAGs	Glycosaminoglycans
HA	Hyaluronic acid
hFKPC	Human fetal kidney precursor cells
hFPSC	Human fetal pancreatic stem cells
HLA	Human leukocyte antigen
HE	Haematoxylin and eosin
ICC	Immunocytochemistry
IF	Immunofluorescence
IHC	Immunohistochemistry
MMP	Matrix metalloproteinase
MT	Masson's trichrome
PAA	Peracetic acid
PBMC	Peripheral blood mononuclear cells
PSG	Pig skin gel
qPCR	Quantitative polymerase chain reaction
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor
α-gal	Galactose-alpha-1,3-galactose

1. INTRODUCTION

The failure of or damage to tissues and organs often caused by major health problems, accidents and so on is having a significant impact on health expenditure [1]. Treatments other than drugs include surgical repair, the implantation of artificial prostheses, mechanical devices and transplantation with autologous, allogeneic or xenogeneic tissues and organs.

1.1 Transplantation

Transplantation is the procedure by which a healthy tissue or organ explanted from a deceased or living donor is implanted to replace the damaged or non-functional tissue or organ in the recipient. During end-stage organ failure, transplantation with healthy organs will improve the quality of life of patients [2]. The common organs or tissues transplanted in clinics today are the skin, heart, lungs, kidneys, blood vessels, pancreas, islets of Langerhans, liver, small intestine, heart valves and bone marrow [3].

Organs or tissues used in transplantation can be autologous (same person), allogeneic (genetically non-identical person), isogeneic (genetically identical person) and xenogeneic (graft from different species). In humans, an isogenic graft donor is mostly a twin brother or sister. Compared with allogeneic transplants, isogeneic transplants have a greater chance of survival, as they have a genetically identical major histocompatibility complex, but they are rarely performed, due to lack of twin siblings [4].

1.1.1 Autologous transplantation

An autologous transplant is tissue from a healthy site in the same person that is used to replace the damaged tissue. The most frequently used autografts are skin grafts for severe burns and saphenous veins for coronary artery bypass. As autologous tissue shares the same antigenic components, it is not rejected and immune suppression is not required for patients receiving autografts. Autografts are regarded as the gold standard in transplantation surgery. However, it is always difficult to obtain suitable transplantable quality grafts from the same person. Edwards et al. reported that 30% of arterial bypass patients had unsuitable saphenous veins [5]. Another problem with autografts is that it requires an operation to explant the graft.

1.1.2 Allogeneic transplantation

Allogeneic transplantation involves the use of tissues or organs from deceased, brain-dead or living, genetically non-identical individuals. Most transplanted grafts today are allogeneic. Since the recipient's immune system regognizes the allograft as

foreign, a series of cellular and molecular events that are initiated after transplantation may finally result in rejection of the graft. The correct selection of a donor following clinical guidelines (a lower number of human leukocyte antigen (HLA) mismatches, age, gender, race, serological testing for infectious diseases, diabetes and so on) and treatment with immunosuppression improve the time and chance of graft survival [6, 7]. At present, the survival rate one year after transplantation for most organs exceeds 90% and acute rejections can be controlled effectively. However, chronic rejections and mortality or morbidity resulting from immunosuppression persist [8].

1.1.3 Xenogeneic transplantation

Xenogeneic transplantation uses tissues or organs from a different species. Pigs have been regarded as the best organ source because of their similar anatomy, organs of comparable size, early maturity, short gestation time, high litter number, easy breeding in a microbiologically controlled environment and function similar to human organs [9]. The main obstacle in pig-to-human transplantation is the hyperacute rejection initiated by the galactose-alpha-1, 3-galactose (α-gal) epitope. Humans have natural antibodies to α-gal in circulating blood, causing the activation of complement and clotting systems when human blood contacts the transplanted pig organ [10]. However, porcine and bovine heart valves fixed in glutaraldehyde are used in clinical transplantation with success rate of more than 80% [11] in short term studies of up to 5 years. Glutaraldehyde forms a covalent bond and crosslinks with all free amino groups and makes the tissue stable [12]. Currently, several knockout porcine models lacking the enzyme galactosyl transferase, human complement and coagulation-stimulating genes have been produced [13-18]. Xenotransplantation with hearts derived from these pigs and appropriate maintenance resulted in an increase in graft survival rate for over 900 days [19]. Despite the advances in generating these pigs, their use for human transplantations is limited because of the ethical issues and the risk of endogenous porcine retrovirus transfer [20, 21].

1.1.4 Lack of organ donors causing human death

Despite the availability of organs from different sources, the whole world is facing a shortage of organs, where patients awaiting transplantation far outnumber the organs available. The median waiting time has increased substantially, resulting in the death of many patients before they can receive a suitable transplant because of the lack of organ donors [22, 23]. The lack of human organ donors and the problems involved in xenotransplantation have caused researchers to explore alternative solutions.

1.1.5 Alternative solutions to organ transplantation

The generation of artificial organs might be a solution to the problem of donor organ shortage. The most successful example is the dialysis machine that replaces the function of the kidney by removing waste and water from blood. It is used for patients who have lost their kidney function, but it only serves as a bridge until a suitable kidney for transplant is found [24]. Other mechanical devices of this kind include the artificial heart [25], the pacemaker [26], the ventricular assistance machine [27] and artificial lungs [28]. The disadvantages of these alternatives include the risk of adverse reactions to the materials used, infections, everyday care, high cost and longevity.

Tissue engineering emerged as a technology in the 1960s, when biologically compatible materials were used to make skin tissue [29]. The advancements during the past two decades in the fields of immunology, molecular biology and biomaterials laid the foundations in the field of regenerative medicine that runs alongside tissue engineering.

1.2 Tissue engineering

Tissue engineering can be defined as a technology used to create or recreate or reconstruct functional organs or tissues for transplantation [30]. The reconstruction of organs or tissues requires scaffolds, cells and growth nutrients. A scaffold can be defined as a three-dimensional, porous structure that mimics the extracellular matrix (ECM) of a native tissue or organ. Scaffolds are very important, as they provide support for cell attachment and help in subsequent tissue remodelling. Most probably, the best scaffold for any tissue engineering is the natural ECM of respective tissue or organs. The important properties to consider while using any other scaffolds are as follows [31, 32].

1) Architecture: the scaffold should have an architecture and porosity that are able to support cell migration and the transportation of micro molecules.

2) Cyto-compatibility: the scaffold should support the adhesion, proliferation and differentiation of cells in vitro and in vivo.

3) Bioactivity: the scaffold should possess signalling molecules that interact with cells, regulate their function and influence tissue remodelling.

4) Mechanical strength: the scaffold should provide shape, stability and intrinsic biomechanical properties for the anatomical site of implantation.

5) Manufacturing technology: in order to be clinically and commercially feasible, it should be possible to produce the scaffold cost effectively and in accordance with good manufacturing practices (GMP). Easy storage and availability, such as off the shelf, are regarded as an added advantage.

Many varieties of scaffolds generated by a number of methods using natural and synthetic polymers are currently available for tissue engineering. Based on the materials used to make scaffolds, they can be broadly divided into synthetic and biological scaffolds.

1.2.1 Synthetic scaffolds

Synthetic scaffolds made from inorganic or organic materials using different fabrication technologies can have good porosity, physical and mechanical properties. They can be efficiently tailored for the tissue engineering of soft or hard tissues. However,

they may be less biocompatible and, for this reason, they are sometimes surface modified to improve cyto-compatibility. Although the use of fabrication technology produces scaffolds of the required shape, size and mechanical properties, they lack the internal microvasculature, spatial configuration and three-dimensional arrangement of proteins, native carbohydrates and growth factors [33]. The materials used in the generation of synthetic scaffolds can be broadly categorized into synthetic polymers, bioceramics and nanocomposites.

1.2.1.1 Synthetic polymers

Synthetic polymers commonly used in the tissue engineering of scaffolds are polyglycolic acid (PGA), poly-l-lactic acid (PLLA), poly-l-glycolic acid (PLGA) and polycaprolactone (PCL). These polymers are biodegradable and can be effectively fabricated into scaffolds with varying pore sizes, fibre dimensions, mechanical properties and degradation rates. Synthetic polymers are widely used to make scaffolds for tissues like blood vessels, the meniscus and so on [34, 35]. To improve cyto-compatibility, synthetic polymers are also blended with natural polymers (carbohydrates or signalling proteins like vascular endothelial growth factor (VEGF)), followed by crosslinking and fabrication [35-38].

1.2.1.2 Bioceramics

Bioceramics are popularly used in load-bearing tissue engineering applications like bone. They are inorganic compounds and include synthetic hydroxyapatite, bioactive glass and tricalcium phosphate (TCP) [39, 40]. Hydroxyapatite is used as bone filler. However, the scaffolds made using only hydroxyapatite have poor mechanical strength and a low resorption rate [41]. To modulate the mechanical properties and resorption rates, bioceramics can be blended with synthetic polymers to make composites that are used to produce scaffolds [42].

1.2.1.3 Nanocomposites

Nanocomposite fibrous scaffolds are usually generated by the electrospinning of synthetic and natural polymers. The nanofibres that are generated mimic the dimensions of collagen fibres and thereby the ECM environment by having a high surface-to-volume ratio, porosity and mechanical strength. Scaffolds made from nanofibres have been shown to influence stem cell differentiation and proliferation [43-46]. Scaffolds made from nanofibres have been used in the tissue engineering of the trachea and cartilage [47, 48].

1.2.2 Biological scaffolds

Biological scaffolds are generated from biological materials. The composition and structure of these scaffolds depend on the source of material, processing and sterilization [49]. The materials used in the preparation of these scaffolds are briefly described below.

1.2.2.1 Natural polymers

The natural polymers used in scaffold preparation are popularly proteins and polysaccharides. The proteins include silk, collagen, gelatin, fibrinogen, elastin, keratin, actin and myosin. The polysaccharides include cellulose, amylose, dextran, chitosan and glycosaminoglycans. These natural materials are polymerized and fabricated to generate scaffolds for tissue engineering [50].

1.2.2.2 Cell sheets

In culture, cells secrete their own ECM while growing and can be harvested from thermo-responsive polymer without using enzymes at confluence. Several single cell layers can be laminated to generate a thick matrix. The scaffolds generated from cell sheets with relevant sources have been used for the tissue engineering of the cornea, myocardium, blood vessels and skin [51-54]. However, cell sheets have limited applications to thin tissues alone, as making an organ with cell sheets is difficult. Other disadvantages include the high cost and long preparation time.

1.2.2.3 Hydrogels

Hydrogels are semi-liquid hydrophilic materials made by the covalent crosslinking of natural materials (collagen, gelatin, fibrin, alginate, chitosan) or synthetic polymeric materials (polyethylene glycol, polylactic acid) [55]. Hydrogels are good for cell adhesion, cell migration, angiogenesis and controlled degradation to enhance tissue remodelling. However, they have poor mechanical strength because of their high water content [45]. Hydrogels coupled with growth factors, adhesive peptides and ECM proteins are currently being generated to enhance cell survival and tissue remodelling [56].

1.2.2.4 Acellular scaffolds

Human or xenogeneic organs can be engineered by removing immunogenic components (cells) from the organ by processes known as "decellularization". The acellular scaffold generated by decellularization may retain the necessary ECM proteins in spatial and three-dimensional confirmity. The acellular scaffold can thus be an ideal scaffold for cell attachment, growth, migration, differentiation and the transportation of gases, nutrients and regulatory factors. The acellular scaffold also provides mechanical properties similar to the organ or tissue of interest and is non-immunogenic [50]. For these reasons, acellular scaffolds can be regarded as superior to natural polymers, cell sheets and hydrogels.

The current thesis focuses on generating acellular scaffolds and using them to recellularize with human cells for future potential therapeutic strategies. The acellular scaffolds seeded with cells can recreate a functional organ. In clinical transplantations, the recipient cells can be seeded to produce a personalized organ for the patient. The reseeding of cells into scaffolds requires a large number of cells. Functional organs like the lungs, kidneys and liver have a complex, intricate architecture containing billions of cells with multiple cell types arranged in an organized fashion. Suitable cells (stem cells or tissue-specific cells) are first expanded to the required number and then seeded into the scaffold, followed by culture for several days to months in a bioreactor under suitable conditions. The functional organ derived using patient cells following a procedure of this kind can be used for transplantation without immune suppression [57].

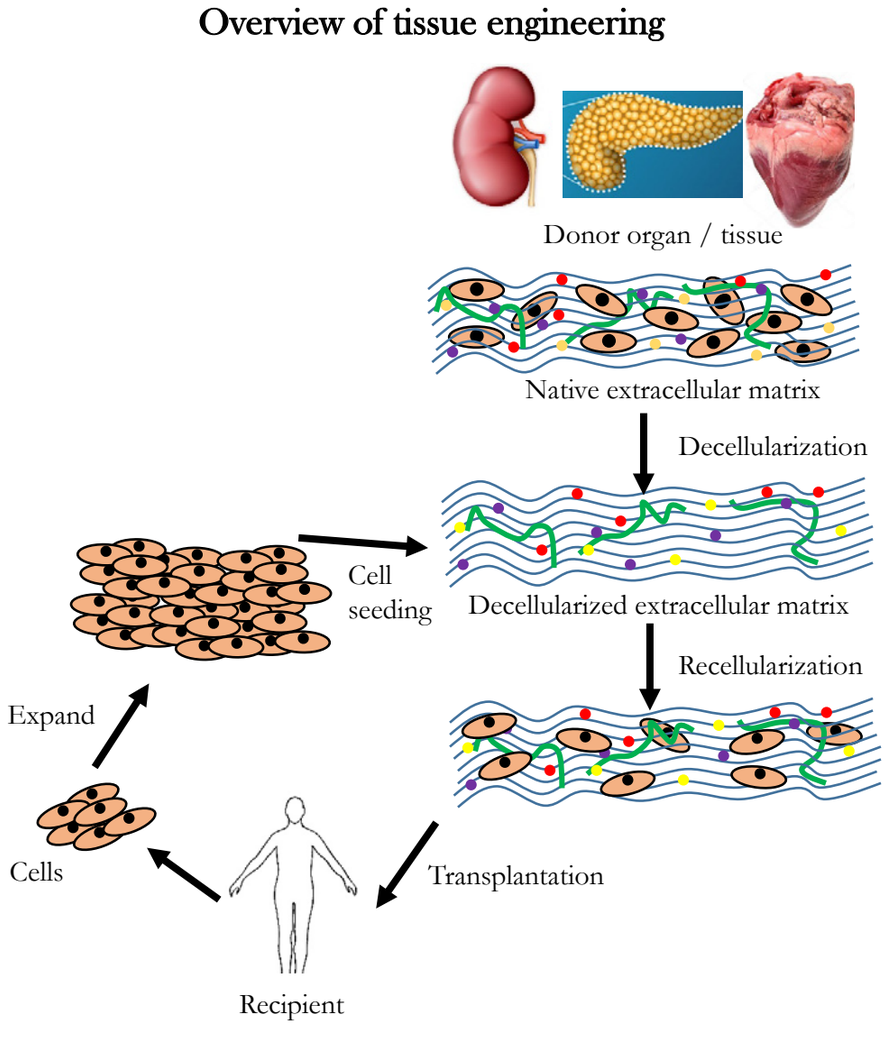

FIGURE 1: The image gives an overview of the tissue engineering process using a decellularization and recellularization method to generate a personalized organ suitable for transplantation.

1.3 ECM

The ECM is the interstitial matrix present between the cells and underlying the cells called basement membrane. In addition to acting as a substrate for cell attachment, the ECM also assists with cell migration and differentiation leading to tissue morphogenesis and the maintenance of homeostasis [58, 59]. The ECM is composed of proteins, polysaccharides, minerals and water. The main proteins and polysaccharides constituting the ECM are collagen, elastin, glycosaminoglycans (GAGs), fibronectin, laminin and associated growth factors. Every organ and tissue is composed of an ECM with a unique composition. The composition of the ECM depends on the local cell type of tissue and dynamic changes that occur throughout development. The cell type and composition of the ECM also determine the biomechanical properties such as elasticity, tensile strength, burst pressure and compression strength. The composition of the ECM is also altered continuously, depending on the conditions [58].

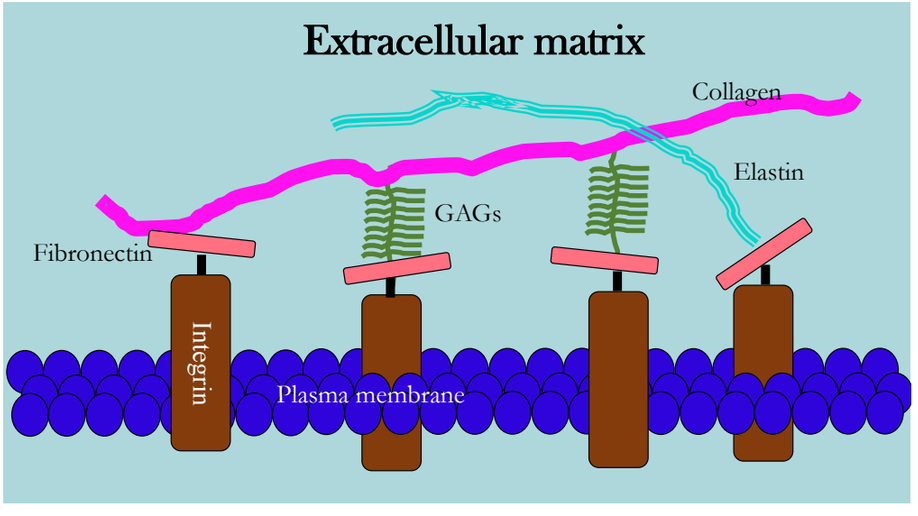

FIGURE 2: The image shows the arrangement of proteins in a three-dimensional configuration while interacting with the plasma membrane of a cell.

1.3.1 Collagen

Collagen is the most abundant protein in the ECM and human body. To date, almost 28 types of collagen have been discovered, formed from 46 different polypeptide chains. Each polypeptide chain has a characteristic feature containing repetitive glycine-X-Y motifs where X and Y are mostly proline and 4-hydroxyproline. Based on the structure and function, collagens can be categorized into fibrillary, network-forming,

fibril-associated, membrane-associated, beaded-filament-forming, anchoring fibrils and endostatin-producing types [60].

Collagen types I, II and III are the main fibrillary collagens and they account for 80-90% of total collagens. These collagens give mechanical strength to tissue. Collagen types I and III are mainly located in skin, muscle, blood vessels, tendon, bone and dentine. Collagen II is mainly located in cartilage [60]. Network-forming collagens, such as collagen IV, are mainly found in the basement membrane.

1.3.2 Elastin

Elastin forms the fibrous component of the ECM that is made of amorphous elastin and microfibrils. Elastin is formed from tropoelastin, a secreted precursor that polymerizes and self-assembles to form insoluble elastin fibre [60]. The hydrophobic domains of elastin mainly contribute to the elastic property of tissue [61]. Microfibrils mainly locate in the periphery of fibre and help in the correct alignment of elastin molecules. The composition of microfibrils includes fibrillin 1, 2 and 3 and large-sized cysteine-rich glycoproteins [62]. Elastic fibres generated during early development are stable compared with adult fibres. Sometimes, organ failure may result from damage to elastic fibres by matrix metalloproteinases (MMPs) and aspartic, cysteine or serine proteases. The optimal elastin fibre formation and function is required for the correct function of arteries, lungs, heart, bladder and skin.

1.3.3 Glycosaminoglycans (GAGs)

GAGs are large molecules with a protein backbone attached by many large polysaccharide side chains. They act as a water-retention component and serve as a lubricant and shock absorber for tissues. Importantly, they participate in cell signalling, proliferation, adhesion, migration, differentiation and apoptosis by interacting with cytokines, chemokines and growth factors in the ECM through polysaccharide side chains or protein core [63-66]. GAGs comprise sulfated and non-sulfated types. The sulfated GAGs are heparin sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate. The non-sulfated GAGs are hyaluronic acid and heparin.

1.3.4 Fibronectin

Fibronectin is a high-molecular-weight glycoprotein present in the ECM of a variety of cell types. Fibronectin is composed of subunits that are covalently linked by disulfide bonds. In its soluble form, fibronectin is present as plasma-fibronectin and cellular-fibronectin molecules. Cells assemble the plasma and secreted cellular fibronectin molecules into a supermolecular fibre and subsequently into a fibrillar state [67, 68]. Fibronectin interacts with collagen and cells via their integrin receptors and helps with tissue formation, repair and remodelling [67, 69].

1.3.5 Laminin

Laminin is also a high-molecular-weight glycoprotein, primarily located in the basement membrane, together with collagen IV. It is a heterotrimeric protein and is able to interact with itself to form polymer [70-72].

Laminins are also able to interact with other ECM proteins and cells and help with adhesion, differentiation, migration and ECM organization. Laminins play a key role during wound healing by providing a substrate for the attachment of epithelial cells. During angiogenesis, they form a component of basement membrane for endothelial cells [71, 73].

1.3.6 Growth factors

In addition to a fibrillar meshwork of proteins and glycans, the ECM contains growth factors secreted by cells that will make it unique for a specific type of organ and site. The wide range of growth factors in the ECM include fibroblast growth factor (FGF), VEGF, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), granulocyte macrophage- colony-stimulating factor (GM-CSF), transforming growth factor ß (TGF-ß) and insulin-like growth factors (IGF). In the ECM, growth factors are usually found in association with GAGs like heparan sulfate. In its three-dimensional confirmation with all the stated molecules, the ECM regulates cell behaviour, proliferation, differentiation, tissue formation, repair and regeneration [74].

1.4 Decellularization

Decellularization is a method of removing cells from an organ or tissue, thereby generating a scaffold with an intricate architecture and a composition similar to the ECM of the native organ. The concept of decellularization and the evaluation of obtained ECM scaffolds began in 1986 with human lung pieces [75]. In recent years, several decellularization protocols for whole organs, simple and complex tissues from human and animal sources have been published [75-80]. The scaffold that remains after complete decellularization can be regarded as minimally immunogenic (as immunogenic cellular components are removed) and can be transplanted without any need for immune suppression [81].

Decellularization can be performed using single physical, chemical and enzymatic methods or a combination of these methods. Most decellularization protocols usually begin with a physical method to lyse the cells, followed by chemical or enzymatic treatments to separate cells and nuclear material from the ECM.

1.4.1 Physical methods

The physical methods used for decellularization are freezing, pressure, sonication and mechanical agitation [82]. In general, the physical agents use mechanical force to disrupt the cells and dislodge them from the surrounding ECM. These methods have an effect on the structure of the ECM. So care must be taken in terms of power and time of application for these methods. The physical treatment alone is unable to decellularize the tissue or organ and washing after the treatment is required to remove the damaged cells.

Rapid freezing causes the formation of ice crystals in cells that disrupt the cell membrane, thereby causing cell lysis. Even though cells are effectively lysed following this method, damage to ECM structures can occur because of ice crystals damaging

the collagen fibres. Although slow freezing and slow thawing may be of some benefit, ECM damage cannot be completely prevented. Freezing has been successfully used for the decellularization of nerves, tendons and ligaments [83-85].

Applying high pressure is also an effective method that destroys the cell membranes in a short time. However, the application of high pressure can damage the collagen fibres in tissue, thereby compromising their mechanical strength. High hydrostatic pressure has been used in decellularization for corneas [86] and blood vessels [87].

Sonication applies acoustic energy to cell membranes and ruptures them. Even though sonication is able to decellularize effectively in a short time, it can cause an increase in temperature which can damage the scaffold. Sonication itself may also damage ECM structures. Sonication has been used successfully to decellularize arteries [88].

Mechanical agitation is performed together with a chemical or enzymatic agent. Agitation helps to remove the superficial cellular material that is loosely attached to the ECM. Agitation also helps chemicals to reach the inner layers, thereby effectively removing cells. Agitation at high speeds can damage the ECM. Agitation has been used successfully to decellularize heart valves [89].

1.4.2 Chemical methods

Chemical methods include the use of acids, alkaline reagents, detergents, solvents, hypotonic and hypertonic buffers. Detergents are widely accepted and used in most decellularization protocols, as their effect on ECM proteins can be controlled by changing the concentration of detergent or choosing another detergent. Typically, detergents cause cell lysis by damaging the phospholipid membrane of cells [90].

1.4.2.1 Acid and alkaline reagents

The acid and alkaline reagents that are mainly used in decellularization include peracetic acid (PAA) and ammonium hydroxide. Acid and alkaline reagents use charge and pH effectively to solubilise the cell membrane, cytoplasmic and nuclear components. At the same time, they cause serious damage to collagen by altering its arrangement and they also remove GAGs from tissue. PAA has been used to decellularize urinary bladder [91, 92] and small intestine submucosa with mixed results [93, 94]. In addition, PAA acts as a sterilizing agent by entering micro-organisms and oxidizing their enzymes [94].

1.4.2.2 Detergents

Detergents consist of a hydrophobic tail and a hydrophilic head. Based on the charge of the hydrophilic head, detergents are categorized as ionic (cationic and anionic), non-ionic or zwitterionic types. Cell adhesion to the ECM and the arrangement of ECM proteins are based on protein-protein, protein-lipid and lipid-lipid interactions, where protein-protein interactions are the main ones [95, 96]. Because of their amphipathic nature, detergents disrupt or form hydrophobic or hydrophilic interactions with biological molecules, forming a detergent-protein/lipid complex [97]. They thus disrupt protein-protein, protein-lipid and lipid-lipid interactions in the cell-ECM environment. This leads to the release of cells from the ECM following the rupture of cell membrane, the release of soluble cytoplasmic proteins and damage to cell membrane proteins interacting with the ECM. In general, detergents may be effective against one or two of the protein-protein, protein-lipid or lipid-lipid interactions but not all and a mixture of detergents may therefore be required for the effective removal of cellular material from the ECM.

The ionic detergents used in decellularization are mainly sodium dodecyl sulfate (SDS) and sodium deoxycholate (SDC). Ionic detergents are harsh detergents. They are well known for their ability to destroy cell membranes and nuclear material. However, they also damage proteins by destroying protein-protein interactions [98]. SDS is an anionic detergent effectively used in the decellularization of many organs and tissues [93, 99-102]. SDS treatment also damages the native ECM architecture by damaging the arrangement of collagen fibres [103] and also causes the loss of GAGs and important growth factors [82, 104]. SDC is another anionic detergent used in several decellularization protocols. One study showed that SDC-treated scaffolds were better than SDS, as they showed higher metabolic activity when seeded with cells [93]. In some studies, SDC has been used in combination with other detergents or enzymes [104]. SDC has been used in the successful decellularization of rat lungs and heart valves [105, 106]. It has been reported that SDC causes the agglomeration of deoxyribonucleic acid (DNA) [82], thereby making it difficult to remove by washing.

The non-ionic detergents damage lipid-lipid and lipid-protein interactions, leaving protein-protein interactions intact. As a result, they do not denature proteins and leave them in native form. They are regarded as mild detergents [107]. The widely used non-ionic detergent is Triton X-100. Decellularization using Triton X-100 alone has been least successful, as cellular material has been noticed even after long-term treatment [108-111]. The effect of Triton X-100 on the ECM is, however, dependent on the concentration. It has shown mixed results in terms of the loss of GAGs and compromising tensile strength [109, 112, 113]. In a few studies involving the decellularization of whole organs by perfusion, Triton X-100 was used to remove remnant SDS [76, 79, 93, 105].

The zwitterionic detergents exhibit the properties of both ionic and non-ionic detergents. They are harsher than non-ionic detergents and weaker than ionic detergents. The most commonly used zwitterionic detergent is 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO). Decellularization using CHAPSO has shown a loss of nuclei and collagen preservation in blood vessels and lungs. However, in blood vessels, the biomechanical properties were compromised [107] and, in lungs, cytoplasmic remnants were not completely removed [105].

1.4.2.3 Other chemicals

Tri-n-butyl phosphate (TnBP) is an organic solvent and functions by disrupting protein-protein and lipid-protein interactions. TnBP has been used in the decellularization of tendon and ligament [109, 110]. TnBP has shown a mild effect on mechanical properties and ECM proteins. Hypotonic and hypertonic treatment lyses cell membrane by osmosis. However, the removal of cellular remnants requires other treatments. These solutions have been used in the decellularization of blood vessels and ligaments [107, 110, 114].

1.4.3 Enzymatic methods

The enzymatic agents include proteases and endonucleases. Proteases are regarded as harsh decellularization agents and their use is often coupled with extensive ECM damage. The commonly used protease is trypsin, a serine protease and it is often used together with ethylene diamine tetra acetic acid (EDTA). Although trypsin effectively removes cells, its prolonged exposure affects the collagen, elastin and GAG content [115]. Simple tissues such as heart valves have shown complete cell removal with trypsin [89]. In many protocols for complex tissues and organs, trypsin has been used in combination with other detergents [103].

Endonucleases such as deoxyribonuclease I (DNase I) are used to hydrolyze the deoxyribonucleotides, thereby degrading DNA during decellularization. DNase-I treatment maintains collagen, fibronectin and laminin but not GAGs [104]. However, treatment with endonucleases helps to reduce the time for decellularization in complex organs [116]. Even though concern remains about the effective wash-out of DNase after decellularization, one study has shown the absence of functional DNase in decellularized tissue [117]. DNase has been used in the decellularization of several tissues like lungs and heart valves [104].

1.4.4 Additives in decellularization solutions

Protease inhibitors are added to detergent solutions for decellularization to save the ECM structure from proteases released after cell lysis. They might be important while decellularizing tissues or organs with high metabolic activity. The common protease inhibitors that are used are phenyl methyl serine protease (PMSF), aprotonin and leupeptin [82].

Chelating agents such as EDTA are added to decellularization solutions, as they bind the divalent cations Ca2+ and Mg2+, thereby stopping the action of intracellular enzymes. In addition, the divalent cations are required for cell adhesion [118].

To prevent bacterial contamination, antibiotics and bacteriostatic agents are added to decellularization solutions. The common antibiotics that are used are penicillin, streptomycin, amphotericin and gentamycin [82]. The bacteriostatic agent, sodium azide, is also used in some protocols to stop bacterial growth during decellularization [119].

1.4.5 Washing decellularization chemicals

Chemicals and enzymes used for decellularization procedures must be removed for effective recellularization and transplantation, as they are cytotoxic and immunogenic. Extensive washing with distilled water or PBS is used for this purpose. No simple methods currently exist for the quantification of residual decellularization chemicals in decellularized tissues. Although methods like HPLC can be used, they are not widely used because of their technical complexity and the cost involved. One study [120] showed the presence of remaining detergents in decellularized heart valves, indicating that heart valves currently used in the clinic and decellularized using a similar protocol might also contain detergent remnants. The authors noticed a reduction in the amount of detergent only after washing in eight changes of water; this was four times longer than the detergent exposure time. However, in many cases, scaffolds supporting cell attachment and proliferation are regarded as good, non-toxic and suitable for transplantation [120-122].

1.4.6 Sterilization after decellularization

Although performing decellularization in sterile conditions is optimum for future recellularization and transplantation, it is not done in several protocols because of high costs and elaborate maintenance. So, after decellularization, tissues and organs need to be sterilized. Treatment with low concentrations of PAA is commonly used, as it kills bacteria by oxidizing their enzymes [94]. Irradiation using gamma rays is also regarded as an effective method. Although the effect of gamma rays on the structural components of the ECM has been demonstrated, several published papers have reported the successful recellularization of tissues and organs sterilized using this method [123-126]. Using gamma irradiation, one study showed that the residual lipids of the ECM became cytotoxic and increased tissue degradation has been noted during recellularization [127]. Another study showed that exposing the tissues to gamma irradiation at low and high dose for long time altered mechanical properties [125]. Ethylene-oxide and electron-beam irradiation have also been shown to sterilize decellularized grafts effectively. Because of their non-availability in regular research environments, very little research has been performed using these methods. However, both these methods are known to affect the ECM structure, thereby altering biomechanical properties [123]. Supercritical carbon dioxide in combination with PAA has also been used as a sterilization method for porcine dermal matrix. Sterilization was achieved after 30 min of exposure, with a minimal alteration in biomechanical properties [128].

1.4.7 Verification of decellularization

The verification of decellularized tissues or organs is essential, as any remnants of immunogenic compounds can cause a significant immune response, leading to organ failure [104]. However, no guidelines that confirm a decellularized tissue or organ currently exist. Decellularization is assessed by taking a biopsy from the tissue and processing and analysing using histological methods. Haematoxylin and eosin (HE) staining, that stains nuclei blue is widely used. Masson's

trichrome (MT) staining is another histology method that stains nuclei blue/black, cytoplasm red and collagen blue. Other histology methods that stain nuclei, such as Movat's pentachrome, safranin O staining, can also be used [82]. In addition, the removal of cytoplasmic components can be determined by immunohistochemistry (IHC) using specific antibodies. Staining for proteins like actin, vimentin, α-Gal is used [110, 129]. A decellularization method showing the absence of nuclei and cytoplasmic proteins in decellularized tissues is generally considered successful. Although not widely used, the scanning and transmission electron microscopy (SEM & TEM) of decellularized tissues can also provide a deeper insight into the presence of cells, cellular remnants, cytoplasmic proteins and organelles [82]. The problem with using SEM & TEM is, visualization at high resolution gives detail information of ECM and the components present but covers a small area and vice versa. In addition, very small samples of less than 1mm are preferred for TEM, thus whether this small size samples are representative for whole tissue or organ needs to be determined.

Qualitative and quantitative methods for DNA are also widely used in verifying decellularization. Qualitative methods include histology staining using DNA binding dyes 4´, 6-diamidino-2-phenylindole (DAPI) or Hoechst. The isolation of DNA and quantification using spectrophotometric or fluorometric methods is also highly regarded [119]. One study showed that decellularized tissues containing less than 50 ng/mg dry weight and a DNA length of less than 200 base pairs can be regarded as decellularized and these tissues do not initiate an immune response after transplantation [130]. The length of DNA can be estimated by running isolated DNA on agarose gel electrophoresis.

1.4.8 Characterization of decellularized ECM

The characterization of decellularized ECM includes an assessment of biomechanics, ECM proteins and growth factors. The characterization of decellularized ECM helps us to understand the effect of detergents on ECM structure and function. Characterization also determines the suitability of the tissue or organ for recellularization. ECM proteins and growth factors can be assessed qualitatively using IHC staining and quantitatively using dye-based assays or antibody-based methods like ELISA, Western blot and luminex. Dye-based methods are mostly used to quantify collagen, elastin and GAGs, while fibronectin, laminin and specific collagen types are detected using IHC staining. ELISA, Western blot and luminex are popular for the quantification of growth factors [101, 119, 131]. Though the decellularized ECM is believed to contain proteins that can function, no direct evidences exist that prove the function of ECM proteins in vitro.

Biomechanical properties are very important for any tissue or organ as the decellularized tissue with poor biomechanical properties may result in graft damage if transplanted. The common biomechanical tests currently used for most organs include compression, tensile testing, burst pressure test and suture retention. The type of test that is chosen depends on the type of ECM and the function of the organ. All these tests involve the application of forces of different magnitudes, directions and frequencies, while recording the displacement or until the organ or tissue fails. The results are compared with the corresponding native tissue to determine whether the decellularized tissue or organ has satisfactory mechanical properties [101, 119, 131].

Although all decellularization methods using various compounds affect the ECM proteins, structure and biomechanical properties, the balanced use of detergents, enzymatic agents and physical methods can effectively remove donor cells from a tissue, without major alterations in structure and arrangement of ECM components and biomechanics [82, 130, 132].

1.5 Recellularization

Recellularization is a procedure for seeding cells back into the decellularized organ or tissue to achieve function. The recellularization of organs or tissues requires a renewable cell source, a cell-seeding strategy, an organ- or tissue-specific bioreactor that provides favourable conditions and a culture medium [133]. The conditions required include continuous perfusion with growth media (vitamins, minerals and amino acids), the optimum temperature (37°C), gases (oxygen and carbon dioxide), pressure and maintaining a slightly basic pH between 7.2 and 7.4. Different types of cell are required for the recellularization of different organs. Although simple tissues require one or two cell types, functional organs require multiple cell types. Since millions of cells are required for recellularization, obtaining a sufficient number of autologous cells from the recipient is not possible in most instances. For example, the recellularization of a human liver requires 10 billion hepatocytes, which is 5-10% of the mass of a human liver [134]. So, use of stem cells with a high proliferative capacity is required. Although significant work has been done to establish effective recellularization protocols, some success in the clinical studies has been achieved for simple tissues like blood vessels [53], urethra [135], bladder [136], heart valves [137-139] and skin [140, 141]. Extensive research on functional organs like the lungs [77], kidneys [142] and liver [79] has revealed partial recellularization and partial success in animal transplantations. The different stem cells used for recellularization are embryonic, fetal, adult, mesenchymal and induced pluripotent cells.

1.5.1 Embryonic stem cells (ESCs)

ESCs are obtained from embryos prior to implantation in the uterus. ESCs are regarded as undifferentiated and pluripotent, possessing the ability to form any organ [143]. In addition, they have high proliferation and differentiation capacities in the presence of the appropriate stimuli in vitro [144]. The numbers of cells required for recellularization can therefore be achieved with ESC cultures. However, the clinical application of these cells is difficult, because of several ethical hurdles and safety issues. Nevertheless, after appropriate ethical approval, they can be explored in experimental investigations using ESCs obtained from animal embryos [145]. The

use of ESCs has been mainly reported in the recellularization of decellularized kidneys and lungs. In kidneys, they have mainly repopulated the vascular and glomerular areas but occasionally also the tubular compartment [146-148]. In the lung, they have mainly repopulated alveolar spaces [149]. It has been reported that, after seeding into acellular organs, ESCs lose their pluripotency and begin to differentiate towards renal and endothelial lineages [146, 148]. The differentiation of ESCs is highly dependent on ECM signals and the loss of these during decellularization prevents effective organ-specific differentiation [133, 150]. One study has shown that the pre-differentiation of ESCs into committed progenitor cells directs them to an organ-specific type where mesendodermal cells derived from ESCs only differentiated into cardiac marker-expressing type cells when seeded onto decellularized cardiac scaffolds [151].

1.5.2 Fetal stem cells

Fetal stem cells can be derived from fetal blood and bone marrow, as well as from the organ of interest. Fetal tissues or organs possess large numbers of multipotent progenitor cells [152]. In addition, they have a high proliferation capacity, together with the expression of relevant phenotypic markers and function. Fetal cells from the first trimester are considered immature and express fewer HLA I and II molecules [153]. It has also been reported in mouse and rat transplantation studies that fetal cells are less immunogenic [153, 154]. Fetal cells from the first trimester have also been used in human transplantation to treat patients with Parkinson disease without immune suppression successfully [155, 156]. Fetal liver cells from the second trimester have been used in the treatment of liver cirrhosis and acute liver failure patients [157]. The patients transplanted with fetal cells showed marked clinical and biochemical improvements. In the tested animal models with immune suppression, the human fetal cells engrafted into the damage site differentiated into functional cell types and were involved in tissue repair [158]. However, the use of fetal cells also has some complex ethical and unresolved issues in some countries but fewer compared with ESCs. Fetal cells have also produced successful results by the attachment and expression of phenotypic and functional markers when seeded onto decellularized rat lungs [77, 131], liver [159-161] and kidneys [142].

1.5.3 Adult stem cells

Adult stem cells can be obtained from bone marrow, blood, fat and various organs or tissues. Bone marrow is a reliable source of stem cells in adults. Although they possess a proliferative capacity, current knowledge of their differentiation capacities to all required cell types is limited. Bone marrow stem cells have been used in the tissue engineering of heart valves [162]. The use of adipogenic stem cells in recellularization has recently gained importance because of easy retrieval. These cells have shown promise in the recellularization of kidneys and lungs. A study of recellularized kidneys reported the expression of endothelial and functional renal cell markers [163]. In lungs, they differentiated into pericytes and stabilised the endothelial layer [164].

Organ-specific cells obtained from biopsies or donor organs have also been used. They can be ideal for use in recellularization, as they constitute the phenotypic and functional capacities of respective organs. However, they cannot be obtained in large enough numbers and, since they have a very limited proliferation capacity, expansion in vitro is difficult. Adult organ-specific cells obtained from the liver [79, 165, 166], lungs [101] and kidneys [100] have been used in recellularization. Although these organs were not functionally evaluated, the cells attached to the decellularized matrix and expressed related phenotypic markers.

1.5.4 Mesenchymal stem cells (MSC)

MSCs are the multipotent adult stem cells that are located in bone marrow, adipose tissue, amniotic fluid, cord blood, olfactory mucosa and Wharton's jelly [133]. In addition, MSCs are also known to be present in adult organs like the lungs and liver [167, 168]. MSCs have a high proliferation capacity and are well known to differentiate into osteoblasts, chondrocytes, myocytes and fat cells, but they have also been shown to differentiate into several other cell types like airway epithelial cells, renal tubular cells and cardiomyocytes [168-172]. MSCs are also known to be involved in tissue repair and secrete cytokines and chemokines to aid in stromal support and integration. MSCs have been used in the recellularization of several organs and tissues with mixed success [117, 173, 174]. Although the source of MSCs also has an effect on their ability to differentiate, the enormous amount of literature demonstrates their potential to differentiate into many cell types from all sources [133].

1.5.5 Induced pluripotent stem cells (iPSC)

The iPSCs are adult cells reprogrammed into an embryonic state by the nuclear transfer of OCT4, SOX2, NANOG and LIN28 genes [175]. These cells possess an excellent proliferation capacity and differentiate to several cell types in the presence of the appropriate stimuli. This strategy provides an easy cell source from adult patients and the opportunity to create organ-specific cell types in the required numbers. The iPSCs have been used in recellularization of the lungs, heart and kidneys [176-178]. Recellularization with iPSCs has also revealed the expression of phenotypic and functional markers, but the biochemical function of these cells is not known. In comparison, iPSCs showed decreased adhesion to scaffolds compared with MSCs [179]. More studies using iPSCs are required to predict their use in recellularization.

1.5.6 Support cells

Support cells include the mural, stromal, immune and interstitial cell types. Depending on the type of organ, the seeding of these cell types together with organ-specific cells might improve in remodelling and complete function [133]. Mural cells include pericytes and vascular smooth muscle cells. They are involved mainly in the formation of vasculature [180]. The addition of these cells to tissue-engineered skeletal muscle improved the formation and stabilisation of vessels [181]. Kupffer cells are the immune cells of the liver. The improved adhesion and viability of hepatocytes was noted when hepatocytes were seeded together with Kupffer cells and pericytes [160].

Moreover, improved migration was seen when hepatocytes were seeded with MSCs [182].

1.5.7 Preconditioning

Since the decellularization protocols currently used for any organ or tissue affect and cause alterations in scaffold composition which may affect recellularization efficiency, the preconditioning or coating of decellularized organs or tissues has been employed to improve the attachment and proliferation of incoming stem cells [133, 183]. Preconditioning with conditioned media, culture media, ECM proteins (collagen I, collagen IV, laminin and fibronectin), growth factors, matrigel and antibodies is thought to increase cell attachment, differentiation and proliferation. The preconditioning of decellularized rat lungs with conditioned media showed an improvement in the attachment and distribution of ESCs [184]. The preconditioning of decellularized ovine aortic valves with fibronectin and stromal cell-derived factor-1α (SDF-1α) improved the re-endothelialization and decreased the immune response-related degeneration of grafts after transplantation [185]. Decellularized bone scaffolds coated with collagen I, hydroxyapatite and SDF-1α improved osteogenesis with MSCs [186]. When conjugated to decellularized tissue, antibodies improve the chances of the selective binding of cells to tissue. Heart valves preconditioned with CD133 antibodies onto decellularized valves showed improved recellularization with CD133-expressing cells that subsequently became endothelial and smooth muscle cells [187, 188].

1.5.8 Techniques in cell seeding

The technique of seeding cells in organs or tissue influences their distribution and viability. Depending on the type of organ and tissue, various techniques are employed. The different techniques are passive, dynamic (rotation and pressure), perfusion, gravity, magnetic and electrostatic methods. In the passive method, generally used for membranous tissues and organ pieces, cells suspended in a small volume of medium are aspirated using a pipette and dropped over the whole surface or the tissue itself is incubated in a cell suspension [189]. For organs, cells are injected via the vasculature using a syringe [190]. This method is very simple, easy to perform and will not expose the cells to any damaging force [191]. However, the homogeneous distribution of cells throughout a scaffold is not always possible. In addition, the optimum incubation time for cell attachment is not known and cells may not attach if short incubation times are used. In addition, the seeded cells may also remain as clusters around a small lumen [192].

Dynamic seeding uses a force that increases the efficiency of cells to attach, distribute and penetrate the scaffold. Rotation is one technique that is applied to hollow organs like the trachea, blood vessels and small intestine. After filling with cell suspension, the tissue is rotated slowly for several hours in an incubator. This method has been used successfully for seeding endothelial cells on a vascular graft where 90% of the seeded cells remained attached [193]. Heart valves also showed improved cell adhesion and monolayer distribution by rotation after cell seeding [194]. The other method using force is centrifugal seeding where rotation at high speeds is performed. This method improves seeding efficiency and induces cell penetration. Although cell viability is not affected, the cell morphology is altered at high speeds, forcing researchers to use low centrifugation speeds with reduced efficiency. This method is popular for seeding cells on scaffolds for cartilage and bone [195, 196]. In other methods, positive or negative pressure (vacuum) has also been shown to improve cell attachment, distribution and penetration. A vacuum has mostly been used in the recellularization of porous scaffolds and hollow organs [197, 198]. However, vacuum seeding has also been used in the recellularization of kidneys through the ureter, where improved seeding efficiency was noted [142]. The spraying of cells is another method for the effective homogeneous seeding of cells. To spray cells, air is applied at low pressure near the nozzle carrying the cell suspension. The spraying of epithelial cells onto decellularized small intestine generated a homogeneous layer without any signs of cell aggregation [199].

Cell seeding by perfusion is a widely used technique for the recellularization of organs. Using the syringe or peristaltic pump at specified flow rates, a cell suspension is infused into the organ through the vasculature, followed by static incubation to enable cell attachment. An increase in cell adhesion was noted when hepatocytes were infused into decellularized rat liver by perfusion in comparison to direct infusion by syringe [190]. Cell density in suspension, flow rate and incubation time for attachment are factors that influence the efficiency of this method [133]. Several researchers have shown that multiple infusions serially at 10- to 15-minute intervals resulted in a significant increase in the number of hepatocytes attached to decellularized livers [79, 165, 200]. Although flow rates for cell seeding ranging from 0.2 mL/min to 25 mL/min have been used, low flow rates ranging from 0.2 to 3 mL/min have shown good results, with more and uniform cell attachment [131, 146, 178]. Although the optimal cell density for cell seeding in any organ is not known, seeding at high density increases the chance of forming cell clumps that in turn occlude small vessels causing tissue necrosis [79, 133, 190].

Gravity cell seeding has been used to seed endothelial and epithelial cells into blood vessels, the trachea and lungs [77, 201]. In this method, cells suspended at low density are allowed to perfuse through the organ using gravitational force, followed by incubation to enable cell attachment. In the lungs, this method produced complete seeding with endothelial cells at the lumen of vessels and with epithelial cells to the terminal bronchioles and alveoli.

Magnetic cell seeding uses magnetic nanoparticles that are able to bind specifically to a cell based on coated antibody. In the presence of a temporary magnetic field, cells spread and attach to the graft. This strategy has been used in the seeding of cells for blood vessels, where an increase in cell attachment was seen while maintaining viability. However, the clinical

application of this technology requires verification [202, 203].

The electrostatic seeding of cells has been mainly used for blood vessels, where a temporary positive charge is applied on the luminal side to the expanded negative charge. This method has been shown to increase seeding efficiency, achieve morphology maturation and reduce thrombosis [204-206].

1.5.9 Bioreactor culture

Bioreactors play a crucial role in the maintenance of organs or tissues during recellularization. In tissue engineering, the function of a bioreactor is to keep the tissue or organ in optimal conditions (sterility, temperature, humidity, pressure, pH, gases and nutrient media) to support cell survival, proliferation and differentiation for several weeks. The ideal bioreactor should also provide ports for mass (media and cells) transfer and information about tissue formation, control air bubble formation, be cost-effective, allow the re-use of the system, be easy to handle in a sterile environment, withstand multiple autoclaving and offer easy assembly and disassembly. All tissues or organs of approximately more than 1 cm3 require a bioreactor system, as gases are unable to diffuse more than 200 to 300 μm without the cells dying of hypoxia [133, 207-210]. The design of bioreactors depends on the type and function of organs. In comparison with a static culture, a bioreactor culture improves cell attachment, penetration, distribution, differentiation and the production of ECM proteins in several tissues and organs [211-213]. Although no bioreactor currently known provides all the necessary conditions for any organ, several bioreactors and culture systems have been developed that enable the control of some parameters. Depending on the aim and conditions to monitor, the available bioreactors range from very simple to very complex with sensor and control systems. However, most of them are yet quite expensive.

To have a flow of medium containing nutrients, bioreactor systems usually use a peristaltic pump with a pulsatile delivery of solutions through the vasculature. This pulsatile force creates pressure on the walls, mimicking arterial flow, and helps to improve cell attachment. However, one study opted to perfuse hearts at only eight-hour intervals, as the authors noted the wash-out of most cells in continuous perfusion [177]. To prevent this wash-out effect and since the growth media used in perfusion have less viscosity compared with blood, many researchers use perfusion speeds of between 2 and 5 mL/min. However, successful recellularization in terms of cell attachment and proliferation has also been reported at high speeds of 20 mL/min [76, 133].

1.5.10 Verification and characterization of recellularization

The presence of cells and their distribution in the organ or tissue can be examined using HE staining. The phenotype and functionality of cells in the recellularized tissue or organ can be verified by IHC or immunofluorescence (IF) staining for specific markers using antibodies, by the quantification of gene expression using quantitative polymerase chain reaction (qPCR) and by the quantification of specific expressed proteins in the tissue by Western blot. The function of cells in recellularized tissues or organs can be evaluated using a variety of functional analysis for different cell types, for example biochemical assays like glucose metabolism for the islets of pancreas and albumin and urea production for the liver. For tissues that constantly experience mechanical forces like the heart, blood vessels and cartilage, performing biomechanical tests like compliance, tensile strength and burst pressure might indicate their potential in transplantation. DNA quantification will also give an idea of cell load in recellularized tissue in comparison to its corresponding native tissue.

1.6 Immunogenicity of tissue-engineered organs

Ideally, tissue-engineered organs should not elicit an immune response on transplantation, as immunogenic donor cellular material is removed and further seeded with non-immunogenic recipient cells. It has been postulated that the transplantation of only decellularized organs can be performed, as the body may serve as a bioreactor and recellularization may occur in vivo. However, the immune reaction depends on the dose of antigen and the site of transplantation. Although decellularized tissues have shown a significant reduction in DNA and HLA I and II molecules [131, 149], adverse immune reactions can still occur because of denuded blood vessels and the infiltration of inflammatory cells into tissue [214-217].

Small intestine submucosa decellularized using PAA and transplanted into rats with ventral abdominal wall defect showed the infiltration of polymorphonuclear leukocytes and the tissue was degraded in 90 days by the simultaneous deposition of the host's site-specific tissue [218, 219]. In contrast, porcine dermis decellularized using Triton X-100 and SDC when implanted into monkeys showed only an initial minimal inflammatory response that disappeared with time. In addition, tissue remodelling was minimal and the skin was vascularised and infiltrated with fibroblasts [220]. This difference may be due to differences in decellularization efficiency where PAA is less effective compared with Triton X-100 and SDC.

For whole organs, rat kidneys decellularized using SDS when implanted into rats showed the partial recellularization of glomeruli and the animals survived up to two months. However, along with several deaths, the kidneys showed atrophy, shape deformation, thrombosis, intimal hyperplasia, the infiltration of inflammatory cells and fibrosis [221]. Similar results were reported when decellularized porcine hearts were transplanted into pigs, resulting in thrombosis, hematoma formation and infiltration by inflammatory cells [222]. However, decellularized mouse pancreas did not show any immune response when implanted under mouse skin [223]. Homogenates of decellularized horse artery caused an immune response, where antibody production to collagen VI was found when injected intra-peritoneally into mice [224].

The immunogenicity results from recellularized organ or tissue have not been well reported in the literature. The

endothelialization of the vasculature by seeding with the host endothelial cells is, however, believed to be advantageous in organ transplantation, as the endothelium acts as a gate-keeper and prevents inflammatory cell infiltration into tissue as reviewed [225]. When implanted after recellularization with endothelial cells, decellularized rat hearts showed reduced thrombogencity and improved contractile function of the left ventricle [226].

1.7 Organs sources in tissue engineering

Using non-humans as an organ source for tissue engineering is beneficial as it reduces the waiting time for a suitable organ and increases the organ pool. Although primates are the closest species, the ethical issues involved in handling primates and the size of organs inhibit their use in research. Pigs are regarded as the next choice of source, as they are physiologically relatively similar to humans, easy to breed, have a short gestation, can be grown in clean conditions and have organ sizes similar to those of humans. Rats and mice organs are considered best for testing hypotheses and new concepts in tissue engineering applications due to their small size, easy breeding, easy handling, less maintenance and anatomy similar to humans. Although tissues and organs from cows, horses and dogs can also be used for tissue engineering, they are not widely used, due to problems with variation in size, ECM composition, biomechanical problems, ethical issues, growth conditions and breeding problems. However, skin, heart valves, blood vessels, small intestine, cartilage, pericardium and corneas from these animals have been used in decellularization and recellularization [227-231].

1.8 Clinical application of tissue-engineered organs

Although significant work has been done to establish effective protocols for decellularization and recellularization, limited success has been achieved in the clinic with tissue-engineered transplants. The main problems when translating to clinical use are ethical clearance and production issues in good manufacturing practices. The tissues engineered by decellularization and recellularization that have shown success in the clinic are muscle and heart valves [232]. Decellularized dermis, bladder, small intestine, pericardium, heart valves, bone and cartilage from human, porcine or bovine sources have also been clinically transplanted for skin, heart valves, knee joints and bone damages with varying outcomes [233].

1.9 Challenges in translation of tissue-engineered products to the clinic

The tissue-engineered products in clinical practice is obstructed for several procedural and regulatory reasons. They include the maintenance of sterility throughout the procedure, preventing viral or bacterial transfer, the storage and transport of recellularized organs, the minimal manipulation of recipient cells during culture, less preparation time, the potential for GMP production, low production costs and the stringent evaluation process prior to approval for transplantation. Large animal studies, clinical trials, funding and industry collaboration are also of the utmost importance. In addition, the product should overcome challenging advanced therapy medicinal product (ATMP) hurdles to obtain a licence for marketing [234, 235].

2. AIMS

The problem of organ shortage and the potential patient benefits of transplantation with personalized tissues and organs initiated the present study.

Considering the facts

1) The ECM plays an important role and guides tissue remodelling

2) Methods of decellularization and recellularization can be used to remove donor cells and seed recipient cells in tissues and organs

3) Finding the optimum cell source, cell type and cell number is necessary for any recellularization

4) The routine clinical use of engineered tissues requires minimal preparation time and the minimal manipulation of cells,

we aimed to develop novel strategies for the tissue engineering of simple and complex tissues and whole organs with clinical potential, using decellularization and recellularization. Specifically, the aims were:

I. to design a method for the decellularization of human saphenous veins and to perform recellularization using blood in a custom-designed bioreactor system (Paper I)

II. to decellularize porcine skin, generate a skin gel using pulverized acellular skin and hyaluronic acid and then evaluate its potential in the treatment of skin wounds in mice (Paper II)

III. to design a method for the isolation and decellularization of porcine pancreas and evaluate its potential for recellularization with human fetal stem cells (Paper III)

IV. to design a method for the decellularization of porcine kidneys and evaluate its potential for recellularization with human fetal stem cells (Paper IV).

3. METHODOLOGICAL CONSIDERATIONS

3.1 Ethical approval

The human saphenous veins used in Paper I were the remnants of bypass surgery at Sahlgrenska University Hospital, Gothenburg. The gal/gal knockout porcine skin used in Paper II was purchased from Avantea, Italy. The porcine pancreas and kidney were retrieved from dead pigs at animal facility in Gothenburg University. The decellularization and recellularization procedure for tissues and organs from human and animal sources in Papers I-IV was performed according to the ethical approval (Dnr: 229-15) given by the regional ethical vetting board in Gothenburg. The wound healing experiments in mice were performed according to the ethical approval (Dnr: 208-2013) given by the animal research vetting board in Gothenburg. The use of fetal pancreatic stem cells in Paper III complied with the ethical approval (Dnr: 428/02) given by Karolinska Institute. The use of fetal kidney stem cells in Paper IV complied with the ethical approval (Dnr: 198-11) given by the regional ethical vetting board in Gothenburg. The blood collection from healthy donors in Papers I & II and the use of adult kidney biopsies in Paper IV complied with the ethical approval (Dnr: 250-11) given by the regional ethical vetting board in Gothenburg. The adult pancreas for positive control for immunohistochemistry in Paper III were purchased as prepared slides from Abcam.

3.2 Organ harvest

The successful tissue engineering of organs requires good dissection, preserving intact vasculature and appropriate cannulation. Failure to do so causes the leakage of solutions resulting in incomplete perfusion and/or organ damage. The human saphenous veins used in Paper I were left over after bypass surgery at Sahlgrenska University Hospital, Gothenburg. The gal/gal knockout porcine skin used in Paper II was purchased from Avantea, Italy. For harvest of whole pig organs, the blood anti-coagulant heparin was first injected to prevent the formation of thrombus in the vasculature. A considerable amount of time was spent in Paper III on establishing an easy and reliable method for dissecting pig pancreas that favours complete perfusion. The pancreas was dissected, keeping the splenic, duodenal and connection lobes, together with the duodenum, aorta and portal vein. In Paper IV, pig kidneys were dissected by keeping the renal artery, renal

vein and ureter intact.

3.3 Decellularization

The purpose of decellularization is to remove immunogenic cellular material from tissue while preserving ECM proteins and growth factors. Although all decellularization chemicals and procedures also have an effect on the ECM, a fine balance when choosing detergent type, concentration, exposure time and mode of detergent exposure can result in tissue with a preserved structure, ECM proteins and growth factors. However, this increases the time required for decellularization. Considering the tissue type, thickness, amount of cellular material and density of ECM proteins, a strong or weak ionic detergent (SDS or SDC) in combination with a weak non-ionic detergent, Triton X-100, was used. The enzyme DNase was used at the end to remove nuclear material.

As perfusion rather than agitation establishes good contact between the detergents and small-diameter blood vessels, the saphenous vein in Paper I was decellularized by perfusion at pressure to facilitate a uniform effect throughout the vein wall. Since veins are simple tissues with thin walls, a weak non-ionic detergent, Triton X-100, together with TnBP and DNase, was used. In Paper II, for porcine skin, which is a relatively thick tissue with dense collagen, a strong ionic detergent, SDS, was applied at high agitation speed of 200 rpm. SDS was used alone, as it has high penetration capacity and is able to remove cellular and nuclear material effectively [82, 130]. Extensive washing with distilled water was performed post treatment to remove SDS remnants, as they can be cytotoxic [236].

The pancreas comprises a tissue with high exocrine activity, where the enzymes released during decellularization can damage the organ. For this reason, in Paper III, porcine pancreata were decellularized at cold temperature (4°C) using the perfusion of a weak non-ionic detergent, Triton X-100, and SDC in one solution. For the decellularization of rat and porcine kidneys, SDS in a range of 0.05-2% was primarily reported in the literature for effective cell removal [147, 148, 237]. Considering the complex and intricate architecture of nephrons, dense ECM, large amount of cellular material and support cell survival for recellularization [238], weak, non-ionic and ionic detergents were preferred for kidney decellularization in Paper IV. Triton X-100, SDC and DNase were perfused through the renal artery and ureter for continous cycles. The degassing of solutions to remove air bubbles and dissolved gases was thought to be an advantage for pancreas and kidney decellularization, as they contain small blood vessels that can be blocked by air bubbles from detergents, leading to incomplete perfusion. As a result, detergent solutions were first passed through the degasser before perfusion into the pancreas and kidney.

3.4 Characterization of decellularization

Since the suboptimal decellularization of tissues and organs may result in deleterious immune responses, it is very important to verify the decellularization process. To verify decellularization, we employed standard histological methods in Papers I-IV and DNA quantification in Papers II-IV. The histology methods used were HE, MT and VVG staining. After HE staining, the nuclei are purple and the ECM is bright red. After MT staining, the nuclei are black and the collagen is blue. After VVG staining, the nuclei and elastin are black and the collagen is pink.

In Paper IV, the effect of decellularization solutions on ECM proteins was evaluated by IHC staining for fibronectin and laminin. The ECM proteins, collagen, elastin and sulfated GAGs, were isolated, separated by complexing with dye and quantified using spectrophotometric methods in Papers II-IV.

3.5 Sterilization of decellularized tissues

Since the tissues and organs may be exposed to bacteria and fungi from decellularization solutions and non-sterile organ handling, sterilization is required in order to proceed for recellularization. The decellularized tissues were sterilized using PAA treatment in Papers I, III and IV and by gamma irradiation in Paper II for skin powder. The pulverized, decellularized skin was irradiated following a standard method at 25,000 KGy for 3 min 25 seconds. The human vein and pieces of porcine pancreas and kidneys were sterilized in 0.1% PAA for 1-2 h. However, the effect of the chosen sterilization method on tissue was not evaluated.

3.6 Preconditioning

Preconditioning was chosen as a method for the treatment of scaffolds, as it could enhance the attachment and differentiation of incoming cells [133, 183]. For this reason, in Paper I, the decellularized vein was preconditioned by incubation in endothelial medium to improve the adhesion of endothelial cells. However, in this project, preconditioning was only used as an additional step before recellularization without evaluating the effects of the chosen treatment.

3.7 Bioreactor

To our knowledge, a simple bioreactor system for vein recellularization specifying all the minor details has not previously been described. However, some complex and expensive systems that are used for tubular organs are available [239]. As a result, in Paper I, a bioreactor system was specially designed for the recellularization of human saphenous vein. This bioreactor includes the advantages of easy handling, the opportunity for autoclaving, preparation from commercially available parts, reusability and at relatively low cost. In addition, with some modifications to increase the number of ports, it will permit the monitoring of gases and nutrients. The disadvantage is that the vein is attached in a fixed position and cannot be rotated continuously, which might otherwise help to achieve the more even distribution of cells.

3.8 Cell culture and characterization

Since human fetal organs can be regarded as a good source of organ-specific stem cells with high proliferation and

differentiation potential [240, 241], they have been used for the recellularization of porcine pancreas and porcine kidneys. Human fetal pancreatic stem cells (hFPSC) in Paper III and human fetal kidney precursor cells (hFKPC) in Paper IV were cultured from the pancreas and kidneys of legally aborted fetuses. The cells were cultured in standard flasks in pancreas differentiation medium for hFPSC and in endothelial and epithelial media for hFKPC. To characterize the cellular phenotype, standard flow cytometry and immunocytochemistry (ICC) methods using antibodies that bind cells expressing the marker were used. The cells expressing markers were detected by passing them through a laser in flow cytometry or visualizing them directly by a microscope in ICC.

3.9 Identification of stem cell markers

The isolation of a stem cell population with the potential to proliferate and differentiate to the required cell type on stimulation will be a milestone for recellularization. Identifying these markers may advance the field of cell therapy and facilitate the translation of tissue-engineered organs to a reality in clinical transplantation. As a result, in Paper IV, we focused on identifying putative stem cell markers in fetal kidneys and comparing them with adult kidneys. A literature search uncovered several transcription factors and very limited cell surface markers to identify progenitor cells in kidneys [242]. The transcription factors cannot be used in the isolation of stem cells as they are expressed within the nucleus. Some proposed cell surface markers are CD133, neural cell adhesion molecule (NCAM) 1, frizzled class receptor (FZD) 7, FZD2, delta-like 1 homologue (DLK-1), activin receptor IIB (ACVRIIB) and epithelial cell adhesion molecule (EPCAM) [243-246]. Despite the mixed results in the maintenance of cultures and the differentiation to functional epithelial types, the results do not help to decide on reliable markers [245, 247]. Together with known markers, we therefore examined the presence and distribution of ephrin (Eph) receptors that are known to be important regulators of pattern formation, cell interaction, cell migration and the maintenance of stem cell niches in many tissues and organs during development [248]. Based on a study showing the expression of several Eph receptors in various human tissues, we focused in particular on EphA6, EphA7, EphB2 and EphB3 [249] by IHC staining in fetal kidneys and compared them with adult kidneys.

3.10 Recellularization

The purpose of recellularization is to make the acellular organ functional again by seeding cells in the scaffold and culturing them in a bioreactor with specific media that enhance the attachment, distribution, infiltration and differentiation of seeded cells. The recellularization of veins was previously performed using human umbilical vein endothelial cells [250] and MSC [251]. However, we chose blood perfusion followed by endothelial media in Paper I, as it has been reported that there are circulating endothelial progenitor cells (EPC) in blood [252]. In addition, the perfusion of blood is an easy procedure for the endothelialization of grafts, as in vitro expansion of cells is not required which is a potential risk of genetic changes in cells. In order to maintain a physiological balance of blood constituents in vitro and improve the adherence of cells, blood was supplemented with glucose maintenance between 3-9 mmol/L, growth factors VEGF and basic FGF, platelet inhibitor acetyl salicylic acid and Steen's solution.

In Papers III and IV, pieces of acellular pancreas and kidneys were recellularized using a standard static culture method. As we noticed that the ratio of plastic-to-tissue surface area and the density of tissue influence cell attachment and infiltration (unpublished data), pancreas and kidney tissues were placed on transwells with the size same as tissue. As the histology of acellular kidneys showed a dense ECM, kidney slices in Paper IV were treated with MMPs which are known to alter the structure of the matrix by degrading the interactions between ECM proteins like collagen [253], leading to improved cell infiltration. Culture on transwells will enable better gaseous exchange and provide media contact for cells attached to the bottom side of tissue. Our own experience indicates that static culture methods are best performed using transwells.

3.11 Characterization of recellularization

The standard and known methods of histology, IHC or IF and qPCR, were used to visualize the presence of cells, phenotype markers expressed and gene expression respectively. HE staining to detect the presence of cells was used in Papers I, III and IV. IHC and IF were used to detect the expression of endothelial, epithelial, exocrine, endocrine and functional proteins in Papers III and IV. To understand cell differentiation and tissue remodelling, the expression of genes involved in kidney development was detected in recellularized kidneys by qPCR in Paper IV.

3.12 Animal experiments

Advanced wound healing strategies that restore the physiological and anatomic functions of regenerating skin will benefit patients with chronic and acute skin wounds [254, 255]. Several skin grafts and dermal substitutes are currently available using xenogeneic collagen, synthetic layers with or without cells, acellular allografts and autologous cell cultured grafts. Despite their promise in several treatments, the main disadvantages include a short shelf life, high cost and a long and demanding manufacturing process [256]. We therefore focused on exploring the feasibility of an off-the-shelf product that can be hydrated when required and, in addition, can be rapidly personalized with autologous cells. So, in Paper II, decellularized porcine skin was cut into pieces, lyophilized and pulverized in a cryomill to store as an off-the-shelf product. The powder was brought to gel-like constituency by mixing powder with hyaluronic acid (HA), as it helps in wound healing and retaining moisture in wounds [257, 258]. In addition, using ECM as a gel can provide a more open matrix that facilitates migration, proliferation, ECM turnover and remodelling [259]. To test the proof of concept, the gel alone or gel with human PBMC was used to treat full-thickness skin wounds in athymic mice, while using

untreated and HA-treated mice as controls. The healing and regenerated skin was characterized by measuring wound size, HE staining to see the skin architecture and IF staining by human and mouse CD31 for angiogenesis. The presence of human cells in the wounds were identified by IHC and qPCR for human mitochondria protein and cytochrome B genes expression respectively.

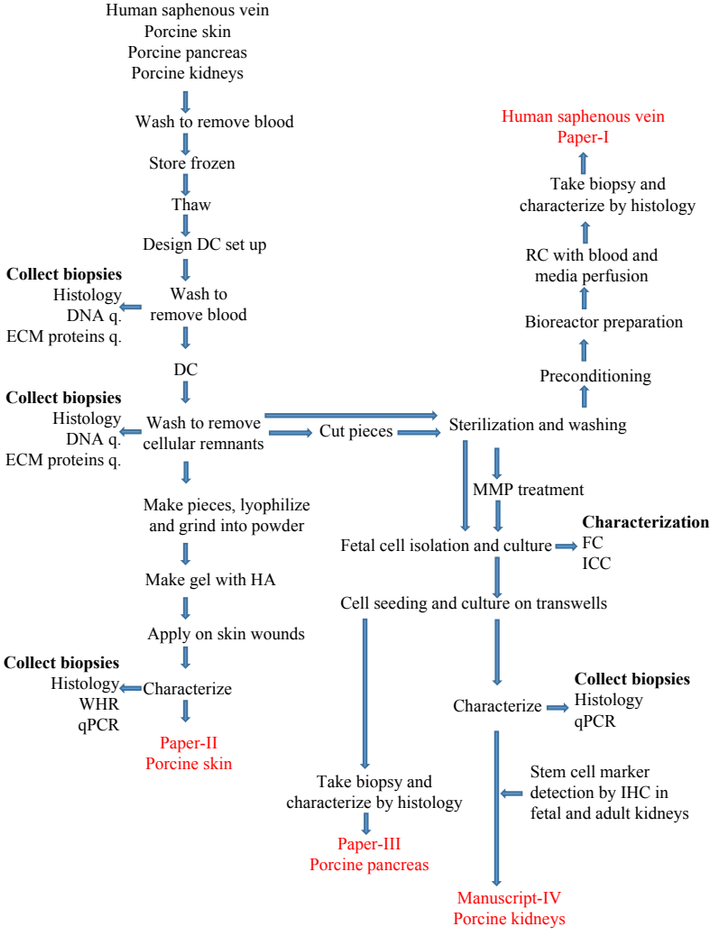

FIGURE 3: Flow chart illustrating the processing of human saphenous vein, porcine skin, porcine pancreas and porcine kidneys for decellularization and recellularization. (DC – decellularization; ECM – extracellular matrix; HA – hyaluronic acid; WHR – wound healing rate; qPCR – quantitative polymerase chain reaction; MMP – matrix metalloproteinase; FC – flow cytometer; ICC –immunocytochemistry; IHC – immunohistochemistry

4. RESULTS AND DISCUSSION

4.1 Tissue engineering of human saphenous veins (Paper I)

We found from HE staining that the decellularization treatment resulted in the loss of nuclei and that recellularization by perfusion with blood revealed the presence of cells at the luminal surface. We speculate that using pressure together with perfusion might be a strategy for effective and reproducible decellularization for small-diameter veins of less than 8 mm internal diameter. Even though the detailed mechanism by which recellularization has occurred, the cell types involved and the function of the endothelium in recellularized veins have not been evaluated, we speculate that EPC present in the circulating peripheral blood might have been attracted by molecules of the ECM. On verification of a cellular phenotype, veins de- and recellularized using the method in the proposed conditions could be considered in clinical use. The endothelium plays a critical role in the functional maintenance of blood vessels by allowing the selective infiltration of cells into the matrix where smooth muscle cells mainly help with vasoconstriction [225].

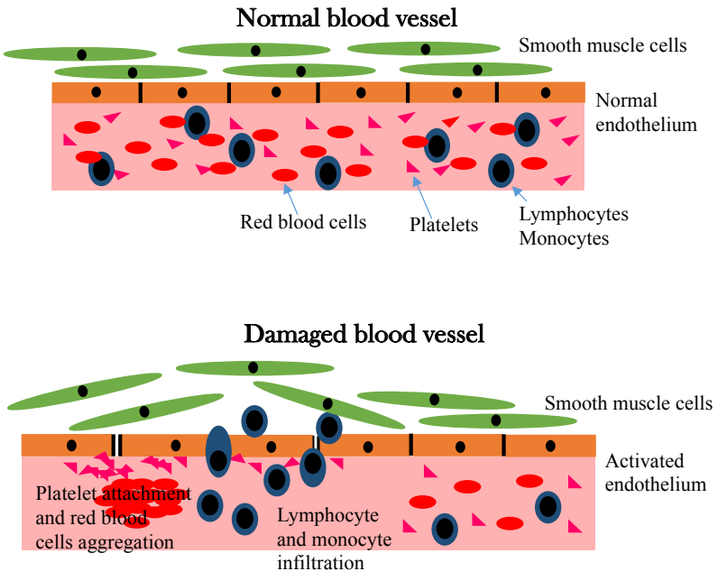

FIGURE 4: The endothelium in normal blood vessels inhibits the attachment of blood cells, while the activated endothelium in damaged blood vessels recruits blood cells and allows the infiltration of inflammatory cells, causing thrombosis by the aggregation of red blood cells.

Endothelial cells synthesise several anti-thrombogenic compounds like heparans, thrombomodulin, thrombin, anti-thrombin and nitric oxide that inhibit platelet aggregation and thrombus formation [260]. Damage to the endothelium causes the exposure of underlying collagens and other thrombogenic proteins, triggering the infiltration of inflammatory cells into the smooth muscle cell layer. This results in a cascade of inflammatory reactions, leading to thrombosis or damage to blood vessels by thickening, weakening, narrowing or scarring [261].

4.2 Tissue engineering of porcine skin (Paper II)

This study was designed to make a gel with powdered pig skin and evaluate its role in wound healing for mice with full-thickness skin wounds. We noticed that pig skin gel alone and gel with human PBMC produced complete wound closure in 15 days as compared to 25 days in untreated and HA-treated groups.

The histology of regenerated skin for all groups showed that gel alone and gel with human PBMC groups had dense collagen and the regenerated skin was similar to normal epidermis. The dense collagen can be due to that the ECM gel contained collagen fibres. In previous studies, skin gel for wound application was made using collagen powder [259]. Porcine skin collagen made into a gel by polymerization with dextran, polar amino acids, cysteine, aminoguanidine and EDTA showed a 72% reduction in wound size for patients with diabetic foot ulcers [262]. However, we propose that using the ECM is better than using collagen, as hydrated ECM powder provides proteins and structures in native form that can be critical in wound healing. In diabetic patients with chronic wounds, applying ECM gels may support healing by supplying collagen, as their native collagen is heavily glycosylated and its deposition at wounds is reduced [263].

The qPCR showed the expression of the human cytochrome B gene in most mice with human cells and IHC staining showed human mitochondria in regenerating skin, indicating the presence of living human cells even up to 25 days.

The impact of human PBMCs on wound healing was understood by noting an increase in the number of human and mouse blood vessels in the regenerating skin using gel with the human PBMC group. We speculate that EPC could have resulted in this angiogenesis. Even though the direct application of PBMC on wounds is new, studies have reported improved angiogenesis and reduced scarring when a secretome of living or irradiated PBMC with hydrogel was applied to skin wounds in pigs and mice [264, 265]. There have several reports with encouraging results in terms of skin regeneration following the addition of stem cells and recombinant proteins to wounds [266-269].

We propose that our method of directly applying PBMC with gel could be regarded as superior, as it has the advantage of using natural ECM and the short preparation time of 1 hour. All these allow clinicians to consider its use in acute conditions. Further, the tedious and expensive procedure of isolating and culturing stem cells or making PBMC secretome is not required.

4.3 Tissue engineering of porcine pancreas (Paper III)

We found that the whole porcine pancreas, can be successfully decellularized at 4°C, while retaining the histological architecture and some ECM proteins. The decellularized ECM also supported the adhesion and multiplication of hFPSC and expressed exocrine and endocrine markers.

The aorta connects to the pancreas via the coeliac and superior mesenteric arteries and we therefore chose the aorta as an inlet to obtain the complete perfusion of the pancreas rather than the previously suggested pancreatic duct and superior mesenteric artery [270]. In addition, choosing the aorta simplifies perfusion with only one cannulation or else a demanding cannulation using a sealing ring would be required to perfuse both arteries using one cannula [271]. The same study also suggested that the individual cannulation of both arteries resulted in incomplete perfusion in one of three pancreata due to the resistance in flow caused by occluded arterial side branches. To obtain complete organ perfusion, the duodenum was kept while dissecting the pancreas, as the superior and inferior pancreaticoduodenal arteries connect the duodenum and pancreas [271].

Although our decellularization protocol exposes a mix of ionic and non-ionic detergents for only a short period of eight hours, similar results for decellularization were shown for the human pancreas where detergent exposure lasted for 48 h with a non-ionic detergent in ammonium hydroxide [272]. Our results strongly recommend the use of a cold temperature and protease inhibitors during decellularization to protect the ECM from the proteases and lipases released from exocrine cells. This fact was supported by 1) the presence of the ECM with ruptured type and cytoplasmic remnants in the pancreas decellularized at room temperature and 2) the addition of pancreatic extract digested cells under culture for 10 min, indicating the presence of active enzymes. The presence of cytoplasmic remnants can cause the inhibition of infiltrating cells, the remodelling of the ECM and an inflammatory response if transplanted [273-276].

The various cell types reported for pancreas recellularization are, amniotic fluid stem cells, islets and MSCs [277-279], all showing insulin production. For recellularization, we preferred to test with cells from a whole fetal pancreas, as cells from an adult pancreas have limited growth [280] and differentiation capacities [281, 282] in vitro. Fetal pancreas-derived cells have the advantages of high proliferation and differentiation and contain stem cell populations for all pancreatic cell types [283]. In addition, the presence of exocrine cells together with endocrine cells is required for the optimal development and function of the pancreas [270].

On characterization, pieces of pancreas recellularized with fetal cells showed attached cells and expressed markers of exocrine,

endocrine, proliferating and progenitor types. The expression of the markers glucagon, c-peptide (by-product of proinsulin to insulin) and α-amylase indicates the presence and function of endocrine cells (alpha and beta) and exocrine cells respectively. The expression of pancreatic duodenal homeobox-1 (PDX1) indicates the presence of pancreatic progenitor cells. PDX1 is a prominent marker of pancreatic progenitor cells and the cells expressing PDX1 are known to differentiate into ductal, exocrine and endocrine types in vitro [284]. Further, the presence of proliferating cells was indicated by the expression of proliferating cell nuclear antigen (PCNA). PCNA is an essential factor for DNA polymerase and is only expressed during DNA synthesis and repair [285, 286].

4.4 Tissue engineering of porcine kidneys (Paper IV)

This study aimed to determine the potential of hFKPC in the regeneration of acellular porcine kidneys and, at the same time, to find the putative stem cell markers in fetal kidneys that can be used for kidney regeneration and in future recellularization experiments. We found that the decellularized kidneys showed the attachment and infiltration of hFKPC, as well as the increased expression of genes involved in kidney development. We also found from screening stem cell markers that EphA7 might be an interesting stem cell marker involved in kidney regeneration and is a potential candidate to test in future kidney regeneration experiments.

Like previous results with SDS, the decellularization of kidneys by the perfusion of Triton X-100, SDC and DNase removed all nuclear material [238]. A noticeable detrimental effect was only seen in the amount of GAGs but not in the structure of the ECM or the retention of the ECM proteins, fibronectin, laminin, collagen and elastin. Decellularization through the renal artery and ureter was not performed in most studies of kidneys. However, it has been suggested that the seeding of cells through the ureter and renal artery is important for the complete distribution of cells [142]. Even though it is surprising how the decellularization solutions are successful in damaging all the cells in tubular compartments and removing cellular remnants from tissue, the use of SDS in all these studies that have a high penetration capacity may be the reason for success. In our optimisation experiments for decellularization, perfusion through the renal artery did not result in complete cell removal from tubular compartments and we therefore decellularized kidneys by perfusion through both the renal artery and the ureter.

The kidneys contain more than 26 different cell types, all originating from two progenitor populations of the ureteric bud and metanephric mesenchyme [287, 288]. Recellularization studies performed to date for any kidney used endothelial, tubular epithelial, rat neonatal, murine embryonic cells and iPSC [142, 146, 148, 178, 289]. Although neonatal, embryonic and iPSC are good strategies, using only endothelial, tubular and epithelial cell types may not result in a recellularized kidney as they may not have the potency to differentiate into all kidney cell types. In particular, the use of organ-specific stem cells for recellularization might be important for organs with heterogeneous cell types, as the acellular ECM contains cues that may direct the growth and differentiation of stem cells [290]. So, considering the advantages of proliferation, heterogeneity, ECM cues and the clinical relevance of using human rather than animal cells, hFKPC were used for recellularization in this study.

The culture and characterization of fetal cells in endothelial and epithelial media showed the continued expression of stem cell markers DLK-1, CD133, EPCAM, together with EphA7 and EphA6. Although the recellularized pieces showed cell infiltration, many cells were seen growing attached to the surface of the retained ECM resembling kidney structures. These cells in turn showed the expression of epithelial cell markers, CK8 and CK18, endothelial progenitor cell marker, CD133, stem cell marker, DLK-1, Eph receptors, EphA7 and EphB3, glomerular podocyte marker, podocin, and the proliferation cell marker, PCNA. Interestingly, the qPCR analysis showed the increased expression of transcription factors involved in kidney development indicating the active role of seeded cells in kidney formation and the positive effect of ECM on signalling the seeded cells to involve in tissue remodelling.

The staining for stem cell markers found that, in fetal kidneys, Eph receptors, EphA6 and EphB3, are only expressed in the capsule, while EphA7 is strongly expressed in the capsule and mesenchyme. However, none of these were detected in adult kidneys. In addition, the known stem cell markers of the kidney, CD133 and DLK-1, were also expressed in the capsule and mesenchyme. EPCAM was only seen in tubules and ducts. Only DLK-1 and EPCAM were seen expressed in adult kidneys. So, based on the expression pattern of EphA7 in the mesenchyme of fetal kidneys, we postulate that EphA7 is a probable stem cell marker. However, the potential of cells expressing EphA7 in proliferation, differentiation and regeneration needs to be verified in isolated cell culture experiments in vitro, kidney remodelling or regeneration experiments in vivo and ex vivo. Strengthening the postulation, one piece of evidence shows that a truncated form of EphA7 is a key regulator for reprogramming mouse embryonic fibroblasts and the induced expression of pluripotency markers Nanog and Oct3/4.

5. CONCLUSION

In conclusion, all decellularization protocols designed in Studies I-IV using enzymatic agents, ionic and non-ionic detergents can be successfully used as the removal of nuclei was noted in all studies.

The in house designed bioreactor system and the perfusion with whole peripheral blood and endothelial medium might be a strategy for the recellularization of veins.

The pig skin gel containing human PBMC is able to effectively induce angiogenesis and collagen deposition and displays faster epidermis formation during wound healing in mice. All these facts indicate its potential in wound treatment and encourage testing it as filler in large animal wound healing studies and clinical human trials.

The use of human fetal stem cells in pancreas and kidney recellularization studies showed that fetal cells attach, proliferate and express phenotypic and functional markers of respective cell types. The characterization of gene expression in kidney pieces demonstrated increased expression of genes involved in kidney regeneration indicating the response of cells to the ECM. In the future, human fetal cells might act as an ideal cell source in whole organ recellularization.

6. SUMMARY

The decellularization and recellularization of tissues and organs is a promising method to generate personalized organs which could solve the problems of organ shortage and the use of life-long immunosuppression in the field of transplantation. Intense research over the last two decades has resulted in the development of several protocols for the decellularization of several tissues and organs. The development of protocols for recellularization and clinically relevant models is on soft pedal because of a lack of knowledge and access to appropriate cell types, cell sources, bioreactors and so on.

In Paper I, we present a step-by-step method for the decellularization of saphenous veins and recellularization using peripheral blood in an in house designed bioreactor system. The recellularized veins displayed the presence of cells at the lumen. This study contributes to the field by providing a detailed method for the preparation of perfusion set-ups, bioreactors and the de- and recellularization of saphenous veins.

In Paper II, we developed a decellularization method for porcine skin and made it into a gel using pulverization, followed by the addition of hyaluronic acid and keratinocyte medium. The skin gel alone or skin gel mixed with human PBMC was tested in mice with full-thickness skin wounds. Improved collagen deposition, a faster healing rate, increased angiogenesis and the improved histology of regenerated skin were noted in mice treated with gel and PBMC. The skin powder can be stored as an off-the-shelf product that can be hydrated and mixed with PBMC in less than one hour. This study contributes to the field by introducing a novel method for making skin gel that has shown promise in enhanced wound healing and may help patients with acute skin burns and ulcers.

In Paper III, we developed a dissection and decellularization method for porcine pancreas using non-ionic and weak ionic detergents at cold temperature and tested its potential for regeneration with hFPSC. We also demonstrated that the decellularization of the pancreas at cold temperatures is important in order to inhibit the action of exocrine enzymes on the ECM. The initial recellularization with pieces of pancreas showed cell attachment, proliferation and the expression of exocrine and endocrine markers. This study contributes to the field by presenting a promising method for the dissection, perfusion and decellularization of the pancreas.

In Paper IV, we developed a decellularization method for porcine kidneys using non-ionic and weak ionic detergents and tested its potential in regeneration with hFKPC. The characterization of recellularized pieces showed the phenotypic morphology of developing kidneys and an increase in the expression of genes involved in kidney development. This shows that hFKPC is a promising cell source in kidney regeneration. The characterization of stem cell markers in fetal, cell culture and recellularized kidneys gave the impression that the ephrin receptor, EphA7, might be a novel stem cell marker with a potential role in kidney regeneration. Cell expansion to large numbers and characterization, whole-organ recellularization in a bioreactor, functional tests of recellularized organs and animal transplantation studies are required for the studies in Papers III and IV before testing in clinical studies. This study contributes to the field by presenting an alternative method for kidney decellularization, MMP treatment as a strategy for the recellularization of organs with a dense ECM and a postulation to evaluate EphA7 cells in kidney regeneration.

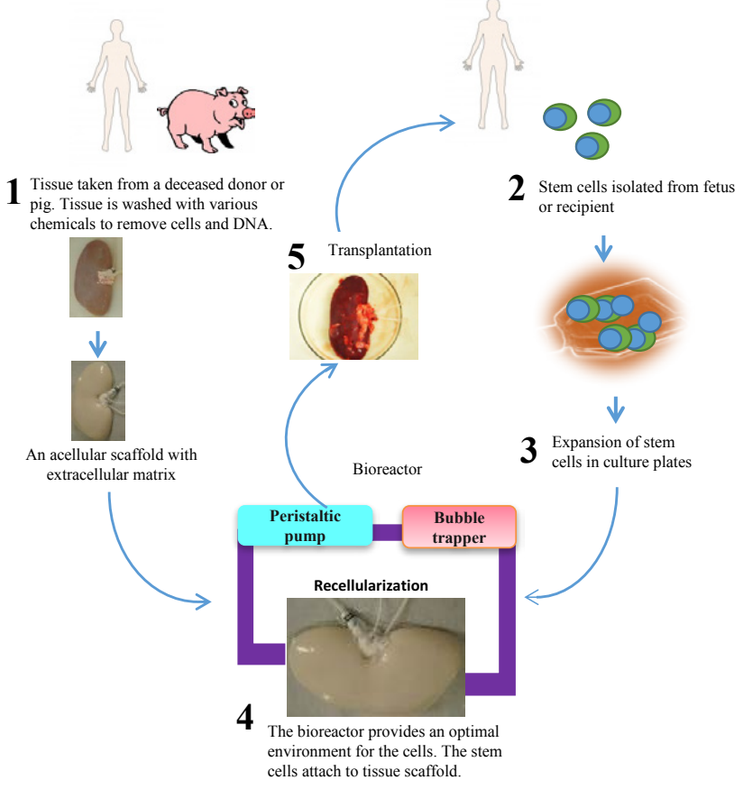

FIGURE 5: The flowchart demonstrating the steps involved in tissue engineering of personalized organ.

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