

Tissue engineering of the kidney using a whole organ decellularisation approach

PhD Thesis

Ming He¹ *BSc MBBS MRCS*

1st Supervisor:

Professor Molly M Stevens¹

¹Department of Bioengineering
Department of Materials
Imperial College London
South Kensington
London SW7 2AZ

2nd Supervisor:

Professor Alun H Davies²

Department of Surgery and Cancer
Imperial College London
²Charing Cross Hospital
St Dunstan's Road
London W6 8RP

Declaration of originality

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Abstract

End stage renal failure is associated with major morbidity and mortality, and renal transplantation is the optimal form of renal replacement therapy. However, since the number of organs available for transplantation is limited, an additional (e.g. bioengineered) source of organs for transplantation is both attractive and necessary. Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences towards the development of functional replacement tissues for clinical use. Within this field, the technique of whole organ decellularisation allows the preservation of the native vasculature and the complete 3-dimensional macro- and micro-architecture of the organ extracellular matrix (ECM) to create a whole organ ECM bioscaffold. This approach has been employed in the animal model to create viable and partially functional solid organ constructs, some of which have been implanted *in vivo*, including in the kidney. However, a number of major technical factors and challenges exist before clinical application can be considered, such as the pre-optimisation and standardisation of both decellularisation and recellularisation protocols. The current applications and methods in using xenogeneic whole organ ECM scaffolds to create potentially functional bio-artificial organ constructs for surgical implantation have been reviewed here; comparison of specific trends reveals the need for systematic rationalisation throughout the field.

On this basis and using the rat model, the first aim of this project was to optimise the decellularisation process for whole kidney ECM bioscaffolds with regards to two fundamental parameters: concentration of decellularising agent and duration of perfusion, using the most common single decellularising agent drawn from the literature i.e. sodium dodecyl sulphate. Optimisation was determined with regards to structural and functional characteristics of the ECM bio-scaffold as assessed by histology, immunohistochemistry, quantitative assays of DNA and sulfated glycosaminoglycan content, and growth factor

quantification. Secondly, recellularisation of the whole kidney bioscaffolds produced using this more optimised protocol was carried out to yield a viable and implantable organ graft/construct, with both primary renal cells and also adult-derived mesenchymal stem cells. Thirdly, both the decellularised and recellularised kidney constructs were implanted *in vivo* using the renal transplant model in order to investigate the long-term viability and function of the graft construct. This showed long-term viability and biocompatibility of the acellular (decellularised) kidney scaffold when implanted *in vivo*, but poor viability of all scaffolds (acellular or recellularised) when implanted in combination with vascular perfusion due to a postulated pro-thrombotic mechanism.

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Chapter 1

Introduction

1.1 General background

1.1.1 End stage renal failure and renal transplantation

End stage renal failure is associated with major morbidity and mortality, and has been increasing in both incidence and prevalence since 1990. A person aged 60 or more requiring renal replacement therapy (RRT) on haemodialysis has a life expectancy of 5 years or less, as compared to 20 years for the general population [1]. In comparison, renal transplantation has been shown to be the optimal form of RRT (whether in terms of survival, quality of life or cost-effectiveness) but is restricted by the limited pool of organs available for transplantation from deceased donors. According to the UK Renal Registry, there were 54,824 adult patients receiving RRT on 31/12/2012 in the UK but the total number of kidney transplants performed in 2012 was limited to only 2,901 [1]. This number has only increased by 5% from the previous year, and 10-20% over the last decade despite strategies to improve transplantation rates such as utilising organs from living donors and donors after circulatory death. Currently, only about 20% of the patients awaiting a transplant in the UK are transplanted per year, with a median waiting time of approximately 3 years and 2-3% mortality whilst on the waiting list [2]. The relatively static or even slightly decreasing trend in numbers of deceased donor organs available for transplantation can be attributed to a static number of deceased donor organs available overall but also a slight decrease in deceased donation rates over the last decade [3]. While there is ongoing debate to address this issue with strategies both within a wider social or legislative context (e.g. so-called 'opt-out' or 'presumed consent' organ donation [4]) and others more specifically applicable to the issue of renal transplantation (e.g. living kidney donation, altruistic kidney donation), these strategies can be either controversial or not without additional clinical risk (e.g. to the

living donor). Nor will these strategies necessarily be able to sufficiently deal with the very large disparity between numbers of renal transplants performed and those on the transplant waiting list, not to mention the additional tens of thousands of patients who are in end stage renal failure who may currently not satisfy the stringent eligibility criteria for transplantation but who may still significantly benefit from such a procedure were the source of organs to become much more numerous or even 'on demand'. Hence, an additional or alternative (e.g. bioengineered) source of organs for transplantation or implantation is both attractive and necessary.

1.1.2 Tissue engineering approaches to renal replacement

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences towards the development of functional replacement tissues for clinical use. Many developments within this field employ the seeding and cultivation of cells onto acellular scaffolds, although control and utilisation of the types of cell, scaffold material, interactions between the two and the optimal conditions for the regeneration of functional tissue replacement are complex and vary from the nano- to the macro-scale [5]. However, recent innovations in the field such as whole organ decellularisation may lead to a successful regenerative medicine approach and bioengineered solution to the pressing clinical need of organ replacement [6-8].

A relatively new approach to 'whole organ' engineering based on the technique of whole organ decellularisation allows the preservation of the native vasculature and the complete 3-dimensional (3D) macro- and micro-architecture of the organ extracellular matrix (ECM). This technique actually utilises the intrinsic vasculature to decellularise the entire organ by perfusion of the decellularising agent to efficiently penetrate the tissue and remove cellular material. The decellularised bioscaffold thus created preserves the intrinsic structure and properties of the ECM [5] with its natural organisational complexity in order to promote

recellularisation and direct cellular differentiation on a whole organ level. The inherent vascular network is also critical in allowing perfusion (i.e. delivery of nutrients and oxygen) to sustain cell culture in complex 3D constructs on a level which was previously not feasible. This approach has been successfully employed in the major solid organs to create viable and partially functional organ constructs, some of which have been implanted *in vivo* [9-14]. In particular recent work on the regenerated kidney has demonstrated up to 10% functionality in terms of urine production and creatinine clearance [14].

A number of other approaches have been taken to attempt to artificially replace the functions of native kidney. These range from bioengineered tubular structures which supplement haemofiltration *in vivo* analogous to clinical haemofiltration and haemodialysis devices [15, 16] to the developmental biology approach with *de novo* organogenesis in animal models [17, 18], as well as other applied experimental approaches using renal cells and ECM to form 3D tubular and glomerular-like structures *in vitro* and *in vivo* [19-21]. However, a technique such as whole organ decellularisation that can yield whole-organ ECM bioscaffolds of the appropriate scale and size on demand (e.g. from xenogeneic sources), which are capable of supporting recellularisation on a complex 3D level and sustaining appropriate cellular and tissue functionality to produce an implantable and potentially permanent graft, could indeed provide the ideal shortcut to bioengineering a kidney organ replacement. This potential construct also has the advantage of being structurally and histologically similar to the original organ in question and allows relatively easy surgical manipulation (with preservation of the intact vascular pedicle for direct anastomosis at the orthotopic or conventional transplantation site). Furthermore, if regeneration of whole organ bioscaffolds could be successfully carried out with host-derived cell populations, e.g. mesenchymal stem cells, then further potential advances in bypassing the barrier of immune rejection and significant benefits to patients could be gained.

1.2 The extracellular matrix (ECM)

The extracellular matrix is the naturally occurring scaffold material secreted and manufactured by the resident cells of each tissue and organ. The complex three-dimensional organisation of the ECM and its components are dictated by the tissue from which the ECM is derived and can be considered specific to that tissue or organ [22-24], however individual components are common throughout most tissues such as collagen, laminin, fibronectin and hyaluronic acid, as well as various growth factors. The structural and functional molecules of the ECM are in a state of dynamic equilibrium within the surrounding micro-environment [25], and also provide the means by which cells communicate with each other and the external environment [26, 27]. By definition the ECM possesses the ideal characteristics of the tissue engineered scaffold or biomaterial (in addition to the functions already described), being biocompatible, a supportive medium for blood vessels, nerves and lymphatics and for the diffusion of nutrients from the blood, as well as being able to undergo constructive remodelling and degradation within the body's own systems [28-30].

1.2.1 Key components of the ECM

Most of the 'structural' molecules of the ECM such as collagen in fact perform a dual function as they often contain peptides, e.g. RGD in fibronectin, that promote adhesion of different cell types, and they may also be considered to act as a controlled release vehicle for growth factors due to their naturally rapid degradation *in vivo*. The most abundant protein within ECM is collagen and the primary structural type within mammalian tissues is collagen type I. There are over 20 types of collagen identified and it is a well characterised protein ubiquitous across both the animal and plant kingdoms [31]; it has a well conserved amino acid sequence and is hence well tolerated as a material for tissue repair or replacement. Other types of collagen exist in naturally occurring ECM at much lower levels. Collagen type IV in particular exists within basement membranes of vascular structures

where it acts as a ligand for endothelial cells and hence is also an important component of the kidney ECM. It also acts as a 'bridge' between functional proteins and glycosaminoglycans to larger structural proteins such as collagen I [32].

Fibronectin is the next most common type of ECM protein and exists within both basement membrane and submucosal structures [33, 34]. It exists in both tissue and solution isoforms and contains ligands for adhesion of many cell types [35, 36]. Laminin is an adhesion protein which also has an important role to play in basement membranes and vascular structures. It has a trimeric cross-linked polypeptide structure and exists in numerous forms depending on the combination of the different peptide chains (e.g. $\alpha 1$, $\beta 1$, $\gamma 1$) [37, 38]. Glycosaminoglycans (GAGs) are a group of proteins which have various functions such as growth factor and cytokine binding, water retention and gel properties of ECM. GAG components of ECM consist of chondroitin sulphates, heparin, heparan sulphate, and hyaluronic acid [39]; many growth factors (e.g. fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF)) and cell surface receptors have heparin binding properties which explain the importance of GAGs within the ECM. All of these key proteins, the diversity of their types, and combinations within a unique spatial distribution that leads to the specificity of the ECM for any particular tissue type, exemplify the difficulty in recreating this composite material *in vivo*.

1.2.2 The properties of ECM

1.2.2.1 Bioinductive properties of the ECM

The ability of the ECM scaffold to facilitate and integrate cellular and external environmental cues is the consequence of its bioinductive properties, allowing the constructive remodelling of tissue after the *in vivo* implantation of ECM scaffolds [40-42]. This remodelling cannot be solely attributed to characteristics such viscoelastic behaviour, biomechanical properties or host cell attachment through collagen, fibronectin and laminin ligands within the ECM. Growth factors such as vascular endothelial growth factor (VEGF) [43], basic fibroblast growth factor (bFGF) [44, 45], and transforming growth factor beta (TGF- β)[45, 46] are responsible for crucial events of remodelling such as angiogenesis, abundant host cell infiltration, mitogenesis and deposition and organisation of new host ECM ([Table 1](#)). These can survive tissue processing and terminal sterilisation [44, 46, 47] to exert an effect on tissue remodelling and are released during the degradation of the ECM scaffold [36, 43-47]. Indeed the degradation process itself, which is mediated by enzymatic and cellular processes, may be considered as a mechanism for controlled release of the ECM constituent molecules. The process of degradation and growth factor release continues until the scaffold is completely degraded. Degradation products of the molecules that constituted the ECM may mediate a subsequent series of remodelling events. These subsequent events include the release of cryptic peptides that initiate and sustain the recruitment of circulating, bone marrow derived undifferentiated progenitor cells that actively participate in long-term tissue remodelling [48, 49], the generation of antimicrobial peptides that protect the remodelling site from pathogens [50-55], other peptides that modulate angiogenesis and the recruitment of endothelial cells over periods up to 6-8 weeks [56]. Hence the bioinductive effects of ECM scaffolds are sustained in correlation with its ability to undergo *in vivo* degradation. Conversely, ECM scaffolds that resist or retard the degradation process may elicit a chronic inflammatory response [57].

Table 1 (Adapted from [58])

Bioinductive growth factors found within the ECMs and their functions.

Growth Factor	Function
Vascular Endothelial Growth Factor (VEGF)	Regulates angiogenesis by controlling blood vessel formation and growth
Platelet Derived Growth Factor (PDGF)	Deposition of granulation tissue and stimulation of angiogenesis
Bone Morphogenetic Protein (BMP)	Stimulates formation of bone and cartilage
Keratinocyte Growth Factor (KGF)	Epithelialisation of wounds during healing
Fibroblast Growth Factor (FGF)	Induces growth of fibroblasts and endothelial cells during wound healing
Transforming Growth Factor (TGF-beta)	Reorganization of matrix molecules to improve dermal architecture and reduce scarring

1.2.2.2 Biomechanical properties of the ECM

The mechanical properties of the ECM can be predominantly understood from the combination of its collagen fibre architecture and kinematics. The tissue from which an ECM scaffold is harvested will define its structural characteristics (such as fibre size, orientation and alignment) and mechanical properties. For instance, small intestine submucosa (SIS) derived ECM has been shown to have a preferred fibre alignment in a spiral arrangement along the longitudinal axis of the small intestine [23, 59, 60] and it is likely that this structural arrangement facilitates dilation and retraction of the small intestine during peristalsis and transport of intraluminal contents. However, with the exception of the SIS and urinary bladder mucosa derived ECM (UBM) [22, 61], there has as yet been almost no systematic assessment of the biomechanical properties of different ECM scaffold materials, or the effect of processing methods on these properties. An understanding of the collagen fibre structure from each organ would be important in order to closely match the scaffold mechanical properties to those of the intended target organ. However, ECM materials can also be manipulated into different types of products for implantation e.g. sheets [62], powder [63], or gels [64, 65] and the latter types provide alternative properties that may facilitate clinical use such as injection of the ECM via minimally invasive procedures [66]. In this case, the bioinductive properties and the retention of these during the processing steps are considered of greater importance.

1.2.2.3 Degradation and constructive modelling of the ECM

The bioinductive properties of ECM scaffolds depend crucially on the efficient and effective degradation of the scaffold material and facilitate the constructive remodelling of injured tissue. The species of origin, tissue of origin, and processing techniques during the production of the ECM scaffold can differ markedly and factors such as method of decellularisation, use of chemical crosslinking agents, and means of sterilisation can affect

the degradability and host response to the scaffold material [67]. One method of quantitative evaluation of the degradation process is with the use of radioactive labelling with isotope carbon-14 (^{14}C), which can be stably incorporated into organic compounds and has a long half-life of 5,760 years. Since type I collagen is the predominant protein within ECM scaffolds and is rich in proline, ^{14}C -labelled proline is used as the marker for degradation and can be tracked over time using accelerator mass spectrometry [66].

Quantitative studies of ^{14}C -labelled SIS used in augmentation cystoplasty [68] and Achilles tendon reconstruction[69] procedures have shown that 50% or more of the ECM scaffold is degraded and removed from the implantation site by 28 days. The degradation products are excreted via the urine with no recycling to other tissues. Furthermore, the replacement of the degraded SIS with functional (and similar to normal) host tissue occurred without loss of function e.g. bladder or tendon rupture suggest a rapid infiltration and/or proliferation of functional host cells at the remodelling site with deposition of new ECM.

However the involvement of biomechanical factors was also shown to be critical in both these models of degradation analysis. In the augmentation cystoplasty model, if the urinary bladder was prevented from physiological filling and emptying by the presence of a Foley catheter, scar tissue replaces the scaffold material instead of functional smooth muscle cells [40]. Similarly, if the Achilles tendon interpositional graft repair animal was not allowed to weight bear post-operatively in a progressive and controlled fashion on the limb in question, the scaffold degraded into loose connective tissue that easily ruptured subsequently [28]. Environmental factors may also play an important role in determining *in situ* regeneration or transformation of implanted tissues, such as that seen in the formation of host-derived tracheal tissue within a long segment of allogenic aortic graft transplanted as a potential conduit for tracheal replacement [70, 71], whereby the original aortic graft is 'transformed' into cartilage-bearing tracheal-like tissue. Hence understanding of the additional factors that modulate remodelling is also essential in order to utilise ECM scaffolds effectively.

1.2.2.4 Host response to xenogeneic ECM

Most ECM scaffold biomaterials and commercially available surgical implants are of xenogeneic origin, such as a porcine or bovine source with a few types from human allogeneic tissue [72]. Non-human biomaterials have been used in humans for many years without evidence of adverse host immunological outcomes e.g. porcine heart valve replacements, porcine and bovine insulin for the treatment of diabetes mellitus. Xeno- and allo-geneic cellular antigens are by definition recognised as foreign by the host and induce an inflammatory or immune-mediated rejection response; however components of the ECM are generally conserved among species and tolerated well. While no systematic comprehensive studies have been made to characterise the host immune response towards the full range of ECM scaffold materials, various studies have been conducted on SIS ECM.

With SIS ECM, complement activation or cell mediated rejection response does not occur even with the small amounts of natural gal-epitope that are present [73, 74] and this can be further prevented with either use of transgenic gal-knockout pigs for tissue harvesting or treatment of harvested ECM with galactosidase.

In terms of the cell mediated immune response, the Th1 lymphocyte phenotype is associated with macrophage activation, complement fixation, and CD8+ cytotoxic cell differentiation and activation of the Th1 pathway is implicated in transplant rejection [75, 76]. In contrast, activation of the Th2 lymphocyte pathway does not lead to these events, differs markedly both in the profile of cytokines and antibody isotypes produced and is hence associated with transplant acceptance [77, 78]. Implantation studies of SIS ECM in mice have shown that a Th2 type immune response occurs, similar to that elicited by syngeneic muscle tissue [79, 80].

The likely innate immune-mediated inflammatory response to ECM materials and bio-scaffolds also plays an important role in their degradation *in situ* and subsequent

constructive remodelling process, and this can be demonstrated by the macrophage phenotype response. Macrophages are phenotypically and functionally plastic, but can be broadly characterised into a polarised M1/M2 phenotype classification which forms a parallel to the Th1/Th2 response in that M1 macrophages are associated with a 'pro-inflammatory' and M2 with a constructive remodelling response [81]. The constructive remodelling process can be correlated with the ability of the implanted material to direct the macrophage phenotype and ECM has been shown to promote the switch from M1 to M2 following implantation [82, 83]. However, use of chemical crosslinking agents that may interfere with the macrophage-mediated breakdown of ECM material inhibits the beneficial M2 phenotype and leads to chronic inflammation and fibrosis [84].

Hence it can be seen that the overall host immune response for any particular ECM material will be variable and dependent on the diversity of tissue sources and processing methods used in its preparation.

1.2.3 Clinical relevance and use of the ECM as a scaffold material

There are already many forms of naturally derived ECM scaffold biomaterials available in a variety of formats for surgical implant applications. Examples of clinical products such as mesh materials composed of ECM harvested from SIS, UBM, dermis, pericardium and heart valves from several species are listed in [Table 2](#) [72].

SIS ECM has been used in more than one million human patients in reconstructive procedures involving a variety of tissues including the integument [85, 86], body wall [87], urinary bladder [88-90], rotator cuff [91-94], intestine [95], urethra [96, 97], ureter [98, 99] and diaphragm [100, 101]. While the outcomes for selected clinical applications have been mixed, generally the outcome has been very positive. The reasons for disparity of outcome may include patient selection, surgical technique, and lack of understanding of the optimal use of an inductive scaffold in certain tissues or applications.

The use of ECM scaffold is increasing within tissue engineering and regenerative medicine, with recent applications such as the use of three-dimensional whole organ decellularised scaffolds. The properties of the ECM in influencing cell mitogenesis and chemotaxis, cell differentiation, inducing host constructive remodelling may be further enhanced by the three-dimensional ultrastructure and surface topology. However, as the properties of the ECM are dependent on tissue processing methods, these are also critical determinants of clinical success.

Table 2 (Adapted from [72])

Examples of clinical products composed of decellularised tissues.

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

1.2.4 Decellularisation and production of ECM scaffolds

Due to the complexity of the three-dimensional organisation and composition of all the structural and functional molecules of the ECM, and the fact that these have not yet been fully characterised (or indeed for each type of tissue), it is not possible as yet to synthesise this biomaterial in the laboratory. Hence ECM scaffolds are produced by the process of decellularisation of naturally derived tissues, and this can be achieved by a variety of agents and techniques [72].

The effectiveness of a particular agent or method in achieving decellularisation depends on factors such as tissue cellularity, density, lipid content, thickness and the intended clinical application of the decellularised tissue. The complexity and length of the decellularisation protocol is correlated with the degree of structural and biological conservation required for the post-processed tissue, especially for composite tissues and whole organs. Any agent or method will cause some disruption of the ECM composition and ultrastructure, and minimisation of these effects is desirable as complete avoidance is not possible.

Table 3 gives a) an overview of some commonly used decellularising agents and b) methods of application and their effects on the ECM [72].

Table 3a

Commonly used decellularising agents and their effects on the ECM (adapted from [72]).

Agent	Mode of action	Effects on ECM
Chemical Agents		
<i>Acids and bases</i>	Solubilizes cytoplasmic components of cells, disrupts nucleic acids, tend to denature proteins	May damage collagen, GAG, and growth factors
<i>Hypotonic and hypertonic solutions</i>	Cell lysis by osmotic shock, disrupt DNA-protein interactions	Effectively lyses cells, but does not effectively remove cellular residues
<i>Non-ionic detergents</i>	Disrupt DNA-protein interactions, disrupt lipid-lipid and lipid-protein interactions and to a lesser degree protein-protein interactions	
- Triton X-100		Mixed results with efficacy dependent on tissue, more effective cell removal from thin tissues, some disruption of ultrastructure and removal of GAG, less effective than SDS
<i>Ionic detergents</i>	Solubilize cell and nucleic membranes, tend to denature proteins	
- Sodium dodecyl sulfate (SDS)		Effectively removes nuclear remnants and cytoplasmic proteins from dense tissues, tends to disrupt ultrastructure, removes GAG and growth factors and damages collagen
- Sodium deoxycholate		Mixed results with efficacy dependent on tissue thickness, some disruption of ultrastructure and removal of GAG
- Triton X-200		More effectively removes cells from thin tissues but with greater disruption of ultrastructure compared to other detergents
<i>Zwitterionic detergents</i>	Exhibit properties of non-ionic and ionic detergents	
- CHAPS		Effectively removes cells with mild disruption of ultrastructure in thin tissues
- Sulfobetaine-10 and -16 (SB-10, SB-16)		Effectively removes cells with mild disruption of ultrastructure in thin tissues
<i>Solvents</i>		
- Alcohols	Cell lysis by dehydration, solubilize and remove lipids	Effectively removes cells from dense tissues and inactivates pyrogens, but crosslinks and precipitates proteins, including collagen
- Acetone	Cell lysis by dehydration, solubilizes and removes lipids	Effectively removes cells from dense tissues and inactivates pyrogens, but crosslinks and precipitates proteins, including collagen
- Tributyl phosphate (TBP)	Forms stable complexes with metals, disrupts protein-protein interactions	Mixed results with efficacy dependent on tissue, dense tissues lost collagen but impact on mechanical properties was minimal
Biologic Agents		
<i>Enzymes – Nucleases</i>	Catalyze the hydrolysis of ribonucleotide and deoxyribonucleotide chain	Difficult to remove from the tissue, could invoke an immune response
- Trypsin	Cleaves peptide bonds on the C-side of Arg and Lys	Prolonged exposure can disrupt ECM ultrastructure, removes ECM components such as collagen, laminin, fibronectin, elastin, and GAG, slower removal of GAG compared to detergents
- Dispace	Cleaves specific peptides, mainly fibronectin and collagen IV	Prolonged exposure can disrupt ECM ultrastructure, removes ECM components such as fibronectin and collagen IV

<i>Chelating Agents (EDTA, EGTA)</i>	Chelating agents bind metallic ions, thereby disrupting cell adhesion to ECM	Typically used with enzymatic methods (e.g. trypsin) but can be used with other agents, ineffective when used alone
Physical and Miscellaneous Agents		
Temperature (freezing and thawing)	Intracellular ice crystals disrupt cell membrane	Ice crystal formation can disrupt or fracture ECM
Direct application of force	Removal of tissue eliminates cells and force can burst remaining cells	Force can directly damage ECM
Pressure	Pressure can burst cells and aid in removal of cellular material	Pressure can disrupt ECM
Electroporation	Pulsed electrical fields disrupt cell membrane	Electrical field oscillation can disrupt ECM

Table 3b

Techniques to apply decellularising agents and their effects (adapted from [72]).

Agent	Mode of action	Effects on ECM
Perfusion	Facilitates chemical exposure and removal of cellular material	Pressure associated with perfusion can disrupt ECM
Pressure gradient across tissue	Facilitates chemical exposure and removal of cellular material	Pressure gradient can disrupt ECM
Supercritical fluid	Pressure can burst cells, supercritical fluid facilitates chemical exposure and removal of cellular material	Pressure necessary for supercritical phase can disrupt ECM
Agitation	Can lyse cells, but more commonly used to facilitate chemical exposure and removal of cellular material	Aggressive agitation or sonication can disrupt ECM

1.2.5 Sterilisation of decellularised ECM

It is necessary to sterilise ECM scaffolds prior to *in vitro* use or implantation, as well as the removal of pyrogens (such as endotoxins, intact viral and bacterial DNA) that may be present. Biological scaffolds may be sterilised by simple treatments such as incubation with acids or solvents, or other treatments such as ethylene oxide exposure, gamma irradiation and electron beam irradiation. While the simple methods may lack penetration, the latter treatments are known to affect ECM ultrastructure and mechanical properties [61, 102, 103], and newer methods such as supercritical carbon dioxide requires further investigation [104]. In particular, ethylene oxide can cause undesirable host immune responses that impair function of the scaffold post-implantation [105], and irradiation causes degradation of the ECM by denaturation of key proteins such as collagen even at relatively low doses. Hence this necessary process is another factor that can critically influence the properties of the bio-scaffold, and must be taken into account during the wider-scale standardisation and quality assurance of such ECM products prior to clinical translation.

1.2.6 Evaluation and characterisation of decellularised ECM

While it is not possible to remove 100% of cellular material from the ECM, it is possible to quantitatively assay cell components such as DNA, mitochondria and membrane phospholipids. Residual cellular and nuclear material may contribute to cytocompatibility problems *in vitro* and adverse host responses *in vivo* [105-109].

Based on studies in which a constructive remodelling response has been observed *in vivo* and adverse cell and host responses avoided, the following minimum criteria apply for the achievement of decellularisation:

- <50ng dsDNA per mg ECM dry weight
- <200 bp DNA fragment length
- lack of visible nuclear material in tissue sections stained with 4',6-diamidino-2-phenylindole (DAPI) or haematoxylin & eosin (H&E)

Other characterisation modalities are used to assess the structural, biomechanical and biological properties of ECM depending on which are relevant to the appropriate tissue or application in question. These vary from assays of protein component content (e.g. collagens, sGAG, growth factors), histology and/or immunohistochemistry to assess presence and distribution of key structural proteins, biomechanical testing (including specialised tests e.g. respiratory capacitance), other specialised imaging to demonstrate 3-dimensional architecture from the microscopic level (e.g. electron microscopy) to demonstrating the preservation of specialised features such as vascular or airway networks (e.g. micro-computed tomography, vascular corrosion resin casting).

1.2.7 Limitations of ECM (in tissue engineering applications)

One key issue with the use of ECM and bioscaffolds based on this material is related to its being derived from natural tissues by the process of decellularisation of the specific tissue [6], due to the complexity of the three-dimensional organisation and composition of all the structural and functional molecules of the ECM.

Not only are there a wide range of agents available to achieve decellularisation, including chemical agents (e.g. acids and bases, hypo-/hyper-tonic solutions, ionic/non-ionic/zwitterionic detergents, solvents), biological agents (e.g. enzymes, chelating agents), physical methods (e.g. temperature, direct force, pressure and electroporation), there are also different means of applying these agents [72]. The choice and/or combination of these agents and methods are often specific to the tissue and application in question and far from being fully optimised as yet. These techniques also vary in their mechanism of action and associated effect on the ECM [66], especially deleterious ones that may affect the native composition and ultrastructure, thus impacting on the ability of the scaffold to support and influence cell behaviour and tissue regeneration. Also, while it is more straightforward to assess the structural integrity of ECM bioscaffolds with a range of tests ranging from quantitative assays of major protein components to imaging of 3-dimensional architecture and biomechanical testing, it is more challenging to assess the bioinductive properties of ECM molecules – both their preservation and especially *in situ* biological function [67].

In addition, there can be significant differences in the quality of the end product due to the biological origin of ECM materials and there are significant species, strain, age and sex differences to be found within these groups – and sometimes batch variability even between groups controlled for these factors. Age in particular is an independent factor for significant deterioration in the quality of decellularised ECM materials [110].

1.3 Whole organ decellularisation

1.3.1 Perfusion decellularisation and use of the vascular network

This is a relatively new technique that uses anterograde or retrograde perfusion via the intrinsic vascular network as a means of applying the decellularising agent while largely preserving the three-dimensional architecture of the organ from which the ECM has been isolated. It is the only technique to allow decellularisation of thick 3-D tissue sections or complete organs in a way that was not previously possible. Since the vascular network exists to minimise the diffusion distance for oxygen to cells, this is a particularly efficient way of delivering decellularising agents throughout the tissue and transporting cellular material from the tissue. The vascular network remains intact even after full decellularisation has been achieved, hence offering a route for the efficient delivery and penetration of cells and nutrients into the ECM scaffold during the process of recellularisation to create a functional organ construct. (See [Figure 1](#) for the schematic diagram of the life cycle of whole organ decellularisation and potential clinical applications.)

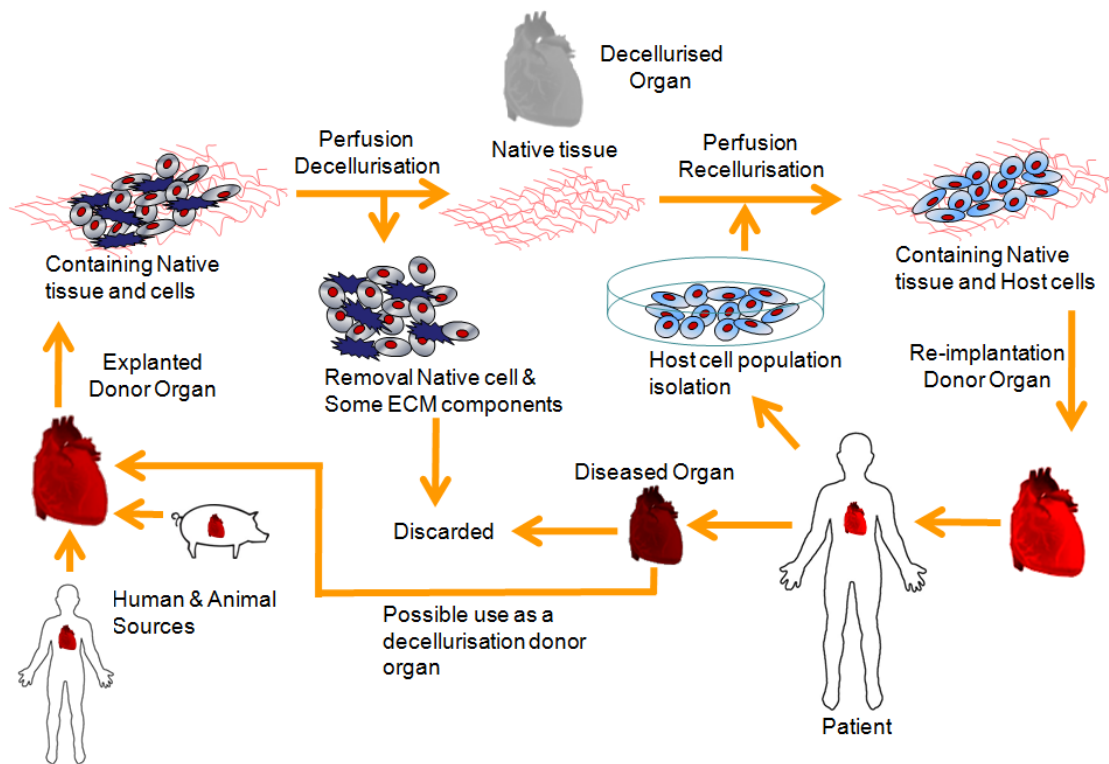


Figure 1 The life cycle of whole organ decellularisation and its potential clinical application(s).
Reproduced from [129].

1.3.2 An overview of whole organ decellularisation

1.3.2.1 The heart

The first report in which whole organ decellularisation was successfully performed was on the heart [9]. The aorta of a rat heart was cannulated to allow retrograde coronary perfusion with heparinised PBS with adenosine, 1% SDS and 1% Triton-X100 in sequence alternating with rinsing with deionised water between steps. This produced a decellularised scaffold that appeared similar to the heart but had a translucent white appearance throughout ([Figure 2](#)). Re-implantation of the acellular heart scaffold onto the aorta demonstrated an intact and patent vascular network ([Figure 3](#)). Recellularisation of the heart scaffold with cardiomyocytes under electro-physiological stimulation formed a construct capable of muscular contraction.

Another study has decellularised porcine hearts to demonstrate the extrapolation of this technique in larger organs/scaffolds with potential clinical applications [111], although only non-perfusion cell seeding with cardiomyocytes was investigated i.e. using lyophilised cardiac ECM rather than intact whole organ recellularisation cell culture hence only porcine heart decellularisation was demonstrated on a whole organ level.

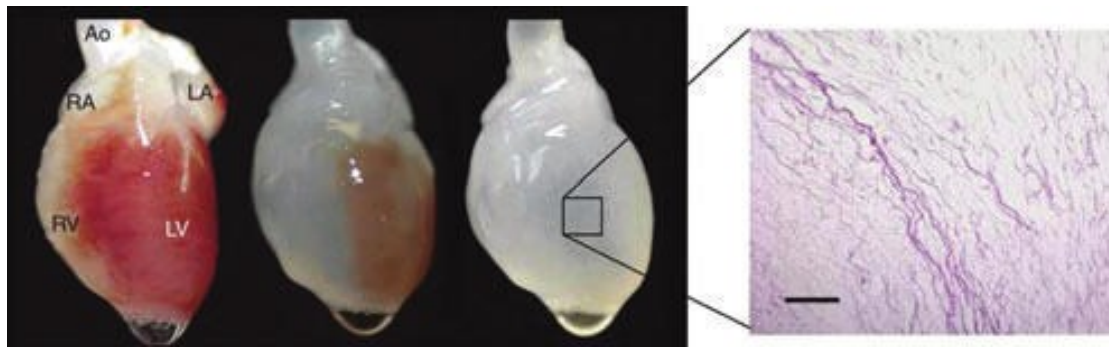


Figure 2 The heart in stages of increasing decellularisation over time with 1% SDS as the decellularising agent, with the end result of a translucent appearing heart-like scaffold structure. The panel on the right shows histological evidence (H&E staining) of decellularisation. Reproduced from [9].

Ao = aorta; RA = right atrium; RV = right ventricle; LA = left atrium; LV = left ventricle.

Scale bar = 200 μ m.

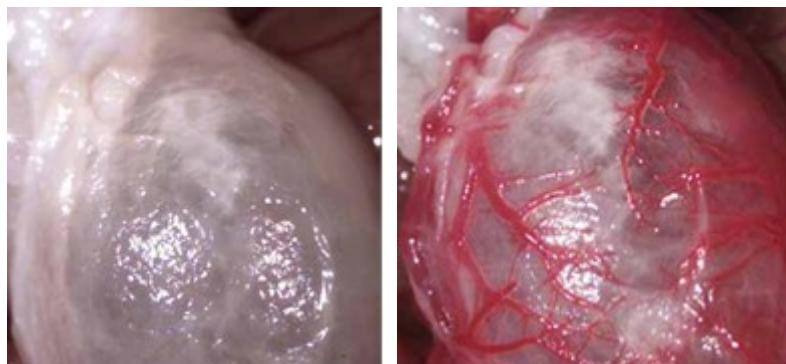


Figure 3 Coronary perfusion and evidence of intact coronary vasculature (right) after orthotopic implantation of the decellularised heart scaffold (left) and unclamping of the aorta (using the rat model). Reproduced from [9].

1.3.2.2 The lungs

A number of groups have investigated the use of perfusion decellularisation in the lungs [10-12, 112-115], which contains two accessible compartments separated by a short diffusion distance i.e. the vascular and the airway systems, by using varying protocols of vascular perfusion alone [10, 12], or in combination with endotracheal instillation [11, 113], bronchial/tracheal instillation alone [114, 115], or perfusion of the airway compartment [112]. The decellularising agents used were also far more varied from protocol to protocol, from SDS at different concentrations [10, 12, 102] to 8mM CHAPS [11], to a combination of Triton-X100/ deoxycholate/DNAse [113, 115]. All groups demonstrated preservation of the major components of the ECM and the micro- and macro-architecture of the lung. Recellularisation was achieved with all the reports, although Petersen *et al* mimicked physiological conditions within the bioreactor by ventilation of the airway compartment at negative pressure at 1 breath/min, and maintained pulmonary artery pressure at 20mmHg or below [11]. Orthotopic implantation of these tissue engineered lung constructs demonstrated active gas exchange occurring via sampling of pulmonary arterial and venous blood. However, results in this study and Ott *et al* showed vascular leakage and pulmonary oedema after a few hours implantation, indicating damage to the microvasculature from the decellularisation process. In a follow-up study to Ott *et al*, Song *et al* demonstrated further optimisation of graft preservation and oxygenative function post-implantation for as long as 7 days [12]. Recellularisation was performed with mixtures of whole lung cell isolates in four cases [10-12, 113] but one group used a homogeneous mouse embryonic stem cell population [112], which showed that the matrix was capable of promoting site-appropriate differentiation without any other specific differentiation cues, while another group particularly investigated seeding efficiencies using murine ESCs and conditioned media but did not carry out perfusion culture [115]. Wallis *et al* compared the effects of different detergent protocols for decellularisation and utilised MSCs for cell seeding but again did not

carry out perfusion culture [114], although slices of the seeded lung scaffolds were cultured for up to 14 days.

1.3.2.3 The liver

Liver decellularisation has been performed by antegrade perfusion via the portal vein with SDS and Triton X-100, either alone or in combination [13, 116-118], although another study used trypsin/EGTA [119] and Baptista *et al* used Triton X-100 in combination with ammonium hydroxide [120, 123]. Decellularisation was demonstrated with haematoxylin and eosin and evidence of DNA removal, and retention of key ECM constituents with preservation of microvasculature and ECM ultrastructure. Hepatocytes were seeded onto the acellular liver scaffold via portal vein perfusion and were shown to be functional in four studies [13, 118-120] by showing evidence of synthesis of lactate dehydrogenase, albumin and urea after heterotopic implantation. In particular, two studies attempted to scale up this approach in order to further approximate human-scale engineered organ constructs by use of ferret [120] and porcine [118] livers, and both studies seeded the bio-scaffolds with human liver cells which were shown to be viable and compatible on these xenogeneic scaffolds.

1.3.2.4 The kidney

The kidney may also be considered to have two compartments – the vascular and the urothelial cell-lined pelvi-ureteric system. One group have notably decellularised whole kidneys with 1% w/v SDS and then achieved recellularisation through the ureteric and vascular compartments with primary renal and endothelial cells respectively [14]. This recent work on the regenerated kidney has demonstrated up to 10% functionality in terms of urine production and creatinine clearance. The recellularised construct was also

implanted *in vivo* for a short 'test period' (several hours) and demonstrated urine flow through the implant ureter *in vivo*.

Several other groups have decellularised whole kidneys by the perfusion method with preservation of the vascular network and complete cell removal [121-125]. Decellularising agents vary from 1% v/v Triton or SDS [122-125] to 3% v/v Triton with DNase and additional 4% w/v SDS [121]. The preservation and presence of ECM constituents such as laminin and collagen IV were also demonstrated, as well as collagen I, fibronectin and heparin sulfate in a separate study which did not employ perfusion [126]. The latter study juxtaposed fetal cells in fresh renal explants with the ECM scaffolds by layering fetal kidney tissue against the ECM, which supported developmental renal phenotypes in these cells after they migrated into the ECM. In two studies, decellularised rat kidneys scaffolds were reseeded with murine embryonic stem cells, and the renal ECM was shown to support the renal differentiation of the embryonic stem cells [121, 125]. One study did not investigate recellularisation but rather use of human kidneys unsuitable for transplant as a potential source of whole organ scaffold decellularisation with successful whole kidney decellularisation and removal of cellular antigens [124].

1.3.2.5 Other tissues/organs

The technique of whole organ decellularisation is also expanding into other tissues and organs such as the small bowel and skeletal muscle, especially as it has the potential to allow the development of a viable replacement in the absence of any current clinical alternative e.g. intestinal failure and large muscle defects due to trauma. Totonelli *et al* have demonstrated the application of this technique to small bowel [127] and shown the viability of amniotic fetal stem cells seeded onto the decellularised scaffold and its angiogenic properties, although perfusion recellularisation was not performed. Perniconi *et al* have decellularised whole skeletal muscles derived from rats by an incubation method and

further, have implanted non-perfused acellular scaffolds *in vivo* within an equivalent muscle defect to show infiltration of inflammatory and stem cells within the scaffolds [128].

1.3.3 Comparison of methods and protocols

The current applications and methods in using xenogeneic whole organ ECM scaffolds to create potentially functional bio-artificial organ constructs for surgical implantation [9-14, 111-128] have been systematically reviewed as follows, and comparison made of specific trends within this new and developing technique to give a concise overview of existing experimental work across all the relevant organ systems as a tool for researchers [129].

1.3.3.1 Common trends seen within published protocols

The main parameters of interest for the perfusion decellularisation and recellularisation protocols are compared and summarised in [Tables 4a-d](#) and [5a-b](#) respectively.

[Table 4a](#) shows the different animal species and strains used, with the most common being rat, although there is an intentional move towards larger species in order to scale up the technique to approach more human-sized organ constructs.

[Table 4b](#) displays the most common decellularising agent(s) used, with the predominance of SDS whether alone or in combination with Triton X-100. When 'other agent' is quoted, there is a far wider disparity between protocols which almost vary from paper to paper (9 out of 24 in total). There are also other marked differences in protocols such as the additional use of physical methods (freezing) and whether DNase or RNase is also employed in combination with the main decellularising agent (full details in [Tables 6 and 7](#)).

[Table 4c](#) summarises the decellularisation pump setup parameters i.e. flow rate (or pressure) and duration of decellularisation (for the entire protocol, including periods of non-perfusion if relevant). While flow rates vary widely depending on the animal species, generally the rates adopted tend to be low and sub-physiological vascular flow rates, while

flow pressures tend to mimic physiological in situ ones. The total duration of the decellularisation protocol gives an indication of how widely varying the protocols can be from group to group, whether in terms of the additional steps/methods used e.g. freezing, storage phases within the decellularisation process, and the flow rates and concentrations applied with even the same decellularising agents. The most marked contrast may be seen between De Kock *et al* and Uygun *et al* in work on the liver bio-scaffold (1-2 hours vs. up to 5 days), especially as the former quote directly from the latter as a baseline for developing their more streamlined protocol.

Table 4d summarises the characterisation of the decellularised organ scaffold and the techniques used; there is a common 'panel' of tests to demonstrate a variety of important structural properties preserved in the ECM that are necessary for both the effectiveness of the bio-scaffold for subsequent recellularisation and the decellularisation process itself. As can be seen, groups vary in how much of this 'panel' of tests are completed. Not shown are any 'functional' tests of ECM bio-activity as this is limited so far to single growth factor assays and in two groups only [113, 119].

Table 4a

Summary of species and strains used for decellularisation.

Organ	Authors	Species (strain)
Heart	Ott et al, 2008	Rat (Fischer 344)
Heart	Wainwright et al, 2010	Pig
Lung	Ott et al, 2010	Rat (Sprague Dawley)
Lung	Petersen et al, 2010	Rat (Fischer 344)
Lung	Cortiella et al, 2010	Rat
Lung	Price et al, 2010	Mouse (C57BL/6)
Lung	Song et al, 2011	Rat (Sprague Dawley)
Lung	Wallis et al, 2013	Mouse (BALB/C)
Lung	Lecht et al, 2014	Rat (Sprague Dawley)
Liver	Uygun et al, 2010	Rat (Lewis)
Liver	Shupe et al, 2010	Rat (Fischer 344)
Liver	Soto-Gutierrez et al, 2011	Rat (Sprague Dawley)
Liver	Baptista et al, 2011	Ferret
Liver	De Kock et al, 2011	Rat (Sprague Dawley)
Liver	Barakat et al, 2011	Pig (Yorkshire)
Kidney	Ross et al, 2009	Rat (Sprague Dawley)
Kidney	Liu et al, 2009	Rat (Wistar)
Liver, Kidney, Pancreas, Small bowel	Baptista et al, 2009	Ferret liver Pig kidney/pancreas/ small bowel
Kidney	Song et al, 2013	Rat (Sprague Dawley)
Kidney	Orlando et al, 2013	Human
Kidney	Bonandrini et al, 2014	Rat (Sprague Dawley)
Small bowel	Totonelli et al, 2012	Rat (Sprague Dawley)
Skeletal muscle	Perniconi et al, 2012	Mouse (BALB/C)

Table 4b

Summary of decellularisation agents used.

Organ	Authors	SDS	SDS + Triton	Other
Heart	Ott et al, 2008	(+)	+	-
Heart	Wainwright et al, 2010	-	-	+
Lung	Ott et al, 2010	(+)	+	-
Lung	Petersen et al, 2010	-	-	+
Lung	Cortiella et al, 2010	+	-	-
Lung	Price et al, 2010	-	-	+
Lung	Song et al, 2011	(+)	+	-
Lung	Wallis et al, 2013	(+)	+	+
Lung	Lecht et al, 2014	-	-	+
Liver	Uygun et al, 2010	(+)	+	-
Liver	Shupe et al, 2010	(+)	+	-
Liver	Soto-Gutierrez et al, 2011	-	-	+
Liver	Baptista et al, 2011	-	-	+
Liver	De Kock et al, 2011	(+)	+	-
Liver	Barakat et al, 2011	+	-	-
Kidney	Ross et al, 2009	(+)	+	-
Kidney	Liu et al, 2009	(+)	+	-
Liver, Kidney, Pancreas, Small bowel	Baptista et al, 2009	-	-	+
Kidney	Song et al, 2013	(+)	+	-
Kidney	Orlando et al, 2013	+	-	-
Kidney	Bonandrini et al, 2014	+	-	-
Small bowel	Totonelli et al, 2012	-	-	+
Skeletal muscle	Perniconi et al, 2012	+	-	-

Table 4c

Summary of conditions used for perfusion decellularisation.

Organ	Authors	Flow rate	Perfusion pressure	Duration of decellularisation (perfusion)
Heart	Ott et al, 2008	-	77.5mm Hg	12-13 hours
Heart	Wainwright et al, 2010	1.3L/min	-	6-7 hours
Lung	Ott et al, 2010	-	30mm Hg	2-3 hours
Lung	Petersen et al, 2010	-	<20mm Hg	(Total 500ml perfused)
Lung	Cortiella et al, 2010	N/A	N/A	N/A
Lung	Price et al, 2010	N/A	N/A	N/A
Lung	Song et al, 2011	-	80cm H ₂ O	2-3 hours
Lung	Wallis et al, 2013	N/A	N/A	2-3 days
Lung	Lecht et al, 2014	N/A	N/A	6 days
Liver	Uygun et al, 2010	1ml/min	-	5 days
Liver	Shupe et al, 2010	5ml/min	-	(Total 300ml x 4 perfused)
Liver	Soto-Gutierrez et al, 2011	8ml/min	-	20-26 hours
Liver	Baptista et al, 2011	5ml/min	-	(Total 40x + 50x volume of liver)
Liver	De Kock et al, 2011	30ml/min	-	1-2 hours
Liver	Barakat et al, 2011	-	80mm Hg	2-3 days
Kidney	Ross et al, 2009	-	100mm Hg	(Not stated)
Kidney	Liu et al, 2009	-	100cm H ₂ O	12-13 hours
Liver, Kidney, Pancreas, Small bowel	Baptista et al, 2009	10-60 ml/min	-	24 hours
Kidney	Song et al, 2013	-	30mm Hg	12-13 hours
Kidney	Orlando et al, 2013	12ml/min	-	12 + 48 hours
Kidney	Bonandrini et al, 2014	-	-	-
Small bowel	Totonelli et al, 2012	0.6 ml/hour	-	31 hours (per DET cycle)
Skeletal muscle	Perniconi et al, 2012	N/A	N/A	24-48 hours

Table 4d

Summary of decellularised whole organ scaffold characterisation and techniques.

Organ	Authors	Histology/ IHC	EM	Quantitative assay for DNA/ECM components	Vascular characterisation	Mechanical testing
Heart	Ott et al, 2008	+/+	+	-	Corrosion casting + implantation	+
Heart	Wainwright et al, 2010	+/+	+	+	-	+
Lung	Ott et al, 2010	+/-	+	-	-	+
Lung	Petersen et al, 2010	+/+	+	-	Micro-CT	+
Lung	Cortiella et al, 2010	-/- (IF)	-	(Gel electrophoresis for DNA)	-	-
Lung	Song et al, 2011	-	-	-	-	-
Lung	Price et al, 2010	+/+	+	+	-	+
Lung	Wallis et al, 2013	+/-IF	-	(Western blot analyses)	-	+
Lung	Lecht et al, 2014	-	-	(Western blot analyses)	-	-
Liver	Uygun et al, 2010	+/+	+	+	Corrosion casting	-
Liver	Shupe et al, 2010	+/+	-	-	-	-
Liver	Soto-Gutierrez et al, 2011	+/+	+	(Gel electrophoresis for DNA)	Corrosion casting	-
Liver	Baptista et al, 2011	+/+	+	+	Fluoroscopy + confocal microscopy	-
Liver	De Kock et al, 2011	+/+	+	-	Corrosion casting	-
Liver	Barakat et al, 2011	+/+	-	-	Corrosion casting + fluoroscopy	-
Kidney	Ross et al, 2009	+/+	+	-	-	-
Kidney	Liu et al, 2009	+/- (IF)	+	-	-	-
Liver, Kidney, Kidney, Pancreas, Small bowel	Baptista et al, 2009	+/-	-	-	Fluoroscopy	-
Kidney	Song et al, 2013	+/+	+	+	-	-
Kidney	Orlando et al, 2013	+/+	+	+	-	-
Kidney	Bonandrini et al, 2014	-	-	(ELISA of ECM protein interactions)	Micro-CT	-
Small bowel	Totonelli et al, 2012	+/+	+	+	-	+
Skeletal muscle	Perniconi et al, 2012	+/+	-	-	-	-

IHC = immunohistochemistry

IF = immunofluorescence

EM = electron microscopy

Table 5a summarises the four main parameters in recellularisation protocols: cell population, cell numbers, route of seeding and perfusion rate. The bioreactor conditions tend to be similar i.e. incubator conditions, and duration of continuous culture is on average 7 days (with one group moving onto a longer period [12]), and hence these parameters have been omitted from the table. Full details can be found in Table 7. Cell populations used have been commonly the mixed population native cells isolated from fetal or neonatal organs as the initial proof of concept, but work is now moving onto human cell populations and stem cell populations to demonstrate bio-compatibility and the bio-inductive properties of the ECM scaffold. Cell numbers are by necessity high e.g. 50-100 million on rat-derived scaffolds but exponentially higher on larger species, however this is usually still only 10-20% of normal physiological cell numbers. The route of seeding can depend on the physiological compartments available (other than the vascular) e.g. the airway, and also direct injection is possible, but most researchers utilise the vascular route. There has been some comparison between a continuously perfused seeding process and a bolus intravascular injection, with some evidence that the latter affords higher engraftment [119].

Table 5b displays those techniques for characterisation of the recellularised organ constructs (where applicable), and these are usually a combination of histology-based techniques and functional testing specific to the organ/tissue in question.

Table 5a

Summary of conditions used for whole organ scaffold recellularisation.

Organ	Authors	Cell population(s)	Cell numbers (million)	Seeding route (bolus injection/perfusion/other)	Perfusion flow rate/pressure
Heart	Ott et al, 2008	Rat cardiocytes	50-75	Intramural injection	6ml/min
Lung	Ott et al, 2010	Human carcinomatous alveolar cells	91.25+/- 31.72	Tracheal perfusion	10-15mm Hg
Lung	Petersen et al, 2010	Rat lung epithelial cells	100	Airway injection	1-5ml/min
Lung	Cortiella et al, 2010	Murine ESCs	2	Tracheal injection	(Non-perfusion)
Lung	Price et al, 2010	Murine fetal lung cells	3	Infusion in culture medium	(Non-perfusion)
Lung	Song et al, 2011	Rat fetal lung cells	Not stated	Tracheal perfusion	10-15 mm Hg
Lung	Wallis et al, 2013	Rat MSCs	2		
		Murine C10 lung epithelial cells			
Lung	Lecht et al, 2014	Murine ESCs	10		
Liver	Uygun et al, 2010	Rat hepatocytes	50	Bolus intravascular injection x 4	Not stated
Liver	Shupe et al, 2010	Rat liver progenitor cells	1	Inferior vena caval injection	Not perfused or cultured
Liver	Soto-Gutierrez et al, 2011	Murine hepatocytes	10-50	Intraparenchymal injection vs steady perfusion vs bolus injection	2ml/min
Liver	Baptista et al, 2011	Human fetal liver cells	70	Portal vein perfusion	3ml/min
Liver	Barakat et al, 2011	Human fetal stellate cells + human fetal hepatocytes	350 + 1000	Portal vein perfusion	90ml/min
Kidney	Ross et al, 2009	Murine ESCs	2	Arterial + ureteric injection	(Non-perfusion)
Kidney	Song et al, 2013	Rat renal cells	60.71+/- 11.67	Arterial + ureteric perfusion	1.5ml/min
Kidney	Bonandrini et al, 2014	Murine ESCs	10		
Liver, Kidney, Pancreas, Small bowel	Baptista et al, 2009	Human HepG2 cells	30	Intravascular injection	6ml/min

Table 5b

Summary of recellularised organ construct characterisation and techniques.

Organ	Authors	Histology/ IF	TUNEL	EM	Functional assessment	<i>In vivo</i> implantation
Heart	Ott et al, 2008	+/+	+	+	LV pressure monitoring + live video (contractility)	-
Lung	Ott et al, 2010	+/+	-	+	Dynamic lung fn tests, blood gases, live video	+
Lung	Petersen et al, 2010	+/+	+	+	Compliance testing, blood gases	+
Lung	Cortiella et al, 2010	-/+	+	-	-	-
Lung	Price et al, 2010	-/+	-	-	-	-
Lung	Wallis et al, 2013	-/+	-	-	-	-
Lung	Lecht et al, 2014	-/+	-	-	-	-
Liver	Uygun et al, 2010	+/+	+	+	LDH/urea/albumin assay RT-PCR of enzymes	+
Liver	Shupe et al, 2010	+/-	-	-	-	-
Liver	Soto-Gutierrez et al, 2011	+/+	-	-	Albumin/cytochrome/ ammonia assay	-
Liver	Baptista et al, 2011	+/+	+	-	Urea/albumin/prostacylin assays	-
Liver	Barakat et al, 2011	+/- (IHC)	+	-	Platelet deposition studies Urea/albumin/lactate assays	+
Kidney	Ross et al, 2009	+/- (IHC)	-	-	-	-
Kidney	Song et al, 2013	+/+	-	+	Glucose/albumin/urea + creatinine content of urine	+
Kidney	Bonandrini et al, 2014	+/+	-	-	-	-
Liver, Kidney, Pancreas, Small bowel	Baptista et al, 2009	+/- (IHC)	-	-	-	-

TUNEL = Terminal deoxynucleotidyl transferase dUTP nick end labeling

1.3.3.2 Summary of trends seen within whole organ decellularisation work extant

In terms of decellularisation protocols, these vary widely in many aspects e.g. strain/species of animal used, decellularisation agents and parameters (plus post-decellularisation sterilisation) to achieve the whole organ ECM scaffold required for recellularisation. On one hand this is an indication of the universal applicability of the principle of perfusion decellularisation to all animal tissues, and while variations in the optimal protocol can also be attributed to differences in the nature of each type of organ/tissue and its specific structure and composition, it also appears that a complete and systematic rationale for determining the optimal perfusion decellularisation process has not yet been determined. Some studies have started to formally assess optimisation of decellularisation protocols e.g. scaffold properties with varying number of decellularisation 'cycles' [127], or comparison of different decellularising agents on the same tissue [114], while only one study reports direct optimisation from a baseline protocol taken from another study [117]. Characterisation of the structural components of the ECM have yielded similar methods and results across most of the groups, but only two group have attempted to assess the presence of the (presumed) bioinductive molecules present in the ECM with a quantification assay of two specific growth factors [113, 119]. This is an area that requires further investigation in all the organs/tissues of relevance as regulatory and standardisation issues (e.g. quality control, release criteria) are fundamental to the clinical translation and commercialisation of these ECM products. ECM products are known to vary from batch to batch (even animal to animal) and hence standardisation of basic parameters (species/strain, sex, age, weight) need to be maintained in order to achieve meaningful comparison in outcomes, or from group to group. These donor factors, and others relating to the use of biological agents within certain protocols, may require a degree of dynamicity within the decellularisation process, but currently the extreme disparity between some protocols suggests the need for a more evidence-based approach. Pre-optimisation and standard operating procedures are even more critical when

it is likely that post-processing testing of the ECM bio-scaffold (and the recellularised construct) is likely to be limited in scope in those cases destined for implantation. Minimum standardised biological requirements within whole organ ECM scaffolds (e.g. threshold DNA levels, structural integrity growth factor levels/activity) should also be established in order to maintain biological quality and clinical safety.

Regarding the recellularisation process, most groups use a similar anatomical approach to the vascular perfusion setup for circulation of medium during continuous culture and also use appropriate physiological adjuncts to help stimulate constructive remodelling (e.g. in the heart and lung models). Most groups have investigated the use of a mixed population of organ-specific cells isolated from neonatal or fetal animals in order to seed the acellular ECM scaffold as proof of concept and also to achieve the full extent of cellular variety and density required to populate a whole organ scaffold. Several groups have also attempted reseeded with embryonic stem cells, which have demonstrated proliferation and differentiation specific to organ-specific bioinductive cues within the ECM scaffold. In addition, human cell populations have also been seeded onto the xenogeneic scaffolds to demonstrate biocompatibility. Again there are quite widely varying parameters for the recellularisation protocol between groups in terms of cell numbers and culture conditions and this is an area where further systematic optimisation is required. A physiological degree of cellular engraftment has not yet been achieved with any group, partly because of the very large cell numbers required. Finally, functional assessment of recellularised constructs depends on the degree of recellularisation achieved and varies from organ to organ and whether the graft is suitable for implantation in vivo. Generally only very short-term viability and implantation in vivo have been achieved so far (e.g. 6 hours) but one study has expanded this into a 7-day implantation period after improving graft preservation [12].

Tables 6 and 7 (pages 53 – 57) summarise more complete details of the decellularisation and recellularisation protocols used in work published from the groups above.

1.3.3.3 Further work (including need for optimisation) and surgical implications

It has been shown so far that whole organ decellularisation can yield whole organ ECM bio-scaffolds capable of supporting recellularisation on a complex 3D level and sustaining appropriate cellular and tissue functionality. These bio-artificial organ constructs also allow relatively easy surgical manipulation, especially as they preserve the intact vascular pedicle for direct anastomosis at the orthotopic implantation site. The intact and inherent vasculature permits direct perfusion of the tissue and maintain provision of nutrients and oxygen to the implanted graft, in an exact parallel to the transplant procedure. As such, this technique could provide an ideal short-cut to bio-engineering an organ replacement that is structurally and histologically similar to the original organ in question, uses established surgical procedures for implantation, is able to potentially support cellular function to the same degree, is biologically compatible and comes from a widely available source (e.g. porcine or other xenogeneic organs) [130].

Currently there are limitations to all the tissue engineered organ constructs produced so far in terms of functionality and viability in vivo, varying from achieving sufficient or comparable to normal cellular density, achieving full cellular and tissue differentiation, and assessment of long-term viability, functionality and constructive remodelling of organ grafts implanted in vivo. It would also be necessary to expand these techniques on larger animal organs which are closer in size and functionality to human ones. These are issues to be addressed and optimised before work can proceed to the clinical stage, in conjunction with the regulatory issues which guide cell choice (such as those from the US Food and Drug Administration regarding the use of autologous human cells applied in a non-homologous location, or the use of allogeneic cells in virtually all cases), as well as a common language for reporting outcomes in trials.

In addition, it would be important to investigate the possibility of organ regeneration from an alternative cell source such as a stem cell population. Possibilities for this may be

embryonic stem cells as has been tried in the lung and kidney, or mesenchymal stem cells which could be harvested in large numbers for tissue engineering applications even in the adult e.g. bone marrow. If eventually autologous cells such as mesenchymal stem cells from the recipient alone could be used to create a functional organ graft, there could be significant clinical implications in overcoming the immune barrier and problem of rejection, with further great potential benefits to the patient.

1.4 Aims and scope of thesis: using a whole organ decellularisation approach in the kidney

While the potential clinical implications of a regenerative medicine approach to bioengineering organ replacements using the technique of whole organ decellularisation are extremely exciting, there are a number of major technical factors and challenges that exist before clinical application can be considered. One critical group of pre-optimisation parameters lies with the production of the ECM bioscaffold and the decellularisation process. Both donor factors and the decellularisation parameters must be standardised and rationalised to yield consistent and optimised bioscaffolds. A few studies have started to assess or compare effects of perfusion decellularisation protocols in a more systematic manner e.g. different decellularising agents [114], duration/exposure to the decellularising agent [127]; however, it has been shown in a recent systematic review that currently there is extreme disparity between different decellularisation protocols in the literature that strongly suggests the need for a more evidence-based approach [129].

On this basis and using the rat model which is both the most common in the whole organ decellularisation literature [129] and also for feasibility and comparison of data, the first aim of this project is to optimise the decellularisation process for whole kidney ECM bioscaffolds with regards to two fundamental parameters: concentration of decellularising agent and duration of perfusion, using the most commonly used single decellularising agent drawn from the literature i.e. sodium dodecyl sulphate. Optimisation can be determined with regards to structural and functional characteristics of the ECM bio-scaffold as assessed by histology, immunohistochemistry (IHC), quantitative assays of DNA and sulfated glycosaminoglycan (sGAG) content, and growth factor quantification.

Secondly, in a continuation and extension of other work, the aim is to carry out recellularisation of the whole kidney bioscaffolds produced using a more optimised protocol (as specified above) to yield a functionally significant and implantable organ graft/construct. Recellularisation may be attempted with use of mixed primary renal cell population derived

from neonatal kidneys as with Song *et al* [14] as this is the most likely to yield a potentially functional construct, but it would also be meaningful to consider recellularisation of the kidney ECM scaffold with an adult stem cell population, to investigate the possibility of renal regeneration from an alternative cell source. An appropriate choice of cell population may be mesenchymal stem cells which can differentiate into functional nephrons following injection into the nephrogenic site of developing rat embryos [131, 132] and are furthermore involved in the repair and recovery of renal function after acute renal injury [133, 134]. This could have significant clinical implications such that eventually autologous cells such as mesenchymal stem cells from the recipient alone could be used to create a functional organ graft, thus overcoming the immune barrier and problem of rejection, with further great potential benefits to the patient.

Thirdly and finally, the aim is to implant both the decellularised and recellularised kidney constructs (both primary renal cell and mesenchymal stem cell types) *in vivo* using the renal transplant model. In a potential advance on other implantation studies so far, since the kidney is unique in that a single organ may sustain life normally, it may be possible to maintain *in vivo* implantation of the recellularised grafts for much longer periods than has been possible with the heart/lungs/liver, yielding valuable information on the long-term viability and function of the graft construct, and the possible effect of the ECM bioscaffold on constructive remodelling *in vivo*.

Table 6

Summary of the decellularisation protocols used in the literature.

Organ	Authors	Tissue harvesting (species/strain/age, explantation details)	Perfusion type	Agent(s) + duration	Flow rate/pressure	Sterilisation	Characterisation of decellularised scaffold
H	Ott et al, 2008	12 week old Fischer 344 rats Systemic heparinisation	Retrograde coronary perfusion via ascending aorta	Heparinised PBS + 10µM adenosine for 15 min + 1% SDS for 12 hours + 1% Triton-X100 for 30 min	77.4 mm Hg coronary arterial pressure	PBS + 100u/ml pen-strep for 124 hours	Histology + immunofluorescence of ECM – collagen I + III, laminin, fibronectin Aortic valve – Evans blue perfusion SEM/TEM of ECM architecture Vascular corrosion resin casting + heterotopic transplantation Mechanical (stress-strain/ tangential modulus) testing
H	Wainwright et al, 2010	Adult pigs (strain not specified)	Retrograde coronary perfusion via ascending aorta	Frozen at -80°C for 16 h then thawed at room temperature (RT) + hypotonic type 1 water for 15 min + 0.02% trypsin/0.05% EDTA/0.05% NaN3 for 2 h + 3% Triton X-100/0.05% EDTA/0.05% NaN3 for 2 h + 4% deoxycholic acid for 2 h	1-1.3 L/min	0.1% peracetic acid/4% ethanol for 4 hours at 1.7 L/min	Histology + IHC/immunofluorescence of ECM – DAPI, elastin, collagens (I, III, IV), GAGs DNA/GAG/elastin quantitative assays SEM of ECM architecture Mechanical (ball burst) testing
L	Ott et al, 2010	12 week old male Sprague Dawley rats Systemic heparinisation	Retrograde pulmonary arterial perfusion	Heparinised PBS for 15 min + 0.1% SDS for 120 min + 1% Triton-X100 for 10 min	30 mm Hg pulmonary arterial pressure	PBS + 100u/ml pen-strep for 72 hours	Histology of ECM – collagen, proteoglycans, elastin + Morphometry and stereology of alveoli TEM of ECM alveolar architecture Dynamic lung testing – compliance + vital capacity
L	Petersen et al, 2010	3 month old Fischer 344 rats Intraperitoneal heparin	Antegrade perfusion via right ventricle	PBS + 50u/ml + 1µg/ml SNP + 8mM CHAPS/1M NaCl/25 mM EDTA in PBS until total 500ml of fluid perfused + 90u/ml benzonase	<20 mm Hg pulmonary arterial pressure	PBS + 10% pen-strep (duration not stated)	Histology + immunofluorescence of ECM – elastin, collagen I + IV, laminin, fibronectin DNA/collagen/GAG/elastin quantitative assays SEM/TEM of ECM architecture Micro-CT of vascular + alveolar architecture Mechanical (stress-strain/ compliance) testing
L	Cortiella et al, 2010	Rats (age/strain not specified)	Mechanical agitation within	Frozen at -70°C for then thawed at 40°C/flash	(2.5 rpm rotational speed)	PBS + pen-strep + amphotericin	Immunofluorescence – DAPI, MHC-1 Gel electrophoresis of

			bioreactor + initial freezing process	frozen x 4 times + 1% SDS with continuous circulation for 5 weeks + DNase/RNase	within bioreactor)	cin for 24 hours	DNA content
L	Price et al, 2010	2-3 month old C57BL/6 female mice	Injection via right ventricle + trachea	0.1% Triton X-100 2% sodium deoxycholate DNase Repeated injection/incubation steps – not continuous perfusion	N/A	All solutions sterile with added pen-strep	Histology + immunofluorescence of ECM – collagen, elastin, DAPI, laminin Collagen/GAG/laminin/elastin quantitative assays SEM of ECM architecture Pulmonary function tests
L	Song et al, 2012	Male Sprague-Dawley rats (260-280g) Systemic heparinisation	Antegrade pulmonary arterial perfusion	Heparinised PBS for 15 min + 0.1% SDS for 120 min + 1% Triton-X100 for 10 min	80 cm H ₂ O	PBS + 100u/ml pen-strep for 72 hours	(Not performed)
L	Wallis et al, 2013	8-24 week female mice (BALB/C)	‘Rinsing’ via trachea + instillation via right ventricle	Protocol 1 with 0.1% Triton X-for 24 h then 3% deoxycholate for 24 h + DNase x 1h + rinsing steps; Protocol 2 with 0.1% SDS for 24 h (x2) + Triton X-100 for 1 h; Protocol 3 with 8mM CHAPS for 24 h (x2) + DNase for 1h	N/A	PBS + pen-strep during ‘rinsing’ steps + at end of each protocol	Histology + immunofluorescence of ECM – collagen I + IV, laminin, fibronectin, smooth muscle actin Western blot analyses for collagen I + IV, laminin, fibronectin, other ECM proteins Dynamic mechanical testing – resistance, compliance
L	Lecht et al, 2014	Adult male + female Sprague Dawley rats	(Incubation /rotation system)	4% sodium deoxycholate/3 % Triton X-100, solution replaced at 0.5% lower dilution/day decreasing to 0.5% sodium deoxycholate	N/A	Storage in DMEM with antibiotic/anti-mycotic	(Western blot analyses of ECM protein interactions only)
Lv	Uygun et al, 2010	Female adult (150-200g) Lewis rats Intra-cardiac heparin	Antegrade perfusion via portal vein	Initial 20ml 0.9% saline then frozen at -80°C for 4 hours then thawed at 4°C + PBS at 1ml/min overnight + 0.01% SDS for 72 h + 0.1% SDS for 24 h + 1% SDS for 24 h + 1% Triton-X100 for 30 min	1ml/min	PBS + 0.1% peracetic acid for 3 hours	Histology + immunofluorescence of ECM – collagen I + IV, laminin, fibronectin DNA/collagen/GAG quantitative assays SEM of ECM architecture Vascular corrosion resin casting
Lv	Shupe et al, 2010	Adult Fischer 344 rats	Retrograde perfusion via inferior vena cava	Initial 100ml PBS + 1%, 2%, 3% Triton X-100 in sequence until 300ml each perfused + 0.1% SDS until 300 ml perfused	5ml/min	300ml PBS	Histology + immunofluorescence of ECM – collagen IV, laminin, DAPI
Lv	Soto-Gutierrez et al,	Male adult (250-300g) Sprague-	Retrograde perfusion via inferior	Initial 5-10ml PBS/heparin then frozen at -	8ml/min	2MRad gamma irradiation	Histology + immunohistochemistry (IHC) of ECM – collagen

	2011	Dawley rats Systemic heparinisation	vena cava	80°C for 24 hours then thawed at RT + 0.02% trypsin/0.05% EGTA for 2 h +3% Triton X- 100/0.05% EGTA for 18-24 h			IV, laminin, fibronectin, DAPI Gel electrophoresis of DNA content SEM/TEM of ECM architecture Vascular corrosion resin casting
Lv	Baptista et al, 2011	Ferret (age/strain/sex not specified)	Antegrade perfusion via portal vein	Initial dH ₂ O (40x volume of liver) + 1% Triton X- 100/0.1% NH ₄ OH (50x volume of liver)	5ml/min	Not stated	IHC + Western blotting - collagen I /III/IV, laminin, fibronectin DNA/collagen/GAG quantitative assays SEM of ECM architecture Fluoroscopy + confocal microscopy of vasculature
Lv	De Kock et al, 2011	Male adult (250-300g) Sprague- Dawley rats Systemic heparinisation	Antegrade perfusion via portal vein	Initial Krebs- Henseleit buffer for 15 min + 1% Triton X- 100 for 30 min + 1% SDS for 30 min	30ml/min	N/A	Histology + IHC of ECM – collagen I + IV, laminin, fibronectin, VEGF SEM of ECM architecture Vascular corrosion resin casting
Lv	Barakat et al, 2011	Yorkshire swine (25- 35kg)	Antegrade perfusion via portal vein	Initial 6-10L dH ₂ O + 20L 0.25% SDS (store in 0.25% SDS for 48 hours) + 40L 0.5% SDS over 2-3 h + 20L dH ₂ O + 10% formalin + 40L PBS	80mm Hg	10% formalin	Histology + IHC of ECM – collagen I + IV, laminin, fibronectin Vascular corrosion resin casting + fluoroscopy
K	Ross et al, 2009	Male adult (250-300g) Sprague- Dawley rats Systemic heparinisation	Antegrade perfusion via renal artery	Initially saline + SNP + 3% Triton- X100, DNase + 3% Triton-X100 + 4% SDS (durations not stated)	100 mm Hg	Not stated	Histology + IHC of ECM – collagen IV, laminin SEM of ECM architecture
K	Liu et al, 2009	12 week (180- 220g) Wistar rats	Antegrade perfusion via renal artery	Initial PBS/heparin for 15 min + 1% SDS for 12 hours + 1% Triton-X100 for 30 min	100 cm H ₂ O	PBS + pen- strep for 48 hours	Histology + immunofluorescence of ECM – DAPI SEM of ECM architecture
K	Song et al, 2013	Sprague Dawley rats Systemic heparinisation	Antegrade perfusion via renal artery	Heparinised PBS for 15 min + 1% SDS for 12 h + deionised water/ 15 min + Triton X-100 for 30 min	30 mm Hg	PBS + pen/strep/ amphoteri cin at 1.5 ml/min for 96 h	Histology + IHC of ECM – collagen IV, laminin, elastin TEM of ECM architecture DNA/collagen quantitative assays
K	Orlando et al, 2013	Human (rejected for transplants)	Antegrade perfusion via renal artery + perfusion via ureter	Distilled water at 12 ml/min for 12 h + 0.5% SDS for 48 h then PBS rinse at 6 ml/min for 5 d	12 ml/min	N/A	Histology + immunohistochemistry/ IF of ECM – collagen IV, laminin, agrin, nidogen and antigenic markers SEM of ECM architecture DNA/sGAG quantification assays
K	Bonan- drini et al, 2014	Male adult (250-400g) Sprague Dawley rats	Antegrade perfusion via renal artery	1% SDS in PBS for 17 h + washed in distilled water	0.4 ml/min (62 to 106 mm Hg)	1% pen strep for 2 h	Histology/IF of ECM – collagen IV, laminin, fibronectin SEM of ECM architecture micro-CT of vasculature

Lv K P Sb	Baptista et al, 2009	Ferret liver Pig kidney/pancreas/ small bowel	Not stated	1% Triton X-100/0.1% Ammonium hydroxide for 24 h or until translucent	10-60ml/min	Not stated	Histology Fluoroscopy of vasculature
Sb	Totonelli et al, 2012	Adult (320-350g) Sprague-Dawley rats	Antegrade perfusion via superior mesenteric artery	Detergent-enzymatic treatment (DET): Deionised water for 24 h + 0.4% sodium deoxycholate for 4 hours + 2000kU DNase for 3 h (1-4 cycles)	0.6ml/hour	N/A	Histology + IHC of ECM – MHC-II, SMA, vimentin, MNF116 DNA/collagen/GAG quantitative assays SEM/TEM of ECM architecture Mechanical testing – stress/strain
Sm	Perniconi et al, 2012	Adult BALB/C mice	(Incubation / rotation method)	1% SDS for 24 or 48 h	(N/A)	PBS washes	Histology + immunofluorescence of ECM – actin, laminin, fibronectin

H = heart

L = lungs

Lv = liver

K = kidney

P = pancreas

Sb = small bowel

Sm = skeletal muscle

Table 7

Summary of the recellularisation protocols used in the literature.

Organ	Authors	Bioreactor setup/special conditions (in addition to vascular perfusion)	Cell population/ numbers + reseeding	Culture conditions	Characterisation of organ construct and functionality
Heart	Ott et al, 2008	Cannulation of left atrium and ascending aorta –pulsatile distension of left ventricle + compliance loop attached to ascending aorta to reproduce physiological preload, afterload and intraventricular pressure Coordinated electrical stimulation at 5-20V via electrodes	50-75 million rat neonatal cardiocytes isolated and seeded via intramural injection + 2 million rat aortic endothelial cells via perfusion	Culture medium (CM) perfused at atrial flow rate (FR) 20ml/min, coronary FR 6ml/min 37°C 5% CO ₂ incubator Duration of culture: 4-8 days	Histology (engraftment) + immunofluorescence – TUNEL, a-actin, MHC, vWF, connexin-43 TEM of ultra-architecture Perfusion with CMFDA to demonstrate endothelialisation LV pressure monitoring + live video to demonstrate contractile activity
Heart	Wainwright et al, 2010	(Non-perfusion culture system: lyophilised sheets of cardiac ECM used)	500000 cells/cm ² white leghorn chicken embryonic cardiomyocytes isolated	37°C 5% CO ₂ incubator Duration of culture: 4 days	Immunofluorescence – a-actinin + b-tubulin to identify cardiomyocytes
Lung	Ott et al, 2010	Perfusion system through pulmonary artery, left atrium and trachea; pulmonary vein drained to equilibration chamber Trachea connected to gas ventilation chamber Alternating air and fluid (culture medium) perfusion	91.25+/- 31.72 million carcinomatous human alveolar basal epithelial cells by gravity perfusion through trachea + 66.57+/- 18.22 human umbilical cord venous endothelial cells (HUVECs) by gravity perfusion of pulmonary artery and vein; 308.57+/- 146.9 million rat fetal lung cells isolated + HUVECs seeded via same method	CM perfused at 10-15 mm Hg constant pressure with negative pressure ventilation 37°C 5% CO ₂ incubator Duration of culture: 5-9 days	Histology (engraftment) + immunofluorescence – caveolin-1, T1a, Ttf1, vimentin, pro-SPC TEM of ultra-architecture Immunoblotting – SP-A, SP-C Morphometry + stereology of lung architecture <i>In vitro</i> function testing – blood gas values, dynamic lung function tests Orthotopic transplantation into syngeneic male rats – fluoroscopy and live video, blood gas values
Lung	Petersen et al, 2010	Pulmonary artery cannulation for perfusion loop 'Breathing loop'/negative pressure ventilation via trachea cannulation + syringe pump	100 million rat neonatal lung epithelial cells isolated and injected into airway compartment + 30 million lung vascular endothelial cells via artery	CM perfused at 1-5ml/min with negative pressure ventilation at 1 breath/min 37°C 5% CO ₂ incubator Duration of culture: 4-8 days	Histology (engraftment, TUNEL) + immunofluorescence – CD-31, CCSP, pro-SPC, aquaporin-5, a-actin, Cy-14. TEM of ultra-architecture Stress-strain + compliance testing Orthotopic transplantation into syngeneic male Fischer 344 rats + blood gas values tested to demonstrate gas exchange
Lung	Cortiella et al, 2010	(Non-perfusion bioreactor: rotating	2 million murine embryonic stem	CM circulated by pump action	Immunofluorescence – DAPI, live/dead staining,

		chamber system)	cells (ESCs) injected into trachea (1 million cells into each side/lung scaffold)	at speed of 2rpm 37°C 5% CO ₂ incubator Duration of culture: 14-21 days	TUNEL, cytokeratin 18, CD31, pro-SPC, laminin, collagen IV
Lung	Price et al, 2010	(Non-perfusion bioreactor: scaffolds suspended in CM flasks) Tracheal cannulation for ventilation loop	3 million fetal lung cells isolated from day E17 gestation B6 mice infused into CM	Mechanical ventilation at 180 breaths/min (300ul volume) 37°C 5% CO ₂ incubator Duration of culture: 7 days	Immunofluorescence – DAPI, cytokeratin 18, CD31, pro-SPC, aquaporin-5, CCSP, CD45, CD11b, vimentin
Lung	Song et al, 2011	Perfusion system through pulmonary artery, left atrium and trachea; pulmonary vein drained to equilibration chamber Trachea connected to gas ventilation chamber Alternating air and fluid (culture medium) perfusion	Rat fetal lung cells + HUVECs (numbers not stated)	CM perfused at 1-5ml/min with negative pressure ventilation at 1 breath/min 37°C 5% CO ₂ incubator Duration of culture: 7-10 days	Histology + IHC – TTF-1, pro-SPC, CC10, CD31, vimentin Morphometry + stereology of lung architecture <i>In vitro</i> function testing – blood gas values, dynamic lung function tests Orthotopic transplantation into syngeneic male rats up to 14 days – fluoroscopy and live video, blood gas values
Lung	Wallis et al, 2013	Seeding via intratracheal injection Non-perfusion culture system: slices of lung scaffold used)	2 million adult male C57Bl/6 MSCs <u>or</u> C10 murine lung epithelial cells	N/A	IF – DAPI, Ki67, Casp3
Lung	Lecht et al, 2014	Seeding via left bronchus (cannulated) but no cell culture/perfusion	10 million murine ESCs seeded via left bronchus	N/A	Quantification of cells seeded using fluorescent labelling – microplate reading + microscopy based method
Liver	Uygun et al, 2010	Portal vein cannulation for perfusion loop	50 million rat hepatocytes isolated and seeded via bolus intravascular injection x 4 + 40 million cardiac endothelial cells via perfusion	Perfusion FR not stated 37°C 5% CO ₂ incubator Duration of culture: 1-5 days	Histology (engraftment) + immunofluorescence – TUNEL, albumin, G6pc, Ugt1a LDH release quantification Urea + albumin synthesis quantification RT-PCR of drug metabolism enzymes Heterotopic transplantation into syngeneic rats + fresh blood perfusion model to test viability and hepatocyte function
Liver	Shupe et al, 2010	Inferior vena cava (IVC) cannulation but no cell culture/perfusion	1 million rat liver progenitor cells WB344 via IVC injection	N/A	Histology
Liver	Soto-Gutierrez et al, 2011	Portal vein cannulation for perfusion loop	10-50 million murine adult hepatocytes injected directly into parenchyma vs perfusion in CM at 2ml/min vs perfused in	Perfusion FR 2ml/min 37°C 5% CO ₂ incubator Duration of culture: 7 days	Liver

			multiple boluses		
Liver	Baptista et al, 2011	Portal vein cannulation for perfusion loop	70 million human fetal liver cells + 30 million HUVECs co-infusion via portal vein	Perfusion FR 3ml/min for seeding x 16 hours; Perfusion FR 0.5ml/min 37°C 5% CO ₂ incubator Duration of culture: 7 days	Histology + immunofluorescence – TUNEL, Ki-67 Urea/albumin/prostacyclin synthesis quantification Platelet deposition studies
Liver	Barakat et al, 2011	Posterior segments only (x3) Portal vein cannulation for perfusion loop	350 million human fetal stellate cells + 1 billion human fetal hepatocytes via portal vein	Perfusion FR 90ml/min 37°C 5% CO ₂ incubator Duration of culture: 3-13 days	Histology + IHC – CK-18, CK-19, AFP, CYP3A4, TUNEL, Ki-67 Urea/albumin/lactate synthesis quantification Orthotopic transplantation - histology
Kidney	Ross et al, 2009	Renal artery cannulation for perfusion loop, also cannulation of ureter	2 million murine pluripotent ES cells injected via artery or ureter	Sections of seeded graft cultured for 3-10 days 37°C 5% CO ₂ incubator Perfusion of whole organ at 120/80 mm Hg for 6-10 days	Histology (engraftment + morphology) + IHC – pancytokeratin, Pax-2, Ksp-cadherin, Ki-67 RT-PCR of Pax-2 + Ksp-cadherin expression
Kidney	Song et al, 2013	Renal artery cannulation for perfusion loop, ureter cannulated for cell seeding	60.71 +/- 11.67 million rat neonatal primary renal cells via ureter at -40 cm H ₂ O pressure + 50.67 +/- 12.84 million HUVECs through arterial cannula	Perfusion FR 1 ml/min for HUVEC seeding; Perfusion FR 1.5 ml/min 37°C 5% CO ₂ incubator; silicone tube oxygenator Duration of culture: 7 days	Histology/IHC + immunofluorescence – CD31, DAPI, podocin, Na/K-ATPase, E-cadherin, nephrin, aquaporin 1, β 1-integrin TEM of ultra-architecture urine flow/creatinine clearance/vascular resistance quantification Orthotopic transplantation into NIHHRNU-M rats for 'test period'
Kidney	Bonandrini et al, 2014	Renal artery cannulation for perfusion loop	12 million murine ESCs infused over 30 mins via renal artery	Perfusion FR not stated 37°C 5% CO ₂ incubator Duration of culture: 24 or 72 h	Histology + IHC – DAPI, WGA agglutinin, Oct 4, NCAM, Tie-2, CD31
Liver, Kidney, Pancreas, Small bowel	Baptista et al, 2009	Recellularisation performed on ferret liver via portal vein cannulation and perfusion	30 million human HepG2 cells + 30 million mouse endothelial cells injected intravascularly	Perfusion FR 6ml/min 37°C 5% CO ₂ incubator Duration of culture: 7 days	Histology IHC – albumin, vWF, Ki-67

Chapter 2

Optimisation of two key parameters in perfusion decellularisation of whole kidney bioscaffolds

2.1 Introduction

A relatively new approach to ‘whole organ’ engineering based on the technique of whole organ decellularisation allows the preservation of the native vasculature and the complete 3-dimensional (3D) macro- and micro-architecture of the organ extracellular matrix (ECM). This technique utilises the intrinsic vasculature to decellularise the entire organ by vascular perfusion of the decellularising agent to efficiently penetrate the tissue and remove cellular material. The decellularised bioscaffold thus created preserves the intrinsic structure and properties of the ECM [5] with its natural organisational complexity and 3D architecture in order to promote recellularisation and direct cellular differentiation on a whole organ level. The inherent vascular network is critical in allowing perfusion (i.e. delivery of nutrients and oxygen) to sustain cell culture in complex 3-D constructs on a level which was previously not feasible. This approach has been successfully employed in the major solid organs to create viable and partially functional organ constructs, some of which have been implanted in vivo [9-14]. In particular recent work on the regenerated kidney has demonstrated up to 10% functionality in terms of urine production and creatinine clearance [14].

Although the potential clinical implications of this are extremely exciting, there are a number of major technical factors and challenges that exist before clinical application can be considered. One critical group of pre-optimisation parameters lies with the production of the ECM bioscaffold and the decellularisation process. While the tissue- and organ-specific functional and bio-inductive molecules of the ECM are intrinsic to the success of the recellularisation and regenerative process, the quality of the ECM not only varies with each donor but also with each protocol applied (and the different steps or agents therein) [129].

Both donor factors and the decellularisation parameters must be standardised and rationalised to yield consistent and optimised bioscaffolds. Pre-optimisation and standard operating procedures are even more critical when it is likely that post-processing testing of the ECM bioscaffold (and the recellularised construct) is likely to be limited in scope in those cases destined for implantation. Minimum standardized biological requirements within whole-organ ECM scaffolds (e.g. threshold DNA levels and structural integrity, growth factor levels/activity) should also be established to maintain biological quality and clinical safety. A few studies have started to assess or compare effects of perfusion decellularisation protocols in a more systematic manner e.g. different decellularising agents [114], duration/exposure to the decellularising agent [127]; however, it has been shown in a recent systematic review [129] that currently there is extreme disparity between different decellularisation protocols in the literature even when the tissues (organs) under investigation are the same type and from the same species (e.g. rat liver). Hence both potential clinical safety and application and the current status quo in this field strongly suggest the need for a more evidence-based approach.

On this basis, the first aim of this thesis was to optimise the decellularisation process for whole kidney ECM bioscaffolds with regards to two fundamental parameters: concentration of decellularising agent and duration of perfusion. This was done using the most commonly used single decellularising agent drawn from the literature i.e. sodium dodecyl sulphate [129], which also has an extremely wide application as a detergent/decellularising agent in the background context of naturally derived tissue decellularisation and ECM bioscaffolds. Optimisation was determined with regards to structural and functional characteristics of the ECM bio-scaffold as assessed by histology, immunohistochemistry (IHC), quantitative assays of DNA and sulfated glycosaminoglycan (sGAG) content, and growth factor quantification – and optimisation was continued to the point where a plateau effect was observed in one or more of these modalities.

2.2 Materials and materials

2.2.1 Tissue harvesting

Male Wistar rats (350-375 g, Charles River, UK) were used for kidney harvest for whole organ decellularisation immediately after euthanasia of the animal by cervical dislocation. All animal work was performed in accordance with the Animals (Scientific Procedures) Act 1986 and approved by the institutional Animal Welfare Ethical Review Body (AWERB) at Imperial College London.

A median laparotomy was performed and both kidneys were removed *en bloc* with abdominal aorta and inferior vena cava. The renal arteries were accessed and cannulated via the aorta with a 25G vascular cannula (BE Neoflon, Sweden) and perfused with phosphate buffered saline (PBS) containing a vasodilator (sodium nitroprusside, SNP, Sigma, UK) by manual injection until a uniform blanching indicative of even distribution of perfusate was observed, after which each kidney was perfused with PBS without vasodilator. The perfusion/irrigation sequence was: 10 ml of 10 µg/ml SNP in PBS; 20 ml of 1 µg/ml SNP in PBS; and then 30 ml PBS alone.

Once irrigation of the kidney to remove any traces of blood within the vasculature was completed as above, the left and right kidneys were separated by individual dissection and ligation of the renal arteries, preserving an aortic cuff in order to secure the vascular cannula. The renal veins were similarly ligated from the inferior vena cava, but without the caval cuff (and un-cannulated) to act as the outflow.

2.2.2 Whole kidney decellularisation and setup

Rat kidneys were suspended within a reservoir (glass beaker) and perfused continuously through the arterial cannula ([Figure 4](#)) at a flow rate of 10 ml/min via a peristaltic pump for different time periods (initially 24 hours/8 hours/4 hours, plus a further optimisation series – see Tables 8a + 8b). The direction of fluid flow was: **inflow** through free end of tubing in

reservoir → cannulated renal artery (via peristaltic pump) → **outflow** into reservoir through free end of renal vein. The effluent fluid was then recirculated from the reservoir.

The perfusate (decellularising agent) was sodium dodecyl sulfate (SDS) (Sigma, UK) at differing concentrations (0.125% - 1.0% w/v in distilled water). Decellularised kidneys were washed with PBS for 1 hour before either sterilisation or freezing for storage at -80°C (for characterisation studies).

Decellularised whole kidney bioscaffolds for subsequent recellularisation were sterilised by perfusion with 1% w/v antibiotic-antimycotic (Gibco®, Invitrogen, UK) in sterile PBS for 18 hours at 2 ml/min inside a cell culture hood.

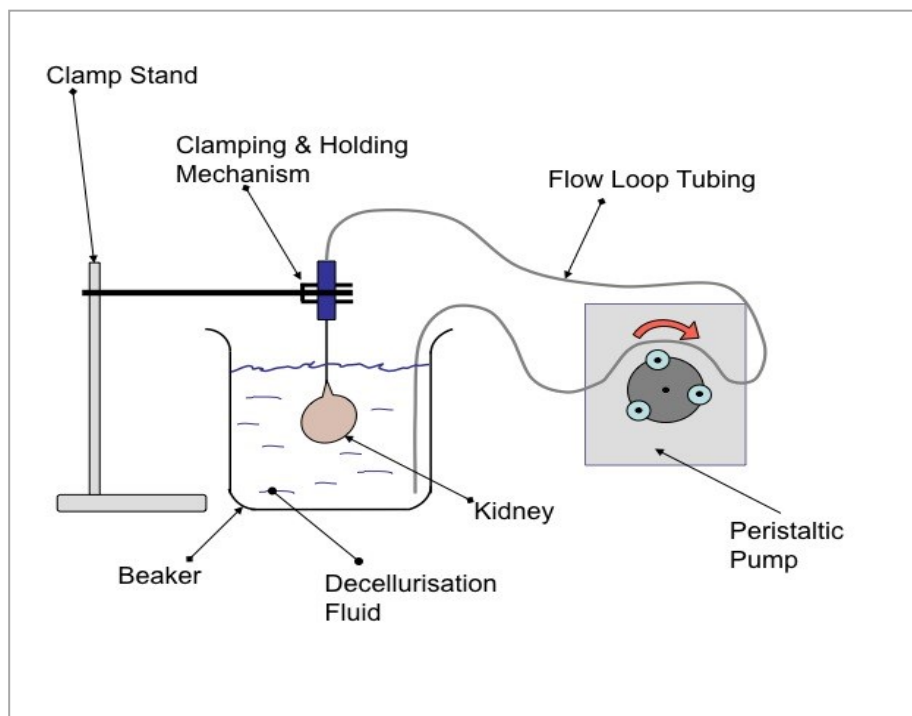


Figure 4 Perfusion decellularisation of whole kidneys – schematic of pump setup.

2.2.3 Histology and immunohistochemistry (IHC)

All samples were fixed in 10% v/v buffered neutral formalin by immersion for at least 24 hours and samples were stained with haematoxylin and eosin (H+E) for light microscopy to assess the degree of cell removal and preservation of the ECM architecture within the whole kidney bioscaffold.

IHC was performed to assess retention of the key ECM and basement membrane proteins collagens I and IV, laminin and fibronectin (using antibodies ab34710, ab6586, ab11575, ab23751 respectively – all Abcam, UK). All samples were treated identically with regards to fixation, antigen retrieval, antibody staining, blocking and development.

Paraffin-embedded sections were dewaxed and submerged in methanol and 1% v/v hydrogen peroxide for 10 minutes to block endogenous peroxidases. Heat-mediated antigen retrieval was performed in a 0.01M sodium citrate buffer (Sigma, UK), pH 6.0 using a microwave at high power for 10 minutes. Avidin/biotin (blocking kit, Dako, UK) blocking was performed according to manufacturer's protocol. Primary antibodies were incubated with the sections overnight at 4°C. Primary antibody dilutions were made in blocking solution (5% v/v goat serum in PBS) and were as follows: collagen I 1:250, collagen IV 1:500, laminin 1:50, and fibronectin 1:500. After primary antibody attachment, sections were washed twice in 0.1% v/v TWEEN (Sigma, UK) in PBS, pH 7.4. Hydrogen peroxidase conjugated secondary antibody (goat anti-rabbit, Vector Labs, UK) was applied at 1:100 dilution for 1 hour at room temperature, after which the sections were washed twice with 0.01% v/v TWEEN. Slides were developed using 3,3'-diaminobenzidine (DAB staining kit, Dako, UK) until good staining intensity was observed (between 2 to 10 minutes), then the staining process was terminated. Species (rabbit) immunoglobulin and PBS served as negative controls. Haematoxylin was used for nuclear counterstaining to finish.

2.2.4 DNA and sGAG quantification

Approximately half of a whole rat kidney bioscaffold was suspended within 5 ml of papain solution and digested for 24 h at 60°C until no visible solid material remained. The papain solution consisted of 55 mM L-cysteine hydrochloride, 80 mM EDTA, and 2.5 units/ml papain (all Sigma, UK) at pH 6.0. After digestion, the ECM solution was then freeze-dried for 24h using the Heto PowerDry LL1500 freeze dryer (Thermo Scientific, UK) to ascertain the dry weight of digested solution. The freeze-dried kidney ECM was then re-suspended in either deionised water or assay buffer for DNA and sGAG quantification assays.

The total amount of DNA was quantified using the PicoGreen® dsDNA assay (Invitrogen, UK) according to the manufacturer's instructions. Briefly: a standard curve of DNA solution (0 – 1000 ng/ml) was prepared in conjunction with a 1:10 dilution of all sample solutions with assay buffer (Tris-EDTA, as supplied). All samples were measured in duplicate after adding an equal volume of PicoGreen substrate (as supplied) within a black 96 micro well plate, using a microplate reader (Spectramax M5, Molecular Devices, LLC, USA) at 485/535 nm (excitation/emission wavelengths).

The total amount of sGAG was quantified using the Blyscan™ sGAG assay (Biocolor, UK) according to the manufacturer's instructions. Briefly: a standard curve of sGAG solution (0 – 5 mg/ml) was prepared with a 1:10 dilution of all sample solutions in deionised water. Blyscan dye reagent (as supplied) was added to all samples and agitated for 30 minutes, then these were centrifuged at 12,000 rotations per minute for 10 minutes. Sample tubes were drained while taking care to preserve any precipitate/pellet formed at the bottom. Dissociation reagent (as supplied) was added and tubes were vortexed to release the dye, then centrifuged at 12,000 rpm for 5 minutes. Samples were measured in duplicate within a clear 96 micro well plate, using a microplate reader (Spectramax M5, Molecular Devices, LLC, USA) at 656 nm.

Each assay was performed in duplicate three times.

2.2.5 Growth factor extraction and quantification assays

Approximately half of a whole rat kidney or whole rat kidney bioscaffold was minced then suspended in ~3.75 ml of urea–heparin extraction buffer.

The extraction buffer consisted of 2 M urea and 5 mg/ml heparin in 50 mM Tris at pH 7.4 with protease inhibitors (protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 10 mM N-ethylmaleimide) (all Sigma, UK).

The extraction mixture was agitated at 4°C for 20 to 24 hours and then centrifuged at 5,000 x g for 30 minutes. Supernatants were collected, and 6 ml of urea–heparin extraction buffer was added to each pellet. Pellets with extraction buffer were again agitated at 4°C for 20 to 24 h, centrifuged at 5,000 x g for 30 min, and supernatants were collected. Supernatants from first and second extractions were combined then dialysed against Barnstead filtered water (total of three changes of dialysis water, 80 to 100 volumes per change) in Spectra/Por 3500 MWCO dialysis membrane (Spectrum Laboratories, USA). The final dialysed extracts were frozen in aliquots at -80°C until time of assay.

The concentration of total protein in each dialyzed extract was determined by the BCA protein assay (Pierce, Thermo Scientific, UK) following the manufacturer's protocol. Concentrations of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in urea–heparin extracts of kidney samples were determined with the Quantikine Human FGF basic Immunoassay and the Quantikine Rat VEGF Immunoassay (both R&D Systems, UK) respectively. Manufacturer's instructions were followed for both growth factor assays.

Briefly: all reagents, standard, sample, and control solutions were prepared as directed. Assay Diluent (as supplied) was added to each well of the micro plate strips (as supplied) to which the standard, control and sample solutions were then added in turn. The wells used were covered with an adhesive strip and incubated on a horizontal orbital shaker at room temperature for 2 hours. Washing using Wash Buffer (as supplied) was then performed for

each well, with complete removal of liquid with each wash step, and repeated 4 or 5 times (depending on the assay required). Growth factor Conjugate (as supplied) was added to each well and incubated for 1 or 2 hours on a shaker at room temperature (duration depending on the assay required). Washing of each well 4 or 5 times as above was repeated. Finally, Substrate Solution (as supplied) was added to each well and incubated for 30 minutes at room temperature without agitation but protected from light. Stop Solution (as supplied) was added to each well before reading the absorbance using a micro plate reader (Spectramax M5, Molecular Devices, LLC, USA) at 450 nm with wavelength correction at 540 nm.

Each growth factor assay was performed in duplicate three times.

It should be noted that growth factor assays measured the concentration of each growth factor protein and did not measure growth factor activity.

2.2.6 Vascular resin corrosion casting

Cannulation of the renal artery was performed followed by injection of 0.5-1 ml polymer mixture depending on the kidney size using Batson's no. 17 anatomic corrosion casting kit (Polysciences Inc., USA) as recommended by the manufacturer.

Briefly: the total required volume of Base Solution A (as supplied, with 2% w/v red pigment added prior to use) was divided into two equal quantities and the Catalyst solution (as supplied) or Promoter C solution (as supplied) was added to each quantity i.e. separately. The Catalyst and Promoter C solutions may be adjusted in concentration to produce a faster or slower polymerisation during the curing process. Typically 10 ml (5 ml + 5 ml) of Base Solution A was used with 3 ml of Catalyst and 3 drops of Promoter C.

Polymerisation took 20-30 min at 4°C and photographic images were taken during the injection process over 10-30 seconds to highlight the preservation of the intrinsic vascular network.

2.2.7 3-D Histology (Histocutter) and reconstruction of images

Whole decellularised rat kidney bioscaffolds were fixed in 4% w/v paraformaldehyde then samples were processed and embedded using a modified tissue processing technique in order to utilise native tissue auto-fluorescence for imaging purposes. The procedure consisted of the following stages (outlined below): dehydration, clearing, staining, and embedding of tissue, then cutting/imaging of sample blocks using a fully automated system which includes a rotary microtome, microscope, and camera (with computer/operating software). The steps within each stage are outlined as follows:

Dehydration

Tissue was immersed in a graded ethanol series: 50% for 1 hour, 70% for 1 hour, 90% for 1 hour, 100% for 1.5 hours, and 100% for 1.5 hours.

Clearing

Subsequent to dehydration, tissue was placed in sealed glass vials (one sample per vial) with xylene for 1 hour (10x sample volume) after which vial contents were replaced with fresh xylene, then sealed and left at 4°C overnight.

Staining

Within a hood, xylene was removed and replaced with the same volume of 0.5% w/v Sudan IV (Red) in xylene. Tissue was immersed in this solution for 4 hours at room temperature within sealed vials. Then the Sudan IV solution was removed and replaced with same volume of 2% w/w Sudan II (Blue) in stearic acid, which has been pre-prepared as a solution at 75°C. Tissue was immersed in this solution (within open vials) for 4 hours in a vacuum oven at 600 mBar and 75°C.

Embedding

Sudan Blue/stearic acid solution was removed and replaced with the same volume of 2% w/w Sudan II (Blue) in 10% w/w stearic acid/72% w/w paraffin/15% w/w vybar wax which has been pre-prepared as a solution at 75°C. Tissue was immersed in this solution (within open vials) overnight in a vacuum oven at 600 mBar and 75°C.

Using pre-heated steel moulds and the same Sudan Blue/stearic/paraffin/vybar solution, the tissue sample was manually embedded within the mould and allowed to set at room temperature for at least 1 day before cutting.

Cutting of samples/Histocutter

Embedded sample block(s) were mounted individually onto the Histocutter apparatus which consisted of: fully automated rotary microtome, stage, microscope and fluorescent lamp, camera and software (Cutter Master CW (Java)). Details of all components are listed in the Appendix (Section 7.1).

Automated continuous cutting of samples was set and controlled via the Cutter Master CW software and a tissue sample may be fully processed in its entirety as required (i.e. up to the total block thickness). As each slice was cut (at 1–5 μm thickness as required) the block face was imaged automatically via the camera and the individual image was stored within the data set. For each image/slice, different filters may be applied to obtain multiple wavelength data e.g. UV (UV/purple excitation, blue emission), Texas Red HYQ (green excitation, red emission). Details of filters used/available are listed in the Appendix (Section 7.1).

For the protocol utilised in this project, embedded whole rat kidney bioscaffolds were mounted on the microtome and processed in individual blocks with continuous cutting at 5 μm thickness and a total of 800-1200 slices, depending on sample thickness. For native tissue imaging of decellularised scaffolds, the Texas Red HYQ filter was applied to obtain auto-fluorescent images of the whole kidney ECM.

Projection images were then reconstructed using Amira software (FEI, USA) with image resolution of 7.804 $\mu\text{m}/\text{voxel}$.

2.2.8 Statistical analysis

Results are reported as mean $\pm$ standard error. Statistical analysis was performed with analysis of variance followed by Mann Whitney two-sample test using the Statistical Package for Social Sciences (SPSS) for Windows Version 20 software. A p -value of < 0.05 was considered significant.

2.3 Results: Optimisation of perfusion decellularisation in whole rat kidneys

2.3.1 Initial decellularisation studies using 1% SDS

Cadaveric whole rat kidneys were successfully decellularised using the perfusion method via the renal artery with SDS initially at 1% w/v concentration; SDS was chosen for its empirical efficacy of decellularisation as shown in the literature – it was the most common single decellularising agent utilised in the field of whole organ decellularisation[129]. While some studies in the kidney incorporate the use of Triton-X100 as a subsidiary decellularising agent[14, 121], SDS alone was found to be effective in providing adequate decellularisation as evidenced by histology and DNA quantification studies, as consistent with a number of other studies which used SDS alone, whether in the kidney[125] or in other organs[129] within whole organ decellularisation. Successfully decellularised whole kidney bioscaffolds have a white translucent appearance, while preserving the outline and architecture of the whole kidney ([Figure 5](#)) and were at least robust enough for manual handling.

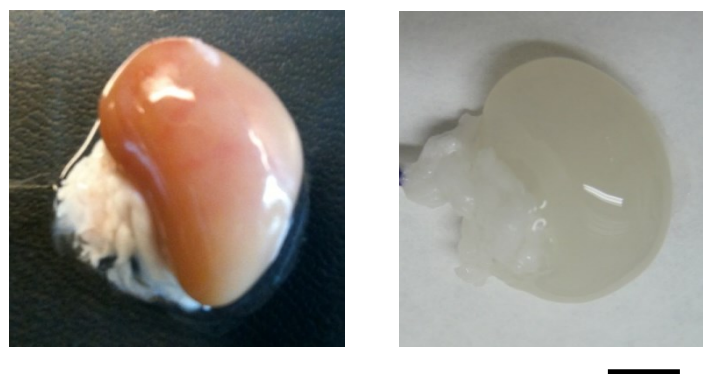


Figure 5 Whole rat kidney (**left**) and completely decellularised whole rat kidney bioscaffold (**right**).

Scale bar = 5 mm.

Initial investigations were performed for different durations of continuous perfusion at 10 ml/min with 1% w/v SDS i.e. for 24 hours, 8 hours and 4 hours ([Table 8a](#)), which all yielded well decellularised bioscaffolds on histological analysis ([Figure 6](#)). Successful decellularisation was characterised by a combination of histology and IHC, DNA and sGAG quantification, and growth factor (vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)) quantification.

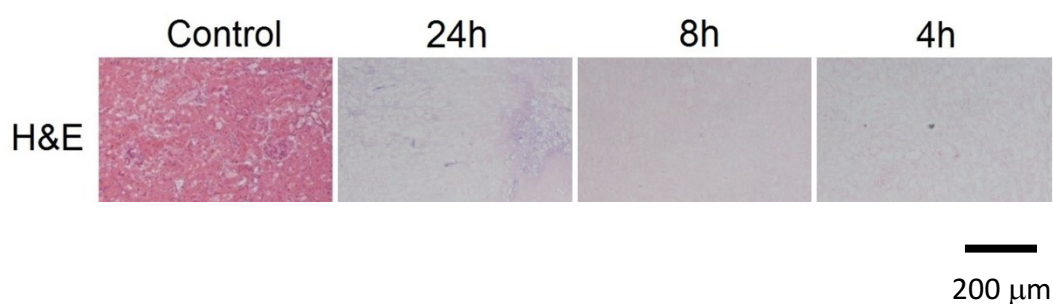


Figure 6 Histology (H+E staining) of whole kidney bioscaffolds decellularised with 1% w/v SDS and continuous perfusion (at 10ml/min) for different time periods – 24 hours, 8 hours and 4 hours. No obvious difference in decellularisation was observed. Control = normal kidney. Representative images from $n \geq 10$ kidneys.

The 1% w/v SDS x 4 hours of constant perfusion (followed by washing with PBS) was denoted the baseline protocol for further comparisons ([Table 8a](#)). Based on both the common micelle concentration of SDS being approximately 0.23% (w/v) and the need to minimise the disruptive effects of any decellularising agent and SDS in particular on ECM ultrastructure and growth factor content[20], it was postulated that much lower concentrations of SDS would be equally effective in terms of decellularisation and may well yield significant benefits in terms of preservation of ECM components and in particular growth factors.

Hence two further series of protocols were carried out in decreasing concentrations of SDS (0.5%, 0.25%, and 0.125% w/v) and for two durations of perfusion (i.e. 4 hours and 8 hours, [Table 8b](#)). A longer duration of perfusion, i.e. 8 hours, was additionally trialled in order to test whether a lower concentration of SDS might therefore require a longer exposure. All further decellularisation protocols were characterised similarly to the baseline protocol using histology and IHC, DNA/sGAG/VEGF/bFGF quantification assays. These results are described in Sections 2.3.2 – 2.3.3.

Table 8a

Initial decellularisation protocols using SDS at 1% w/v and decreasing durations of continuous perfusion from 24 hours to 4 hours (at 10ml/min).

SDS concentration (% w/v)	Duration (hours)	Duration (hours)	Duration (hours)
1.0	24	8	4

Table 8b

Further optimisation protocols using SDS at decreasing concentrations (1% to 0.125% w/v) and different durations, i.e. 4 hours and 8 hours, of continuous perfusion (at 10ml/min).

SDS concentration (% w/v)	Duration (hours)	Duration (hours)
0.125	4	8
0.25	4	8
0.5	4	8
1.0 (index group)	4	-

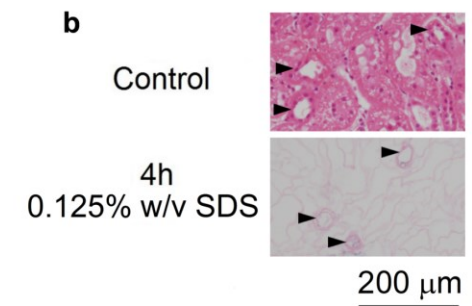
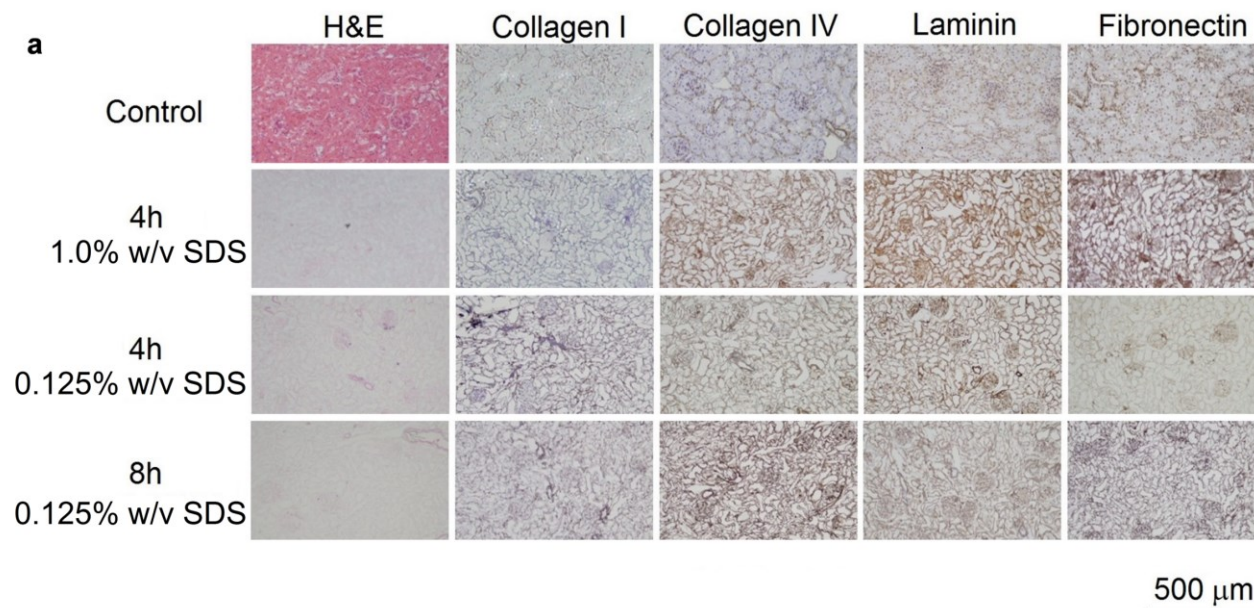
2.3.2 Optimisation of decellularisation: concentration of single decellularising agent

2.3.2.1 Histology and IHC

Histological analysis of perfusion decellularised kidney bioscaffolds shows preservation of the structure and architecture of the renal ECM and the arterial network within the acellular cortical and medullary parenchyma ([Figure 7a](#)), as has been previously shown[14, 121, 124, 125]. The presence of micro-vasculature which is also well preserved after decellularisation can be seen on histology analysis ([Figure 7b](#)). Immunohistochemical staining for key ECM components (collagen I, collagen IV, laminin and fibronectin) shows a physiological distribution as compared to normal kidney tissue ([Figure 7a](#)). There was no significant difference observed between all the protocols (variation in % w/v SDS or duration of perfusion), nor in comparison with the baseline protocol, in terms of the histological appearance and IHC staining.

Figure 7 (a) Histology (H+E staining) and IHC for ECM components (collagen I, collagen IV, laminin, and fibronectin) in decellularised whole rat kidney bioscaffolds.

Selected representative SDS decellularisation protocols (0.125% x 4 hours, 0.125% x 8 hours) are shown compared with baseline (1% SDS x 4 hours) and control (i.e. normal kidney). No gross differences between these protocols were observed, nor between any of the other protocols compared. **(b)** Histology (H+E staining) of normal kidney and decellularised whole rat kidney bioscaffold (0.125% x 4 hours) shows preservation of micro-vasculature within the tissue (black arrows) similar in calibre and structure after decellularisation. For all samples shown, $n \geq 6$.



2.3.2.2 Quantification Assays

For all protocols, DNA levels in the decellularised kidney bioscaffolds ([Figure 8c](#)) were no more than 0.02% of normal kidney DNA content and are comparable to the less than 50 ng dsDNA per mg ECM dry weight which is the recommended level based on studies that show a constructive remodelling response in vivo in conjunction with the avoidance of adverse cell and host responses[20].

There was no significant difference across groups for different concentrations of SDS and preservation of sGAG for either the 4 hour or 8 hour series, but a significant difference was seen between the groups with an at least two-fold increase in levels of sGAG with the shorter exposure to SDS ($p < 0.005$) ([Figure 8a, b](#)).

Levels of VEGF and bFGF ([Figure 8d-f, g-i](#)) preserved within the kidney ECM showed no difference across groups for concentrations for the 8 hour levels for both growth factors, which were in fact almost negligible for both. However, the levels of both growth factors were well preserved compared to normal kidney in the 4 hour series ([8d-e, g-h](#)), with the highest levels preserved at the lowest concentration of SDS exposure i.e. 0.125% w/v for both VEGF and bFGF ($p < 0.05$). A clear trend of increasing levels of bFGF preserved as the concentration of SDS decreases can also be seen; however a plateau effect can be observed with both VEGF and bFGF as the difference seen between levels with 0.25% w/v SDS and those with 0.125% w/v was not of significance ($p = 0.256$ for both). Group variations were noted in the levels obtained for all the quantification assays, which are likely to be due to batch variation.

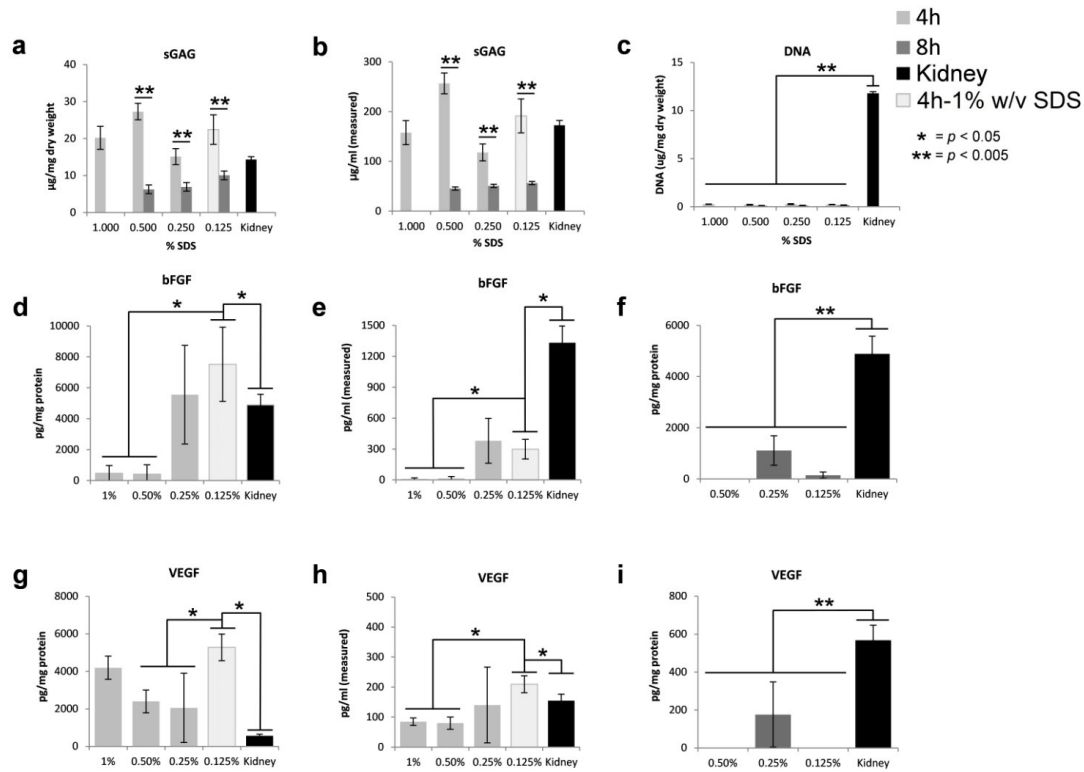


Figure 8 Characterisation of whole rat kidney ECM bioscaffolds for retention of sGAG, removal of DNA and growth factors (VEGF, bFGF) with quantification assays. **(a, b)** Quantification of sGAG retention within whole kidney ECM shows an at least 2-fold increase in the level of sGAG with the 4 hour protocols, as compared to the 8 hour protocols ($p < 0.005$). **(c)** DNA quantification shows no more than 0.02% of normal kidney DNA levels ($p < 0.005$) remaining on the decellularised kidney bioscaffolds and no significant difference seen between the 4 hour and 8 hour protocols. **(d-i)** Quantification of bFGF and VEGF levels shows virtually negligible levels preserved in the 8 hour protocols for both **(f, i)** but significant increasing levels preserved in the 4 hour protocols **(d, e, g, h)** with the decreasing concentration of SDS, and in particular with 0.125% x 4 hours **(d, g)** ($p < 0.05$). A plateauing off in the absolute measured levels of both bFGF and VEGF with decreasing concentrations of SDS **(e, h)** suggests that further decreases in the SDS concentration may not yield significantly increased preservation of growth factors.

For all samples shown, $n \geq 6$.

2.3.3 Optimisation of decellularisation: duration of perfusion decellularisation

2.3.3.1 Histology and IHC

As stated previously (Section 2.3.2), there was no significant difference observed between all the protocols (variation in % w/v SDS or duration of perfusion), nor in comparison with the baseline protocol, in terms of the histological appearance and IHC staining.

2.3.3.2 Quantification Assays

For all protocols, DNA levels in the decellularised kidney bioscaffolds ([Figure 8c](#)) were no more than 0.02% of normal kidney DNA content as reported above – hence there was no significant difference in the removal of DNA between the shorter (4 hour) and longer (8 hour) exposures.

However, quantification of sGAG levels ([Figure 8a, b](#)) show an at least two-fold increase in the average levels of sGAG preserved between the 4 hour and 8 hour protocols ($p < 0.005$), suggesting a distinct correlation between the loss of sGAG within the ECM bioscaffolds and the increased time exposure to SDS. It was not possible to assess any further possible benefit in the preservation of sGAG levels with a lower exposure (i.e. <4 hours) due to the inconsistency of decellularisation achieved with shorter durations.

Levels of VEGF and bFGF ([Figure 8d-f, g-i](#)) preserved within the kidney ECM showed a similar result in that the 8 hour levels for both growth factors were again significantly decreased compared to both normal kidney and the 4 hour protocols, and were in fact almost negligible for both growth factors. The trend of increasing levels of growth factors preserved as the concentration of SDS decreases with the 4 hour group has already discussed.

2.3.4 Assessment of 3-D vasculature and architecture of whole kidney bioscaffolds

Preservation of the vasculature within decellularised whole kidney scaffolds may be assessed both microscopically on histology and on a 3-D/whole organ basis:

Routine histological analysis with H+E staining of decellularised scaffold sections show the distinct appearance of micro-vasculature within the ECM which is well-preserved down to the capillary level ([Figure 7b](#)), in direct comparison to normal kidney tissue. The preservation of the arterial network within the whole kidney bioscaffolds was also qualitatively demonstrated with injection of a resin polymer for vascular casting ([Figure 9](#)). However the actual resin cast produced with this technique proved to be too brittle for further imaging or technical manipulation.

In addition, a new histological technique known as the Histocutter has been investigated within this thesis. This was originally built at the Devers Eye Institute in Portland (USA) and further developed here at Imperial College London (UK), and is a technique whereby the entire tissue sample/block may be continuously imaged by the automated coordination of microtome cutting and block face imaging (i.e. after each slice), thus building up a differential data set of images that may be further reconstructed using the appropriate software e.g. Amira (FEI), analogous to other 3-D imaging techniques such as micro-computed tomography (micro-CT). Internal architecture and structures within the whole decellularised kidney scaffold may be clearly seen on a whole organ level from the gross/anatomical level down to the finer microscopic details ([Figure 10](#)). This includes the native vasculature and the pelvi-calyceal system of the kidney, and is not limited to the vasculature only, as has been previously demonstrated by using the micro-CT technique in the kidney scaffold[125].

This simultaneous imaging of the internal 3-D structure and architecture of the whole kidney ECM bioscaffold in its entirety has not been demonstrated previously. Radiological imaging is more limited by the necessity for contrast material and a route or 'system' (e.g. vascular,

airway) for this to be introduced and to permeate into the tissue sample, whereas one major advantage of the Histocutter is being able to utilise the auto-fluorescent properties of native tissue and hence all the structural features of the ECM may be imaged without recourse to any special additional measures. However, one disadvantage of this technique is that the entire sample block is 'consumed' during the imaging process due to the continuous cutting (slices are discarded as the block face is imaged, not individual slices) and hence cannot be preserved for further testing.

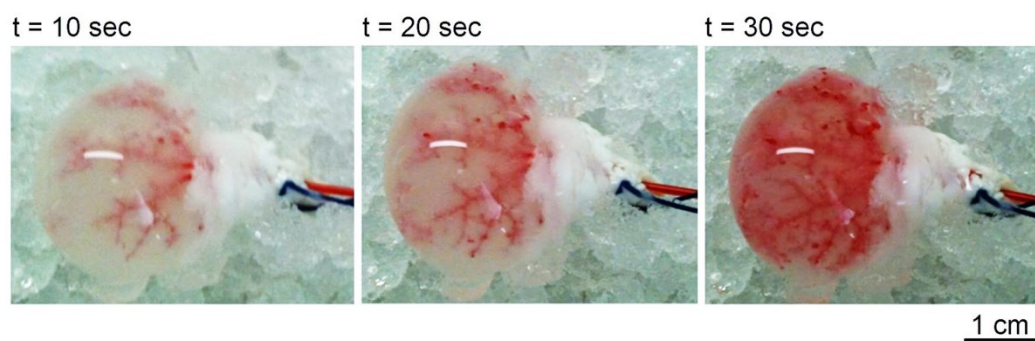


Figure 9

Vascular casting of SDS decellularised whole kidney bioscaffolds (using 0.125% SDS x 4 hours perfusion) shows preservation of intrinsic vasculature on injection of resin into cannulated renal artery (over 10-30 seconds). For all samples shown, $n \geq 6$.

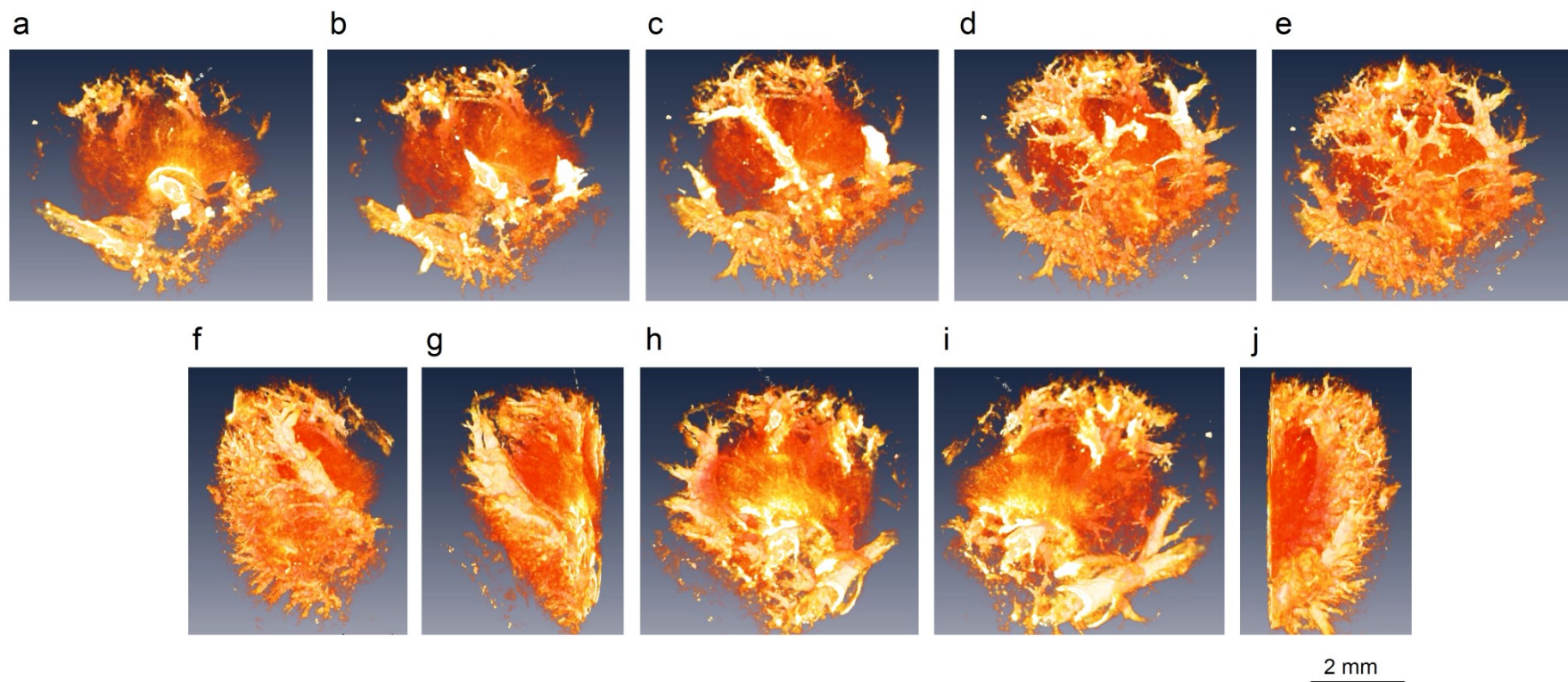


Figure 10 Reconstruction of 3-D histology (Histocutter) images of whole decellularised kidney bioscaffolds (using 0.125% SDS x 4 hours perfusion).

(a-e) The 3-D kidney scaffold seen at different depths/thicknesses (seen at 0.5 mm intervals) within the tissue to demonstrate the internal architecture of the whole kidney ECM. (f-j) Rotation of the whole scaffold structure. For all samples, $n \geq 3$.

2.4 Discussion

Various groups have demonstrated the feasibility of using the whole organ decellularisation approach to derive bioengineered (partially) functional organ constructs for surgical implantation[10-14]. While there is great interest in the recellularisation potential of these bioscaffolds, this potential should be maximised by optimisation of the quality of the ECM preserved after perfusion decellularisation – and there is a need for systematic and rationalised protocols that are comparable across the field, in addition to standardisation for the purposes of quality control and regulation[129]. Currently most groups are utilising disparate decellularisation protocols with marked variations even in work on the same organ and species[13, 116-119], although there are a few common trends observed, and a few studies have started to take a more rationalised approach[114, 116, 127].

Hence the work above represents an evidence-based approach to determining a more optimised protocol using a single agent (SDS) with continuous perfusion as defined by characterisation of ECM components and their preservation within the kidney bioscaffolds. This yielded an improved protocol (0.125% w/v SDS perfused for 4 hours) which uses a far lower concentration of SDS than other work on whole kidney decellularisation[14, 121, 124, 125] while being equally effective in achieving removal of cellular components and DNA, and which is consistent with the general underlying principle of minimising exposure to the decellularising agent that is well established within the ECM literature. Other studies in the rat model have utilised mostly 1% w/v SDS for at least 12 hours[14, 122, 125] except in one case, which utilised 3-4% w/v SDS for durations unspecified[121]. The one study on human kidneys used 0.5% w/v SDS for 48 hours[124], but the species difference makes it less directly comparable, and a specific rationale for the choice of concentration was not stated. While two studies have performed quantification assays of ECM components (e.g. collagen) to assess preservation levels[14, 124], nonetheless these have not been correlated with varying the parameters or conditions for decellularisation.

In contrast, this work has been able to demonstrate a clear relationship between decreasing exposure to SDS (i.e. concentration) and preservation of *in situ* growth factors, down to the level where further decreases in the SDS concentration may not yield significantly increased preservation, which has not been previously shown. It is likely that these findings should also be applicable in general for any whole organ bioscaffolds and for any species, rather than being specific to the rat kidney as such, which also leads the way to further optimisation possibilities.

In addition to the optimisation aspects of the decellularisation process, new and existing imaging techniques for assessing the 3-D vasculature and architecture of the decellularised whole kidney bioscaffold have also been investigated. Vascular corrosion resin casting is a well-established anatomical technique which was initially employed in whole kidney bioscaffolds to give a 'global' qualitative indication of the preservation of the vascular network ([Figure 9](#)); however, reliably reproducing the finer structural details and quantitative assessment was not readily possible, due to a combination of the small calibre of the anatomy involved and the resulting resin cast being extremely brittle when the surrounding tissue was removed by the corrosion process. The qualitative images obtained with this technique were similar to simple injection of dye or contrast material throughout the whole kidney bioscaffold, which has been demonstrated previously[122, 123]. Two other studies have successfully imaged the 3-D whole organ vasculature with a 3-D radiological method (i.e. micro-CT) which can produce reconstructed images of both the complete vascular network within the kidney [125] and the bronchial network in the lung after decellularisation[11], which might overcome these issues of corrosion casting.

However, it has also been possible to investigate here a new histological technique called the Histocutter whereby the entire tissue sample/block (rather than selective slices as with traditional histology) may be completely imaged by building up a differential series of images (typically around one thousand in total) by automated cutting in a continuous

process analogous to micro-CT. The collective images may be further reconstructed and analysed using the appropriate software e.g. Amira (FEI). This has allowed the reproducible and ready visualisation of the internal 3-D architecture of the entire kidney ECM, including the vasculature ([Figure 10](#)). The further advantage of this system is that all the ECM scaffold internal architecture is visualised simultaneously by use of native tissue auto-fluorescence, rather than 'highlighting' of each system or compartment with radiological contrast material as required with micro-CT (and which is possible only if this is accessible to perfusion with contrast e.g. the vasculature). This simultaneous visualisation of the internal architecture of the entire ECM bioscaffold without compartmental selection has not been previously demonstrated. A further advance would be to increase the quantitative capability of the software system to make reliable measurements of the finer structures visualised.

2.5 Further work

In the work outlined above two fundamental parameters within the perfusion decellularisation process have been examined more systematically, however there are many other parameters that exist to be considered – such as other choices of decellularising agent (which may also depend on type of tissue being decellularised), additional steps in the protocol e.g. freezing, use of other biological agents, tissue harvesting factors, continuous perfusion versus non-continuous, varying perfusion rates, perfusion pressure versus perfusion rate, and last but not least, post-processing sterilisation methods[129]. All these will inevitably affect the quality of the ECM bioscaffold to a greater or lesser degree, and these effects are as yet unquantified in a systematic manner for whole organ bioscaffolds. In addition, while growth factor levels may be better preserved by lowering the concentrations of the decellularising agent used (as have been demonstrated), functional tests of bioactivity should also be utilised for more complete characterisation, although this is a challenging area within the study of ECM bioscaffolds. These are all areas for future improvement and should play a critical part in developing safe, reliable and consistent whole organ bioscaffolds if they are to be taken to the clinical stage in the development of regenerated organ replacements.

More specifically, while the principle of these findings should be widely applicable i.e. using the lowest concentration of SDS and for the shortest duration of perfusion possible, in order to further tailor these findings towards a clinical application, an adjustment of the exact value of either parameter may be required in practice with much larger organs (and from different species). In that case the parameter most likely to be varied would be perfusion rate – which surely should be based on the physiological rate for the species/organ in question to achieve adequate ‘perfusion’, or rather penetration into the tissue by the decellularising agent. Hence, a similar optimisation study to this one should be carried out on appropriate tissues for human application e.g. porcine, cadaveric.

Chapter 3

Recellularisation of whole kidney bioscaffolds

3.1 Introduction

Recellularisation of whole organ bioscaffolds have been successfully attempted with the heart, liver, lung and kidney, predominantly in the rat model but also in larger animals such as pig[116] by a few groups. Most groups have investigated the use of a mixed primary population of cells native to the organ or tissue in question to achieve the full extent of cell diversity necessary to populate a whole organ scaffold and proof of concept in terms of achieving a degree of functionality has been shown in the heart[9], lung[10-12], liver[13, 118-120], and the kidney [14].

Several groups have attempted seeding with embryonic stem cells (ESCs) in the lung[112] and kidney[121, 125] and have demonstrated proliferation and differentiation in response to organ-specific bioinductive cues within the ECM bioscaffold but no obvious evidence of functionality, however both cell seeding numbers were low (i.e. 2 to 12 million cells per scaffold) and it may also be that the *in vitro* culture duration was insufficient to observe this (7 or 14-21 days). While much larger cell seeding numbers were used in the primary cell recellularisation studies – ranging from 50 to 100 million used per protocol – nonetheless this was still far from achieving physiological tissue numbers, and the *in vitro* culture period was similar in duration, ranging from 3 to 13 days (median culture duration: 7 days).

A few studies have also used human cells on the decellularised whole organ bioscaffold, such as human carcinomatous alveolar basal epithelial cells and human umbilical cord vein endothelial cells (in combination) in the lung (rat-derived scaffold)[10], and human fetal stellate cells and fetal hepatocytes in the liver (porcine scaffold)[118, 120]. These studies confirm the biocompatibility and viability of human-derived cell lines on these ECM

bioscaffolds, and hence the potential clinical application of regenerating whole organs using appropriately sized xeno-derived bioscaffolds with human (possibly host-derived) cells.

Recellularisation i.e. cell seeding of whole organ scaffolds have been achieved using a variety of simple methods ranging from direct intra-parenchymal or intra-mural injection into the scaffold[9, 119] to using the intravascular route, sometimes in conjunction with another cellular compartment where there is an appropriate conduit/compartment such as in the lung (trachea/airway)[10, 11] and kidney (ureter/urinary)[14]. Use of the intravascular route (+/- the trachea/ureter) varies from injection of cells (sometimes in divided bolus doses) in a relatively dense suspension (e.g. injection volume 1-2 ml) to continuous infusion of cells in a proportionately much larger volume of culture medium (e.g. 10-50 ml). One group has directly investigated the comparative density of cellular engraftment between the three main methods of intra-parenchymal injection, intra-vascular injection, and intravascular continuous infusion; the intra-vascular injection method (using divided doses in multiple injections) was found to be the most efficient in seeding with least cell loss[119]. It would also seem likely that this principle may apply to injection versus infusion when utilising a different compartment such as the airway or the urinary, but no specific study has been carried out to corroborate this as yet.

After recellularisation, *in vitro* culture is carried out similarly across the studies extant within a bioreactor setup. This is usually based on the seeded bioscaffold suspended (via the intravascular perfusion cannula) within a sealed compartment or flask and continuous perfusion of the bioscaffold with culture medium can be easily achieved by attaching the intravascular cannula to a tubing and peristaltic pump system (outside the main compartment or flask), similar to the decellularisation setup. The culture medium may be recirculated within the main chamber and changed periodically or it may be pumped in a unidirectional manner from an outside source and then drained from the main chamber. The

main compartment can then be contained within a standard 37°C 5% incubator, with the peristaltic pump either inside or outside the incubator.

There may be other adjuncts required with this basic setup, depending on the organ system involved; in particular, pulsatile distension of the left ventricle and other physiological pressures reproduced by a compliance loop in conjunction with coordinated electrical stimulation using electrodes directly inserted into the heart bioscaffold during *in vitro* culture[9] and a negative pressure ventilation system to mimic physiological breathing conditions for the lung[11]. However, these extra bioreactor requirements may not have their equivalents in all organs and tissue and none have been typically utilised in the liver and kidney studies.

Finally, a smaller number of studies have implanted the whole organ constructs *in vivo* for a limited duration, typically 6 hours[10, 11, 13, 14] although one group achieved a much longer implantation period of 7 days[12]. For implantation, the recellularised grafts were co-seeded with endothelial cells for lining the vascular network to allow blood flow *in vivo*. These aspects will be discussed further in the subsequent chapter.

In this study, the aim was to recellularise whole kidney bioscaffolds (decellularised using the optimised protocol as outlined previously) using two cell populations, primary renal cells and mesenchymal stem cells. The aim is to demonstrate proof of concept with a primary cell population and investigate the viability of an 'adult-derived stem cell' population as an alternative cell source for recellularisation. More specific aspects of the recellularisation protocol for both cell groups are discussed further below. The further aim was to implant both types of recellularised construct *in vivo*. Kidney constructs prepared for implantation used a modified protocol (incorporating endothelial cell culture) and the implantation study which will be all discussed in the following chapter.

3.1.1 Primary renal cells (PRC)

As already mentioned, one group has attempted recellularisation of whole kidney bioscaffolds using primary renal cells which has yielded constructs with up to 10% functionality as defined by creatinine clearance[14]. Specifically their protocol utilised ~60 million primary renal cells derived from day 3 neonatal Sprague-Dawley rats, and the cell suspension after collagen digestion of renal tissues was directly seeded onto the scaffolds without prior *in vitro* culture or passaging. The ureteric compartment or route for seeding of cells utilised by cannulating the ureter (in addition to the renal artery) and 'perfusing' the urinary compartment of the kidney bioscaffold via a pressure differential method whereby the organ chamber was subjected to a -40 cm H₂O pressure. In contrast, Ross *et al* directly injected cells (but ESCs in this case) into either the renal artery or the ureter[111] to investigate the differential effects of cellular engraftment depending on seeding method. They observed that cells were not uniformly dispersed throughout the kidney on a consistent basis with the latter method, and conjectured that the papillary architecture of the rat kidney may be hindering the dispersion of cells through the ureteric route in comparison to intra-arterial seeding, where cells were much more uniformly seeded and highly retained (95% versus 50% in the ureteric seeding group). However, this was noted to be more of an empirical observation especially as relatively low cell numbers were used (2 million in total). A further study using ESCs (10 million cells) in the kidney also used the arterial seeding route[125].

The recellularisation protocol for this project uses a mixed primary renal cell population isolated from day 15-20 old Wistar rats, with the cells having been cultured and passaged after the initial isolation, and also using similar numbers of cells (i.e. ~50 million) to Song *et al*[14]. However, it differs by utilising an intravascular injection seeding protocol, and using multiple bolus injections (i.e. divided doses), as has been shown to be the most efficient seeding method in terms of cellular engraftment and density[119, 121]. The seeded

constructs were cultured *in vitro* with continuous perfusion of culture medium for a period of 7 days and were further characterised by viability testing and histological and immunohistochemical analysis.

3.1.2 Mesenchymal stem cells (MSCs)

While only one group so far has investigated the effects of seeding MSCs onto whole organ bioscaffolds[114] (to assess comparative cellular engraftment effects only) and only three have investigated the effects of seeding ESCs[112, 121, 125], MSCs may provide some key properties as an alternative cell population that may be advantageous for a regenerative medicine strategy towards organ replacement. Not only have they been shown to have some nephrogenic properties both developmentally[131, 132] and in response to acute kidney injury[133, 134], they may be derived and expanded *in vitro* in large numbers from the bone marrow in the adult and hence could be potentially isolated from the host (patient), and the recellularised organ constructs thus produced could be implanted into the host without causing any undesirable immunogenic response. Hence they may be considered a potential 'adult-derived' stem cell population with feasibility for clinical application.

This protocol utilises adult bone marrow-derived mesenchymal stem cells isolated from young adult Wistar rats at the same stage of maturity as those selected for the decellularised whole kidney bioscaffolds. In terms of cell numbers used, seeding method, bioreactor setup, *in vitro* culture conditions, and subsequent characterisation, the same protocol as for PRCs above was followed.

3.2 Materials and materials

3.2.1 Primary renal cell (PRC) isolation

Day 15-20 Wistar rats were used for PRC isolation. The protocol was adapted from Joraku *et al*[129]. Animals were euthanized by cervical dislocation prior to tissue harvesting.

Kidneys were removed under aseptic conditions and placed in sterile Krebs-Ringer (K-R) buffer (Sigma, UK) with 20% v/v antibiotic-antimycotic for at least 30 minutes. All connective tissue including the capsule was removed using sharp dissection and the remaining kidney tissue minced within a cell culture hood until a gel-like consistency was achieved. The minced tissue was suspended in fresh K-R solution, typically 4 kidneys per 25 ml buffer solution, and digested with collagenase in a water bath at 37°C (Liberase TM Research Grade, Roche, UK) according to the manufacturer's recommendations.

The digested tissue suspension was filtered through a 100 µm cell strainer (BD Falcon®, Corning UK) and neutralised with an equal volume of DMEM culture medium. This solution was then centrifuged at 1200 rpm for 5 minutes.

The supernatant was discarded and cell pellets re-suspended and plated in 50% DMEM and 50% K-SFM Medium with bovine pituitary extract and epidermal growth factor with the addition of 5% v/v FBS and 1% v/v AA. Cells were passaged when they reached 95% confluence but utilised as soon as sufficient numbers were obtained, typically at the P1/P2 stage.

3.2.2 Mesenchymal stem cell (MSC) isolation

Male Wistar rats (350-375g) were used for MSC isolation. Animals were euthanized by cervical dislocation prior to tissue harvesting.

Whole intact femurs were removed under aseptic conditions and placed in sterile PBS with 20% v/v antibiotic-antimycotic for at least 30 minutes. Both epiphyseal ends of the each bone were removed with a sterile bone nibbler. Each femur was manually irrigated through

the diaphysis with fresh culture medium (α -MEM) using a 20ml syringe and green (21G) needle. The resulting cell suspension was plated out in tissue culture plastic flasks in α -MEM with the addition of 10% v/v FBS and 1% v/v AA.

MSCs were passaged when they reached 95% confluence and typically used for recellularisation at the P2 stage.

3.2.3. Recellularised kidney bioscaffold perfusion and bioreactor setup

Decellularised whole kidney bioscaffolds (as produced with improved optimised conditions as outlined in Chapter 2 i.e. 0.125% SDS x 4 hours) designated for subsequent recellularisation were sterilised by continuous perfusion with 1% v/v antibiotic-antimycotic (Gibco®, Invitrogen, UK) in sterile PBS for 18 hours at 2 ml/min within the same system described below and placed inside a cell culture hood, prior to seeding with the appropriate cell population (either PRCs or MSCs).

The recellularised whole kidney bioscaffold was suspended in the main chamber of a sterile three-port flask via the cannulated renal artery and also within the reservoir of culture medium (150-180 ml volume), enabling the sterile culture of the recellularised grafts. The perfusion system also included a sterile exchange membrane (BugStopper, Whatman, UK) and a peristaltic pump ([Figure 11](#)). The culture medium was recirculated from the reservoir within the main chamber and the tubing system was sealed for sterility. The system was placed inside a standard 37°C 5% CO₂ incubator for temperature and carbon dioxide control. The graft was continuously perfused via the renal artery at 2 ml/min. The culture medium was changed every 48 hours, the first change occurring 24 hours after initial recellularisation (seeding), then on day 3 and day 5 post-recellularisation during the total *in vitro* culture period of 7 days.

The perfusate consisted of either i) 50% DMEM and 50% Keratinocyte Serum-Free Medium (K-SFM) with bovine pituitary extract and epidermal growth factor with the addition of 5%

v/v fetal bovine serum (FBS) and 1% v/v antibiotic-antimycotic (AA) for primary renal cells, or
ii) α -MEM with the addition of 10% v/v FBS and 1% v/v AA (all Gibco®, Invitrogen, UK) for
mesenchymal stem cells.

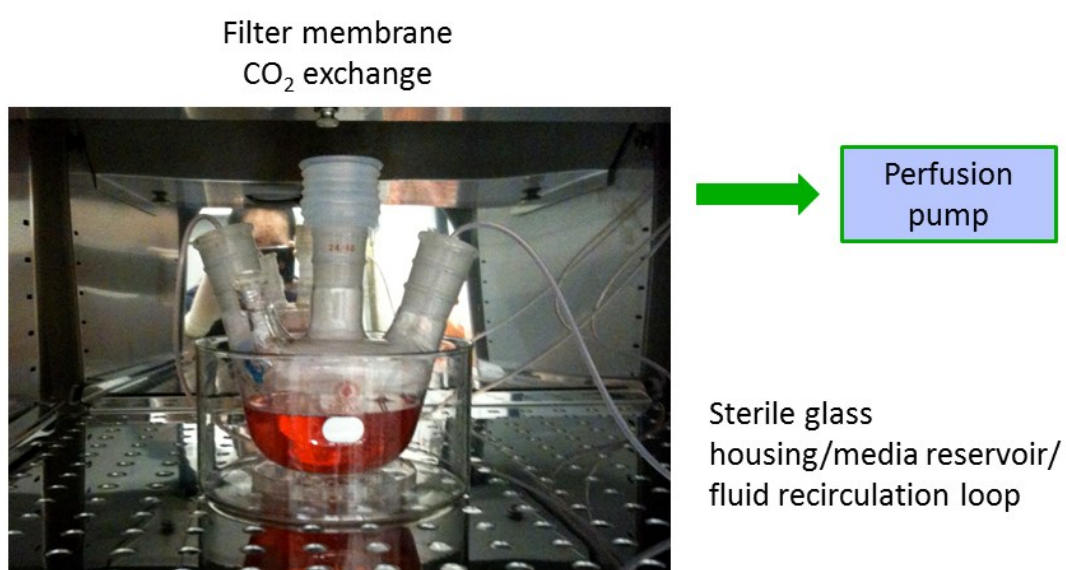


Figure 11 Schematic of bioreactor for perfusion recellularisation.

3.2.4 Recellularisation of whole kidney bioscaffolds – seeding of cells

After sterilisation, the decellularised kidney matrix was seeded with $\sim 50 \times 10^6$ cells (either primary renal cells or mesenchymal stem cells). This was performed by making multiple intravascular bolus injections (typically $3 \times \sim 17$ million cells) via the cannulated renal artery with an injection volume of 1 ml per injection over 60 seconds. An interval of 60 minutes was left in between each injection step to allow cell attachment.

When the injection seeding process was completed, the recellularised scaffold was connected to the perfusion circuit within the bioreactor setup as above.

3.2.5 LIVE/DEAD™ staining

The tissue sample consisted of recellularised kidney construct (either with PRCs or MSCs) hemi-sectioned through the midline in a clamshell fashion. The sample was washed with sterile PBS, and then completely immersed in the working solution (20 µl of Ethd-1 and 10 µl of calcein (LIVE/DEAD™ kit, Invitrogen, UK) in 10 ml of sterile PBS) for an incubation period of 10-15 minutes at room temperature while being protected from light. Then the working solution was aspirated and the sample was washed 3 times with fresh sterile PBS and examined using fluorescent microscopy for analysis.

3.2.6 AlamarBlue® assay

A working solution of 1:10 alamarBlue® reagent (Invitrogen, UK) in culture medium was added directly in order to completely immerse the tissue sample which was recellularised kidney construct (either with PRCs or MSCs). Samples and controls (i.e. 1:10 alamarBlue® in culture medium without tissue sample) were incubated for 4 hours while protected from light at 37°C in a standard 5% CO₂ incubator. After this, 100 µl aliquots from each tissue sample or control were transferred in triplicate into a 96 well plate for analysis using a micro plate reader (SpectraMax M5, Molecular Devices, USA) measuring fluorescence (set at an excitation wavelength of 540nm and emission at 590nm).

Results were reported as mean +/- standard error.

3.2.7 Histology and immunohistochemistry

All samples were fixed in 10% v/v buffered neutral formalin for at least 24 hours, and samples were stained with haematoxylin and eosin (H+E) for light microscopy to evaluate cellular engraftment on the whole kidney bioscaffold.

IHC was performed to characterise renal cell types and their distribution on the recellularised kidney bioscaffolds and the protocol used was the same as in Chapter 2 for characterisation of decellularised whole kidney bioscaffolds.

Primary antibodies and dilutions used were as follows: aquaporin-1, aquaporin-2, synaptopodin, and von Willebrand Factor (ab15080, ab15116, 101883, ab6994 respectively, all Abcam, UK) – all at 1:500 dilution. Secondary antibodies and dilutions were the same as previously stated.

3.2.8 3-D Histology (histocutter) and reconstruction of images

Whole recellularised kidney constructs were fixed, processed and embedded for automated cutting on the HistoCutter setup (including subsequent image processing) using the same protocol as previously outlined (Section 2.2.7).

Recellularised kidney constructs were also stained with DAPI (prior to tissue processing) in order to assess cellular engraftment and hence a UV filter was also applied during image collection (in conjunction with the Texas Red filter). Images for each filter were saved in parallel (not in combination) and reconstructed accordingly using Amira software (FEI, USA).

3.3 Results: Recellularisation of whole kidney bioscaffolds

Using the intravascular multiple injection seeding method and bioreactor setup as described above, two types of recellularised kidney construct were successfully cultured *in vitro* using primary renal cells (PRCs) isolated from 4 week old Wistar rats and adult rat bone marrow-derived mesenchymal stem cells (MSCs). The continuous perfusion of culture medium through the vascular network allowed the viable culture of the whole kidney scaffold in its 3-dimensional entirety as demonstrated by both imaging (fluorescent microscopy with LIVE/DEAD staining) and an assay of metabolic function (alamarBlue® assay). Cellular seeding and engraftment after 7 days was also widespread, as consistent with the distribution of the vascular network, and both PRC- and MSC-recellularised kidney constructs showed similar features in terms of histological appearance and cellular density.

Figure 12 shows the macroscopic appearance of the acellular kidney scaffold and the recellularised kidney scaffold (at day 1 immediately post-seeding, and at day 7 at termination of perfusion culture). There were no gross differences in appearance seen between the different types of construct using PRCs or MSCs for recellularisation.

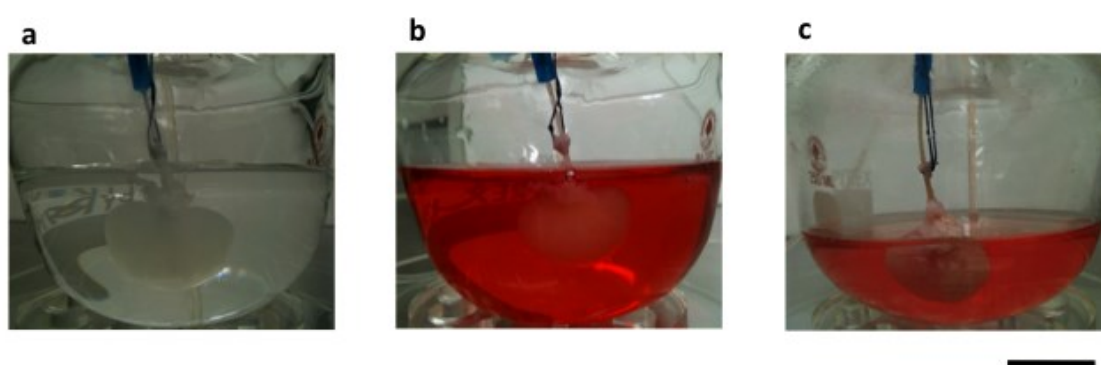


Figure 12 Recellularised kidney construct within bioreactor setup.

(a) Day 0 (sterilised acellular kidney scaffold). (b) Day 1. (c) Day 7 (end of *in vitro* culture period).

Scale bar = 1 cm.

3.3.1 PRC- recellularised constructs

Cellular viability tests ([Figure 13a, b](#)) after 7 days of *in vitro* culture demonstrate continued and extensive viability throughout the 3-D kidney construct, with obvious cellular 'patterning' following the vascular network and also ECM structural cues as observed with LIVE/DEAD™ staining.

Histological analysis showed cellular engraftment dispersed throughout the kidney ECM bioscaffolds and was not restricted to the vascular compartment, as has been shown with other studies which employed the purely intravascular route for cell seeding[13, 119, 125]. In addition, histological sections taken at different levels throughout the recellularised scaffolds consistently demonstrate the presence of cells throughout the specimen, which are represented in [Figure 14](#). Areas of recellularisation comparable to normal histological density were present throughout and distinctive 'glomerular-like' structures are easily observed ([Figure 14](#)). Localised areas of cellular non-engraftment were noted in one or two specimens and appeared histologically similar to the acellular bioscaffolds and probably corresponded to areas of localised vascular non-perfusion, hence likely due to non-seeding of cells initially.

IHC of renal cell markers (aquaporin 1 and aquaporin 2 for tubular cells, synaptopodin and von Willebrand factor for glomerular epithelial and endothelial cells respectively) demonstrated the presence of mixed cell types throughout the recellularised construct ([Figure 15](#)), although there was no obvious appearance of histological differentiation or regional localisation of different renal cell types on the recellularised kidney scaffold to suggest functional renal structures.

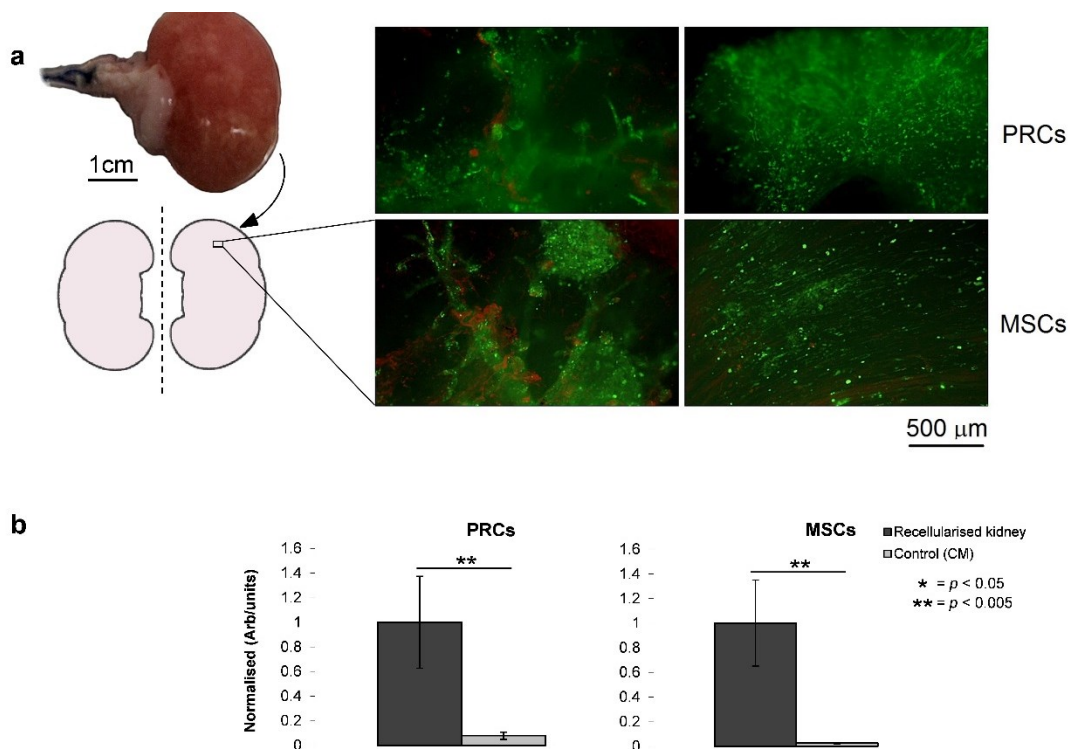


Figure 13 Viability testing in whole kidney bioscaffolds recellularised with primary renal cells (PRCs) and MSCs. **(a)** Photograph of a recellularised kidney construct which is hemi-sected to obtain fluorescence-microscopy images of LIVE/DEAD™ staining and for testing with Alamar Blue assay after 7 days of perfusion culture. LIVE/DEAD™ staining of the cut surface of both PRC- and MSC-recellularised constructs show dense distribution of viable cells throughout the kidney bioscaffolds with obvious cellular ‘patterning’ following the vascular network and ECM structural cues. **(b)** AlamarBlue® assay demonstrated a highly visible colorimetric change indicative of cellular viability with both the PRC- and MSC-recellularised constructs as compared to the control which is culture medium (CM), which was confirmed on fluorescence reading by spectrophotometer (** $p < 0.005$). For all samples, $n \geq 3$.

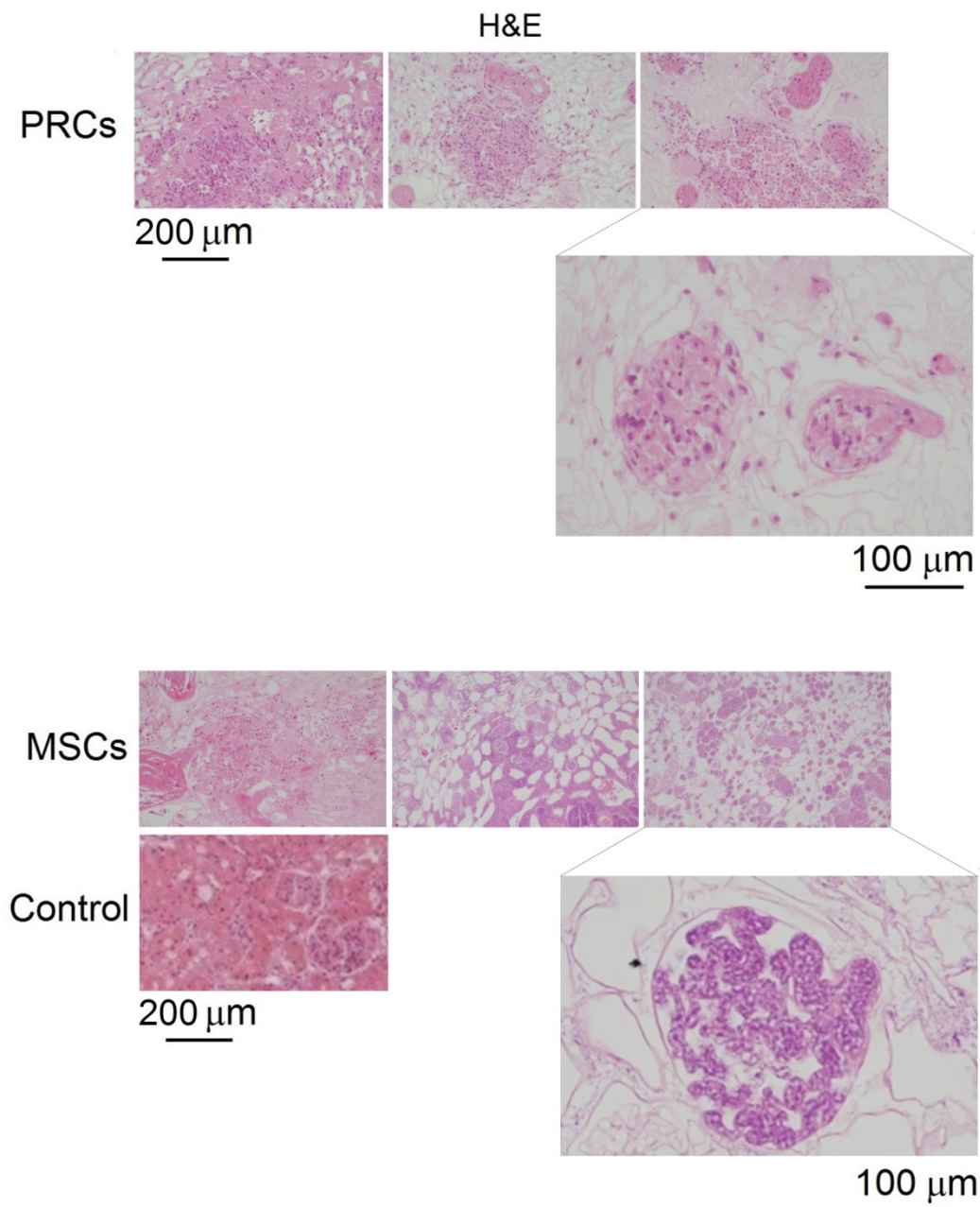


Figure 14 Histology (H+E) of recellularised whole rat kidney bioscaffolds.

H+E staining of the recellularised constructs shows cellular engraftment dispersed throughout the kidney bioscaffolds. Areas of recellularisation comparable to normal histological density are present throughout and distinctive 'glomerular-like' structures are observed. Similar findings are seen in both PRC- and MSC- recellularised constructs. Control = normal kidney.

For all samples, $n \geq 3$.

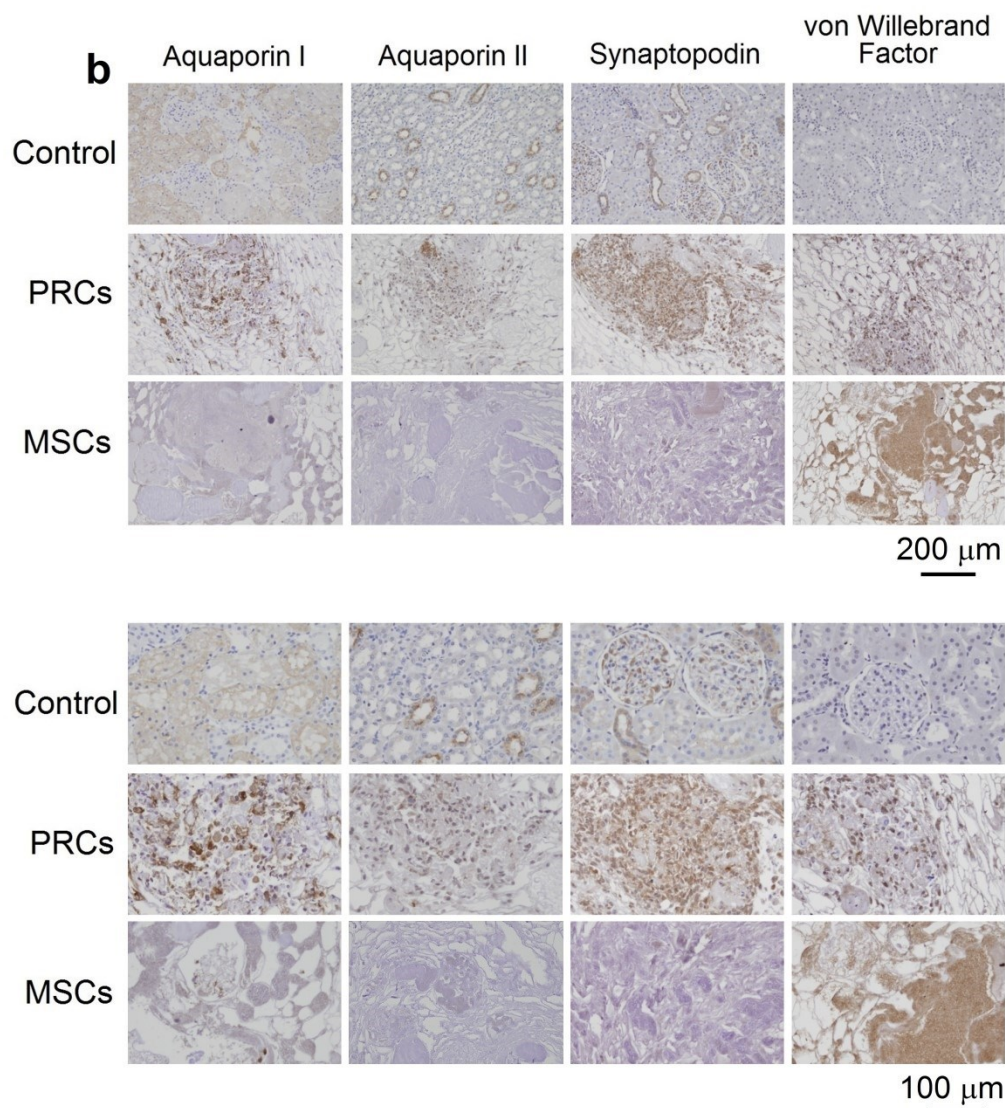


Figure 15 IHC for renal cell markers in recellularised whole rat kidney bioscaffolds.

IHC for renal cell markers (aquaporin 1, aquaporin 2, synaptopodin, and von Willebrand Factor for glomerular epithelial and endothelial cells respectively) demonstrates presence of mixed cell types throughout PRC-recellularised constructs although histological differentiation or localisation was not noted. Control = normal kidney. In MSC-recellularised constructs, IHC for renal cell markers were only significantly positive for von Willebrand factor.

For all samples, $n \geq 3$.

3.3.2 MSC-recellularised constructs

Cellular viability tests ([Figure 13a, b](#)) after 7 days of *in vitro* culture were similar to the PRC constructs: extensive viability throughout the 3-D kidney construct, with obvious cellular 'patterning' following the vascular network and also ECM structural cues on LIVE/DEAD™ staining. Histological analysis again showed cellular engraftment dispersed throughout the kidney ECM bioscaffolds with areas of recellularisation comparable to normal histological density present throughout and distinctive 'glomerular-like' structures ([Figure 14](#)).

IHC of renal cell markers (aquaporin 1 and aquaporin 2 for tubular cells, synaptopodin, and von Willebrand factor) were only strongly positive for von Willebrand factor as an endothelial cell marker ([Figure 15](#)).

3.3.3 Assessment of 3-D architecture of whole kidney recellularised constructs

Using the 3-D histology (Histocutter) technique, the internal architecture and cellular engraftment of the whole recellularised kidney scaffold can be visualised in its entirety (Figure 16). Whole kidney scaffolds recellularised with mesenchymal stems cells and cultured for 7 days with continuous perfusion were processed and analysed with this method. The areas in blue denote DAPI staining and hence show the distribution of cells within the kidney scaffold, which is shown in orange (auto-fluorescence) as before.

As can be seen on routine histology, there is a widespread distribution of cells throughout the scaffold from the central hilar region (where cells are seeded by intra-arterial injection) to the periphery of the scaffold and the glomerular compartment, although the resolution of the finer cellular details may require further refinement of this technique and subsequent image processing/reconstruction.

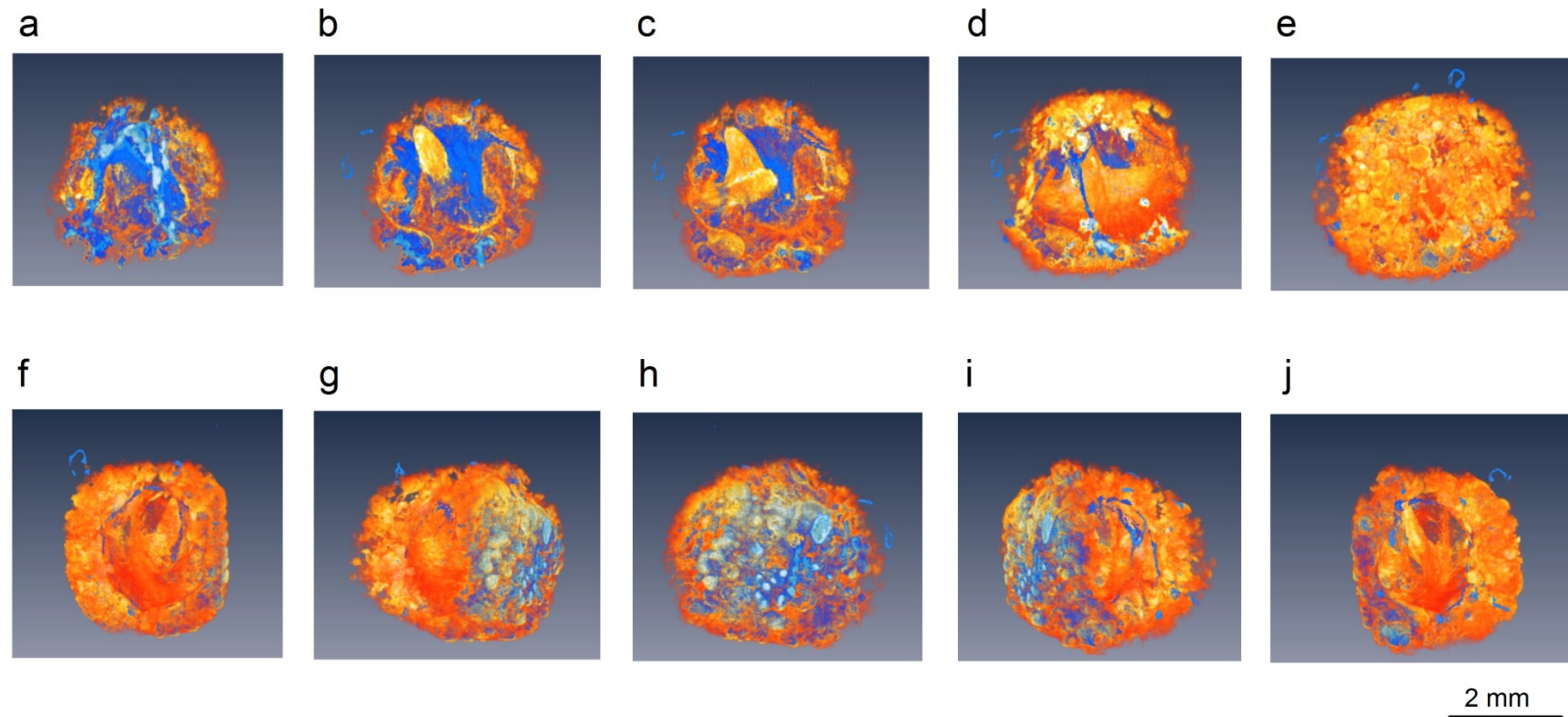


Figure 16

Reconstruction of 3-D histology (Histocutter) images of whole recellularised kidney constructs using MSCs after 7 days of perfusion culture.

(a-e) The 3-D recellularised kidney scaffold seen at different depths/thicknesses (shown at 1 mm intervals) within the tissue to demonstrate the internal architecture of the whole kidney ECM and cellular engraftment and distribution. (f-j) Rotation of the whole recellularised scaffold structure. Blue = DAPI staining. For all samples, $n \geq 3$.

3.4 Discussion

Two types of recellularised whole kidney scaffolds have been successfully cultured *in vitro* for a period of 7 days, using either primary renal cells or mesenchymal stem cells, and viability testing in combination with histological analysis have demonstrated high degrees of cellular engraftment throughout the specimens with formation of renal-like structures in response to the ECM architecture and structural cues within the kidney scaffold. However, in comparison with the one main study on recellularisation of whole kidneys using primary renal cells [14], no obvious evidence of significant renal-appropriate differentiation or distribution to suggest functionality of the kidney construct was found in this work. This can be probably attributed to two main groups of factors: variations in the specific recellularisation protocol used and the general lack of systematic pre-optimisation for recellularisation studies currently.

The key differences of note within the recellularisation protocols adopted within our study and the above mentioned were route/method of cell seeding and *in vitro* culture conditions (particularly medium supplementation), and age of animals from which cells were isolated. However, there are also some differences in exact equipment utilised, sterilisation setup and duration, strain of rat used and the decellularisation protocol. These key differences were specifically: arterial versus ureteric seeding for the primary renal cells (although arterial perfusion was utilised for the endothelialisation process hence this could also be considered dual compartment seeding), and 2-3 day old neonatal rat renal cells as opposed to 2-3 week old. As the authors have stated, such immature kidney cells required *in vitro* maturation with culture medium supplementation with glucocorticoids and catecholamines. (For clarity, the underlined conditions which are highlighted for comparison are derived from the study by Song *et al* [14].)

Dual compartment seeding, especially when endothelialisation is required, may be more appropriate in delivering cells to the urinary compartment, especially if there is good

preservation of basement membranes within the tubular structures. However, in view of only limited evidence or comparative data regarding seeding methods from two studies already discussed [119, 121] which would suggest that the intravascular injection route was the most efficient, and given the relatively 'leaky' and permeable nature of the decellularised ECM kidney scaffold, the initial choice was made for the intravascular injection route for seeding and subsequent experiments conducted in this study/thesis were kept consistent with this. One further recent study also used the arterial route for seeding (murine ESCs) but with a slow perfusion rate over 30 minutes [125] as opposed to much faster and/or concentrated cell seeding employed by Song *et al* [14], as well as Ross *et al* [121] and the work outlined in this thesis i.e. typically over 1-2 minutes. Overall, as cell types and numbers vary greatly between the previous studies, and all authors report good distribution and engraftment of seeded cell populations within the whole kidney bioscaffold (and this study is consistent with this general finding) with very limited explicit comparative rationalisation, it is currently difficult to specify which seeding route and perfusion parameters are optimal without further comparative studies.

Regarding culture medium supplementation secondary to choice of neonatal age of cells isolated, Song *et al* mention the use of glucocorticoids and catecholamines in order to aid *in vitro* maturation of the neonatal cells but this is not further detailed (e.g. actual substances, dosing regimen), and a simpler protocol was chosen in this study in order to assess purely the effects of the whole kidney ECM might produce on recellularisation – and the additional *in vitro* maturation may not be necessary with primary renal cells derived from older subjects (i.e. 2-3 weeks of age) which have more mature or developed kidney tubular systems. Also, the authors do not state a clear rationale for the age choice of these immature renal cells, although the general improved viability and greater preservation of varied differentiation of younger cell lineages may be inferred.

In the MSC-recellularisation work from this study, very similar results to the PRC-recellularisation constructs were found regarding cellular engraftment and distribution within the recellularised kidney bioscaffolds, with some 'renal-like' cell formations that appear to follow ECM architecture and structural cues. While renal functionality might not be expected at this current stage, and mature renal cell markers were not expressed in the MSC constructs, it may be valuable to explore expression of glomerular and epithelial precursor cell markers e.g. Pax-2, as were shown to be expressed with ESCs seeded onto whole kidney bioscaffolds [121]. No other studies within whole kidney decellularisation have investigated the use of MSCs as a cell population for recellularisation, and these initial results appear to indicate widespread engraftment and high viability/compatibility with the whole kidney bioscaffold. However, issues of optimisation of recellularisation parameters (seeding route and method, *in vitro* culture and supplementation in order to promote directed differentiation into reno-typic cell lineages, testing of compatibility between human cells and xenogenic bioscaffolds, considerations of cell source and isolation, etc) are as yet to be investigated and rationalized.

As a follow-on from the recellularisation studies conducted here, since both the PRC- and MSC- recellularised kidney constructs were found to be highly viable after 7 days *in vitro* culture, the aim was to implant both types *in vivo* (with decellularised or 'acellular' kidney bioscaffold as a control group) in order to assess long-term *in vivo* viability and to investigate the possible regenerative effects of constructive remodeling, and whether this would aid further renal differentiation and functionality in either type of recellularised construct. This will be discussed in detail in the following chapter.

3.5 Further work

Despite the promising and exciting findings from some preliminary studies, there is a very limited number of studies on recellularisation of whole organ bioscaffolds, and when subdivided further into organ systems (and species i.e. small and large animal models), it can be clearly seen that there is a lack of systematic pre-optimisation of virtually all recellularisation parameters. While these may be less easily controllable or systematically assessed than perhaps those for decellularisation, nonetheless systematic pre-optimisation should be essential in order to guarantee clinical quality and safety. Parameters include: seeding route and mechanism, *in vitro* culture conditions (bioreactor setup, culture medium +/- appropriate supplementation, perfusion rate, duration of culture), choice of cell populations (including endothelial cells) and total numbers used for recellularisation, and even choice (source) of decellularised organ bioscaffold.

The seeding route and mechanism may be the most straightforward to assess. The predominant study in the kidney suggests use of the ureteric route and has demonstrated proof of concept with a partially (up to 10%) functional graft, hence it would be interesting to compare intravascular/intra-arterial seeding with ureteric using the exact same protocol and setup, including the pressure differential method for drawing cells onto the scaffold that is employed, to see the differential effects on cellular engraftment and differentiation. While the authors quote the pressure used of -40 cm H₂O as being physiologically compatible with tubular basement membranes[14], this may also be a variable to be considered. With regards to the intra-arterial seeding route, it is not absolutely clear whether single compartment seeding is not suitable for the kidney given the limited data extant. There are subsidiary issues such as exact rate and volume/concentration of cells being used, and the time allowed for cell attachment before culture medium perfusion is commenced.

Culture conditions may well depend on the cell population used, and additional supplementation quoted in the above study was concerned with *in vitro* maturation of

neonatal renal cells, however this may well not be a relevant issue in clinical application since using neonatal human cells in this context would be currently unlikely. A stem cell population would be more promising, and an adult-derived stem population (especially if patient-derived) would be the most desirable in terms of biological (and legislative) feasibility. Current options might include induced pluripotent stem cells [135-7] and mesenchymal stem cells derived from e.g. bone marrow.

Finally but not least, the choice of decellularised scaffold should be considered for clinical application at least in terms of appropriate size e.g. human cadaveric (such as from donated organs considered not suitable for transplant), porcine, and the cell population for recellularisation should be optimised in conjunction with this scaffold. Recellularisation studies of cells from different species should be carried out on each type of scaffold, although biocompatibility is generally expected due to the properties of the ECM scaffold [61]. Recellularisation optimisation studies should also be conducted in conjunction with optimisation of decellularisation protocols. One study has made a simple comparison between three different detergent protocols but demonstrated that there was no significant difference seen between any of these in terms of cellular engraftment or apoptosis in the recellularised constructs [114]. A logical continuation of the optimisation of decellularisation work described in Chapter 2 would be to assess any differential effects of improved growth factor preservation within the scaffold on cell proliferation and differentiation when seeded on the kidney scaffolds, using both primary renal cells and mesenchymal stem cells.

Chapter 4

Implantation of recellularised kidney constructs in the *in vivo* rat model

4.1 Introduction

As previously reviewed, several groups have successfully surgically implanted recellularised organ constructs *in vivo* [10-14], and also demonstrated survival and functionality of these constructs, at least to a partial degree. Constructs vary in organ type (three studies on the lung, one each on liver and kidney) but all except one group demonstrated only very limited durations of graft survival, typically up to 6 hours, mainly due to tissue oedema and congestion. All groups empirically note 'leakiness' of constructs compared to normal tissue, and certainly vascular resistance was noted to be significantly higher in the recellularised graft compared to normal kidney, but higher still in the decellularised scaffold [14].

However, one group has successfully implanted a unilateral recellularised lung construct for up to 14 days [12], although they noted decline in oxygenation and compliance from day 1 onwards, with no significant difference in these parameters from the control group (unilateral pneumonectomy) from about day 7, suggesting graft function and survival were limited to around this period. They attributed the improved graft survival to a modified graft preservation protocol including dry ventilation with positive end-expiratory pressure and hyperosmolar perfusion before transplantation (during *in vitro* culture), and a conservative weaning protocol from mechanical ventilation; however, these strategies may be mostly limited to the specific context of recellularised lung grafts.

In the single *in vivo* study on the kidney [14], the implanted construct (recellularised with primary renal cells) was anastomosed to renal vessels *in situ* in order to establish vascular patency and free urine/fluid drainage through the graft ureter (which was left open-ended) was also observed during the 'test period' (exact duration not specified). In this case, the animal was not recovered in order to assess long-term graft survival and/or function.

Similarly and with all the studies so far, it has not been possible to assess any potential effects of constructive remodeling *in vivo*, mostly because of the extremely short implantation periods, although the longer-implantation lung study has demonstrated the presence of surfactant being produced during the implantation period as a possible indication of lung matrix remodeling during the implantation period [12].

4.1.1 The effects of the *in vivo* environment on constructive remodelling

Since ECM is a material secreted by native cells in their natural environment, it contains all the structural and bioinductive molecules necessary to perform its function as a scaffold i.e. a supportive medium and a means of dynamic equilibration and communication between cells, which also allows them to perform their designated function within specific tissue types and to respond to their environment. Another key feature of ECM relevant to this dynamic equilibration is its ability to be degraded and (re-)secreted by the cells inhabiting this scaffold, and obviously this occurs constantly in normal physiological conditions in conjunction with cell death and renewal. This process or ability for ECM to be absorbed/degraded may also be seen as a form of true assimilation, especially when applied to ECM scaffold materials used in a regenerative medicine context, as these are broken down *in situ* (when implanted *in vivo*) and new ECM is produced by local cells without an adverse immune reaction – which is the underlying mechanism of constructive remodelling. Constructive remodelling has been demonstrated in different models of degradation analysis of implanted ECM material using radio-labelled SIS as already discussed i.e. in repair of the bladder (augmentation cystoplasty) [68] and Achilles tendon reconstruction [67]. In both these cases, there was functional replacement of the SIS implant material with no significant loss of function and appropriate degradation of the ECM scaffold. Similarly, aortic grafts (both decellularised and cryopreserved grafts) implanted in sheep as a conduit for potential tracheal replacement showed *in situ* transformation of the aortic graft tissue to cartilage

bearing tracheal-like tissue [69, 70]. In whole organ decellularisation work, decellularised whole skeletal muscle scaffolds (without recellularisation) have been implanted within an equivalent muscle defect and show infiltration of inflammatory and stem cells to demonstrate a potentially transformative ‘pro-myogenic’ response *in vivo* [128] after a period of 2 to 4 weeks, especially when the decellularised muscle scaffold was transplanted in athymic mice to avoid leucocyte-mediated graft-host responses. Indeed, it might be interesting to speculate on the hypothetical properties of a whole organ decellularised bioscaffold implanted *in vivo* – in theory it could even lead to complete *in situ* regeneration by the appropriate recruitment and directed differentiation of cells, at least if its structural and bioinductive properties were to have been perfectly preserved within the decellularisation process [130].

4.1.2 Experimental setup

In this study so far, perfusion recellularisation has been carried out with *in vitro* culture to produce kidney constructs with both primary renal cells and MSCs, which have shown highly viable dense engraftment of cells forming some ‘renal-like’ structures within the ECM bio-scaffold (Figures 14, 15). While these PRC- and MSC- recellularised constructs do not appear obviously histologically differentiated or functional after 7 days of *in vitro* culture, the processes underlying constructive remodelling secondary to the complex physiological environmental cues *in vivo* could encourage further renal differentiation and regeneration, as suggested by the limited evidence from the lung implantation study [12] but also other ECM implantation studies such as those on bladder/tendon/tracheal replacement [67-70] . Hence the aim is to implant these constructs *in vivo* for longer periods than previously studied in order to assess long-term graft survival and any potential transformative or regenerative effect from long-term implantation.

For this study, the animal model used will be similar to that of renal transplantation in the rat, and this may be carried out using microsurgical techniques. With regards to the immune tolerance of the graft, either an inbred or an immunocompromised e.g. athymic strain should be used for the implant recipients. For this study, an inbred strain (i.e. Lewis) was chosen for absolute consistency of cell and scaffold immune compatibility (using the same strain as the source for all decellularised scaffolds, cells for recellularisation, and for the implant recipients), and to avoid additional complications during the post-operative phase as possible e.g. higher infection risk with athymic animals.

One kidney construct was to be implanted per recipient, allowing the remaining kidney to sustain normal renal function and physiology, and three types of kidney graft were intended to be implanted – PRC-recellularised, MSC-recellularised, and acellular (decellularised) whole kidney scaffolds. Surgical implantation was performed exactly analogous to the orthotopic transplant procedure with direct anastomoses of renal vessels and ureter, after performing native nephrectomy on that side. The duration of implantation for all three types of graft was planned for two separate time periods: 7 days, and 6 weeks ([Table 9](#)). Interim graft survival (for the recellularised constructs) may be assessed by contrast imaging of the renal vasculature e.g. CT angiogram to assess ongoing perfusion. At the end of the implantation period, the explanted grafts would be tested for viability e.g. TUNEL testing, and assessed histologically for evidence of renal differentiation.

Table 9**Numbers of animals (rats) required:**

In accordance with the principle of the '3Rs' (Replacement, Reduction, and Refinement) as outlined in the Animals (Scientific Procedures) Act 1986, animal numbers were minimised for this preliminary study. For each test group, $n = 3$ minimum per group with allowance for mortality (renal transplant procedure severity: moderate) hence $n = 4$ is proposed as the maximum required per group.

	Experimental group		
Time period	PRC-recellularised graft	MSC-recellularised graft	Acellular graft
7 days	4	4	4
42 days	4	4	4
	24		

(Figures stated = number of animals)

4.2 Materials and materials

4.3.1 Endothelial cell culture

Lewis rat-derived primary vein endothelial cells were obtained directly from a commercial source (Cell Biologics, Chicago, USA); the cell line was cultured and expanded *in vitro* prior to recellularisation work. Cells were cultured in Animal Endothelial Cell Medium with Supplement Kit (M1266, Cell Biologics, USA) which contained the following supplements: epidermal growth factor, vascular endothelial growth factor, L-glutamine, fetal bovine serum, antibiotic-antimycotic (all as supplied). Cells were typically utilised at the P2/P3 stage or frozen until required.

4.3.2 Recellularisation of whole kidney bioscaffolds – endothelial cell co-seeding

After sterilisation, the decellularised kidney matrix was seeded with $\sim 50 \times 10^6$ cells (either primary renal cells or mesenchymal stem cells). This was performed by making multiple intravascular bolus injections (typically $3 \times \sim 17$ million cells) via the cannulated renal artery with an injection volume of ~ 1 ml per injection over 60 seconds. An interval of 60 minutes was left in between each injection step to allow cell attachment.

After the main cell population was seeded, 20×10^6 Lewis primary vein endothelial cells (R2133-LS, Cell Biologics, USA) was similarly injected into the cannulated renal artery, suspended in a total volume of 2 ml of culture medium. Another interval of 60 minutes was allowed after this injection step.

When the injection seeding process was completed, the recellularised scaffold was connected to the perfusion circuit within the bioreactor setup as previously (see chapter 3 for details). The culture medium for these co-seeded constructs in the bioreactor reservoir was a 50:50 mixture of either the culture medium for PRCs or for MSCs (Section 3.2.1) and the endothelial cell culture medium (as below).

4.3.3 Recellularised graft preparation

Acellular kidney grafts (decellularised kidney scaffolds):

Whole Lewis rat cadaveric kidneys were perfusion decellularised as previously described (Sections 2.2.1 – 2.2.2). Briefly, male Lewis rats weighing 350 to 400 g were euthanized by cervical dislocation; kidneys were explanted and cannulated with a 24G cannula and perfused with phosphate-buffered saline (PBS) with sodium nitroprusside, then perfused with 0.125% w/v sodium dodecyl sulphate (constant rate, 10 ml/min) for 4 hours, then washed with PBS for 1 hour. PBS with 1% antibiotic-antimycotic was then perfused for 18 hours (constant rate, 2 ml/min). Decellularised whole kidney scaffolds were then placed in sterile PBS for immediate implantation. The graft was flushed with heparinised saline (10 i.u./ml) prior to vascular anastomoses.

Recellularised kidney grafts:

After perfusion decellularisation of whole Lewis rat cadaveric kidneys as previously described (Sections 2.2.1 – 2.2.2.), recellularisation of whole kidney scaffolds using either primary renal cells or mesenchymal stem cells in conjunction with endothelial cells was carried out as described above (Section 4.3.1) and *in vitro* culture was carried out in the bioreactor setup as previously described (Section 3.2.1). On completion of the 7-day culture period, kidney constructs were removed from the bioreactor circuit and placed in sterile PBS for immediate implantation. The graft was flushed with heparinised saline (10 i.u./ml) prior to vascular anastomoses. All grafts were treated identically during the implantation process.

4.3.4 Surgical (renal transplant) implantation procedure (Figure 17)

All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 and approved by the institutional Animal Welfare Ethical Review Body (AWERB) at Imperial College London. Adult male Lewis rats weighing 350 to 400g were used for all implantation procedures. All types of kidney graft were treated identically as regards the surgical implantation.

General anaesthetic was administered by intraperitoneal injection (ketamine 75mg/kg + medetomidine 0.5 mg/kg) and subjects were monitored with pulse oximetry and given supplementary oxygen via a Bain's circuit. At the end of the procedure, a reversal agent (atipamezole 1 mg/kg) was given subcutaneously. Subjects were also given saline (5 ml) and analgesic (carprofen 5 mg/kg) subcutaneously after administration of anaesthetic prior to commencing the procedure.

A median laparotomy was performed and the left kidney dissected from Gerota's fascia and peri-renal fat. Subsequent to this step, the procedure was carried out with the use of a dissection microscope (Olympus, UK). The left recipient renal artery, renal vein and ureter were identified and mobilised up to the level of the left adrenal artery, which was preserved. The left renal artery and vein were clamped using single microvascular clamps (Mercian, UK), then ligated transversely close to the renal hilum. The ureter was ligated (without clamping) proximally close to the kidney. The kidney was then removed. The segments of renal artery and vein distal to the clamp were flushed with heparinised saline (10 i.u./ml).

The recellularised kidney graft (or control decellularised scaffold) was placed in the renal cavity and surgical end-to-end anastomoses of the renal vein, renal artery and ureter were performed (in that order), using a double vascular clamp with frame (Mercian, UK). For the vessels, a continuous suturing technique using 10/0 nylon was used, and for the ureter, an interrupted technique with 10/0 nylon. The recipient artery and vein were unclamped and patency of anastomoses was confirmed. After haemostasis was confirmed, the peri-renal fat

was closed over the kidney with 6/0 prolene and then the abdomen was closed *en masse* with 3/0 vicryl interrupted sutures, then 3/0 silk interrupted mattress sutures to the skin. (All sutures were supplied by Ethilon, UK.)

All subjects were recovered and monitored clinically post-operatively in the same manner.

All received analgesia (carprofen 5 mg/kg subcutaneously) for at least 3 days post-operatively or until this was assessed to be no longer required.

Figure 17 shows aspects of the microsurgical procedure.

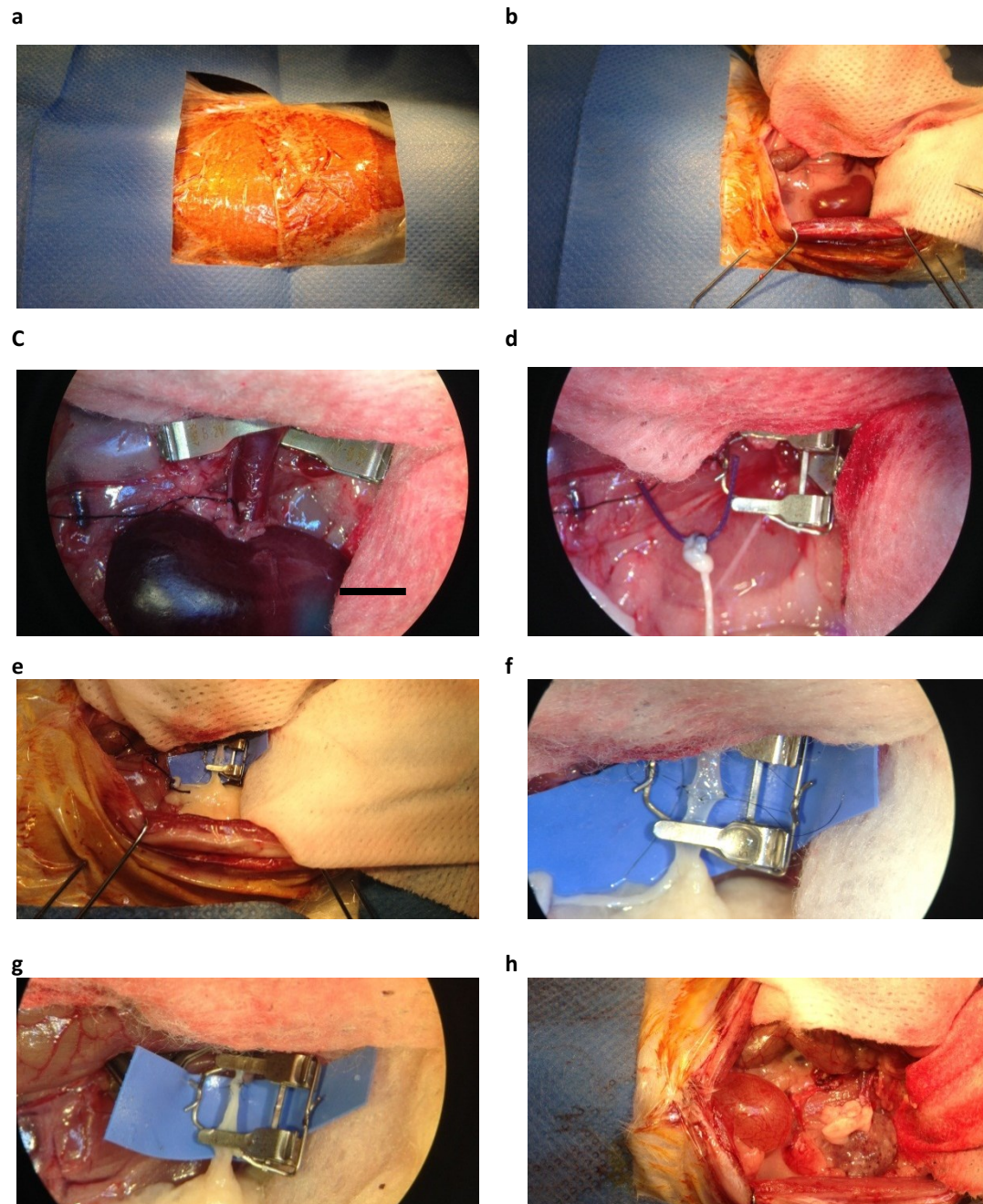


Figure 17 Surgical procedure for implantation of kidney grafts (acellular kidney scaffolds or recellularised kidney constructs) (a) Anterior abdominal wall, draped and prepped (subject's left side is to the bottom of image); (b) left native kidney exposed *in situ* within the peri-renal fat; (c) microscope view: mobilised and clamped renal vessels (vein is anterior/partially obscuring artery); (d) microscope view: native kidney has been removed, double vascular clamp applied to recipient vein (partially retracted), kidney graft at bottom with graft renal artery visible (suture marker); (e) double clamp applied to both renal vein segments for end-to-end anastomosis; (f) microscope view: renal vein end-to-end continuous suture anastomosis in progress with stay sutures either side; (g) microscope view: completed renal artery anastomosis (also end-to-end continuous suture); (h) perfusion of kidney graft with both patent anastomoses. Scale bar (microscope view only) = 5 mm.

4.3.5 Explantation of whole kidney grafts

Implanted kidney constructs or acellular scaffold grafts were removed at the designated time point (7 days or 6 weeks post-operatively) immediately after euthanasia of subjects. A median laparotomy was performed and the abdominal cavity inspected for evidence of post-operative complications or any other findings. The kidney implant was identified and dissected free from the surrounding fat and removed completely after ligation of the renal vessels and ureter.

4.3.6 Histology and immunohistochemistry

All samples were fixed in 10% v/v buffered neutral formalin for at least 24 hours, and samples were stained with haematoxylin and eosin (H+E) for light microscopy to evaluate cellular engraftment on the whole kidney bioscaffold. IHC was performed to characterise inflammatory cell types and their infiltration of the explanted acellular kidney scaffolds and the protocol used was the same previously (Section 2.2.3). Primary antibodies and dilutions used were as follows: CD34 at 1:100 and CD68 at 1 μ g/ml (ab64480 and ab125212 respectively, both Abcam, UK).

4.3 Results

Surgical *in vivo* implantation of three types of kidney graft was planned: acellular kidney scaffolds, PRC-recellularised constructs, and MSC-recellularised constructs, and for two durations (7 days and 6 weeks). The surgical procedure is exactly analogous to that for orthotopic renal transplantation and this was successfully carried out in a number of cases using the Lewis (an inbred strain) rat model. Tissue handling and surgical manipulation of both acellular and recellularised grafts were straightforward, with no significant modification of the transplant procedure required. Constructs were noted to be slightly less robust in terms of tissue handling, and the vascular structures in the acellular scaffold in particular were slightly more fragile, but overall there was no significant difference to surgical manipulation. Well-perfused grafts did not lead to significant haemorrhage or extravasation of blood due to the 'leakiness' of the vascular network that has been previously noted, even in the acellular scaffolds.

However, one major adverse finding became apparent early on in those cases where vascular patency was established by anastomosing renal vessels from graft to host/recipient with subsequent immediate perfusion of the implanted graft (i.e. as immediately observed). In these cases, a consistent pattern of early mortality in the recipient occurred. This ranged from about 2 hours to 12 hours (overnight) post-recovery. Although sample numbers were very small due to the need to minimise mortality and pre-empt futile procedures, it was empirically noted that the endothelialised or recellularised grafts were associated with a longer period of survival compared to the acellular scaffold i.e. the two groups were observed to have been polarised to either mortality within about 2 hours or mortality between 8 to 16 hours (overnight).

When the initial cases of premature mortality were observed (which were in acellular grafts that were immediately well-perfused), it was postulated that this was likely due to rapid thrombosis of the graft leading to a thrombo-embolic cause of death. Hence an acellular

graft was implanted in exactly the same manner but deliberately not allowing perfusion of vessels (i.e. non-patent vascular anastomoses), in order to test this hypothesis. This resulted in no obvious adverse effects to the recipient apart from those associated with the surgical procedure. The animal recovered quickly both from the anaesthetic and in general, and was assessed to be healthy for the duration of the implantation period (up to 6 weeks), and no intra-abdominal or systemic complication was observed at time of explantation. Hence the only experimental group to be completed in terms of graft implantation and survival was the acellular control group with non-perfusion although one fully recellularised and two endothelialised (acellular) grafts were also implanted (resulting in non-viable subjects). The perfused 'cellular' graft cases (whether endothelial graft alone or fully recellularised) were empirically noted to survive longer post-operatively than the perfused acellular group.

Table 10 shows details and numbers of constructs successfully implanted.

Each test group will be further discussed individually below.

Table 10

Final completed experimental groups and numbers.

	Experimental group			
Time period	PRC- recellularised graft	MSC- recellularised graft	Acellular graft	
			Non-perfused	Perfused +/- LVECs
7 days	-	(1)	4	(3 + 2)
42 days	-	-	4	-
	8 (+ 6)			

LVECs = Lewis vein endothelial cells

() = non-viable

4.3.1 Acellular kidney bioscaffolds (control group)

These implants were carried out both perfused and non-perfused, and either completely acellular or endothelialised. The only group to survive to the designated time endpoint(s) was the acellular non-perfused group.

Initially three well-perfused acellular grafts were implanted (individually), however none of these consistently survived beyond 2 to 3 hours post-operatively. The recipients were recovered from the general anaesthetic and were initially stable cardiovascularly and on pulse oximetry monitoring. However, there was a subsequent rapid deterioration with a significant decrease in oxygenation levels prior to their demise. On exploration of the abdomen immediately post-mortem, there was no evidence of bleeding or other surgical complication. The graft was noted to be thrombosed, presumed pre-mortem.

The non-perfused acellular grafts were then implanted with survival of all recipients until the designated time endpoint. There were no adverse effects seen with these cases except for the initial post-operative period, after which they were assessed to be normal and healthy in appearance and behaviour with the appropriate weight gain. These grafts were implanted for both time periods being assessed, 7 days and 6 weeks. At explantation, exploration of the abdomen revealed no obvious evidence of surgical complication, the kidney graft (scaffold) was clearly visible in the renal region within the peri-renal fat. The size of the graft was visibly different in the two time periods, being close to the original size at 7 days and significantly shrunken at 6 weeks. Viability testing was not carried in these grafts due to their being non-perfused and completely acellular originally. The histological analysis of these grafts follows in Section 4.3.4.

Endothelialisation only of the acellular scaffold was carried out in order to attempt perfusion of the acellular graft while decreasing the risk of graft thrombosis, and two endothelialised grafts were implanted. However, these cases also led to early mortality, although this

appeared to be at a later stage than with the completely acellular grafts, both cases surviving for at least 6 hours post-operatively.

(Endothelialised non-perfused scaffolds were not tested.)

4.3.2 PRC- recellularised constructs

These implants were not carried out due to the consistent problem with severe thrombo-embolic complications secondary to successful perfusion of grafts.

4.3.3 MSC-recellularised constructs

One MSC-recellularised graft (with endothelialisation) was implanted surgically with vascular perfusion. This also appeared to follow the same pattern of early mortality overnight following implantation, due to presumed thrombo-embolic complications. On exploration of the abdomen post-mortem, there was no evidence of any other obvious cause of death such as bleeding or other surgical complication.

4.3.4 Histology and IHC

Histological analysis of the explanted acellular grafts at 7 days and 6 weeks showed a consistent dense inflammatory infiltrate ([Figure 18](#)) persisting from implantation (or between 0 and 7 days post-implantation) until at least 6 weeks. While the microscopic appearance of the infiltrate itself is not significantly different between the two time points, there is clearly a very significant decrease in overall size of the sample/scaffold. The histological appearance also shows that the densest areas of inflammatory infiltration is in a sort of peripheral zone or 'band', suggesting the gradual influx of white cells possibly via diapedesis from the surrounding tissues into the substance of the kidney ECM scaffold. These appearances are all consistent with an inflammatory-mediated ECM degradation process.

IHC confirms the presence of multi-lineage inflammatory cells (both CD34 and CD68 positive) within the dense infiltrate on the acellular scaffolds post-implantation ([Figure 19](#)). Similar appearances are seen in both the 7 day and 6 week samples in terms of antibody binding and distribution within areas of the dense infiltrate, particularly around the periphery, except that the tissue sample is significantly diminished in the later samples, as already noted.

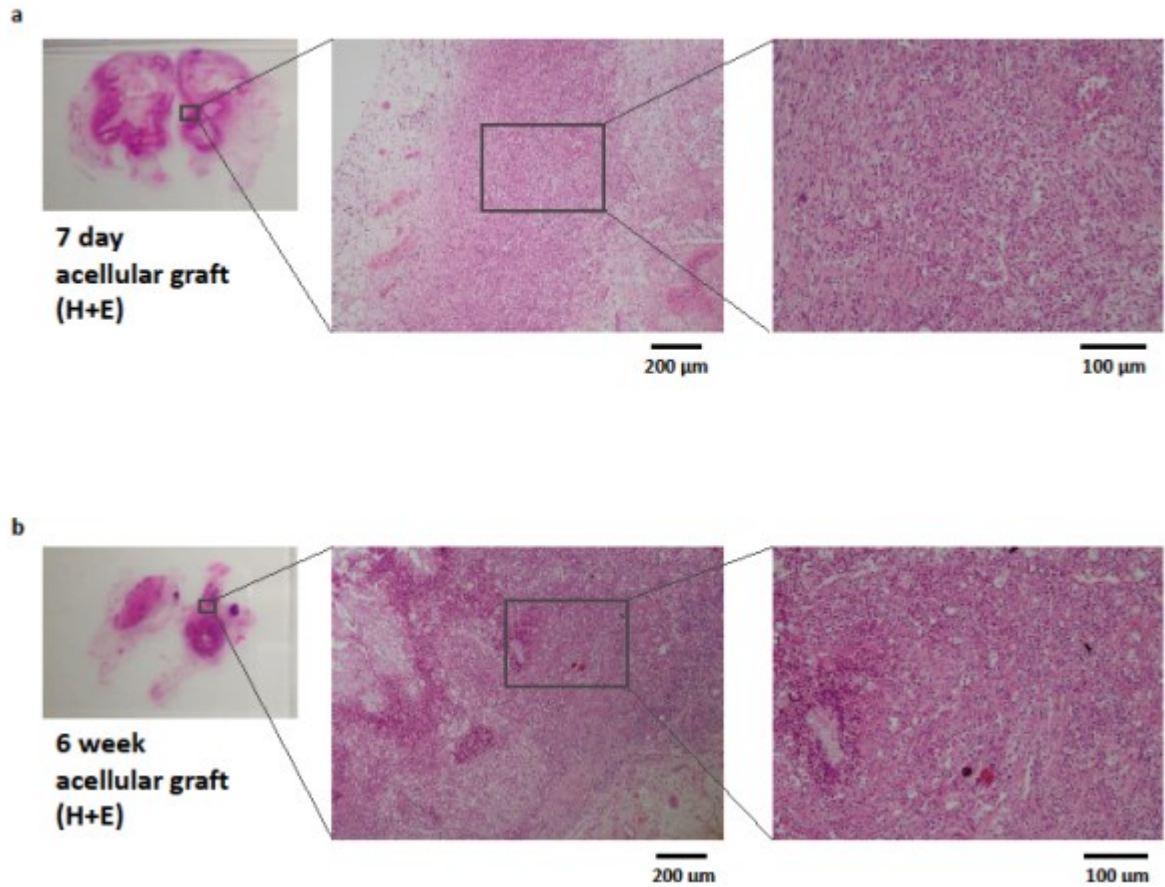


Figure 18 Histology (H + E staining) of the explanted acellular grafts at 7 days and 6 weeks.

(a) Whole acellular graft explanted at 7 days, duplicated sample shown in hemi-section as a 'clamshell'. There is a dense cellular infiltrate seen throughout the sample where the kidney outline is still apparent i.e. superiorly and less present in areas where the ECM scaffold has already been degraded i.e. the inferior pole.

(b) Whole acellular graft explanted at 6 weeks, duplicated sample shown in hemi-section. While the microscopic cellular infiltration is very similar in appearance to the 7 day grafts, the overall size of the graft/scaffold is significantly shrunken, consistent with the ECM degradation process.

For all samples, $n \geq 3$.

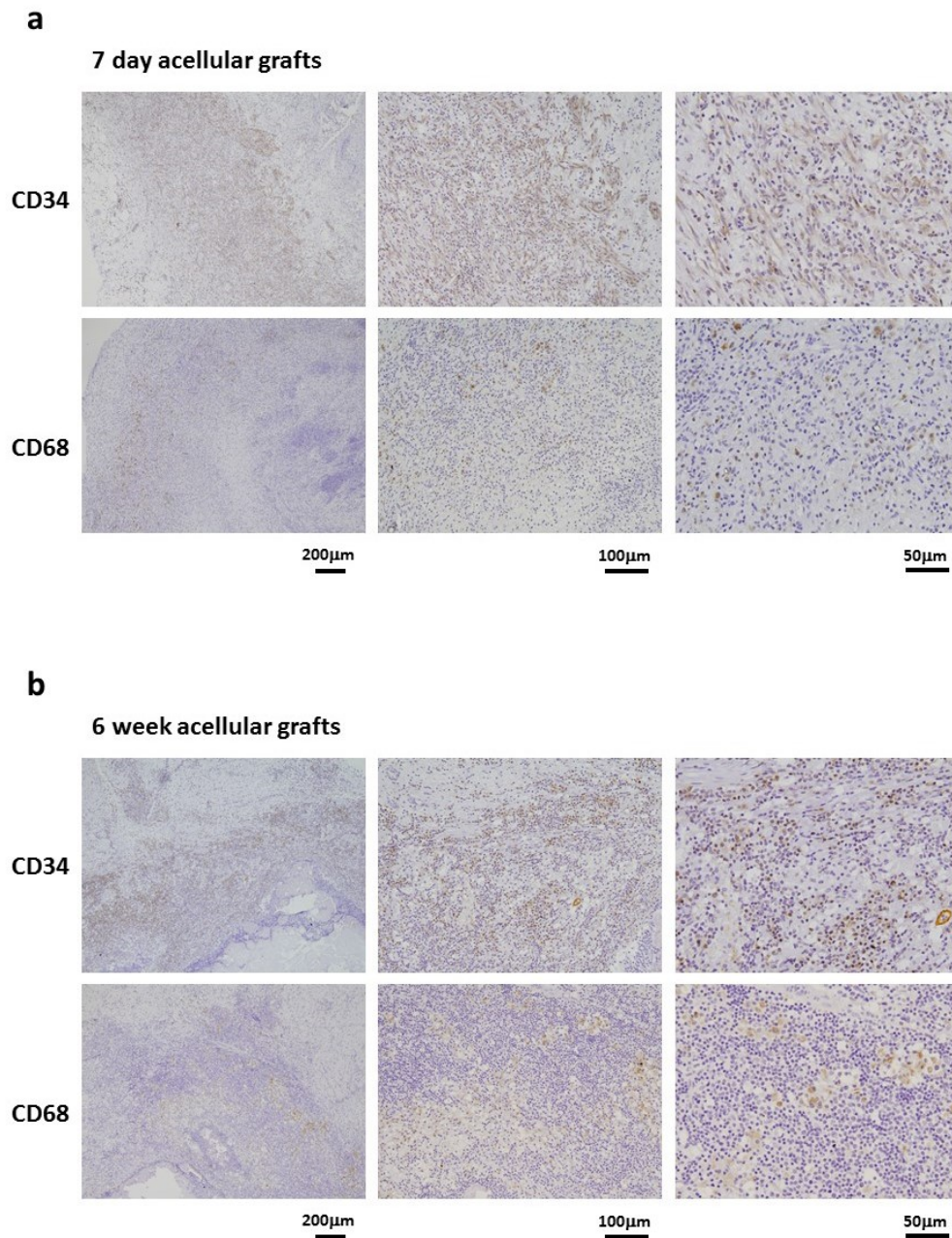


Figure 19 IHC for inflammatory markers in the explanted acellular grafts at 7 days and 6 weeks.

(a) Whole acellular graft explanted at 7 days. CD34 and CD68 antibody binding are both strongly positive in the densely cellular 'peripheral zone' of the graft.

(b) Whole acellular graft explanted at 6 weeks. Similar appearances in the dense inflammatory infiltrate although the whole sample is more than halved in size (Figure 18).

For all samples, $n \geq 3$.

4.4. Discussion

It has been shown that surgical implantation of both acellular and recellularised whole kidney grafts in the rat model is technically very achievable using microsurgical techniques with whole organ decellularisation. The anatomical preservation of vascular structures as well as whole organ scaffold architecture make these grafts easy to handle and surgically manipulate. There is very little, if any, modification of established transplantation procedures required. Even the acellular scaffold tissue is robust enough to allow surgical handling and suturing, and the 'leakiness' of the scaffold did not lead to any significant bleeding or extravasation. In this study, a more orthodox microsurgical technique was used whereby vascular anastomosis is established with end-to-end suturing, as compared with the 'modified cuff' technique employed in the single other kidney *in vivo* study by Song *et al*[14]. This suturing technique may possibly provide improved vascular patency in the long term due to less foreign material at the site of the anastomosis and hence minimizing the potential for stenosis; while this was not tested specifically due to other issues, certainly immediate vascular patency and perfusion was demonstrated.

Within this study, one major adverse issue was encountered with regards to the early thrombosis of perfused grafts, even leading to a presumed thrombo-embolic cause of death in the recipients. This is not an issue that has been highlighted previously as the recipients of kidney grafts were not allowed to 'recover' from surgery in Song *et al*, although the reasons for this are not specified. The only other longer term implantation study (which was in the lung) did not appear to have encountered this problem, nor did they employ systemic anti-coagulation [12] which might be another strategy to counter graft thrombosis. However, the lung construct that was implanted did differ in terms of being partially functional and hence presumably more closely approximating normal tissue in its properties, including being at least significantly less thrombogenic. The lung graft in this study only survived *in vivo* for 7 to 14 days and hence slower graft thrombosis could also have contributed to its decline,

although this is not specifically mentioned, nor any issues with recipient mortality secondary to implantation [12].

Thrombogenesis is certainly an important physiological issue with regards to vascular perfusion and tissue/graft implantation, and it may be that any tissue that is sub-normal (especially with regards to the vasculature) would lead to this problem. Systemic heparinisation could be attempted in order to overcome this issue, but this would be challenging to maintain in the rat model if animals were to be allowed to recover normally post-operatively, as well as the potentially long duration this may be required. It is also debatable whether even high levels of systemic anticoagulation would fully pre-empt the very severe acute thrombosis i.e. leading to mortality within 2-3 hours post-operatively seen with the acellular grafts. Therefore these findings may suggest that further optimisation of the recellularisation process resulting in a more functional and physiologically normal tissue construct is necessary. The empirical findings in this work that perfused 'cellular' grafts were associated with a longer period of survival (and likely perfusion) than the completely acellular grafts are consistent with the hypothesis that this is due to the cellular grafts being closer to normal tissue and hence less thrombogenic. While the regenerative properties of whole organ ECM bioscaffolds implanted *in vivo* (with perfusion) have been speculated upon, the current study would seem to indicate that they remain theoretical at present.

In this study, the only test group to be successfully implanted was the non-perfused acellular group (as non-perfused recellularised grafts were considered futile), and these did show the acellular whole kidney bioscaffold to be host-compatible and to undergo immune-mediated ECM degradation in a consistent and predictable manner. Histology and IHC of all implanted grafts at 1 week and 6 weeks post-implantation show a dense multi-lineage inflammatory infiltrate associated with a significant decrease in the overall size of the kidney (ECM) graft, consistent with an immune-tolerant degradation process which would be desirable in any tissue engineered grafts of ECM origin, especially as a vital adjunct to the process of

constructive remodeling. Since the other test groups involving recellularised constructs could not be completed, any regenerative effects of constructive remodeling to be seen with recellularised whole kidney bioscaffolds must also remain theoretical at present. The single study with a 'test' implantation in the kidney performed by Song *et al* demonstrated the functionality of their recellularised construct with perfusion *in vivo*, as demonstrated by the observation of 'urine production' (draining via the construct ureter) during the implantation period [14]. Long-term viability or regeneration of renal tissue *in vivo* was not apparently investigated.

Non-perfused acellular scaffolds have been previously implanted in skeletal muscle[128], and they also showed an inflammatory response and cellular infiltration. However in that study, the muscle scaffolds were explanted after only 2 weeks and at least some normal muscle tissue remained at the implantation site as a potential source of muscle and progenitor cells to migrate into the ECM scaffold, in combination with avoidance of normal leucocyte-mediated inflammatory responses by using athymic mice. In this thesis, the acellular kidney scaffolds implanted without perfusion were degraded in a consistent manner, without replacement of the ECM, over a much longer period (i.e. up to 6 weeks). It would seem likely that the kidney scaffold would have been completely degraded at an even later time point and hence demonstrates the biocompatibility of the ECM bioscaffold.

4.5 Further work

Since perfusion is the critical issue for these whole organ recellularised constructs to serve their function *in vivo*, and while the anatomical considerations for achieving this are clearly present, further difficulties exist in maintaining perfusion in a normal physiological manner. Although the issue of graft thrombosis leading to subsequent systemic thrombo-embolic complications was not previously documented in the limited number of *in vivo* studies extant, one study notably maintained graft survival of a lung construct without employing systemic heparinisation for a period of 7 to 14 days [12]. However, the causes of graft loss after this period were not specifically explored. Perhaps key differences between the early mortality observed in our study (and presumed acute graft thrombosis) and the longer survival in the study by Song *et al* lie in the partially functional graft that they achieved prior to implantation, as well as their specific lung ventilation strategies to aid graft survival. Undoubtedly a more functional construct would approach normal physiological tissue more closely, and hence allow more normal vascular flow and perfusion.

While adjuncts and strategies for improving anti-coagulation in the animal model and ensuring optimal surgical technique will be helpful during the pre-clinical stage of testing these whole organ recellularised constructs *in vivo*, graft survival long term surely depends most on achieving optimal recellularisation and cellular/tissue functionality. The closer a bioengineered graft approaches the organ it is intended to replace, the more likely it will be to achieve this purpose. While ECM scaffold materials have already been employed clinically in many applications, these have been essentially structural and not predominantly 'physiological' in function, nor have they been perfused with their own inherent vasculature. While cellular infiltration and tissue 'transformation' of the implanted graft into the native tissue in question (e.g. muscle, tendon, cartilage) do occur after implantation *in vivo* [67-70], there are still adjacent sources of these native cells *in situ* i.e. the defect or lack of the tissue is not complete, in contrast to solid organ replacement.

Hence, it would seem that optimisation of the recellularisation process to achieve constructs of greater functionality is of primary importance, despite the theoretical interest and potential in implanting just whole organ ECM scaffolds *in vivo*. From our study, it would seem that cell recruitment and subsequent differentiation into appropriate tissue purely from *in vivo* bioinductive cues acting on the whole organ ECM bioscaffold are not really feasible both in terms of maintaining vascular perfusion (given their severe thrombogenicity), and non-perfusion merely leads to an immune-mediated degradation process as would be expected.

Chapter 5

Conclusions and future work

5.1 Optimisation of parameters in perfusion decellularisation of whole kidney

The current applications and methods in using xenogeneic whole organ ECM scaffolds to create potentially functional bio-artificial organ constructs for surgical implantation [9-14, 111-128] have been systematically reviewed in this thesis. After comparison of methods and specific trends within this new and developing technique [129], it has been shown that there is a need for a more evidence-based approach as well as a need for pre-optimisation and standard operating procedures, in order to yield consistent and optimised bioscaffolds potentially destined for clinical application.

On this basis, the aim was to optimise the decellularisation process for whole kidney ECM bioscaffolds with regards to two fundamental parameters: concentration of decellularising agent and duration of perfusion, using the most commonly used single decellularising agent drawn from the literature i.e. sodium dodecyl sulphate [129]. Optimisation was determined with regards to structural and functional characteristics of the ECM bio-scaffold as assessed by histology, immunohistochemistry, quantitative assays of DNA and sGAG content, and growth factor quantification.

This yielded an improved protocol (0.125% w/v SDS perfused for 4 hours) which uses a far lower concentration of SDS than other work on whole kidney decellularisation [14, 121, 124, 125] while being equally effective in achieving removal of cellular components and DNA. This has also demonstrated a clear relationship between decreasing exposure to SDS and preservation of *in situ* growth factors, down to the level where further decreases in the SDS concentration may not yield significantly increased preservation, which has not been previously shown. It is likely that these findings should also be applicable in general for any whole organ bioscaffolds and for any species, rather than being specific to the rat kidney as

such. This also leads the way to further optimisation possibilities, such as a parallel study in decellularisation of human or porcine kidneys for bioscaffolds that may be used for clinical application. The perfusion method itself seems ideally suited to the 'up-grading' process of moving towards larger human-sized organs and there seems to be no barrier to this being directly achievable – indeed, successful whole organ decellularisation of larger organs has already been demonstrated in a number of studies as previously outlined. However, optimised and systemically assessed protocols should be developed in all species and types of tissues suitable for clinical application.

There are many other decellularisation parameters that exist to be considered – such as other choices of decellularising agent (although since different types of tissue respond optimally to different agents, not all will need to be considered for the kidney), additional steps in the protocol e.g. freezing, use of other biological agents, tissue harvesting factors, continuous perfusion versus non-continuous, varying perfusion rates, perfusion pressure versus perfusion rate, total duration of protocols applied, and last but not least, post-processing sterilisation methods [129]. All these will inevitably affect the quality of the ECM bioscaffold to a greater or lesser degree, and these effects are as yet unquantified in a systematic manner for whole organ bioscaffolds. In addition, while growth factor levels may be better preserved by lowering the concentrations of the decellularising agent used (as have been demonstrated), functional tests of bioactivity should also be utilised for more complete characterisation. Although this is a challenging area within the study of ECM bioscaffolds, there are some combined applied ECM and developmental approaches to demonstrate the effects of ECM components on the kidney using embryonic organ culture [138, 139].

Another consideration or requirement of decellularisation which may require greater stringency in the clinical arena is that of antigen removal – while decellularisation is assumed

to be the mainstay underlying immune-compatibility in xeno-derived ECM materials, more detailed and standardised assessment of antigen removal will be necessary [140, 141].

These are all areas for future improvement and should play a critical part in developing safe, reliable and consistent whole organ bioscaffolds if they are to be taken to the clinical stage in the development of regenerated organ replacements.

5.2 Optimisation of recellularisation of whole kidney bioscaffolds

In this study, the aim was to recellularise whole kidney bioscaffolds (decellularised using the optimised protocol as outlined previously) using two cell populations, primary renal cells and mesenchymal stem cells, in order to demonstrate proof of concept with a primary cell population and investigate the viability of an 'adult-derived stem cell' population as an alternative cell source for recellularisation. Similar numbers of cells (i.e. ~50 million) to other recellularisation studies using large primary cell populations[129] and also an intravascular injection seeding protocol as has been shown to be the most efficient seeding method in terms of cellular engraftment and density[119, 121], were chosen and utilised for this recellularisation protocol. Both types of recellularised kidney construct were successfully cultured *in vitro* for a period of 7 days. Viability testing with histological analysis have demonstrated high degrees of cellular engraftment throughout the specimens with formation of renal-like structures in response to the ECM architecture and structural cues within the kidney scaffold, however no complex renal differentiation as to suggest any functionality was observed at this stage.

There are many variations in the recellularisation protocols used throughout the literature and there is also a lack of systematic pre-optimisation for recellularisation studies currently. Parameters for rationalisation and optimisation include: seeding route and mechanism, *in vitro* culture conditions (bioreactor setup, culture medium +/- appropriate supplementation, perfusion rate, and duration of culture), choice of cell populations (including endothelial cells) and total numbers used for recellularisation, and choice of optimised decellularised organ bioscaffold. Regarding the last factor, certainly a logical continuation of the decellularisation optimisation work that has been carried out here would be to investigate the differential effects (if any) of cellular engraftment or differentiation due to the different decellularisation protocols that have been investigated in Chapter 2. One study has also investigated additional 'priming' or pre-conditioning of the whole organ decellularised

scaffold with ECM components e.g. laminin, fibronectin, in order to promote cellular adhesion (of ESCs) to the decellularised lung scaffold[115].

The seeding route and mechanism may be the most straightforward to assess. The predominant study in the kidney suggests use of the ureteric route and has demonstrated proof of concept with a partially (up to 10%) functional graft[12], hence it would be interesting to compare intravascular/intra-arterial seeding with ureteric using the exact same protocol and setup in order to see the differential effects on cellular engraftment and differentiation. Other studies have previously shown that the intravascular injection method was the most efficient for seeding[119, 121], but the data overall is very limited.

In this thesis, the use of adult marrow-derived mesenchymal stem cells in order to ascertain viability and cellular engraftment on the whole kidney scaffold has also been investigated. MSC-recellularised constructs show similar appearances to the PRC-recellularised scaffolds in terms of dense cellular engraftment, but may require further cellular and differentiation cues to further develop any renal cell attributes. They remain a potential option for recellularisation strategies in clinical application. There are also as yet many unexplored areas in cellular and scaffold pre-conditioning or manipulation that could promote recellularisation in whole organ decellularised scaffolds, in conjunction with choice(s) of cell population used.

Recellularisation remains the most complex and challenging area for investigation in order to achieve significantly functional whole organ grafts suitable for *in vivo* implantation, even at the level of the small animal model – these limitations are literally amplified exponentially in order to achieve a bioengineered organ replacement with potential clinical application. However, perhaps once these challenges are resolved in principle with a small animal model, it may become more simply a ‘technical’ issue in up-scaling these techniques for the clinical context.

5.3 Implantation of recellularised kidney constructs in the *in vivo* rat model

It has been shown here that surgical implantation of both acellular and recellularised whole kidney grafts in the rat model is technically very achievable using microsurgical techniques with whole organ decellularisation. The anatomical preservation of vascular structures as well as the whole organ scaffold architecture make these grafts easy to handle and surgically manipulate with minimal modification of established transplantation procedures required. Vascular patency and immediate perfusion can be achieved with surgical anastomosis of the renal vessels at the orthotopic site. In this study the primary consideration that arose after perfusion was acute thrombosis of the graft, leading to severe thrombo-embolic complications. However, these empirical findings that 'cellular' grafts were associated with a longer period of survival (and likely perfusion) than the completely acellular grafts may imply that these are more closely approximating normal tissue and hence less thrombogenic. This would also be consistent with the only *in vivo* study with a longer-term implantation period (up to 14 days) where the recellularised lung construct was initially partially functional (i.e. closer to normal tissue)[12].

While ensuring optimal surgical technique and strategies for improving anti-coagulation in the animal model will be helpful during the pre-clinical stage of testing these whole organ recellularised constructs *in vivo*, graft survival long term surely depends most on achieving optimal recellularisation and cellular/tissue functionality. Hence, it would seem that optimisation of the recellularisation process to achieve constructs of greater functionality is the primary concern.

5.4 Future considerations for whole organ decellularisation work in the kidney

Whole organ decellularisation can yield whole kidney ECM bio-scaffolds capable of supporting recellularisation on a complex 3D level and sustaining partial cellular and tissue functionality, as shown in one study[14]. These findings have also been demonstrated here with successfully decellularised whole rat kidneys, furthermore using an optimised protocol in order to better preserve key ECM components; these kidney scaffolds were then recellularised and cultured *in vitro*, demonstrating high levels of cellular engraftment and viability. These bio-artificial organ constructs also allow relatively easy surgical manipulation, especially as they preserve the intact vascular pedicle for direct anastomosis at the orthotopic implantation site, in an exact parallel to the transplant procedure, as has been demonstrated as well.

However, all aspects of this technique still require further optimisation in order to achieve fully functional organ constructs that may be implanted *in vivo* long term, from the decellularisation process to recellularisation and understanding of cell-scaffold interactions so as to promote cellular differentiation and function, which are currently mostly unexplored. Innovations even in decellularisation (which is long-established) and scaffold production are still arising e.g. use of targeted apoptosis rather than chemical or biomechanical decellularising agents[142]. Whole organ 3-D imaging is also in its early stages, with limited use of traditional resin casting and radiological methods e.g. micro-CT. Newer techniques also remain to be explored and optimised further e.g. 3-D histology (Histocutter) as we have started to use, or perhaps even other innovative technology to obtain high-resolution information from a complex system e.g. CLARITY hydrogel technique [143].

Additional aims or milestones involved in developing the recellularisation process towards clinical application would be selection of the appropriate cell population(s) e.g. fetal mixed native cell populations would be unlikely to be a feasible option, appropriate cell culture

techniques for expanding and handling *in vitro* potentially very large numbers of cells (i.e. billions) in order to achieve physiological levels of recellularisation, immunogenic factors (although if the cell population is patient-derived e.g. autologous MSCs, then this would be less important), and developing standardized quality checks of organ function, (non-)toxicity, and sterility prior to implantation.

Nonetheless, whole organ decellularisation could provide an ideal short-cut to bio-engineering an organ replacement that is structurally and histologically similar to the original organ in question, uses established surgical procedures for implantation, is able to potentially support cellular function to the same degree, is biologically compatible and may be potentially bioengineered 'on demand' using patient-derived cells for regeneration.

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7) Appendices

7.1 3-D histology (Histocutter) setup and components

(Courtesy of Amanda Wilson, Imperial College London)

Camera = Apogee U-Series (from Apogee Instruments Inc) ALTA U16 s/n 091629 4096 x 4096 array, 9 x 9 micron pixels

http://ccd.com/alta_u16.html

Camera ports: Power | USB 2 | Serial A | Serial B | Aux (8-pin)

Lamp = X-Cite Q series 120PC (EXFO) Mercury Arc lamp

2000 hr lamp lifetime - no alignment required for lamp change (bulb replaced Jul 2012)

<http://www.ldgi-xcite.com/products-xcite-120-pc.php>

3mm X 1500mm X-Cite 120 liquid light guide (last replaced Nov 2012)

From Imsol Image Solutions <http://www.imsol.co.uk>

Stage = LG Motion Ltd model # AXY2512b-bx 0032544 (originally supplied by Velmexm, USA).

The size is 1' 3/4" x 1' 3/4" (i.e. just under 13 inches square; matches bench (Imperial scale)

Contact Gary Livingstone/Leyton Chapfield at LG Motion Ltd: Tel: +44 (0) 1256 365600 Email: info@lgmotion.co.uk

Microtome Leica RM2265 Fully automated rotary microtome

<http://www.leica-microsystems.com/biosystems/products/total-histology/sectioning/rotary-microtomes/details/product/leica-rm2265/>

Ref 43136072 | equip number 10899594 | serial number 595

Microscope Nikon AZ100

http://www.nikoninstruments.com/en_GB/Products/Microscope-Systems/Upright-Microscopes/Biological/AZ100-Multizoom

MNH85200 AZ-Plan Fluor 2x N.A. 0.2, W.D. 45mm £1,589 MNH85400 AZ-Plan Apo 4x N.A 0.4, W.D 20mm £2,115

Software = Cutter Master CW(Java) Image stack 4096 x 4096 pixels, 8bit (256 grey levels), PNGs (non-lossy compressed images).

Microscope Filter Sets

SLOT 1: FITC HQ (blue/green excitation, green emission) 41001

C128429 ex = HHQ480/40X (Bandpass 460-500) dichroic = 505 (Long Pass) em = HQ535/50m (Bandpass 510-560)

SLOT 2: Texas Red HYQ (green excitation, red emission) 41004

C128430 ex = 560/55 (Bandpass 532-587) dichroic = 595 (Long Pass) em = 645/75 (Bandpass 608-683)

SLOT 3: UV (uv/purple excitation, blue emission) XF403 filter set

Ex = 365QM35 (Bandpass 348-383) Dichroic = 410 (Long Pass) Em = 450QM60 (Bandpass 420-480)

SLOT 4: TEMPORARY CY5 CUBE

Fluorochrome notes:

FITC fluorochrome ex 490/em 525 TR fluorochrome ex596/em615 Rhodamine B dye absorption peak 544 (although excited from ~450 to 580), emission 566 (although emits from ~ 520 - 700) Dapi fluorochrome ex350/em 470 Alizarin Red: ex 530-560, em 580 Calcein: ex 495, em 515. All values for wavelengths in nm.

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Comparison of Methods for Whole-Organ Decellularization in Tissue Engineering of Bioartificial Organs

Ming He, MBBS,¹ and Anthony Callanan, Ph.D.^{1,2}

Organ transplantation is now a well-established procedure for the treatment of end-stage organ failure due to various causes, but is a victim of its own success in that there is a growing disparity in numbers between the donor organ pool available for transplantation and the patients eligible for such a procedure; hence, an alternative solution to the limited donor organ pool is both desirable and necessary. Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of functional replacement tissues for clinical use. A recent innovation in tissue and organ engineering is the technique of whole-organ decellularization, which allows the production of complex three-dimensional extracellular matrix (ECM) bioscaffolds of the entire organ with preservation of the intrinsic vascular network. These bioscaffolds can then be recellularized to create potentially functional organ constructs as a regenerative medicine strategy for organ replacement. We review the current applications and methods in using xenogeneic whole-organ ECM scaffolds to create potentially functional bioartificial organ constructs for surgical implantation, and present a comparison of specific trends within this new and developing technique.

Introduction

ORGAN TRANSPLANTATION IS NOW a well-established procedure for the treatment of end-stage organ failure due to various causes, but is a victim of its own success in that there is a growing disparity in numbers between the donor organ pool available for transplantation and the patients eligible for such a procedure, with a high mortality rate in those who are on the waiting list. While there is ongoing debate to address this issue with strategies, both within a wider social or legislative context (e.g., so-called opt-out or presumed consent organ donation¹) and others more specifically applicable to certain groups (e.g., living kidney donation), these strategies can be either controversial or not without additional clinical risk (e.g., to the living donor). An alternative solution to the limited donor organ pool is both desirable and necessary.

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of functional replacement tissues for clinical use. Many developments within this field employ the seeding and cultivation of cells onto acellular scaffolds; however, control and utilization of the types of cell, scaffold material, interactions between the two, and the optimal conditions for the regeneration of functional tissue replacement are complex and vary from the nano- to macroscale.² One group of biological scaffold materials that are already commonly in

use for a variety of reconstructive surgical applications is that derived from the extracellular matrix (ECM).³ The ECM is secreted by the resident cells of each tissue and organ, and scaffolds derived from this material are produced by the process of decellularization of the specific tissue.⁴

A recent innovation in decellularization is the generation of whole-organ ECM scaffolds.^{5–9} This is a new technique in the application of decellularization agents (e.g., detergents) by using antegrade or retrograde perfusion of the inherent vascular network within the organ. This technique has led to the production of so-called bioartificial scaffolds^{10,11} that preserve the three-dimensional (3D) ECM structure of an organ in its entirety.^{10–27} This complete 3D structure gives site-specific architecture and composition, which control function at a local level within the organ. This scaffold can then be subsequently repopulated with cells using the same perfusion technique to produce organ regeneration to a functional degree. This type of scaffold combines the natural advantages and properties of the ECM in promoting and regulating cell proliferation and differentiation, the organizational and architectural complexity of the whole organ, as well as the supportive vascular network for the provision of oxygen and nutrients required for 3D tissue metabolism. This work has been carried out with preliminary success in animal studies of the heart,¹⁰ the lungs,^{11,14,21} and the liver,¹⁷ and the decellularized whole-organ scaffold has also been

¹Department of Bioengineering and Materials, Institute of Biomedical Engineering, Imperial College London, London, United Kingdom.

²Centre for Applied Biomedical Engineering Research (CABER), Department of Mechanical, Aeronautical and Biomedical Engineering, and Materials and Surface Science Institute, University of Limerick, Ireland.

produced in the kidney,^{18–20} as well other tissues such as small bowel²⁶ and skeletal muscle.²⁷

We review the current applications and methods in using xenogeneic whole-organ ECM scaffolds to create potentially functional bioartificial organ constructs for surgical implantation, and compare specific trends within this new and developing technique to give a concise overview of existing experimental work across all the relevant organ systems as a tool for researchers.

The ECM

The ECM is the naturally occurring scaffold material secreted and manufactured by the resident cells of each tissue and organ. The complex 3D organization of the ECM and its components are dictated by the tissue from which the ECM is derived and can be considered specific to that tissue or organ^{28–30}; however, individual components are common throughout most tissues such as collagen, laminin, fibronectin, and hyaluronic acid. The structural and functional molecules of the ECM are in a state of dynamic equilibrium within the surrounding microenvironment,³¹ and also provide the means by which cells communicate with each other and the external environment.^{32,33} By definition, the ECM possesses the ideal characteristics of the tissue-engineered scaffold or biomaterial (in addition to the functions already described), being biocompatible, a supportive medium for blood vessels, nerves, and lymphatics and for the diffusion of nutrients from the blood, as well as being able to undergo constructive remodeling and degradation within the body's own systems.^{34–36}

The Properties of ECM

Bioinductive properties of ECM

The ability of the ECM scaffold to facilitate and integrate cellular and external environmental cues is the consequence of its bioinductive properties, allowing the constructive remodeling of tissue after the *in vivo* implantation of ECM scaffolds.^{37–39} This remodeling cannot be solely attributed to characteristics such as viscoelastic behavior, biomechanical properties, or host cell attachment through collagen, fibronectin, and laminin ligands within the ECM. Growth factors such as vascular endothelial growth factor,⁴⁰ basic fibroblast growth factor,^{41,42} and transforming growth factor-beta^{44,45} are responsible for crucial events of remodeling such as angiogenesis, abundant host cell infiltration, mitogenesis, and deposition and organization of new host ECM. These can survive tissue processing and terminal sterilization^{41,43,44} to exert an effect on tissue remodeling and are released during the degradation of the ECM scaffold.^{40–45} Indeed, the degradation process itself, which is mediated by enzymatic and cellular processes, may be considered as a mechanism for controlled release of the ECM constituent molecules. The process of degradation and growth factor release continues until the scaffold is completely degraded. Degradation products of the molecules that constituted the ECM may mediate a subsequent series of remodeling events. These subsequent events include the release of cryptic peptides that initiate and sustain the recruitment of circulating, bone marrow-derived undifferentiated progenitor cells that actively participate in long-term tissue remodeling,^{46,47} the

generation of antimicrobial peptides that protect the remodeling site from pathogens,^{48–53} other peptides that modulate angiogenesis, and the recruitment of endothelial cells over periods up to 6–8 weeks.⁵⁴

Biomechanical properties of ECM

The mechanical properties of the ECM can be predominantly understood from the combination of its collagen fiber architecture and kinematics. The tissue from which an ECM scaffold is harvested will define its structural characteristics (such as fiber size, orientation, and alignment) and mechanical properties. For instance, small intestinal submucosa (SIS)-derived ECM has been shown to have a preferred fiber alignment in a spiral arrangement along the longitudinal axis of the small intestine,^{40,55,56} and it is likely that this structural arrangement facilitates dilation and retraction of the small intestine during peristalsis and transport of intraluminal contents. An understanding of the collagen fiber structure from each organ is important to closely match the scaffold mechanical properties to those of the intended target organ.

Degradation and constructive remodeling of ECM

The bioinductive properties of ECM scaffolds depend crucially on the efficient and effective degradation of the scaffold material and facilitate the constructive remodeling of injured tissue. The species of origin, tissue of origin, and processing techniques during the production of the ECM scaffold can differ markedly and factors such as method of decellularization, use of chemical crosslinking agents, and means of sterilization can affect the degradability and host response to the scaffold material.⁵⁷ Quantitative studies of ¹⁴C-labeled SIS used in augmentation cystoplasty⁵⁸ and Achilles tendon reconstruction⁵⁹ procedures have shown that 50% or more of the ECM scaffold is degraded and removed from the implantation site by 28 days. The degradation products are excreted via the urine with no recycling to other tissues. Furthermore, the replacement of the degraded SIS with functional (and similar to normal) host tissue occurred without the loss-of-function, for example, bladder or tendon rupture suggests a rapid infiltration and/or proliferation of functional host cells at the remodeling site with deposition of new ECM. In these two models of degradation analysis, there was also the critical involvement of biomechanical factors such as physiological bladder filling and emptying³⁸ or progressive weight bearing postoperatively in the tendon repair model.³⁴ Environmental factors may also play an important role in determining *in situ* regeneration or transformation of implanted tissues, such as that seen in the formation of host-derived tracheal tissue within a long segment of allogenic aortic graft transplanted as a potential conduit for tracheal replacement.^{60,61} Hence, understanding of the additional factors that modulate remodeling is also essential to utilize ECM scaffolds effectively.

Host immune response to xenogeneic ECM

Most ECM scaffold biomaterials and commercially available surgical implants are of xenogeneic origin, such as a porcine or bovine source with a few types from human allogeneic tissue.³ Nonhuman biomaterials have been used in humans for many years without evidence of adverse

host immunological outcomes, for example, porcine heart valve replacements and porcine and bovine insulin for the treatment of diabetes mellitus. Xeno- and allogeneic cellular antigens (e.g., cell membrane Gal epitope) are by definition recognized as foreign by the host and can induce a hyperacute rejection response after organ xenotransplantation; however, components of the ECM are generally conserved among species and tolerated well. With the SIS ECM, complement activation or cell-mediated rejection response does not occur even with the small amounts of Gal epitope that are present,^{62,63} and this could be further prevented with either use of transgenic gal knockout animals for tissue harvesting or treatment of harvested ECM with galactosidase.

In terms of the cell-mediated immune response, the Th1 lymphocyte phenotype is associated with macrophage activation, complement fixation, and CD8+ cytotoxic cell differentiation, and activation of the Th1 pathway is implicated in transplant rejection.^{64,65} In contrast, activation of the Th2 lymphocyte pathway does not lead to these events, differs markedly both in the profile of cytokines and antibody isotypes produced, and is hence associated with transplant acceptance.^{66,67} Implantation studies of the SIS ECM in mice have shown that a Th2 type immune response occurs, similar to that elicited by syngeneic muscle tissue.^{68,69}

The likely innate immune-mediated inflammatory response to ECM materials and bioscaffolds also plays an important role in their degradation *in situ* and subsequent constructive remodeling process, and this can be demonstrated by the macrophage phenotype response. Macrophages are phenotypically and functionally plastic, but can be broadly characterized into a polarized M1/M2 phenotype classification, which forms a parallel to the Th1/Th2 response in that M1 macrophages are associated with a proinflammatory and M2 with a constructive remodeling response.⁷⁰ The constructive remodeling process can be correlated with the ability of the implanted material to direct the macrophage phenotype, and the ECM has been shown to promote the switch from M1 to M2 after implantation.^{71,72} However, the use of chemical crosslinking agents that may interfere with the macrophage-mediated breakdown of the ECM material inhibits the beneficial M2 phenotype and leads to chronic inflammation and fibrosis.⁷³

Hence, it can be seen that the overall host immune response for any particular ECM material will be variable and dependent on the diversity of tissue sources and processing methods used in its preparation.

Decellularization and Processing of ECM Scaffolds

Because of the complexity of the 3D organization and composition of all the structural and functional molecules of the ECM that have not yet been fully characterized (and for each type of tissue), it is not currently possible to synthesize this biomaterial in the laboratory. Hence, ECM scaffolds are produced by the process of decellularization of naturally derived tissues, and this can be achieved by a variety of agents and techniques.³ The complexity and length of the decellularization protocol are correlated with the degree of structural and biological conservation required for the postprocessed tissue, especially for composite tissues and whole organs. Any agent or method will cause some disruption of the ECM composition and ultrastructure, and

minimization of these effects is desirable as complete avoidance is not yet feasible. While it is not possible to remove 100% of cellular material from the ECM, it is possible to quantitatively assay cell components such as DNA, mitochondria, and membrane phospholipids. Residual cellular and nuclear material may contribute to cytocompatibility problems *in vitro* and adverse host responses *in vivo*.^{74–77} It is also necessary to sterilize ECM scaffolds before *in vitro* use or implantation, as well as the removal of pyrogens (such as endotoxins and intact viral and bacterial DNA) that may be present.

Biological scaffolds may be sterilized by simple treatments such as incubation with acids or solvents, or other treatments such as ethylene oxide exposure, gamma-radiation, and electron beam irradiation. While the simple methods can lack penetration or may damage key ECM components,⁷⁸ the latter ones can affect ECM ultrastructure and mechanical properties,^{79–81} and newer methods such as supercritical carbon dioxide requires further investigation.⁸² Hence, this necessary process is another factor that can critically influence the properties of the bioscaffold, and must be taken into account during the wider-scale standardization and quality assurance of such ECM products before clinical translation.

Whole-Organ Decellularization: Perfusion-Decellularization

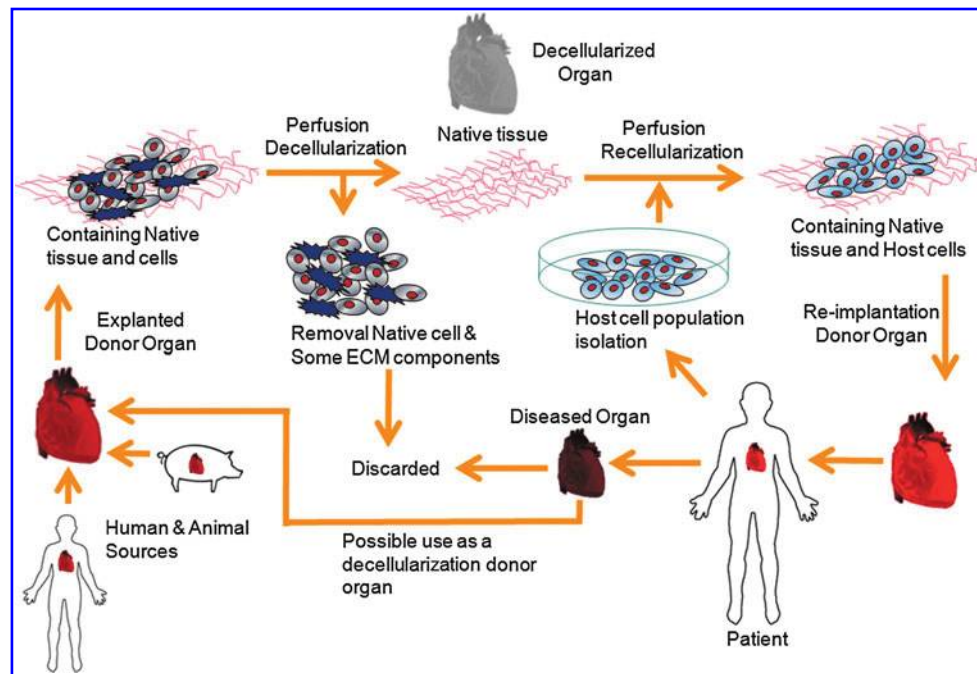
This is a relatively new technique that uses anterograde or retrograde perfusion via the intrinsic vascular network as a means of applying the decellularizing agent while largely preserving the 3D architecture of the organ from which the ECM has been isolated. It is the only technique to allow decellularization of thick 3D tissue sections or complete organs in a way that was not previously possible. Since the vascular network exists to minimize the diffusion distance for oxygen to cells, this is a particularly efficient way of delivering decellularizing agents throughout the tissue and transporting the cellular material from the tissue. The vascular network remains intact even after full decellularization has been achieved, hence offering a route for the efficient delivery and penetration of cells and nutrients into the ECM scaffold during the process of recellularization to create a functional organ construct (see Fig. 1 for the schematic diagram of the life cycle of whole-organ decellularization and potential clinical applications).

An Overview of Whole-Organ Decellularization Work

The heart

The first report in which whole-organ decellularization was successfully performed was on the heart.¹⁰ The aorta of a rat heart was cannulated to allow retrograde coronary perfusion with heparinized phosphate-buffered saline with adenosine, 1% sodium dodecyl sulfate (SDS), and 1% Triton X-100 in sequence alternating with rinsing with deionized water between steps. This produced a decellularized scaffold that was structurally similar to the heart, but had a translucent white appearance throughout (Fig. 2). Reimplantation of the acellular heart scaffold onto the aorta demonstrated an intact and patent vascular network (Fig. 3). Recellularization of the heart scaffold with cardiomyocytes under electrophysiological stimulation formed a construct capable of muscular contraction.

FIG. 1. Schematic diagram of the life cycle of whole-organ decellularization and potential clinical applications. Color images available online at www.liebertpub.com/teb



The lungs

A number of groups have investigated the use of perfusion decellularization in the lungs,^{11,13–15,21} which contain two accessible compartments separated by a short diffusion distance, that is, the vascular and the airway systems, by using varying protocols of vascular perfusion alone,^{11,21} or in combination with endotracheal instillation,^{14,15} or perfusion of the airway compartment.¹³ The decellularizing agents used were also far more varied from protocol to protocol, from SDS at different concentrations^{11,13,21} to 8 mM CHAPS,¹⁴ to a combination of Triton X-100/deoxycholate/DNAse/bleach.¹⁵ All groups demonstrated preservation of the major components of the ECM and the micro- and macroarchitecture of the lung. Recellularization was achieved with all the reports, although Petersen *et al.* mimicked physiological conditions within the bioreactor by ventilation of the airway compartment at negative pressure at 1 breath/min, and maintained pulmonary artery pressure at 20 mmHg or below. Orthotopic implantation of these tissue-engineered lung constructs demonstrated active gas exchange occurring via sampling of pulmonary arterial and venous blood. However, results in this study and Ott *et al.*

showed vascular leakage and pulmonary edema after a few hours of implantation, indicating damage to the microvasculature from the decellularization process. In a follow-up study to Ott *et al.*, Song *et al.* demonstrated further optimization of graft preservation and oxygenative function post-implantation for as long as 7 days.²¹ Recellularization was performed with mixtures of whole-lung cell isolates in four cases,^{11,14,15,21} but one group used a homogeneous mouse embryonic stem cell population,¹³ which showed that the matrix was capable of promoting site-appropriate differentiation without any other specific differentiation cues.

The liver

Liver decellularization has been performed by antegrade perfusion via the portal vein with SDS and Triton X-100, either alone or in combination,^{16,17,23,24} although another study used trypsin/EGTA,²⁵ and Baptista *et al.* used Triton X-100 in combination with ammonium hydroxide.^{18,22} Decellularization was demonstrated with histology and evidence of DNA removal, and retention of key ECM constituents with preservation of microvasculature and ECM

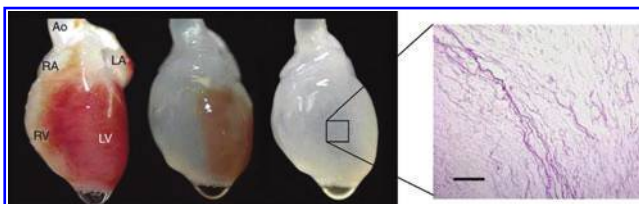


FIG. 2. The heart in stages of increasing decellularization over time with 1% sodium dodecyl sulfate as the decellularizing agent, with the end result of a translucent appearing heart-like scaffold structure. The panel on the right shows histological evidence (H&E staining) of decellularization. Reproduced from [8]. Scale bar=200 μ m. Color images available online at www.liebertpub.com/teb



FIG. 3. Coronary perfusion and evidence of intact coronary vasculature (right) after orthotopic implantation of the decellularized heart scaffold (left) and unclamping of the aorta. Reproduced from [8]. Color images available online at www.liebertpub.com/teb

TABLE 1a. SUMMARY OF SPECIES AND STRAIN USED FOR DECELLULARIZATION

<i>Organ</i>	<i>Authors</i>	<i>Species (strain)</i>
Heart	Ott <i>et al.</i> , 2008	Rat (Fischer 344)
Heart	Wainwright <i>et al.</i> , 2010	Pig
Lung	Ott <i>et al.</i> , 2010	Rat (Sprague Dawley)
Lung	Petersen <i>et al.</i> , 2010	Rat (Fischer 344)
Lung	Cortiella <i>et al.</i> , 2010	Rat
Lung	Price <i>et al.</i> , 2010	Mouse (C57BL/6)
Lung	Song <i>et al.</i> , 2011	Rat (Sprague-Dawley)
Liver	Uygun <i>et al.</i> , 2010	Rat (Lewis)
Liver	Shupe <i>et al.</i> , 2010	Rat (Fischer 344)
Liver	Soto-Gutierrez <i>et al.</i> , 2011	Rat (Sprague-Dawley)
Liver	Baptista <i>et al.</i> , 2011	Ferret
Liver	De Kock <i>et al.</i> , 2011	Rat (Sprague-Dawley)
Liver	Barakat <i>et al.</i> , 2011	Pig (Yorkshire)
Kidney	Ross <i>et al.</i> , 2009	Rat (Sprague-Dawley)
Kidney	Liu <i>et al.</i> , 2009	Rat (Wistar)
Liver, Kidney, Pancreas, Small bowel	Baptista <i>et al.</i> , 2009	Ferret liver Pig kidney/pancreas/small bowel
Small bowel	Totonelli <i>et al.</i> , 2012	Rat (Sprague-Dawley)
Skeletal muscle	Perniconi <i>et al.</i> , 2012	Mouse (BALB/C)

TABLE 1b. SUMMARY OF DECELLULARIZATION AGENTS USED

<i>Organ</i>	<i>Authors</i>	<i>SDS</i>	<i>SDS + Triton</i>	<i>Other</i>
Heart	Ott <i>et al.</i> , 2008	(+)	+	–
Heart	Wainwright <i>et al.</i> , 2010	–	–	+
Lung	Ott <i>et al.</i> , 2010	(+)	+	–
Lung	Petersen <i>et al.</i> , 2010	–	–	+
Lung	Cortiella <i>et al.</i> , 2010	+	–	–
Lung	Price <i>et al.</i> , 2010	–	–	+
Lung	Song <i>et al.</i> , 2011	(+)	+	–
Liver	Uygun <i>et al.</i> , 2010	(+)	+	–
Liver	Shupe <i>et al.</i> , 2010	(+)	+	–
Liver	Soto-Gutierrez <i>et al.</i> , 2011	–	–	+
Liver	Baptista <i>et al.</i> , 2011	–	–	+
Liver	De Kock <i>et al.</i> , 2011	(+)	+	–
Liver	Barakat <i>et al.</i> , 2011	+	–	–
Kidney	Ross <i>et al.</i> , 2009	(+)	+	–
Kidney	Liu <i>et al.</i> , 2009	(+)	+	–
Liver, Kidney, Pancreas, Small bowel	Baptista <i>et al.</i> , 2009	–	–	+
Small bowel	Totonelli <i>et al.</i> , 2012	–	–	+
Skeletal muscle	Perniconi <i>et al.</i> , 2012	+	–	–

SDS, sodium dodecyl sulfate.

ultrastructure. Hepatocytes were seeded onto the acellular liver scaffold via portal vein perfusion and were shown to be functional in four studies^{17,22,24,25} by showing evidence of synthesis of lactate dehydrogenase, albumin, and urea after heterotopic implantation. In particular, two studies attempt to scale-up this approach to further approximate human-scale engineered organ constructs by use of ferret²² and porcine²⁴ livers, and both studies seeded the bioscaffolds with human liver cells, which were shown to be compatible on these xenogeneic scaffolds.

The kidney

Several groups have decellularized whole kidneys by the perfusion method with preservation of the vascular network and complete cell removal.^{18–20} Decellularizing agents vary from 1% Triton or SDS^{18,19} to 3% Triton with DNase and

additional 4% SDS.²⁰ The preservation and presence of ECM constituents such as laminin and collagen IV were also demonstrated, as well as collagen I, fibronectin, and heparin sulfate in a separate study, which did not employ perfusion.⁸³ The latter study juxtaposed fetal cells in fresh renal explants with the ECM scaffolds by layering fetal kidney tissue against the ECM, which supported developmental renal phenotypes in these cells after they migrated into the ECM. In the study by Ross *et al.*, decellularized rat kidneys scaffolds were reseeded with murine embryonic stem cells, and the renal ECM was shown to support the renal differentiation of the embryonic stem cells.

Other tissues/organs

The technique of whole-organ decellularization is also expanding into other tissues and organs such as the small

TABLE 1c. SUMMARY OF CONDITIONS USED FOR PERFUSION DECELLULARIZATION

Organ	Authors	Flow rate	Perfusion pressure	Duration of decellularization (perfusion)
Heart	Ott <i>et al.</i> , 2008	-	77.5 mm Hg	12–13 h
Heart	Wainwright <i>et al.</i> , 2010	1.3L/min	-	6–7 h
Lung	Ott <i>et al.</i> , 2010	-	30 mm Hg	2–3 h
Lung	Petersen <i>et al.</i> , 2010	-	<20 mm Hg	(Total 500 mL perfused)
Lung	Cortiella <i>et al.</i> , 2010	N/A	N/A	N/A
Lung	Price <i>et al.</i> , 2010	N/A	N/A	N/A
Lung	Song <i>et al.</i> , 2011	-	80 cm H ₂ O	2–3 h
Liver	Uygun <i>et al.</i> , 2010	1 mL/min	-	5 days
Liver	Shupe <i>et al.</i> , 2010	5 mL/min	-	(Total 300 mL × 4 perfused)
Liver	Soto-Gutierrez <i>et al.</i> , 2011	8 mL/min	-	20–26 h
Liver	Baptista <i>et al.</i> , 2011	5 mL/min	-	(Total 40 × + 50 × volume of the liver)
Liver	De Kock <i>et al.</i> , 2011	30 mL/min	-	1–2 h
Liver	Barakat <i>et al.</i> , 2011	-	80 mm Hg	2–3 days
Kidney	Ross <i>et al.</i> , 2009	-	100 mm Hg	(Not stated)
Kidney	Liu <i>et al.</i> , 2009	-	100 cm H ₂ O	12–13 h
Liver, Kidney, Pancreas, Small bowel	Baptista <i>et al.</i> , 2009	10–60 mL/min	-	24 h
Small bowel	Totonelli <i>et al.</i> , 2012	0.6 mL/h	-	31 h (per DET cycle)
Skeletal muscle	Perniconi <i>et al.</i> , 2012	N/A	N/A	24–48 h

TABLE 1d. SUMMARY OF DECELLULARIZED WHOLE-ORGAN SCAFFOLD CHARACTERIZATION AND TECHNIQUES

Organ	Authors	Histology/ IHC	EM	Quantitative assay for DNA/ECM components	Vascular characterization	Mechanical testing
Heart	Ott <i>et al.</i> , 2008	+ / +	+	-	Corrosion casting + implantation	+
Heart	Wainwright <i>et al.</i> , 2010	+ / +	+	+	-	+
Lung	Ott <i>et al.</i> , 2010	+ / -	+	-	-	+
Lung	Petersen <i>et al.</i> , 2010	+ / +	+	-	Micro-CT	+
Lung	Cortiella <i>et al.</i> , 2010	- / - (IF)	-	(Gel electrophoresis for DNA)	-	-
Lung	Song <i>et al.</i> , 2011	-	-	-	-	-
Lung	Price <i>et al.</i> , 2010	+ / +	+	+	-	+
Liver	Uygun <i>et al.</i> , 2010	+ / +	+	+	Corrosion casting	-
Liver	Shupe <i>et al.</i> , 2010	+ / +	-	-	-	-
Liver	Soto-Gutierrez <i>et al.</i> , 2011	+ / +	+	(Gel electrophoresis for DNA)	Corrosion casting	-
Liver	Baptista <i>et al.</i> , 2011	+ / +	+	+	Fluoroscopy + confocal microscopy	-
Liver	De Kock <i>et al.</i> , 2011	+ / +	+	-	Corrosion casting	-
Liver	Barakat <i>et al.</i> , 2011	+ / +	-	-	Corrosion casting + fluoroscopy	-
Kidney	Ross <i>et al.</i> , 2009	+ / +	+	-	-	-
Kidney	Liu <i>et al.</i> , 2009	+ / - (IF)	+	-	-	-
Liver, Kidney, Pancreas, Small bowel	Baptista <i>et al.</i> , 2009	+ / -	-	-	Fluoroscopy	-
Small bowel	Totonelli <i>et al.</i> , 2012	+ / +	+	+	-	+
Skeletal muscle	Perniconi <i>et al.</i> , 2012	+ / +	-	-	-	-

IHC, immunohistochemistry; EM, electron microscopy; IF, immunofluorescence; ECM, extracellular matrix.

bowel and skeletal muscle, especially as it has the potential to allow the development of a viable replacement in the absence of any current clinical alternative, for example, intestinal failure and large-muscle defects due to trauma. Totonelli *et al.* have demonstrated the application of this technique to small bowel²⁶ and shown the viability of amniotic fetal stem cells seeded onto the decellularized scaffold

and its angiogenic properties, although perfusion recellularization was not performed. Perniconi *et al.* have decellularized whole skeletal muscles derived from rats by an incubation method, and further have implanted the acellular scaffolds *in vivo* within an equivalent muscle defect to show infiltration of inflammatory and stem cells within the scaffolds.²⁷

TABLE 2a. SUMMARY OF CONDITIONS USED FOR WHOLE-ORGAN SCAFFOLD RECELLULARIZATION

Organ	Authors	Cell population(s)	Cell numbers (million)	Seeding route (bolus injection/perfusion/other)	Perfusion flow rate/pressure
Heart	Ott <i>et al.</i> , 2008	Rat cardiocytes	50–75	Intramural injection	6 mL/min
Lung	Ott <i>et al.</i> , 2010	Human carcinomatous alveolar cells	91.25 ± 31.72	Tracheal perfusion	10–15 mm Hg
Lung	Petersen <i>et al.</i> , 2010	Rat lung epithelial cells	100	Airway injection	1–5 mL/min
Lung	Cortiella <i>et al.</i> , 2010	Murine ESCs	2	Tracheal injection	(Nonperfusion)
Lung	Price <i>et al.</i> , 2010	Murine fetal lung cells	3	Infusion in culture medium	(Nonperfusion)
Lung	Song <i>et al.</i> , 2011	Rat fetal lung cells	Not stated	Tracheal perfusion	10–15 mm Hg
Liver	Uygun <i>et al.</i> , 2010	Rat hepatocytes	50	Bolus intravascular injection × 4	Not stated
Liver	Shupe <i>et al.</i> , 2010	Rat liver progenitor cells	1	Inferior vena caval injection	Not perfused or cultured
Liver	Soto-Gutierrez <i>et al.</i> , 2011	Murine hepatocytes	10–50	Intraparenchymal injection vs. steady perfusion vs. bolus injection	2 mL/min
Liver	Baptista <i>et al.</i> , 2011	Human fetal liver cells	70	Portal vein perfusion	3 mL/min
Liver	Barakat <i>et al.</i> , 2011	Human fetal stellate cells + human fetal hepatocytes	350 + 1000	Portal vein perfusion	90 mL/min
Kidney	Ross <i>et al.</i> , 2009	Murine ESCs	2	Arterial + ureteric injection	(Nonperfusion)
Liver, Kidney, Pancreas, Small bowel	Baptista <i>et al.</i> , 2009	Human HepG2 cells	30	Intravascular injection	6 mL/min

ESC, embryonic stem cell.

TABLE 2b. SUMMARY OF RECELLULARIZED ORGAN CONSTRUCT CHARACTERIZATION AND TECHNIQUES

Organ	Authors	Histology/IF	TUNEL	EM	Functional assessment	In vivo implantation
Heart	Ott <i>et al.</i> , 2008	+ / +	+	+	LV pressure monitoring + live video (contractility)	–
Lung	Ott <i>et al.</i> , 2010	+ / +	–	+	Dynamic lung fn tests, blood gases, live video	+
Lung	Petersen <i>et al.</i> , 2010	+ / +	+	+	Compliance testing, blood gases	+
Lung	Cortiella <i>et al.</i> , 2010	– / +	+	–	–	–
Lung	Price <i>et al.</i> , 2010	– / +	–	–	–	–
Liver	Uygun <i>et al.</i> , 2010	+ / +	+	+	LDH/urea/albumin assay RT-PCR of enzymes	+
Liver	Shupe <i>et al.</i> , 2010	+ / –	–	–	–	–
Liver	Soto-Gutierrez <i>et al.</i> , 2011	+ / +	–	–	Albumin/cytochrome/ ammonia assay	–
Liver	Baptista <i>et al.</i> , 2011	+ / +	+	–	Urea/albumin/prostacylin assays Platelet deposition studies	–
Liver	Barakat <i>et al.</i> , 2011	+ / – (IHC)	+	–	Urea/albumin/lactate assays	+
Kidney	Ross <i>et al.</i> , 2009	+ / – (IHC)	–	–	–	–
Liver, Kidney, Pancreas, Small bowel	Baptista <i>et al.</i> , 2009	+ / – (IHC)	–	–	–	–

LV, left ventricle; LDH, lactate dehydrogenase; RT-PCR, reverse transcriptase–polymerase chain reaction.

Common trends seen within published protocols

The main parameters of interest for the perfusion decellularization and recellularization protocols are compared and summarized in Tables 1a–d and 2a and b, respectively.

Table 1a shows the different animal species and strains used, with the most common being rat, although there is an intentional move toward larger species to scale-up the technique to approach more human-sized organ constructs.

Table 1b displays the most common decellularizing agents used, with the predominance of SDS whether alone or in combination with Triton X-100. When another agent is quoted, there is a far wider disparity between protocols that almost vary from article to article (7 out of 18 in total). There are also other marked differences in protocols such as the additional use of physical methods (freezing) and whether DNase or RNase is also employed in combination with the main decellularizing agent (full details in Appendix Tables A1 and A2).

Table 1c summarizes the decellularization pump setup parameters, that is, flow rate (or pressure) and duration of decellularization (for the entire protocol, including periods of nonperfusion if relevant). While flow rates vary widely depending on the animal species, generally the rates adopted tend to be low and subphysiological vascular flow rates, while flow pressures tend to mimic physiological *in situ* ones. The total duration of the decellularization protocol gives an indication of how widely varying the protocols can be from group to group, whether in terms of the additional steps/methods used, for example, freezing, storage phases within the decellularization process, and the flow rates and concentrations applied with even the same decellularizing agents. The most marked contrast may be seen between De Kock *et al.* and Uygun *et al.* in work on the liver bioscaffold (1–2 h vs. up to 5 days), especially as the former quote directly from the latter as a baseline for developing their more streamlined protocol.

Table 1d summarizes the characterization of the decellularized organ scaffold and the techniques used; there is a common panel of tests to demonstrate a variety of important structural properties preserved in the ECM that are necessary for both the effectiveness of the bioscaffold for subsequent recellularization and the decellularization process itself. As can be seen, groups vary in how much of this panel of tests are completed. Not shown are any functional tests of ECM bioactivity, as this is limited so far to single growth factor assays and in two groups only.^{23,25}

Table 2a summarizes the four main parameters in the recellularization protocols: cell population, cell numbers, route of seeding, and perfusion rate. The bioreactor conditions tend to be similar, that is, incubator conditions, and duration of continuous culture is on average 7 days (with one group moving onto a longer period²¹), and hence these parameters have been omitted from the table. Full details can be found in Appendix Table A2. Cell populations used have been commonly the mixed population-native cells isolated from fetal or neonatal organs as the initial proof of concept, but work is now moving onto human cell populations and stem cell populations to demonstrate biocompatibility and the bioinductive properties of the ECM scaffold. Cell numbers are by necessity high, for example, 50–100 million on rat-derived scaffolds, but exponentially higher on larger species; however, this is usually still only 10%–20% of normal physiological cell

numbers. The route of seeding can depend on the physiological compartments available (other than the vascular), for example, the airway, and also direct injection is possible, but most researchers utilize the vascular route. There has been some comparison between a continuously perfused seeding process and a bolus intravascular injection, with some evidence that the latter affords higher engraftment.¹⁷

Table 2b displays those techniques for characterization of the recellularized organ constructs (where applicable), and these are usually a combination of histology-based techniques and functional testing specific to the organ/tissue in question.

Summary

In terms of decellularization protocols, these vary widely in many aspects, for example, strain/species of animal used, decellularization agents and other parameters (plus postdecellularization sterilization) to achieve the whole-organ ECM scaffold required for recellularization. On one hand, this is an indication of the universal applicability of the principle of perfusion decellularization to all animal tissues, and while variations in the optimal protocol can also be attributed to differences in the nature of each type of organ/tissue and its specific structure and composition, it also appears that a complete and systematic rationale for determining the optimal perfusion decellularization process has not yet been achieved. Some studies have started to formally assess achieved of decellularization protocols, for example, scaffold properties with varying number of decellularization cycles,²⁶ while only one study reports direct optimization from a baseline protocol taken from another study.²³ Characterization of the structural components of the ECM has yielded similar methods and results across most of the groups, but only two groups have attempted to assess the presence of the (presumed) bioinductive molecules present in the ECM with a quantification assay of two specific growth factors.^{23,25} This is an area that requires further investigation in all the organs/tissues of relevance, as regulatory and standardization issues (e.g., quality control and release criteria) are fundamental to the clinical translation and commercialization of these ECM products. ECM products are known to vary from batch to batch (even animal to animal), and hence standardization of basic parameters (species/strain, gender, age, and weight) needs to be maintained to achieve meaningful comparison in outcomes, or from group to group. These donor factors, and others relating to the use of biological agents within certain protocols, may require a degree of dynamicity within the decellularization process, but currently the extreme disparity between some protocols suggests the need for a more evidence-based approach. Preoptimization and standard operating procedures are even more critical when it is likely that post-processing testing of the ECM bioscaffold (and the recellularized construct) is likely to be limited in scope in those cases destined for implantation. Minimum standardized biological requirements within whole-organ ECM scaffolds (e.g., threshold DNA levels and structural integrity growth factor levels/activity) should also be established to maintain biological quality and clinical safety.

Regarding the recellularization process, most groups use a similar anatomical approach to the vascular perfusion setup for circulation of medium during continuous culture and

also use appropriate physiological adjuncts to help stimulate constructive remodeling (e.g., in the heart and lung models). Most groups have investigated the use of a mixed population of organ-specific cells isolated from neonatal or fetal animals to seed the acellular ECM scaffold as proof of concept and also to achieve the full extent of cellular variety and density required to populate a whole-organ scaffold. Several groups have also attempted reseeding with embryonic stem cells, which have demonstrated proliferation and differentiation specific to organ-specific bioinductive cues within the ECM scaffold. In addition, human cell populations have also been seeded onto the xenogeneic scaffolds to demonstrate biocompatibility. Again, there are quite widely varying parameters for the recellularization protocol between groups in terms of cell numbers and culture conditions, and this is an area where further systematic optimization is required. A physiological degree of cellular engraftment has not yet been achieved with any group, partly because of the very large cell numbers required. Finally, functional assessment of recellularized constructs depends on the degree of recellularization achieved and varies from organ to organ and whether the graft is suitable for implantation *in vivo*. Generally, only very short-term viability and implantation *in vivo* have been achieved so far (e.g., 6 h), but one study has expanded this into a 7-day implantation period after improving graft preservation.²¹

Appendix Tables A1 and A2 summarize further details of the decellularization and recellularization protocols used in the work published from the groups above.

Future Work and Considerations: Surgical Implantation of Bioartificial Organ Constructs

It has been shown so far that whole-organ decellularization can yield whole-organ ECM bioscaffolds capable of supporting recellularization on a complex 3D level and sustaining appropriate cellular and tissue functionality. These bioartificial organ constructs also allow relatively easy surgical manipulation, especially as they preserve the intact vascular pedicle for direct anastomosis at the orthotopic implantation site. The intact and inherent vasculature permits direct perfusion of the tissue and maintains provision of nutrients and oxygen to the implanted graft, in an exact parallel to the transplant procedure. As such, this technique could provide an ideal shortcut to bioengineering an organ replacement that is structurally and histologically similar to the original organ in question, uses established surgical procedures for implantation, is able to potentially support cellular function to the same degree, is biologically compatible, and comes from a widely available source (e.g., porcine or other xenogeneic organs).⁷

Currently, there are limitations to all the tissue-engineered organ constructs produced so far in terms of functionality and viability *in vivo*, varying from achieving sufficient or comparable to normal cellular density, achieving full cellular and tissue differentiation, and assessment of long-term viability, functionality, and constructive remodeling of organ grafts implanted *in vivo*. It would also be necessary to expand these techniques on larger-animal organs, which are closer in size and functionality to human ones. These are issues to be addressed and optimized before work can proceed to the clinical stage, in conjunction with the regulatory

issues that guide cell choice (such as those as by the U.S. Food and Drug Administration regarding the use of autologous human cells applied in a nonhomologous location, or the use of allogeneic cells in virtually all cases), as well as a common language for reporting outcomes in trials.

In addition, it would be important to investigate the possibility of organ regeneration from an alternative cell source such as a stem cell population. Possibilities for this may be embryonic stem cells as has been tried in the lung or mesenchymal stem cells, which can differentiate into functional nephrons after injection into the nephrogenic site of developing rat embryos,⁸⁴ and are furthermore involved in the repair and recovery of renal function after acute renal injury.^{85,86} If eventually autologous cells such as mesenchymal stem cells from the recipient alone could be used to create a functional organ graft, there could be significant clinical implications in overcoming the immune barrier and problem of rejection, with further great potential benefits to the patient.

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Address correspondence to:

Ming He, MBBS

Department of Bioengineering and Materials

Institute of Biomedical Engineering

Imperial College London

London SW7 2AZ

United Kingdom

E-mail: m.he10@imperial.ac.uk

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APPENDIX TABLE A1. SUMMARY OF THE DECELLULARIZATION PROTOCOLS USED IN THE LITERATURE

Decellularization protocol							
Organ	Authors	Tissue harvesting (species/strain/age, explantation details)	Perfusion type	Agent(s) + duration	Flow rate/pressure	Sterilization	Characterization of decellularised scaffold
Heart	Ott <i>et al.</i> , 2008	12-week-old Fischer 344 rats Systemic heparinization	Retrograde coronary perfusion via ascending aorta	Heparinized PBS + 10 μ M adenosine for 15 mins + 1% SDS for 12h + 1% Triton X-100 for 30 mins	77.4 mm Hg coronary arterial pressure	PBS + 100 μ /mL pen-strep for 124h	Histology + immunofluorescence of ECM-collagen I + III, laminin, fibronectin Aortic valve-Evans blue perfusion SEM/TEM of ECM architecture Vascular corrosion resin casting + heterotopic transplantation Mechanical (stress-strain/tangential modulus) testing Histology + IHC/immunofluorescence of ECM-DAPI, elastin, collagens (I, III, IV), GAGs DNA/GAG/elastin quantitative assays SEM of ECM architecture Mechanical (ball burst) testing Histology of ECM-collagen, proteoglycans, elastin + Morphometry and stereology of alveoli TEM of ECM alveolar architecture
Heart	Wainwright <i>et al.</i> , 2010	Adult pigs (strain not specified)	Retrograde coronary perfusion via ascending aorta	Frozen at -80°C for 16 h then thawed at room temperature (RT) + hypotonic type 1 water for 15 mins + 0.02% trypsin/0.05% EDTA/0.05% NaN ₃ for 2 h + 3% Triton X-100/0.05% EDTA/0.05% NaN ₃ for 2 h + 4% deoxycholic acid for 2 h Heparinized PBS for 15 mins + 0.1% SDS for 120 mins + 1% Triton-X100 for 10 mins	1–1.3 L/min	0.1% peracetic acid/4% ethanol for 4 h at 1.7 L/min	Dynamic lung testing–compliance + vital capacity Histology + immunofluorescence of ECM–elastin, collagen I + IV, laminin, fibronectin DNA/collagen/GAG/elastin quantitative assays SEM/TEM of ECM architecture Micro-CT of vascular + alveolar architecture Mechanical (stress-strain/compliance) testing Immunofluorescence–DAPI, MHC-1 Gel electrophoresis of DNA content
Lung	Ott <i>et al.</i> , 2010	12-week-old male Sprague-Dawley rats Systemic heparinization	Retrograde pulmonary arterial perfusion		30 mm Hg pulmonary arterial pressure	PBS + 100u/mL pen-strep for 72h	
Lung	Petersen <i>et al.</i> , 2010	3-month-old Fischer 344 rats Intrapertitoneal heparin	Antegrade perfusion via right ventricle	PBS + 50 μ /mL + 1 μ g/mL SNP + 8 mM CHAPS/1M NaCl/25 mM EDTA in PBS until total 500 mL of fluid perfused + 90 μ /mL benzonase	<20 mm Hg pulmonary arterial pressure	PBS + 10% pen-strep (duration not stated)	
Lung	Cortiella <i>et al.</i> , 2010	Rats (age/strain not specified)	Mechanical agitation within bioreactor + initial freezing process	Frozen at -70°C for then thawed at 40°C /flash frozen $\times$ 4 times + 1% SDS with continuous circulation for 5 weeks + DNase/RNase 0.1% Triton X-100 2% sodium deoxycholate DNase Repeated injection/incubation steps—not continuous perfusion	(2.5 rpm rotational speed within bioreactor)	PBS + pen-strep + amphotericin for 24 h	
Lung	Price <i>et al.</i> , 2010	2–3-month-old C57BL/6 female mice	Injection via right ventricle + trachea		N/A	All solutions sterile with added pen-strep	Histology + immunofluorescence of ECM-collagen, elastin, DAPI, laminin Collagen/GAG/laminin/elastin quantitative assays SEM of ECM architecture Pulmonary function tests (Not performed)
Lung	Song <i>et al.</i> , 2011	Male Sprague-Dawley rats (260–280 g) Systemic heparinization	Antegrade pulmonary arterial perfusion	Heparinized PBS for 15 mins + 0.1% SDS for 120 mins + 1% Triton-X100 for 10 mins	80 cm H ₂ O	PBS + 100u/mL pen-strep for 72h	
Liver	Uygun <i>et al.</i> , 2010	Female adult (150–200 g) Lewis rats	Antegrade perfusion via portal vein	Initial 20 mL 0.9% saline then frozen at -80°C for 4 h then thawed at	1 mL/min	PBS + 0.1% peracetic acid for 3 h	Histology + immunofluorescence of ECM-collagen I + IV, laminin,

(continued)

(continued)

APPENDIX TABLE A1. (CONTINUED)

Decellularization protocol						
Organ	Authors	Tissue harvesting (species/strain/age, explantation details)	Perfusion type	Agent(s) + duration	Flow rate/pressure	Sterilization
		Intracardiac heparin		4°C + PBS at 1 mL/min overnight + 0.01% SDS for 72 h + 0.1% SDS for 24 h + 1% SDS for 24 h + 1% Triton-X100 for 30 mins		Characterization of decellularised scaffold
Liver	Shupe <i>et al.</i> , 2010	Adult Fischer 344 rats	Retrograde perfusion via inferior vena cava	Initial 100 mL PBS + 1%, 2%, 3% Triton X-100 in sequence until 300 mL each perfused + 0.1% SDS until 300 mL	5 mL/min	300 mL PBS
Liver	Soto-Gutierrez <i>et al.</i> , 2011	Male adult (250–300 g) Sprague-Dawley rats Systemic heparinization	Retrograde perfusion via inferior vena cava	Initial 5–10 mL PBS/heparin, and then frozen at –80°C for 24 h then thawed at RT + 0.02% trypsin/0.05% EGTA for 2 h + 3% Triton X-100/0.05% EGTA for 18–24 h	8 mL/min	2 M Rad gamma irradiation
Liver	Baptista <i>et al.</i> , 2011	Ferret (age/strain/ gender not specified)	Antegrade perfusion via portal vein	Initial dH ₂ O (40× volume of liver) + 1% Triton X-100/0.1% NH ₄ OH (50× volume of liver)	5 mL/min	Not stated
Liver	De Kock <i>et al.</i> , 2011	Male adult (250–300 g) Sprague-Dawley rats Systemic heparinization	Antegrade perfusion via portal vein	Initial Krebs-Henseleit buffer for 15 mins + 1% Triton X-100 for 30 mins + 1% SDS for 30 mins	30 mL/min	N/A
Liver	Barakat <i>et al.</i> , 2011	Yorkshire swine (25–35 kg)	Antegrade perfusion via portal vein	Initial 6–10 L dH ₂ O + 20 L 0.25% SDS (store in 0.25% SDS for 48 h) + 40 L 0.5% SDS over 2–3 h + 20 L dH ₂ O + 10% formalin + 40 L PBS Initially saline + SNP + 3% Triton-X100, DNase + 3% Triton-X100 + 4% SDS (durations not stated)	80 mm Hg	10% formalin
Kidney	Ross <i>et al.</i> , 2009	Male adult (250–300 g) Sprague-Dawley rats Systemic heparinization	Antegrade perfusion via renal artery	Initially saline + SNP + 3% Triton-X100, DNase + 3% Triton-X100 + 4% SDS (durations not stated)	100 mm Hg	Not stated
Kidney	Liu <i>et al.</i> , 2009	12-week (180–220 g) Wistar rats	Antegrade perfusion via renal artery	Initial PBS/heparin for 15 mins + 1% SDS for 12 h + 1% Triton-X100 for 30 mins	100 cm H ₂ O	PBS + pen-strep for 48 h
Liver, Kidney, Pancreas, Small bowel	Baptista <i>et al.</i> , 2009	Ferret liver Pig kidney/pancreas/ small bowel	Not stated	1% Triton X-100/0.1% Ammonium hydroxide for 24 h or until translucent	10–60 mL/min	Not stated
Small bowel	Totonelli <i>et al.</i> , 2012	Adult (320–350 g) Sprague-Dawley rats	Antegrade perfusion via superior mesenteric artery	Detergent-enzymatic treatment (DET): Deionized water for 24 h + 0.4% sodium deoxycholate for 4 h + 2000 kU DNase for 3 h (1–4 cycles)	0.6 mL/h	N/A
Skeletal muscle	Perniconi <i>et al.</i> , 2012	Adult BALB/C mice	(Incubation/ rotation method)	1% SDS for 24 or 48 h	(N/A)	PBS washes

PBS, phosphate-buffered saline; ECM, extracellular matrix; SDS, sodium dodecyl sulfate; SNP, sodium nitroprusside; SEM, scanning electron microscopy; TEM, transmission electron microscopy; N/A, not applicable; GAG, glycosaminoglycan; VEGF, vascular endothelial growth factor.

APPENDIX TABLE A2. SUMMARY OF THE RECELLULARIZATION PROTOCOLS USED IN THE LITERATURE

Recellularization protocol				
Organ	Authors	Bioreactor setup/special conditions (in addition to vascular perfusion)	Cell population/numbers + reseeding	Culture conditions
Heart	Ott <i>et al.</i> , 2008	Cannulation of left atrium and ascending aorta—pulsatile distension of left ventricle + compliance loop attached to ascending aorta to reproduce physiological preload, after-load and intraventricular pressure. Coordinated electrical stimulation at 5–20 V via electrodes	50–75 million rat neonatal cardiocytes isolated and seeded via intramural injection + 2 million rat aortic endothelial cells via perfusion	Culture medium (CM) perfused at atrial flow rate (FR) 20 mL/min, coronary FR 6 mL/min 37°C 5% CO ₂ incubator Duration of culture: 4–8 days
Heart	Wainwright <i>et al.</i> , 2010	(Nonperfusion culture system: lyophilized sheets of cardiac ECM used)	500000 cells/cm ² white leghorn chicken embryonic cardiomyocytes isolated	37°C 5% CO ₂ incubator Duration of culture: 4 days
Lung	Ott <i>et al.</i> , 2010	Perfusion system through pulmonary artery, left atrium, and trachea; pulmonary vein drained to equilibration chamber Trachea connected to gas ventilation chamber. Alternating air and fluid (culture medium) perfusion	91.25 ± 31.72 million carcinomatous human alveolar basal epithelial cells by gravity perfusion through trachea + 66.57 ± 18.22 human umbilical cord venous endothelial cells (HUVECs) by gravity perfusion of pulmonary artery and vein; 308.57 ± 146.9 million rat fetal lung cells isolated + HUVECs seeded via same method	CM perfused at 10–15 mm Hg constant pressure with negative pressure ventilation 37°C 5% CO ₂ incubator Duration of culture: 5–9 days
Lung	Petersen <i>et al.</i> , 2010	Pulmonary artery cannulation for perfusion loop Breathing loop/negative pressure ventilation via trachea cannulation + syringe pump	100 million rat neonatal lung epithelial cells isolated and injected into airway compartment + 30 million lung vascular endothelial cells via artery	CM perfused at 1–5 mL/min with negative pressure ventilation at 1 breath/min 37°C 5% CO ₂ incubator Duration of culture: 4–8 days
Lung	Cortiella <i>et al.</i> , 2010	(Nonperfusion bioreactor: rotating chamber system)	2 million murine embryonic stem cells (ESCs) injected into trachea (1 million cells into each side/lung scaffold)	CM circulated by pump action at speed of 2 rpm 37°C 5% CO ₂ incubator Duration of culture: 14–21 days Mechanical ventilation at 180 breaths/min (300 µL volume) 37°C 5% CO ₂ incubator
Lung	Price <i>et al.</i> , 2010	(Nonperfusion bioreactor: scaffolds suspended in CM flasks) Tracheal cannulation for ventilation loop	3 million fetal lung cells isolated from day E17 gestation B6 mice infused into CM	Duration of culture: 7 days CM perfused at 1–5 mL/min with negative pressure ventilation at 1 breath/min 37°C 5% CO ₂ incubator Duration of culture: 7–10 days
Lung	Song <i>et al.</i> , 2011	Perfusion system through pulmonary artery, left atrium, and trachea; pulmonary vein drained to equilibration chamber Trachea connected to gas ventilation chamber. Alternating air and fluid (culture medium) perfusion loop	Rat fetal lung cells + HUVECs (numbers not stated)	Duration of culture: 7 days CM perfused at 1–5 mL/min with negative pressure ventilation at 1 breath/min 37°C 5% CO ₂ incubator
Liver	Uygun <i>et al.</i> , 2010	Portal vein cannulation for perfusion loop	50 million rat hepatocytes isolated and seeded via bolus intravascular injection × 4 + 40 million cardiac endothelial cells via perfusion	Perfusion FR not stated 37°C 5% CO ₂ incubator Duration of culture: 1–5 days

Characterization of organ construct and functionality

Histology (engraftment) + immunofluorescence—TUNEL, α -actin, MHC, vWF, connexin-43
TEM of ultra-architecture
Perfusion with CMFDA to demonstrate endothelialization
LV pressure monitoring + live video to demonstrate contractile activity
Immunofluorescence— α -actinin + β -tubulin to identify cardiomyocytes

Histology (engraftment) + immunofluorescence—caveolin-1, T1 α , Ttf1, vimentin, pro-SPC
TEM of ultra-architecture
Immunoblotting-SP-A, SP-C
Morphometry + stereology of lung architecture
In vitro function testing—blood gas values, dynamic lung function tests
Orthotopic transplantation into syngeneic male rats—fluorescopy and live video, blood gas values

Histology (engraftment, TUNEL) + immunofluorescence—CD-31, CCSP, pro-SPC, aquaporin-5, α -actin, Cy-14.
TEM of ultra-architecture
Stress-strain + compliance testing
Orthotopic transplantation into syngeneic male Fischer 344 rats + blood gas values tested to demonstrate gas exchange

Immunofluorescence—DAPI, live/dead staining, TUNEL, cytokeratin 18, CD31, pro-SPC, laminin, collagen IV
Immunofluorescence—DAPI, cytokeratin 18, CD31, pro-SPC, aquaporin-5, CCSP, CD45, CD11b, vimentin

Histology + IHC—TTF-1, pro-SPC, CC10, CD31, vimentin
Morphometry + stereology of lung architecture
In vitro function testing—blood gas values, dynamic lung function tests
Orthotopic transplantation into syngeneic male rats up to 14 days—fluorescopy and live video, blood gas values

Histology (engraftment) + immunofluorescence—TUNEL, albumin, G6pc, Ugt1a
LDH release quantification
Urea + albumin synthesis quantification
RT-PCR of drug metabolism enzymes
Heterotopic transplantation into syngeneic rats + fresh blood perfusion model to test viability and hepatocyte function

(continued)

APPENDIX TABLE A2. (CONTINUED)

Recellularization protocol					
Organ	Authors	Bioreactor setup/special conditions (in addition to vascular perfusion)	Cell population/numbers + reseeding	Culture conditions	Characterization of organ construct and functionality
Liver	Shupe <i>et al.</i> , 2010	Inferior vena cava (IVC) cannulation, but no continuous perfusion	1 million rat liver progenitor cells WB344 via IVC injection	-	Histology
Liver	Soto-Gutierrez <i>et al.</i> , 2011	Portal vein cannulation for perfusion loop culture performed	10–50 million murine adult hepatocytes injected directly into parenchyma vs. perfusion in CM at 2 mL/min vs. perfused in multiple boluses	Perfusion FR 2 mL/min 37°C 5% CO ₂ incubator Duration of culture: 7 days	Histology (engraftment) + immunofluorescence—DAPI, albumin, Ki67 Albumin synthesis/cytochrome activity / ammonia metabolism quantification assays
Liver	Baptista <i>et al.</i> , 2011	Portal vein cannulation for perfusion loop	70 million human fetal liver cells + 30 million HUVECs coinfusion via portal vein	Perfusion FR 3 mL/min for seeding × 16 h; Perfusion FR 0.5 mL/min 37°C 5% CO ₂ incubator Duration of culture: 7 days	Histology + immunofluorescence—TUNEL, Ki-67 Urea/albumin/prostacyclin synthesis quantification Platelet deposition studies
Liver	De Kock <i>et al.</i> , 2011	N/A	N/A	N/A	N/A
Liver	Barakat <i>et al.</i> , 2011	Posterior segments only (x3) Portal vein cannulation for perfusion loop	350 million human fetal stellate cells + 1 billion human fetal hepatocytes via portal vein	Perfusion FR 90 mL/min 37°C 5% CO ₂ incubator Duration of culture: 3–13 days	Histology + IHC—CK-18, CK-19, AFP, CYP3A4, TUNEL, Ki-67
Kidney	Ross <i>et al.</i> , 2009	Renal artery cannulation for perfusion loop, also cannulation of ureter	2 million murine pluripotent ES cells injected via artery or ureter	Sections of seeded graft cultured for 3–10 days 37°C 5% CO ₂ incubator Perfusion of whole organ at 120/80 mm Hg for 6–10 days (N/A)	Urea/albumin/lactate synthesis quantification Orthotopic transplantation - histology Histology (engraftment + morphology) + IHC—pancytokeratin, Pax-2, Ksp-cadherin, Ki-67 RT-PCR of Pax-2 + Ksp-cadherin expression
Kidney	Liu <i>et al.</i> , 2009	(N/A)	(N/A)	(N/A)	(N/A)
Liver, Kidney, Pancreas, Small bowel	Baptista <i>et al.</i> , 2009	Recellularization performed on ferret liver via portal vein cannulation and perfusion	30 million human HepG2 cells + 30 million mouse endothelial cells injected intravascularly	Perfusion FR 6 mL/min 37°C 5% CO ₂ incubator Duration of culture: 7 days	Histology IHC—albumin, vWF, Ki-67
Small bowel	Totonelli <i>et al.</i> , 2012	N/A	N/A	N/A	N/A
Skeletal muscle	Perniconi <i>et al.</i> , 2012	N/A	N/A	N/A	N/A

RT-PCR, reverse transcriptase–polymerase chain reaction; LDH, lactate dehydrogenase.



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**Optimisation of SDS exposure on preservation of ECM characteristics in whole organ
decellularisation of rat kidneys**

M He^a, A Callanan^b, K Lagaras^a, JAM Steele^a, MM Stevens^{*a}

Authors

Dr Ming He, MBBS MRCS

m.he10@imperial.ac.uk

+44 (0)20 7589 5111 ext. 56725

Dr Anthony Callanan, PhD

anthony.callanan@ed.ac.uk

+44 (0)131 650 4860

Mr Konstantinos Lagaras, MSc

konstantinos.lagaras11@imperial.ac.uk

+44 (0)20 7589 5111 ext. 56725

Mr Joseph AM Steele, MSc

j.steele11@imperial.ac.uk

+44 (0)20 7589 5111 ext. 56725

Professor Molly M Stevens*, PhD

m.stevens@imperial.ac.uk

+44 (0)20 7594 6804

*Corresponding author

^aDepartment of Bioengineering, Department of Materials and Institute of Biomedical Engineering

Imperial College London, London, United Kingdom

^bInstitute for BioEngineering (IBioE), School of Engineering

University of Edinburgh, Edinburgh, United Kingdom

Abstract

Renal transplantation is well established as the optimal form of renal replacement therapy but is restricted by the limited pool of organs available for transplantation. The whole organ decellularisation approach is leading the way for a regenerative medicine solution towards bioengineered organ replacements. However, systematic pre-optimisation of both decellularisation and recellularisation parameters is essential prior to any potential clinical application and should be the next stage in the evolution of whole organ decellularisation as a potential strategy for bioengineered organ replacements. Here we have systematically assessed two fundamental parameters (concentration and duration of perfusion) with regards to the effects of differing exposure to the most commonly used single decellularising agent (sodium dodecyl sulphate/SDS) in the perfusion decellularisation process for whole rat kidney ECM bioscaffolds, with findings showing improved preservation of both structural and functional components of the whole kidney ECM bioscaffold. Whole kidney bioscaffolds based on our enhanced protocol were successfully recellularised with rat primary renal cells and mesenchymal stromal cells. These findings should be widely applicable to decellularised whole organ bioscaffolds and their optimisation in the development of regenerated organ replacements for transplantation.

Introduction

End stage renal failure is associated with major morbidity and mortality[1]. Renal transplantation is well established as the optimal form of renal replacement therapy (RRT) but is restricted by the limited pool of organs available for transplantation from deceased donors. According to the UK Renal Registry, there were 56940 adult patients receiving RRT on 31/12/2013 in the UK but the total number of kidney transplants performed in 2011 was 2930[1]. While a number of other approaches have been taken to attempt to artificially replace the functions of native kidney[2-8], recent innovations in the field of tissue engineering such as whole organ decellularisation may lead to a successful regenerative medicine approach and bioengineered solution to this pressing clinical need[9-11]. Whole organ decellularisation provides a relatively new approach to ‘whole organ’ engineering and this technique utilises the intrinsic vasculature to perfuse the decellularising agent and efficiently penetrate tissue and remove cellular material. The decellularised extracellular matrix (ECM) bioscaffold thus created preserves important intrinsic properties of the ECM[12] along with its natural organisational complexity in order to promote recellularisation and cellular differentiation on a whole organ level. The inherent vasculature is also critical in allowing perfusion to sustain cell culture in complex 3-D constructs. This approach has been successfully employed in the major solid organs to create viable and partially functional organ constructs, some of which have been implanted *in vivo*[13-18]. In particular, recent work on the regenerated rat kidney has demonstrated up to 10% functionality in terms of urine production and creatinine clearance[18].

While the potential clinical implications are extremely exciting, a number of major technical factors and challenges exist before clinical application can be considered. One critical group of pre-optimisation parameters lies with the production of the ECM bioscaffold and the

decellularisation process. While the tissue- and organ-specific functional and bio-inductive molecules of the ECM are intrinsic to the success of the recellularisation and regenerative process, the quality of the ECM not only varies with each donor but also with each protocol applied[19]. A few studies have started to assess or compare effects of perfusion decellularisation protocols in a more systematic manner e.g. different decellularising agents[20], duration/exposure to the decellularising agent[21], but not in the rat kidney model. We have also shown in a recent systematic review that currently there can be significant disparity between decellularisation protocols in the literature even for the same organs/species, strongly suggesting the need for a more widespread evidence-based approach[22]. There are many parameters that exist to be considered – such as choice of decellularising agent (which also depends on type of tissue being decellularised), concentration and duration of perfusion (i.e. exposure) of decellularising agent on the tissue/ECM, additional steps in the protocol (e.g. freezing, use of biological agents), tissue harvesting factors, perfusion versus non-continuous, perfusion pressure versus perfusion rate, and last but not least, post-processing sterilisation methods.

On this basis, we aimed to optimise two fundamental parameters (i.e. concentration of decellularising agent and duration of perfusion) in order to assess the effects of differential exposure to decellularisation agent for whole rat kidney ECM bioscaffolds, using the most commonly reported single decellularising agent i.e. sodium dodecyl sulphate (SDS)[22]. Optimisation was determined with regards to structural and functional characteristics of the ECM bio-scaffold as assessed by histology, immunohistochemistry (IHC), quantitative assays of DNA and sulfated glycosaminoglycan (sGAG) content, and growth factor quantification – and optimisation was continued until a ‘plateau’ effect (i.e. no further comparative benefit) was observed in one or more of these modalities. We have also performed perfusion-based

recellularisation of these whole rat kidney ECM bioscaffolds with rat primary renal cells and mesenchymal stromal cells to demonstrate high levels of cellular engraftment and cellular viability with *in vitro* perfusion culture, and formation of renal-like structures on histology.

Methods and materials

Decellurisation of whole kidneys

1. Tissue harvesting

Male Wistar rats (350-375 g, Charles River/UK) were chosen for kidney harvest for perfusion decellularisation. Briefly, renal arteries were cannulated immediately after euthanasia of the animal and perfused with phosphate buffered saline (PBS) with vasodilator (10 ml of 10 µg/ml sodium nitroprusside in PBS, Sigma/UK, followed by 20 ml at 1 µg/ml) until a uniform blanching was observed, after which each kidney was perfused with 30 ml PBS without vasodilator.

2. Whole kidney decellularisation

Kidneys were suspended within a reservoir and perfused continuously via the arterial cannula at 10 ml/min, recirculating the total volume of 400ml of decellularising solution throughout the decellularisation process. The perfusate was sodium dodecyl sulfate (Sigma/UK) at differing concentrations and durations (see Tables 1a + 1b). After decellularisation, kidney bioscaffolds were perfused with PBS for 1 hour. Those for subsequent recellularisation were sterilised with 1% w/v antibiotic-antimycotic (Gibco®, Invitrogen/UK) in sterile PBS for 18 hours at 2 ml/min.

3. Histology and immunohistochemistry (IHC)

All histological samples were fixed in 10% v/v buffered neutral formalin and stained with haematoxylin and eosin (H+E) for light microscopy.

IHC was performed to assess retention of key ECM and basement membrane proteins collagens I and IV, laminin and fibronectin (all Abcam/UK). All samples were treated identically with regards to fixation, antigen retrieval, antibody staining, blocking and development. Briefly, paraffin-embedded sections were dewaxed and blocked with 1% v/v hydrogen peroxide in methanol for 10 minutes. Heat-mediated antigen retrieval was performed in 0.01M sodium citrate buffer (Sigma/UK), pH 6.0, followed by Avidin-Biotin blocking (Dako/UK). Sections were blocked for 1 hour with 5% v/v goat serum in PBS, and primary antibodies were incubated overnight at 4°C. Primary antibody dilutions in blocking solution (5% v/v goat serum in PBS) were: collagen I 1:250, collagen IV 1:500, laminin 1:50, and fibronectin 1:500 (Abcam/UK). After washing sections in 0.1% v/v TWEEN (Sigma/UK) in PBS, pH 7.4, hydrogen peroxidase conjugated secondary antibody (Abcam/UK) was applied at 1:100 for 1 h at room temperature then washed again in TWEEN solution. Slides were developed using 3,3'-diaminobenzidine (Dako/UK) until good staining intensity was observed. Species immunoglobulin and PBS served as negative controls. Haematoxylin was used for nuclear counterstaining.

4. DNA/sGAG quantification

Approximately half of a whole, unfixed rat kidney or kidney bioscaffold was suspended within 5 ml of papain solution (55 mM L-cysteine hydrochloride, 80 mM EDTA, 2.5 units/ml papain, pH 6.0, all Sigma/UK) for 24 h at 60°C until no visible solid material remained. After digestion, the solution was freeze-dried for 24h using the

Heto PowerDry LL1500 freeze dryer (Thermo Scientific/UK) to ascertain the dry weight.

Total DNA content was quantified using the Picogreen® dsDNA assay (Invitrogen/UK) and total sGAG content was quantified using the Blyscan™ sGAG assay (Biocolor/UK) according to the manufacturer’s instructions. Each assay was performed in duplicate and three times in total.

5. Growth Factor extraction and quantification assays

Approximately half of a whole, unfixed rat kidney or kidney bioscaffold was suspended in ~3.75 ml of urea–heparin extraction buffer (2 M urea and 5 mg/ml heparin in 50 mM Tris with protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 10 mM N-ethylmaleimide at pH 7.4, all Sigma/UK). The extraction mixture was rocked at 4°C for 20-24 hours then centrifuged at 5,000 x g for 30 minutes; supernatants were collected. 6 ml of extraction buffer was added to each pellet. These were again rocked at 4°C for 20-24 hours, centrifuged at 5,000 x g for 30 minutes, and supernatants collected. Supernatants from both extractions were dialyzed against ultrapure filtered water (total of three changes of dialysis water, 80-100 volumes/change) in Spectra/Por 3500 MWCO dialysis membrane (Spectrum Lab./USA). Extracts were frozen in aliquots at -80°C until assayed. Total protein concentration in each dialyzed extract was determined by the BCA protein assay (Pierce, Thermo Scientific/UK) following the manufacturer’s protocol. Basic fibroblast growth factor and vascular endothelial growth factor concentrations were determined with the Quantikine Human FGF basic Immunoassay and Quantikine Rat VEGF Immunoassay (R&D Systems/UK) respectively, following the manufacturer’s

protocols. Each growth factor assay was performed in duplicate and three times in total.

It should be noted that each growth factor assay measured growth factor protein concentration and did not measure growth factor activity.

6. Vascular resin corrosion casting

A 0.5-1.0 ml volume of polymer mixture, depending on the kidney size, was injected via the arterial cannula using Batson's no. 17 anatomic corrosion casting kit (Polysciences Inc./USA) as recommended by the manufacturer. Polymerisation took 20-30 minutes at 4°C and photographic images were taken during the injection process over 10-30 seconds.

7. Statistical analysis

Results were reported as mean $\pm$ standard error. Statistical analysis was performed with analysis of variance which demonstrated non-parametric distribution of data followed by Mann Whitney two sample test. A p -value of < 0.05 was considered significant.

Recellularisation of the whole kidney bioscaffold

1. Recellularised kidney graft perfusion – system setup

After sterilisation, the decellularised kidney bioscaffold was seeded with 50×10^6 cells in divided multistep bolus injections via the arterial cannula at 45-60 minute intervals. Typically three injections of approximately 16×10^6 cells at 32×10^6 cells/ml were used. The recellularised kidney bioscaffold was then suspended in the main chamber of a sterile three-port flask immersed within the reservoir of culture medium (150-180 ml volume). The perfusion system also included a sterile exchange

membrane (BugStopper, Whatman/UK) and a peristaltic pump. The system was placed inside a standard 37°C 5% CO₂ incubator for temperature and CO₂ control. The graft was continuously perfused at 1.5 ml/min. The culture medium was changed every 48 hours throughout the 7 day culture period (the first change occurring at 24 hours post-seeding).

The perfusate consisted of either i) 50% DMEM and 50% Keratinocyte Serum-Free Medium (K-SFM) with bovine pituitary extract (BPE) and epidermal growth factor (EGF) with the addition of 5% v/v foetal bovine serum (FBS) and 1% v/v antibiotic-antimycotic (AA) for primary renal cells, or ii) α -MEM with the addition of 10% v/v FBS and 1% v/v AA (all Gibco®, Invitrogen/UK) for mesenchymal stromal cells.

2. Primary renal cell (PRC) isolation

Four-week-old Wistar rats were used for PRC isolation (protocol adapted from Joraku *et al*[16]). Briefly, minced kidney tissue from culled animals was digested in collagenase solution (Liberase TM Research Grade, Roche/UK) according to the manufacturer's recommendations. The digested tissue suspension was filtered through a 100 μ m cell strainer (BD Falcon®, Corning/UK) and neutralised with an equal volume of DMEM culture medium. This solution was then centrifuged at 1200 rpm for 5 minutes. The resulting cell pellets were re-suspended and plated in 50% DMEM + 50% K-SFM with BPE/EGF with the addition of 5% v/v FBS and 1% v/v AA. PRCs were passaged when they reached 95% confluence and typically used for recellularisation at P2 stage.

3. Mesenchymal stromal cell (MSC) isolation

Male Wistar rats (350-375g) were used for MSC isolation. Briefly, sterile femurs from culled rats were irrigated (by manual injection) through the diaphysis with culture

medium (α -MEM) and the resulting cell suspension plated in α -MEM with 10% v/v FBS and 1% v/v AA. MSCs were passaged when they reached 95% confluence and typically used for recellularisation at the P2 stage.

4. LIVE/DEAD™ staining

The tissue sample was washed with PBS, and then completely immersed in the working solution (20 μ l of Ethd-1 and 10 μ l of calcein (Invitrogen/UK) in 10 ml of sterile PBS). This solution was incubated with the tissue sample for a period of 10-15 minutes while being protected from light, then aspirated. The sample was washed three times with PBS and examined using fluorescent microscopy.

5. AlamarBlue® assay

A working solution of 1:10 alamarBlue® reagent (Invitrogen/UK) in culture medium was added directly to the tissue to completely immerse the sample. Samples and controls (working solution without tissue sample) were incubated for 4 hours at 37°C in 5% CO₂ incubator. Each assay was performed in triplicate. Results were reported as mean $\pm$ standard error.

6. Histology and IHC

All samples were fixed in 10% v/v buffered neutral formalin, and samples were stained with haematoxylin and eosin (H+E) for light microscopy.

IHC was performed to characterise cell types and their distribution on the recellularised kidney bioscaffolds; the protocol is the same as above. Primary antibody dilutions were: aquaporin-1, aquaporin-2, synaptopodin, and von Willebrand Factor (all Abcam, UK) – all at 1:500.

For all experimental work as described above, $n \geq 3$.

Results

Optimisation of perfusion decellularisation in whole rat kidneys

We have decellularised cadaveric whole rat kidneys using perfusion decellularisation via the renal artery with SDS initially at 1% w/v concentration performed for decreasing durations of continuous perfusion (24 hours down to 4 hours) at 10 ml/min with 1% w/v SDS (Table 1a), which all yielded well decellularised bioscaffolds on histological analysis (Supplementary Fig. 1). Decellularisation was characterised by: histology and IHC, DNA and sGAG quantification, and growth factor (vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)) quantification. The 1% w/v SDS x 4 hours protocol was denoted the baseline protocol for further comparisons. Two further series of protocols were carried out in decreasing concentrations of SDS (0.5%, 0.25%, and 0.125% w/v) and for two durations of perfusion (4 hours and 8 hours, Table 1b). All further decellularisation protocols were characterised similarly to the baseline protocol.

Histology and IHC

Histological analysis of perfusion decellularised kidney bioscaffolds shows preservation of the structure and architecture of the renal ECM within the acellular parenchyma (Fig. 1), as has been previously shown[18, 23, 24]. The preservation of the arterial network within the whole kidney bioscaffolds was also demonstrated with injection of a resin polymer for vascular casting (Fig. 2). Immunohistochemical staining for key ECM components (collagen I, collagen IV, laminin and fibronectin) shows a physiological distribution as compared to the normal kidney tissue control (Fig. 1). There were no gross differences observed between any of the protocols (variation in % SDS or duration of perfusion) in terms of the histological

appearance and IHC staining (see also Supplementary Fig. 2 for full range of conditions analysed).

Quantification Assays

For all protocols, DNA levels in the decellularised kidney bioscaffolds (Fig. 3c) were $\leq 0.02\%$ of normal kidney DNA content. Quantification of sGAG levels (Fig. 3a, b) show an at least two-fold increase in the average levels of sGAG preserved between the 4 hour and 8 hour protocols ($p < 0.005$).

Levels of VEGF and bFGF preserved within the kidney ECM (Fig. 3d-f, g-i) were similar in that the 8 hour levels for both growth factors were significantly decreased compared to both normal kidney and the 4 hour protocols, and were in fact almost negligible for both. However, the levels of both growth factors were well preserved compared to normal kidney in the 4 hour series (3d-e, g-h), with the highest levels of preservation seen at the lowest concentration of SDS exposure i.e. 0.125% w/v in both VEGF and bFGF ($p < 0.05$). A clear trend of increasing levels of bFGF preserved as the concentration of SDS decreases can also be seen; however a plateau effect can be observed with both VEGF and bFGF as the difference observed between levels with 0.25% w/v SDS and those with 0.125% w/v was not significant ($p = 0.256$ for both). Group variations were noted in the levels obtained for all the quantification assays which are likely to be due to batch variation.

Recellularisation of whole kidney bioscaffolds

To recellularise the acellular whole kidney bioscaffolds for *in vitro* culture, these were injected intravascularly with two cell populations in parallel studies: primary renal cells (PRCs) isolated from 4 week old Wistar rats and adult Wistar rat bone marrow-derived

mesenchymal stromal cells (MSCs). After the cell seeding process, perfusion was maintained at 1.5ml/min for the designated 7 day culture period.

PRC-recellularised constructs

Cellular viability tests (Fig. 4a, b) after 7 days of *in vitro* culture demonstrate continued and extensive viability throughout the 3-D kidney construct, with obvious cellular ‘patterning’ following the vascular network and also ECM structural cues on LIVE/DEAD™ staining. Histological analysis showed cellular engraftment dispersed throughout the kidney ECM bioscaffolds which was not restricted to the vascular compartment. Focal areas of recellularisation comparable to normal histological density were present throughout and distinctive ‘glomerular-like’ structures are easily observed (Fig. 5a). IHC of renal cell markers (aquaporin 1 and aquaporin 2 for tubular cells, synaptopodin and von Willebrand factor for glomerular epithelial and endothelial cells respectively) demonstrated the presence of mixed cell types throughout the recellularised construct (Fig. 5b).

MSC-recellularised constructs

Cellular viability tests (Fig. 4a, b) after 7 days of *in vitro* culture were similar to the PRC constructs: extensive viability throughout the 3-D kidney construct, with obvious cellular ‘patterning’ following the vascular network and also ECM structural cues on LIVE/DEAD™ staining. Histological analysis again showed cellular engraftment dispersed throughout the kidney ECM bioscaffolds with focal areas of recellularisation comparable to normal histological density present throughout and distinctive ‘glomerular-like’ structures (Fig. 5a). IHC of renal cell markers (as above) were only strongly positive for von Willebrand factor as an endothelial cell marker (Fig. 5b).

Discussion

Various groups have demonstrated the feasibility of using the whole organ decellularisation approach to derive bioengineered (partially) functional organ constructs for surgical implantation[14-18]. While there is great interest in the recellularisation potential of these bioscaffolds, this potential should be maximised by optimisation of the quality of the ECM preserved after perfusion decellularisation. Both donor factors and decellularisation parameters should be standardised and rationalised to yield consistent and optimised bioscaffolds. Pre-optimisation and standard operating procedures are even more critical when it is likely that post-processing testing of the ECM bioscaffold (and the recellularised construct) is likely to be limited in scope in those cases destined for implantation. This should be the next stage in the evolution of whole organ decellularisation as a potential strategy for bioengineered organ replacements.

Currently, most groups are utilising disparate decellularisation protocols with marked variations even in work on the same organ and species[17, 25-29]. Hence we present here an evidence-based approach to determining a more systematically assessed protocol for whole rat kidney bioscaffolds using a single agent (SDS) with continuous perfusion, as defined by preservation of essential ECM components within the bioscaffold. The choice of SDS was derived from a systematic review of the whole organ decellularisation literature and SDS being the pre-dominant decellularising agent of choice, sometimes in combination with Triton-X100 or other agents[22]. SDS was utilised alone within this study as it has been well documented to produce complete decellularisation within ECM literature and also for purposes of clarity.

SDS ($\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3\text{Na}$) is an ionising detergent with molecule amphiphilic properties and critical micelle concentration (CMC) is 8.2 mM or approximately 0.23% w/v[30]; it is not pH-

sensitive between pH 5-10. It acts by solubilising cell membranes and nucleic membranes, with very effective removal of nuclear remnants and cytoplasmic proteins. However it can also denature proteins, remove GAG and growth factors, and cause damage to collagen and the ultrastructure of basement membranes[19] – which could have subsequent detrimental effects on recellularisation. Whole organ decellularisation with its underlying technique of vascular perfusion lends itself well to the principle of minimising SDS exposure since the native vascular system allows the maximum efficiency in decellularising agent delivery and penetration, especially to dense tissues. Historical protocols traditionally utilising SDS at 1% or even higher are not necessarily relevant in this context. Indeed, concentrations above the CMC lead to micellar formation of SDS molecules and subsequent plateauing of increased detergent efficacy. In addition, SDS residuals may be highly adherent and cytotoxic (with subsequent detrimental effects on recellularisation), and hence it would be important for these to be quantified in future work e.g. quantification assays of solubilised decellularised scaffolds using a colorimetric assay method [31]. Currently this is a significant limitation of all extant whole organ decellularisation work utilising SDS.

Our results have yielded an improved protocol (0.125% w/v SDS perfused for 4 hours) which uses a far lower concentration of SDS than other work on whole rat kidney decellularisation[18, 23] while being at least as effective in achieving removal of cellular components and DNA. The minimum concentration of SDS previously utilised for whole rat kidney decellularisation was typically 1%[18], although one study has used 0.5% in porcine kidneys[24], and the concentration can be variable up to as high as 4%[23]; no explicit justification (or systematic comparative characterisation of decellularised scaffolds) for any specific conditions is typically provided in any study except for *de facto* demonstration of ‘successful decellularisation’.

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3 VEGF and bFGF are both highly expressed within the kidney and are vital to both cellular
4 proliferation and kidney development. VEGF is expressed by virtually every endothelial cell
5 within the kidney[32], which is particularly relevant to the highly intricate vasculature of the
6 renal nephron; bFGF is involved in critical functions such as mitogenesis[33]. These two
7 growth factors were selected for their importance and ubiquity within the kidney for
8 quantitative assessment. Here we have also demonstrated a clear relationship between
9 decreasing exposure to SDS (i.e. concentration) and preservation of *in situ* growth factors,
10 down to the level where further decreases in the SDS concentration may not yield
11 significantly increased preservation, which has not been previously shown for any whole
12 organ bioscaffolds.
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26 In line with other kidney studies[18, 23], after confirmation of successful decellularisation,
27 we have recellularised the whole rat kidney bioscaffolds (by intravascular injection) with
28 PRCs and continuous *in vitro* perfusion and culture for 7 days, leading to viable constructs.
29 Histological analysis showed focal areas of dense cellular engraftment with some response
30 to kidney ECM structural cues although IHC analysis did not show appropriately localised
31 glomerular or tubular cells. We have also achieved comparable findings in recellularisation
32 of whole rat kidney bioscaffolds with MSCs, as they are a primary cell population of
33 relevance in potential clinical application. MSCs have been observed to have nephrogenic
34 potential[34-37] and as they are both sufficiently numerous in the adult and a potentially
35 host-derived (and hence immune-tolerant) cell population, they may play a crucial role in
36 the clinical translation of bioengineered whole organ constructs. As there was no evidence
37 of renal cellular differentiation with histological analysis, we did not proceed with renal
38 functional testing. However, since Song *et al* recently demonstrated urine production *in*
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vitro with their recellularisation protocol[18], it is possible that recellularisation with PRCs can lead to potentially functional renal regeneration on a whole kidney bioscaffold.

While we have examined here two fundamental parameters within the decellularisation process using SDS for whole rat kidneys, there are many other parameters of significance employed within decellularisation protocols as stated before – such as other decellularising agents (relevant to the type of tissue in question), additional processes e.g. freezing, use of additional agents, tissue harvesting factors, perfusion factors, and post-processing sterilisation methods[22]. All these will inevitably affect the quality of the ECM bioscaffold to a greater or lesser degree, and these effects have yet to be systematically quantified for whole rat kidneys or other organs/species. In addition, while growth factor levels may be better preserved by reducing the concentrations of the decellularising agent used (as we have demonstrated), functional tests of bioactivity should also be considered for more complete characterisation, such as comparative recellularisation studies related to different decellularisation parameters[20]. This is another current limitation and major area for future improvement. All these factors should play a critical part in developing safe, reliable and consistent whole organ bioscaffolds if they are to be taken to the clinical stage in the development of regenerated organ replacements.

In this study, systematic assessment of perfusion decellularisation parameters for whole rat kidneys with SDS has led to improved preservation of sGAG and growth factors along with minimised exposure to the decellularising agent and its detrimental effects; these whole rat kidney bioscaffolds were successfully recellularised with primary renal cells and MSCs. These findings may be applicable to other decellularised whole organ/species, and may lead the way to further optimisation and standardisation in the development of regenerated organ replacements for transplantation.

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Table 1

SDS concentration (% w/v)	Duration (hours)	Duration (hours)	Duration (hours)
1.0	24	8	4

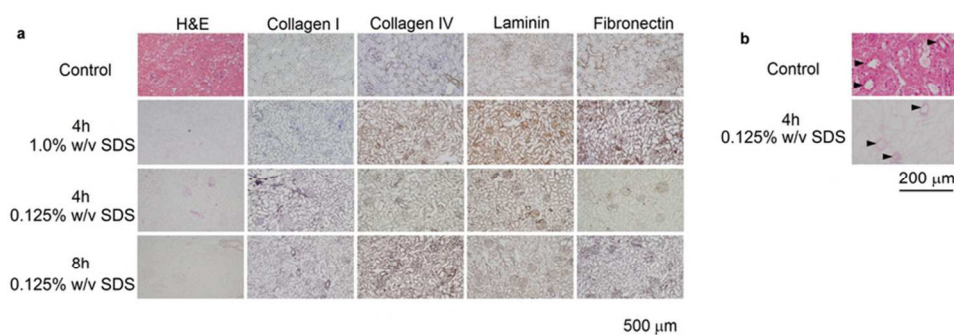
1a

Initial decellularisation protocols using SDS at 1% w/v and decreasing durations of continuous perfusion from 24 hours to 4 hours (at 10 ml/min).

SDS concentration (% w/v)	Duration (hours)	Duration (hours)
0.125	4	8
0.25	4	8
0.5	4	8
1.0 (index group)	4	-

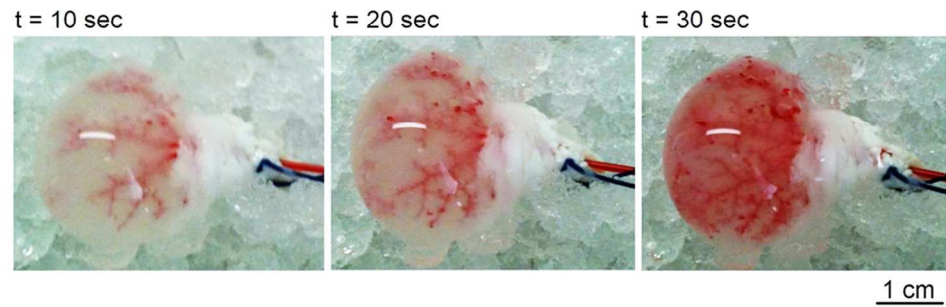
1b

Further optimisation protocols using SDS at decreasing concentrations (1% to 0.125% w/v) and different durations, i.e. 4 hours and 8 hours, of continuous perfusion (at 10 ml/min).



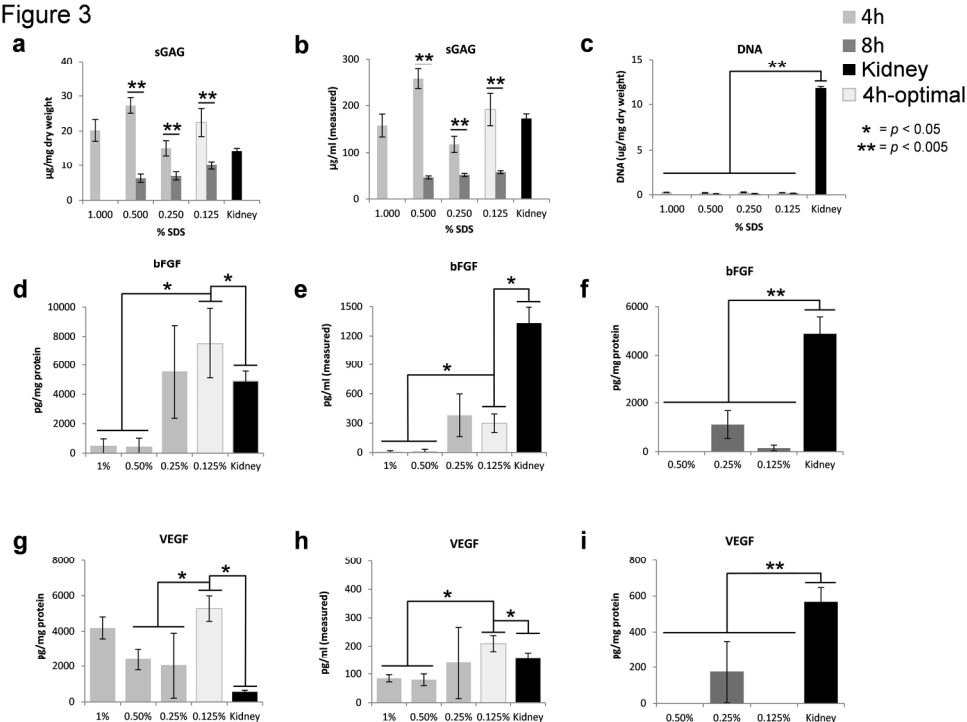
- (a) Histology (H+E staining) and IHC for ECM components (collagen I, collagen IV, laminin, and fibronectin) in decellularised whole rat kidney bioscaffolds. Selected representative SDS decellularisation protocols (0.125% x 4 hours, 0.125% x 8 hours) are shown compared with baseline (1% SDS x 4 hours) and control (i.e. normal kidney). No gross differences between these protocols were observed, nor between any of the other protocols compared.
- (b) Histology (H+E staining) of normal kidney and decellularised whole rat kidney bioscaffold (0.125% x 4 hours) shows preservation of micro-vasculature within the tissue (black arrows) similar in calibre and structure after decellularisation.
- 79x28mm (300 x 300 DPI)

Figure 2



Vascular casting of SDS decellularised (0.125% x 4 hours) whole kidney bioscaffolds shows preservation of intrinsic vasculature on injection of resin into cannulated renal artery (over 10-30 seconds).
99x38mm (300 x 300 DPI)

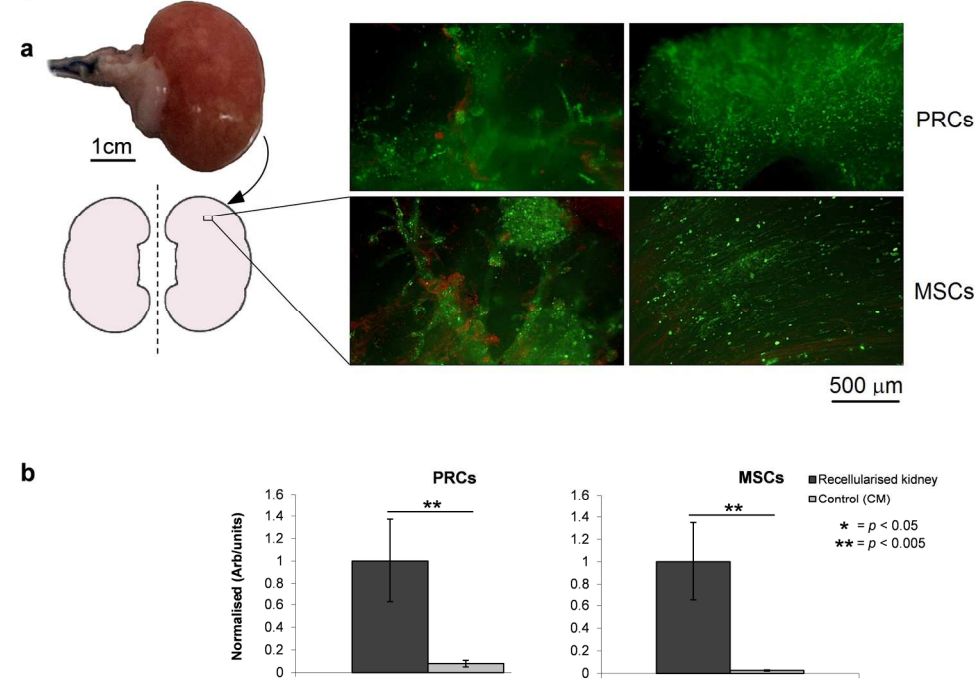
Figure 3



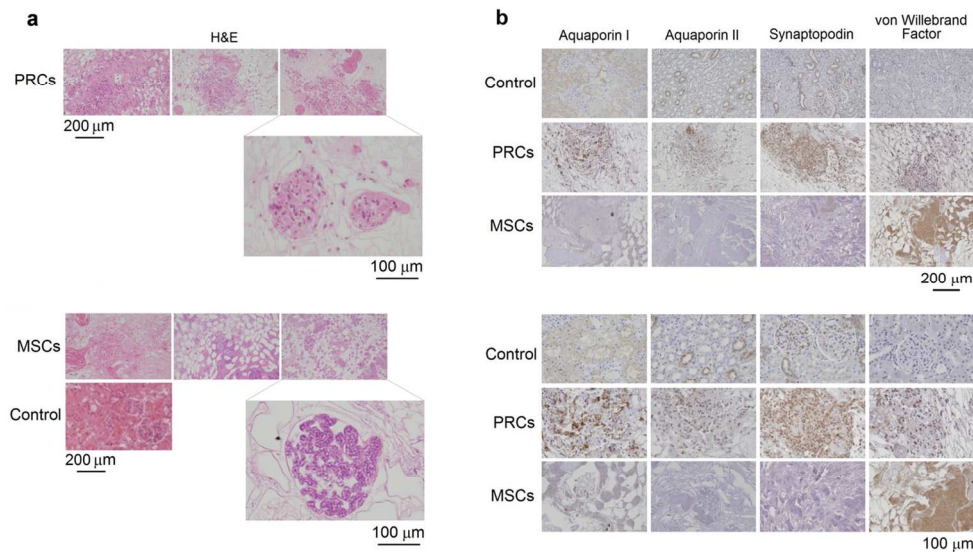
Characterisation of whole rat kidney ECM bioscaffolds for retention of sGAG, removal of DNA and growth factors (VEGF, bFGF) with quantification assays. (a, b) Quantification of sGAG retention within whole kidney ECM shows an at least 2-fold increase in the level of sGAG with the 4 hour protocols, as compared to the 8 hour protocols ($p < 0.005$). (c) DNA quantification shows no more than 0.02% of normal kidney DNA levels ($p < 0.005$) remaining on the decellularised kidney bioscaffolds and no significant difference seen between the 4 hour and 8 hour protocols. (d-i) Quantification of bFGF and VEGF levels shows virtually negligible levels preserved in the 8 hour protocols for both (f, i) but significant increasing levels preserved in the 4 hour protocols (d, e, g, h) with the decreasing concentration of SDS, and in particular with 0.125% x 4 hours (d, g) ($p < 0.05$). A plateauing off in the absolute measured levels of both bFGF and VEGF with decreasing concentrations of SDS (e, h) suggests that further decreases in the SDS concentration may not yield significantly increased preservation of growth factors.

201x159mm (300 x 300 DPI)

Figure 4



Viability testing in whole kidney bioscaffolds recellularised with primary renal cells (PRCs) and MSCs. (a) Photograph of a recellularised kidney construct which is hemi-sected to obtain fluorescence-microscopy images of LIVE/DEAD staining and for testing with Alamar Blue assay. LIVE/DEAD staining of the cut surface of both PRC- and MSC-recellularised constructs show dense distribution of viable cells throughout the kidney bioscaffolds with obvious cellular 'patterning' following the vascular network and ECM structural cues. (b) Alamar Blue assay demonstrated a highly visible colorimetric change indicative of cellular viability with both the PRC- and MSC-recellularised constructs, which was confirmed on fluorescence reading by spectrophotometer ($p < 0.005$).
193x145mm (300 x 300 DPI)



Histology and IHC for renal cell markers in recellularised whole rat kidney bioscaffolds. (a) H+E staining of the recellularised constructs shows cellular engraftment dispersed throughout the kidney bioscaffolds. Areas of recellularisation comparable to normal histological density are present throughout and distinctive 'glomerular-like' structures are observed. Similar findings are seen in both PRC- and MSC- recellularised constructs. Control = normal kidney. (b) IHC for renal cell markers (aquaporin 1, aquaporin 2, synaptopodin, and von Willebrand Factor for glomerular epithelial and endothelial cells respectively) demonstrates presence of mixed cell types throughout PRC-recellularised constructs although histological differentiation or localisation was not noted. Control = normal kidney. In MSC-recellularised constructs, IHC for renal cell markers were only significantly positive for von Willebrand factor.

127x74mm (300 x 300 DPI)