Translational process engineering for tissue engineered hollow organ advanced therapy investigational medicinal products

A thesis submitted to University College London for the degree of

Doctor of Engineering

by

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STATEMENT OF ORIGINALITY

I, Toby Joseph Proctor, confirm that the work presented in this thesis is my own. Where information has been derived from other sources or through a collaborative means, I confirm that this has been indicated in the thesis.

Print: Toby Joseph Proctor

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ABSTRACT

Tissue engineering has experienced increasing exposure and translational success in recent times, with tissue engineered products accounting for more than a quarter of approved advanced therapy medicinal products within Europe. Hollow organs represent a key target for developing novel therapies, typified by the recent success reported for tissue engineered hemilaryngeal replacements in a preclinical study. This thesis investigates the translational process engineering required to progress from a preclinical, good laboratory practice (GLP) process, to one that is compliant with good manufacturing practice (GMP) guidelines and suitable for clinical manufacture.

A GLP decellularisation protocol was translated to conform to GMP-guidelines by modifying the existing standard operating procedure, and adapting an off-the-shelf bioreactor to form a closed-system for aseptic processing. The process was successfully validated for aseptic operation and decellularisation efficacy evaluated, relative to the preclinical process. The decellularised, human hemilarynx scaffolds produced were demonstrated to support bone marrow mesenchymal stromal cells (BM-MSCs) up to product release. A bespoke, modular bioreactor was designed and fabricated to enable manufacture of hemilarynges at scale. The bioreactor was successfully validated for aseptic use, whilst biocompatibility testing indicated no preclusion to use with BM-MSCs or epithelial cells. Proof-of-principle data supported the concept of epithelial sheet production inside the bioreactor, utilising a sheet-specific cassette.

The bioreactor was retrospectively adapted to enable closed-system decellularisation processing of a third tissue-type, juvenile oesophagus. Acellular scaffold biomolecular composition and biomechanics were characterised, preceding implantation in a large-animal model. A second, bespoke bioreactor was designed, manufactured and employed to improve the manufacturing process.

The combined human larynx data supported the award of a clinical trial authorisation, whilst the oesophageal work is now transitioning to a pivotal animal study. These findings support the application of bespoke bioreactor systems in process closure and translation towards robust, regulatory compliant, manufacturing processes for tissue engineered products.
**IMPACT STATEMENT**

The research presented hereafter has, already, exhibited an impact outside of the originally intended thesis framework. The data presented in Chapter 2.2 and Chapter 4 have been submitted to the Medicines and Healthcare products Regulatory Agency as part of two investigational medicinal product dossiers, which have subsequently resulted in the award of clinical trial authorisations (EudraCT: 2013-004359-18 and EudraCT: 2015-002108-10). The data generated by these trials will provide information on the safety and efficacy of the respective advanced therapy investigational medicinal products, providing a foundation for subsequent pivotal studies. Should the therapies eventually receive marketing authorisation, they will provide a solution to the existing unmet clinical need associated with their respective indications.

Additionally, the modular bioreactor described in Chapter 4 has been recently employed in the manufacture of a tissue engineered tracheal replacement for a paediatric patient, as an unlicensed medicine for human use, by the Centre for Cell, Gene and Tissue Therapeutics (MHRA MIA(IMP) 11149). The patient has, at time of print, experienced a gross improvement in quality of life.
ACKNOWLEDGEMENTS

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<th>Description</th>
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<tr>
<td>A/A</td>
<td>Antibiotic-Antimycotic</td>
</tr>
<tr>
<td>ALI</td>
<td>Air-liquid interface</td>
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<tr>
<td>ATMP</td>
<td>Advanced therapy medicinal product</td>
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<tr>
<td>ATIMP</td>
<td>Advanced therapy investigational medicinal product</td>
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<tr>
<td>autoDET</td>
<td>Automated detergent enzymatic treatment decellularisation process</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BM-MSC</td>
<td>Bone marrow mesenchymal stromal cell</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CAD</td>
<td>Computer aided design</td>
</tr>
<tr>
<td>CAM</td>
<td>Computer aided manufacture</td>
</tr>
<tr>
<td>CAT</td>
<td>Committee for Advanced Therapies</td>
</tr>
<tr>
<td>CCGTT</td>
<td>Centre for Cell, Gene and Tissue Therapeutics</td>
</tr>
<tr>
<td>CHMP</td>
<td>Committee for Medicinal Products for Human Use</td>
</tr>
<tr>
<td>CTA</td>
<td>Clinical trials authorisation</td>
</tr>
<tr>
<td>CQA</td>
<td>Critical quality attribute</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEM</td>
<td>Detergent-enzymatic method</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DS</td>
<td>Design specification</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
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<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
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<tr>
<td>GLP</td>
<td>Good laboratory practice</td>
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<td>GTMP</td>
<td>Gene therapy medicinal product</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
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<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HDT</td>
<td>Heat deflection temperature</td>
</tr>
<tr>
<td>HTA</td>
<td>Human Tissue Authority</td>
</tr>
<tr>
<td>ID</td>
<td>Inner diameter</td>
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<tr>
<td>IMPD</td>
<td>Investigational medicinal product dossier</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KI</td>
<td>Potassium iodide</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption/ionization time-of-flight</td>
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<tr>
<td>MHRA</td>
<td>Medicines and Healthcare products Regulatory Agency</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal stromal cell</td>
</tr>
<tr>
<td>NHSBT</td>
<td>National Health Service Blood and Transplant</td>
</tr>
<tr>
<td>NZW</td>
<td>New Zealand White</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyetherimide</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethyl methacrylate</td>
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<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>sCTMP</td>
<td>Somatic cell therapy medicinal product</td>
</tr>
<tr>
<td>SDC</td>
<td>Sodium deoxycholate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>sGAG</td>
<td>Sulphated glycosaminoglycan</td>
</tr>
<tr>
<td>SM</td>
<td>Starting material</td>
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<tr>
<td>SOP</td>
<td>Standard operating procedure</td>
</tr>
<tr>
<td>TE</td>
<td>Tissue engineering</td>
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<tr>
<td>TEP</td>
<td>Tissue engineered product</td>
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<tr>
<td>TERM</td>
<td>Tissue engineering regenerative medicine</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
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<tr>
<td>3WSC</td>
<td>Three-way stop-cock</td>
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1 INTRODUCTION

1.1 REGENERATIVE MEDICINE AND TISSUE ENGINEERING

Encompassing pharmaceutical, biopharmaceutical/biotech, medical device and cell therapy platform technologies, regenerative medicine is focussed upon the restoration and/or establishment of normal physiological function through the replacement or regeneration of cells, tissues or organs (Mason and Dunnill, 2008). The application of tissue engineered, cellular, and genetic therapies is an exciting and rapidly expanding field within regenerative medicine that is being driven by advancements in biochemistry, developmental and cellular biology, immunology, materials science, physics, medicine and biotechnology (Schenke-Layland and Walles, 2013). Through ever increasing collaboration across these fields of expertise, there is a growing trend of clinical translation throughout almost all clinical specialities (Harrison et al., 2014, Huang et al., 2015, Wobma and Vunjak-Novakovic, 2016, Gomes et al., 2017). This is, in turn, reflected by an increasing global market value that is set to increase from $18.9 billion in 2016 to greater than $53.7 billion in 2021 (Kelly Scientific Publications, 2017). Following the recent U.S. Food and Drug Administration (FDA) approval of the first cell mediated gene therapies, Kymriah™ (tisagenlecleucel; Novartis) and Yescarta™ (axicabtagene ciloleucel; Kite Pharma), it may be that the inflection point for the industry has now been passed.

One branch of regenerative medicine that is currently exhibiting promise is tissue engineering (TE). First proposed as a distinct subject field in communications with the National Science Foundation during the 1980s (Viola, 2003), this interdisciplinary field has been defined as one that “applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function” (Langer and Vacanti, 1993). TE was originally purported to consist of three basic functional approaches: the application of isolated cells or cellular substitutes, the use of tissue-inducing substances such as growth factors, and the employment of biologic and synthetic matrices either with or without cellular contribution (Langer and Vacanti, 1993). Both cells and matrices may then be further classified in accordance with their origin. Allogeneic therapies involve the administration of agents to a recipient from a genetically distinct donor, or pool thereof, from the same species. Alternatively, autologous treatments are isolated from and administered to the same individual. Starting materials (SMs) may also be procured from different species to the recipient, such as animal derived bio-prosthetic cardiovascular valves. These are referred to as xenogeneic. Furthermore, matrices may be synthetically or biologically derived; with each category possessing both benefits and caveats.
Although initially conceived of as a method for addressing the ever increasing tissue and organ shortage, conceptual and technical innovation has increased the potential range of TE applications and resulted in the development of cell-containing life support systems, such as extracorporeal bioartificial kidneys (Humes et al., 2004) and livers (Sauer et al., 2003), as well as tissue units capable of diagnostic, efficacy and toxicology screening (Chang et al., 2010, Schreiter et al., 2012). The field has continued to expand, and now interfaces with clinical medicine, developmental biology, biochemical and mechanical engineering, materials science and plethora of fields in between. Given the inherent manner in which contributory fields and sub-fields are intertwined, it is perhaps unsurprising that TE and regenerative medicine are considered by different experts to be both simultaneously synonymous and a subcategory of one another (Nerem et al., 2002, Viola, 2003). Whilst the nomenclature remains debatable it is perhaps more appropriate to utilise the umbrella terminology of tissue engineering regenerative medicine (TERM).

Early regeneration efforts in TERM were largely focussed upon flat tissues, such as the bioengineered skin developed in the early 1980s (Green et al., 1979, Banks-Schlegel and Green, 1980, Burke et al., 1981, O'Connor et al., 1981). The techniques employed here provided the foundations for what would become the first cell-based tissue engineered products (Parenteau, 1999), including Epicel® (Wright et al., 1998), Apligraf® (Eaglstein and Falanga, 1997)and Dermagraft® (Hansbrough et al., 1992). Subsequent advancements within TERM saw the successful implementation of autologous chondrocyte implantation for the treatment of cartilage defects (Brittberg et al., 1994). This was shortly followed by the first marketing authorisation for a product for cartilage repair, Carticel®, in 1997 (Department of Health and Human Services Biologics License No. 1233).

These initial targets represent the primary level of organ/tissue complexity and so it is perhaps unsurprising that they have been in clinical use, both pre- and post-marketing authorisation, for almost three decades. Subsequent increments in functional complexity results in three further discrete categories of TE targets: (1) hollow tubular organs, including blood vessels, the trachea and urethra; (2) hollow non-tubular organs such as the bladder and uterus; and (3) solid organs, including highly functionally and architecturally intricate structures like the heart, kidney and liver (Shafiee and Atala, 2017). Given their enormous complexity, generation of functional solid organs currently remains at the outer boundaries of what is achievable through research science. However, ever quickening progress has been made in the development of hollow tubular and non-tubular organs alike.
Development of TERM approaches for pathological indications of the bladder remain archetypal for hollow non-tubular organs. Promising results have been observed in murine (Atala et al., 1992, Atala et al., 1993, Lai et al., 2002, Danielsson et al., 2006), rat (Schoeller et al., 2001, Kanematsu et al., 2003), porcine (Fraser et al., 2004, Schultheiss et al., 2005) and canine (Yoo et al., 1998, Oberpenning et al., 1999, Sievert et al., 2006) models using a variety of synthetic and biologically derived biomaterials, in combination with autologous and allogeneic cell types. Subsequently, these have formed the scientific basis for 18 clinical studies comprising of a total of 169 patients (Gasanz et al., 2018). Despite this modest number of early studies there remains a lack of a consensus regarding the most appropriate approach, with both positive and negative outcomes being reported for biological (small-intestinal submucosa) and synthetic (collagen-polyglycolic acid) constructs (Atala et al., 2006, Caione et al., 2012, Schaefer et al., 2013, Joseph et al., 2014, Zhang and Liao, 2014). For further information, both Adamowicz et al. (2017) and Horst et al. (2013) provide a suitable insight into this particular field of TERM.

Of the aforementioned subsets, hollow tubular organs have been the most frequently adopted as suitable for tissue engineered regeneration. Less structurally and functionally complex than some hollow non-tubular organs, they represent the next logical progression when pushing the boundaries of TERM. Whilst there have been promising advances in the clinical application of both blood vessels (Shin'oaka et al., 2005, Lawson et al., 2016) and the urethra (Osman et al., 2014), development of upper-airway tissue engineered replacements has perhaps been the more notable within this subfield and is widely reported as one of the frontrunners of TERM. Whether or not this is an accurate reflection, it can be attributed to the physiological nature of the airway as well as the pathological indications underlying the required treatments. Unlike vasculature tissue engineered replacements, which may consist of a short length of vessel or a patch for augmentation, upper airway interventions require the replacement of large proportions of the diseased organ. Patients are typically suffering end-stage disease without suitable conventional alternatives, in contrast to interventions that are used to address either morbidity or to mitigate long-term risk of mortality.

Following a series of reported clinical successes within the literature (Macchiarini et al., 2008, Elliott et al., 2012), two clinical trials have recently been initiated within the UK to formally investigate the safety and potential efficacy of tissue engineered upper-airway replacements, separately targeting the larynx (EudraCT: 2013-004359-18) and trachea (EudraCT: 2015-002108-10). The establishment of formal clinical trials not only underlines the potential that these interventions possess, but also highlights the increasing awareness from both clinicians and researchers that there is a necessity to
established robust manufacturing processes that facilitate the smooth and effective translation. Through thorough process engineering and an increasing adoption of good manufacturing practice (GMP), the “minimum [quality] standard[s] that a medicines manufacturer must meet in their production processes” (European Medicines Agency, 2019), it is increasingly likely that scalable manufacture of tissue engineered organ replacements may be realised.

1.2 EUROPEAN REGULATION OF ADVANCED THERAPIES

In accordance with the expansion of TERM both academically and clinically, there has in recent years been an acknowledgement from national and international bodies that there is a growing need for oversight and regulation of potential medicines conceived through the knowledge generated within this field. Regulatory bodies whose traditional jurisdiction encompassed chemical drug substances, therapeutic antibodies and biosimilars are now required to ensure the appropriate regulation and control of potentially novel therapies generated by expanding research into regenerative medicine.

Within the European Union regulation of both human and animal health is overseen by the European Medicines Agency (EMA), which reports directly to the European Commission. The Agency are responsible for authorising advanced therapy medicines for human use that are founded upon the application of genes, cells or tissues. Formal classification of such medicines as advanced therapy medicinal products (ATMPs) is in accordance with the criteria outlined in Directive 2001/83/EC, as amended by Article 17 of Regulation (EC) No 1394/2007 (European Commission, 2007).

1.2.1 Advanced therapy medicinal product classification

ATMP classification identifies whether medicinal products satisfy the definition of gene therapy medicinal products (GTMPs), somatic cell therapy medicinal products (sCTMPs), tissue engineered products (TEPs) or combined ATMPs as outlined in Directive 2001/83/EC, as amended by Regulation (EC) No 1394/2007 (referred to hereafter as ‘the ATMP Regulation’). The defining characteristics of GTMPs, sCTMPs, TEPs and combined ATMPs, as outlined in the ATMP Regulation, are covered in Table 1.1. For further clarification on these definitions, and associated topics, the EMA has published a reflection paper on classification of ATMPs (European Medicines Agency, 2015).
Table 1.1 Advanced therapy medicinal product definition - Adapted from Regulation (EC) No 1394/2007

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<tr>
<th>ATMP Classification</th>
<th>Defining Characteristics (Regulation (EC) No 1394/2007)</th>
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| **Gene Therapy Medicinal Product (GTMP)** | (a) Contains an active substance which contains or consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence.  
(b) The therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence. |
| **Somatic Cell Therapy Medicinal Product (sCTMP)** | (a) Contains or consists of cells or tissues that have been subjected to substantial manipulation so that biological characteristics, physiological functions or structural properties relevant for the intended clinical use have been altered, or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor.  
(b) Is presented as having properties for, or is used in or administered to human beings with a view to treating, preventing or diagnosing a disease through the pharmacological, immunological or metabolic action of its cells or tissues. |
| **Tissue Engineered Product (TEP)** | (a) Contains or consists of engineered cells or tissues.  
(b) Is presented as having properties for, or is used in or administered to human beings with a view to regenerating, repairing or replacing a human tissue. |
| **Combined Advanced Therapy Medicinal Product** | (a) An ATMP that must incorporate, as an integral part of the product, one or more medical devices within the meaning of Article 1(2)(a) of Directive 93/42/EEC or one or more active implantable medical devices within the meaning of Article 1(2)(c) of Directive 90/385/EEC.  
(b) Its cellular or tissue part must contain viable cells or tissues.  
(c) Its cellular or tissue part containing non-viable cells or tissues must be liable to act upon the human body with action that can be considered as primary to that of the devices referred to. |
## ATMP Classification

<table>
<thead>
<tr>
<th>ATMP Classification</th>
<th>Additional Information</th>
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<tr>
<td>Gene Therapy Medicinal Product (GTMP)</td>
<td>GTMPs do not include vaccines against infectious disease. Both characteristics (a) and (b) must be fulfilled for a product to be considered a GTMP.</td>
</tr>
<tr>
<td>Somatic Cell Therapy Medicinal Product (sCTMP)</td>
<td>For the purposes of point (a), the manipulations listed in Annex I to Regulation (EC) No 1394/2007, in particular, shall not be considered as substantial manipulations: cutting, grinding, shaping, centrifugation, soaking in antibiotic or antimicrobial solutions, sterilization, irradiation, cell separation, concentration or purification, filtering, lyophilization, freezing, cryopreservation, and vitrification. This list is, however, non-exhaustive. Both characteristics (a) and (b) must be fulfilled for a product to be considered a sCTMP.</td>
</tr>
<tr>
<td>Tissue Engineered Product (TEP)</td>
<td>A tissue engineered product may contain cells or tissues of human or animal origin, or both. The cells or tissues may be viable or non-viable. It may also contain additional substances, such as cellular products, bio-molecules, biomaterials, chemical substances, scaffolds or matrices. Products containing or consisting exclusively of non-viable human or animal cells and/or tissues, which do not contain any viable cells or tissues and which do not act principally by pharmacological, immunological or metabolic action, are excluded from this definition. Cells or tissues shall be considered ‘engineered’ if they fulfil at least one of the following conditions: (a) the cells or tissues have been subject to substantial manipulation, so that biological characteristics, physiological functions or structural properties relevant for the intended regeneration, repair or replacement are achieved. The manipulations listed in Annex I, in particular, shall not be considered as substantial manipulations, (b) the cells or tissues are not intended to be used for the same essential function or functions in the recipient as in the donor.</td>
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Additional concepts that are worth clarifying to provide a more complete understanding of ATMP classification can be found in Appendix 1.
Whilst formal classification of medicinal products as ATMPs is optional within a European context, it does provide clear benefits in establishing the correct regulatory framework under which the medicine should be regulated. This has implications not only clinically, but also highlights suitable development pathways and how the product fits within the scientific regulatory framework. This can stimulate appropriate dialogue with additional international regulatory bodies, such as the FDA or the Pharmaceuticals and Medical Devices Agency, facilitating global access to safe and effective high quality healthcare. Additionally, and with particular importance to the work undertaken herein, it provides the European classification position on a potential medicine to the appropriate National Competent Authorities. Subsequently, this enables the suitable procedural progression that is necessary for the submission of clinical trial dossiers, ultimately contributing to the award and undertaking of clinical trials.

1.2.2 The current European ATMP landscape

Between 1999 and 2015 there has been an increasing number of ATMP trials registered globally, with advanced therapy trials accounting for more than 10% of all clinical trials during this period (Hanna et al., 2016). Within the last decade alone, since the adoption of the ATMP Regulation, there have been 487 ATMP trial registrations. Moreover, the EMA has granted 270 ATMP classifications and received a total of 19 marketing authorisation applications (European Biopharmaceutical Enterprises, 2017). There are currently seven ATMPs with marketing authorisation within the EU, including Chondrocelect®, Imlygic®, Kymriah®, MACI®, Provenge®, Strimvelis®, and Yescarta® (de Wilde et al., 2018, Gilead Sciences, 2018, Novartis, 2018).

Within the UK there has been a sharp increase in the number of organisations developing ATMPs over the last five years, rising from 22 to 64 in 2017. This has been accompanied by a growing number of clinical trials, with 59 active in 2017 compared to 21 in 2012 (Cell and Gene Therapy Catapult, 2018). Undoubtedly, this highlights the increasing acknowledgement of the potential of advanced therapies to address unmet clinical need.

1.2.3 Tissue Engineered ATMPs

Despite much of the current global focus being directed towards sCTMPs and GTMPs, nearly a quarter of all ATMP clinical trials are investigating TEPs (Hanna et al., 2016). It is perhaps unsurprising then that TEPs account for two of the seven ATMPs with marketing authorisation in Europe. Guidance applications to the EMA have resulted in a gross increase in TEP classification in recent years, and in 2016 TEP classifications rose substantially, both in real terms and as a proportion of total ATMP allocations, when compared to the previous 7 years (Phacilitate, 2017).
As discussed in section 1.1, early stage development of hollow tubular TEPs is currently burgeoning as a consequence of strong foundational TERM research that has been buoyed by a series of landmark clinical cases. In addition to the aforementioned trials addressing pathologies of the larynx and trachea, there are also reports of promising preclinical research investigating potential TEPs for treating oesophageal disease (La Francesca et al., 2018, Urbani et al., 2018). This research represents a key opportunity for the field to build upon existing evidence that tissue engineered advanced therapies can provide a widespread solution to unmet clinical need. Whilst initial ground-breaking clinical interventions have been produced as unlicensed medicines (Macchiarini et al., 2008, Elliott et al., 2012), either as hospital exemptions or through the specials scheme, it is essential that suitable manufacturing processes are developed in order to reliably and reproducibly manufacture ATMPs that meet existing and future demand. This requires effective translation of preclinical work through process engineering that encompasses an appreciation of scale, GMP, process and quality control, as well as the existing regulatory environment. Failure to do so not only restricts the progress of global healthcare but potentially places the patient population at risk, either through restricted access to the best available treatments or directly as a result of medicine safety.

This thesis documents the process engineering that was undertaken as part of the translation of a preclinical manufacturing process for tissue engineered larynx production, to one that complies with GMP guidelines and is suitable for licensure as a medicinal product. It subsequently describes how a bespoke bioreactor, developed for tissue engineered larynx, was then applied to the aforementioned oesophageal process (Urbani et al., 2018). Whilst the respective Chapter introductions provide a more in-depth background overview, the following section provides an introduction to each target organ and current tissue engineering approaches employed address existing pathologies.
1.3 HOLLOW ORGAN TARGETS FOR ATMP DEVELOPMENT

1.3.1 Larynx

1.3.1.1 Laryngeal anatomy and pathology

Superior to the trachea, the larynx is located within the anterior aspect of the neck. This box-like organ is typically 44 mm in length and 36 mm in diameter in the male, and 36 mm by 26 mm in the female (Finucane, 2010). The larynx consists of a mucosa encased assembly of cartilages, ligaments, and muscles. The cartilage portion is comprised of the singular thyroid, cricoid, and epiglottis cartilages, and the paired arytenoids, corniculate and cuneiform (Baiguera, 2013). The thyroid cartilage, which encompasses the airway anteriorly and laterally, plays a largely protective role; whilst the inferior, ring-shaped cricoid cartilage fully encircles the airway. The epiglottis is a fibroelastic cartilage that, whilst attached to the thyroid cartilage, serves to prevent aspiration during swallowing (Baiguera, 2013) The pyramidal paired arytenoids provide an attachment platform for the vocal ligament, whilst the fibroelastic corniculates and elastic cuneiform cartilages structurally support the ary-epiglottic fold. This structure provides a supportive framework for the vocal folds, as well as the thyroarytenoid, lateral cricoarytenoid, and posterior cricoarytenoid muscles.

At a cellular level, nonkeratinizing stratified squamous epithelium covers the supraglottis and true vocal cords. The subglottis, ventricle, and false vocal cords are covered by pseudostratified ciliated respiratory epithelium. Progression between the false and true vocal cords is mediated by an intermediate transitional epithelium. These cellular populations are supported by an extensively branched vascular and lymphatic network, as well as musculature and additional cell types (Baiguera, 2013).

Functionally, the larynx acts as multilevel sphincter that prevents aspiration caused by foreign matter during inhalation. More recently, in an evolutionary context, it facilitates phonation as the primary sound source (Birchall, 2011). It has also been suggested that the larynx may represent an important immunological organ, given the high concentration of immunologically active cells located within its mucosa during the native, non-response state (Gorti et al., 1999, Rees et al., 2003, Barker et al., 2006).
Figure 1.1 Human larynx anatomy. Articulate anterior (A) and lateral (B) aspects. Disarticulated and separated views (C, D). Articulated posterior view (E), epiglottis and hyo-epiglottic ligament (F), and articulated superior view (G) (Agur et al., 2013).
Pathological narrowing, known as stenosis, of the larynx can occur as a result of a range of causes including congenital defect, blunt trauma, inhalation injury, gastroesophageal reflux, autoimmune disease, and endotracheal intubation (Younis et al., 2004, Abdelkafy et al., 2007, Baiguera, 2013). Intubation is cited as the most common cause of stenosis, and is reported to occur in 6-21% of interventions (Omori et al., 2005). Oncologically, laryngeal carcinoma has been reported as the second most abundant malignancy of the head and neck, with 90% of cases reported as smoking/alcohol related squamous cell carcinoma (Duque et al., 2007, Baiguera, 2013).

As is the case with tracheal stenosis, there are a number of treatments currently in practice; however, these are generally considered unacceptable options and there is a clear need for novel therapies. Mild laryngeal stenosis can be addressed with endoscopic treatment options; however these are largely unsuitable for more severe indications. In these instances open surgical procedures, such as laryngotracheal reconstruction are needed. The most widely undertaken of these involves an anterior-posterior cricoid split. This procedure leaves much to be desired though, with reintubation and postoperative tracheostomy often employed to invasive and detrimental effect. In particularly severe stenoses, cricotracheal resection is an option. This, however, tends to result in a wide range of contraindicative complications, detailed by Rutter et al. (2001) and Hart et al. (2009). Oncology patients suffering from minor neoplasms typically undergo localised resection, although this often results in vocal cord defects that cause hoarseness. In advanced neoplastic cases it is necessary to perform highly mutilating total laryngectomies (The Royal College of Surgeons of England, 2000). Postoperatively there is a significant issue with social reintegration, with patients requiring a life-long tracheotomy and vocal rehabilitation being palliative at best. Chemo-radiation offers the only viable nonsurgical intervention at present, although this typically results in high morbidity, 5% mortality, and often entire loss of laryngeal functionality (Baiguera, 2013).

Current laryngotracheal augmentation relies upon autologous cartilage to expand stenosed areas (de Jong et al., 2000). Cartilage source depends largely on graft locality, with auricle and thyroid ala cartilage used for anterior sections. Where wide cricoid distraction is required, rib cartilage is often sourced. Although successful, these procedure are associated with high levels of morbidity and complications (Baiguera, 2013).

Autografts have been utilised post hemilaryngectomy for laryngeal reconstruction using external larynx muscle flaps (Miodonski et al., 1965). Although this procedure has proven capable of preserving speech, it is highly variable in its efficacy and tracheotomy tubes have been required postoperatively in a number of patients. Using a similar technique,
ipsilateral thyrohyoid muscle has been used to reconstruct the glottis, though once again outcomes were varied (Quinn, 1975). More recently, tracheal autotransplantation has been employed to ameliorate hemilaryngectomy defects (Delaere et al., 1998, Delaere et al., 2000, Delaere and Hermans, 2003, Delaere et al., 2011). Although this procedure appears to be safe, reliable, and resulting in good postoperative functionality, it remains unsuitable for oncological indications; following delayed tumour removal and early tumour reoccurrence. Although this procedure has since been improved, it has been limited to the treatment of glottis carcinoma with subglottic extension and cricoid chondrosarcomas by highly expert surgical teams (Delaere and Hermans, 2003, Delaere et al., 2011).

Homografts have been utilised in instances of subglottic stenosis, whereby irradiated trachea have been used for laryngeal reconstruction, resulting in the successful regeneration of epithelialized lumen (Kunachak et al., 2000). Additionally, cryopreserved thyroid/cricoid cartilage has been homografted to facilitate neo-larynx construction following total laryngectomy (Garozzo and Rossi, 1993). There has, however, been just one reported incidence of this technique, and long-term follow up remains unreported. As it stands, all grafting approaches have failed to provide an adequate solution to stenosis due to their lack of true laryngeal structure, and thus functionality.

An alternate option to grafting is total, or partial, laryngeal transplantation. The first instance of this, attempted in 1969 by Kluyskens and Ringoir (1970) resulted in a poor outcome and cancer recurrence. Since then, however, there has been clinical success. In 2001, Strome et al. (2001) reported total larynx replacement with a “pharyngolaryngeal complex with six tracheal rings and adjoining parathyroid glands” (Baiguera, 2013). Although the patients must remain immunosuppressed, the transplant has remained functional and continues to provide an acceptable quality of life. More recently, in 2010, the world’s first true laryngeal transplantation was successfully completed in a patient in the USA (University College London, 2011). Despite initial postoperative monitoring failing to report any complications, it remains to be seen as to what the long term stability of this intervention will be. Unfortunately, this procedure still requires life-long immunosuppression, and so may not be suitable for patients with a history or risk of laryngeal carcinoma; a patient group who, paradoxically, are more likely to require laryngeal replacement/intervention.
1.3.2 Tissue engineering approaches to laryngeal regeneration

Investigatory research has, to date, focussed on a number of varied tissue engineering approaches to tackle laryngeal regeneration. These include, but are perhaps not limited to, the application of synthetic polymeric materials, biomolecular scaffolds, and decellularised scaffolds.

In 2004, Kamil et al. (2004) investigated a laryngotracheal reconstruction utilising a Pluronic F-127 biodegradable scaffold, in a porcine model. Autologous auricular chondrocytes were cultured in combination with the biodegradable Pluronic F-127 to produce viable and structurally stable tissue engineered cartilage. Grafts were seamlessly incorporated within the surrounding native tissue, with no evidence of an inflammatory reaction. Kamil et al. (2004) suggested that whilst the ability to acquire cartilage of desired dimensions with low donor morbidity is advantageous, the costs and time-frame involved still require improvement. In addition to tracheal regeneration, collagen sponge encased Marlex mesh scaffolds have been investigated for subglottic stenosis treatment (Omori et al., 2005). The outlook for this approach appears promising, with confluent epithelial regeneration and good incorporation of the scaffold into native tissue reported in both animal and human investigations (Omori et al., 2004, Omori et al., 2008). Future characterisation of efficacy is still required before widespread clinical adoption, following evidence of granulation and scaffold polymer exposure (Omori et al., 2004).

Biomolecular scaffolds have been investigated to determine whether donor site morbidity can be reduced when harvesting cartilage grafts for laryngotracheal reconstruction (Weidenbecher et al., 2007). Autologous chondrocytes were seeded onto a hyaluronan-based scaffold and cultured within a bioreactor, before being implanted in a rabbit model. Although there were no indications of respiratory stress, histological assessment revealed a nonspecific foreign body response, eventually resulting in complete degradation of the Hyalograft C and graft failure. Subsequent to this, Gilpin et al. (2010) investigated whether fabricating scaffold-free auricular cartilage graft would circumvent a foreign body response. Although implanted grafts did not exhibit histological signs of inflammation or degradation, there was evidence of mechanical failure and migration. Further work is needed to produce biomechanically stronger biomolecular scaffolds that do not elicit immunorejection or inflammation.

Given the success achieved in the application of decellularised tracheal scaffolds for clinical intervention (Elliott et al., 2012), it is unsurprising that a similar approach has been applied to laryngeal regeneration. Following partial hemilaryngectomies,
decellularised porcine-derived extracellular matrix (ECM) scaffolds were employed to reconstruct canine thyroid cartilage and vocal folds (Huber et al., 2003). This approach was highly successful, with histological evidence of epithelialization, thyroid cartilage regeneration, organised submucosal glandular structuring, and some skeletal muscle fibres (Huber et al., 2003). Results revealed ECM-augmented macro- and microscopic regeneration that is superior to standard control procedures, further supporting the notion that ECM remodelling may be employed to regenerate tissue to provide structural and functional characteristics comparable to the native state (Ringel et al., 2006).

Subsequent to these positive results, Ansari et al. (2017) reported on a preclinical study in a porcine model investigating the application of porcine derived hemilarynges seeded with human bone-marrow mesenchymal stromal cells (BM-MSCs). Results were largely positive and, given their foundational importance to the work described hereafter, are discussed further in section 3.1.

1.3.3 Oesophagus

1.3.3.1 Paediatric oesophageal anatomy and pathology

The human oesophagus starts at the distal juncture of the pharynx, posterior to the larynx. It extends through the cervical region, posterior to the trachea, continuing in the posterior thorax before passing through the oesophageal hiatus and terminating in the abdomen at the oesophagogastric junction (Agur et al., 2013). The primary function of the organ is to act as a motile conduit for food and liquids to pass from the mouth to the stomach. Movement of contents is achieved through peristaltic relaxation-constriction of the abluminal muscular compartment, which is innervated by the vagus nerve and the sympathetic cervical and thoracic trunks (Agur et al., 2013). Total length is typically 8-10 cm in neonates (Lander and Newman, 2013). The upper third consists of striated muscle, which transitions to smooth muscle for the lower two thirds.

The lumen of the oesophagus is enclosed by a mucosal layer of nonkeratinizing stratified squamous epithelium, lamina propria and muscularis mucosae. The submucosa supports secretary mucosal glands that are responsible for luminal lubrication, and interposes between the mucosa and the muscularis externa. The muscular compartment consists of an inner layer of circularly oriented muscle fibres, surrounded by an outer longitudinal layer (King, 2002).

Oesophageal replacement or augmentation is indicated by a range of congenital and acquired conditions. Within the paediatric population, the principal indication for augmentation is failed oesophageal anastomosis following treatment of oesophageal atresia (OA) (Totonelli et al., 2013). OA occurs as frequently as 1:4000 live births in the
UK, with approximately 8-10% of pathologies manifesting as long-gap (2-5 cm) (Ron et al., 2009). This equates to a target population of approximately 20 year⁻¹.

The most prevalent presentation of OA, indicated in 86% of cases, is associated with tracheooesophageal fistula (Spitz, 2007). In this instance, the proximal oesophagus terminates blindly in the superior mediastinum; whilst the distal oesophagus forms the tracheooesophageal fistula with the posterior trachea at, or above, the carina (Spitz, 2007). In approximately 7% of incidences, blind termination of the proximal and distal oesophagus occurs without fistula, and in 4% of cases tracheooesophageal fistulas occur without atresia (Spitz, 2007).

Current surgical reconstructive techniques primarily comprise of interposition of either the colon or jejunum (Hamza, 2009), or gastric transposition (Spitz, 2009); whereby the stomach is extended into the thoracic cavity and connected to the oesophagus. In addition to OA, intestinal interposition or gastric pull-up may be used to treat uncommon neoplastic conditions and caustic ingestion, for which incidence is increasing (Spitz et al., 2004, Lupa et al., 2009). However, none of the existing techniques are without postoperative complication. Major causes of morbidity include anastomotic stenosis, which can occur in greater than 50% of patients (Orringer et al., 2000), stricture formation, dysphagia, dysmotility, pulmonary complications, and the potentially carcinogenic effect of gastroesophageal reflux (Totonelli et al., 2013). These, in combination with significant donor site morbidity, are highly detrimental to patients’ quality of life.

1.3.4 Tissue engineering approaches to oesophageal regeneration

Tissue engineered modular organs with distinct physiological functional and structural components, such as the oesophagus, provide a more complex regenerative challenge, and so have remained distant from clinical application (Orlando et al., 2012, Orlando et al., 2013). To address this, both synthetic constructs and those of biological origin have been applied to varying effect.

Collagen scaffolds have resulted in success in animal models. Collagen coupled with vicryl mesh (Shinhar et al., 1998) or supported with silicone stenting (Yamamoto et al., 1999) have been employed in independent canine models to repair long-gap total defects. Both studies reported low mortality, 8.3% and 4.8% respectively, with long term stricture aversion. Regeneration of distinct anatomical compartments, including mucosa and submucosa, was reported; although muscle regeneration was limited to regions proximal to the sites of anastomoses (Yamamoto et al., 1999).
This has since been improved upon by combining synthetic scaffolds with specific and non-specific cell types, before implanting in vivo. Grikscheit et al. (2003) demonstrated that by combining polyglycolic acid poly-L-lactic acid scaffolds with mesenchymal-epithelial organoid units, it was possible to form constructs that demonstrated oesophageal microarchitecture. Following interposition in rats, animals gained weight until euthanised; despite stricture presentation at the upper anastomosis.

Tissue engineered constructs manufactured from porcine small intestinal submucosa have also been widely reported, but with mixed results (Lopes et al., 2006a, Lopes et al., 2006b). When applied as a patch graft, rats gained weight and displayed no signs of dysphagia. This was coupled with long-term regeneration of a typical epithelium and muscular reconstitution. However, when applied as full segmental interpositions, no survival to the 28 day target was observed (Lopes et al., 2006a). Similarly, when porcine small intestine submucosa was employed as a circumferential graft in a porcine model, severe stenoses were reported during post-mortem of 13/14 animals that failed to meet the four week study endpoint (Doede et al., 2009).

Subsequently, positive results have been reported when using decellularised, organ specific matrices. Decellularised rat oesophagus has been demonstrated to support the early formation of epithelium (Ozeki et al., 2006), whilst decellularised porcine oesophagus had no detrimental effect on the viability of human perivascular stem cells (Keane et al., 2013). This approach of orthotopic scaffold sourcing has been applied in vivo, with porcine derived decellularised oesophagi orthotopically implanted in adult pigs with or without omental pre-vascularisation (Luc et al., 2018). Animals received interposition of the abdominal oesophagus to reflect adult population adenocarcinoma indications. Only one of six animals did not experience post-operative complications, including fistula, stricture and abscesses, although the root cause of this was difficult to decipher due to the absence of a sham control. Whilst in vivo study results remain promising, there is clearly further development required prior to the establishment and execution of successful large-animal studies.
1.5 THESIS AIMS

Bespoke bioreactor systems represent a suitable means for translation of pre-GMP processes for manufacturing tissue engineered products, across different stages of the product development cycle. Utilising a modular approach, which enables processing of multiple product types within individual systems, can be used to reduce equipment characterisation and validation burden. The overarching aims of this thesis are as follows:

- Adaptation of an off-the-shelf tissue bioprocessing equipment can be demonstrated to reproduce GLP-compliant hemilarynx decellularisation in a GMP-compliant process, producing decellularised tissue constructs that are capable of supporting BM-MSCs.

- A bespoke bioreactor will be designed and manufactured to improve on the preclinical recellularisation system, providing an aseptically operable modular unit that conforms to the operational requirements of the clinical trial manufacturing centre and has the capacity for recellularisation of both product types used in the GLP-compliant study.

- The novel modular bioreactors use a as a platform technology for closed-system processing additional tissue types, specifically decellularisation of oesophagi to produce constructs for treating oesophageal defects in paediatrics, will be assessed.

- A second, modular bioreactor for tubular tissues will be designed, fabricated and operationally verified to investigate whether it can be used to decellularise oesophagi in an automated, closed-system that replicates the decellularisation efficacy of the process performed in the original bioreactor.
2 MATERIALS AND METHODS

2.1 GENERAL MATERIALS AND METHODS

2.1.1 Tissue procurement and preparation

2.1.1.1 Adult porcine larynx

Adult porcine larynges were procured from Animal Organs for Research (Cheale Meats Ltd, Little Warley, UK), as previously established and described by Partington (2014). Briefly, plucks comprising of heart, larynx, liver, lungs and trachea were retrieved and trimmed on-site to isolate the larynx and trachea, terminating superiorly to the carina. Tissues were shipped to the laboratory on ice where, upon receipt, they were further trimmed to remove excess non-laryngeal tissue. Larynges and tracheae were separated by circumferential incision of the intercartilaginous tissue between the first and second tracheal rings, distal to the cricoid cartilage, using a disposable surgical scalpel (No 22; Swann-Morton, Sheffield, UK). Separated larynges were stored in phosphate buffered saline (PBS; Sigma-Aldrich, Gillingham, UK) containing antibiotic-antimycotic (A/A; 1% v/v; ThermoFisher Scientific, Loughborough, UK), in sterile, low density polyethylene sample bags (VWR, Lutterworth, UK). Tissues that were to be processed within 24 h were stored at 4 °C, otherwise they were frozen at -20 °C.

2.1.1.2 Neonatal and juvenile porcine oesophagus and buccal Tissue

Whole neonatal and juvenile pigs were procured from JSR Newbottle Pigs (JSR Farms Ltd, East Yorkshire, UK). Neonatal animals were aged <12 h and had a mass of <1 kg, whilst juvenile animals were aged 6-8 days with a mass of 2.2-3.8 kg. Farm staff euthanised animals on-site by blunt trauma in accordance with the Red Tractor Assurance Pig Scheme standards. Both female and male pigs were procured. Animals were shipped to the laboratory at ambient temperature, where they were washed to remove debris and haematomata both externally and within the oral cavity.

To obtain buccal mucosa, incisions were made internally along the superior and inferior gingivolabial sulci and extended posteriorly to elevate a full-thickness triangular flap. Starting at the posterior apex, buccal mucosa and a thin strip of muscle were resected by tangential excision. All work was performed using disposable scalpels (No. 22). Excised buccal tissue was stored in PBS containing A/A (1%), ciprofloxacin (1.25 µL mL⁻¹; Fannin, Wellingborough, UK) and gentamicin (0.25 µL mL⁻¹; Amdipharm UK, Basildon, UK) at 4 °C for <24 h, prior to processing.
Subsequently, a midline laparotomy without perforating the viscera was followed by a circumferential separation of the oesophagus inferior to the lower oesophageal sphincter. Subsequently, partial circumferential incision in the second intercostal space was performed to open the thoracic cavity, stopping at the thoracic vertebrae. This was extended with an additional vertical incision to expose the ventral surface of the larynx and upper trachea to allow the heart, larynx, lungs, oesophagus, pharynx and trachea to be excised en bloc. Using a combination of sharp and blunt dissection, the oesophagus was mobilised and transected superior to the upper oesophageal sphincter. The oesophagus was stored in PBS and the same antibiotic-antimycotic mixture as used for buccal excisions, at 4 °C for <24 h before processing.
2.1.2 Cell isolation and expansion

Unless otherwise stated, all cell isolation and expansion operations were performed aseptically using a class II biological safety cabinet where appropriate. All consumables and reagents used were sterile.

2.1.2.1 Cell number and viability approximation

For all cell suspensions, unless otherwise stated, total cell number was counted on an Improved Neubauer haemocytometer (Hawksley, Lancing, UK) and viability assessed using the Trypan Blue exclusion method. Briefly, the cell suspension sample was treated with Trypan Blue (1:1 ratio; ThermoFisher Scientific) and triturated before 10 µL was pipetted adjacent to the cover slip on the haemocytometer, with the liquid drawn under the cover slip by capillary action. Total live cells and dead cells, which do not exclude Trypan Blue, were counted within four sets of 16 squares (Figure 2.1). Of the cells that crossed the boundary lines between squares, only those on the top and right hand side were counted. Mean total live cell and total dead cell numbers were then used to approximate cell population viability, as show in Equation 2.1.

Equation 2.1

\[
Population \ Viability \ (\%) = 100 \cdot \left( \frac{Mean \ total \ live \ cells}{Mean \ total \ cells} \right)
\]

The total number of viable cells counted was then used to estimate the cell concentration of the resuspended pellet (Equation 2.2). Cells were then either cryopreserved or seeded.

Equation 2.2

\[
Cell \ Concentration = Mean \ total \ live \ cells \cdot \text{dilution factor} \cdot \left( \frac{sample \ volume}{resuspension \ volume} \right)
\]
2.1.2.2 Cell cryopreservation

Cell suspensions (≤1 mL) containing 1-5 x 10⁶ cells were pipetted into cryogenic vials (2 mL; Sigma-Aldrich). An equal volume of solution of dimethyl sulfoxide (DMSO; 20% v/v; WAK-Chemie Medical, Steinbach, Germany) in human serum albumin (HSA; 4.5% v/v; Bio Products Laboratory, Elstree, UK) was added and gently triturated. Vials were immediately placed inside a Mr Frosty™ freezing container (ThermoFisher Scientific) filled with isopropyl alcohol (ThermoFisher Scientific) as per the manufacturer’s instructions, which was then placed inside a -80 °C freezer. After 24 h cryogenic vials were transferred to the UCL-RFH Biobank where they were stored in liquid nitrogen.

2.1.2.3 Cell thawing

Cells were transferred from the UCL-RFH Biobank on dry ice, before being thawed in a 37 °C water bath. When the vial contents had liquefied they were added dropwise to a conical tube (15 mL; ThermoFisher Scientific) containing the appropriate medium for culturing the selected cell type. Suspensions were centrifuged for 5 min at 300 x g. Resultant pellets were resuspended in the same medium, to a final volume of 100-1000 µL, and counted as described in section 2.1.2.1.

Figure 2.1 Haemocytometer grid. The blue box encompasses 16 squares (1 mm²).
2.1.3 Biomolecular quantitative analysis

2.1.3.1 Collagen quantification

Total collagen was quantified for native and decellularised tissues. Approximately 25-50 mg of tissue was finely diced into 1 mm³ portions using a disposable surgical scalpel (No. 23). Oesophageal tissue was then dried to a constant mass before proceeding. The total collagen content was extracted using a QuickZyme Total Collagen Assay kit (QuickZyme Biosciences, Leiden, Netherlands), in accordance with the manufacturer's protocol. The assay relies on the detection of hydroxyproline, a non-proteinogenic amino acid that is near ubiquitous in mammalian collagens. Briefly, tissue was hydrolysed for 20 h at 95 °C in hydrochloric acid (6 M; Sigma-Aldrich) at a concentration of 50-300 mg mL⁻¹. The hydrolysed supernatant was then diluted to 4 M using purified water, before being further diluted to ensure the hydrolysate absorbance values fell within the linear region of the associated standard curve. The hydrolysed hydroxyproline was oxidised before being chromogen-coupled. Absorbance at 570 nm was detected using a Tecan Sunrise (Tecan, Männedorf, Switzerland) or Victor X3 (Perkin Elmer, Waltham, USA). Total collagen concentration was determined using the standard curve ranging from 0-300 µg mL⁻¹, before being normalised to the tissue mass and the standard error of the mean (SEM) calculated.

2.1.3.2 DNA quantification

Total DNA quantification was performed for both native and decellularised tissues. Tissue was sampled, diced and dried as before (section 2.1.3.1). DNA was extracted from the tissue using a DNeasy Blood & Tissue Kit (Qiagen, Manchester, UK) as described in the manufacturer's protocol. Briefly, tissue was lysed using proteinase K for 20 h at 56 °C, whilst agitating at 150 rpm. The DNA was then selectively bound to a spin DNeasy Mini spin column membrane and washed by a series of buffer additions. Purified DNA was eluted in a buffer, before being quantified using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific). Total DNA was normalised to the tissue mass and the SEM calculated.

2.1.3.3 Glycosaminoglycan quantification

Total sulphated glycosaminoglycan (sGAG) content was quantified for native and decellularised tissue. Approximately 50 mg of tissue was diced and dried as before (section 2.1.3.1). Extraction was achieved using the Blyscan™ Sulphated Glycosaminoglycan Assay kit (Biocolor, Carrickfergus, UK), as described in the manufacturer's protocol. Briefly, samples were digested in a papain extraction reagent (Sigma-Aldrich) for 20 h at 65°C, whilst agitating at 150 rpm. The resulting supernatant
was sampled and diluted using purified water to ensure the subsequent absorbance values fell within the linear region of the associated standard curve. Samples were incubated for 30 min at room temperature (RT; 16-25 °C), under gentle agitation, with a 1, 9-dimethyl-methylene blue containing reagent to form a precipitating sGAG-dye complex. The complex was pelleted and dissociated using a reagent containing a sodium salt of an anionic surfactant before detecting the absorbance at 656 nm, as before (section 2.1.3.1). Total sGAG concentration was determined using the standard curve ranging from 0-5 µg, before being normalised to the tissue mass and the SEM calculated.

2.1.4 Qualitative tissue analysis

2.1.4.1 Optimal cutting temperature compound embedding and cryosectioning

Tissue samples were fixed for four hours in paraformaldehyde (PFA; 4% m/v; Sigma-Aldrich) before being incrementally dehydrated in sucrose solutions (10%, 15%, 30% (all m/v); Sigma-Aldrich). Samples were stored in sucrose solution (20% m/v) overnight at 4 °C. Following cryoprotection, samples were briefly rinsed with PBS before being acclimatised in optimal cutting temperature (OCT) compound (ThermoFisher Scientific) for 10 min. They were then oriented within embedding moulds containing OCT compound and gently frozen in liquid nitrogen chilled 2-methyl butane. Samples were stored at -80 °C before sectioning on a Cryotome™ FSE cryostat (ThermoFisher Scientific). Sections, 5 µm unless otherwise stated, were taken onto SuperFrost Plus™ adhesions slides (ThermoFisher Scientific) and stored at -20 °C.

2.1.4.2 Histology

For haematoxylin and eosin (H&E) staining, slides were rehydrated in dH2O (10 min, twice) before being stained with Harris haematoxylin (RAL Diagnostics, Martillac, France) for 5-10 min. Slides were dipped in hydrochloric acid (1% v/v; 4M) in ethanol (70% v/v), washed with dH2O (1-2 min), tap water and then dH2O again. Sections were stained with eosin (Sigma-Aldrich) for 10 min, washed with dH2O and incrementally dehydrated in 90%, 95% and 100% (all v/v) ethanol. Slides were cleared with xylene (Sigma-Aldrich) and cover slips mounted with DPX (Merck, Darmstadt, Germany).

2.1.4.3 Scanning electron microscopy

Tissue samples were fixed in Karnovsky fixative, consisting to PFA (2% m/v) and glutaraldehyde (1.5% m/v; Sigma-Aldrich) in sodium phosphate buffer (0.1 M, pH 7.2 hereafter), for >24 h at 4 °C. Samples were washed with sodium phosphate buffer before incubating at 4 °C for 1 h in solution of osmium tetroxide (1% v/v; Sigma-Aldrich) and potassium ferrocyanide (1.5% m/v; Sigma-Aldrich) in potassium phosphate buffer.
Samples were again washed with potassium phosphate buffer, twice, before being incrementally ethanol dehydrated (25%, 50%, 70%, 90%, 100%); ThermoFisher Scientific) at 10 min intervals. Samples were then critical point dried using carbon dioxide. A critical point of approximately 34 °C was achieved at 7.1 x10^6 Pa. Samples were then mounted on aluminium stubs and sputter coated with a gold/palladium alloy using a Gatan ion beam coater (Gatan, Abingdon, UK). Imaging was performed using a Jeol 7401 field emission scanning electron microscope.

### 2.1.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism v7.05 (GraphPad Software, La Jolla, USA). Statistical tests employed are described within the materials and methods for each chapter. Data were assessed for normality, where possible, using a Shapiro-Wilk test. Error bars represent SEM throughout, unless stated otherwise, and significance inferred when $p \leq 0.05$. 
2.2 CHAPTER SPECIFIC MATERIALS AND METHODS – CHAPTER 3

The original decellularisation process flow was provided by T Ansari (Northwick Park Institute for Medical Research, UK) and was comprised of a human-specific protocol resulting from modification of the preclinical, GLP-compliant study standard operating procedure (SOP). This protocol was an adaptation of the porcine SOP that was later published (Ansari et al., 2017).

2.2.1 Decellularisation chamber assembly

An off-the-shelf equipment, Ricordi® chamber (Figure 3.1), was adopted for decellularisation processing (section 3.3). Prior to Ricordi® chamber assembly, a decellularisation kit was compiled within an Aesculap PrimeLine® sterile container (Aesculap AG, Tuttingen, Germany) before being cleaned and autoclaved by the Central Surgical Sterilisation Department at the Royal Free Hospital. The kit composition can be found in Table 2.1

Post sterilisation, the chamber was assembled aseptically within a class II biological safety cabinet. A sterile three-way stop-cock (3WSC) (Becton Dickinson, Franklin Lakes, USA) was added to each of the luer barbs, which were then inserted into the corresponding tubing pieces (Cole-Parmer Instrument Company, St Neots, UK). Two sterile hydrophobic Minisart® 0.2 µm filters (Sartorius Stedim Biotech, Göttingen, Germany) were added in series to the 1/4” luer thread hose barb and terminally capped with an additional 3WSC. The 3/16” tubing was inserted over the two ports on the base of the chamber and the 1/4” tubing on the chamber lid port. Tubing was secured with either nylon cable-ties or appropriately sized unex clips (both VWR, Lutterworth, UK). A 3WSC, followed by two sterile hydrophobic Minisart® 0.2 µm filters (Sartorius Stedim Biotech), was attached to the lateral luer port on the chamber body. An online analogue pressure gauge (RS Components, Corby, UK), measuring -1.0 x 10^5 Pa to 0 Pa, was attached to the filters via a 1/8” BSP to male luer thread connector (EM-Technik, Maxdorf, Germany). The thread of the pressure gauge was wrapped with polytetrafluoroethylene (PTFE) tape (VWR) to minimise leakage. All ports were capped with sterile combi-stoppers (VWR) when not in use, throughout the process.
Table 2.1 Decellularisation kit contents

<table>
<thead>
<tr>
<th>Component</th>
<th>Dimension</th>
<th>Units</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ricordi® Chamber, including O-ring and mesh insert</td>
<td>500 mL</td>
<td>1</td>
<td>Biorep Technologies (Miami, USA)</td>
</tr>
<tr>
<td>Tygon® E-603 non-DEHP vacuum tubing</td>
<td>3/16” inner diameter (ID) x 50-70 mm</td>
<td>2</td>
<td>Cole-Parmer Instrument Company (St Neots, UK)</td>
</tr>
<tr>
<td></td>
<td>1/4” ID x 50-70 mm</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Female luer thread hose barb, stainless steel 303</td>
<td>3/16”</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/4”</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

2.2.2 Decellularisation chamber pressure maintenance

Ricordi® chambers constructed of polyetherimide (PEI; n=3) or stainless steel 303 (SS303; n=2) were assembled as described in section 2.2.1. The PEI chamber had only one port on the base of the chamber. Each chamber was filled with 250 mL of sterile water for irrigation (Baxter Healthcare, Compton, UK) and a vacuum of approximately 200 Pa was pulled using a Telstar 2F-10 rotary vane vacuum pump (Telstar Vacuum Solutions, Terrassa, Spain). The 3WSC connected in-line with the analogue pressure gauge was closed to prevent loss of vacuum, and each chamber was placed within an orbital shaking incubator at 4 °C, 125 rpm and incubated for 72 h. Chambers were removed from the incubator and the 3WSC opened, before reading the pressure gauge. The investigation was repeated (n=3).

2.2.3 Procurement of adult human larynx

Adult larynges were procured from National Health Service Blood and Transplant (NHSBT) under service level agreement between NHSBT and CCGTT. All starting material was procured in accordance with the Human Tissue Authority (HTA) Quality & Safety Regulations for Tissues and Cells under existing HTA licenses. Donors were screened for relevant infectious disease markers, including human immunodeficiency virus, human T cell lymphotropic virus, hepatitis B, hepatitis C, and syphilis (Table 2.2). Screening was performed by a Clinical Pathology Accreditation certified virology and/or
microbiology laboratory. Tissue donors were tested just prior to, or up to 24 h after, death. In each instance, cadaveric organs were retrieved from consenting donors by surgically qualified retrieval personnel. Larynges were rinsed with chlorhexidine before being stored in University of Wisconsin cold storage solution (UW; Bridge to Life (Europe), London, UK) in sterile polypropylene pots (Medfor, Farnborough, UK) and frozen at -80 °C. Organs were stored by NHSBT and released to the CCGTT upon request. Tissues were shipped on dry-ice and immediately transferred to a CCGTT or UCL-Royal Free Hospital Biobank -80 °C freezer upon receipt.

Table 2.2 Infectious disease markers screening specification

<table>
<thead>
<tr>
<th>Assay</th>
<th>Expected Result</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HIV I/II</td>
<td></td>
<td>No laboratory evidence of HIV I/II infection</td>
</tr>
<tr>
<td>HBsAg</td>
<td></td>
<td>No laboratory evidence of HBV infection</td>
</tr>
<tr>
<td>HBcAb</td>
<td>Negative</td>
<td>No laboratory evidence of recent and/or past HBV infection</td>
</tr>
<tr>
<td>Anti-HCV</td>
<td></td>
<td>No laboratory evidence of HCV infection</td>
</tr>
<tr>
<td>RPR (Syphilis)</td>
<td></td>
<td>No laboratory evidence of syphilis infection</td>
</tr>
<tr>
<td>Human T-cell lymphotropic virus I/II (HTLV -1/II) antibody</td>
<td></td>
<td>No laboratory evidence of HTLV I/II infection</td>
</tr>
</tbody>
</table>
2.2.4 Preparation of native larynx for decellularisation

Frozen tissues were thawed at RT for 12 +/- 4 h prior to commencing decellularisation. Temperature was monitored throughout the process using a LIBERO Ti1-S data logger (Elpro-Buchs, Buchs, Switzerland). Transport solution was sampled and used to inoculate BD BACTEC™ Plus Aerobic/F and Plus Anaerobic/F Medium bottles (Becton Dickinson), which were transferred to the Microbiology Department at the Royal Free Hospital for microbiological testing. Excess tissue was removed from the outer surface of the organs using a disposable surgical scalpel (No. 22; Swann-Morton, Sheffield, UK) and blunt tipped, curved surgical scissors (World Precision Instruments, Sarasota, USA). Larynges were bisected along the sagittal plane to yield two hemilarynges.

2.2.5 Vacuum-assisted detergent-enzymatic decellularisation

All reagent solutions were prepared one day prior to commencing the decellularisation process; other than the DNase/RNase solution, which was prepared immediately prior to use. Unless otherwise stated, all solutions had a final volume of 250 mL.

Latrunculin B (Sigma-Aldrich, Gillingham, UK) solution was prepared to a final concentration of 50 nM in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma-Aldrich). KCl (Sigma-Aldrich) solution was prepared to 0.6 M, and KI (Sigma-Aldrich) to 1.0 M, in PBS (Sigma-Aldrich). Triton X-100 (Sigma-Aldrich) and sodium deoxycholate (SDC; Sigma-Aldrich) were combined to prepare a 0.25%/0.25% (v/v; m/v) solution in PBS. A stock solution of magnesium chloride (MgCl₂; 0.005 M; Sigma-Aldrich) and calcium chloride (CaCl₂; 0.005 M; Sigma-Aldrich) was prepared, using PBS, for the DNase/RNase steps. On the day of use, RNase type I (ThermoFisher Scientific) and DNase I (F. Hoffmann-La Roche, Basel, Switzerland) were added to the stock solution containing bags to final concentrations of 0.1 g L⁻¹ and 2000 Kunitz units L⁻¹ respectively. All solutions were sterile filtered using Nalgene™ Rapid-Flow™ Sterile Disposable Filtration Units (ThermoFisher Scientific), with vacuum being pulled using a Telstar 2F-10 rotary vane vacuum pump. Sterile water for irrigation was used for the water steps (Baxter Healthcare).

Sterile solutions were then aseptically transferred to sterile transfer bags using a peristaltic pump (Watson-Marlow, Falmouth, UK), via individual sterile haematology pump segments (Quest Biomedical, Solihull, UK) spiked with bag spikes with removable injection port connected to spike coupler-luer lines (Quest Biomedical). Dependent upon availability, 600 mL transfer bags (Fresenius Kabi, Bad Homburg vor der Höhe, Germany) or 300 mL transfer bags (Terumo, Leuven, Belgium) were used. Bags which were to contain DNase/RNase were spiked, prior to filling, with a bag spike with
removable injection port. All reagent solutions were stored at 4 °C, and removed from the refrigerator to acclimatise to RT at least 1 h prior to use.

Antibiotics and antimycotics were added to the reagent solutions to final concentrations as follows: gentamicin 50 mg L⁻¹, amoxicillin (Ibigen, Aprilia, Italy) 1 mg L⁻¹, amphotericin B (Gilead Sciences, Uxbridge, UK) 5 mg L⁻¹, and penicillin-streptomycin (Sigma-Aldrich) 10 mL L⁻¹. They were added to solutions used in steps one, four and 7 in Table 2.3, for decellularisations one and two (HT1 and HT2). Decellularisations three and four (HT3 and HT4) had no antibiotics or antimycotics used.

Hemilarynges were aseptically placed within the assembled Ricordi® chamber, before the mesh insert (not shown) was located in the recess of the chamber body and the lid fixed in position. Decellularisation proceeded as described in Table 2.3, with reagents being transferred to and from the chamber by attaching the transfer bag and holding it above or below the chamber, thus relying upon gravity. A vacuum was pulled within the chamber, to a pressure of approximately 200 Pa, via the port on the lid using a Telstar 2F-10 rotary vane vacuum pump. All steps were agitated in a variable temperature orbital incubator at 125 rpm.

Additional samples were taken from the waste bags of step 8 and upon completion of the decellularisation. Both sample sets underwent microbiological testing, as before (section 2.2.4). The final sample was also sent for endotoxin testing, provided by the Scottish National Blood Transfusion Service (Edinburgh, UK). Decellularised tissue was removed from the drained chamber and placed in a tamper evident sterile container (Scientific Laboratory Supplies, Hessle, UK), before being covered with sterile HEPES buffered saline solution (Lonza, Basel, Switzerland) containing penicillin-streptomycin (10 mL L⁻¹; unless stated otherwise). Tissues were stored at 4 °C.
<table>
<thead>
<tr>
<th>Step</th>
<th>Day</th>
<th>Reagent</th>
<th>Duration</th>
<th>Temperature</th>
<th>Vacuum (ca. 200 Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Latrunculin B</td>
<td>2 h</td>
<td>37 °C</td>
<td>+</td>
</tr>
<tr>
<td>2/3</td>
<td>1</td>
<td>Sterile water for irrigation</td>
<td>2 x 15 min</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>KCl</td>
<td>2 h</td>
<td>RT</td>
<td>+</td>
</tr>
<tr>
<td>5/6</td>
<td>1</td>
<td>Sterile water for irrigation</td>
<td>2 x 15 min</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>KI</td>
<td>2 h</td>
<td>RT</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>Sterile water for irrigation</td>
<td>12 ± 4 h</td>
<td>RT</td>
<td>-</td>
</tr>
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<td>RT</td>
<td>+</td>
</tr>
<tr>
<td>10/11</td>
<td>2</td>
<td>Sterile water for irrigation</td>
<td>2 x 15 min</td>
<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>KI</td>
<td>2 h</td>
<td>RT</td>
<td>+</td>
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<td>RT</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>DNase/RNase</td>
<td>2 h</td>
<td>37 °C</td>
<td>+</td>
</tr>
<tr>
<td>16/17</td>
<td>2</td>
<td>Sterile water for irrigation</td>
<td>2 x 15 min</td>
<td>RT</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>2</td>
<td>Freeze</td>
<td>12 ± 4 h</td>
<td>-25 ± 5 °C</td>
<td>+</td>
</tr>
<tr>
<td>Step</td>
<td>Day</td>
<td>Reagent</td>
<td>Duration</td>
<td>Temperature</td>
<td>Vacuum (ca. 200 Pa)</td>
</tr>
<tr>
<td>------</td>
<td>-----</td>
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<td>----------------</td>
<td>-------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>19</td>
<td>3</td>
<td>Thaw</td>
<td>12 ± 4 h</td>
<td>RT</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>Triton X-100/SDC</td>
<td>24 h</td>
<td>RT</td>
<td>+</td>
</tr>
<tr>
<td>21/22</td>
<td>4</td>
<td>Sterile water for irrigation</td>
<td>2 x 15 min</td>
<td>RT</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>4</td>
<td>DNase/RNase</td>
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<td>37 °C</td>
<td>+</td>
</tr>
<tr>
<td>24/25</td>
<td>4</td>
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<td>3 x 24 ± 4 h</td>
<td>RT</td>
<td>+</td>
</tr>
</tbody>
</table>

### 2.2.6 Vacuum-assisted detergent-enzymatic decellularisation sterility validation

To validate the sterility of the decellularisation process undertaken in the Ricordi® chamber, chambers were assembled as in section 2.2.1. The laryngeal decellularisation process flow was followed, as described in section 2.2.5 and Table 2.3, with tryptic soy broth (TSB; Cherwell Laboratories, Bicester, UK) used in place of all reagents. Samples, 25 mL, were removed from the waste bag after each reagent change and incubated for 7 d at 20-25 °C, followed by 7 d at 30-35 °C. Contamination/sterility was confirmed by the presence/absence of sample turbidity at 14 d.
2.2.7 Tissue decontamination using chlorhexidine digluconate

Whole adult porcine larynges were procured, processed and stored as described in section 2.1.1.1.

Larynges (n=2) were thawed at RT and incubated in DMEM at 4 °C for 48 h, before one larynx was bisected to yield two hemilarynges. Media were visually inspected for turbidity, an indicator of contamination. Media samples were used to inoculate BD BACTEC™ bottles and sent to the Microbiology Department as before (section 2.2.3), to confirm contamination. Tissues were then submerged in chlorhexidine digluconate solution (20% v/v; ThermoFisher Scientific) for 5 min at RT in individual sterile polypropylene pots. The solution was discarded and tissues covered with sterile saline (Baxter Healthcare) and again incubated at RT for 5 min. This wash step was repeated a total of three times. Tissues were then immersed in DMEM and incubated for 24 h, at RT. Media were visually inspected for turbidity and BD BACTEC™ bottles inoculated as before.

2.2.8 Tissue sterilisation using gamma-irradiation

Hemilarynges, native (n=1) and decellularised (n=2), that had been previously identified as contaminated were shipped at -80 °C in HEPES buffered saline solution to NHSBT, before being gamma-irradiated by Synergy Health (Swindon, UK). Tissues received a 25-40 kGy dose. Irradiated tissues were thawed at 25 °C for ca. 23 h, following receipt at the CCGTT. Tissues were transferred to new sterile pots containing HEPES buffered saline solution and stored at 4 °C. Samples of the HEPES buffered saline solution were tested for sterility by the Microbiology Department at the Royal Free Hospital, as previously described.

2.2.9 Statistical analysis

The two-tailed paired t test was used to compare population mean values.
2.3 CHAPTER SPECIFIC MATERIALS AND METHODS – CHAPTER 4

2.3.1 Cell isolation and expansion

2.3.1.1 Mesenchymal stromal cell isolation and expansion

All use of BM-MSCs described herein was performed using previously isolated and cryopreserved primary cells and so began with cell thawing (section 2.2.3). The isolation process is described for completeness. For all BM-MSC work, unless described otherwise, the term “complete medium” is used to refer to αMEM with GlutaMAX™ (Sigma-Aldrich) and foetal bovine serum (10% v/v; FBS; Sigma-Aldrich).

Human bone marrow (BM) was procured in accordance with the HTA Quality & Safety Regulations for Tissues and Cells under existing HTA licences. Following informed consent, donors were screened for relevant infectious disease markers (Table 2.2) prior to the start of the manufacturing process by a clinical pathology accredited virology and/or microbiology laboratory. BM donors were tested within 30 days of harvest.

Heparinised syringes containing heparin (Wockhardt UK, Wrexham, UK) 20 IU mL\(^{-1}\) of BM to be collected, in sterile saline (Baxter Healthcare, Compton, UK) with a final volume of 1 mL, were sent to the clinical site along with a conical tube (50 mL; ThermoFisher Scientific) containing heparin 20 IU mL\(^{-1}\) of BM to be collected as part of a BM collection kit. At the clinical site BM was procured from the posterior iliac crest, added to the heparin containing conical tube and returned to the CCGTT. Samples were diluted 1:1 with sterile PBS with CaCl\(_2\) and MgCl\(_2\) (Sigma-Aldrich), before layering onto Lymphoprep™ (1:1 ratio; Alere Technologies, Jena, Germany) and centrifuging for 20 min at 600 x g with no break deceleration. The mononuclear cell layer was aspirated and washed with PBS with CaCl\(_2\) and MgCl\(_2\) for 10 min at 400 x g with no brake deceleration. The resultant pellet was resuspended in complete medium and A/A (1%). Cell number and viability was approximated, as described in section 2.1.2.1.

The mononuclear cell fraction was seeded into 25 cm\(^2\) or 75 cm\(^2\) culture flasks at approximately 5.0 x 10\(^4\) cm\(^{-2}\) for passage 0 (P0). Briefly, warmed complete medium and A/A (1%) was added into tissue culture flasks (ThermoFisher Scientific) to a final volume of 0.1-0.2 mL cm\(^{-2}\). Cell suspensions were then gently pipetted into the flask, which was capped and tilted to evenly distribute the cells. Plated cells were cultured under normoxia in a humidified CO\(_2\) (5%) incubator at 37 °C. Medium was replaced at day three by aspirating and disposing of the waste medium, before gently washing with an equal volume of HBSS to remove non-adherent cells. Warmed complete medium was then gently added and the flasks returned to the incubator. Flasks were observed every 2-4
days using phase contrast microscopy (Nikon Eclipse Ti-E; Nikon, Tokyo, Japan) to monitor for microbiological contamination and cell detachment.

Once cultures reached 80% confluency, approximated via phase contrast microscopy, they were either passaged or cryopreserved (section 2.1.2.2). To passage cells, medium was aspirated and flasks washed with warmed HBSS of equal volume. Subsequently, 50-100 µL cm\(^{-2}\) of warmed TrypLE (ThermoFisher Scientific) was gently pipetted into the flask, which was tilted to ensure complete covering of the culture surface. It was then returned to the incubator for 7 min. Cells were dislodged from the tissue culture surface and disaggregated by gentle tapping of the flask, before observing using phase contrast microscopy. If a significant proportion of cells remained attached, flasks were returned to the incubator for a further 2 min. Once the majority of cells were unattached, the TrypLE was quenched with the same volume of HBSS. Quenched suspensions were centrifuged for 5 min at 300 x g, before the pellet was resuspended in warmed complete medium and cell number approximated as before (section 2.1.2.1). Cells were re-seeded at 5 x \(10^3\) cm\(^{-2}\) in culture flasks.

2.3.1.2 Fibroblast isolation and expansion

Fibroblasts and epithelial cells were kindly isolated and expanded by A Tait.

Porcine buccal biopsies, retrieved as described in section 2.1.1.2, were incubated overnight in neutral protease (4 U; Roche Holding, Basel, Switzerland), A/A (1%), ciprofloxacin (1.25 µL mL\(^{-1}\)) and gentamicin (0.25 µL mL\(^{-1}\)) at 4 °C. The epithelium was removed using sterile stainless steel forceps and cryopreserved as described for cells in section 2.1.2.2, with the addition of ROCK inhibitor Y-27632 (10% v/v; Cell Guidance Systems, Babraham, UK). The lamina propria was incubated in dissociation solution for 3 h at 37 °C prior to being dissociated using a gentleMACS™ Octo Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), using program h_Skin_1. The dissociation solution was comprised of collagenase (0.4 U mL\(^{-1}\); Amsbio, Abingdon, UK) with dornase alpha (229 U mL\(^{-1}\); Roche Holding), A/A (1%), ciprofloxacin (1.25 µL mL\(^{-1}\)) and gentamycin (0.25 µL mL\(^{-1}\)). The resultant suspension was washed with low glucose DMEM and passed through a 100 µm cell strainer (Miltenyi Biotec). Cells were plated into T25 flasks (ThermoFisher Scientific) that had been coated with recombinant human fibronectin (MSC Attachment Solution; Biological Industries, Beit-Haemek, Israel), and cultured in MSC NutriStem XF Media plus MSC NutriStem XF Supplement Mix (both Biological Industries) with human pooled plasma lysate (5% v/v; Cook Regentec, Indianapolis, USA). Plated cells were cultured under normoxia in a humidified CO2 (5%) incubator at 37 °C. Media was replaced every three days and cells expanded to 80% confluency, whereupon they were passaged as previously described.
2.3.1.3 Epithelial cell isolation and expansion

Tissue was cut into sections <4 mm$^2$ before being added to TrypLE containing dornase alpha (229 U mL$^{-1}$), A/A (1%), ciprofloxacin (250 µg) and gentamycin (25 µg). Dissociation was achieved using a gentleMACS™ Octo Dissociator (Miltenyi Biotec), using dissociation programme 37_TDK_1. The resultant cellular suspension was washed with DMEM and passed through a 100 µm cell strainer, before being seeded in modified Green’s medium that consisted of DMEM (66%), Ham’s F12 Nutrient Mixture (22%; Sigma-Aldrich), FBS (10%), hydrocortisone (0.4 µg mL$^{-1}$ Bio-Techne, Minneapolis, USA), human recombinant epidermal growth factor (10 µg mL$^{-1}$; Bio-Techne), insulin (5 µg mL$^{-1}$; Novo Nordisk, Bagsværd, Denmark), isoproterenol (250 µg; Merck & Co., Kenilworth, USA), A/A (1%), ciprofloxacin (250 µg) and gentamycin (25 µg) and ROCK inhibitor Y-27632 (10 mM) (Rheinwald and Green, 1975, Aslanova et al., 2015). Plated cells were cultured under normoxia in a humidified CO$_2$ (5%) incubator at 37 °C. Media was replaced every three days and cells expanded to 80% confluency, whereupon they were passaged as previously described. Cells were reseeded in modified Green’s medium.

2.3.2 Cell-scaffold adherence

2.3.2.1 Proof-of-concept

BM-MSCs were membrane labelled using a PKH26 red fluorescent cell linker kit (Sigma-Aldrich), in accordance with the manufacturer’s instructions. Briefly, cells were trypsinised to form a single cell suspension (section 2.3.1.1) and washed once in serum free complete medium before pelleting (300 x g, 5 min). Cells were resuspended in Diluent C before adding PKH26 ethanolic dye solution and triturating. Excess dye was quenched by adding an equal volume of FBS, before washing three times with complete medium.

Cells were counterstained using Hoechst 33342 (1 µg mL$^{-1}$; ThermoFisher Scientific) for 10 min at RT. Cell suspensions were washed to remove excess dye. Staining and counterstaining were qualitatively confirmed by fluorescent microscopy (Leica DMI 4000 B; Leica Microsystems, Wetzlar, Germany).

Punch biopsies (6.0 mm; ThermoFisher Scientific) were used to sample the thyroid cartilage of decellularised human larynx. Biopsies were inserted into 96-well microplates (black with clear bottom; Sigma-Aldrich), with the luminal surface face-down. Stained and unstained BM-MSCs were seeded into wells without biopsies at a density of 1.5 x 10$^5$ cells cm$^{-2}$, which was derived from the density used in the GLP-compliant study. Stained cells were seeded manually, dropwise, onto the upward facing surface of
biopsies at the same density. Suspensions were seeded at multiple points across the biopsy surface in an attempt to yield an even distribution of cells. Unseeded biopsies providing a negative control. Samples were hydrated with complete medium, to a final well volume of 150 μL. A standard curve of stained BM-MSCs, $3.75 \times 10^4 – 6.00 \times 10^5$ cm$^{-2}$, was also plated. Samples were incubated at 37 °C under normoxia in a humidified CO$_2$ (5%) incubator. After 4 h, scaffolds were removed from wells and washed by pipetting 20 μL of PBS (Sigma-Aldrich) across the seeded surface of the biopsies, into individual universal centrifuge tubes (5 mL; ThermoFisher Scientific). Scaffolds were returned to adjacent wells within the 96-well microplate. The PBS was transferred from the universal centrifuge tube to the well from which the corresponding scaffold originated, to ensure that any cells that had become detached during manipulation were included in the assay. Plate fluorescence was quantified using a FLUOstar Galaxy (BMG Labtech, Ortenberg, Germany).

2.3.2.2 Attachment time

BM-MSCs were labelled with PKH26, and punch biopsies isolated, as before (section 2.3.2.1). Biopsies were transferred to ultra-low attachment surface 96-well microplates (Sigma-Aldrich). BM-MSCs were seeded onto the abluminal surface of the biopsies, as before, at a density of $1.5 \times 10^5$ cells cm$^{-2}$ and volumes made up to 150 μL. Biopsies were incubated for 4 h, 8 h and 24 h at 37 °C under normoxia in a humidified CO$_2$ (5%) incubator, in independent microplates. A negative control, containing scaffolds with no cells, was incubated for 4 h. Following incubation scaffolds were removed, washed, and fluorescence read as before (section 2.3.2.1).

2.3.2.3 BM-MSC persistence

Punch biopsies were isolated and seeded with BM-MSCs as before (section 2.3.2.2). Seeded scaffolds were incubated for 4 h at 37 °C under normoxia in a humidified CO$_2$ (5%) incubator, in independent ultra-low attachment surface 96-well microplates. Unseeded scaffolds provided a negative control. All scaffolds were washed and fresh complete medium added. Plates were assayed immediately or incubated for 1 d, 3 d, and 5 d. Following incubation, medium was aspirated and replaced with alamarBlue® (150 μL; 10% in complete medium; ThermoFisher Scientific). After a 4 h incubation at 37 °C under normoxia in a humidified CO$_2$ (5%) incubator, 100 μL was aspirated and transferred to a different well. The scaffold-containing well was topped up with 100 μL of complete medium. Microplate alamarBlue® fluorescence was quantified using the FLUOstar Galaxy, as per the manufacturer’s guidelines ($\lambda_{ex}$ 544 nm; $\lambda_{em}$ 590 nm).
Subsequently, biopsies were digested and total DNA concentration quantified as described in section 2.1.3.2.

2.3.3 A bespoke, modular bioreactor for scaffold recellularisation

2.3.3.1 Design and manufacture

In accordance with the design specification (DS) outlined in Table 4.2, a modular airway ATIMP recellularisation bioreactor was conceived before being drafted using the 3D computer aided design (CAD) software package Autodesk Inventor Professional (Autodesk, San Rafael, USA). Consultation with the UCL Biochemical Engineering Mechanical Engineering Workshop Manager outlined the tolerance capabilities of in-house machinery to ensure the parts were drafted in accord with in-house capabilities. Computer numerical code (CNC) script was generated using computer aided manufacture (CAM) software (Edgecam; Hexagon, Stockholm, Sweden) by the Workshop Manager. For manufacture, PTFE sheets 120 mm x 100 mm x 50 mm and 1200 mm x 1200 mm x 8 mm, as well as polycarbonate (PC) sheets 1000 mm x 1000 mm x 8 mm (all Direct Plastics, Sheffield, UK), were used. Machining was performed by E Herrmann and D Fernandez using a HAAS TM1P CNC Toolroom Mill (Haas Automation, Oxnard, USA). Post-mach ining cleaning of bioreactor parts was achieved by washing with surfactant at 90 °C in a sonication bath for 15 min, before thoroughly rinsing with tap water.

Additional parts required for assembly included M4 x 12 mm, DIN 912, stainless steel A4 socket cap screws (Precision Technology Supplies, East Grinstead, UK) to affix the base plate and M4 x 12 mm, DIN 464 stainless steel A1 knurled thumb screws (Wixroyd International, Cranleigh, UK) for the head plate. The seal between the bioreactor body and base/head plates was provided by a silicone shore 70, USP Class VI, BS154 O-ring (Hooper, Huntingdon, UK). Lateral and head ports were fitted with polyvinylidene fluoride (PVDF) 1/8” G thread to male luer lock DIN EN 20594-1 connectors (EM-Technik, Maxdorf, Germany), which were sealed with silicone shore 70, USP Class VI, BS803 O-rings (Hooper).

2.3.3.2 Aseptic assembly

The recellularisation bioreactor was aseptically assembled by placing the chamber body on the safety cabinet surface, such that the smallest face was in contact with the surface, before inserting BS154 O-rings into the reciprocal grooves using sterile forceps (VWR), whilst ensuring no twisting or extrusion (Figure 2.2 (A-C)). Each luer-lock connector with male 1/8” G thread was held using sterile forceps and connected to a 3WSC to produce 5 units. BS803 O-rings were placed over the threaded end of each connector, and
screwed into the ports on the lateral sides of the chamber body (Figure 2.2 (D, E)). The base plate was aligned with the bottom of the chamber body using the clearance holes as reference points and socket cap screws inserted through the base plate (Figure 2.2 (G, H)). Opposing screws were tightened incrementally to ensure an even seal. The assembly was placed on the base plate and the larynx cassette inserted into the chamber body using sterile forceps (Figure 2.2 (I)). The polycarbonate head plate was placed on the top of the chamber body and aligned as for the base plate. The knurled thumb screws were inserted into the head plate and tightened, as before (Figure 2.2 (J)). The final luer-lock connector with male 1/8” G thread attached to 3WSC was inserted into the head plate, with the 3WSC terminated with a sterile hydrophobic Minisart® 0.2 µm filter (Figure 2.2 (K)). All 3WSCs were closed and ports capped with sterile combi-stoppers.
Figure 2.2 Recellularisation bioreactor aseptic assembly. The sterile chamber body was placed on one of its smaller, lateral sides (A), before inserting BS154 O-rings into the reciprocating glands using sterile forceps (B, C). Luer lock connectors were attached to a 3WSC and OR9.6X2.4 O-rings added (D). These were inserted into the corresponding holes of the lateral sides of the chamber body (E, F). The base plate was aligned with the chamber body (G) and socket cap screws inserted (H). The desired cassette was then lowered into the chamber body (I), before adding the head plate and knurled thumb screws (J). The final luer lock connector with attached 3WSC and filter was added to the head plate (K).
2.3.3.3 Sterility validation

To perform an operational sterility validation, a recellularisation kit was compiled within an Aesculap PrimeLine® sterile container (Aesculap AG, Tuttlingen, Germany) before being cleaned and autoclaved by the Central Surgical Sterilisation Department at the Royal Free Hospital. The kit composition can be found in Table 2.4. The bioreactor was assembled as described in section 2.3.3.2, in a Grade A environment with an uncontrolled background. An additional 3WSC was added to one of the 3WSCs positioned proximal to the base plate. A transfer bag containing TSB was attached to the first 3WSC, whilst a syringe (50 mL) was attached to the 3WSC added secondarily (Figure 2.3 (A)). The 3WSCs were configured to allow TSB to be drawn from the transfer bag to the syringe (Figure 2.3 (B)), before reconfiguring to allow expulsion into the bioreactor (Figure 2.3 (C)). This process was repeated until 120 mL of TSB had been transferred into the bioreactor. After five days of incubation at 37 °C under normoxia in a humidified CO₂ (5%), samples (25 mL) were removed from the bioreactor and incubated for 7 days at 20-25 °C, followed by 7 days at 30-35 °C. Contamination/sterility was confirmed, through visual inspection, by the presence/absence of sample turbidity at 14 days.

2.3.4 Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test was performed on the proof-of-concept data described in section 4.3.1. PKH26 fluorescence intensity as an indicator of cell attachment (section 4.3.2) was analysed by two-way ANOVA with Sidak’s multiple comparisons test to compare scaffold and supernatant values at each time point. A Tukey’s multiple comparison test probed for statistical difference between the fluorescence values at different time points, for scaffold and supernatant. Metabolic activity of populations seeded on scaffolds was compared using a Friedman test with Dunn’s multiple comparisons test (section 4.3.3). The DNA concentration of the seeded scaffolds was then analysed by ordinary one-way ANOVA with and Tukey’s multiple comparisons test. Metabolic activity values for media conditioning experiments were compared using repeated-measures two-way ANOVA with Tukey’s multiple comparisons tests, or two-tailed paired t test.
<table>
<thead>
<tr>
<th>Component</th>
<th>Dimension</th>
<th>Units</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recellularisation bioreactor chamber body</td>
<td>N/A</td>
<td>1</td>
<td>Bespoke</td>
</tr>
<tr>
<td>Recellularisation bioreactor base plate</td>
<td>N/A</td>
<td>1</td>
<td>Bespoke</td>
</tr>
<tr>
<td>Recellularisation bioreactor head plate</td>
<td>N/A</td>
<td>1</td>
<td>Bespoke</td>
</tr>
<tr>
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<td>1</td>
<td>Bespoke</td>
</tr>
<tr>
<td>BS154 SI70 USP VI O-ring</td>
<td>BS154</td>
<td>2</td>
<td>Hooper (Huntingdon, UK)</td>
</tr>
<tr>
<td>BS803 SI70 USP VI O-ring</td>
<td>BS803</td>
<td>5</td>
<td>Hooper (Huntingdon, UK)</td>
</tr>
<tr>
<td>Socket cap screw, A4</td>
<td>M4 x 12 mm, DIN 912</td>
<td>8</td>
<td>Precision Technology Supplies (East Grinstead, UK)</td>
</tr>
<tr>
<td>Knurled thumb screw, A1</td>
<td>M4 x 12 mm, DIN 46</td>
<td>8</td>
<td>Wixroyd International (Cranleigh, UK)</td>
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<td>1/8” G thread to male luer lock connectors</td>
<td>DIN EN 20594-1</td>
<td>5</td>
<td>EM-Technik (Maxdorf, Germany)</td>
</tr>
</tbody>
</table>
Figure 2.3 Aseptic bioreactor filling. The bioreactor was fully assembled before a secondary 3WSC was attached to one of the 3WSCs positioned laterally, proximal to the base plate. A full transfer bag was connected to the primary 3WSC, whilst the terminal unit was attached to a syringe (A). Configuration of the 3WSCs allowed media to be drawn from the transfer bag into the syringe (B). Alteration of the configuration then allowed liquid to be expelled into the bioreactor (C). This process was repeated to fill the bioreactor to the desired final volume. A photograph of the assembly is shown in (D).
2.3.4.1 Temperature mapping

Bioreactors were assembled as described in section 2.3.3.2, and filled with 120 mL of PBS (n=4). A thermocouple, attached to a data logger (TC-08; Pico Technology, St Neots, UK), was inserted into the bioreactor via the port in the head plate, which had been fitted with a luer-lock connector with male 1/8” G thread only. The probe was submerged in the liquid, terminating ca. 5 mm above the cassette. Bioreactors were allowed to acclimatise to 37 °C inside an incubator (Heratherm™ compact microbiological incubator; ThermoFisher Scientific). Bioreactors were then transferred to a second incubator, set at either 18 °C or 25 °C, and temperature monitored for 4 h at five minute intervals.

2.3.4.2 BM-MSC biocompatibility

A sterile recellularisation bioreactor was assembled as described in section 2.3.3.2. A larynx cassette was inserted into the bioreactor body in this instance (Figure 2.2 (I)). The bioreactor was filled with complete medium (120 mL) and incubated at 37 °C under normoxia in a humidified CO₂ (5%) incubator for 7 d. Samples of conditioned complete medium (20 mL) were withdrawn from the bioreactor at 24 h, 72 h, 120 h and 168 h, referred to hereafter as 1 d, 3 d, 5 d and 7 d.

A standard curve of viable cells, determined by Trypan Blue exclusion (section 2.1.2.1), with two-fold increments between 5.0 x 10² and 6.4 x 10⁴ cells cm⁻² (n=4), was plated in complete medium (200 µL) containing 20% (v/v) CellTiter 96® AQueous One Solution Reagent (Promega, Madison, USA) in four 96-well microplates (ThermoFisher Scientific). At either 1 h, 2 h, 3 h or 4 h post-seeding, medium was transferred to a new well and absorbance assayed at 490 nm using a Victor™ X3 microplate reader (PerkinElmer, Waltham, USA). The data obtained were used for assay incubation time and seeding density selection for BM-MSC viability investigation.

BM-MSCs were plated in conditioned complete medium (1 d, 3 d, 5 d, 7 d; 200 µL) in 96-well microplates at a density of 5.0 x 10³ viable cells cm⁻² (n=6). One microplate was seeded for each challenge time-point: 4 h, 8 h, 24 h, 72 h and 120 h. A positive control of BM-MSCs in complete medium (n=6), as well as negative control of BM-MSCs in complete medium and each conditioned medium (n=3) were plated in each microplate. Control complete medium and medium from each conditioning time-point was also included in each plate (n=3) for post-hoc removal of medium background absorbance. Four hours prior to each challenge time-point, 20% of the each well’s volume was replaced with CellTiter 96® AQueous One Solution Reagent. After four hours incubation the supernatant was transferred and absorbance assayed as before.
All absorbance values were adjusted for background absorbance contributed by the complete medium and complete medium from each conditioning time-point, respectively. Absorbance values were normalised to those obtained for BM-MSCs cultured in complete medium, for each challenge time-point respectively.

2.3.4.3 Epithelial cell biocompatibility

Modified Green’s medium (section 2.3.1.3) was conditioned for 17 d in a sterile recellularisation bioreactor. Non-contact co-cultures were established using both unconditioned and bioreactor conditioned media in a Transwell® model (n=4; ThermoFisher Scientific). Fibroblasts were seeded in 24-well microplates at $1.0 \times 10^4$ cells cm$^{-2}$, whilst epithelial cells were seeded onto 0.4 µm Transwell® membranes at $3.6 \times 10^5$ cells cm$^{-2}$. Cell densities had been previously determined by A Tait. Both cell populations were submerged and cultured for three days before medium was aspirated from the epithelial-seeded Transwell®, which was then cultured for 14 days at air-liquid interface (ALI). Medium was replaced three times weekly.

After 17 d, medium (100 µL) containing 20% CellTiter 96® AQueous One Solution Reagent was added to cover epithelial cultures. Medium was aspirated after four hours and absorbance read as before (section 2.3.4.2). Membranes were fixed with paraformaldehyde (4%) before being processed, paraffin embedded, sectioned to 3 µm and stained for histology by the UCL Cancer Institute Core Facility. Sections were either H&E or immunofluorescently stained. For immunofluorescent staining, performed by A Tait, sections were deparaffinised with xylene (Sigma-Aldrich) and rehydrated with water. Heat induced antigen retrieval was performed for 20 min using boiling Tris-EDTA-Tween buffer (pH 9) comprised of Tris base (10 mM; Sigma-Aldrich), EDTA (1 mM; Sigma-Aldrich) and Tween 20 (0.05% v/v; ThermoFisher Scientific). Non-specific binding was blocked using bovine serum albumin (5% m/v in Tween 20 (0.1 % v/v; Sigma-Aldrich)) and stained for E-cadherin (ab15148; Abcam, Cambridge, UK), or cytokeratin 4 (GTX11215; GeneTex, Irvine, USA) and integrin β4 (sc-9090; Santa Cruz Biotechnology, Dallas, USA). Anti-mouse Alexa 488 and anti-rabbit Alexa 633 (ThermoFisher Scientific) were used as secondary antibodies, at a concentration of 1:1000 in 0.1% (v/v) Triton X-100 in PBS. Slides were stained at RT for 1 h, before being counterstained using 4’,6-diamidino-2-phenylindole (DAPI; ThermoFisher Scientific) and mounted using Prolong Gold (ThermoFisher Scientific). Samples were imaged using confocal microscopy (Nikon Eclipse Ti-E; Nikon).
2.3.4.4 Epithelial sheet production proof-of-concept

A bioreactor was sterilised and aseptically assembled as previously described (section 2.3.3.2). Acellular human dermis (Euro Skin Bank, Beverwijk, Netherlands) was washed overnight, on an orbital shaker, in saline at 125 rpm at RT to remove excess glycerol. The dermis was cut to ca. 35 mm x 20 mm and clamped between Pt1.1 and Pt2.1 (Figure 4.16), with the outer surface of the dermis oriented towards Pt1.1. The assembled cassette, with scaffold, was inserted into the chamber body. Porcine epithelial cells and porcine fibroblasts, isolated and expanded as described in sections 2.3.1.2 and 2.3.1.3, were seeded at 3.80 x 10^5 cells cm^{-2} and 1.26 x 10^5 cells cm^{-2} on to the surface of the scaffold. Modified Green's medium, without ROCK inhibitor Y-27632, was added to the chamber to fill the lower compartment, whilst 1 mL was added to the surface of the seeded sheet. The bioreactor was closed and incubated at 37 °C under normoxia in a humidified CO_2 (5%) TEB500 incubator (EBERS Medical Technology, Zaragoza, Spain). After three days the bioreactor was fully drained and medium in the lower compartment replaced to bring the epithelial sheet to ALI. Thereafter medium was perfused through the lower compartment at 26.0 µL min^{-1} for a further 14 days. Samples were removed from the bioreactor, embedded and sectioned as previously described, before staining for cytokeratin 4 and integrin β4 and counterstaining with DAPI.
2.4 CHAPTER SPECIFIC MATERIALS AND METHODS – CHAPTER 5

2.4.1 Detergent-enzymatic treatment protocol adaptation

Reagents containing, or derived from, animal components that may pose a risk of transmitting animal spongiform encephalopathies were replaced. DNase from bovine pancreas was replaced with recombinant DNase I expressed in Chinese hamster ovary cells. NaCl, the DNase solvent employed in the published DET protocol, was replaced with sterile HBSS containing Ca\(^{2+}\) and Mg\(^{2+}\).

An additional H\(_2\)O wash period of 48-72 h was introduced at the beginning and end of the DET process, which itself was extended to incorporate three cycles due to the increased organ size relative to that observed in rat. Table 2.5 outlines the final protocol employed.

Table 2.5 Modified detergent-enzymatic treatment protocol

<table>
<thead>
<tr>
<th>Unit Operation</th>
<th>Reagent</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sterile water for injection (Baxter Healthcare)</td>
<td>48-72 h</td>
</tr>
<tr>
<td>2</td>
<td>SDC (4% in sterile water for injection; Sigma-Aldrich)</td>
<td>4 h ± 5 min</td>
</tr>
<tr>
<td>3</td>
<td>Sterile water for injection</td>
<td>1 h ± 5 min</td>
</tr>
<tr>
<td>4</td>
<td>DNase I (22.5 mg L(^{-1}) in HBSS with Ca(^{2+}) and Mg(^{2+}); Roche Holding; ThermoFisher Scientific)</td>
<td>3 h ± 5 min</td>
</tr>
<tr>
<td>5</td>
<td>Sterile water for injection</td>
<td>12 h ± 4 h</td>
</tr>
<tr>
<td>6</td>
<td>Repeat unit operations 2-4</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Sterile water for injection</td>
<td>48-72 h</td>
</tr>
</tbody>
</table>
2.4.2 Modular bioreactor adaptation

Before assembling the chamber the necessary decellularisation kit components were cleaned using amphoteric disinfectant ClearKlens Tego® 2001 (JohnsonDiversey, Northampton, UK) and autoclave sterilised. The kit included the modular bioreactor components detailed in Table 2.4, plus one sterile female 1/8” BSP to male luer thread connector (EM-Technik), one sterile Bardex® I.C. Foley catheter (12Fr; Bard, Crawley, UK), one sterile marprene tube for LF pump-head (0.38 mm ID; Don Whitley Scientific, Shipley, UK) and two sterile PVDF male luer thread hose barbs (3/32”; Cole-Parmer Instruments).

Following sterilisation, the chamber was aseptically assembled in a Grade A environment with an uncontrolled background, as described in section 2.3.3.2. Notably, one of the upper lateral ports was not fitted with a male luer thread connector and was instead left blank. The laryngeal cassette was located within the chamber and the balloon-terminal of the Foley catheter passed through the open port to allow approximately 20 mm to protrude into the chamber. All luer terminated ports were fitted with a sterile 3WSC and capped with sterile combi stoppers. The female 1/8” BSP to male luer thread connector was inserted into the open end of the Foley catheter, which was then connected in series to a sterile 3WSC and the marprene tubing via the male luer thread hose barbs, with all open connections capped as before (Figure 2.4).

2.4.3 Detergent-enzymatic treatment decellularisation

All reagent solutions were prepared one day prior to commencing the decellularisation process; other than the DNase solution, which was prepared immediately prior to use.

SDC (Sigma-Aldrich) solution was prepared to 4% (m/v) in sterile water for irrigation (Baxter Healthcare), whilst HBSS containing Ca$^{2+}$ and Mg$^{2+}$ (ThermoFisher Scientific) was used for the DNase steps. SDC solution was sterile filtered and all reagents aseptically transferred to 300 mL, 1000 mL or 2000 mL transfer bags (Terumo) as described in section 3.3.1. Bags which were to contain HBSS containing Ca$^{2+}$ and Mg$^{2+}$ were spiked with a bag spike with removable injection port. One hour prior to starting the DNase step, dornase alfa (purified rhDNase; Roche Holding) was added to the HBSS containing Ca$^{2+}$ and Mg$^{2+}$ via the injection port, to a final concentration of 22.5 mg L$^{-1}$. All reagents were stored at RT.

Oesophagi were procured from neonatal or juvenile pigs as described in section 2.1.1.2.

Oesophagi were transferred from storage to the modified bioreactor aseptically. After gently flushing the lumen with 10 mL of sterile saline (Baxter Healthcare), a purse-string
suture using non-absorbable 3/0 silk sutures (Johnson & Johnson, Somerville, USA) was used to fix the proximal end of the oesophagi over the catheter terminal within the bioreactor. The head plate was replaced and the chamber sealed.

Filling and draining of the chamber was achieved by attaching the appropriate reagent or waste bag to one of the lower lateral ports on the bioreactor and holding it either above or below the chamber, as was previously done for the Ricordi® chamber (Figure 3.3). The 3WSC on the head plate was opened to allow air displacement through a sterile hydrophobic Minisart® 0.2 µm filter (Sartorius Stedim Biotech) as necessary. During each reagent step the waste line was attached to the additional port on the head plate 3WSC (Figure 2.4 (B)), such that waste reagent would pass into the waste bag and whilst the chamber remained filled. The reagents were flowed through the catheter and lumen of the oesophagi at 0.5 mL min⁻¹ at RT, using either a my-Control bioreactor controller (Applikon Biotechnology, Delft, Netherlands) or TEB500 incubator. The process flow is outlined in Table 2.5.

Following decellularisation, quantitative biomolecular analysis was performed as described in section 2.1.3.

2.4.4 Qualitative biomolecular analysis

Native and decellularised tissue samples were processed for cryosectioning as described in section 2.1.4.1. For native tissue, 3-5 mm full thickness biopsies were taken at the distal terminus. Equivalent biopsies were taken from decellularised tissue, following discard of the distal 5 mm of the tissue. Discarded tissue was grossly more decellularised than the rest of the organ, and so was deemed unrepresentative overall. H&E staining was performed as described in section 2.1.4.2. For fluorescence immunohistochemistry, samples were blocked with 10% (v/v) goat serum (ThermoFisher Scientific) containing 1% (v/v) bovine serum albumin (Sigma-Aldrich) in tris buffered saline (Sigma-Aldrich) for two hours at RT. They were then incubated at 4 °C overnight with primary antibodies collagen I (1:200; ab90395; Abcam, Cambridge, UK), collagen IV (1:200; ab6586; Abcam) or laminin (1:200; ab11575; Abcam). Sections were washed twice in 0.025% (v/v) Triton-X (Sigma Aldrich, UK) in TBS, before being incubated with secondary antibodies for one hour at RT: goat-anti-rabbit (1:500; a11037, ThermoFisher Scientific) and goat-anti-mouse (1:500; a11032, ThermoFisher Scientific). Stained samples were imaged using an Olympus IX70 inverted fluorescence microscope.
Figure 2.4 Aseptic bioreactor filling and luminal perfusion. See Figure 3.3 for parts key. Dashed, blue arrows indicated fluid flow direction. The bioreactor was aseptically assembled and oesophagi sutured over the Foley catheter terminating inside the bioreactor. The bioreactor was filled by holding reagent bags above the chamber, with displaced air vented through the head plate filter (A). The reagent line was then connected to the Foley catheter and oesophagi perfused via the lumen, with waste passing into the bag attached to the head plate (B). The waste bag was attached to a lower lateral port and chamber drained by opening the head plate vent filter (C).
2.4.5 Sterility validation

Bioreactors were sterilised and assembled as described in section 2.3.3.2. The DET protocol was completed (n=3), with each reagent replaced with TSB (Cherwell Laboratories). Samples (25 mL) were removed from the waste after each unit operation and incubated for 7 days at 20-25 °C, followed by 7 days at 30-35 °C. Contamination/sterility was confirmed by the presence/absence of sample turbidity at 14 days.

2.4.6 Quantitative biomechanical analysis

Native samples were assayed <12 h post-scheduling of donor animals. Decellularised non-irradiated tissue was assayed at 1 ± 0.5 week, or 4 ± 0.5 weeks. Decellularised irradiated tissues were γ-irradiated with a dose of approximately 1.8 kGy within 72 h of process completion, before being assayed or stored for 4 ± 0.5 weeks and then tested. All samples were stored in sterile PBS with A/A (1%) at 4 °C.

Longitudinal and circumferentially oriented samples were cut to provide flat specimens, 5 mm by ≥15 mm, before measuring thickness in triplicate using a dial thickness gauge (Mitutoyo, Kawasaki, Japan). Samples were inserted into a zwickiLine testing machine (Zwick/Roell, Ulm, Germany) with 10.00 mm grip separation and submerged in 35-37 °C water. Samples were preconditioned with 8 cycles of loading-unloading up to 40% strain at a constant rate of 20 mm min⁻¹, before a failure test was performed with the same constant rate. Young’s modulus was interpolated from the loading ramp of the failure test, as in Equation 2.3. Stress and strain at break provided the ultimate tensile stress and ultimate strain.

Equation 2.3

\[ E = \frac{\sigma_{0.40} - \sigma_{0.20}}{\varepsilon_{0.40} - \varepsilon_{0.20}} \]

2.4.7 Statistical analysis

Biomolecular (section 5.4.1) and gross characteristics (section 5.5) data were both compared using two-tailed t tests, with the biomolecular analysis accounting for pairing of values. Repeated-measures two-way ANOVA with Sidak’s multiple comparisons test was used to compare biomolecular quantification across different process (section 5.5.1.1). Biomechanical data were compared using one-way ANOVA with Tukey’s multiple comparisons test (section 5.5.1.2).
2.5 CHAPTER SPECIFIC MATERIALS AND METHODS – CHAPTER 6

2.5.1 A bespoke, modular bioreactor for hollow tissue processing

2.5.1.1 Design and manufacture

Founded on the DS outlined in Table 6.2, a custom bioreactor was conceived and drafted using 3D CAD software package Autodesk Inventor Professional. Consultation with a Mechanical Workshop engineer from the Department of Medical Physics and Biomedical Engineering at University College Hospitals confirmed in-house machining capabilities, and designs were drafted accordingly. CNC script was generated using AlphaCAM Ultimate Mill software (Hexagon) by the Workshop engineer. Polyoxymethylene and PC sheet (Heaven Dowsett & Co., Birmingham, UK) were machined using a Gate Precision Mill (Gate Machinery International, Watford, UK) with an Anilam 3000M 3 axis controller (Heidenhain, Traunreut, Germany). Machining was performed by Workshop engineer Philip Sands.

Additional parts for assembly included M4 stainless steel A2-70 retainer flanges for 6 mm paneling and M4 x 16 mm, DIN 464 stainless steel A1 knurled thumb screws (both Wixroyd International). Head plate sealing was achieved with a silicone shore 70 BS257 O-ring, shaft sealing with silicone shore 70 BS014 O-rings, and silicone shore 70 BS803 O-rings for sealing of luer connectors (all Hooper). Lateral and head ports were fitted with nylon 1/4-28 UNF thread to male luer lock connectors (Cole-Parmer), with PVDF 1/8” G thread to male luer lock DIN EN 20594-1 connectors and female 1/8” BSP to male luer thread connectors (both EM-Technik) used to terminate shaft-luer lock fitting connector Pt1/2 assemblies. PVDF female luer thread hose barbs were sized to fit proximal and distal lumen accordingly (Cole-Parmer). An M5 x 20 stainless steel A4 shoulder screw with hexagon socket (Wixroyd International) was coupled with a corresponding hexagon thin nut, DIN439 stainless steel A4 (Precision Technology Supplies) to clamp the internal clip.

2.5.1.2 Aseptic assembly

All non-sterile components were cleaned using amphoteric disinfectant ClearKlens Tego® 2001 and autoclave sterilised, before assembling the bioreactor in a Grade A environment with an uncontrolled background. The threaded lateral port of the bioreactor chamber was fitted with a nylon 1/4-28 UNF thread to male luer lock connector, interposed with a BS803 O-ring. Shaft sealing BS014 O-rings were located in their respective glands on the Front and Back faces of the chamber body, before crush plates were affixed using knurled thumb screws. The rail cassette clip was positioned in its
corresponding groove in the rail cassette and luer lock fittings Pt1 and Pt2 located in the rail cassette clip and rail cassette, respectively (Figure 6.11). BS803 O-rings were fitted over externally threaded termini using sterile forceps, before female 1/8" BSP to male luer thread connectors were attached. The shoulder screw was passed through the rail cassette clip and engaged with the corresponding nut on the opposing side of the rail cassette. The assembled cassette insert was located inside the chamber body. Crush gauges were inserted into the recesses in the crush plates, with the threaded gauge located on the same side as the rail cassette clip. Both shaft parts had termini fitted with BS803 O-rings and female 1/8" BSP to male luer thread connectors. Shaft Pt1 was passed through the threaded crush gauge, into the chamber and rotated to attach to luer lock fitting connector Pt1. Shaft Pt2 was, likewise, attached to luer lock fitting connector Pt2. Rotation of the threaded crush gauge extended the portion of shaft Pt1 within the chamber body to the desired position, before the shoulder screw was tightened to prevent inadvertent movement of the rail cassette clip thereafter. The head plate was then positioned and affixed with knurled thumb screws, whilst its two clearance holes were fitted with nylon 1/4-28 UNF thread to male luer lock connectors with BS803 O-rings. All luer terminals were terminated with 3WSCs and capped.

2.5.1.3 Leak testing

The hollow tissue bioreactor was assembled as described in section 2.5.1.2. Prior to cassette insertion in the bioreactor, a decellularised juvenile porcine oesophagus was sutured to accordingly sized PVDF female luer thread hose barbs that had been affixed to the luer lock fitting connectors, using non-absorbable 3/0 silk sutures (Figure 6.12 (C)). Medium containing phenol red was slowly passed through the lumen of the oesophagus via a manually operated syringe attached to an external shaft terminus. Subsequently, the abluminal compartment of the chamber was manually filled with PBS and the lumen perfused with phenol red containing medium for 15 min at 5 mL min⁻¹ using a Graseby 3500 Anaesthesia Pump (Smiths Medical International, Ashford, UK). The system was observed for leaking from the luminal to abluminal compartment in both instances.

2.5.2 An automated system for DET decellularisation

2.5.2.1 Hardware design and assembly

A system was conceived to perform the DET decellularisation described in Chapter 5 without the need for manual intervention/operation following initial assembly. Figure 2.5 provides a schematic overview of the assembly. Sterile reagents were retained in Duran® laboratory bottles (1 L and 10 L; ThermoFisher Scientific) that were capped with Omnifit™GL45 caps with four 1/4-28 UNF threaded ports (ThermoFisher Scientific).
Caps were fitted with nylon 1/4-28 UNF thread to male luer lock connectors interposed with a silicone shore 70 BS803 O-ring. Two ports were capped, whilst one was connected to a sterile hydrophobic Minisart® 0.2 μm filter and the other to Tygon E-3603 1.6 mm ID tubing (Saint-Gobain, Courbevoie, France) via a 1/16” PVDF female luer thread hose barb (Cole-Parmer). Tubing from each reagent reservoir was passed through a 2/2 solenoid pinch valve (Asco Numatics Sirai, Bussero, Italy) before being condensed to single fluid path via a three-input two-output manifold constructed of three 3WSCs in series. The two output lines were each passed through a 3/2 solenoid pinch valve before being fitted through 114FDC OEM peristaltic pump that was geared to 30 rpm or 200 rpm (Watson-Marlow Fluid Technology Group, Falmouth, UK). The tubing that passed through the 30 rpm pump was connected to Shaft Pt1 via a 1/16” PVDF female luer thread hose barb. The second line was attached to the head plate via a 1/16” PVDF female luer thread hose barb connected to a 1/4-28 UNF thread to male luer lock connector. The second fitting on the head plate provided a vent via a sterile hydrophobic Minisart® 0.2 μm filter, with control again provided via a 2/2 solenoid pinch valve. Shaft Pt2 was connected to a vented waste reservoir, via the normally open path of a 3/2 solenoid pinch valve. The Tygon E-3603 1.6 mm ID tubing emanating from the lateral port on the bioreactor body passed through the normally closed path of the same 3/2 solenoid pinch valve, and was connected to the waste reservoir via an additional 200 rpm geared 114FDC OEM peristaltic pump.

Hardware activation was controlled using an Arduino Uno R3 microcontroller (Arduino, Somerville, USA) and Adafruit Motor Shields (Adafruit, New York City, USA). Wiring, 18 AWG (Alpha Wire International, Sudbury-on-Thames, UK), was connected using crimp terminals (RS Components) and solderless push connectors (Maplin Electronics, Rotheram, UK). Power was supplied using a 12V 10.83A AC adapter (PowerPax, Theale, UK) and a 5-way power splitter (Maplin Electronics).
Figure 2.5 Automated system hardware assembly. Part key is outlined in Figure 2.6. Vented reagent reservoirs are connected to a 3WSC manifold, with each tubing passing through a pinch valve. Tubing outputs passed through a 3/2 solenoid pinch valve and connected to the luminal or abluminal compartments of the vented bioreactor via 200 rpm and 30 rpm peristaltic pumps, respectively. Second tubing paths from luminal and abluminal compartments were connected to a vented waste reservoir via a 3/2 solenoid pinch valve and peristaltic pump.
Figure 2.6 Automated system hardware key. Components used in the assembly of hardware included: Duran® laboratory bottle (A); female luer thread hose barb (B); 3WSC (C); male luer thread hose barb (D); 114FDC OEM peristaltic pumps geared to 30 rpm or 200 rpm (E); 2/2 solenoid pinch valve (F); 3/2 solenoid pinch valve (G); hollow tissue bioreactor (H); 1/4-28 UNF thread to male luer lock connector (I); hydrophobic Minisart® 0.2 μm filter (J) and Tygon E-3603 1.6 mm ID tubing (K).
2.5.2.2 Programming

The code that formed the programme to control the peristaltic pumps and solenoid valves was written in the open-source Arduino Integrated Development Environment software using a set of C/C++ functions. The sketch was passed directly to the compiler prior to uploading to the microcontroller.

2.5.2.3 System calibration and proof-of-concept

To ensure that each motor was activated in the correct sequence, the programme was altered to reduce each activation period to 10 seconds. The programme was uploaded to the microcontroller and executed, noting the sequence of motor activation.

To determine the activation voltage of solenoid valves, they were attached to a variable DC power supply and voltage increased until the solenoid valve with tubing in situ was actuated. Voltage was then decreased to determine the range over which the valve remained engaged, and eventually the release voltage.

Flowrates were assessed against theoretical values derived from the peristaltic pump duty cycle and the pressure and flow performance information provided by the manufacturer. Tubing was primed with deionised water and run for 10 min at 0.5 mL min\(^{-1}\). Flowrate was determined from the mass of the water and the duty cycle adjusted accordingly. Extended testing for 1 h was performed to allow for refinement that would finalise the duty cycle.

Filling and emptying of the assembled chamber was timed when operating peristaltic pumps at maximum flowrate. Pump flowrate was reduced to prevent bubble formation within reagent lines, before again timing fill and empty periods.

2.5.3 Statistical analysis

Biomolecular data were analysed using a two-tailed paired t test (section 6.4.4.1.1).
3 SYSTEM CLOSURE AND GMP-COMPLIANT PRODUCTION OF DECELLULARISED AIRWAY SCAFFOLDS IN AN OFF-THE-SHELF BIOREACTOR

3.1 INTRODUCTION

Following the landmark, and subsequently controversial, first-in-man clinical application of a tissue engineered airway reported a decade ago (Macchiarini et al., 2008), there has been an increasing focus on clinical translation within the subfield of airway tissue engineering. Initially, this was directed towards the refinement and improved characterisation of the detergent-enzymatic method (DEM) used for the decellularisation of tracheae (Jungebluth et al., 2009). Originally described by Conconi (Conconi et al., 2005), this technique provided the foundations for the breakthrough intervention reported in 2008. Subsequent in vivo investigations in a porcine model supported the notion that decellularised scaffolds produced using a DEM could, when seeded with epithelial cells and mesenchymal stromal cell (MSC) derived chondrocytes, prove clinically effective (Go et al., 2010). Naturally, this led to the investigation of the capability of the modified DEM to produce decellularised human tracheal scaffolds that could be clinically suitable (Baiguera et al., 2010). In addition to generating a construct that had biological and biomechanical properties comparable to native trachea, the authors highlighted, for the first time, an appreciation of the importance of the manufacturing process when delivering clinically applicable TEPs. Whilst the considerations associated with clinical manufacture were limited only to the effect of process duration on timelines and costs, it signified a milestone in the comprehension and application of effective clinical translation within the field.

Buoyed by the progress made in tracheal tissue engineering, as well as a series of promising studies demonstrating the generation and application of decellularised ECM derived matrices in canine (Huber et al., 2003, Ringel et al., 2006) and rat models (Xu et al., 2010), efforts were expanded to address laryngeal regeneration. Directly applying their previously described modified DEM for human trachea (Baiguera et al., 2010), Baiguera and colleagues reported the successful decellularisation of whole human larynges (Baiguera et al., 2011). As predicted, histological analysis revealed complete removal of extra-cartilaginous cell types, leaving behind a small number of chondrocytes within the cricoid and thyroid cartilage. This was echoed by the large, but not complete, removal of detectable DNA. The architecture of the tissue micro- and ultra-structure did not appear to be largely disrupted and the biomechanical profile was similar for native and decellularised tissue. Much like the trachea, the decellularised larynx demonstrated
angiogenic potential when assessed immunohistochemically and in vivo (chicken chorioallantoic membrane assay). These results demonstrated, for the first time, the production of a decellularised human laryngeal construct that possessed the characteristic profile thought to be suitable for producing a functional partial hemilaryngectomy (Baiguera et al., 2011).

Unlike the trachea, the functionality of which is imparted by providing a passive conduit to the lungs, the larynx requires extensive muscular activation to allow for unhindered breathing, speech and prevention of aspiration (Fishman et al., 2011). Having suggested that protocols containing detergent and enzymatic reagents can have a destructive/detrimental effect on the structural, biochemical and biomechanical profiles of decellularised skeletal muscle ECM, Fishman and colleagues compared the efficacy of decellularisation using a non-detergent, non-proteolytic protocol to the proven DEM previously discussed (Fishman et al., 2012). The process, which relied upon subsequent tissue washes in latrunculin B (a toxin that disrupts the cell cytoskeleton), potassium chloride (KCl), potassium iodide (KI), DNase I and hypotonic water, was used to decellularise rabbit cricoarytenoid dorsalis muscles. This muscle is analogous to the posterior cricoarytenoid in humans and is the singular, intrinsic muscle responsible for controlling vocal fold opening. The data generated indicated that non-detergent, non-proteolytic decellularisation resulted in a significantly greater reduction in quantifiable DNA compared to the DEM, whilst both retained microarchitecture similar to that of the native muscle. Although the non-detergent, non-proteolytic decellularisation appeared, histologically, to have a lesser effect on collagen and elastin degradation than the DEM, both protocols induced a significant reduction in quantified total collagen and sGAG content. The acellular scaffolds produced were identified as biocompatible after failing to elicit a rejection response in a xenotransplantation model. Although the findings presented suggested that a non-detergent, non-proteolytic decellularisation protocol may prove beneficial when producing skeletal muscle scaffolds for laryngeal regeneration, it remained unclear how this approach may fair when applied to larger constructs.

Acknowledgement of the caveats associated with each of these approaches led to the development of a combination decellularisation technique that incorporated both detergent and enzyme reagents, as well as those described in the non-detergent, non-proteolytic protocol. It was proposed that this could effectively decellularise both the cartilaginous and muscular components of the larynx. The novel approach, described by Ansari et al. (2017), subjected hemilarynges to a freeze-thaw cycle, before incubating in latrunculin B. Tissues subsequently underwent a series of hypertonic-hypotonic washes and an enzymatic step, before a second freeze-thaw cycle. Samples were incubated in a detergent mixture before enzymes were used to solubilise any remaining nuclear
material. A second detergent step was then used before an extended wash in deionised water concluded the process. All reagent steps were interspersed with deionised water washes to remove any remaining reagent and the protocol was performed under a vacuum of <1 kPa absolute pressure (Ansari et al., 2017).

Histological assessment and DNA quantification were employed to evaluate the extent of decellularisation. H&E staining revealed an absence of localised nuclear material, which was in accord with a reduction in total DNA to <50 ng mg⁻¹ of wet tissue. There appeared to be no substantial difference in the micro-architecture of the tissue following decellularisation, although there was a reduction of approximately 50% in total collagen and total sGAG content. Biomechanical testing revealed no significant difference in the tensile strength or rupture force of cartilage and muscle following decellularisation, nor of the elongation at break of the cartilaginous component. A significant reduction in the elongation at break of the muscle fraction was reported. In combination, these results demonstrated that an effective decellularisation of hemilarynx had been achieved in accordance with the indicators cited by the authors. Moreover, the efficacy of decellularisation appeared comparable to that reported by Baiguera et al. (2011); with the significant benefit of being completed in 8 d, compared to 17 d.

The reported findings were the result of a Medical Research Council funded study (RegenVOX I – G1001539) designed, following consultation with Medicines and Healthcare products Regulatory Agency (MHRA), to provide data supporting a Clinical Trials Authorisation (CTA) application. The study complied with the guidelines outlined in the Good Laboratory Practice Regulations 1999 (UK Parliament, 1999), and conformed to the requirements of EU directives 2004/09/EC and 2004/10/EC (European Commission, 2004a, European Commission, 2004b). The study was intended to generate long-term safety and efficacy data supporting the application of MSC-seeded decellularised scaffolds for partial laryngeal replacement. Whilst not designed to assess long term functionality, the study did address whether the constructs could provide full thickness structural replacements (Ansari et al., 2017).

Following the successful completion of the study it was necessary to generate further data to support a successful CTA application. Primarily, it would be compulsory to establish and validate a GMP-compliant process capable of recapitulating the results of the good laboratory practice (GLP) compliant study. Demonstrating this would provide an essential addition to the investigational medicinal product dossier (IMPD), which in turn would provide the basis of the CTA application.
3.2 AIMS AND OBJECTIVES

3.2.1 Aims

Chapter 2.2 provides an account of the process engineering undertaken to translate the decellularisation operations of the GLP-compliant process, developed by our team and later described by Ansari et al. (2017), to comply with the GMP guidelines for ATMPs outlined in EudraLex – Volume 4 (European Commission, 2017). The aim was to develop a process suitable for manufacturing a small number (n=10) of human, acellular larynx scaffolds that could act as constructs for reseeding and implantation in man, as part of a Phase I/II clinical trial of an advanced therapy investigational medicinal product (ATIMP). The development and validation of this process would produce data that would be incorporated into the IMPD that would form the basis of a CTA application. The intended manufacturing site for the ATIMP was the Centre for Cell, Gene and Tissue Therapeutics (CCGTT) at the Royal Free Hospital, London. As such, the process was designed to operate within the established constraints and quality systems of the CCGTT.

3.2.2 Objectives

- To develop and validate an aseptic system for the decellularisation of human hemilarynges.
- To adapt the decellularisation protocol used in the GLP-compliant study to incorporate quality control measures suitable for ATIMP manufacture, resulting in a GMP-compliant process.
- To validate the GMP-compliant decellularisation of human larynx against the critical quality attributes outlined as comparators of the GLP-compliant process.
3.3 PROCESS CLOSURE AND GMP-COMPLIANCE

The original human-specific, larynx decellularisation SOP provided was developed in accordance with GLP guidelines. It was necessary to modify the process to adhere to GMP guidelines for ATMPs as outlined in EudraLex – Volume 4 (European Commission, 2017), with particular focus on Annex 13 – Manufacture of Investigational Medicinal Products and Annex 1 – Manufacture of Sterile Medicinal Products (European Commission, 2008, European Commission, 2010b). The changes implemented to align with the guidelines were associated with two overarching categories; process closure and materials control.

3.3.1 Process closure and sterility validation

The GLP-compliant decellularisation protocol was performed using an open-system, a desiccator, which was not suitable for clinical manufacture of ATIMPs. To ensure an appropriate replacement was found, a user requirement specification (URS) was outlined (Table 3.1). In addition to the requirements listed, it was also identified that operational ease-of-use was highly desirable.

A Ricordi® chamber was identified as a potentially suitable replacement vessel, following previously successful application by our group clinically (unpublished). Two Ricordi® chamber models, one constructed of PEI and one of SS303 (Figure 3.1), were assessed for suitability against the URS.
Table 3.1 User requirement specification for decellularisation vessel

<table>
<thead>
<tr>
<th>UR#</th>
<th>User Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The vessel must consist of internal geometry and dimensions large enough to accommodate an adult human larynx, plus 250 mL of liquid, with external dimensions less than 0.50 x 0.50 x 0.20 m.</td>
</tr>
<tr>
<td>2</td>
<td>The vessel must be constructed of inert materials that are resistant to degradation by those reagents used in the detergent-enzymatic decellularisation process.</td>
</tr>
<tr>
<td>3</td>
<td>The vessel must be constructed of materials that can be repeatedly autoclaved at 137 °C without degradation or deformation.</td>
</tr>
<tr>
<td>4</td>
<td>The vessel must be constructed of materials that can be suitably operated at the temperatures used in the detergent-enzymatic decellularisation process, without degradation or deformation.</td>
</tr>
<tr>
<td>5</td>
<td>The vessel must form a sealed compartment capable of maintaining a vacuum of approximately 200 Pa for up to 72 h.</td>
</tr>
<tr>
<td>6</td>
<td>The vessel must allow for the exchange of liquid across the internal-external wall in a semi-closed/closed* manner.</td>
</tr>
</tbody>
</table>

* semi-closed defines a process or action that, whilst not entirely closed, has been deemed significantly low risk so as to allow processing in a Grade A environment with a Grade D background.
Figure 3.1 Ricordi® chambers. Both stainless steel 303 (A) and PEI (B) models were assessed for suitability against the URS. The engineering drawing of the stainless steel unit provided the outer dimensions of the vessel. Measurement confirmed these to be the same for the PEI unit. Engineering drawing reproduced with permission from Biorep Technologies.
Both models satisfied user requirement one, with an internal volume capacity of 500 mL and external dimensions of ø 0.132 m by 0.158 m (Figure 3.1).

PEI has been proven suitable for use with a range of detergents and ionic solutions (Baur (ed.), 2016), and so it was deemed that the chamber constructed of the polymer would satisfy user requirement two. Stainless steel also has a well-defined chemical resistance profile (Graco, 2013) and, whilst it is widely acknowledged that it is corroded by chlorides, austenitic stainless steels, such as 303, have improved corrosion resistance. It was considered that the relatively short KCl and MgCl₂ steps used within the decellularisation process represented a low corrosion risk and that the subsequent washes would be adequate to remove residual chloride containing solution. In addition, SS303 Ricordi® chambers have more than 25 years of clinical use with chloride containing enzymatic and hypotonic reagents since they were first developed, supporting their suitability (Ricordi et al., 1988, Ricordi et al., 1992, Ricordi et al., 2016). Subsequently, it was deemed that the SS303 chamber also satisfied user requirement two.

Both the PEI and SS303 models are suitable for repeated autoclave sterilisation cycles and pass user requirement three (Biorep Technologies, 2016). They have maximum operating temperatures significantly greater than 37 °C, the maximum temperature used during the decellularisation process, and minimum service temperatures suitably lower than the decellularisation process minimum of -30 °C (Chemical Retrieval on the Web, 2015, British Stainless Steel Association, 2018); thus meeting user requirement four.

To determine whether user requirement five was met, the vessels’ capacity to maintain a pressure of approximately 200 Pa was investigated as described in section 2.2.2. It was found that the PEI chamber was unable to sustain the required pressure for the desired 72 h. Further investigation revealed the port on the base of the chamber to be the source of pressure equalisation, due to incomplete sealing between the port and tubing. The PEI chamber was discounted from any future application accordingly. The SS303 chamber maintained pressure for the required 72 h.

The primary reason for adopting an alternative decellularisation chamber to that used in the GLP decellularisation SOP was the need to fill and drain the chamber in a semi-closed/closed aseptic manner; captured by user requirement six of the URS. It should be noted that semi-closed, in this instance, defines a process or action that whilst not entirely closed, has been deemed significantly low risk so as to allow processing in a Grade A environment with a Grade D background (ISO 14644-1 (International Organization for Standardization, 2015)). To achieve this it was necessary for reagents to first be transferred into a closed container, before a semi-closed/closed transfer could be made to the decellularisation chamber. Likewise, there was a necessity to empty the
chamber in a semi-closed/closed manner before the next reagent could be added. It was proposed that this could be achieved by using transfer bags to house the reagents and waste. A process flow and methodology for transferring reagents into transfer bags, as well as filling and emptying the chamber, was subsequently developed.

The conceived process, represented in Figure 3.2, was initiated by transferring sterile reagents from open containers into transfer bags via a coupled pump segment and peristaltic pump. Each transfer bag was fitted with a male spike coupler-luer line, whilst the pump segment was spiked with a removable injection port that terminated in a female luer. By connecting and disconnecting the male and female luer terminals, multiple bags could be filled from a single container. The correct volume was added to each bag by measuring the mass of liquid transferred using a balance, in accordance with Equation 3.1.

Equation 3.1

\[
\text{Required Reagent Mass in Bag} = \text{Reagent Density} \times \text{Desired Reagent Volume}
\]

Each bag had its luer terminal capped and was stored accordingly. This process, due to its open nature, had to be performed in a Grade A environment with a Grade B background to adhere to GMP guidelines (European Commission, 2008). By adopting this approach it is possible to transfer all reagents to closed containers in a single session, in a time efficient manner, before storing appropriately.

When filling or emptying of the decellularisation chamber was required, bags were connected to a 3WSC terminal on the base of the chamber via a male spike coupler-luer line and the contents transferred. Transfer bags were held above the Ricordi® chamber to allow contents to drain in slowly, and vice versa (Figure 3.3). Once the transfer was completed, bags were removed and all ports terminated with luer caps. Using this methodology it was possible to perform the decellularisation process in a semi-closed/closed manner.

To substantiate the claim that the novel process flow and methodology supported aseptic semi-closed/closed operation, a media simulation was performed in accordance with the guidelines set out in EudraLex – Volume 4, Annex 1 (European Commission, 2008). To ensure rigorous testing, the entire larynx decellularisation process, as outlined in Table 2.3, was performed using TSB (n=3). This assessed both the bag filling methodology (n=87) and the method of filling/emptying the chamber (n=174). Following incubation for
14 d, all samples were confirmed to be negative for microbial contamination, indicated by the absence of turbidity. A representative sample is shown in Figure 3.4. Temperature was mapped throughout the process to demonstrate that each step within the procedure fell within the temperature limits indicated in Table 2.3. An example report is depicted in Figure 3.5.
Figure 3.2 Transfer bag filling. Each transfer bag was fitted with a male spike coupler-luer line and pump segment spiked with a bag spike with removable injection port, with the opposing end inserted into sterile filtered reagent via a peristaltic pump (A). Empty transfer bags were coupled to the pump segment (B) and filled by operating peristaltic pump (C). The coupler-luer line was disconnected and capped (D), thus allowing for the introduction of a new, empty transfer bag. The process was repeated (A-D) to fill multiple transfer bags. Schematic key is depicted in Figure 3.3.
Figure 3.3 Ricordi® chamber reagent transfer and parts key. Reagents are added by raising the bag above the chamber and opening the three-way stop-cock (3WSC) (A). The bag is moved below the chamber and the 3WSC opened to drain (B). The components used in bag and chamber filling included: bag spike with removable injection port (C); female luer thread hose barb (D); 1/8” BSP to male luer thread connector (E); haematology pump segment (F); male spike coupler-luer line (G); tubing (H); peristaltic pump (I); pressure gauge (J); hydrophobic Minisart® 0.2 µm filter (K); transfer bag (L) and three-way stop-cock (M).
Figure 3.4 Tryptic soy broth media fill turbidity

Figure 3.5 Temperature log for Ricordi® chamber decellularisation media simulation
3.3.2 Materials control

To ensure appropriate control of materials, a series of alterations and requirements were introduced for the procurement and use of raw materials in the decellularisation process. Starting materials, namely human larynx, were procured in accordance with the HTA Quality & Safety Regulations for Tissues and Cells under existing HTA licenses. All tissues were screened for relevant infectious markers (Table 2.2) and only processed following negative microbiology/virology results.

Reagents containing, or derived from, animal components that may pose a risk of transmitting animal spongiform encephalopathies were replaced, as recommended by EU Notice EMA/410/01 rev.3 (European Commission, 2011). This resulted in RNase and DNase, both from bovine pancreas, being replaced with recombinant RNase I expressed in *Pichia* and recombinant DNase I expressed in Chinese hamster ovary cells respectively.

Where suitable, GLP raw materials were replaced by compendium materials. This acted as a secondary driver in the replacement of bovine DNase I with human recombinant DNase I (dornase alfa), and also resulted in the replacement of deionised water with sterile hypotonic water for irrigation. When the introduction of compendium materials was not possible, and/or appropriate, higher grade/specification materials were sought to replace those used in the GLP process. A comparison of the relative grades can be seen in Table 3.2.

To limit the number of reagents requiring preparation and sterile filtration in-house, which increases risk of incorrect solution concentrations and contamination, pre-prepared reagents were introduced. To provide the necessary divalent cations for enzymatic activity, sterile Hank’s Balanced Salt Solution (HBSS) containing Ca$^{2+}$ and Mg$^{2+}$ was used in place of the PBS containing CaCl$_2$ and MgCl$_2$ solution that previously had to be prepared and filtered in-house. Sterile PBS solution was used to prepare the KCl, KI and detergent solutions rather than preparing solution from PBS tablets and deionised water.
Table 3.2 Comparison of material grades used in GLP- and GMP-compliant processes

<table>
<thead>
<tr>
<th>Material</th>
<th>Grade or Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-compliant Process</td>
<td>GMP-compliant Process</td>
</tr>
<tr>
<td>DMEM high glucose</td>
<td>Research (Premium)</td>
</tr>
<tr>
<td>DNase</td>
<td>Research</td>
</tr>
<tr>
<td>HBSS Ca²⁺ Mg²⁺</td>
<td>N/A</td>
</tr>
<tr>
<td>HEPES buffered saline solution</td>
<td>Research</td>
</tr>
<tr>
<td>Latrunculin B from Latruncula magnifica</td>
<td>Research (&gt;80%)</td>
</tr>
<tr>
<td>PBS</td>
<td>Research (N/A)</td>
</tr>
<tr>
<td>KCl</td>
<td>Research (≥99.0%)</td>
</tr>
<tr>
<td>KI</td>
<td>Research (Analytical; ≥99.0%)</td>
</tr>
<tr>
<td>RNase I</td>
<td>Research (purity unspecified)</td>
</tr>
<tr>
<td>SDC</td>
<td>Research (Premium; ≥98%)</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Research</td>
</tr>
</tbody>
</table>
3.4 VACUUM-ASSISTED DETERGENT-ENZYMATIC DECELLULARISATION TRANSLATION VALIDATION

Following the adoption of the newly introduced materials and establishment of process closure, validation of the vacuum-assisted detergent-enzymatic decellularisation process was performed to demonstrate successful translation from a GLP-compliant to GMP-compliant process. The critical quality attributes (CQAs) identified as indicative comparators between the processes were limited to post-decellularisation DNA content (Table 3.3). Histological analysis of nuclear material and maintenance of tissue architecture, as well as relative retention of total collagen and sGAG, were used as non-critical comparators.

Secondarily, CQAs for internal release of the decellularised scaffold for recellularisation, as well as final product release, were proposed. Sterility was to be assessed at both these time points, whilst DNA content of the decellularised tissue and endotoxin contamination were identified as a final release criteria (Table 3.3).

Table 3.3 Critical quality attributes for decellularisation process

<table>
<thead>
<tr>
<th>Critical Quality Attribute</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GLP-GMP Process Comparator</strong></td>
<td></td>
</tr>
<tr>
<td>Post-decellularisation DNA content</td>
<td>&lt;50 ng DNA / mg tissue</td>
</tr>
<tr>
<td><strong>Release Criteria</strong></td>
<td></td>
</tr>
<tr>
<td>Post-decellularisation DNA content</td>
<td>&lt;50 ng DNA / mg tissue</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>&lt;0.5 EU mL⁻¹</td>
</tr>
<tr>
<td>Sterility</td>
<td>Negative</td>
</tr>
</tbody>
</table>
3.4.1 Endotoxin and sterility assessment

Human hemilarynges (n=2) underwent vacuum-assisted detergent-enzymatic decellularisation as previously described (sections 2.2.4 and 2.2.5).

Process sterility was evaluated via microbiological testing, as per section 2.2.4, of samples taken from the pre-decellularisation transport solution, the first overnight H₂O wash (step 8) and the final wash step (see section 2.2.5). Both tissues, HT1 and HT2, returned positive results for microbiological contamination at the first two sampling points. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, performed by the Microbiology Department at the RFH, identified the contaminants. For HT1 *Klebsiella oxytoca* and *Staphylococcus aureus* were identified in the transport solution, with *S. aureus* persisting following A/A treatment. *Candida albicans* was also identified within the waste from step 8 and, although *S. aureus* was eliminated, persisted throughout the remainder of the decellularisation. HT2 was initially contaminated with *Escherichia coli*, *Hafnia alvei* and *Streptococcus salivarius*. Sampling of step 8 waste identified *S. salivarius* as still present, although this was not the case for the final sample. Endotoxin testing was performed on the final wash solution.

To eliminate the identified contamination, HT1 was transferred to a sterile container and washed in PBS containing amphotericin B (5 mg L⁻¹), gentamycin (50 mg L⁻¹), penicillin and streptomycin (10 mL L⁻¹) overnight at RT on a SRT6 roller mixer at 33 rpm (Stuart, Cole-Parmer Instrument Company). This was repeated, with the second incubation also containing amoxicillin (1 mg L⁻¹). Subsequent testing revealed that the sample was still contaminated with *C. albicans*. With no viable bacteria detectable, samples were sent for endotoxin testing. Results were negative (Table 3.4).

Following the introduction of a decontamination step (section 1.1.1), two further vacuum-assisted detergent-enzymatic process validations were performed; HT3 and HT4. To conserve tissue usage, one hemilarynx was divided into thyroid and cricoid cartilage components and processed in independent Ricordi® chambers simultaneously. Antimicrobial compounds were not used during these processes. Both tissues were initially contaminated with *K. oxytoca*, *Enterococcus raffinosus* and *Lactobacillus sp.*, although microbiology samples returned negative following chlorhexidine digluconate decontamination. Samples taken from step 8 were flagged for containing *Serratia marcescens*, which persisted through the remainder of the decellularisation. *K. oxytoca* and *Enterococcus faecum* were also present with the final wash sample. Subsequently, endotoxin testing was not performed.
3.4.2 Tissue decontamination using chlorhexidine digluconate

To reduce/eradicate the bioburden on tissues before initiating the decellularisation process, decontamination using chlorhexidine digluconate solution was investigated. Porcine larynges (n=1) and hemilarynges (n=2) were thawed and contamination confirmed by media turbidity.

Tissues were decontaminated by submersion in chlorhexidine digluconate, washed and incubated in media (section 1.1.1). The incubation media was confirmed to be sterile by turbidity visualisation, inferring successful decontamination of the tissue.

Table 3.4 Endotoxin and sterility results for process validations HT1-HT4

<table>
<thead>
<tr>
<th>Assay</th>
<th>Test Sample</th>
<th>Batch</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endotoxin</td>
<td>Final process wash step</td>
<td>HT2</td>
<td>Pass</td>
</tr>
<tr>
<td></td>
<td>Post A/A wash</td>
<td>HT1</td>
<td></td>
</tr>
<tr>
<td>Sterility</td>
<td>Transportation solution</td>
<td>HT1</td>
<td>Fail</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HT2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HT3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HT4</td>
<td></td>
</tr>
<tr>
<td>Sterility</td>
<td>First overnight H₂O wash (step 8)</td>
<td>HT1</td>
<td>Fail</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HT2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HT3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HT4</td>
<td></td>
</tr>
<tr>
<td>Sterility</td>
<td>Final process wash step</td>
<td>HT1</td>
<td>Fail</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HT2</td>
<td>Pass</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HT3</td>
<td>Fail</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HT4</td>
<td>Fail</td>
</tr>
<tr>
<td>Sterility</td>
<td>Post A/A wash</td>
<td>HT1</td>
<td>Fail</td>
</tr>
</tbody>
</table>
3.4.3 Tissue sterilisation using gamma-irradiation

Irradiated human hemilarynges, confirmed to be contaminated with *C. albicans*, *C. dubliensis*, *Streptococcus parasanguis*, *Streptococcus vestibularis* and *Staphylococcus aureus* prior to irradiation, were assessed for sterility by sampling the fluid in which they had been irradiated (section 2.2.8). All tissues received a calculated dose within the range 29.1-33.4 kGy, and tested negative for bacterial contamination after 10 days of incubation and negative for fungal growth after an extended period of 14 d.

3.4.4 Qualitative analysis

3.4.4.1 Macroscopic observation of larynx pre- and post-decellularisation

Gross observations of thawed whole larynges, pre- and post-removal of excess tissue, as well as hemilarynges pre- and post-decellularisation, were photographed (Figure 3.6). Dissection removed superfluous extralaryngeal tissue to retain the whole larynx only (Figure 3.6 (D-F)). Sagittal bisection yielded two hemilarynges, maintaining the key anatomical features, including the cricothyroid muscle (Figure 3.6 (G)), vocal folds (Figure 3.6 (H)) and the posterior cricoarytenoid muscle. Following decellularisation the larynx maintained the aforementioned features, with a typical whitening indicating decellularisation (Figure 3.6 (I, J)).

3.4.4.2 Histological analysis of decellularisation efficacy

Full thickness punch biopsies were taken from proximal and distal regions of thyroid cartilage, as well as from cricoid cartilage. This allowed assessment of decellularisation efficiency within separate regions of the thyroid cartilage, as well as between the different cartilages. Importantly, it also mediated comparison between the cartilage and muscle components of the tissue. Biopsies were embedded in OCT and sectioned at 5 μm, before staining with H&E (section 2.1.4.2). Native tissue possessed high densities of cells within the mucosa (Figure 3.7 (A)), and a complete distribution of chondrocytes throughout the cartilage (Figure 3.7 (B)). Decellularised, stained sections revealed a complete removal of intact nuclei from the mucosa (Figure 3.7 (C)). There was also a noted absence of nuclear material within the peripheral regions of the cartilage cross sections, although some nuclear material remained within the most central region of the cartilage (Figure 3.7 (D, arrow)).
Figure 3.6 Human larynx pre- and post- dissection, bisection and decellularisation. Thawed larynx maintained significant extra-laryngeal musculature and pharynx (A-C; C, arrow). Tissue was removed to expose the thyroid cartilage (D, arrow) and cricoid cartilage (E, arrow). Cricothyroid muscle was left in situ (G, arrow) and the larynx bisected to expose the undamaged vocal fold (H, arrow). Decellularised tissue (I, J) maintained key macroscopic features including the mucosal layer, musculature and the vocal fold (J, arrow).
Figure 3.7 Qualitative histological evaluation of decellularised human larynx. H&E staining of native tissue sections revealed a high density of cells within the mucosa (A) and distributed within lacunae throughout the cartilage (B). Decellularised larynx tissue demonstrated a complete clearance of nuclear material from the mucosa (C). Whilst nuclei were absent from peripheral lacunae within the cartilage compartment, they remained in situ in centrally positioned lacunae (D, arrow). Magnification is X10 and scale bars represent 100 µm.
3.4.5 Quantitative biomolecular characterisation

Biomolecular content of non-irradiated tissue, pre- and post-decellularisation, was quantified using commercially available assay kits (section 2.1.3). Tissue was biopsied at distinct locations, as described in section 3.4.4.2.

3.4.5.1 Quantitative analysis of DNA concentration

Quantitative analysis of normalised DNA concentration was used to determine the efficacy of the vacuum-assisted detergent-enzymatic decellularisation process in DNA removal from larynx. Decellularisation resulted in a DNA content reduction from 176.78 ± 58.82 ng mg⁻¹ to 25.85 ± 17.27 ng mg⁻¹, a 6.8 fold decrease (Figure 3.8). This confirmed that decellularisation performed using the GMP-compliant process did not impair the removal of DNA from larynx.

Figure 3.8 Quantitative analysis of normalised DNA content in native and decellularised larynx. Analysis revealed a reduction in normalised DNA concentration from 176.78 ± 58.82 ng mg⁻¹ in the native tissue (n=4) to 25.85 ± 17.27 ng mg⁻¹ once decellularised (n=4). Paired analysis revealed a decrease in normalised DNA concentration to <50 ng mg⁻¹ in all but one of the samples, which saw a reduction from 160.5 ng mg⁻¹ to 86.0 ng mg⁻¹.
3.4.5.2 Quantitative analysis of collagen concentration

Normalised collagen concentration was quantitated to investigate the effect of the vacuum-assisted detergent-enzymatic decellularisation process. Analysis revealed comparable collagen concentrations following decellularisation, with a slight decrease from $46.52 \pm 4.87 \, \mu g \, mg^{-1}$ in the native tissue to $44.91 \pm 12.29 \, \mu g \, mg^{-1}$ once decellularised (Figure 3.9). This corresponded to a 96.54% retention in total collagen.

![Graph](image)

Figure 3.9 Quantitative analysis of normalised total collagen content in native and decellularised larynx. Normalised total collagen content appeared comparable (96.54% retention) between native tissue ($46.52 \pm 4.87 \, \mu g \, mg^{-1}$; n=4) and decellularised tissue ($44.91 \pm 12.29 \, \mu g \, mg^{-1}$; n=4). Paired analysis indicated that normalised total collagen concentration both increased and decreased following decellularisation.
3.4.5.3 Quantitative analysis of sGAG concentration

Quantitative analysis of normalised total sGAG content was performed. Following decellularisation, sGAG concentration was reduced from $3.64 \pm 2.07 \, \mu g \, mg^{-1}$ to $2.61 \pm 1.64 \, \mu g \, mg^{-1}$, representing a retention of 71.70% (Figure 3.10). Paired observations provided an explanation for the large deviation observed; highlighting that whilst absolute values were distributed over a wide range, relative variation in concentration following decellularisation appeared similar in three of the four tissues.

![Figure 3.10](image)

Figure 3.10 Quantitative analysis of normalised sGAG content in native and decellularised larynx. Normalised total sGAG content was reduced from $3.64 \pm 2.07 \, \mu g \, mg^{-1} \,(n=4)$ to $2.61 \pm 1.64 \, \mu g \, mg^{-1} \,(n=4)$, following decellularisation. This was equivalent to a 71.70% retention of sGAG. Paired analysis demonstrated that normalised total sGAG concentration increased in three of the four samples assayed.
3.5 DISCUSSION

Following the successful completion of the GLP-compliant study investigating the long-term safety and efficacy of a partial laryngeal replacement with a decellularised allogeneic porcine laryngeal scaffold seeded with human derived MSCs, GMP-compliant process translation was required in order to generate data to contribute to the IMPD that was to be submitted as part of the CTA application. Moreover, if the CTA were to be awarded, the resultant process would form the initial manufacturing process for the first clinical trial of a stem cell based organ replacement (University College London, 2013); thus the steps undertaken were not simply an exercise to demonstrate translation feasibility. The first aspect of the GLP-compliant methodology to be addressed was the decellularisation process. A human-specific larynx decellularisation SOP was provided by the Principle Investigator of the GLP-compliant study and formed the basis for process translation. Whilst the documentation gave a largely comprehensive overview of the process, clarification regarding justification of antimicrobial concentrations and whether new reagent was used for step 18 (Table 2.3) was not provided. Antimicrobial concentrations were maintained as indicated, and a fresh change of reagent provided for step 18.

The SOP indicated that processing was performed within a desiccator, with the lid of the equipment being removed to allow for the exchange of reagents via pipetting. Whilst the desiccator provided a suitable vessel in which to house the tissue in a low pressure environment, the intermittent removal of the lid would prove unsuitable for a clinical manufacturing process. Using such a system, with a significant number of open-processing steps, would require the majority of the process to be performed in a Grade A (ISO Class 5/Class 100) environment with a Grade B (ISO Class 6/Class 1000) background environment (European Commission, 2008). Caveats to processing in this manner include significantly higher manufacturing costs, due to higher facility rental and labour expenditure, as well as an increase in process complexity; typically associated with a greater number of process complications and failures. Subsequently, it was determined that alternative process equipment and methodology were required to enable closed-system manufacture; which in turn would allow processing to be undertaken in a Grade A with a Grade D background, or simply a Grade D environment.

A URS was compiled in order to support the sourcing of a replacement for the desiccator (Table 3.1). Firstly, it was necessary that the vessel would have an internal compartment large enough to accommodate an adult hemilarynx, on average 44 mm in length with a 36 mm antero-posterior diameter in males (Finucane et al., 2010), and 250 mL of reagent. Secondly, the external dimensions must be of a size suitable for comfortable
operation within a 4’ microbiological safety cabinet; preferably small enough to pass in
and out of the cabinet without needing to raise the sash, which in turn results in temporary
disruption of laminar flow. These dimensions were identified as 0.50 x 0.50 x 0.20 m for
the 4’ cabinets used within the CCGTT (Kojair Silver Line, Kojair Tech Oy, Vilppula,
Finland). Following previous operational experience within our group, it was suggested
that a Ricordi® chamber, a vessel developed for the isolation of pancreatic islets, may
be suitable. Measurement of the external dimensions of a 500 mL Ricordi® chamber
confirmed that it would fall within the constraints, as had been indicated in the vessel’s
engineering drawings. Thus user requirement one was satisfied.

User requirements two to four were concerned with ensuring that the replacement vessel
was constructed of materials that were fit for purpose, namely: resistant to degradation
by the decellularisation reagents; suitable for repeated autoclave sterilisation at 137 °C;
and extended operation within a temperature range of -30 °C to 37 °C. Our group
possessed a number of Ricordi® chambers constructed of either PEI or SS303, which
were assessed against the aforementioned criteria. PEI is documented as being largely
inert and has a service temperature of approximately -270 °C to 300 °C, as well as being
listed as suitable for repeated autoclave sterilisation by the manufacturer. Unlike PEI,
stainless steel is susceptible to corrosion by chloride containing solutions and at risk
during the KCl and MgCl₂ containing steps of the decellularisation protocol. It is
documented that austenitic stainless steels, such as SS303, are more resistant to
chloride-mediated corrosion, and thus it was proposed that the minimal contact time,
followed by subsequent washes, would result in a significantly low enough risk such as
to allow processing in a SS303 chamber. As a precaution, it was determined that use of
a SS303 chamber would require regular inspection for corrosion after sterilisation and
processing. Both the autoclave and operational temperature ranges were of little
concern, as SS303 is widely utilised in both cryogenic and high temperature applications.

Investigation of the capacity to maintain a ca. 200 Pa vacuum was undertaken for
assemblies with both the PEI and SS303 chambers. After 72 h, representing the longest
step duration within the protocol (the final wash did not originally consist of any reagent
changes), the PEI assembly had failed to maintain pressure and had instead equalised.
When the vacuum was again applied to the filled chamber, it was observed that
equalisation was occurring through an incomplete seal between the tubing and the barb
on the base of the chamber. This manifested as a stream of small gas bubbles rising
within the chamber contents. This was observed in each of the PEI units within the
laboratory. This was due to the OD of the tubing being too large, clearly abutting the
outer radius on the base of the chamber and causing an incomplete seal. The SS303
units, with barbs oriented perpendicularly to the base of the chamber, had no such issue
and were capable of maintaining a vacuum for 72 h. Subsequently, the PEI units failed to meet user requirement five and so were deemed unsuitable and discounted from future use.

An additional benefit to using the SS303 model included the possession of dual ports on the base of the vessel, which could provide a secondary port should one become blocked. This was proven invaluable during later decellularisation validations, as blockage of the waste port by pieces of tissue was a regular occurrence. It was also noted that the vessel was less likely to be damaged by the robust cleaning process used by the Central Surgical Sterilisation Department at the Royal Free Hospital, who would be responsible for cleaning and sterilising equipment prior to use. The internal surfaces of the chamber were inspected both post-decellularisation and following sterilisation for signs of corrosion. Whilst there were no indications of corrosion at any time, a second Ricordi® chamber was sterilised and stored to provide a back-up; an approach that was recommended for adoption during clinical manufacturing too.

As alluded to in the results section 3.3.1, the principal factor behind adopting a new chamber was the necessity to exchange fluids across the tissue-containing chamber wall in a semi-closed/closed manner. The full assembly of the Ricordi® chamber with accompanying ancillaries, as depicted in Figure 3.3, was proposed as a solution. By fitting the base ports on the chamber with 3WSC terminals it was possible to provide two distinct fluid paths that could be connected to a wide variety of containers, due to the ubiquity of luer connections within the medical and drug manufacturing fields.

The port on the head plate of the Ricordi® chamber, used for pulling the vacuum, was fitted with a 3WSC, two sterile hydrophobic Minisart® 0.2 µm filters and another 3WSC in series. The two filters were to ensure that if any liquid were to be drawn up through the head plate port whilst pulling a vacuum, it would slow its passage to the point that the operator could stop the vacuum pump before drawing liquid into the equipment. The assembly could then be transferred to a microbiological safety cabinet to replace the filters. Whilst the outer 3WSC provided a terminal to which the vacuum pump could be attached, the additional 3WSC was to minimise the risk of pressure equalisation through the series of connections between filters and the terminal 3WSC during steps.

Though many reagents can be sourced in sterile pre-filled containers that are accessible via a luer connection, the specificity of the reagents used within the decellularisation SOP negated this. Instead, a process of aseptically transferring reagents into closed containers with luer access was conceived and validated. By assembling a haematology pump segment in-line with a bag spike with removable injection port, it was possible to fill transfer bags that had been coupled with a spike coupler-luer line using a peristaltic
pump (Figure 3.2). This provided a sterile fluid path that could be used for filling multiple bags of the same reagent with a single assembly, thus greatly increasing throughput and decreasing contamination risk compared to syringe-mediated filling. Where additional reagents were to be added to the transfer bags at a time closer to the decellularisation step, i.e. enzymatic steps, bag spikes with removable injection ports were added to the bags at the time of initial filling. Reagents could then be added, aseptically, by injecting through swabbed ports.

When filling or draining the chamber, reagent containing or empty transfer bags could be simply attached to the base ports via the luer fittings and held above or below the chamber accordingly. Filling and draining in this manner ensured that the chamber pressure did not rapidly fluctuate; reducing the risk of foaming when filling and that of blockage by tissue when draining, which would be probable using a pump.

It is important to highlight that whilst the conceived system represents a great improvement when compared to the opening of the desiccator for reagent exchange, the described operations of bag filling and reagent transfer to/from the Ricordi® chamber are by no means fully closed. There are, however, no guidelines outlined by the EMA or the MHRA clarifying what represents a fully closed system. Instead, the responsibility lies with the manufacturer’s process development team to appropriately minimise the risk of open processing and to indicate the environmental grade for each unit operation. This information is then provided to the MHRA in the IMPD, who take this into consideration when evaluating whether or not to award the CTA. Should contamination issues then arise during manufacture, it is the responsibility of the quality management infrastructure of the manufacturer to perform a root cause analysis of the non-conformance(s) and to take appropriate remedial action.

Acknowledging the contamination risk associated with making a large number of connections using luer fittings in the proposed system, media fill process simulations were performed to validate whether the system could be reliably operated in an aseptic manner. The results indicated that the process could be successfully completed aseptically, even when excessively challenged by performing operations in a Grade A environment with an uncontrolled background environment. Consequently, it was stated in the IMPD that all decellularisation steps would be performed in a Grade A environment within a Grade D laboratory.

An alternative solution, providing even greater reassurance, would have been to introduce sterile welding as a means of connecting transfer bags to the Ricordi® chamber, thus allowing reagent transfer to and from the vessel. This was ruled out during the process development phase, as it would require a significant excess of tubing to be
connected to the chamber to allow for the large number of connections to be made. Another alternative would have been the implementation of aseptic connectors. At the time of process development there was, however, a lack of suitable aseptic connector solutions on the market for handling smaller volumes and processing in the smaller, more spatially limited GMP facilities in which tissue therapies are currently being produced. Moreover, once each single-use connection had been made and reagent transferred, it would have then been necessary to remove the connection using a heat sealer or tube welder. There are now promising signs that suppliers of ATMP manufacturing equipment are addressing this shortcoming, with an aseptic connector that is capable of connecting and reconnecting multiple fluid paths currently under development (patent pending). Realisation of this device would prove highly significant for this process and others, effectively allowing for manufacturing to be accomplished in a Grade D environment with minimal Grade A operations required.

Following the successful demonstration of aseptic operation of the newly described process, a number of changes to the raw materials used in the decellularisation were implemented. Firstly, reagents that were derived from animals at risk of transmitting animal spongiform encephalopathies were replaced. This brought the process into alignment with EU Notice EMA/410/01, and resulted in both DNase and RNase being exchanged.

The RNase that had been used in the GLP-compliant process was a crude mixture of RNase A and RNase I, whereas the replacement was purified recombinant RNase I. This enzyme degrades all RNA dinucleotide bonds to leave a 5’ hydroxyl and a 2’, 3’ cyclic monophosphate, thus theoretically resulting in a more complete degradation than that achieved using the crude mix of specific and non-specific RNases employed in the GLP process. The significance of this on the decellularisation efficacy was deemed low and so was not investigated further.

DNase I derived from bovine pancreas was replaced by a recombinant human DNase I expressed in a Chinese hamster ovary cell line. The enzyme used was a compendium material, dornase alfa, which is marketed as Pulmozyme® in the UK for the management of cystic fibrosis. It was proposed at the time of process development that incorporating materials that had already received marketing authorisations would ensure that the raw materials used had passed suitably stringent quality tests, thus providing reassurance that the highest quality materials were being used for product manufacture. Discussions with the MHRA have since revealed that the current preference is that compendium materials that have not been given explicit permission for use by the manufacturing company should be avoided. This is due to concerns over the potential withdrawal of
provision of compendium materials where permission has not been granted, and the
subsequent implications that this has on the manufacture and provision of medicines.
Whilst this has not proven problematic for manufacturing for the EudraCT: 2013-004359-
18 (RegenVOX II) clinical trial, it would be prudent for the decellularisation to be
assessed for equivalence using a non-drug recombinant human DNase I.

Having introduced the materials alterations and established that the new system was
capable of aseptic operation, the translation was validated by comparing the GLP- and
GMP-compliant processes. Comparison of the efficacy of the GMP-compliant
decellularisation with respect to that reported in the GLP-compliant study was limited to
a small number of characteristics. This was not only a consequence of limited access to
human tissue, but also due to the nature of process validation. Unlike investigative
research, the purpose of process translation and validation is not to further expand
fundamental knowledge of a given topic; instead it is to demonstrate that an altered
process can consistently replicate previously attained results. This is, naturally, more
readily achieved with clearly quantifiable data generated through robust methodologies
than with qualitative approaches that can often be subjective. To this end, only one CQA
was identified as critical to confirming that decellularisation was suitably equivalent
following process translation. Additional characteristics were identified as non-critical
quality attributes, and were assessed to provide greater product characterisation and a
secondary array of comparators. Furthermore, a series of quality control (QC) assays
were introduced to provide a minimal quality profile that must be fulfilled to allow both
release of the decellularised scaffold for recellularisation, and subsequently product
release of the final ATIMP.

A key quantitative indicator of successful decellularisation of tissue is its relative DNA
content, which was highlighted as a CQA to compare the two processes and as a release
criterion for the final product. The immunogenicity of DNA has long been appreciated
and has been indicated as a causative factor for inflammatory responses when
implanting biologically derived scaffolds, correlating with a shift to a pro-inflammatory M1
macrophage phenotype both in vitro and in vivo (Zheng et al., 2005, Keane et al., 2012,
Londono et al., 2017). Guidelines derived from cumulative phenomenological
observations suggest that a reduction to <50 ng dsDNA per mg of dry weight ECM within
biologically derived scaffolds is an indicator that the construct may avoid inducing
adverse host responses in vivo (Crapo et al., 2011).

Analysis of the GMP-compliant decellularised hemilarynges indicated that effective DNA
removal was achieved in the majority of instances, although on one occasion the
normalised DNA concentration exceeded the guideline threshold. This was likely due to
incomplete decellularisation of the cartilaginous component of the tissue, and so a failure to eliminate chondrocytes from the central region of thick cartilage portions such as the thyroid lamina. This was supported by the histological analysis of decellularised tissue cross-sections, which indicated that nuclear material remained present in some centrally positioned lacunae. Sporadically, this was even identifiable as localised nuclei that are indicative of intact cellular structures. The immunological threat that these remaining cells pose is believed to be insignificant due to the dense nature of the cartilage rendering the central portions impenetrable to the majority of immune cells (Smith et al., 2015). For those chondrocytes that do come into contact with the host’s immune system during tissue remodelling, there are data suggesting that they may mediate an immunosuppressive effect and so reduce the likelihood of scaffold morbidity and rejection (Adkisson et al., 2010, Abe et al., 2016, Pereira et al., 2016).

When compared to the DNA removal achieved by the GLP-compliant process, it was evident that the alterations made during translation had no detrimental effect on process efficacy. This fulfilled the single CQA identified for comparison of decellularisation efficacy between the two processes. Subsequently, the threshold proposed by Crapo et al. (2011) was adopted as a release criterion for the final product. Despite having generated data during process development that failed to meet this specification, as well as the previously discussed relevance of DNA as an indicator of potential rejection of cartilaginous constructs, it was necessary to provide a release criterion that could be used to confirm effective decellularisation. This value was adopted in accordance with the general consensus in the literature, as well as its successful use as an effective indicator in the GLP-compliant study. It is proposed that further investigation of the DNA content of cartilage scaffolds and associated immune response, in contrast to the predominantly small-intestine submucosa ECM upon which current guidelines are founded, would greatly benefit this product and tissue engineering in general.

From a technical standpoint, it is also worth considering the methodologies that have been previously employed for DNA quantification both here and in the field more widely. Whilst the column-mediated genomic DNA isolation approach used in this body of research was suitable as a comparator to the GLP-compliant study, which used the same technique; the poor yields that are typically associated with column-based purification assays should be considered when comparing data to those generated using alternative techniques. To address this shortcoming, both column-mediated quantification and direct quantification using fluorescent dye-based methodologies could be employed in future investigations, allowing direct comparison to previously reported data as well as more accurate total DNA quantification.
Non-critical comparators included the relative retention of total collagen and sGAG. The significance of these biomolecules in maintaining the biomechanical integrity of tissue scaffolds is becoming ever more widely appreciated, especially within the field of airway tissue engineering. The correct balance of tensile-strength inferring fibrillar collagens interlinked with hydrated GAG-rich proteoglycans is essential in maintaining a biomechanically sound matrix (Partington et al., 2013, Pauken et al., 2018). Comparison between the respective biomolecular concentrations of native and decellularised tissue can act as a proxy indicator for construct biomechanical integrity. This is particularly valuable for laryngeal tissue engineering, where there is a paucity of physiologically relevant biomechanical tests to assess tissue constructs. From a manufacturing perspective, biomolecular assays that require minimal sample sizes are preferential to those requiring more significant tissue sections for QC assays, such as tensile testing or dynamic mechanical analysis. Furthermore, biomolecular QC assays typically rely on chromogenic absorbance or fluorescence techniques that are more widely available, and thus practical from a manufacturing standpoint, than biomechanical testing.

Comparison of the relative retention of total collagen revealed that the GMP-compliant decellularisation retained a notably greater proportion than that observed in the GLP-compliant study. Mean retention was >96.5% in the novel process, with the largest decrease amongst replicates observed to be 40.63%. This was in stark contrast to the mean loss of approximately 50% of total collagen in the decellularised porcine tissue. It must be emphasised, however, that this does not necessarily indicate that the translated process preferentially retains collagen over the GLP-compliant predecessor. It would require the paired investigation of tissues from the same species, and preferably the same individual organism, for this conclusion to be drawn. The data do, however, suggest the translated decellularisation process does not have a negative effect on total collagen retention with respect to the GLP-compliant process. Whilst total collagen may have been largely retained, it would be beneficial to gain further information regarding the structural architecture of the ECM collagen. This could be achieved using techniques such as scanning electron microscopy (SEM) or sectioning and staining with Picrosirius red. It is also proposed that additional total collagen quantification assays should be employed to confirm the relative contribution of collagen to dried tissue mass. Collagen typically contributes to approximately 60% of dried ECM mass in hyaline cartilage (Sophia Fox et al., 2009), and so the approximate 45% contribution noted in section 3.4.5.2 appears less than expected. This may be due to the nature of the collagen assay employed, which quantifies acid and pepsin-soluble collagen but not covalently bound collagen. By using an alternative method, such as a fluorescent dye-based assay, it may be possible to more accurately quantify total collagen.
Assaying normalised sGAG concentration in the decellularised tissue revealed mean retention to be approximately 70% of that observed in the native sample. Comparatively, the GLP-compliant decellularisation retained 46% and 52%, on average, for muscular and cartilage fractions. This, again, suggested that the translational process had not imparted any detrimental effect on sGAG retention. Interestingly, the 30% loss of GAG observed was comparable to that reported to be associated with the cell membrane (Fishman et al., 2012), and so it may be presumed that a baseline decrease of at least one third of GAG content from decellularised tissue was to be expected. It should be emphasised, however, that the apparent trend of the compiled results may be a misrepresentation resulting from the large loss of sGAG (63%) observed in one of the replicates. Three of the four samples demonstrated an increase in the normalised sGAG concentration, which itself may be explained by the change in relative proportions of constituent components contributing to the mass of the samples being assayed. That is, if one mg of native tissue consists of both ECM and cells then it may be reasonably assumed that one mg of acellular tissue must therefore be comprised of a relatively greater mass of ECM. If a biomolecule, in this instance sGAG, is associated with both the cellular and ECM components then by extension its concentration may increase or decrease dependent on the proportionate association with each of these components. Subsequently, to gain a greater understanding of the sGAG variation following decellularisation would require a greater number of samples and even the introduction of secondary investigatory techniques such as fluorophore-assisted carbohydrate electrophoresis. Again, whilst this may prove useful from a scientific standpoint, it is not necessary for assessment of appropriate translation of the decellularisation process.

In addition to confirming that the GMP-compliant process was capable of consistently decellularising human tissue to a standard akin to that achieved for porcine hemilarynges in the GLP-compliant study, a number of additional assays were introduced to ensure quality standards were achieved during manufacture. Firstly, it was necessary to demonstrate that the scaffold produced was sterile, and so suitable for subsequent recellularisation and eventual release. Testing was performed by the Microbiology Department at the Royal Free Hospital on samples taken before, during and after the decellularisation process. Unsurprisingly, all tissues received were contaminated and could not be sterilised through the application of antibiotics and antimycotics during the first 24 h of the decellularisation. All but one of the tissues remained contaminated throughout the duration of the process, suggesting significant levels of contamination and the potential presence of biofilms. Following further antimicrobial treatment of the HT1 tissue with compounds that the microorganisms had been identified as sensitive to, all bacteria were eradicated from the tissue. The treatment did not, however, successfully eliminate the fungal contamination, despite the strain being identified as susceptible to
the antimycotic amphotericin B. This was likely to be due to the presence of a drug-resistant population of phenotypic variants, which have been identified as capable of persisting and establishing successive biofilms when present within an initial biofilm (LaFleur et al., 2006).

A subsequent decontamination step with the biguanide chlorhexidine digluconate, an antiseptic with proven efficacy against a wide variety of bacteria and fungi alike, was introduced after successful decontamination of porcine hemilarynges had been demonstrated. Despite initially appearing to eliminate viable contaminants from tissues HT3 and HT4, samples were again infected at step 8. It is possible that this was due to the presence of persistor variants within a bacterial biofilm, however it is more likely that the original microbiology results were a false negative caused by the masking effect of remnant antiseptic.

Following the failure to eradicate microbial contaminants using in-house methods, it was determined that gamma-irradiation would suffice as the only suitable method for reliable sterilisation of decellularised scaffolds. Subsequent to irradiation with a dose of 25-40 kGy, the gold standard for terminal sterilisation of medical products (International Atomic Energy Agency, 1990), previously contaminated hemilarynges were confirmed as sterile by the Microbiology Department at the Royal Free Hospital. This asserted that gamma-irradiation sterilisation could provide a reliable means for sterilising decellularised tissue scaffolds prior to recellularisation and eventual product release. These data were in accord with findings reported by Johnson et al. (2017), which demonstrated successful sterilisation of detergent-enzymatic decellularised murine trachea by gamma irradiation.

However, significant degradation of the decellularised cartilage ultrastructure was observed by electron microscopy following irradiation at a standard dose of 25 kGy. Whilst the authors did not undertake biomechanical testing of the irradiated decellularised tissue, they did suggest that the qualitative data generated supported the widely held notion that high levels of gamma irradiation may negatively affect graft viability. In one of the few studies employing physiologically relevant biomechanical evaluation techniques, Uriarte et al. (2014) reported that whole murine decellularised lungs presented raised mechanical impedance when assessed via mechanical ventilation, following gamma irradiation sterilisation. Interestingly, it was concluded that the observed change in biomechanical profile was “not severe” and that the decellularised lung could still be normally ventilated. Despite these data, it is strongly advised that the effects of irradiation on the biomechanical profile of the larynx tissue scaffolds be investigated in the future using physiologically relevant methods. It is possible that irradiation may have short to medium term implications on the ability of the resultant product to maintain a patent airway, and it is the responsibility of the clinical
trial sponsor, manufacturer and ultimately the MHRA to demonstrate that the product remains safe following this amendment to the GLP-compliant process.

Bacterial endotoxin testing was introduced in accordance with the guidelines outlined in the European Pharmacopoeia 2.6.14 (Council of Europe, 2005). Endotoxins are lipopolysaccharides synthesised by gram-negative bacteria and represent the most common pyrogenic contaminant of pharmaceuticals. Testing was only performed following the confirmation of removal of bacterial contamination from the decellularised tissues. Results indicated that both HT1 and HT2 had endotoxin levels lower than the threshold value of 0.5 EU mL\(^{-1}\). HT3 and HT4 were not tested due to the inability to eradicate the known bacterial contamination. There were no difficulties in sampling and outsourcing to the designated test provider, Scottish National Blood Transfusion Service, who were identified as a suitable supplier for the ensuing clinical trial. For clinical manufacture it was noted that due to the relatively short recellularisation period and shelf life of the ATIMP, the turnaround time for endotoxin detection and sterility confirmation would require a two-stage product release. Neither test would form part of the conditional product release, but instead would be included in the final release approximately 14 days later.
3.6 CONCLUSION

The distinct objectives outlined for the successful translation of the GLP- to GMP-compliant process required the development and validation of a process that would enable aseptic decellularisation of human hemilarynges to produce acellular scaffolds comparable to those reported in the GLP-compliant study. A semi-closed/closed system comprised of an off-the-shelf chamber and ancillary fittings, combined with an aseptic method for exchanging reagents, was developed and successfully validated for sterility. Additional QC requirements concerning endotoxin and microbial contamination were introduced into the process to ensure alignment with the regulations outlined for the manufacture of ATIMPs and ATMPs. Having investigated several in-house decontamination techniques, including treatment with antimicrobials and antiseptics, it was concluded that the only reliable method for ensuring product sterility was gold standard gamma-irradiation. Comparison between the acellular scaffolds produced by the two processes confirmed that the alterations implemented to ensure GMP-compliance had no deleterious effect on the product, with respect to the outlined CQAs.

By successfully meeting each of the original objectives, a process for GMP-compliant production of acellular hemilarynx scaffolds was established. To demonstrate that these could then be implemented in the manufacture of ATIMPs, specifically for application in the clinical trial proposed to succeed the GLP-compliant study, would require demonstration that the scaffolds could then be cellularised in a GMP-compliant process.
4 DESIGN, MANUFACTURE AND VALIDATION OF A MODULAR BIOREACTOR FOR CELLULAR CULTURE OF MULTIPLE PRODUCTS FOR AIRWAY ATIMP MANUFACTURE

4.1 INTRODUCTION

During the GLP-compliant study (RegenVOX I – G1001539) the therapeutic intervention under investigation was comprised of two distinct products. The first of these was an acellular, porcine hemilarynx scaffold that was seeded with human BM-MSCs; whilst the second consisted of a split thickness, acellular human dermis that was seeded with human keratinocytes and fibroblasts (Ansari et al., 2017). The interventional strategy dictated that the reseeded laryngeal construct first be embedded into a muscular pocket for one month to promote vascularisation of the product (stage one), before being orthotopically implanted into an artificial, full-thickness defect in the cricothyroid cartilage of each animal (stage two). During this second stage, the cellularised epithelial sheet was sutured to the vascularised luminal side of the laryngeal implant. In vivo assessment was performed via blood and serum analyses, bronchoscopic evaluation, mucosal brushings and vocal recordings; before computed tomography scanning, histology, immunohistochemical analyses and in situ hybridisation for Alu sequence detection were completed following explantation.

The study reported that the implanted products did not produce any clinically adverse effects, however one animal was terminated early due to an ear infection that was worsened by the immunosuppression regimen. Bronchoscopies revealed mildly inflamed, vascularised mucosal surfaces on the implants at two weeks, though it was postulated that the implanted epithelial sheets detached thereafter; indicated by the presence of an accumulation of white material at the anterior commissure in all animals. Staining of brushings failed to identify human epithelia, whilst porcine specific staining indicated the presence of autologous epithelial cells as early as two weeks. This was perhaps unsurprising after the potential detachment of the implanted sheet, and is in accord with reported native re-epithelialisation in other large animal studies (Grevemeyer et al., 2014). Vocalisation recordings of the animals highlighted a general decrease in vocalisation intensity, coupled with an increase in pitch. Anecdotally, vocalisations were hoarse and rough following surgery. Ex vivo assessment of the larynges confirmed the regeneration of the luminal mucosa, macro- and microscopically, although the epithelial sheet could not be identified histologically in any full-term animal. All cells within the contiguous, revascularised, stratified squamous epithelial layer were identified as...
porcine. Moreover, each explant confirmed the presence of vocal folds or “straps” that had regenerated to varying degrees. Assessment of the laryngeal, cartilaginous component revealed that the construct had displaced and relocated from the original implantation site, although this was attributed to the surgical reconstruction approach used. There was evidence that the cartilage was undergoing remodelling, although in one instance ossification characteristics were identified. To gain an improved understanding of the remodelling would require longer studies, preferably in a small animal model with a larger number of animals. Despite the absence of a uniform response across the cohort, the study did achieve its overarching aim of providing a semi-functional tissue engineered hemilaryngeal replacement that maintained airway patency, whilst reinstating swallowing and vocalisation capacity.

Subsequent to the positive results reported in the GLP-compliant study, as well as the demonstration of successful production of acellular human hemilarynx constructs in a GMP-compliant manner (Chapter 2.2), translation of the scaffold seeding and culturing process was required for IMPD completion and CTA application submission. It was determined that the tissue engineered epithelial sheet would not form part of the proposed clinical trial, and that efforts would instead be focused upon the cartilaginous product employed in the GLP-compliant study. The concept of later utilising a tissue engineered epithelium was not completely abandoned however, and instead underwent further process development in preparation for application as a distinct ATIMP.
4.2 AIMS AND OBJECTIVES

4.2.1 Aims

Chapter 4 discusses the design and development of a novel, modular bioreactor system for recapitulating the seeding and culture processes described in the GLP-compliant study, in a manner that would be amenable to small scale clinical manufacture. The aim of the work was to first confirm that the acellular hemilarynx scaffolds generated using the translated, GMP-compliant decellularisation process were capable of supporting the adherence of BM-MSCs until the predetermined product release date. Subsequent work focused on designing and developing a bespoke bioreactor that would fulfil the user requirements outlined for clinical manufacture, having identified the GLP-compliant bioreactor as unsuitable. It would be necessary to demonstrate that the novel bioreactor could be operated in a GMP-compliant manner, and that it did not exhibit a detrimental impact on the cell types with which it was proposed for use. The data produced would again form an integral part of the IMPD for the clinical trial application.

4.2.2 Objectives

- To generate proof-of-concept data demonstrating that human BM-MSCs could successfully adhere to the acellular tissue constructs produced by the GMP-compliant decellularisation process.
- To confirm the presence of the seeded cells on the scaffold after the predetermined clinical cellularisation period of five days.
- To design a bespoke bioreactor system that provides a seeding, culture and delivery device for the hemilarynx product described in the GLP-compliant process.
- To expand system functionality to allow for process development work to be performed in the bioreactor for the development of a tissue engineered epithelial sheet.
- To validate the fabricated system for aseptic operation and demonstrate its suitability for use with the proposed cell types.
4.3 CELL-SCAFFOLD ADHERENCE

4.3.1 Proof-of-concept

To provide proof-of-concept data demonstrating that BM-MSCs could successfully adhere to decellularised hemilarynx tissue that had been produced using the GMP-compliant process, BM-MSCs were stained with PKH26 dye and seeded onto decellularised punch biopsies (n=9; section 2.3.2.1). Staining of BM-MSCs was confirmed by a 2.25-fold increase in fluorescence intensity, as well as by qualitative fluorescence microscopy (data not shown), when seeded onto a two-dimensional tissue culture plastic surface (Figure 4.1). Fluorescence intensity of stained cells seeded onto the scaffolds revealed a significant ($p < 0.0001$) increase in the three-dimensional surface model relative to the unstained, unseeded control, as well as both the stained and unstained two-dimensional conditions (Figure 4.1). Non-adhered cells that were washed from the surface of the scaffolds also demonstrated a significant ($p < 0.0001$) reduction in fluorescence intensity, compared to their scaffold counterpart.

![Figure 4.1](image-url)

Figure 4.1 Decellularised scaffold adherence proof of concept. MSC staining with PKH26 was confirmed by a 2.25-fold increase in fluorescence intensity, when assayed on a 2D substrate (tissue culture plastic; n=9). Stained MSCs seeded onto decellularised larynx scaffolds (3D) resulted in a significant ($p < 0.0001$) increase in fluorescence intensity relative to the unstained control and both 2D conditions. Non-adhered cells that had been washed from the scaffolds (3D*) also exhibited a significant decrease in fluorescence intensity relative to the respective scaffolds with adhered MSCs.
4.3.2 Attachment time

Investigation of attachment time of PKH26 labelled BM-MSCs seeded onto decellularised scaffolds (section 2.3.2.2) revealed no significant difference in the fluorescence intensity of seeded scaffolds or non-adhered BM-MSCs across the three time points: 4 h, 8 h and 24 h (n=6; Figure 4.2). There was a significant difference ($p < 0.05$) in fluorescence intensity between the seeded scaffold and non-adhered cells at the 8 h time point, as well as significant differences ($p < 0.01$) at the 4 h and 24 h time points.

![Figure 4.2 Cell-scaffold adherence time-course. PKH26 stained MSCs seeded onto decellularised larynx scaffolds, before non-adhered cells were washed off, revealed no statistically significant difference in the fluorescence intensity of either the adhered MSCs on the scaffold or non-adhered cells in the supernatant. There was a significant difference ($p < 0.05$) between the fluorescence exhibited by the seeded scaffold and supernatant at 8 h post-seeding. Significant differences ($p < 0.01$) were observed at the 4 h and 24 h time points.](image-url)
4.3.3 BM-MSC persistence

Investigation of whether metabolically active BM-MSCs persisted on the decellularised scaffolds (section 2.3.2.3) indicated a viable population remained up to 5 d (n=6; Figure 4.3). There was an observable increase in fluorescence intensity, an indicator of total population metabolic activity, between 4 h post-seeding (0 d) and both 1-5 d post-seeding.

![Fluorescence intensity vs. Adherence Time](image)

Figure 4.3 Metabolically active MSC persistence. Fluorescence intensity, representing total population metabolic activity, increased in MSC seeded decellularised larynx scaffold samples between 0 d and 5 d post-seeding (n=6). There was a notable increase in fluorescence intensity between 0 d and 1-5 d.

Subsequent quantification of the DNA content of the seeded scaffolds (section 2.3.2.3) revealed a significant ($p < 0.0001$) decrease in normalised DNA concentration between 0 d and 1 d, 0 d and 3 d, as well as 0 d and 5 d ($p < 0.001$; Figure 4.4). A non-statistically significant increase in DNA concentration was observed between 1 d, 3 d and subsequently 5 d.

SEM provided a qualitative affirmation of the presence of seeded BM-MSCs on the surface of the decellularised scaffolds at 4 h, 1 d, 3 d and 5 d post-seeding (Figure 4.5; section 2.1.4.3). Whilst the surface of the unseeded scaffolds were evidently devoid of human cells, there did appear to be a small contamination with yeast cells that were sporadically identified on the tissue surface (Figure 4.5 (B)).
Figure 4.4 DNA content of MSC seeded scaffolds. Quantification of the DNA concentration of the MSC seeded scaffolds indicated a significant difference ($p < 0.0001$) between 0 d and 1 d, as well as 0 d and 3 d post-seeding. The decrease between 0 d and 5 d was also significant ($p < 0.001$). DNA concentration did appear to increase from 1 d through to 5 d post-seeding.
Figure 4.5 Scanning electron photomicrographs of seeded decellularised larynx. Photomicrographs were taken at X40 and X320 magnification, respectively, for unseeded scaffolds (A, B), as well as at 4 h (C, D), 1 d (E, F), 3 d (G, H) and 5 d (I, J) post-seeding. MSCs were clearly identifiable in all but the negative control photomicrographs. Yeast were identified on the unseeded scaffolds at X320 magnification (B, arrow). Scale bars represent 250 µm and 50 µm for X40 and X320 magnification, respectively.
4.4 DESIGN OF A BESPOKE, MODULAR BIOREACTOR FOR SCAFFOLD RECELLULARISATION

4.4.1 User requirement specification and design specification

The seeding and recellularisation of decellularised scaffolds undertaken during the GLP-compliant process was performed in a custom-built bioreactor that had been developed for the study (Figure 4.6 (A)). The bioreactor was constructed of polymethyl methacrylate (PMMA) sheet that had been cut to size and bonded with adhesive to form a cuboidal chamber large enough to house the tissue that was to be recellularised. Two PMMA strips were bonded to opposing, internal lateral surfaces of the chamber; abutting the base of the chamber, such that they formed parallel shelves (Figure 4.6 (B, arrows)). These provided surfaces upon which a stainless steel mesh was placed to support the scaffold inside the bioreactor (not shown). Two clearance holes were located on the opposing chamber walls that were orthogonal to the shelves. One hole was located in the proximal third of the chamber wall, relative to the chamber base, and one in the distal third (Figure 4.6 (C and D)). The proximal hole was positioned such that liquid within the chamber would drain to just below the surface of a scaffold placed within the bioreactor. If allowed to freely drain on a flat surface, the chamber would not fully empty; thus preventing the internal environment from drying out if the proximal fluid path was inadvertently left open. Each port was bonded, externally, with a threaded cylinder of PMMA that would enable the connection of a threaded luer connector and subsequent 3WSC to control the inflow and efflux of medium. An O-ring was located on the outer surface of the chamber body that mated with the head plate, which itself had a centrally positioned clearance hole and threaded PMMA cylinder to fit a connector allowing for gas exchange. The seal between the body and the head plate was achieved by locating the whole chamber in an external chassis that had a clamping plate, which applied a force to the two parts (Figure 4.6 (A)).
Figure 4.6 GLP-compliant process recellularisation bioreactor. The chamber body and head plate were constructed of bonded PMMA sheet (A). The body was located within a chassis that facilitated compression and sealing of the head plate against the chamber body. Internally the chamber had a shelf feature (B, arrows) on which tissue could be supported. Lateral ports allowed for the attachment of fittings and exchange of medium (C, D).
The bioreactor was able to house the tissue construct in a closed system, allowing independent influx and efflux paths for medium as well as a port for gas exchange. It had the additional benefit of being constructed of transparent PMMA, thus allowing for non-invasive assessment of media volume and sterility. There were, however, a number of caveats that rendered it unsuitable for GMP-compliant manufacturing. Firstly, it was not suitable for in-house cleaning and sterilisation by the Central Surgical Sterilisation Department at the Royal Free Hospital. This was due to the use of an adhesive that could not withstand the pressure and temperature experienced during this process, as well as the PMMA itself having a heat deflection temperature (HDT) of ca. 106 °C (UL, 2018a). Secondly, the secondary bonding of features, internally and externally, resulted in a series of rough surfaces and joints that were at greater risk of harbouring contamination sources. Thirdly, the depth of the tissue once located in situ in the bioreactor required the operator to pass the pipette deep into the chamber whilst seeding, thus increasing the risk of contamination. This was also a risk when moving the tissue in or out of the chamber. Finally, the bioreactor was not particularly user friendly; with removal of the clamping plate required to open the lid, the bioreactor body was awkwardly located in a minor recess in the chassis that was difficult to appropriately align. The full assembly was also excessively heavy due to the use of steel and high pressure laminate (Trespa International, Weert, Netherlands) in the chassis, and possessed a number of sharp edges that put both the operator and surrounding consumables and equipment at risk of damage.

To address the shortcomings of the existing chamber, and to produce a bioreactor that was suitable for GMP-compliant ATIMP production for the proposed clinical trial, a bioreactor URS was compiled. It was proposed that a single bioreactor be designed to accommodate both TEP types employed in the GLP-compliant study; a decellularised hemilarynx and a tissue engineered epithelial sheet. The URS was subdivided accordingly (Table 4.1), and formed the basis for the subsequent DS (Table 4.2).

In addition to the requirements outlined in the URS and DS, stringent timeline constraints were introduced. A 12 month target date was recommended for completion of design, fabrication and validation of the novel recellularisation bioreactor, to align with the manufacture and surgery dates anticipated for the first patient in the clinical trial, pending the award of the CTA.
Table 4.1 URS for GMP-compliant recellularisation bioreactor

<table>
<thead>
<tr>
<th>UR#</th>
<th>User Requirement – Generic</th>
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<tbody>
<tr>
<td>1</td>
<td>The bioreactor should be suitable for both larynx and epithelial sheet recellularisation.</td>
</tr>
<tr>
<td>2</td>
<td>Constructed from biocompatible materials.</td>
</tr>
<tr>
<td>3</td>
<td>Constructed of materials that can be repeatedly cleaned and autoclaved at 137 °C without degradation or deformation.</td>
</tr>
<tr>
<td>4</td>
<td>Constructed of materials that can be suitably operated at 37 ± 0.5 °C, without degradation or deformation.</td>
</tr>
<tr>
<td>5</td>
<td>Constructed of inert materials that are resistant to degradation by those reagents used in the recellularisation process.</td>
</tr>
<tr>
<td>6</td>
<td>Minimise risk of contamination during cell seeding and product delivery.</td>
</tr>
<tr>
<td>7</td>
<td>Allow for the exchange of liquid across the internal-external wall in a semi-closed/closed* manner.</td>
</tr>
<tr>
<td>8</td>
<td>Single-use functionality.</td>
</tr>
<tr>
<td>9</td>
<td>Function as both the recellularisation equipment and the primary packaging for the final product.</td>
</tr>
<tr>
<td>10</td>
<td>Suitable for user-friendly product removal from packaging upon receipt at the clinical site.</td>
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<table>
<thead>
<tr>
<th>User Requirement – Larynx</th>
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<tbody>
<tr>
<td>11</td>
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<td>12</td>
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<table>
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<tr>
<th>User Requirement – Epithelial sheet</th>
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<td>13</td>
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<td>14</td>
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<td>15</td>
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* semi-closed defines a process or action that, whilst not entirely closed, has been deemed significantly low risk so as to allow processing in a Grade A environment with a Grade D background.
Table 4.2: Design specification for a novel, GMP-compliant recellularisation bioreactor for multiple airway ATIMPs

<table>
<thead>
<tr>
<th>UR#</th>
<th>DS#</th>
<th>Design Specification</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>1.1</td>
<td>A modular approach should be applied, whereby a main chamber body can accommodate independent cassettes with product specific functionality.</td>
</tr>
<tr>
<td>2</td>
<td>2.1</td>
<td>The materials that come into contact with the starting material or final product, as well as those which contact the supporting medium, must have proven examples of biocompatible application with human BM-MSCs and human epithelial cells.</td>
</tr>
<tr>
<td>2</td>
<td>2.2</td>
<td>The final product should be demonstrated to be free of leachables that negatively impact the viability of human BM-MSCs or human epithelial cells.</td>
</tr>
<tr>
<td>2</td>
<td>2.3</td>
<td>The final product should be demonstrated to be free of leachables that negatively impact the viability and maturation of human epithelial cells.</td>
</tr>
<tr>
<td>3</td>
<td>3.1</td>
<td>The materials used for construction must have a HDT/deflection temperature under load (DTUL) of &gt;137 °C.</td>
</tr>
<tr>
<td>4</td>
<td>4.1</td>
<td>Satisfied by DS#3.1</td>
</tr>
<tr>
<td>5</td>
<td>5.1</td>
<td>The materials used for construction should have proven application with media used for culturing human cells.</td>
</tr>
<tr>
<td>6</td>
<td>6.1</td>
<td>The modular cassettes should allow for seeding outside of the bioreactor main body, with a low contamination risk associated with the method of return back to the main body.</td>
</tr>
<tr>
<td>6</td>
<td>6.2</td>
<td>Alternatively, if seeding constructs whilst located inside the bioreactor, cassettes should position tissue scaffolds such that they do not require excessive movement of pipettes inside the main chamber body.</td>
</tr>
<tr>
<td>7</td>
<td>7.1</td>
<td>The bioreactor must enable fluid transfer via accommodation/incorporation of luer fittings.</td>
</tr>
<tr>
<td>UR#</td>
<td>DS#</td>
<td>Design Specification</td>
</tr>
<tr>
<td>-----</td>
<td>-----</td>
<td>----------------------</td>
</tr>
<tr>
<td>8</td>
<td>8.1</td>
<td>The bioreactor, including ancillaries, must cost less than £2500.</td>
</tr>
<tr>
<td>9</td>
<td>9.1</td>
<td>The bioreactor assembly must possess external dimensions of less than 33.0 cm X 16.5 cm X 31.0 cm, to align with the existing transportation packaging in the CCGTT.</td>
</tr>
<tr>
<td></td>
<td>9.2</td>
<td>The bioreactor must be suitable for affixing a primary packaging label to.</td>
</tr>
<tr>
<td></td>
<td>9.3</td>
<td>The bioreactor must be capable of being fully closed, such that it does not leak in transit if positioned &gt;90 ° (any axis) beyond the normal operating position.</td>
</tr>
<tr>
<td></td>
<td>9.4</td>
<td>The bioreactor must possess a means of non-invasively observing the media within, as an indicator of sterility.</td>
</tr>
<tr>
<td>10</td>
<td>10.1</td>
<td>It must be possible to remove the respective cassettes, or product, from the bioreactor using forceps, in an aseptic manner.</td>
</tr>
<tr>
<td>11</td>
<td>11.1</td>
<td>Internal dimensions must be greater than 44 mm x 36 mm x 18 mm.</td>
</tr>
<tr>
<td>12</td>
<td>12.1</td>
<td>The scaffold must be supported to allow medium to contact the majority of tissue surfaces.</td>
</tr>
<tr>
<td>13</td>
<td>13.1</td>
<td>The cassette module must be capable of circumferential application of pressure about a central area that is exposed for cell culture.</td>
</tr>
<tr>
<td>14</td>
<td>14.1</td>
<td>The central area of the envisaged cassette module exposed for cell culture must be greater than 30 mm x 20 mm.</td>
</tr>
<tr>
<td>15</td>
<td>15.1</td>
<td>The cassette module must orient the epithelial sheet such that both surfaces can be exposed to medium, before medium is removed from one side and replaced with air.</td>
</tr>
</tbody>
</table>
4.4.2 Conceptualisation and design

Following the relative functional success of the bioreactor employed for recellularisation during the GLP-compliant process, it was conceived that the novel bioreactor should be founded upon the basic characteristics of the GLP-compliant chamber. These included a shelf-like system for supporting the cassette modules outlined in the DS (DS#1.1, Table 4.2), lateral ports to facilitate media transfer, and a port for gas exchange. It was also highlighted that alignment of the GMP-compliant bioreactor design with that of the GLP-compliant process would provide greater continuity across the two processes, which would be preferable from the perspective of the regulatory assessors of the IMPD.

To avoid the use of adhesives required to bond materials together, it was proposed that the novel bioreactor be manufactured using computer numerical control (CNC) machining to fabricate the individual parts from single polymer sheets. This manufacturing approach would provide a wide range of engineering polymers from which a suitable material could be selected. These materials typically have well characterised property profiles that can prove beneficial for both the end user and also the fabricator, who can identify a polymer proxy with which they have previous experience, and so can model their machining strategy upon. This is an important quality assurance consideration that is often overlooked by designers, and was to be addressed by working closely with the manufacturer. By adopting machining as the fabrication method, it would also be possible to produce small to medium batch volumes in both a cost and time effective manner. The production volumes required for process development, and subsequent clinical processing, were well within the capabilities of typical machining manufacturers.

The bioreactor used in the GLP-compliant study was fabricated by the UCL Biochemical Engineering Mechanical Engineering Workshop. Following positive feedback on the diligence and flexibility of the design-manufacture process experience, and in light of the benefits conferred by having manufactured the previous bioreactor and thus being aware of the scope and constraints of the project, the Biochemical Engineering Mechanical Engineering Workshop were engaged for fabricating the novel bioreactor.

4.4.2.1 Material selection

A wide range of materials have been reported for the construction of tissue engineering bioreactors, both in the literature and on the market. These include PC (Kalyanaraman and Boyce, 2007, Harvard Apparatus, 2013, SKE Research Equipment, 2014b), PMMA (Song et al., 2012), polyether ether ketone (PEEK) (Harvard Apparatus, 2013), polysulfone (Wendt et al., 2003, Macchiarini et al., 2008, Harvard Apparatus, N/A), PTFE
(Wendt et al., 2003, Macchiarini et al., 2008, Harvard Apparatus, N/A), silicone (SKE Research Equipment, 2014a, SKE Research Equipment, 2014b), and stainless steel (Ott et al., 2010, Harvard Apparatus, N/A). Discussions were held with the Biochemical Engineering Mechanical Engineering Workshop Manager to enquire whether the Workshop had experience fabricating with these materials. Based on previous fabrication experience, the following candidate materials were proposed: polyamide (PA), polycarbonate (PC), polyether ether ketone (PEEK) and PTFE. Each polymer was assessed against the material specific requirements outlined in the DS. The results of this are outlined in Table 4.3.

Reviewing the contemporary literature provided evidence that suggested each proposed material had both positive and negative attributes contributing to its suitability profile. There was clear evidence that PC and PTFE were biocompatible with both epithelial cell and BM-MSC populations (Table 4.3), whilst there was an absence of published data concerning interactions between each cell type and PA/PEEK. Whilst this did not prevent the application of PA/PEEK, with the final materials due to undergo in-house biocompatibility investigations, it preferentially favoured selection of PC and/or PTFE.

HDT/DTUL values were compared across the materials to provide a proxy indicator of whether subsequently constructed bioreactors could be repeatedly cleaned and autoclaved at 137 °C without degradation or deformation. PA and PEEK both possess HDT/DTUL values in excess of the 137 °C threshold, whilst the PC value was borderline. PTFE is a very soft fluoropolymer and has an exceptionally low HDT (Table 4.3). Upon further reflection, it was proposed that HDT/DTUL may not represent a suitable indicator of whether a material may be repeatedly cleaned and autoclaved without a detrimental effect on the respective part. It did not take into account the effect of design aspects such as nominal thickness or minimum feature resolution or material characteristics such as brittleness, which could also render a material unsuitable. The Workshop Manager suggested, from previous experience, that each material would be suitably resistant to thermal deformation during autoclaving as long as there were no particularly thin features within the design. It was highlighted, however, that PC may experience a loss of transparency and increase in brittleness with repeated exposure to high temperatures. It was subsequently determined that PC would be used to provide a transparent viewing port through which the bioreactor contents could be non-invasively monitored, rather than being used to construct the chamber body.

Finally, the Workshop Manager indicated whether or not he believed a bioreactor could be constructed from each material for <£2500. Whilst it was not possible to provide an accurate approximation of cost without any additional information on the design or total
number of units to be produced, it was possible to rule-out the use of PEEK based on anecdotal raw material costs. It was ventured that the other candidate materials could fall within the pre-determined cost range.

With PEEK having been withdrawn due to cost, PTFE and PC were selected for part fabrication in the first instance. Although PA was indicated to be more cost effective, PTFE was selected to be the primary material choice for producing the chamber body and inserts based upon its proven biocompatibility record (Table 4.3).

Silicone (shore 70) was selected as the material of choice for sourced O-rings. This was based on proven biocompatible use, as well as the identification of a supplier that could readily provide units manufactured from USP Class VI compliant raw materials. Silicone also meets the temperature requirements outlined in the DS, with an operating temperature range of -100 °C to 260 °C (Hooper, N/A).

The following sections describe the conception, design, and drafting of each of the individual bioreactor parts. Whilst this design process was highly fluid, resulting from the progression of all parts simultaneously, each has been discussed individually hereafter to ensure a full, but concise description can be effectively provided and understood.
<table>
<thead>
<tr>
<th>DS# and Summary</th>
<th>Candidate Material</th>
<th>Supporting Evidence</th>
<th>Suitability (Pass/Indeterminate/Fail)</th>
</tr>
</thead>
</table>
| 2.1/5.1; Proven *in vitro* biocompatibility with human BM-MSCs and human epithelial cells. | PA | • Endometrial MSCs shown to proliferate on PA 6 meshes (Su et al., 2014).  
• Viable BM-MSCs observed on flocked PA scaffolds up to 28 d (Steck et al., 2010). | No evidence of application of PA with epithelial cells at the time of material investigation. Given general acceptance of biocompatibility within the field, PA may be a suitable fabrication material. |
|                  | PC | • BM-MSCs adhere to and proliferate on PC, demonstrating apoptotic levels and cytoskeletal morphology similar to tissue culture plastic cells at 7 d and 28 d, respectively (Wang et al., 2012).  
• Corneal epithelial cells expanded on PC membranes and formed tight junctions at 10 d (Feng et al., 2012). | Clear evidence of biocompatibility for both BM-MSCs and epithelial cells, therefore deemed appropriate for construction. |
<p>|                  | PEEK | • Adipose tissue derived MSCs adhere to and proliferated on PEEK up to 28 d. Cells were capable of adipogenesis and osteogenesis (Waser-Althaus et al., 2014). | No evidence of application of PEEK with epithelial cells at the time of material investigation. Given general acceptance of biocompatibility within the field, PEEK may be a suitable fabrication material. |</p>
<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM-MSCs</td>
<td>Adhered to PTFE patches, demonstrating viability at 7 d (Hodonsky et al., 2015).</td>
<td>Biocompatibility with both target cell types, and so suitability, confirmed.</td>
</tr>
<tr>
<td>UC-MSCs</td>
<td>Adhere and proliferate on PTFE, maintaining high viability at 72 h (Hollweck et al., 2010).</td>
<td></td>
</tr>
<tr>
<td>Retinal epithelial cells</td>
<td>Adhere to and proliferate on plasma-treated PTFE up to 15 d. Cell-cell tight junctions were observed, indicative of a mature epithelial phenotype (Krishna et al., 2011).</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material</th>
<th>HDT Temperature</th>
<th>Achievability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PA</strong></td>
<td>200 °C (UL, 2018b)</td>
<td>Pass</td>
</tr>
<tr>
<td><strong>PC</strong></td>
<td>127 °C (General Electric)</td>
<td>Indeterminate</td>
</tr>
<tr>
<td><strong>PEEK</strong></td>
<td>152 °C (Vicrex, 2018)</td>
<td>Pass</td>
</tr>
<tr>
<td><strong>PTFE</strong></td>
<td>45 °C (SpecialChem, 2018)</td>
<td>Fail</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material</th>
<th>Total Bioreactor Cost</th>
<th>Achievability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PA</strong></td>
<td>Achievable (Herrmann, 2014)</td>
<td>Pass</td>
</tr>
<tr>
<td><strong>PC</strong></td>
<td>Achievable (Herrmann, 2014)</td>
<td>Pass</td>
</tr>
<tr>
<td><strong>PEEK</strong></td>
<td>Unachievable (Herrmann, 2014)</td>
<td>Fail</td>
</tr>
<tr>
<td><strong>PTFE</strong></td>
<td>Achievable (Herrmann, 2014)</td>
<td>Pass</td>
</tr>
</tbody>
</table>
4.4.2.2 Chamber body

The initial concept design proposed for the chamber body maintained the aforementioned features present in the GLP-compliant bioreactor. It was conceived that the parallel shelves previously described could be extended to all four internal faces of the chamber body to provide a circumferential ledge (Figure 4.7). This could provide a mating face against which a seal could be formed by an inserted cassette, with gasket or O-ring, thus creating two independent compartments within the chamber body. Additional clearance holes were located on opposing lateral sides of the bioreactor, such that both the upper and lower compartments had both an influx and efflux path. These holes were located on the midline of the lateral faces, to provide symmetric flow distribution through each compartment. All holes were to be tapped within the chamber walls rather than having externally protruding features, which were at a greater risk of being broken due to feature weakness. This would also reduce the footprint of the bioreactor, in line with DS#9.1.

It was indicated by the Workshop that PTFE sheet could be provided for fabrication in incremental depths up to 50 mm. Based upon the lateral depth of an adult male hemilarynx it was assessed that a sheet depth of 50 mm would be adequate. To comfortably accommodate the maximum tissue dimensions, an internal working area in the XY plane was set at 80.00 mm x 60.00 mm (Figure 4.7). The internal, circumferential ledge was drafted at 7.50 mm depth in the X and Y axes to provide sufficient surface area for an inserted cassette to rest upon. Moreover, it was large enough for a gland accommodating O-rings with standard cross-sections up to 3.53 mm.

The depth from the head plate mating face of the reactor body to the ledge was 29.00 mm. This depth was the greatest that could be achieved with the existing tooling in the Workshop, without unnecessarily high risk of deviating from the programmed cutting path during manufacture. Although it was possible to purchase additional cutters that could achieve a greater maximum machining depth, and so allow for the production of a shelled single-piece chamber body, this would have been detrimental to the fabrication schedule. As a secondary consideration, using a larger cutter would also have resulted in larger internal radii, which may have subsequently required an increase in the internal X and Y axes dimensions to maintain the same working area. It was determined that a cutter with a 4.00 mm radius and 29.00 mm cutting depth would be adequate for accommodating the maximum anticipated tissue depth of 18 mm. The chamber body was machined from the opposing face to produce clearance and form the lower portion of the internal chamber. This void would hold medium during culture of both larynx and epithelial sheet constructs, and would be terminated with an affixed base plate (section 4.4.2.4).
O-rings were proposed as an adequate means of effectively sealing the chamber body when affixing the base and head plates. They were preferred to gaskets, being more readily available and cost effective. A BS154 O-ring was identified as being large enough to encompass the opening in the top of the chamber body, and a corresponding gland was machined at a 5.00 mm offset. The gland width was 2.62 mm and depth 2.20 mm. It should be noted that the gland dimensions were trialled in an offcut prior to final machining, to ensure an appropriate fit.

The outer dimensions of the chamber body were then machined to 120.00 mm x 100.00 mm, with 4.00 radii on the Z-axis vertices. This allowed suitable clearance between the gland feature and outer edge for machining holes for affixing the base and head plates, which were subsequently centred along an 8.00 mm offset from the outer perimeter. Eight holes were machined in the Z-axis, two on each side of the chamber body. Holes were positioned 25.00 mm and 20.00 mm offset from the midline of the 120.00 mm and 100.00 mm sides, respectively, to ensure an even application of force could be applied between the base/head plate and chamber body. Each hole was tapped with an M4 x 0.7 thread to 10.00 mm. The O-ring gland and threaded holes were symmetrically reciprocated on the base and head plate abutting faces.

Holes for luer connections were cut and tapped on the front and back of the chamber body with a BSP 1/8" G thread to a depth of 9.0 mm, before drilling to clearance at 4.00 mm. The hole cut to provide access to the lower portion of the internal chamber was centred 7.00 mm from the base of the chamber body, on the midline. This was to ensure adequate clearance of the luer connector from the base plane during assembly and operation.
Figure 4.7 GMP-compliant recellularisation bioreactor body drafted design.

Dimension units: mm
4.4.2.3 Cassette insert for larynx

Design of the cassette insert for seeding and maintaining the larynx scaffold during recellularisation was influenced by DS#6.1, DS#6.2, DS#10.1 and DS#12.1. Early conceptual ideas were proposed, whereby the tissue scaffold could be held suspended within the bioreactor by a clamp-like assembly, thus ensuring that medium could fully cover both the luminal and abluminal surfaces of the construct. It was noted, however, that this was likely to cause damage to the tissue during the clamping process, whilst also expected to be overly complex and unamenable to simple removal of the tissue from the bioreactor by the end-user (DS#10.1). It was determined that to satisfy each DS, whilst placing a strong focus on user-friendly operation for both the production scientist and the ATIMP receiving clinician, a strong focus on functional simplicity was to be adopted.

Applying this approach, a perforated cassette plate was designed to support the tissue (Figure 4.8). This functioned on the same principles as the mesh support used during the GLP-compliant study, providing a high density of pores through which medium could pass and contact the scaffold. The perforated area was matched to the dimensions of the lower portion of the internal chamber, 65.00 mm x 45.00 mm. Thickness was constrained to a minimum of 3.00 mm by the material softness and fabrication technique. Walls were extended in the Z-axis to 29.00 mm on the 60.00 mm edge (Figure 4.8), thus allowing inflow and efflux from the chamber body ports when the cassette is located in situ. Matching the depth from head plate to ledge ensured that the cassette would be located flush against the chamber body ledge when the head plate was affixed, whilst simultaneously providing an extended body that could be held to insert and remove the cassette from the chamber body in accordance with DS#6.1 (Figure 2.2 (I)). Lugs were machined and fitted to aid this process (Figure 4.8). The cassette was machined to produce a sliding, clearance fit when inserted into the chamber body.
Figure 4.8 GMP-compliant recellularisation bioreactor larynx cassette design.

Dimension units: mm
4.4.2.4 Base plate and head plate

Following the adoption of the clearance design for the chamber body (section 4.4.2.2), a base plate was designed (Figure 4.9). Clearance holes were machined with a diameter of 4.20 mm and a 7.20 mm diameter countersunk to 4.50 mm, to accommodate M4 x 12 mm (DIN 912) socket cap screws. This would ensure that, once affixed, the bioreactor would position flush against any flat surface upon which it was placed. Without incorporating a countersink it could be possible that the bioreactor would not rest level upon a surface, due to differential tightening and thus positioning of screws in the Z-axis. Holes were positioned to align with the corresponding holes on the chamber body, and parts were fabricated from both PC and PTFE.

The head plate was designed and machined to mirror the base plate, without the countersink (Figure 4.10). The lack of countersink provided a surface against which the M4 x 12 mm knurled thumb screws could abut when affixing the head plate to the chamber body. Additionally, a clearance hole was cut in the Z axis at the plate’s centre point and tapped to form a BSP 1/8” G thread; providing what would be the port for gas exchange. The head plate was machined from PC, thus providing a means of non-invasively observing the bioreactor’s contents during operation (DS#9.4).
Figure 4.9 GMP-compliant recellularisation bioreactor base plate.

Dimension units: mm
Figure 4.10 GMP-compliant recellularisation bioreactor head plate.

Dimension units: mm
4.4.2.5 Cassette insert for epithelial sheet

The cassette insert for seeding and culturing an epithelial sheet was designed by modifying the cassette that had been produced to support the larynx. It was ventured that maintaining a degree of similarity between the designs would facilitate operator familiarity and increase functional ease-of-use.

The perforated surface of the cassette insert for the larynx was replaced by a 50.00 mm x 25.00 mm clearance through the cassette (DS#14.1; Figure 4.11 and Figure 4.12). The cassette was split in the Z plane to consist of two parts that could hold the epithelial sheet in situ, preventing the sheet from curling/retracting by locating and clamping it between the mating faces of the two parts. An O-ring between the mating faces was proposed as a means of evenly applying pressure about the centrally exposed culture area (DS#13.1), whilst minimising the risk of the construct tearing. An appropriately sized O-ring that would encompass the culture area was identified (BS032) and the reciprocating gland machined at a 2.00 mm offset to 1.50 mm depth into Pt2 of the cassette (Figure 4.12).

To create distinct compartments within the chamber body, and thus fulfil the requirement of having a liquid-gas differential on either side of the mucosal sheet (DS#15.1), the design of the cassette incorporated an additional O-ring to form a seal between the assembled cassette and the ledge upon which it was supported. It was essential for the O-ring to be located between the outer perimeter of the cassette and that of the internal O-ring gland located in Pt2. Moreover, it was necessary to locate the screws that would clamp the two parts together between these two features, thus forming a complete seal between the two compartments. A BS041 was selected and the corresponding gland machined on the bottom face of Pt2 at a 2.00 mm offset from the cassette outer perimeter, with a width of 1.80 mm and depth of 1.50 mm (Figure 4.12).

Ten, 3.00 mm, clearance holes with 5.50 mm x 3.50 mm countersinks were located and machined into the bottom surface of Pt2. It was proposed that a large number of fittings would ensure a strong, but even, compression of the mating faces and thus epithelial sheet. Corresponding holes were machined into the bottom of Pt1, before being tapped with a M2.5 x 0.45 thread to 7.00 mm depth. As with the cassette insert for larynx, the cassette was machined to a produce a sliding, clearance fit when inserted into the chamber body.
Figure 4.11 GMP-compliant recellularisation bioreactor epithelial cassette Pt1 design.

Dimension units: mm
Figure 4.12 GMP-compliant recellularisation bioreactor epithelial cassette Pt2 design.

Dimension units: mm.
A secondary cassette insert for seeding and culturing epithelium was designed for process development work (Figure 4.13 and Figure 4.14). Founded on the same mechanism and principles as the original epithelial cassette, it instead had three distinct clearances through the cassette in the Z-axis. This would allow for the production of smaller cell seeded surface areas, in triplicate. It was proposed that scaling-down the cell seeding area required less substrate; a significant benefit given the complications experienced in procurement of acellular dermal matrix.

By working within the geometric constraints imposed by maintaining the position of the clearance holes in Pt2, each clearance for culturing epithelium was machined to 25.00 mm x 10.00 mm. BS021 O-rings were identified as appropriate for encompassing the clearance holes, whilst also allowing an appropriate clearance between each hole’s gland. Each gland was designed and machined at a 2.00 mm offset, with a width of 1.80 mm and depth of 1.50 mm.
Figure 4.13 GMP-compliant recellularisation bioreactor epithelial cassette Pt1.1 design.

Dimension units: mm
Figure 4.14 GMP-compliant recellularisation bioreactor epithelial cassette Pt2.1 design.

Dimension units: mm
4.4.3 Fabrication

All drafted designs were assessed for fabrication feasibility by a member of the Workshop prior to final submission of the designs. As an aid to understanding how the parts would interact with one another, 3D models were rendered using Fusion 360 software package (Autodesk). Assembled and exploded views of the chamber body with larynx cassette, base and head plates, plus O-rings and fittings are depicted in Figure 4.16. Equivalent views are also shown for both epithelial cassette assemblies.

Appropriate quantities of raw materials were purchased for the construction of six, full, bioreactor assemblies. Fabrication of all parts was successfully completed and fit checked internally by the Workshop. Figure 4.17 demonstrates a fully assembled bioreactor with larynx cassette, in addition to each of the individual cassettes.

4.4.4 Sterility validation

To validate the sterility of bioreactor assembly and filling, sterilised bioreactors were assembled and filled with TSB (section 2.3.3.3). A complete absence of sample turbidity was noted following incubation, indicating that the bioreactors could be aseptically assembled and filled.
Figure 4.16 GMP-compliant recellularisation bioreactor assembly render. Assembled bioreactor, including larynx cassette and fittings (A). Exploded render demonstrates part alignment (B). Assembled epithelial cassettes within single and triplicate culture areas (C, D). Explosion indicates alignment of constituent parts (E, F).
Figure 4.17 GMP-compliant recellularisation bioreactor assembly. Fully assembled bioreactor fabricated from PTFE, with PC head plate (A). Assembly includes larynx cassette, plus fittings, capped 3WSCs and filter. Fabricated larynx and epithelial cassettes demonstrate respective geometries and culture areas (B).
4.4.5 Temperature mapping

Monitoring of the internal temperature of the bioreactor revealed a decrease from a maximum of 37.1 °C to a minimum of 26.7 °C when incubated in an ambient temperature of 25 °C (n=4; Figure 4.18). Internal temperature was shown to decrease further, to 24.4 °C, when the ambient temperature was 18 °C (n=3). The vertical line plotted in Figure 4.18 represents the point at which bioreactors were transferred from 37 °C to ambient temperature.

Figure 4.18 Internal temperature profile of assembled bioreactor. The internal temperature of assembled bioreactors was recorded at five minute intervals, reporting minimum temperatures of 26.7 °C and 24.4 °C when ambient temperature was 25 °C and 18 °C respectively. Error bars represent 1 SD.
4.4.6 BM-MSC biocompatibility

Metabolic activity of cells cultured in bioreactor conditioned medium was assayed and compared to an unconditioned control population to assess whether any leachables present in the conditioned medium had an effect on gross population metabolic activity.

To determine the optimal conditions for subsequent experimentation, BM-MSCs were seeded at densities ranging from 5.0 x 10^2 to 6.4 x 10^4 cells cm^{-2}, before metabolic activity was assessed at 1 h, 2 h, 3 h and 4 h. It was determined that a density of 5.0 x 10^3 viable cells cm^{-2} incubated with a metabolic assay substrate for 4 h would provide a suitable starting point for subsequent investigation (n=4; data not shown).

BM-MSCs were cultured in either unconditioned media or that which had been bioreactor conditioned for 1 d, 3 d, 5 d or 7 d; with metabolic activity assessed at time points between 4 h, 8 h, 1 d, 3 d and 5 d post-seeding. There was no difference in the metabolic activity of cells cultured in unconditioned or bioreactor conditioned medium at any point following seeding (Figure 4.19 (A)). This was reflected in the fold-change in metabolic activity of each conditioning period, relative to the unconditioned control (Figure 4.19 (B)), which was plotted to demonstrate the variability at each culture period time point with greater resolution. Mean metabolic activity increased throughout the duration of the culture, significantly between 8 h and 24 h (\(p<0.01\)) and significantly thereafter (24 h vs 72 h, 72 h vs 120 h; \(p<0.0001\)).
Figure 4.19 MSC metabolic activity in bioreactor conditioned medium. MSCs cultured for periods between 4 h and 120 h exhibited no significant difference in metabolic activity when cultured in medium that had been bioreactor conditioned for 1 d, 3 d, 5 d or 7 d, relative to unconditioned medium (A). Mean metabolic activity increased significantly between 8 h and 24 h ($p < 0.01$) and significantly thereafter (24 h vs 72 h, 72 h vs 120 h; $p < 0.0001$). Fold-change in the metabolic activity of populations cultured in variably conditioned medium fluctuated relative to the unconditioned control (B). Only at the 24 h culture time point did all conditions result in a unidirectional change in the magnitude of metabolic activity.
4.4.7 Epithelial cell biocompatibility

Comparison of the metabolic activity of porcine epithelial cells co-cultured in a Transwell® model with porcine fibroblasts in unconditioned or bioreactor conditioned medium did not conclusively indicate whether conditioned medium had any gross effect on metabolic activity. There was no significant difference between the population means ($p < 0.05$), though the data exhibited a large spread about the unconditioned median, 0.837 (0.045-1.865), and conditioned median, 0.763 (0.059-1.506). It was proposed that this was due to biological variability. Histological and immunofluorescence mediated observation revealed stratification of squamous epithelial cells that demonstrated the formation of adherens junctions and were supported by a population of basal cells (Figure 4.20). Epithelial cells appeared to be undergoing intermediate and/or terminal differentiation, indicated by the presence of cytokeratin 4.
Figure 4.20 Epithelial cell stratification and differentiation. H&E staining demonstrated clear stratification of squamous cells cultured in both bioreactor conditioned medium (A) and control medium (B). Immunofluorescent staining for integrin β4 demonstrated the presence of basal cell layer for both medium conditions (red; C, D). Cytokeratin 4 detection indicated epithelial cell differentiation in each epithelium (green; C, D). Samples for each medium condition were positively stained for E-cadherin, which confirmed the formation of adherens junctions between cells (E, F). Immunofluorescently stained cells were counterstained with DAPI. Magnification is X20 and scale bars represent 20 µm.
4.4.8 Epithelial sheet production proof-of-concept

Porcine epithelial cells and fibroblasts were co-cultured on an acellular dermal substrate to assess the feasibility of using the novel bioreactor, with epithelial cassette, to produce a tissue engineered epithelial sheet (n=2). Immunofluorescent staining of sheet cross-sections revealed a layer of basal cells (integrin β4 positive), although there was no evidence of differentiating squamous epithelial cells (cytokeratin 4 positive; Figure 4.21). There was significant autofluorescence exhibited by the acellular dermal substrate on which the cells had been cultured.

Figure 4.21 Epithelium production in bioreactor with process development epithelial cassette. Immunofluorescent staining of cross-sections of porcine epithelial cell and fibroblast co-cultures seeded on an acellular dermal matrix revealed the formation of a layer of basal cells expressing integrin β4 (red). There was no evidence of stratification or differentiation of epithelial cells, probed for using cytokeratin 4 staining. Cells were counterstained with DAPI. Significant autofluorescence of the substrate was observed. Magnification is X40.
4.5 DISCUSSION

Chapter 3 provided an account of the production of an acellular hemilaryngeal scaffold in a GMP-compliant process translated from the GLP-compliant study employed during the large animal preclinical study. However, the generation of this construct represented only the first phase of the manufacturing process of the final ATIMP, which must then be cellularised with BM-MSCs before product release. During the GLP-compliant process there was an absence of notable product characterisation or QC testing in place. This was, in part, due to the more relaxed requirements of operating under GLP guidelines, but also a consequence of the complexity of producing tissue engineered organs. Complete characterisation of the interactions between cells and the scaffold substrates on which they are seeded, in addition to the effects of cell source, subtype and the conditions they are exposed to during expansion and differentiation phases, remain a distant goal. Qualifying the fate of cells once implanted in vivo in a meaningful manner to provide an indicator of product quality is separated by yet another order of magnitude in complexity. Instead, as is more often the case with medicine as opposed to pure science, the pressure of unmet clinical need dictates that therapeutic products are rapidly brought to market once they have been proven safe and more efficacious than the existing standard of treatment. Whilst the lack of complete characterisation and understanding of a product may be unnerving to a research and development scientist, it is widely accepted by those working at the translational interface that a fine line between mechanistic understanding and product delivery must be trodden to ensure that patients receive the best available treatment. For complex organ tissue engineering, in vivo application of the novel treatment in a suitable large animal model represents the current gold standard for generating the safety and efficacy data required by the regulators to award a CTA. The preclinical work, consisting of a preliminary pilot study and the GLP-compliant study, was designed with this knowledge in hand. The primary outcomes of safety and functionality in vivo were met, and the study provided adequate animal data for the IMPD. Subsequently, the translational work described in Chapter 4 was designed and executed in the same vain; working towards fulfilling CTA application oriented goals (section 4.2.2) rather than extensive product characterisation.

Following the successful generation of acellular larynx scaffolds, described in Chapter 3, it was necessary to demonstrate that the translation to a GMP-compliant process did not yield scaffolds that were incapable of supporting recellularisation. BM-MSCs were labelled with PKH26, a fluorescent linker dye that possess an aliphatic tail that integrates with the cell membrane. The dye was chosen based upon its proven application with human BM-MSCs in vitro (Dos Santos et al., 2010) and in vivo (Yang et al., 2008), and its proven fluorescence stability that meant it could be used to identify cells beyond the
product defined cellularisation period of five days (Poon, 2000). Whilst staining was confirmed by microscopy, comparative measurement of fluorescence relative to the control population in a 96-well microplate format reported an absolute value only 2.25-fold greater than the control population, rather than the expected 100-fold increase (Figure 4.1). Furthermore, fluorescence intensity was an order of magnitude less than the corresponding values observed in the standard curve, and so meaningful quantification could not be performed as the absolute values fell outside of those reported for the lowest seeding density. It was proposed, however, that the staining would be adequate for distinguishing cells seeded onto decellularised scaffolds compared to unseeded controls. This was confirmed by a significant ($p < 0.0001$) increase in seeded scaffold fluorescence intensity compared to the unseeded control. Surprisingly, the absolute fluorescence was far greater than the sum of the stained population seeded on the 2D surface of the microplate and the scaffold background; although this may be due to the amplification of fluorescent signal on a 3D scaffold compared to a monolayer (Tecan Group, 2012). The fluorescence values of the supernatant used to wash the scaffolds indicated that there was a population of cells that had not adhered, although this was not quantified due to the aforementioned standard curve issues. Whilst repeating the experiment with an alternate or improved cell staining procedure could have provided quantitative data regarding the seeding efficiency, the information gained fulfilled the proof-of-concept objective of the experiment; thus paving the way for further investigations.

Having proven that BM-MSCs remained on the scaffolds following a four hour adherence period, PKH26 labelled cells were seeded on to scaffolds to assess whether increasing the adherence period would result in a greater retention of cells on the constructs. There was no statistical difference in the fluorescent signals of cell seeded scaffolds at four, eight or 24 hours post-seeding, nor between the corresponding wash solutions (Figure 4.2). This implied that there would be no improvement on the number of cells adhering to the scaffold conferred by maintaining a static culture beyond four hours. This was relatively important at the time of investigation, at which point no decision had been made regarding static vs non-static culture, providing an indicator as to when medium flow might be initiated. It was subsequently decided that a static culture would be used, to align with the GLP process.

To align the GMP- with the GLP-compliant process methodology, a period of five days post-seeding was indicated as the maximum culture period of cells on scaffold ex vivo prior to surgical implantation. It was therefore necessary to demonstrate BM-MSCs could persist on the acellular construct throughout this time. It was reasoned that PKH26 may no longer provide a suitable means of monitoring cell adherence to the scaffold, in light
of the variable staining efficiencies that had been achieved thus far. Instead, cell presence was investigated using a metabolic resazurin based assay, alamarBlue®. Metabolically active cells reduce resazurin to resorufin, which may then be identified by the corresponding increase in the fluorescence of the cell surrounding medium. When seeded scaffolds were assayed at one, three and five days post-seeding, there was a clear increase in the fluorescent signal compared to that observed at four hours (Figure 4.3). The maintenance of metabolic activity throughout the five day period was indicative of the persistence of a viable population of cells on the scaffold.

Following metabolic assaying, seeded scaffolds were digested and normalised DNA concentration quantified (Figure 4.4). Whilst this was not necessary to support the demonstration of persistence of cells on the constructs, it did provide additional information from samples that would have otherwise been disposed of. The data indicated a significant decrease ($p < 0.0001$) in the DNA concentration of seeded scaffolds from the day of seeding (0 d) and those assayed one day post-seeding. Thereafter an increasing trend in normalised DNA concentration was observed. From these results it could be tentatively hypothesised that there is a sharp drop in the number of adherent cells on the scaffold in the first 24 hours, whilst the subsequent increase in DNA concentration might indicate a proliferating population. However, to substantiate these claims would require the application of a reliable cell quantification assay that does not rely on inferred quantification from monolayer standard curves; which by no means provide a fair comparison for the cell character profile of cells seeded on complex 3D, biologically derived scaffolds rather than tissue culture plastic. DNA quantification is the clearest indicator of proliferating cells, yet whilst more sensitive DNA quantification assays than that employed here are available (BrdU and 3H-thymidine assays), they are largely unsuitable for accurate quantification due to noise from potential DNA contamination from the scaffold on which the cells have been seeded. One approach that has proven successful is whole construct sectioning and cell enumeration (Thevenot et al., 2008). Whilst the biopsy approach applied for the investigations described thus far is amenable to such an assay, and is suggested as a prime candidate for any future adherence investigations, it was beyond the scope of the translation objectives previously outlined and so not investigated further.

It is important to highlight that whilst cell adherence and subsequent population dynamics may be of interest in a research and development context, the translational nature of the research undertaken mandated only that the presence of cells at the point of product release be demonstrated. This would ensure that products be classified as ATMPs, see Table 1.1, and must therefore be manufactured in accordance with GMP guidelines for ATMPs outlined in EudraLex – Volume 4 (European Commission, 2017). Production
processes would, subsequently, be subject to more stringent quality control than non-ATMP product manufacturing quality requirements; which is, of course, in the greatest interest of the patient, manufacturer and industry as a whole.

Qualitative confirmation of the persistence of cells on the constructs was provided by SEM (Figure 4.5). The photomicrographs clearly demonstrated the presence of cells on the surface of the scaffolds up to five days post-seeding, where none had previously been detectable on the unseeded decellularised construct surface. Combined with the proof-of-concept, attachment time and persistence investigations, these data fulfil the first two objectives outlined at the beginning of this Chapter and support the claim that the acellular scaffolds produced by the GMP-compliant decellularisation process are capable of supporting BM-MSCs for a five day period *ex vivo*.

Subsequent to having proven scaffold suitability in a scaled-down format, it was necessary to identify and apply a system for use with larger tissue constructs. During the GLP-compliant study a bespoke bioreactor had been developed and used for the seeding of decellularised hemilarynges (Figure 4.6). The bioreactor was, however, unsuitable for GMP-compliant manufacture due to a number of factors. These included material incompatibility with the established cleaning and sterilisation process that was to be used for equipment employed in final ATIMP manufacture, as well as possessing design features that were not user friendly and increased the risk of contamination during operation. An alternative system was required to resolve these shortcomings and a URS was compiled to highlight the necessary attributes for a suitable replacement (Table 4.1). Searching existing solutions on the market revealed a lack of suitable options that could address the specific characteristics highlighted, and so it was proposed that a bespoke solution be designed and fabricated to address this.

Firstly a design specification was outlined to reflect the requirements in the URS (Table 4.2). A novel, bespoke bioreactor was designed to replicate the general form of the unit used in the GLP-compliant study, with the addition of cassette modules that could confer product specific functionality. Independent cassettes for supporting larynx and epithelial cell seeding and construct support were designed. The dimensional clearance incorporated into the cassette designs allowed for effortless transfer of the parts to and from the bioreactor (DS#10.1), facilitating simple seeding outside of the chamber body (DS#6.1). Seeding could also be achieved inside of the bioreactor in a low risk manner due to the cassettes positioning the constructs in close proximity to the bioreactor opening, thus minimising the need to pass potentially contaminating pipettes deep into the chamber body (DS#6.2).
The larynx cassette design consisted of a perforated support structure that would enable medium to contact both the luminal and abluminal surfaces of the scaffold (DS#12.1), with access ports located on the upper and lower portions of two opposing lateral sides of the chamber body. Positioned orthogonally to the extruded sides of the cassettes, these access ports were designed to accommodate commonly used luer lock fittings (DS#7.1), thus allowing easy transfer of fluid to and from the chamber in a semi-closed manner. The dimensions of the cassette, and thus corresponding internal dimensions of the chamber body, were large enough to accommodate a hemilarynx that was 20% larger than the male average (DS#11.1). The external dimensions were small enough to ensure that the equipment could be accommodated in the existing transportation packaging used by the manufacturer (DS#9.1), and so fulfil the role of primary packaging and culture device. They were, however, still large enough to affix primary package labelling to, in accordance with the Eudralex – Volume 4, Annex 13 guidelines ((European Commission, 2010b); DS#9.2; Figure 2.2 (J, K)).

Whilst it had initially been envisaged that the chamber body could be fabricated from a shelled polymer sheet, in-house limitations dictated that the body would need to be hollowed from opposing faces and later sealed with base and head plates. Sealing of the chamber was achieved using O-rings that would abut against the base and head plates once assembled. Glands throughout the system were designed to accommodate standard O-ring sizes, thus reducing potential procurement issues associated with sourcing bespoke sized seals. By inserting O-rings between the luer lock fittings and chamber body and head plate, followed by capped 3WSCs, it was possible to fully close the chamber. This would ensure that there was no leakage during normal operation or transport of the bioreactor (DS#9.3), which would represent a serious contamination threat. Potential contamination of the bioreactor contents could be monitored by observing the product and media within the bioreactor through a transparent head plate (DS#9.4).

Although it had been decided that the tissue engineered epithelium used in the GLP-compliant study would not form part of the proposed treatment to be investigated by the clinical trial, a secondary cassette accommodating the seeding and culture of epithelial cells on a sheet-like matrix was designed. Not only would this prove useful for further process development of the epithelial sheet, but would also demonstrate that modular functionality could facilitate the production of multiple products using a single, bespoke bioreactor. Proof-of-concept demonstration of this approach could then pave the way for further functional expansion of the designed bioreactor, thus reducing the burden of equipment characterisation and sterility validation. The epithelium supporting cassette was designed to fit the already drafted internal dimensions of the chamber body. The
cassette consisted of two parts that could be screwed together to locate the epithelial substrate sheet, with a centrally exposed area for seeding and culture (DS#14.1). An O-ring was positioned between the two parts to prevent tearing of the membrane whilst holding it flat, with a number of clamping screws offset from its gland circumference to ensure an even distribution of sealing force (DS#13.1). Once inserted into the chamber body a seal would be formed between the O-ring located in the base of the cassette and the supporting ledge within the chamber body, thus generating two distinct compartments. This would allow for submerged culture, exposing both faces of the epithelial sheet to medium, followed by removal of the medium from the epithelial side of the sheet and ALI culture (DS#15.1).

Initial suggestions for material selection for fabrication were based on those used widely in marketed products and in the literature. The Workshop had previous experience with bioreactor manufacture and it was envisaged that using materials that the fabricator had previous experience with could prove beneficial to the final product. Several engineering polymer candidates were proposed, including PA, PC, PEEK and PTFE. Each was assessed against the material specific points within the DS (Table 4.3). Biocompatibility profiling, formed upon evidence reported in the published literature, indicated PC and PTFE may be more suitable for application with BM-MSCs and epithelial cells than PA and PEEK (DS#2.1, DS#5.1). Combining this with suitable cost and physical properties lead to the selection of PC and PTFE for part fabrication (DS#3.1, DS#4.1).

Fabrication was successfully completed, with all parts assessed for fit by the fabricator prior to release. The cost of each bioreactor was <£1000, fulfilling the requirement for a completely assembled bioreactor to cost <£2500 (data not shown; DS#8.1). It was highlighted by the fabrication engineers within the Workshop that despite the high quality of the final parts, they had experienced significant difficulty when machining the PTFE components. This was due to the softness of PTFE, resulting in location issues and slippage when clamping the raw material for machining. This had the potential to negatively impact the cost and production timelines for fabrication, and would need to be considered for future design projects.

Bioreactors were assembled using the larynx cassette, and sterility of assembly and filling validated. Negative results indicated that the design process had yielded a system that could be aseptically assembled and operated. Whilst not overtly outlined in the DS, the design process had been undertaken with a strong emphasis on user-friendliness for both GMP manufacturing scientist and product-receiving surgeon. The ease with which the bioreactors could be aseptically assembled, in an A Grade environment with an uncontrolled background, was evidence that this approach had been a success. This
was reinforced by the negative results obtained from subsequent, biannual sterility validations performed by non-specialist GMP manufacturing scientists (data not shown).

As highlighted in the URS, it was necessary that the novel bioreactor act as both the culture device and the primary packaging for the described products. It is not atypical that products manufactured within the CCGTT are released and shipped with a broad temperature range, once it has been risk assessed that doing so would not be detrimental to the quality of the product. This was also to be the case for the recellularised larynx product, which the IMPD indicated would be shipped at 18-25 °C. Temperature mapping of the bioreactor was performed to characterise the temperature experienced by products shipped at the upper and lower limits of the range, over the duration of the four hour product shelf life. It revealed that when exposed to an ambient temperature of 18 °C for four hours, the internal temperature would fall to no less than 24 °C (Figure 4.18). Observing the temperature at the median of the product shelf life indicated a value of 27.4 °C. With the proposed clinical site for the trial located comfortably within two hours of the manufacturing site, this timeframe provides a more representative example of the temperatures likely to be experienced by the product. To give insight on any effects this might have on the BM-MSCs on the seeded larynx would require significant further investigation, whilst the effects that this might have on product quality and therapeutic efficacy is beyond the scope of moral and achievable enquiry.

Despite reports in the literature suggesting that both PC and PTFE did not exhibit harmful effects towards either BM-MSCs or epithelial cells, it was essential that the fabricated bioreactors be assessed in-house. Whilst not part of the QC assays performed for conditional or final product release, a cell cytotoxicity assay was performed to assess whether the bioreactor exhibited any effect on cell viability. The main concern was that leachable impurities or chemical remnants from the cleaning and sterilisation process may be passed from the bioreactor to cells via the surrounding medium during normal operation, reducing total population viability. To investigate this, medium was conditioned in sterile bioreactors inside normoxic, humidified CO₂ (5%) incubators at 37 °C, before BM-MSCs were cultured in the conditioned medium in a scaled-down format. To probe for potential time-dependent leaching of harmful impurities from the bioreactor, a series of increasing conditioning periods were used. Conditioning was even performed up to 7 days, to increase the likelihood of leachables entering the medium. Cell populations were then cultured in each conditioned medium for increasing periods to assess whether there were any exposure-time dependent effects on viability, which was assessed using a metabolic activity assay. There was no statistical difference between the metabolic activity of BM-MSCs assayed at four hour or 8 hour exposure times, for any of the medium conditioning periods, when compared to cells grown in unconditioned
control medium. Four and 8 hour incubation times were investigated as they broadly provide distinction between cells undergoing apoptosis and necrosis, respectively (Riss, 2003). None of the subsequently assayed culture periods indicated a difference between any of the conditioned media and the control, and there was a general increase in total metabolic activity across the five days (Figure 4.19). These data indicate that the bioreactors did not leach impurities into the medium that had an effect on the viability of BM-MSCs, thus affirming their suitability for supporting cell persistence throughout the duration of the proposed culture period.

Similar investigations were undertaken for epithelial cells to assess cell specific biocompatibility. Due to a shortage of human tissue biopsies, porcine epithelial cells were co-cultured with porcine fibroblasts as a proxy for the human counterpart. Whilst this was not ideal, it would provide a first insight into any general effects on viability and could be repeated using primary human cells when the data were required for the tissue engineered epithelial sheet IMPD. As a consequence of the slower population growth exhibited by epithelial cells, as well as the greater seeding density and subsequent cell numbers required for each replicate, a single conditioning time period was investigated. Following the positive BM-MSC results, worst-case conditions were investigated. Co-cultures were cultured for a total of 17 days, in medium that had been bioreactor conditioned for the same period. The results indicated that there was no significant decline in mean population metabolic activity relative to the control medium, although a decline was evident in two of the replicates. The spread of the values was attributed to variation across donor animals, with each condition pairing associated with an individual donor. To gain a greater appreciation of the effect of donor source on the data generated, as well as insight into the effects of media conditioning on metabolic activity, would require repeat experimentation with cell populations from individual animals. Given the lack of evidence suggesting a gross loss of viability, it is proposed that more thorough investigation is postponed until a human tissue source becomes available.

The ultimate function of the tissue engineered epithelial sheet is to provide a barrier construct that can be applied to the luminal surface of implanted ATIMP airways, which is proposed to help prevent microbial colonisation (Elliott et al., 2012, Martinod et al., 2013) To investigate whether the generation of an epithelium would be inhibited by leachables from the bioreactor, non-contact co-cultures of porcine epithelial cells and fibroblasts were generated using a Transwell® model. Comparison of the histologically and immunofluorescently stained cross-sections grown in each medium revealed no difference in the formation of stratified squamous epithelium consisting of a layer of basal cells supporting differentiating epithelial cells. The formation of adherens junctions
between cells confirmed the contiguity of the epithelium, which is essential for effective barrier functionality (Niessen, 2007).

As a proof-of-concept investigation, co-cultures of porcine epithelial cells and fibroblasts were cultured on acellular dermal matrix within the bioreactor using the epithelial cassette designed for process development. It demonstrated that location of the substrate sheet in the epithelial cassette was feasible and that the cassette maintained a flat sheet that did not tear or aggregate over the 17 days of investigation. Some difficulty arose during the replenishment of medium on day three, when generating the ALI. After draining the bioreactor, filling the lower compartment of the chamber without generating an air bubble that then abutted the underside of the sheet proved difficult and required the operator to orient the whole bioreactor at an angle during filling. Though it was possible to remove the bubble and ensure medium contacted the sheet, this was not user friendly and could not likely be used to reliably produce a tissue engineered epithelium. It was also highlighted that filling the bioreactor in this manner may result in the generation of a positive pressure gradient from the lower to upper compartment, due to overfilling. The effect that this may then have on the generation of a typical epithelium remains unknown and so should either be avoided or investigated. It may be envisaged that subsequent iterations of the bioreactor system have pressure sensors coupled to the upper and lower compartments to ensure that there is no differential. To prevent bubble formation, it is proposed that the medium is continuously perfused through the lower compartment rather than draining and then replenishing. Staining cross-sections of the cellularised matrix at 17 days revealed the formation of a layer of basal cells expressing integrin β4 (Figure 4.21). There was, however, no evidence of stratification or of epithelial differentiation indicated by cytokeratin 4. This could be due to a number of factors. Firstly, it may have been that the bioreactor conditions were not amenable to extensive epithelial cell growth. This was unlikely to be due to nutrient or oxygen depletion though, as the medium was perfused through the bioreactor at a rate that equated to a full medium exchange over 72 h and was housed in a gas-permeable transfer bag within the incubator. It may have been due to the introduction of the acellular dermal substrate, although partially differentiated epithelium has been observed to form on this in a CellCrown™ model within our lab (unpublished data). Alternatively, it may be a consequence of co-culturing epithelial cells with non-growth arrested fibroblasts, which could have outgrown the slower growing epithelial cells on the matrix. Considering the positive results that were demonstrated for differentiated epithelium in the Transwell® model, it is most likely that absence of differentiating epithelium be attributed to the latter two explanations rather than an inherent bioreactor factor. It is suggested that the investigation be repeated with growth arrested fibroblasts, as well as a CellCrown™ model control, to clarify this. This work is currently on hold, however, due to independent
concerns regarding the inability of the acellular dermal matrix to successfully vascularise and persist in vivo (unpublished data). An alternative, thinner substrate is currently being sought.

4.6 CONCLUSION

Subsequent to the previously documented translation of the decellularisation process, the objectives for this Chapter addressed the requirements for translating the recellularisation component of the GLP-compliant process. Initially, it was demonstrated, in a scale-down model, that acellular human hemilaryngeal scaffolds produced using GMP-compliant decellularisation were capable of supporting human BM-MSCs. Seeded cell populations remained viable throughout a five day period, which had been predetermined as the time frame that would be applied during clinical manufacture. To enable recellularisation at scale, a bespoke bioreactor was designed and fabricated; improving upon the GLP-bioreactor and incorporating a range of user specific requirements. The novel bioreactor was validated for aseptic assembly and operation, before successfully demonstrating biocompatibility with BM-MSCs expanded on tissue culture plastic. Secondary functionality that targeted culturing of an epithelial sheet product was investigated, in anticipation of potential future ATIMP development. The bioreactor was proven to have no observable detrimental effect on normal epithelium development, when investigated using bioreactor conditioned medium in a Transwell® model. Direct culturing of epithelial cells within the bioreactor supported the development of a population of basal cells, although future investigation may require the adoption of a more suitable substrate matrix.
5 FUNCTIONAL MODIFICATION OF A MODULAR BIOREACTOR FOR EARLY STAGE TRANSLATION AND CLOSED-SYSTEM PRODUCTION OF ACELLULAR OESOPHAGEAL SCAFFOLDS FOR PRECLINICAL STUDIES

5.1 INTRODUCTION

The data presented in Chapter 4 demonstrated that by designing novel bioreactor systems it is possible to directly address user needs in a specific and tailored manner, particularly when they are unmet by existing solutions on the market. An integral concept applied during this approach was the generation of a modular system that allowed for the expansion of functionality to two different product types, namely three-dimensional scaffolds and two-dimensional sheet constructs. The supporting data confirmed that the system could be aseptically operated in a user-friendly manner and that the construction materials exhibited no detriment to the multiple cell types used for the different products. Having been designed with consideration of GMP-guidelines and the practicalities associated with clean-room operations, the bespoke system represents a platform that has the potential to provide a tissue engineering translational bioprocessing solution for a variety of different construct models. To demonstrate further expansion of the multi-functional system, it was proposed that the system be adapted for application with tubular TEPs. A tissue engineered oesophageal product for paediatric indications was proposed as a model system for investigation, due to its multi-layered functional and structural complexity (Camilli, 2017), unmet clinical need (Ure et al., 1995, Ludman and Spitz, 2003), paucity of suitable manufacturing systems on the market and recent generation of promising results reported by colleagues in a small-animal model (Urbani et al., 2018).

Urbani et al. (2018) described the generation of a tubular construct that was comprised of both the muscular and epithelial compartments of the oesophagus. Acellular scaffolds were produced from donor rat oesophagi using a detergent-enzymatic treatment (DET) protocol that had been previously described for producing decellularised intestine and hepatic tissue in rat (Totonelli et al., 2012, Maghsoudlou et al., 2013, Maghsoudlou et al., 2016). The tissue generated was acellular and did not exhibit immunogenicity in vivo. The microarchitecture was comparable between the native and decellularised tissue and retained native-like distribution of important biomolecules, including collagen I, collagen IV and laminin. Importantly, the biomechanical profile post-decellularisation reflected that of the native tissue, which was also evident in the quantified elastin and sGAG content. Sterilised scaffolds were then co-seeded with human mesoangioblasts and mouse
fibroblasts to repopulate the abluminal muscular component of the oesophagus, before being dynamically cultured in a modified glass bioreactor (Crowley, 2015). The resultant scaffolds demonstrated cell density and distribution similar to the native organ, spatially integrating with the scaffold architecture and differentiating to generate mature smooth muscle cells. Seeding of enteric nervous system precursor cells resulted in a proliferating population of naïve murine neural crest cells that matured to form neurons and glial cells that established connections with neighbouring muscle cells. Importantly, they oriented within the tissue to form two concentric rings, which form part of the nerve circuitry that controls peristalsis in the native organ. The capacity of the acellular scaffold to support the establishment of a luminal epithelium was described, demonstrating the necessary barrier characteristics required for oesophageal tissue engineering. The resultant, dynamically cultured construct was then implanted in a rat model in a two-stage surgical approach that pre-vascularised the scaffold in the omentum before subsequent heterotopic implantation. The host inflammatory response was typical of biologically derived acellular scaffolds, with a clear macrophage and neutrophil infiltrate.

Appreciation of the requirements for effective clinical translation played a significant role in the planning and execution of the described proof-of-principle investigation. It was acknowledged that the cell types required could be sourced from locations that are readily accessible during the standard care of the target patient population, with biopsies processed in a manner that is amenable to GMP translation (Urbani et al., 2018). The group also highlighted the need for a closed-system bioreactor that would improve on the existing equipment employed, facilitating the process scale-up and scale-out necessary for preclinical large-animal investigation and clinical production respectively. The positive results described by colleagues, combined with those reported in Chapter 4, justified the application of the modular bioreactor to produce acellular oesophageal scaffolds for larger animal, preclinical studies.
5.2 AIMS AND OBJECTIVES

5.2.1 Aims

The investigations planned and executed in this Chapter were intended to demonstrate that the functionality of the modular bioreactor described in Chapter 4 could be expanded to include the production of acellular oesophageal scaffolds for tissue engineering applications. It was proposed that this could be coupled with the scale-up work required following the findings reported by Urbani et al. (2018), generating and characterising acellular porcine oesophagi before implantation as part of a surgical feasibility study. As a prelude to the pivotal preclinical study, process development would incorporate several aspects of GMP translation preparation that would facilitate a smooth transition to a GMP-compliant process in preparation for the eventual Phase I/II clinical trial.

5.2.2 Objectives

- To adapt the modular bioreactor previously described to enable aseptic, semi-closed/closed-system perfusion operation.
- To generate and characterise the biomolecular and biomechanical profiles of acellular porcine oesophageal scaffolds.
- To produce acellular porcine oesophageal constructs suitable for implantation in a large-animal model.
5.3 MODULAR BIOREACTOR ADAPTATION FOR TUBULAR CONSTRUCTS

The modular bioreactor first described in Chapter 4 was modified to accommodate tubular tissues, namely neonatal and juvenile porcine oesophagus. The published DET decellularisation protocol relied on luminal perfusion of the oesophagus and so an approach that could reproduce this within the bioreactor was required. To avoid further fabrication and permanent alteration of the existing design it was proposed that a pipe-like structure that passed through one of the existing ports, to which tubular tissues could be attached, may provide a suitable solution. It would be essential that the conduit formed a seal with the existing through hole, to ensure that a semi-closed/closed-system could be maintained.

It was proposed that a Foley catheter would resolve the identified requirements. In addition to the standard lumen, Foley catheters possess a balloon that is inflated to maintain the catheter opening *in situ* during typical usage. It was proposed that the catheter may be passed inside the bioreactor and balloon inflated to form a seal between the inner and outer environments. Suturing the oesophagus to the internal terminus and attaching a luer fitting to the external would then allow for fluid to be perfused from outside of the bioreactor. Catheters are supplied sterile by manufacturers, thus reducing the total sterilisation load.

A Foley catheter with an OD of 4.0 mm was procured and the proposed sealing mechanism investigated. Inflation successfully formed a seal and maintained the catheter opening in position within the bioreactor. It was also demonstrated that due to the matching of sizing of the catheter OD and the OD of the clearance holes (4.00 mm; Figure 4.6), even without inflation there was no evidence of leakage from the bioreactor. Inspection of the Foley catheters revealed all units to be sized ≥4.0 mm, accounting for the sealing observed. The external terminal of the catheter was fitted with a female 1/8” BSP to male luer thread connector and 3WSC, thus allowing easy fluid path connection.

To assess whether the adapted assembly could be operated as a semi-closed/closed-system a TSB sterility validation of the DET process in the modified unit was performed (section 2.4.5). The results confirmed sterility, indicating that the modified system achieved semi-closed/closed-system processing (data not shown).
5.4 DETERGENT-ENZYMATIC TREATMENT DECELLULARISATION OF NEONATAL PORCINE OESOPHAGUS

Having confirmed that the modified semi-closed/closed-system bioreactor could be aseptically assembled and operated, the revised DET was performed to decellularise oesophagi from neonatal (<24 h) pigs. Gross observation revealed decellularised, pale oesophagi that were comparable in size to the native tissue (Figure 5.1 (A, C)). By using a my-Control controller unit, or TEB500 incubator, oesophagi were processed in batch sizes ranging from 1-5 tissues (Figure 5.1 (B)).

5.4.1 Qualitative analysis of decellularised neonatal porcine oesophagus

Histological examination demonstrated a complete clearance of intact nuclei from the tissue following decellularisation (Figure 5.2). The tissue microarchitecture was preserved following decellularisation, with mucosa, submucosa and muscularis externa compartments still clearly identifiable. Fibril-forming collagen type I was ubiquitously distributed throughout the ECM pre- and post decellularisation (Figure 5.3 (A, B)). Collagen type IV and laminin immunofluorescence staining revealed intact basement membranes were also preserved following decellularisation (Figure 5.3 (C-F)).

Figure 5.1 Semi-closed/closed-system oesophagus decellularisation. Native porcine oesophagus inside a modified, semi-closed/closed-system bioreactor (A). Multiple bioreactors could be connected to a single pump unit to enable scaled-out decellularisation of multiple tissues (B). Decellularised oesophagi were pale and appeared similar in size to the native precursor (C).
Figure 5.2 Qualitative evaluation of decellularised neonatal porcine oesophagus. H&E staining of native tissue revealed cells distributed throughout the mucosa (m), submucosa (sm) and muscularis externa (me) when observed at X10 (A) and X20 magnification (B). Decellularised oesophagus demonstrated complete clearance of intact cells throughout the scaffold layers (C, D). Distinct structural layers were still evident, although the mucosa appeared distended. Scale bars represent 100 µm and 50 µm for X10 and X20 magnification.
Figure 5.3 Qualitative analysis of biomolecular distribution in native and decellularised neonatal porcine oesophagus. Immunofluorescence revealed ubiquitous distribution of collagen type I in native (A) and decellularised oesophagus (B). Collagen type IV, native (C), was preserved following decellularisation (D). Laminin staining of native tissue (E) exhibited significant autofluorescence. Laminin was still present in the basement membrane of decellularised oesophagus (F). Submucosa immunofluorescence reflected the microarchitecture observed in Figure 5.2 (C). Scale bars represent 50 µm.
5.4.2 Quantitative analysis of decellularised neonatal porcine oesophagus

Quantitative analysis of DNA content was applied to assess the efficacy of decellularisation. Relative concentration significantly decreased from 543.09 ± 114.11 ng mg⁻¹ in the native tissue (n=6) to 35.48 ± 10.36 ng mg⁻¹ once decellularised (Figure 5.4; n=6; p <0.05). This represented a 93.47% reduction in DNA concentration. In all but one sample, DNA was reduced to <50 ng mg⁻¹.

Normalised total collagen concentration reported a significant increase following decellularisation, from 109.98 ± 2.39 µg mg⁻¹ (n=4) to 189.98 ± 18.10 µg mg⁻¹ (Figure 5.5; p <0.05; n=4). The relative increase was attributed to the loss of cell and non-collagen biomolecule mass.

Quantification of normalised sGAG content in the decellularised oesophagus revealed a drop from 8.58 ± 0.489 µg mg⁻¹ (n=4) in the native tissue to 6.07 ± 1.27 µg mg⁻¹ (n=4; Figure 5.6). This corresponded to a 70.75% retention of sGAG.

Figure 5.4 Quantitative analysis of normalised DNA content in native and decellularised neonatal porcine oesophagus. Analysis revealed a significant (p <0.05) reduction in normalised DNA concentration from 543.09 ± 114.11 ng mg⁻¹ in the native tissue (n=6) to 35.48 ± 10.36 ng mg⁻¹ once decellularised (n=6). Paired analysis revealed a decrease in normalised DNA concentration to <50 ng mg⁻¹ in all but one of the samples, which saw a reduction from 288.80 ng mg⁻¹ to 85.34 ng mg⁻¹.
Figure 5.5 Quantitative analysis of normalised total collagen content in native and decellularised oesophagus. The data indicated a significant ($p < 0.05$) increase in relative total collagen content per $\mu$g of tissue, from $109.98 \pm 2.39 \mu$g mg$^{-1}$ ($n=4$) to $189.98 \pm 18.10 \mu$g mg$^{-1}$ ($n=4$). Paired analysis indicated that normalised total collagen concentration increased in all instances following decellularisation. The relative rise is attributed to the loss of cellular and non-collagen biomolecular mass.

Figure 5.6 Quantitative analysis of normalised sGAG content in native and decellularised oesophagus. Normalised total sGAG content was reduced from $8.58 \pm 0.489 \mu$g mg$^{-1}$ ($n=4$) to $6.07 \pm 1.27 \mu$g mg$^{-1}$ ($n=4$), following decellularisation. This was equivalent to a 70.75% retention of sGAG. Paired analysis demonstrated that normalised total sGAG concentration decreased in all samples assayed.
5.4.3 In vivo evaluation of decellularised neonatal porcine oesophagus

Decellularised oesophagi were evaluated in vivo using a New Zealand White (NZW) rabbit, large-animal model as part of an exploratory study investigating the surgical procedure and model for oesophageal replacement, as well as pre- and post-operative care. The rabbit model was selected primarily due to its clinical relevance, with the adult rabbit oesophagus comparable in size to that of human neonates. Acellular irradiated oesophagi were cervically implanted following trachea mobilisation, oesophageal resection and complete anastomosis of scaffolds <2 cm (Figure 5.7; n=8). All clinical work was performed by E Hannon and F Scottoni, whilst aftercare was provide by Biological Services Unit personnel and members of the clinical team and non-clinical colleagues from the UCL Institute of Child Health.

The study confirmed the feasibility of the rabbit model, with 7/8 experiencing no complications or distress immediately after recovery from anaesthesia. However, respiratory distress was observed in three animals at days one, three or 8 post-op. Oesophageal obstruction at day 9 (n=2), scaffold failure (n=1) at day five and massive weight loss incompatible with life at day 7 (n=1) led to early scheduling of animals. The outcome was attributed to a series of confounding factors, including sub-optimal stent selection failing to maintain patency, poor implant vascularisation and the lack of gastrostomy.

Additionally, it was reported that the acellular oesophagi procured from neonatal pigs were too small for the recipient animals; resulting in difficulties in forming adequate anastomoses during surgery, poor suture retention and excessive graft tension. The thickness of the oesophageal wall was also of concern and it was suggested that procurement of larger oesophagi was required.
Figure 5.7 Acellular porcine oesophagus implantation. New Zealand White rabbits received irradiated, acellular porcine oesophagi in a single stage procedure. Longitudinal incision allowed loop mobilisation of the trachea (A), before 1-2 cm of cervical oesophagus was resected (B). Scaffolds were heterotopically located (C) and complete anastomosis performed (D). Images courtesy of E Hannon (unpublished).
5.5 DETERGENT-ENZYMATIC TREATMENT DECELLULARISATION OF JUVENILE PORCINE OESOPHAGUS

To resolve the animal model tissue size issue, oesophagi were retrieved from donor animals that had a mass closer to the recipient rabbit range (2.8-3.2 kg). Juvenile pigs, 6-8 days old, were procured and oesophagi retrieved as previously described (section 2.1.1.2). External dimensions of retrieved organs were comparable between neonates and juveniles (Figure 5.8), however the oesophageal wall was notably more substantial in the older animals. This was clearly evident when mounting the oesophagi during the decellularisation process (Figure 5.9).

![Graph showing gross biological characteristics of neonatal and juvenile porcine oesophagus]

Figure 5.8 Gross biological characteristics of neonatal and juvenile porcine oesophagus. There was no notable difference in retrieved oesophagus dimensions, despite a significant ($p < 0.0001$) increase in juvenile animal mass. (Neonate length, n=22; juvenile length, n=59. Neonate distal/proximal diameter, n=13; juvenile distal/proximal diameter, n=50. Neonate mass, n=3; juvenile mass, n=48.) Error bars represent 1 SD.
Figure 5.9 Comparison of neonatal and juvenile porcine oesophagus *in situ* during decellularisation. Neonatal oesophagi (A, C) consistently possessed a notably thinner wall than that observed in juvenile donors (B, D). Here, destructive sampling of the distal oesophagus greatly reduced the length of the neonatal organ (A).
5.5.1 Quantitative analysis of decellularised juvenile porcine oesophagus

5.5.1.1 Biomolecular characterisation

To demonstrate that larger oesophagi could produce acellular scaffolds with comparable decellularisation efficacy to the neonatal tissue, juvenile oesophagi were retrieved, decellularised and key biomolecular content quantified. A shortened decellularisation protocol, consisting of two rather than three DET cycles, was also investigated. Both semi-closed/closed-system processes were compared to an open process control decellularisation of juvenile oesophagi.

Quantitative analysis of DNA concentration revealed a significant ($p < 0.01$) decrease across all decellularisation processes. Open-system processing reported a drop from $3164.07 \pm 616.56 \text{ ng mg}^{-1}$ to $150.47 \pm 38.67 \text{ ng mg}^{-1}$ once decellularised ($n=3$). Whilst none of the samples exhibited DNA concentrations $<50 \text{ ng mg}^{-1}$, mean reduction was 95.24%. Semi-closed/closed-system decellularisation produced a 92.56% removal of DNA, from $2146.85 \pm 393.57 \text{ ng mg}^{-1}$ to $159.76 \pm 41.14 \text{ ng mg}^{-1}$ ($n=5$). Reducing the number of DET cycles to two resulted in a reduction from $2580.15 \pm 559.79 \text{ ng mg}^{-1}$ to $72.88 \pm 21.30 \text{ ng mg}^{-1}$ ($n=4$), equivalent to a 97.18% decrease. Whilst two cycle processing corresponded to an almost 5% greater removal of DNA than three cycles of DET, there was no statistically significant difference between any of the decellularised values.

Assessment of total collagen indicated that the relative concentration increased following decellularisation in all processing systems. A significant increase ($p < 0.05$) was reported for open-system decellularisation, from $111.36 \pm 8.36 \mu \text{g mg}^{-1}$ to $189.00 \pm 18.41 \mu \text{g mg}^{-1}$ ($n=3$). When operating in a closed-system a significant increase ($p < 0.01$) from $129.23 \pm 3.93 \mu \text{g mg}^{-1}$ to $190.47 \pm 13.74 \mu \text{g mg}^{-1}$ ($n=4$) was reported. The collagen concentration when two DET cycles were used in a semi-closed/closed-system rose from $125.93 \pm 4.19 \mu \text{g mg}^{-1}$ to $246.76 \pm 27.26 \mu \text{g mg}^{-1}$ ($n=4$).

When sGAG was quantified following decellularisation, it was shown to decrease by 83.87%, 89.11% and 83.25% for open-system processing, semi-closed/closed-system two DET cycle and semi-closed/closed-system three DET cycle processes respectively. Open process decellularisation with three DET cycles yielded a significant ($p < 0.01$) decrease in sGAG from $8.93 \pm 0.39 \mu \text{g mg}^{-1}$ to $1.44 \pm 0.22 \mu \text{g mg}^{-1}$ ($n=3$), whilst closing the process yielded a significant ($p < 0.001$) reduction from $10.47 \pm 1.31 \mu \text{g mg}^{-1}$ to $1.14 \pm 0.28 \mu \text{g mg}^{-1}$ ($n=4$). Reducing the number of DET cycles to two also corresponded with a significant ($p < 0.001$) decrease from $9.91 \pm 1.09 \mu \text{g mg}^{-1}$ to $1.66 \pm 0.57 \mu \text{g mg}^{-1}$ ($n=4$).
Figure 5.10 Quantitative analysis of normalised DNA content in juvenile porcine oesophagus, decellularised using open, closed and shortened processes. All processes reported a significant ($p < 0.01$) decrease in DNA content following decellularisation. Open process decellularisation with three DET cycles produced a decrease from $3164.07 \pm 616.56$ ng mg$^{-1}$ in the native tissue ($n=3$) to $150.47 \pm 38.67$ ng mg$^{-1}$ once decellularised ($n=3$). For the closed, three cycle process, content dropped from $2146.85 \pm 393.57$ ng mg$^{-1}$ ($n=5$) to $159.76 \pm 41.14$ ng mg$^{-1}$ ($n=5$). Two DET cycles in a semi-closed/closed-system produced a decrease from $2580.15 \pm 559.79$ ng mg$^{-1}$ ($n=4$) to $72.88 \pm 21.30$ ng mg$^{-1}$ ($n=4$). Reduction of DNA content to <50 ng mg$^{-1}$ was observed in 0%, 50% and 40% of replicates, respectively.
Figure 5.11 Quantitative analysis of normalised total collagen content in juvenile porcine oesophagus, decellularised using open, semi-closed/closed and shortened processes. Open process decellularisation with three DET cycles yielded a significant ($p < 0.05$) increase from $111.36 \pm 8.36 \mu g \text{ mg}^{-1}$ in the native tissue ($n=3$) to $189.00 \pm 18.41 \mu g \text{ mg}^{-1}$ once decellularised ($n=3$). For the closed, three cycle process, content increase significantly ($p < 0.01$) from $129.23 \pm 3.93 \mu g \text{ mg}^{-1}$ ($n=4$) to $190.47 \pm 13.74 \mu g \text{ mg}^{-1}$ ($n=4$). Two DET cycles in a closed system produced an increase from $125.93 \pm 4.19 \mu g \text{ mg}^{-1}$ ($n=4$) to $246.76 \pm 27.26 \mu g \text{ mg}^{-1}$ ($n=4$). The increase in collagen concentration in all observed samples was attributed to the relative increase in total mass contribution, following the loss of cellular mass.
Figure 5.12 Quantitative analysis of normalised sGAG content in juvenile porcine oesophagus, decellularised using open, semi-closed/closed and shortened processes.

Open process decellularisation with three DET cycles yielded a significant \( (p < 0.01) \) decrease from \( 8.93 \pm 0.39 \mu g \text{ mg}^{-1} \) in the native tissue \( (n=3) \) to \( 1.44 \pm 0.22 \mu g \text{ mg}^{-1} \) once decellularised \( (n=3) \). For the closed, three cycle process, content decreased significantly \( (p < 0.001) \) from \( 10.47 \pm 1.31 \mu g \text{ mg}^{-1} \) \( (n=4) \) to \( 1.14 \pm 0.28 \mu g \text{ mg}^{-1} \) \( (n=4) \). Two DET cycles in a closed system produced a significant \( (p < 0.001) \) decrease from \( 9.91 \pm 1.09 \mu g \text{ mg}^{-1} \) \( (n=4) \) to \( 1.66 \pm 0.57 \mu g \text{ mg}^{-1} \) \( (n=4) \). Corresponding sGAG retention was 16.13\%, 10.89\% and 16.75\% respectively.
5.5.1.2 Biomechanical characterisation

Oesophagus flat specimens, oriented in the longitudinal or circumferential axis, were sampled from native tissue and decellularised tissue. Acellular samples were assayed at one and four weeks, with and without gamma-irradiation. Comparison of the stiffness, ultimate stress and ultimate strain of tissue biopsied from the proximal and distal thirds of the organ revealed no difference between sites in all but two conditions (Table 5.1). Whilst there was a significant difference ($p < 0.05$) in the ultimate strain of decellularised, irradiated circumferential proximal and distal sections immediately post-irradiation and after four weeks storage, proximal and distal biopsies were treated as single populations thereafter to provide an overview of total oesophagus biomechanics.

Comparison of longitudinal stiffness revealed an increase in Young's Modulus from 0.676 ± 0.059 MPa to 1.051 ± 0.160 MPa one week after decellularisation, which was maintained after a further three weeks of storage in PBS at 4 °C (1.045 ± 0.207 MPa; Figure 5.13). The ultimate tensile stress remained largely unchanged following decellularisation (0.855 ± 0.123 MPa vs 0.718 ± 0.169 MPa), although after four weeks storage it increased significantly relative to storage for one week (1.497 ± 0.281 MPa; $p < 0.05$). Ultimate strain of the longitudinal specimens was similar for all conditions (1.069 ± 0.054 vs 0.8913 ± 0.083 vs 1.057 ± 0.035).

Stiffness of circumferentially oriented samples increased significantly following decellularisation and gamma-irradiation, from 0.821 ± 0.078 MPa to 39.294 ± 6.038 MPa (Figure 5.14; $p < 0.0001$). Storage for a further three weeks resulted in a significant decrease in the stiffness to 3.263 ± 0.699 MPa ($p < 0.001$), which was not significantly different from the native tissue. Ultimate tensile stress increased significantly following decellularisation and irradiation, at the four week time point (0.400 ± 0.038 MPa vs 0.986 ± 0.314 MPa; $p < 0.01$). The ultimate stress exhibited by irradiated, decellularised tissue after one week was intermediate to the native and four week values (0.561 ± 0.066 MPa). Ultimate strain decreased progressively, but non-significantly, from 1.415 ± 0.072 to 1.185 ± 0.066, and then 1.060 ± 0.120.
Table 5.1 Comparison of biomechanical properties of proximal and distal juvenile porcine oesophagus

<table>
<thead>
<tr>
<th>Storage Duration (weeks)</th>
<th>Section Position</th>
<th>Biomechanical Characteristic</th>
<th>Proximal</th>
<th>Distal</th>
<th>P Value</th>
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<td>Circumferential</td>
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<td>Ultimate Strain (mm/mm)</td>
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<td>Longitudinal</td>
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<td>0.765 ± 0.037</td>
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<td>Ultimate Strain (mm/mm)</td>
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<td>Longitudinal</td>
<td>Stiffness (MPa)</td>
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Construct Condition

- Native
- Decellularised, Non-irradiated
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<th>Section Position</th>
<th>Storage Duration (weeks)</th>
<th>Biomechanical Characteristic</th>
<th>Proximal</th>
<th>Distal</th>
<th>$P$ Value</th>
</tr>
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<tbody>
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<td>Decellularised, Irradiated</td>
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<td>Stiffness (MPa)</td>
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<td></td>
<td>Ultimate Stress (MPa)</td>
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<td>0.564 ± 0.116</td>
<td>0.9998</td>
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<td></td>
<td>Ultimate Strain (mm/mm)</td>
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<td>1.316 ± 0.078</td>
<td>0.0349</td>
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<tr>
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<td></td>
<td>4</td>
<td>Stiffness (MPa)</td>
<td>4.754 ± 0.409</td>
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<td>Ultimate Stress (MPa)</td>
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<td>Ultimate Strain (mm/mm)</td>
<td>0.843 ± 0.029</td>
<td>1.276 ± 0.155</td>
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Figure 5.13 Biomechanical properties of native and decellularised, non-irradiated juvenile porcine oesophagus. Longitudinal sections of decellularised tissue exhibited higher stiffness relative to native tissue (1.051 ± 0.160 MPa vs 0.676 ± 0.059 MPa; n=5), which did not significantly alter following four weeks storage at 4 °C (1.045 ± 0.207 MPa; n=5). Ultimate tensile stress was not notably affected following decellularisation (0.855 ± 0.123 MPa vs 0.718 ± 0.169 MPa; n=5), although after four weeks storage it significantly increased (1.497 ± 0.281 MPa; \( p < 0.05 \); n=5). Ultimate strain was unaffected following decellularisation and subsequent storage (1.069 ± 0.054 vs 0.891 ± 0.083 vs 1.057 ± 0.035; n=5).
Figure 5.14 Biomechanical properties of native and decellularised, irradiated juvenile porcine oesophagus. Circumferential sections of decellularised tissue exhibited a significant increase in stiffness following irradiation (0.821 ± 0.078 MPa vs 39.294 ± 6.038 MPa; \( p < 0.0001; n=6, n=11 \)). After four weeks storage this decreased significantly (39.294 ± 6.038 vs 3.263 ± 0.699 MPa; \( p <0.001; n=11, n=6 \)). Ultimate tensile stress of irradiated decellularised tissue was similar to native samples (0.561 ± 0.066 vs 0.400 ± 0.038 MPa; \( n=11, n=6 \)), however there was a significant increase in ultimate stress relative to the native, after four weeks storage at 4 °C (0.986 ± 0.314 MPa vs 0.400 ± 0.038 MPa; \( p <0.01; n=6, n=6 \)). Whilst there appeared to be a decrease in ultimate strain following decellularisation and irradiation, and subsequent storage, this was not statistically significant (1.415 ± 0.072 vs 1.185 ± 0.066 vs 1.060 ± 0.120; \( n=6, n=11, n=6 \)).
5.5.2 *In vivo* evaluation of decellularised juvenile porcine oesophagus

Decellularised scaffolds, produced using the semi-closed/closed-system three cycle process, were implanted to assess whether porcine oesophageal constructs generated from larger donors were more suitable for *in vivo* application in the large-animal model. Scaffolds were stented with 4 x 20 mm polydioxanone stents (ELLA-CS, Hradec Kralove, Czech Republic) and animals gastrostomised (n=3), with 13 animals also receiving a vasculature supporting muscle flap around the implant. Subsequently, a two-stage surgical approach was adopted, wherein primary heterotopic implantation in a muscle pedicle flap (Figure 5.15 (A)) to vascularise was followed by mobilisation of the flap and orthotopic implantation after 3-4 weeks (Figure 5.15 (B); n=2)). Macroscopic observation indicated a well vascularised graft at stage two, and was reported to demonstrate better handling properties and retention of sutures, resulting in improved anastomoses (Hannon et al., 2017). Overall, animal survival was improved and the muscle flap provided effective mitigation of oesophageal leakage.

![Figure 5.15](image)

*Figure 5.15* *In vivo* implantation of juvenile porcine oesophagus. Acellular porcine oesophagi from juvenile donors were stented and implanted in New Zealand White rabbits in a two-stage procedure. Stage one, heterotopic implantation of stented scaffolds resulted in macroscopic vascularisation of implants that was identifiable at stage two (A). Mobilisation of the pedicle flap enabled orthotopic implantation and anastomosis, whilst maintaining a vascular supply (B). Images reproduced with permission (Hannon et al., 2017).
5.6 MANUFACTURING SYSTEM SUITABILITY

Accumulation of anecdotal evidence from oesophageal decellularisation processes (n>60) enabled evaluation of the adaptation of the modular bioreactor for processing of tubular constructs. Functionally, the modified system was capable of aseptically perfusing tubular constructs in a semi-closed/closed-system. Bioreactor assembly, filling, perfusion and draining could be comfortably achieved with minimal training, as demonstrated by assessing operators that had no previous experience with the recellularisation bioreactor.

The system was not without caveats, however. During assembly, insertion of the Foley catheter into the bioreactor posed a moderate contamination risk due to the high level of manipulation and the tight fit of the catheter within the port. Subsequent location of the native oesophagus over the catheter opening and suturing in situ required greater than average dexterity and posed a high risk of contamination due to excessive handling within and immediately above the bioreactor body. It was observed that inadequate suturing would result in the scaffold being shed from the catheter in process, as a consequence of luminal perfusion. The bioreactor would then require opening and sutures reinforcing to resolve the issue. On a small number of occasions operator error led to over pressurisation of the chamber, when the waste line was not (fully) opened. This resulted in expulsion of the catheter from the lateral port, compromising system sterility.

Notably, the geometric constraints of the existing chamber rendered it unsuitable for processing tissues >60 mm in length. Locating oesophagi greater than 60 mm in length within the chamber required curvature of the tissue (Figure 5.9 (B)). This resulted in flow restriction along the length of the oesophagus, which subsequently caused incomplete decellularisation of regions of the tissue (Figure 5.16). Moreover, it was observed that tissue was deformed during the process such that it no longer formed a straight conduit post-decellularisation (Figure 5.16). Whilst this may be of little consequence when destructively characterising the scaffolds, it has clear implications for seeding and implantation investigation.
Figure 5.16 Gross morphology of decellularised juvenile oesophagus. Decellularisation of oesophagi >60 mm in length resulted in gross deformation of the construct, as tissues were positioned in a non-linear fashion inside the bioreactor. This resulted in incomplete and/or un-even decellularisation throughout the tissue, indicated by un-paled areas.
5.7 DISCUSSION

Building on its previous success, Chapter 5 sought to expand the functionality of the bioreactor to incorporate processing of tubular tissues. Colleagues recently demonstrated that rat oesophagi could be decellularised and recellularised to reconstitute cell populations across the distinct compartments of the native tissue (Urbani et al., 2018). They provided proof-of-principle evidence supporting the notion that biologically derived TEPs may one day provide a solution to the unmet clinical need associated with severe congenital and acquired oesophageal defects in paediatrics. To build upon these promising data, larger animal pilot studies were required to demonstrate reproducible product generation in preparation of preclinical studies using non-xenogeneic components. The modular bioreactor, having been validated for GMP-compliant airway product manufacture (Chapter 4), represented a proven system that could be adapted to facilitate the tissue decellularisation of larger oesophagi in a closed-system. This would simultaneously demonstrate the beginnings of GMP translation of the oesophageal production process in earnest, whilst also expanding the functionality of the modular bioreactor.

Adaptation of the modular bioreactor to accommodate oesophagi was dependent on developing a means of perfusing decellularisation reagents through the lumen of the tissue, whilst maintaining a closed-system. To avoid the need for bioreactor altering fabrication, it was proposed that a conduit that could be passed through one of the existing ports may provide a solution. It would be necessary that the part be fitted with a luer connector at the external terminus, could readily support oesophagus attachment internally and could form a seal between the internal and external environments. A Foley catheter was identified as a means of fulfilling all requirements, with the inflatable balloon located adjacent to the internal terminal capable of sealing the through port. Leak testing the bioreactor with the catheter in situ revealed that it was not necessary to inflate the balloon to maintain a liquid-tight unit; instead the compliant body of the catheter formed a complete seal when passed through the bioreactor wall. The external terminus of the catheter was readily fitted with a luer connector, and the resultant closed-system was successfully validated for sterile assembly and DET decellularisation operation using TSB. Whilst decellularisation process efficacy was yet to be investigated, the sterility investigation demonstrated the flexibility of the modular bioreactor for semi-closed/closed-system adaptation and aseptic operation. This would prove vitally important should the system be adopted for GMP-compliant processing.

Neonatal pigs were selected as a donor source for oesophagi for investigation in a NZW rabbit large-animal model. A robust procurement chain was established with JSR
Newbottle Pigs (JSR Farms Ltd) that enabled regular sourcing of neonatal donors, and a suitable retrieval approach was established for recovering donor tissues. Oesophagi were mounted inside the modular bioreactor and decellularised using a modified DET decellularisation protocol. The methodology applied during the small-animal study relied on two cycles of DET (Urbani et al., 2018), a process that had previously proven efficacious in decellularising rat intestine and hepatic tissue, as well as oesophagus from 12-16 week old pigs (Maghsoudlou et al., 2013, Totonelli et al., 2013, Maghsoudlou et al., 2016). To address the increased size of porcine derived oesophagi relative to that of the rat, the process was adapted to incorporate an additional DET cycle and was preceded and ended with an extended perfusion of water. Reagents were altered to facilitate GMP-compliant translation at a later date, removing those containing animal components that may pose a risk of transmitting animal spongiform encephalopathies. As has been alluded to in Chapter 2.2, whilst the adoption of compendium DNase may ensure its quality, it should be replace with a non-drug recombinant human DNase in future iterations of the decellularisation protocol. NaCl solution was replaced with HBSS containing Ca²⁺ and Mg²⁺, in accordance with the evidence suggesting greatly improved DNA hydrolysis in the presence of divalent cations (Melgar and Goldthwait, 1968, Price, 1972, Pan et al., 1998). Sterile reagents were sourced where possible, to reduce the cost and risk associated with in-house sterilisation of reagents.

Characterisation of decellularised neonatal porcine oesophagi demonstrated effective removal of cells and DNA from the tissue, when assessed using histology and biomolecular quantification. Microarchitecture was comparable between the native and decellularised tissue, as had been previously shown in the small-animal study, although there was a noted expansion of the mucosal layer. This was deemed to be insignificant however, as the existing re-epithelialisation strategy relies on removing the donor mucosa prior to seeding. Qualitative immunostaining of processed samples revealed ubiquitous maintenance of collagen I, a fibrillar collagen that is an essential component of compliant tissue ECM (Bosman and Stamenkovic, 2003). It also demonstrated the maintenance of collagen IV and laminin, both key constituents of the basement membrane (Kefalides, 2005, LeBleu et al., 2007), which would prove beneficial for effective re-epithelialisation of decellularised constructs. Whilst the immunostaining performed was able to demonstrate the maintenance of the assayed biomolecules, from a technical aspect it was sub-optimal. Higher quality images could have been produce using a red-fluorescent secondary antibody, due to the reduced scaffold autofluorescence of the red portion of the electromagnetic spectrum relative to the green region. Quantification of total collagen and sGAG content was also performed. Normalised total collagen concentration increased significantly following decellularisation. Whilst initially unexpected, it is proposed that this observation is due to
the relatively greater contribution of collagen to total ECM mass following the removal of the non-collagenous contributors to mass, i.e. cells. This is in accord with the findings reported by Totonelli et al. (2013) and with the data generated following the decellularisation of cartilaginous larynx tissue (Chapter 2.2), which has a significantly smaller cell density within the ECM and so does not experience the same relative increase in total collagen concentration. Normalised sGAG concentration decreased, as has been previously reported for DET decellularisation of oesophagus (Totonelli et al., 2013, Urbani et al., 2017, Urbani et al., 2018).

Having successfully demonstrated a reproducible process for decellularising neonatal porcine oesophagi in closed-system, constructs were sterilised using gamma-irradiation and implanted in a NZW rabbit, large-animal model. Whilst the majority interventions were complete without complication, all animals failed to meet the target survival date. Oesophageal obstruction and tearing, as well as severe weight loss, accounted for 50% of mortality. Both the implant and post-operative care regimen were evidently suboptimal.

To address the shortcomings of the acellular neonatal porcine oesophagi, it was proposed that matching the mass range of donor animals to that of the recipient NZW rabbit population would provide more appropriately sized tissue constructs for implantation. Subsequently, juvenile donors aged 6-8 days with a mass range of 2.4-3.8 kg were procured and oesophagi retrieved. Characterisation of the gross external dimensions of oesophagi indicated no difference in total length or OD at the proximal and distal termini. There was, however, a notable difference in the wall thickness of oesophagi that was discernible when mounting tissues within the bioreactor; although this was not quantified due to the destructive nature of the test and the relative value of animal derived tissue. An increase in total wall thickness was expected as the muscular compartment of the oesophagus grew post-natally.

To ensure that the larger tissue could still be effectively decellularised, standard key biomolecules were quantified. Processed scaffolds demonstrated a >92% reduction in DNA content, which was <1% less than the reduction observed in neonatal oesophagi. This led to the evaluation of a shortened decellularisation process that consisted of just two DET cycles, as was employed in the small-animal model. Reducing the number of cycles of DET would not only reduce the cost associated with reagents and staffing, but would have significant cost implications if the process was used for GMP-compliant manufacture. Evaluation of DNA removal in the two cycle process reported a >97% reduction, whilst both closed-system processes yielded comparable removal of DNA to the open-system process control (>95%). This clearly supported preference of two cycle decellularisation, rather than three cycle, for future processes.
Quantification of total collagen revealed comparable compositions between scaffolds produced with the different processes. All samples exhibited the increase in relative contribution to total mass that was observed in the neonatal tissues. Assaying oesophageal sGAG content revealed a sharp decrease across all replicates, with a mean reduction of >85% reported. Whilst a reduction was to be expected, it did not correlate with the mean reduction of <30% in the neonatal oesophagus. Whilst it is possible that the neonatal results are anomalous, this is unlikely given the number of replicates (n=4) that were processed as multiple batches (n=2). An alternative explanation may be that the decellularisation process has resulted in degradation of sGAGs to smaller sulphated disaccharides that cannot be detected by the 1, 9-dimethylmethylene blue assay employed. A greater understanding of the GAG profile of the native and decellularised tissue could be generated by using additional assays including commercial carbazole assays or high-performance liquid chromatography, a comparison of which is provided by Frazier et al. (2008). Ultimately, both collagen and GAG assays are typically employed to provide preliminary proxy indications of the biomechanical properties of scaffolds where physiologically relevant biomechanical profiling cannot be readily assayed. Given the relatively simple structural function of the oesophagus in vivo, it was conceivable that the relevant biomechanical properties of the scaffolds could be appropriately investigated.

The biomechanical properties of juvenile porcine oesophagi were evaluated pre- and post-decellularisation using a uniaxial tensile test until failure. Native tissue was assayed within 12 h of euthanasia and within 4 h of retrieval, to provide a more accurate reflection of the true native biomechanics. Importantly, both longitudinal and circumferentially oriented specimens were investigated to account for the full range of physiological movement that oesophagi experience in vivo. In man, the muscularis externa of the proximal oesophagus is dominated by striated muscle, whilst the distal transitions to smooth muscle. This is reflected in the porcine oesophagus (La Francesca et al., 2018), and so a comparison of the stiffness, ultimate stress and ultimate strain of tissue biopsied from the upper and lower thirds of the organ was undertaken. It indicated that in the majority of instances there was no discernible difference in the biomechanical profiles of the distinct regions, in both longitudinal and circumferential aspects. There was, however, a statistically significant increase in the ultimate strain experienced by the distal oesophagus relative to the proximal, following decellularisation and irradiation. To enable effective comparison of the implications of decellularisation and gamma-irradiation on the oesophagus, and in light of their broadly similar profiles, proximal and distal values were subsequently combined.
Following decellularisation, longitudinal specimens identified an increase in the stiffness of the oesophagus. This was expected and in accord with published Young's modulus data in similarly sized rabbit oesophagi that had been decellularised using a DET protocol (Urbani et al., 2017). Whilst stiffness appeared unchanged following storage up to four weeks, there was a statistically significant increase in the ultimate stress of the decellularised tissue between the first and fourth week of storage. This increase in strength may be due to ECM alterations that occur during the degradation process. Comparison of the ultimate strain of native and decellularised tissues did not reveal any difference between the conditions.

When oesophagi were irradiated following decellularisation, circumferential samples reported a statistically significant increase in stiffness relative to the native, non-irradiated organ. This sharp increase is likely due to irradiation induced cross-sectional enlargement and gross condensation of collagen fibrils within the ECM, as has been previously reported (Leontiou et al., 1993, Gouk et al., 2008). Stiffness did, however, drastically decrease after further storage at 4 °C. Combined, these data mirror the increase-decrease cycling in stiffness that was also reported for rabbit oesophagi when comparing native tissue to that stored for two weeks and six months at 4 °C (Urbani et al., 2017). Ultimate stress increased greatly at the four week time point, as had been observed for non-irradiated samples. Again there appeared to be no significant effect on the ultimate strain of the tissue across the samples, although the data did suggest a decrease following decellularisation and subsequent storage. Combining these data began to provide a characteristic profile of the processed tissue, although limiting starting material led to incomplete longitudinal and circumferential profiles across all conditions. Characterisation could be improved further through the implementation of additional assays such as biaxial relaxation testing or the suture retention test described by Pellegata et al. (2013), which may provide clinically relevant data to surgical colleagues. It will be important to investigate the optimal storage regimen for DET decellularised porcine oesophagi, as has been described in rabbit by Urbani et al. (2017), in accord with the varying biomechanical properties, to identify the most appropriate time-frame for subsequent cellularisation or implantation.

The ultimate goal of switching to larger donors was to provide constructs that were more suitable for the existing large-animal model. The irradiated decellularised scaffolds provided to the surgical team were reported to be superior to those derived from neonatal donors; more appropriately matching the resected tissue dimensions, as well as being more amenable to manipulation and formation of complete anastomoses that did not leak. Combining this with a series of surgical and post-operative improvements,
including stenting, gastrostomy, muscle-wrap and two-stage implantation, coincided with improved gross outcomes (Hannon et al., 2017).

Whilst the data generated supported the successful production of acellular constructs that were suitable of implantation in the existing large-animal model, there were a number of issues associated with the scaffold production process. Although it did not represent a significant limitation during the process development phase, during which large portions of native tissue were being removed and assayed to provide baseline data, internal chamber dimensions were clearly unsuitable for processing organs >60 mm in length. This required rectifying prior to seeding investigations and additional animal studies, to ensure maximum output from retrieved donor tissues. Moreover, though the data supported the proof-of-concept expansion of the modular bioreactor to incorporate processing of tubular organs, it was evident that the system was sub-optimal. Efficient processing of tubular constructs would likely be better achieved using a bespoke bioreactor system with features that reduced the operational contamination risk and increased user ease-of-use.

5.8 CONCLUSION

Following the functional adaptation of the modular bioreactor for processing tubular tissues, it was demonstrated that the system could be aseptically operated in a semi-closed/closed manner. Porcine oesophagi, derived from neonatal and juvenile donors, provided a model system for demonstrating that the bioreactor could be used for hollow organ tissue engineering applications. Decellularising oesophagi using a modified DET process simultaneously demonstrated scale-up of a protocol that colleagues had utilised to produce rat scaffolds in a proof-of-principle study, with a long-term goal of addressing indications in paediatric oesophageal pathologies. Characterisation of the constructs confirmed decellularisation, with key biomolecule and biomechanical profiles reflecting those reported by colleagues in rat and rabbit models. In vivo implantation of the final constructs appeared positive, although further work is required to investigate construct recellularisation and long-term graft integration. Whilst each of the original objectives were met, it became evident that the adaptation of the modular bioreactor did not provide an optimal solution for the long-term production of acellular tubular constructs for tissue engineering. The positive progress in the initial animal studies has resulted in the award of funding for a pivotal preclinical large-animal study, and so an improved system is recommended for the production of acellular oesophageal scaffolds.
6 DESIGN, MANUFACTURE AND PROOF-OF-CONCEPT OF A NOVEL, MODULAR BIOREACTOR FOR HOLLOW TISSUE PROCESSING

6.1 INTRODUCTION

Subsequent to the findings on the suitability of the modular bioreactor system to produce acellular oesophageal constructs for in vivo implantation (Chapter 5), it was evident that an improved system was required to scale-up decellularised scaffolds and to refine the manufacturing process. Chapter 6 provides an account of the design and realisation of a novel bioreactor, specifically tailored to the requirements of perfusion decellularisation processing hollow tissues, which was subsequently paired with a bespoke system that enables automated, closed-system decellularisation of juvenile porcine oesophagi.

6.2 AIMS AND OBJECTIVES

6.2.1 Aims

The purpose of the work undertaken was, primarily, to produce a novel bioreactor that could accommodate and decellularise hollow tissues that could not be suitably processed using the modular bioreactor, as described in Chapter 5. Secondarily, it was intended that the new bioreactor form a constituent part of a larger system that would enable fully closed processing. This system would also be automated, to reduce the requirement for manual operation and reduce the risk of operator error.

6.2.2 Objectives

- To design and fabricate a novel, modular bioreactor that could be used to process small to medium sized hollow tissue constructs.
- To assemble a system from off-the-shelf components that enables closed-system processing.
- To automate the system assembly, such that juvenile porcine oesophagi can be decellularised without requiring an operator to change reagents.
6.3 DESIGN OF A BIOREACTOR FOR HOLLOW TISSUE PROCESSING

6.3.1 User requirement specification and design specification

Following identification of the sub-optimal characteristics of the initial bioreactor system employed during the decellularisation of oesophagi, primarily internal dimension restrictions and oesophagus attachment approach, a URS was composed for a novel, modular bioreactor system (Table 6.1). The URS was generated in accordance with the underlying principle of modular functionality within a closed-system. In addition to decellularisation processing, it was proposed that the system be suitable for recellularisation of oesophageal constructs. This was highlighted during the small-animal model studies as an integral factor in effective translation to clinic, via a GMP-compliant manufacturing process, that would require the design and manufacture of a closed-system bioreactor (Urbani et al., 2018).

In keeping with previously demonstrated multi-functionality, it would be beneficial if the novel bioreactor system could be utilised for processing additional hollow tissues such as vessels, pulmonary structures and larger gastrointestinal tract constituents. Processing would not be limited to decellularisation alone, but may also include recellularisation. This influenced the final dimensions and material requirements of the bioreactor, as outlined in the DS (Table 6.2).
Table 6.1 User requirement specification for hollow tissue bioreactor

<table>
<thead>
<tr>
<th>UR#</th>
<th>User Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Consist of a closed-system that forms distinct, luminal and abluminal compartments once constructs are <em>in situ</em>.</td>
</tr>
<tr>
<td>2</td>
<td>Allow for liquid transfer across the internal-external wall in a semi-closed/closed* manner.</td>
</tr>
<tr>
<td>3</td>
<td>Employ a modular system for construct mounting outside of the bioreactor chamber.</td>
</tr>
<tr>
<td>4</td>
<td>Variable length mounting mechanism for constructs ranging from 40-120 mm.</td>
</tr>
<tr>
<td>5</td>
<td>Mechanism to rotate mounted construct through 360° along its Z-axis.</td>
</tr>
<tr>
<td>6</td>
<td>User-friendly insertion/removal of construct in/from the chamber at manufacturing and clinical sites.</td>
</tr>
<tr>
<td>7</td>
<td>Minimise risk of contamination during cell seeding and product delivery.</td>
</tr>
<tr>
<td>8</td>
<td>Function as primary packaging.</td>
</tr>
<tr>
<td>9</td>
<td>Single-use functionality.</td>
</tr>
<tr>
<td>10</td>
<td>Constructed of biocompatible materials.</td>
</tr>
<tr>
<td>11</td>
<td>Constructed of materials that can be suitably operated at 16-37 ± 0.5 °C, without degradation or deformation.</td>
</tr>
<tr>
<td>12</td>
<td>Constructed of inert materials that are resistant to degradation by those reagents used in the decellularisation/recellularisation process.</td>
</tr>
<tr>
<td>13</td>
<td>Constructed of materials that are suitable for repeated sterilisation by autoclaving at 121 °C.</td>
</tr>
</tbody>
</table>

* semi-closed defines a process or action that, whilst not entirely closed, has been deemed significantly low risk so as to allow processing in a Grade A environment with a Grade D background.
Table 6.2 Design specification for hollow tissue bioreactor

<table>
<thead>
<tr>
<th>UR#</th>
<th>DS#</th>
<th>Design Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.1</td>
<td>The bioreactor should possess conduits that connect to both proximal and distal tissue termini, within a closed chamber.</td>
</tr>
<tr>
<td>1</td>
<td>1.2</td>
<td>A liquid-tight seal should be formed between tissue and conduits.</td>
</tr>
<tr>
<td>2</td>
<td>2.1</td>
<td>Chamber body should accommodate luer fittings.</td>
</tr>
<tr>
<td>3</td>
<td>3.1</td>
<td>Employ a cassette that tissue can be mounted to outside of the bioreactor, before placing inside the chamber and forming a sealed conduit with the outer environment.</td>
</tr>
<tr>
<td>4</td>
<td>4.1</td>
<td>A single cassette must allow variation of size for mounting tissue, ranging from 40-120 mm in length.</td>
</tr>
<tr>
<td>4</td>
<td>4.2</td>
<td>The cassette must accommodate constructs up to 30 mm OD.</td>
</tr>
<tr>
<td>4</td>
<td>4.3</td>
<td>A locator mechanism is required to prevent inadvertent length variation following mounting.</td>
</tr>
<tr>
<td>5</td>
<td>5.1</td>
<td>The cassette must enable torsion-free rotation of mounted tissue, both inside and outside of the chamber body.</td>
</tr>
<tr>
<td>6</td>
<td>6.1</td>
<td>It must be possible to easily insert/remove the cassette or product into/from the bioreactor using forceps, in an aseptic manner.</td>
</tr>
<tr>
<td>7</td>
<td>7.1</td>
<td>The cassette should allow for seeding outside of the bioreactor main body, with a low contamination risk associated with returning it to the main body.</td>
</tr>
<tr>
<td>7</td>
<td>7.2</td>
<td>Alternatively, if seeding constructs whilst located inside the bioreactor, cassettes should position tissue scaffolds such that they do not require excessive movement of pipettes inside the main chamber body.</td>
</tr>
<tr>
<td>8</td>
<td>8.1</td>
<td>The bioreactor assembly must possess external dimensions of less than 33.0 cm x 16.5 cm x 31.0 cm, to align with the existing transportation packaging in the CCGTT.</td>
</tr>
<tr>
<td>9</td>
<td>9.1</td>
<td>The bioreactor, including ancillaries, must cost less than £2500.</td>
</tr>
</tbody>
</table>
6.3.2 Conceptualisation and design

Following the successful design and realisation of the modular bioreactor first described in Chapter 4, it was proposed that CNC machining again be used for fabrication. Due to contemporary timeline and capacity constraints associated the Workshop previously used, it was necessary to identify and engage with an alternate fabricator. It was important that the new workshop be amenable to a highly interactive approach, given the prototypical nature of the design and manufacture process. Whilst it was important that the workshop had the capacity to engage in an iterative design-fabrication cycle, absolute capacity did would not exceed 10 units per year. It was noted that it would be highly beneficial if the fabricators had previous experience with manufacturing parts for use within the biomedical industry, as had previously been the case. Subsequently, the Mechanical Workshop within the Department of Medical Physics and Biomedical Engineering at University College Hospitals was approached and assessed against the aforementioned requirements, before being engaged.

The operational mechanism that was conceived for realising the mechanistic points of the DS (DS#1.1-DS#7.2) was founded on a cassette system that allowed tissue to be mounted outside of the bioreactor body, before transferring inside the chamber and attaching to the conduits that would enable luminal perfusion. Alignment of a threaded portion of the external conduit with an external gauge would allow fine control of the position of the conduit within the bioreactor body, and thus the strain of the mounted construct. Simultaneous rotation of the gauge and conduit, at both ends of the supported tissue, would allow for 360 degree rotation of the construct.

<table>
<thead>
<tr>
<th>UR#</th>
<th>DS#</th>
<th>Design Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10.1</td>
<td>The materials that come into contact with the starting material or the final product, as well as those which contact the supporting medium, must have proven examples of biocompatible application with at least one cell type typically used in hollow organ tissue engineering applications.</td>
</tr>
<tr>
<td>11</td>
<td>11.1</td>
<td>The materials used for fabrication must have a continuous operating temperature range that encompasses 16-37 ± 0.5 °C.</td>
</tr>
<tr>
<td>12</td>
<td>12.1</td>
<td>Constituent materials must possess broadly inert profiles, preferably with proven application with media used for culturing human cells.</td>
</tr>
<tr>
<td>13</td>
<td>13.1</td>
<td>The fabrication materials must have proven application in situations that involve repeated sterilisation by steam at 121 °C.</td>
</tr>
</tbody>
</table>
6.3.2.1 Material selection

In light of the fabrication complications reported during the manufacture of the modular bioreactor described in Chapter 4, it was determined that PTFE would not be a suitable material from which to manufacture the novel bioreactor. This decision was also influenced by the reduced fabrication capability of the Mechanical Workshop within the Department of Medical Physics and Biomedical Engineering at University College Hospitals (section 2.5.1.1). Given the Workshop’s propensity to work with hard metals, it was proposed that a material with similar machining properties to stainless steel be investigated. The candidate material would also have to suitably address DS#9.1-DS#13.1.

Searching anecdotal literature and consultation with the Workshop fabrication engineer indicated that polyoxymethylene (POM) is extremely amenable to traditional machining approaches and would be within the capabilities of the Workshop to fabricate from. It also possess a range of desirable physical properties including resistance to a broad range solvents (Quadrant, N/A), low friction, low wear and impact resistance (British Plastics Federation, 2018). It also demonstrates low absorption and excellent dimensional stability, which are highly desirable for maintaining tolerances in aqueous operational environments such as bioreactors. Moving parts are aided by natural lubricity, and the thermoplastic has a wide continuous operating temperature range from -50 °C to 90 °C (DS#11.1; (British Plastics Federation, 2018)).

Whilst POM has a proven history of application within the medical industry, it was necessary to demonstrate evidence supporting its suitability for use with cells typically used in tissue engineering applications. This was succinctly shown by Penick et al. (2005), whom investigated the effects of POM conditioned medium on BM-MSC proliferation and differentiation. Having subjected machined POM to up to 20 autoclave cycles (DS#13.1), the resultant conditioned medium had no discernible effect on the proliferation or chondrogenic differentiation of BM-MSCs; demonstrating material stability in medium and biocompatibility (DS#10.1, DS#12.1). Combined with the aforementioned physical properties of POM, this supported its suitability for bioreactor fabrication.

Silicone was again used as the material of choice for O-rings, in accordance with section 4.4.2.1. As described for the design and drafting of the previous modular bioreactor, conception and realisation of the design was a concomitant process. Each part has been described in a linear fashion, however, to provide a comprehensible description.
6.3.2.2 Chamber body

The principle mechanism for providing distinct luminal and abluminal compartments was similar to that employed by tubular construct bioreactors described elsewhere (Asnaghi et al., 2009, Pellegata et al., 2012, Crowley, 2015, Pellegata et al., 2015, Urbani et al., 2018). The proposed system would support hollow tissue constructs on collinear shafts that extended from internal to external environments, bounded by an outer bioreactor body.

The chamber was designed with a high aspect ratio, with internal dimensions large enough to accommodate a cassette that would support tissue up to 120 mm in length with an OD of ≤30 mm (Figure 6.1). The chamber was intended to be machined from 50 mm sheet POM to a maximum internal depth of 40.00 mm. Internal radii in the Z-axis were 10.00 mm, enabling deviation-free machining to the required depth. The base of the internal chamber was drafted from a nominal depth of 35.00 mm to a drainage port at 40.00 mm. The port consisted of a 5.00 mm OD hole machined to 3.50 mm in the Z-axis, which was then intersected by an orthogonal 3.00 mm clearance hole along the X-axis. This was drilled and tapped to receive a 1/4-28 UNF thread to 8.00 mm. The tapped hole was centred at an 8.00 mm offset from the base, to ensure adequate clearance for the connector during assembly and operation.

Front and back faces, as well as the head face, were designed to accommodate retainer flanges for locating opposing crush plates and the head plate (Figure 6.1). A clearance hole of 12.78 mm through the front and back faces was drafted for the shafts that were to act as conduits across the bioreactor body wall. Clearance holes were centred on the midline between left and right faces, offset 14.00 mm from the head face. This was to provide clearance for tissues up to 30 mm OD. Glands were drafted with a 15.77 mm diameter to a depth of 1.91 mm, in accordance with existing guidelines for BS014 O-ring use in oscillating shaft applications (Parker Hannifin Corporation, 2007). The seal achieved using a BS014 O-ring, corresponding gland, and 12.47 mm rod was confirmed using an offcut model prior to final fabrication.

Radii were machined at non-mating vertices to reduce the risk posed by sharp edges.
Figure 6.1 Hollow tissue bioreactor body drafted design.

Dimension units: mm
6.3.2.3 Cassette insert and adapters

The cassette insert and adapters were key to the operational mechanism that would accommodate tissues of varied length, using a single cassette, as well as rotation about the collinear axis of mounted hollow tissues. The cassette (Figure 6.2) was designed to incorporate a sliding clip part (Figure 6.3) that facilitated length variation, whilst connectors associated with the variable clip (Figure 6.4) and rail cassette (Figure 6.5) provided adapter fittings for affixing variably sized hose barbs. The connectors were free to rotate uniaxially within the locating recesses of the rail cassette and clip, thus driving tissue rotation. They also provide the connection between the mounted hollow tissue and conduit passing through the bioreactor body wall.

The rail cassette and internal dimensions of the bioreactor body (Figure 6.1) were co-developed and drafted to ensure correct fit. The cassette possessed two grooves that determined the maximum linear movement that could be achieved by the associated clip part. One groove acted as an initial locator feature for the clip (Figure 6.2 (Front and A)), whilst the second ensured that the clip remained engaged with the rail by means of screw-nut pairing (Figure 6.2 (Top and Isometric)). The length of the grooves was determined by the minimum length of tissue to be mounted (DS#4.1) and the size of female 1/8” BSP to male luer thread connectors and luer thread hose barbs. Graduations were marked on the Top facing X-Y surface of the cassette to provide an indication of the strain of mounted tissue.

Whilst the base of the cassette was planar to allow for stable positioning on level surfaces, the opposing surface was curved to provide clearance for mounted tubular constructs ≤30 mm (Figure 6.2 (Front)). A clearance groove located on the midline of the X-Y plane, at the lowest point of curvature, was designed to prevent pooling of reagent when draining the chamber with the cassette in situ. Cassette tolerances were to be machined to allow for a sliding, clearance fit when inserted into the chamber body.

The clip was designed with a cross sectional profile (X-Z plane) that would align with the mating surface of the rail cassette. Whilst the width of the clip matched that of the rail cassette, the assembled cassette and clip would not abut the head plate when located within the bioreactor body. Instead, clearance was allowed for a cap head screw to be located in the clip (Figure 6.3 (Top)) to prevent rotational movement of luer lock fitting Pt1 once assembled (Figure 6.4). A clearance hole, collinear to the corresponding clearance hole in the bioreactor body, located luer lock fitting Pt1. A second clearance hole, in the Z-axis, was designed to accommodate the aforementioned screw-nut pairing for maintaining engagement between the clip and rail cassette. This could be tightened to prevent inadvertent movement of the clip following desired positioning.
The luer lock fitting Pt1 (Figure 6.4) was designed to provide a connection between shaft Pt1 and a female 1/8” BSP to male luer thread connector. This was achieved with an internal left hand M8 x 1.25 thread and external BSP 1/8” G thread, respectively. A left hand thread was tapped to prevent loosening of the fitting during mounted tissue rotation. The outer dimensions of the connector were in accordance with the corresponding clearance hole of the clip.

Luer lock fitting Pt2 (Figure 6.5) was similarly designed to connect shaft Pt2 and a female 1/8” BSP to male luer thread connector. A left hand thread was tapped to prevent loosening of the fitting during mounted tissue rotation. The outer dimensions of the part were designed against the corresponding recess in the rail cassette, with clearance to allow free rotation.
Figure 6.2 Hollow tissue bioreactor rail cassette drafted design.

Dimension units: mm
Figure 6.3 Hollow tissue bioreactor rail cassette clip drafted design.

Dimension units: mm
Figure 6.4 Hollow tissue bioreactor cassette luer lock fitting Pt1 drafted design.

Dimension units: mm
Figure 6.5 Hollow tissue bioreactor cassette luer lock fitting Pt2 drafted design.

Dimension units: mm
6.3.2.4 Shafts and gauges

To enable both linear and rotational movement of the shafts once assembled through the bioreactor body wall (DS4.1 and DS#5.1), a seal was designed using existing guidelines for oscillating shaft applications (Parker Hannifin Corporation, 2007). Only one of the collinear shafts was required to move linearly, which would be driven by the rotation of a threaded gauge that aligned with a corresponding threaded section of the shaft. Shafts were designed such that termini that were to be positioned internally would connect to luer lock connector fittings, which in turn were joined to female 1/8” BSP to male luer thread connectors that terminated with luer thread hose barbs. This assembly mechanism would allow for tissue to be mounted outside of the bioreactor, before placing inside the chamber and forming a sealed conduit (DS#1.2 and DS#3.1).

A gland crush plate was designed to abut the Front and Back surfaces of the bioreactor body, ensuring that O-ring BS014 did not extrude during operation (Figure 6.6). The X-Z plane profile dimensions of the plate corresponded to that of the front/back surface of the bioreactor body. A 12.78 mm clearance hole was collinearly located on the centre axis of the clearance hole on the Front of the bioreactor body. Clearance holes were positioned to allow clamping of the plate using knurled thumb screws and corresponding retainer flanges located in the bioreactor body. The plate was designed with a 40.00 mm diameter recess centred on the 12.78 mm clearance hole to accommodate the crush gauge (Figure 6.7) that would control the linear and rotational movement of shaft Pt1 (Figure 6.8) and the rotational movement of shaft Pt2 (Figure 6.9). A slot was machined to allow insertion and removal of the crush gauge in the Z-axis.

The crush gauge was designed to provide an internally threaded gauge that would drive linear movement of shaft Pt1 by engaging with its threaded section. The length of the part was drafted to 60.00 mm to ensure manual rotation could be easily achieved (Figure 6.7). The 40.00 mm OD terminus was notched on the outer surface to aid manual rotation, whilst incremental markings on the Front face provided an indicator of degrees of rotation. The clearance hole in Figure 6.7 was drafted at 15.00 mm, providing a pilot hole for subsequent tapping.

Shaft Pt1 was designed to form a conduit connecting the environment external to the bioreactor with the luminal compartment of the mounted tissue construct (Figure 6.8). The terminus that was positioned within the bioreactor was to be tapped with a left hand, M8 x 1.25 thread to correspond to luer lock fitting Pt 1. The external terminus was drafted with a BSP 1/8” G thread. The shaft body was externally threaded to M14 x 2.00, at a 26.00 mm offset from the external terminus. The thread length of 90.00 mm provided a corresponding 90.00 mm minimum range of movement, although only 80 mm of
movement was required to accommodate the tissue size target range (DS#4.1). The shaft bore was drafted to 4.00 mm, to ensure that it did not restrict flow.

Shaft Pt2 was limited to 100.00 mm length, with no threaded region, as it was only required for construct rotation and not linear variation. The OD of 12.47 mm conformed to the sealing requirements previously outlined. Bore design, as well as threading of external and internal termini matched that of shaft Pt1.
Figure 6.6 Hollow tissue bioreactor gland crush plate drafted design.

Dimension units: mm
Figure 6.7 Hollow tissue bioreactor crush gauge drafted design.

Dimension units: mm
Figure 6.8 Hollow tissue bioreactor shaft Pt1 drafted design.

Dimension units: mm
Figure 6.9 Hollow tissue bioreactor shaft Pt2 drafted design.

Dimension units: mm
6.3.2.5 Head plate

The head plate was drafted such that the mating face profile matched that of the Top face of the bioreactor body (Figure 6.10). Non-threaded clearance holes with a 6.80 mm diameter were designed to allow knurled thumb screws to engage with corresponding retainer flanges in the bioreactor body. Holes were not countersunk and instead provided a surface against which the shoulder of the thumb screws could abut. Two 1/4-28 UNF, Z-axis clearance holes were drafted at a 41.00 mm offset from one end of the head plate, each centred at 7.50 mm offset from the Y-axis centreline. The bottom surface of the head plate had a centred 195.00 mm x 44.00 mm profile extruded to 5.00 mm. This was designed to sit within the internal of the bioreactor body, with a seal formed by compression of a BS257 O-ring located between the shoulder created by the extrusion and the top surface of the bioreactor body. An extrusion was designed to hollow the section of head plate that would locate within the bioreactor, to allow for mounting of tissues ≤30 mm (DS#4.2).
Figure 6.10 Hollow tissue bioreactor head plate drafted design.

Dimension units: mm
6.3.3 Fabrication

Drafted designs were evaluated for fabrication feasibility by a member of the Workshop prior to manufacture. To help understanding of how parts would interact with one another, 3D models were rendered using Fusion 360 software package (Figure 6.11).

Raw materials were sourced and a single bioreactor assembly prototype fabricated, to allow for evaluation and reiteration prior to the production of additional units. It was highlighted by the Workshop engineer that there was a significant chance of the shaft-driving thread mechanism seizing during operation, due to wear between interacting threads machined from the same material. This could be overcome by ensuring that one of the threaded components was harder than the other, and so a threaded brass insert was sourced and located within the crush gauge. This can be observed as a darkened region in the gauge shown in the fully assembled bioreactor depicted in Figure 6.12. All parts were assembled and checked for fit by the Workshop prior to release.

6.3.4 Leak testing

A decellularised juvenile porcine oesophagus was mounted in the cassette before inserting into the bioreactor body and connecting the shafts to the corresponding luer lock fitting connectors to form a conduit with the oesophageal lumen (Figure 6.12). No leak was detected along the entirety of the conduit when medium was passed through the lumen. Moreover, no leaks were noted from any connection when the abluminal compartment was filled and the oesophagus perfused (data not shown).
Figure 6.11 Hollow tissue bioreactor assembly render. Assembled bioreactor (A) and exploded render demonstrate part alignment (B). Cassette insert part alignment is also demonstrated (C).
Figure 6.12 Hollow tissue bioreactor assembly. Fully assembled bioreactor fabricated from polyoxymethylene, without polycarbonate head plate (A-C). An acellular juvenile porcine oesophagus is suture mounted in situ. No leaks were detected when medium was passed through the lumen of the construct (C).
6.4 DESIGN OF AN AUTOMATED SYSTEM FOR JUVENILE PORCINE OESOPHAGUS DECELLULARISATION

6.4.1 User requirement specification and design specification

To reduce the requirement for manual intervention and operation during the decellularisation process, and to reduce the risk of human error compromising process sterility, it was proposed that an automated DET decellularisation process (autoDET) be developed. The system would be developed as a proof-of-principle prototype to assess feasibility and deliverable benefit. The URS and DS are outlined in Table 6.3 and Table 6.4 respectively.

Table 6.3 User requirement specification for automated decellularisation system

<table>
<thead>
<tr>
<th>UR#</th>
<th>User Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The assembly should form a fully closed-system.</td>
</tr>
<tr>
<td>2</td>
<td>Following initial set-up, the system should be capable of completing a full decellularisation process without intermediate intervention.</td>
</tr>
<tr>
<td>3</td>
<td>Decellularisation efficacy should be comparable to the manual process.</td>
</tr>
</tbody>
</table>
Table 6.4 Design specification for automated decellularisation system

<table>
<thead>
<tr>
<th>UR#</th>
<th>DS#</th>
<th>Design Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.1</td>
<td>Reagents and waste should be maintained in vented reservoirs that can be connected to the bioreactor assembly via luer connections.</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>Control of fluid movement should not compromise system closure.</td>
</tr>
<tr>
<td>2</td>
<td>2.1</td>
<td>The assembled system should consists of four subsystems, including reagent/waste reservoirs, the hollow tissue bioreactor, fluid movement hardware and a controller unit.</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>The controller unit must determine the sequence and timing of fluid movement to replicate the filling, perfusion and draining that is performed during the manual decellularisation process.</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
<td>Following initialisation, the process should not require user intervention to execute any of the processing steps.</td>
</tr>
<tr>
<td>3</td>
<td>3.1</td>
<td>DNA reduction efficacy, as a function of starting DNA concentration, must be within 10% of the manual process.</td>
</tr>
<tr>
<td>N/A</td>
<td>4.1</td>
<td>Opening-closure of fluid paths should be via valves that do not directly contact reagents.</td>
</tr>
</tbody>
</table>
6.4.2 Conceptualisation and design

6.4.2.1 Process flow

The conceived system for automated decellularisation followed the typical process flow that has been outlined for perfusion-based decellularisation systems (Pellegata et al., 2012, Guyette et al., 2014, Price et al., 2015). Reagents were maintained in three distinct vented reservoirs, with a fluid line leading from each reservoir into a manifold constructed from three 3WSCs connected in series (Figure 6.13). Each line was independently opened/closed by a solenoid pinch valve, before the manifold condensed the three input lines to two outputs that were mounted in a 3/2 solenoid pinch valve. The normally-closed output path passed through a high-geared peristaltic pump that fed into the abluminal compartment of the hollow tissue bioreactor, whilst the normally-open passed through a low-geared peristatic pump and affixed to the conduit that connected to the luminal compartment of the mounted tissue. Waste lines from the luminal and abluminal compartments connected the bioreactor to a waste reservoir, via a 3/2 solenoid pinch valve. The line from the abluminal compartment passed through the normally closed path of the valve and via a high-geared peristaltic pump. A vent filter was attached to the head plate of the bioreactor via a pinch valve, which was opened to allowing pressure equalisation during filling and draining of the abluminal compartment of the bioreactor (Figure 6.13 (A and C)). Sequential operation of pumps and valves was controlled by a microcontroller. A detailed schematic representation of the system hardware is shown in Figure 2.6, whilst Figure 6.13 details the final process flow.
Figure 6.13 Operational fluid flow of autoDET. During the filling phase (A), reagents are drawn from sterile, vented reservoirs (a) through an open pinch valve (b) and manifold set (c) by the peristaltic operation of a high-geared pump (d). As reagents fill the bioreactor, air is displaced via an open, filtered vent (e). The luminal line to the waste reservoir (f) is open throughout the process. During the decellularisation phase (B) the reagent is routed via the low-geared pump (g) and the filtered vent is closed. The draining phase (C) re-opens the filtered vent and reagent withdrawn from the chamber via a second high-geared pump (h). A cleaning phase (D) flushes the manifold with water, between each reagent change.
6.4.2.2 Control system and programme

To provide greater reliability and stability, a microcontroller approach was adopted in preference to a personal computer platform. This had the added benefit of decreasing the footprint of the controller system, which is becoming ever more important with laboratory bench space at a premium. Actuation and timing of motors and solenoids, both relative and absolute, was controlled by an ATmega328 microcontroller, mounted on an open source Arduino Uno r3 board. This was connected to a series of modular boards for driving motors.

The programme consisted of a series of repeated operations that controlled each phase of the process. Table 6.5 broadly outlines the respective actuation for each phase. Timing of operations was controlled using the “millis” function that utilises the internal timing capacity of the microcontroller. The code for the autoDET programme, which consisted of two cycles, is located on the appended CD.

All actuators and circuitry were initially assembled and housed in a plastic chassis to provide a compact system that could be easily moved (Figure 6.14). This proved restrictive when introducing additional hardware, as well as posing a significant overheating risk, and so was disassembled. Hardware was subsequently mounted on a polystyrene board to allow for ease of access and effective heat dissipation.
Table 6.5 Hardware actuation during autoDET execution

<table>
<thead>
<tr>
<th>Motor</th>
<th>Resting Status</th>
<th>autoDET Phase Actuation</th>
<th>Filling</th>
<th>Decellularisation</th>
<th>Draining</th>
<th>Cleaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase/SDC 2/2 solenoid pinch valve</td>
<td>Closed</td>
<td>+/-*</td>
<td>+/-*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water 2/2 solenoid pinch valve</td>
<td>Open</td>
<td>+/-*</td>
<td>+/-*</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3/2 solenoid pinch valve</td>
<td>N/A</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>High/geared peristaltic pump</td>
<td>Off</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Low/geared peristaltic pump</td>
<td>Off</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Filter 2/2 solenoid pinch valve</td>
<td>Closed</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Waste 3/2 solenoid pinch valve</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Waste peristaltic pump</td>
<td>Off</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* Actuation dependent on reagent step.
Figure 6.14 Electronic hardware for autoDET. Front (A) and reverse (B) views of the hardware supporting chassis demonstrate solenoid valve and peristaltic pump positioning. Power inlet can be seen in the front view (A).
6.4.3 Tissue-free functional assessment

The autoDET programme was altered such that each phase of actuation was limited to 10 s. The sequence of hardware actuation for a full decellularisation process was assessed against the theoretical order. The process was stopped and code altered to correct any errors that arose.

Solenoid pinch valve activation voltages and peristaltic pump flow rates were determined as described in section 2.5.2.3.

Subsequently the autoDET hardware, including bioreactor and reagent/waste reservoirs, was assembled. The full autoDET programme was run, with all reagents replaced with water. The system was observed during the transition period between each phase, confirming that all solenoids and motors were actuated in the correct sequence (Table 6.5). It also allowed for refinement of the timing of filling and draining phases for each reagent, with the programme script altered accordingly.

6.4.4 Decellularisation proof-of-concept

6.4.4.1 Gross functional assessment

Juvenile porcine oesophagi were mounted within the hollow tissue bioreactor and decellularised using the autoDET. Whilst assembly of the bioreactor could be readily performed in a manner that was at lower risk of contamination than the original modular bioreactor (Chapter 5), it was notably slower; taking approximately one hour to fully assemble the system and mount the tissue. Great care had to be taken when connecting each shaft with its corresponding luer lock fitting connector, to prevent inadvertent torsion of the mounted oesophagus. Mounted tissue strain could be readily controlled without risk of increased tension.

Observation during decellularisation indicated no lateral translation of the oesophagi termini from the hose barbs on which they were mounted. There was no evidence of blockages throughout the process, although this was to be expected given the relatively large bore sizes employed throughout the system.

Notably, it was observed that positioning of the waste reservoir had a significant effect on the patency of the mounted oesophagus. When the reservoir was positioned at a negative height to the bioreactor, the relative pressure differential resulted in collapse of the oesophagus lumen (Figure 6.15 (A)). This was rectified by positioning the waste reservoir within ca. 10 cm of the bioreactor, in the Z-axis (Figure 6.15 (B)).
Gross evaluation of decellularised oesophagi revealed pale constructs that appeared, macroscopically, to be uniformly decellularised. Importantly, constructs did not exhibit the curvature that was typically observed following decellularisation in the original modular bioreactor (Figure 6.16).

Figure 6.15 Juvenile porcine oesophagus autoDET. Positioning of the waste vessel >30 cm below the bioreactor chamber resulted in a negative pressure that induced collapse of the oesophagus lumen during processing (A). This was remediated by raising the waste vessel to ca. 10 cm below the chamber, resulting in a patent lumen (B).
Figure 6.16 Comparison of autoDET and conventionally decellularised juvenile porcine oesophagus. Organs decellularised using the autoDET yielded scaffolds that were grossly pale and straight (above) relative to conventionality decellularised tissue (below), which exhibited a degree of curvature due to the physical constraints of the original modular bioreactor.
6.4.4.1.1 Quantitative analysis of autoDET decellularised juvenile porcine oesophagus

Quantitative analysis of DNA concentration was employed to evaluate the efficacy of decellularisation (Figure 6.17). Normalised content significantly decreased from 2184.58 ± 597.24 ng mg⁻¹ to 171.15 ± 94.04 ng mg⁻¹ \((p <0.05; n=4)\), corresponding to a 92.17% reduction in DNA. This was comfortably within 10% of the DNA reduction efficacy of all previously reported manual decellularisations, which ranged from 92.56-97.18%.

Assessment of total collagen revealed a significant increase in relative concentration from 127.28 ± 9.88 µg mg⁻¹ to 285.13 ± 41.91 µg mg⁻¹ \((p <0.05; n=4)\). This was in accord with the previously reported trend (section 5.5.1.1).

Quantification of sGAG indicated a reduction from 15.53 ± 4.35 µg mg⁻¹ to 5.70 ± 1.00 µg mg⁻¹ \((n=4)\) following decellularisation (Figure 6.18). This represented a mean 36.70% retention of sGAGs. It was evident that the native data were substantially skewed, with a skewness value of 1.232. When the potential outlier datum, with a value of 27.7 µg mg⁻¹, was excluded from the data, sGAG retention was 53.49%.

Figure 6.17 Quantitative analysis of normalised DNA content in native and autoDET decellularised juvenile porcine oesophagus. Quantification revealed a significant reduction in DNA content from 2184.58 ± 597.24 ng mg⁻¹ to 171.15 ± 94.04 ng mg⁻¹ \((p <0.05; n=4)\). Reduction of DNA to <50 ng mg⁻¹ was observed in one replicate.
Figure 6.19 Quantitative analysis of normalised total collagen content in native and autoDET decellularised juvenile porcine oesophagus. Following decellularisation, total collagen concentration increased significantly from $127.28 \pm 9.88 \, \mu g \, mg^{-1}$ to $285.13 \pm 41.91 \, \mu g \, mg^{-1}$ ($p < 0.05; n=4$).

Figure 6.18 Quantitative analysis of normalised sGAG content in native and autoDET decellularised juvenile porcine oesophagus. Quantification of sGAG content identified a decrease from $15.53 \pm 4.35 \, \mu g \, mg^{-1}$ to $5.70 \pm 1.00 \, \mu g \, mg^{-1}$ (n=4) following decellularisation. This represented a 63.30% reduction in sGAG concentration.
6.5 DISCUSSION

Chapter 5 provided an account of the adaptation of the bespoke, modular bioreactor to enable perfusion decellularisation of oesophageal tissue. Whilst the data generated indicated that the modified DET protocol could be implemented in a semi-closed/closed-system to effectively decellularise juvenile porcine oesophagi, the resultant scaffolds were sub-optimal due to significant alteration of their macroscopic structure. Several concerns were also raised with respect to the suitability of the system for eventual translation and potential ATMP production; with a particular focus on contamination risk from excessive manipulation during bioreactor assembly, as well as sterility compromise due to operator error. To address these limitations, it was proposed that an alternative system be employed. Following the success demonstrated by the application of a bespoke bioreactor in Chapter 4, as well as the relatively poor breadth of commercially available options, it was proposed that a bespoke bioreactor be designed and fabricated specifically for processing hollow tissues.

A URS, and subsequently DS, were compiled for a novel, modular bioreactor. The design process sought to implement the principle of cassette-mediated modularity that had previously proven effective in reducing contamination risk at the point of in situ tissue manipulation. Whilst the work described in Chapter 6 was firmly cemented in the process development phase, with colleagues only recently publishing the first proof-of-principle data, implementing a modular system with the potential for functional expansion also reduces the potential validation burden.

The conceived mechanism that enabled processing of hollow tissue of varying dimensions relied on a sliding cassette and connector approach. It was envisaged that tissue would first be mounted outside of the bioreactor body in a cassette in a relaxed state, with a strain value less than one, with fittings inserted in both openings to the luminal compartment of the tissue (DS#1.1 and DS#7.1). The cassette would then be transferred into the bioreactor body, before conduits that transverse the bioreactor wall were fitted to the connectors such that the lumen could be perfused (DS#3.1). By employing an appropriate sealing mechanism, it would be possible to maintain a closed chamber whilst allowing both linear and rotational movement of the conduit inside the bioreactor (DS#3.1). Linear motion would be driven by engagement of a threaded portion of the conduit with a corresponding threaded gauge. This would allow for fine movement of the conduit, and subsequently mounted tissue, that was imparted by the pitch of the engaged threads and the rotation of the gauge. Once the tissue was correctly positioned the variable components of the cassette would then be fixed in position by a series of screws and/or bolts and nuts (DS#4.3). The entire bioreactor would then be closed and
decellularisation performed by luminal perfusion of mounted tissues submerged in the respective perfusate.

Utilising the experience gained during the design and production of the first modular bioreactor, dimensional clearances that allowed ease of cassette transfer to and from the bioreactor (DS#6.1) were incorporated, along with luer fittings for fluid transfer through the bioreactor assembly (DS#2.1). The cassette and connector parts, in addition to the bioreactor body, were designed and drafted in accordance with the tissue dimension range that had been proposed to allow for future application with tissues larger than juvenile porcine oesophagi (DS#4.1 and DS#4.2). Whilst the mechanism for altering the length of mounted tissue within the bioreactor allowed for fine control, it did result in the final bioreactor assembly exceeding the external dimensions outlined in the DS (DS#8.1). This was deemed to be of minor concern during the current, prototyping phase of bioreactor development. The design specifications addressing dimension variability were prioritised instead, and it was highlighted that DS#8.1 could be resolved in subsequent iterations.

Following design completion, bioreactor parts were successfully fabricated from POM. Although PTFE had comfortably addressed the specification requirements outlined for the original modular bioreactor, it had proven difficult to manipulate and reproducibly machine. POM, with a characteristic profile that makes it highly amenable to traditional machining approaches, has broadly been applied within the medical industry and represented a promising alternative. Moreover, it had also been demonstrated to possess properties that rendered it suitable for bioreactor fabrication; primarily proven biocompatibility after repeated autoclave sterilisation, with no notable effects on BM-MSC viability or assayed differentiation capacity (DS#10.1-DS#13.1; (Penick et al., 2005)).

Final costing for bioreactor manufacture and ancillaries came to more than the target cost of £2500 (DS#9.1). This was anticipated during design and fabrication, due to the iterative nature of the process. Enquiry with the Workshop indicated that subsequent unit fabrication could be comfortably performed at a lower cost, in accord with bulk purchase of raw materials and establishment of the CAM programme. Equipment costing for recent manufacture of large hollow organ TEPs has been reported to be within the region of $3200, equivalent to approximately £2150 (Culme-Seymour et al., 2016). This demonstrates that the cost of producing the novel bioreactor is comparable to that previously employed. Equipment represents a small proportion of the total manufacturing costs, which in turn only constituted 2.3-15.8% of complete treatment cost. Although the
target of £2500 for a novel bioreactor and ancillaries was exceeded, this is by no means a barrier to future iteration and eventual clinical application.

To qualitatively assess ease of use, as well as test for leaks in the assembled system, a previously decellularised juvenile oesophagus was mounted in an aseptically assembled bioreactor. Assembly of parts and tissue mounting were achieved without issue, and the mounted construct was demonstrated to be liquid-tight at the juncture between tissue and fittings. Importantly, filling the entire bioreactor and perfusing the tissue demonstrated that the full assembly could be operated without leaking. Secondarily, it was confirmed that the in situ construct could be readily rotated without macroscopically identifiable torsion.

Having designed and fabricated a bioreactor that was grossly suitable for accommodating oesophagi that were too large for the original modular bioreactor, it was proposed that a fully closed-system be developed and implemented for oesophagus processing. The resultant assembly was characteristic of those used for perfusion decellularisation; consisting of a series of reagents in reservoirs that were pumped into the associated bioreactor, before passing into a waste reservoir (DS#2.1). Each reagent was housed in a distinct, vented reservoir (DS#1.1) that was independently opened/closed by a solenoid pinch valve. These valves, used throughout the system, allowed for line control without directly contacting reagents (DS#1.2 and DS#4.1), thus eliminating any concerns regarding valve material biocompatibility. The resting state of each valve was paired to the corresponding usage within the autoDET, such that lines that were open for the majority of the time were normally open and vice versa. This reduced the power requirement of the system and minimised heat dissipation concerns.

Reagents were either pumped into the bioreactor to submerge the mounted tissue, via a line that passed through the normally closed path of a 3/2 pinch valve and high-geared peristaltic pump, or through the oesophageal lumen via the normally open pinch valve path and a low-geared peristaltic pump. Different gearing provided specific flow rate ranges for each compartment, perfusing mounted constructs at 0.5 mL min⁻¹ and filling the abluminal compartment of the bioreactor at the maximum feasible rate. Perfusate from the lumen passed directly to waste, whilst a high-geared peristaltic pump was used to minimise the time required to empty the bioreactor during reagent changes.

Actuation of the pumps and valves was controlled by a microcontroller with a programme that was written to execute a two cycle autoDET (DS#2.2). Adopting a microcontroller approach eliminated the requirement for using a personal computer or laptop during operation, which improved system reliability due to the greater operational stability of microcontrollers compared to computers. It had the additional benefit of reducing the
footprint of the assembly which, if paired with a battery power source, could be operated as a mobile, standalone system. This approach could be beneficial for future use in a cleanroom environment, where bench space is at a premium and laptops and personal computers pose a serious contamination risk.

Due to the prototypical nature of the system there were a number of significant improvements that could be made. The most significant of these would be totally independent system operation following process initiation. In its current iteration the system required manual addition of DNase to the respective reservoir prior to the enzymatic decellularisation phase, thus failing to meet DS#2.3. This was due to the storage requirements of the reagent, which would lose activity if stored at RT in solution from initial set up. This could be overcome by introducing a temperature controlling Peltier device, which could refrigerate the DNase solution before raising the temperature when required for perfusion. This could also provide the option for performing enzymatic phases at physiological temperatures, to improve activity. Additional improvements might include the introduction of a real-time display providing information regarding the system operation, including time elapsed/remaining and decellularisation phase. One further improvement that could greatly benefit the system is an emergency stop switch. This would allow an operator to stop hardware actuation in order to rectify any acute system issues, before continuing the autoDET from the point at which it had been halted.

The novel bioreactor and autoDET system were combined and used to decellularised juvenile porcine oesophagi to demonstrate system feasibility, following tissue-free system refinement to optimise timings and actuation voltages. Observation of the effects of waste reservoir positioning on luminal occlusion highlighted the importance of system pressure. Altering the position of the waste reservoir was a rudimentary approach for rectifying the problem that was over reliant on operator presence and intervention. It is proposed that future reiterations incorporate a pressure sensor feedback loop that controls flow rate as a function of the pre- and post-tissue differential.

Biomolecular quantification of the decellularised tissue indicated that the efficacy of DNA removal was comparable to that achieved in the manual process (DS#3.1). Collagen and sGAG quantification revealed an increase and decrease in concentration, respectively, in accord with the trend observed for the manual approach. Further characterisation of the scaffolds, including biomechanical investigation and in vivo assessment, is required to confidently conclude that there is no difference in the general profile of tissue decellularised manually or using the autoDET. Should this prove that there is no notable difference between the two products, then transition to automated decellularisation of oesophagi may be implemented in future in vivo studies.
6.6 CONCLUSION

In light of the inadequacies of the original bioreactor to provide a suitable system for producing acellular oesophagi, a novel bioreactor was designed and fabricated. The modular bioreactor was capable of accommodating hollow tissues of various lengths using cassette mediated functionality, was easy to assemble and operate, and formed a semi-closed/closed environment for processing. Manufacturing costs did exceed the anticipated budget, however, and further actions are required to resolve packaging/shipping methods. The bioreactor was combined with a bespoke system that automated juvenile porcine oesophagus decellularisation using the two cycle DET process. Decellularisation efficacy was comparable to the manual process, as indicated by DNA removal. Further characterisation and system refinement are required to achieve complete system closure and optimise the process prior to implementation in a large-animal study.
7 SUMMATIVE DISCUSSION

7.1 STATUS QUO

Tissue engineering has experienced a surge in both interest and progress in recent years (Fisher and Mauck, 2013, Harrison et al., 2014, Huang et al., 2015, Wobma and Vunjak-Novakovic, 2016), and is often touted as an exciting and innovative approach to resolving widespread unmet clinical need. This was epitomised by the furore surrounding the first-in-man application of a tissue engineered airway, a decade ago (Macchiarini et al., 2008). The scrutiny that this case and subsequent clinical interventions has since come under, highlights the importance of due diligence and therapy development via formal clinical trials with regulatory oversight. The importance of this cannot be overstated when developing novel therapeutic approaches and patient wellbeing may be effected. Within the EU, ATMP regulations are established and maintained by the European Commision, with advice provided by EMA. National Competent Authorities, the MHRA within the UK, are then responsible for enforcing issued legislation and harmonising Directives with existing national legislation. This provides a pathway for the development of quality assured medicines that are safe for human use.

Utilising this framework, and following the successful completion of a large-animal preclinical study, data were generated to support a CTA application proposing evaluation of the safety and efficacy of a TEP partial laryngeal replacement as part of a Phase I/II clinical trial. To achieve this, it was necessary to translate the manufacturing process used in the GLP-compliant study to one that complied with the GMP guidelines (European Commission, 2008, European Commission, 2017).

7.2 TRANSLATIONAL PROCESS ENGINEERING

The manufacturing process employed was broadly divided into two sub-processes; decellularisation and recellularisation. Initial alterations were focussed on introducing materials control, and thus raw materials were altered to reduce the requirement for in-house sterilisation and to adopt compendium materials where possible. Reagents that posed a risk of transmitting animal spongiform encephalopathies and were not certified by the European Directorate for the Quality of Medicines and Healthcare, namely bovine derived DNase and RNase, were replaced with human recombinant alternatives.

The decellularisation system described for the GLP-compliant study relied extensively on open-system processing that was not suitable for GMP-compliant manufacture. To address this, steps were taken to close the process; including housing of both
reagents/waste and the tissue itself, and the means by which fluid was transferred to and from the tissue containing equipment. By adapting an off-the-shelf equipment that satisfied the URS, and had previous clinical use (unpublished), it was possible to rapidly form a suitable system for processing. Sterility validation confirmed that assembly and decellularisation operations could be performed aseptically, allaying concerns that utilising a luer lock system instead of sterile welding may risk sterility compromise. Whilst sterile welding was not practical due to the large number of reagents changes used throughout the decellularisation process, and the consequential necessity to have a significant excess of tubing for welding, it is envisaged that future processes may adopt on-off aseptic connectors to reduce in-process contamination risk.

Whilst it was been proven that the process could be aseptically executed, SM was typically contaminated upon receipt. A decontamination approach using liquid reagents was investigated as a means of eliminating the bioburden without altering the gross biomechanics of the tissue. This approach was inadequate at consistently sterilising tissue, and posed a significant risk of reporting false negative results. Gold-standard gamma-irradiation was adopted for sterilising tissue, although the biomechanical implications of this were not investigated due to the absence of a physiologically relevant testing regimen. Though graft failure due to reduced biomechanical strength was deemed to be low-risk by the clinical team, owing to the size and anatomical location proposed for investigation in the RegenVOX clinical trial, it is essential that appropriate investigation of this is performed.

When comparing larynx tissue that had been processed using the translated process to its native counterpart, it exhibited the expected, qualitative, characteristic profile. Tissue was macroscopically palled and largely void of cellular material when assessed histologically. Quantifying DNA concentration, widely reported as perhaps the key indicator of effective decellularisation, revealed a decrease to less than the threshold that has been proposed as clinically acceptable (Crapo et al., 2011). Whilst the relevance of this value in cartilage tissue engineering remains questionable due to the location and nature of chondrocytes in vivo (Smith et al., 2015), it did provide a quantifiable release criterion. Assessment of total collagen and sGAG content was also collected, to provide greater understanding of the decellularised product and reflect on the decellularisation profile relative to the results generated during the GLP-study. These assays would be performed for information only when clinically processing. The relationship between the respective biomolecules and the biomechanical properties of the construct, for which they provide a rudimentary proxy (Partington et al., 2013), is complex and could not feasibly be used as a fair status indicator. When respective biomolecular composition was compared to that the GLP-study results, it indicated that process translation had had
no apparent detrimental effect on decellularisation efficacy. DNA reduction was comparable, whilst total collagen and sGAG retention appeared to have been improved. This assessment can only provide a loose comparison, however, due to the change of donor species. Whilst decellularisation of porcine tissue using the translated process would allow for a direct comparison, it would not have provided an ultimate indication of system suitability for processing human tissue for clinical purposes. The timeline requirements for submitting the IMPD for CTA, as well as knowledge of the needs patient population in the UK, justified translation validations being performed directly with human tissue. For these reasons, as well as questionable manufacturing and clinical benefit from the generated data, further histological and immunofluorescent characterisation of the decellularised tissue was not performed.

Having demonstrated that acellular scaffolds could be manufactured using the translated, GMP-compliant decellularisation process; it was essential that the decellularised tissue could support BM-MSCs, as had been employed in the GLP-compliant study. A population of fluorescently labelled cells were shown to have adhered to the scaffolds four hours post-seeding, though the proportion of seeded cells that had adhered was not quantified due to experimental failings. The data generated were, however, a suitable confirmation of proof-of-concept. Investigation of whether relative adherence correlated with an increase in adherence time indicated there was no notable improvement in adherence after four hours. Quantification of the relative proportion of adherent cells could be investigated with additional human SM, as has been performed by our group for decellularised tracheae (unpublished). The maximum period of time that cells would be cultured on scaffolds, ex vivo was limited to five days, as defined by the GLP-compliant study protocol. Assaying metabolic activity of scaffold seeded BM-MSCs indicated the maintenance of a viable cell population throughout this period. This was qualitatively supported by SEM micrographs.

Whilst the decellularised scaffolds had been proven suitable for clinical investigation, the bioreactor used in the GLP-compliant process was not appropriate for GMP-compliant tissue recellularisation at scale. A novel bioreactor was designed to a URS that aligned with GMP-compliant manufacture. The bespoke design incorporated cassette-mediated modularity, to accommodate both full scale hemilarynx constructs and flat matrices that could be used for producing two-dimensional TEPs; such as the epithelial sheet employed in the preclinical study. The cost of fabricating the bioreactor from PC and PTFE allowed each unit to be single-use; reducing the risk of repeated autoclave sterilisation causing tolerance alteration, which would impact functional operation. Once validated for aseptic operation, medium conditioning investigation demonstrated that the bioreactor did not leach chemicals that effected BM-MSC or epithelial cell viability, during
normal operating conditions. Potential effects on BM-MSC differentiation capacity were not investigated due to the brevity of the recellularisation period, and wider uncertainties regarding \textit{in vivo} cell fate. However, it was demonstrated that epithelial populations could establish stratified squamous epithelium in conditioned medium in a scale-down model. Proof-of-concept demonstration of epithelium production within the bioreactor highlighted the need for operational refinement to prevent bubble introduction to the non-seeded compartment, as well as the adoption of a thinner substrate to support seeded cell population. A continuous perfusion approach is proposed to resolve bubble formation, whilst colleagues are currently investigating suitable substrate alternatives.

The data generated, demonstrating the translation of the decellularisation and recellularisation process, were incorporated into the IMPD that was submitted to the MHRA as part of the CTA application. This resulted in the subsequent award of the CTA, EudraCT: 2013-004359-18. The data concerning the novel, modular bioreactor were also submitted as part of a successful application for a clinical trial investigating tissue engineered tracheal replacement (EudraCT: 2015-002108-10). The bioreactor has recently been employed in the manufacture of a tissue engineered tracheal replacement for a paediatric patient, as an unlicensed medicine for human use, by the Centre for Cell, Gene and Tissue Therapeutics (MHRA MIA(IMP) 11149). The patient has, at time of print, experienced an improvement in quality of life (unpublished).

\section*{7.3 Functional Expansion of a Modular Bioreactor}

Having performed proof-of-concept tissue engineered epithelial sheet production, as well validation of GMP-compliant larynx construct cellularisation and clinical manufacture of a tissue engineered trachea, it was proposed that the modular bioreactor could be adapted for decellularising hollow tissues to demonstrate its capacity as a platform device for processing multiple tissue engineered products. A process for decellularising rat oesophagi, recently published by colleagues (Urbani et al., 2018), was adopted as a model system for demonstrating this; simultaneously translating the rat protocol for scale-up, in preparation of larger-animal studies.

Minor reagent changes were implemented to remove materials which posed a risk of transmitting animal spongiform encephalopathies and to improve DNase activity, whilst also reducing the requirement for in-house sterilisation. The bioreactor was crudely modified, introducing a catheter as conduit across the chamber wall that would enable closed-system, perfusion decellularisation. Oesophagi from neonatal pigs were successfully decellularised, indicated by DNA removal, and exhibited similar biomolecular distribution to the native tissue. Quantitative profiling of total collagen and
sGAG revealed a relative increase and decrease, respectively, which was comparable to published DET data (Totonelli et al., 2013, Urbani et al., 2017, Urbani et al., 2018). Though the classic characterisation profile indicated the successful production of a typical, acellular scaffold; when orthotopically implanted in a NZW rabbit model, all recipients failed to survive to the target date.

A combination of surgical and pre- and post-operative care refinements were implemented, in combination with matching donor and recipient mass, to address the in vivo failures. Standard, quantitative biomolecular characterisation of the processed scaffolds from larger donors indicated decellularisation efficacy was comparable between oesophagi procured from neonatal and juvenile pigs, even when reducing the number of DET cycles from three to two. When provided to the surgical team for implantation, it was reported that the larger scaffolds were more suitably sized for manipulation and anastomosis formation following resection. Together with the aforementioned refinements, this resulted in an improved gross outcome (Hannon et al., 2017).

Biomechanical tests were undertaken to expand the characterisation profile of the decellularised porcine oesophagus, and to provide a greater understanding of the decellularised product. Decellularisation resulted in an increase in construct stiffness, as was anticipated following similar results reported in rabbit oesophagi decellularised using DET (Urbani et al., 2017). This increase was greatly exacerbated by gamma-irradiation, which has been previously reported in accord with alteration of collagen fibres (Gouk et al., 2008). Stiffness and ultimate stress also increased. Interestingly, tissue stiffness decreased to a level that was not statistically significantly different to the native tissue following four weeks refrigerated storage. A similar trend has been reported by Urbani et al. (2017), and so this may represent the typical biomechanical profile of the decellularised oesophagus during the degradation. Further investigation is required to elucidate the underlying mechanisms behind this, although resources may be more appropriately directed towards more clinically relevant biomechanical assays, such as the suture retention test (ISO 7198:2016; (International Organization for Standardization, 2016)).

Ultimately, the combined data demonstrated that the modular bioreactor could be employed for closed-system decellularisation of juvenile porcine oesophagus, which early in vivo data suggest may provide a suitable foundation for developing a tissue engineered oesophageal replacement. Prior to recellularisation studies, recapitulating the small-animal model findings presented by Urbani et al. (2018), it was proposed that the decellularisation process be translated to a bioreactor that was specific to hollow
tissue processing. This was driven by sub-optimal bioreactor operation, and an unsuitable macroscopic architecture of decellularised tissues greater than 60 mm in length.

7.4 A NOVEL, MODULAR BIOREACTOR FOR HOLLOW TISSUES

Addressing the shortcomings of the original modular bioreactor, a novel modular bioreactor was designed and fabricated. Although the prototype failed to meet the external dimension and costing specifications outlined in the DS, it fulfilled the majority of user requirements and represented a successful first realisation of the concept mechanism for accommodating hollow tissues of varying dimensions in a single cassette, modular bioreactor. The bioreactor was subsequently paired with a bespoke system to automate the DET decellularisation process, thus reducing the requirement for costly manual, operator intervention and risk of human error; both of which have been highlighted as barriers to robust bioprocess manufacturing (Williams et al., 2012, Brindley et al., 2013). Juvenile porcine oesophagi were decellularised using the novel system, with biomolecular profiles comparable to those produced using the manual process. Whilst only a small number of data were generated, early indications are that the system could, upon refinement and further product characterisation, be used for automated oesophagus decellularisation in the upcoming preclinical study in a large-animal model.

7.5 LIMITATIONS AND FUTURE PERSPECTIVES

Within a translational framework, the work undertaken regarding the development of a GMP-compliant decellularisation and recellularisation process for manufacturing a laryngeal TEP is complete, following the award of the CTA (EudraCT: 2013-004359-18). This does not, however, justify halting further product characterisation. The primary avenue for further investigation should be developing and/or implementing a physiologically relevant biomechanical testing regimen for the decellularised constructs. Although the Phase I/II trial is designed to minimise the risk posed by potentially suboptimal product biomechanics, future implantation of larger constructs would require structural assurance. Current approaches typically investigate biomechanics immediately after tissue decellularisation, or after a storage period (Partington et al., 2013, Urbani et al., 2017). However, clinical evidence, from more widely investigated tracheal replacement, indicates that in the majority of incidences the critical period occurs during the remodelling response, typified by malacia and necessary stenting (Elliott et al., 2012, Gonfiotti et al., 2014, Hamilton et al., 2015). Subsequently, it is necessary that an approach that accounts for this is employed.
Translation of the DET decellularisation process to one that is suitable for GLP-compliant investigation in a large-animal model, and eventually a GMP-compliant manufacturing process, requires significant additional development. Following the generation of promising data by the autoDET process, the automated decellularisation system represents a key target for improvement.

Firstly, the system should be expanded to include a temperature controlled reservoir for enzymatic reagents. This would allow for complete system automation and independence following initialisation. A series of probes should be introduced to monitor key process parameters, including temperature, flow rate and pressure differential across the luminal-abluminal wall of mounted tissue. The data from these would provide detailed process characterisation, which ultimately provides a robust indicator of the product profile (Williams et al., 2012). Whilst the standalone nature of the existing system ensures that process cannot be inadvertently altered during operation, it is restrictive in allowing emergency intervention should any issues arise. Introducing a process parameter display that can be remotely accessed, in combination with an electronic alert system and emergency pause function, would allow real-time monitoring of the process that could resolve this. Appropriate modification to the programme to ensure compliance with EudraLex – Volume 4, Annex 11 guidelines for Computerised Systems should be implemented to pre-empt GMP translation (European Commission, 2010a).

Aside from refinement of the decellularisation system, it would be beneficial to investigate whether the bioreactor could be gamma-irradiated with the decellularised tissue in situ. This would eliminate the risk of terminally sterilising the tissue and subsequently having to manipulate it prior to reseeding. The desirability of this, itself, is dependent on proving that the decellularised oesophagus can be seeded and cultured to recapitulate the results reported in the small-animal proof-of-concept model (Urbani et al., 2018). Demonstrating that the seeded scaffold could then be cryopreserved, before successfully thawing and continuing culture (Urbani et al., 2017), would represent a significant milestone in tissue engineering bioprocessing.
8 CONCLUSION

The field of tissue engineering remains at the cutting edge of medical science. Whilst it has the potential to provide superior solutions for widespread unmet clinical need, it is essential that the development of medicines, in the form of ATMPs, is undertaken with due diligence and in accord with the governing regulations. Rigorous and transparent product development via the proper pathways not only ensures patient safety, but demonstrates, to colleagues and non-specialists alike, the high standards that professionals within the field adhere to. This thesis has documented the process engineering that was undertaken to translate a GLP-compliant decellularisation-recellularisation methodology, to a GMP-compliant manufacturing process for the production of a tissue engineered partial larynx ATIMP (EudraCT: 2013-004359-18). Hemilarynges were reproducibly decellularised in a semi-closed, off-the-shelf bioreactor to yield acellular scaffolds that were terminally sterilised. These scaffolds were demonstrated to support a seeded population of viable BM-MSCs for a minimum of five days ex vivo. A bespoke modular bioreactor was designed to enable manufacture of recellularised hemilarynges at scale, before being validate for aseptic operation and biocompatibility. Proof-of-concept production of a tissue engineered epithelial sheet for airway applications, within the modular bioreactor, was also demonstrated. Adaptation of the bioreactor facilitated tertiary functionality, and the bioreactor was demonstrated as a chamber for decellularising porcine oesophagi in semi-closed system. The resultant, acellular constructs were successfully implanted in a large-animal model. A second modular bioreactor was designed to improve the oesophageal construct manufacturing process, and was combined with bespoke automated decellularisation system. Data generated by the prototype system demonstrated that oesophagi could be decellularised with an efficacy comparable to the manual process, whilst significantly reducing the requirement for operator processing. Subsequent to further development, the system will be used for in an upcoming preclinical, large animal study that is to form the basis for an associated IMPD and CTA application. This, in turn, will provide a stepping stone on the path towards a pivotal clinical trial, marketing authorisation, and eventual for routine clinical use.
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APPENDIX 1

In addition to the ATMP classification definitions provided in Table 1.1, there are several additional concepts that are worth clarifying to provide a more complete understanding of ATMP classification. Firstly, it is noted that when a product contains both allogeneic and autologous cells and/or tissues that it be classified as an allogeneic product. Secondly, should a product satisfy the defining criteria of both a sCTMP and TEP, then that product shall be classified as a TEP; whilst should the product also satisfy the GTMP criteria, then it is demarcated as a GTMP. When attempting to differentiate between sCTMP and TEP designation, the primary function of the product acts as the distinguishing feature. If the product is intended for prevention, diagnosis and/or treatment achieved through immunological, pharmacological and/or metabolic modes of action then the product is categorised as a sCTMP. TEPs are, alternatively, associated with the regeneration, repair and/or replacement of human tissue. Ultimately, the Committee for Advanced Therapies classification of products is founded upon both the claimed mode of action and the claimed intended function of the product; whereby the mode of action claim is sufficiently substantiated either by data provided by the applicant or by current scientific knowledge/consensus. Finally, it is worth highlighting two key components associated with the concept of substantial manipulation, with regard to sCTMPs and TEPs. Firstly, it is evident that the expansion of cells is classified as substantial manipulation. The proliferation of cells achieved through cell culture is regarded as an alteration of their biological and structural characteristics due to subsequent changes in functionality and/or phenotype. Secondly, should cells be isolated from a tissue through its digestion then, regardless of further processing, the cells are considered to have undergone substantial manipulation and so may be identified as an ATMP. Conversely, should the tissue alone be the target of the isolation then, following the removal of the cellular component, the medicinal product is not classified as an ATMP. Instead the product is identified as a medical device and is regulated under the associated framework (European Medicines Agency, 2015).