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Development of a Supercritical CO_2 Decellularization Technology

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"The only constant in life is change"

Heraclitus

Abstract

Decellularization of mammalian tissue describes the process by which cellular materials are removed from the extracellular matrix (ECM, Hinderer, Layland and Schenke-Layland, 2016). Current decellularization methodologies utilise a multitude of decellularization agents, such as detergents, solvents and biological enzymes, to achieve successful decellularization, which has been demonstrated with a number of different organs (i.e. heart (Ott *et al.*, 2008), lung (Petersen *et al.*, 2012), kidney (Song *et al.*, 2013) and liver (Uygun *et al.*, 2010; Mazza *et al.*, 2015)). However, the use of these common decellularization agents causes damage to the derived ECM ultrastructure. Furthermore, these agents are often retained within the tissue following the decellularization process (Faulk *et al.*, 2014; White *et al.*, 2016), which may negatively impact potential downstream application.

In this thesis, a new $scCO_2$ and $scCO_2$ hybrid decellularization methodology was developed for liver and aorta tissue, respectively. $ScCO_2$ effectively removed genomic material by -75% and -48% from liver and aorta tissue (respectively) as confirmed by DNA quantification and histological analysis. Interestingly, the required $scCO_2$ exposure duration for decellularization was longer for aorta (72 h) compared to liver (48 h), suggesting that differences in tissue structure might affect decellularization efficacy by $scCO_2$. Furthermore, decellularization by $scCO_2$ was inhibited if tissue water content was removed, indicating that moisture content is likely mechanistically involved in the process of decellularization by $scCO_2$. While 48 h $scCO_2$ decellularization reduced DNA content of liver tissue by -75%, aorta tissue required an additional 24 h exposure to $scCO_2$ and a further 1 h incubation in either Trypsin/EDTA, Triton-x-100, SDC or LS-54 to achieve a similar level of DNA reduction. ECM proteins, such as glycosaminoglycans and collagen, were retained following the $scCO_2$ only method (liver) and the $scCO_2$ hybrid method (aorta).

When tested for biocompatibility *in vitro*, the $scCO_2$ decellularized liver and aorta ECM scaffold resulted in a non-cytotoxic response when exposed to HepG2 and 3T3 cells (respectively). In contrast, the hybrid $scCO_2$ decellularization methodology caused cytotoxicity on both cell lines tested (HepG2 and 3T3) and requires further development/optimization to reduce residual toxicity caused by the addition of Trypsin/EDTA, Triton-x-100, SDC or LS-54.

This thesis demonstrates an in depth characterization of the response of mammalian tissue to $scCO_2$, which resulted in the development of a novel, $scCO_2$ based decellularization technology for liver and aorta tissue. The effects of $scCO_2$ on liver tissue were more pronounced than those observed on aorta, suggesting that differences in tissue structure might influence the efficacy of $scCO_2$ to decellularize mammalian tissue. Combinations of $scCO_2$ with commonly used decellularization agents (i.e. hybrid method) further improved the removal of cellular material for aorta but not liver tissue. Taken together, this work demonstrates the potential utility for sCO_2 as a deceullarization agent. However, the hybrid $scCO_2$ method described herein requires further modification to improve biocompatibility in downstream applications.

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Chapter 2

Abbreviations

A	Allograft
AB	Alcian Blue
atm	Standard atmosphere
bp	Base-pairs
C_2H_6	Ethane
CH_4	Methane
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CO_2	Carbon dioxide
DAPI	4',6-Diamidino-2-Phenylindole
DIZG	Deutsches institut fuer Zell- und Gewebeersatz
DMMB	1,9-dimethylmethylene blue
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dsDNA	Double stranded DNA
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
EU	European union
FBS	Foetal bovine serum
g	Gram(s)
gDNA	Genomic DNA
h	Hour(s)
HepG2	Hepatocellular carcinoma cell line
H&E	Haematoxylin and eosin
H_2CO_3	Carbonic acid
H_2O_2	Hydrogen peroxide
H_20	Water
HCl	Hydrochloric acid
kb	Kilo-bases
M	Molar (mol/l)
MEM	Minimum Essential Medium Eagle
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mm	Millimetre
mM	Millimolar
MSCs	Mesenchymal stem cells
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
N	Nitrogen
n	Number of replicates

N	Number of experiments
NaCl	Sodium chloride
NEAA	Non-essential amino acids
ng	Nanogram(s)
NH_3	Ammonia
nm	Nanometre(s)
O_2	Oxygen
PAA	Peracetic acid
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
pH	Potential of hydrogen
psi	Pounds-force per square inch
PSR	Picro Sirius Red
rcf	Relative centrifugal force
RNase	Ribonuclease
rpm	Revolutions per minute
$scCO_2$	Supercritical carbon dioxide
SCF	Supercritical fluid
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulphate
sGAG	Sulphated Glycosaminoglycans
SIS	Small intestinal submucosa
TAE-buffer	Tris-acetic acid-EDTA buffer
TE-buffer	Tris-EDTA buffer
T/E	Trypsin/EDTA
Tof-SIMS	Time-of-Flight secondary ion mass spectrometry
Tris	Tris (hydroxymethyl) aminomethane
V	Volt(s)
v/v	Volume per volume
w/v	Weight per volume
X	Xenograft
%	Percent
°C	Degree Celsius
µg	Microgram(s)
3T3	Mouse embryonic fibroblast cell line

1. Preface

The use of extracellular matrix (ECM) bioscaffolds for regenerative medicine applications, such as wound healing (Murray *et al.*, 2019) and soft tissue reconstruction (Donnely, Griffin and Butler, 2019), has gained a lot of interest in recent years. Allogeneic and xenogeneic ECM products are commercially available and approved for clinical use in the EU and USA (some examples include: human dermis ECM (Flex HD, Ethicon, USA), porcine dermis ECM (Permacol, Covidien, USA) and equine pericardium ECM (ORTHadapt, Pegasus Biologics, USA)). This demonstrates how the biochemical and biophysical cues of ECM bioscaffolds can stimulate localised regeneration in patients (Badylak, 2005).

The success of utilizing ECM bioscaffolds for regeneration of skin or reconstruction of breast tissue (Naranjo *et al.*, 2016) has raised the question as to whether ECM bioscaffolds could also be used for the regeneration of more complex organs. To date, full organ decellularization has been achieved on major organs such heart (Ott *et al.*, 2008), lung (Petersen *et al.*, 2012), kidney (Song *et al.*, 2013) and liver (Uygun *et al.*, 2010; Mazza *et al.*, 2015). Whilst promising, decellularization of organs requires a multitude of decellularization agents, some of which remain in the tissue and have a subsequent negative impact on the biochemical and biophysical cues of the ECM (Faulk *et al.*, 2014; White *et al.*, 2016). This makes the realisation of a functioning organ derived from a detergent decellularized ECM bioscaffold difficult to produce.

Therefore, alternative methods, such as the use of super critical carbon dioxide ($scCO_2$), to decellularize mammalian tissue are being developed as a potential solution to avoid the damage and residual toxicity currently caused by using harsh decellularization agents.

This thesis focused on the development of an alternative decellularization method utilizing $scCO_2$ to produce an ECM bioscaffold from mammalian tissue.

2. Introduction

2.1. THE EXTRACELLULAR MATRIX (ECM)

The extracellular matrix (ECM) is the non-cellular, tissue specific, structural scaffold that is synthesised and secreted by cells (Rozario and DeSimone, 2009). The ECM is present in all biological tissues and organs, from pre-natal tissue development onwards (Frantz, Stewart and Weaver, 2010). The biochemical and biophysical properties of the ECM strongly influence the cellular microenvironment (Rozario and DeSimone, 2009). For example, the ECM provides a foundation for cell anchorage and cell support, which interact via transmembrane receptors such as integrins (Harburger and Calderwood, 2009) or syndecans (Xian, Gopal and Couchman, 2010). The ECM is also influential to cellular proliferation and differentiation, tissue morphogenesis and organ homeostasis (Badylak, 2002; Frantz, Stewart and Weaver, 2010; Bonnans, Chou and Werb, 2014; Mouw, Ou and Weaver, 2015). The ECM interacts with the surrounding cells via signalling molecules and subsequently undergoes constant remodelling in response to injury, mechanical stress and disease (Lu *et al.*, 2011; Bonnans, Chou and Werb, 2014; Jakeman, Williams and Brautigam, 2014). Seminal work by Bissell *et al.,* 1982 described this underlying two-way interaction between the cell and its microenvironment (i.e. including the ECM) as "dynamic reciprocity" (Nelson, Bissell and Gov, 1982). The ECM therefore serves a crucial role throughout the lifespan of all organisms, from pre- and post-natal development to adulthood.

2.1.1. Extracellular matrix components

Whilst crucial to tissue structure and function, proteins of the ECM comprise less than 1% of the proteome of any given tissue (Hynes and Naba, 2012). The ECM is composed of the following key macromolecules:

- Fibrillar collagens provide the structure of the tissue with tensile strength (Hynes and Naba, 2012; Bonnans, Chou and Werb, 2014)

- Elastin provides the ECM with elasticity, therefore enabling flexibility of the tissue (Rozario and DeSimone, 2009)
- Glycoproteins (i.e. fibronectin, vitronectin, laminin) are relevant for the assembly of the ECM and interaction with cells, as well as serving as a growth factor storage (Bonnans, Chou and Werb, 2014)
- Proteoglycans (i.e. aggrecan, versican, perlecan) hydrate the ECM by binding to water and growth factors within the tissue (Bonnans, Chou and Werb, 2014)

It is noteworthy that the amino acid sequences of ECM macromolecules are highly conserved across species, highlighting the xenogeneic therapeutic potential for regenerative medicine and tissue engineering (van der Rest & Garrone 1991).

2.1.2. Extracellular matrix characteristics

Several characteristics of the ECM influence cell behaviour and therefore tissue phenotype. *In vivo*, cells are exposed to secreted proteins from the ECM and adhere directly to the ECM (Watt and Huck, 2013). Structural characteristics of the ECM, such as stiffness and topography (explained in detail below), are transmitted to the cell to regulate stem-cell behaviour by creating a niche that is favoured by specific cell fates. Cells sense these mechanical signals (i.e. forces) from the ECM via transmembrane receptors, such as integrins (Harburger and Calderwood, 2009) or syndecans (Xian, Gopal and Couchman, 2010), which, subsequently influence cell behaviour (Watt and Huck, 2013).

ECM stiffness is modulated by the function of the tissue, the age of the animal and the presence of disease (Watt & Huck 2013). To demonstrate the impact of surface stiffness on cell behaviour and differentiation, Engler *et al.*, 2006 and Gilbert *et al.*, 2011 demonstrated that culturing mesenchymal stem cells (MSCs) in the same serum conditions on a soft (representative of brain), a stiff (representative of muscle) or rigid (representative of bone) matrix directed stem cell fate to neurons, myoblasts and osteoblasts, respectively.

ECM topography varies based on the structure of the tissue or organ (Lu *et al.*, 2011) and can regulate stem cell fate (Unadkat *et al.*, 2011; Watt and Huck, 2013). A

mathematical algorithm has demonstrated a correlation between topographical features (e.g. shapes and sizes) with cellular responses of human mesenchymal stromal cells. However, identification of a topography that induces a specific cellular response remains challenging (Unadkat *et al.*, 2011).

2.1.3. Biomedical uses of extracellular matrix bioscaffolds

Acellular bioscaffolds derived from ECM present ideal natural scaffolds for personalized whole organ engineering (Soto-gutierrez *et al.*, 2010). Transplantation, of any tissue or organ, is accompanied with major risks, such as graft versus host immune response (rejection of the donor organ; Song and Ott, 2011) or transmission of diseases from the donor to the recipient (Park *et al.*, 2013). The increasingly longer-living population is also inadvertently creating a severe organ shortage for patients in need of a transplant (Abouna, 2008). Using an ECM-derived tissue scaffold is a promising alternative that can minimize these risks (Badylak, Freytes and Gilbert, 2009). Repopulating an ECM bioscaffold with the patient's own stem cells could dramatically reduce the risk of organ rejection or donor-to-host disease transmission. However, such technology has thus far only been used for tissue grafting and is yet to be utilized as an alternative to whole organ transplantation.

The benefits of using ECM bioscaffolds for replacement or reconstruction of damaged tissue are well established and have already been shown to be successful in many pre-clinical animal models (Huber *et al.*, 2003; Badylak *et al.*, 2005; Gilbert *et al.*, 2008) as well as clinical studies in humans (Sclafani *et al.*, 2000; Leventhal and Pribitkin, 2008; Macchiarini *et al.*, 2008; Salzberg, Dunavant and Nocera, 2013). The success of this approach is likely due to the preserved unique three-dimensional structure and architecture of the ECM bioscaffold and the presence of proteins and growth factors (Badylak, 2004). These biophysical and biochemical cues from the ECM can then direct cells to repair/regenerate locally at the site of the scaffold.

Transplantable ECM bioscaffolds for clinical use are typically derived from dermis (Vecchia *et al.*, 1999; Sclafani *et al.*, 2000; Rawlani *et al.*, 2011; Macadam and Lennox, 2012), urinary bladder (Rommer, Peric and Wong, 2013; Sasse, Ackerman and Brandt, 2013; Valerio *et al.*, 2015) and small intestinal submucosa (SIS)

(Sandusky, Lantz and Badylak, 1995; Robotin-Johnson *et al.*, 1998; Vecchia *et al.*, 1999). Furthermore, the sources of these ECM bioscaffolds for medical applications are generally human (allografts), bovine or porcine (xenografts).

Pig derived organs are excellent for xenotransplantation due to the very similar internal anatomy of pigs and humans (Bassols *et al.*, 2014). However, xenotransplantation of tissues or organs is particularly dangerous due to the high risk of rejection by the host recipient as well as transmission of zoonotic disease from the donor animal to the human recipient (Boneva, Folks and Louisa, 2001). For instance, organs and tissues from pigs express the alpha-gal epitope, which, if transplanted into humans causes an immune rejection due to the natural production of anti-Gal antibodies in humans (Galili, 2005). This creates an immunological barrier between the species and means xenotransplantation of organs from pigs to humans is not possible. The expression of alpha-gal however is restricted to the cellular material, and is not present in the ECM. Hence, the decellularization of porcine derived tissues and subsequent use of the ECM scaffold for organ regeneration (using the patients cells) for use in humans, avoids the alpha-gal/anti-Gal immune rejection normally seen with xenotransplantation. Furthermore, the amino acid sequences of ECM components are highly conserved across species and are unlikely to transmit zoonotic diseases if properly sterilized (van der Rest and Garrone, 1991). This enables xenotransplantation of decellularized ECM-derived products with only a low risk of an unwanted immune response (van der Rest and Garrone, 1991).

In vivo studies on a dog model revealed that xenograft implanted small intestine mucosa (SIS; porcine derived) ECM scaffolds (previously decellularized and sterilized) stimulated re-modelling of the tissue with no adverse immune response (Rickey *et al.*, 2000). The transplanted ECM bioscaffold degraded within a few months of transplantation (Rickey *et al.*, 2000). The use of non-human ECM for transplantation has the additional advantage that it can be readily manufactured, enabling patient treatment to proceed quicker and most likely at a reduced cost.

Biomedical applications of ECM bioscaffolds are being globally recognised. However, although many ECM based products are being marketed in the USA, less

products have been approved in Europe due to stringent regulatory restrictions (Smart *et al.*, 2014).

Table 1 Selected examples of commercially available ECM products, their use and availability *(allograft (A); xenograft (X))*

Product & Supplier	**Material**	**Form**	**Use**	**EU (Yes/No)**
Alloderm *LifeCell*	Human dermis (A)	Dry Sheet	Breast reconstruction, dermal soft tissue repair	No
Strattice *LifeCell*	Porcine derived matrix (X)	Dry Sheet	Soft tissue reconstruction i.e. hernia, abdominal wall	Yes
Permacol *Covidien*	Porcine dermis (X)	Hydrated Sheet	Soft tissue reconstruction i.e. hernia	Yes
Biodesign *Cook medical*	Porcine SIS (X)	Dry Sheet	Reinforcement and support of weak and soft tissue	No
Xen Matrix *Bard Davol*	Porcine derived matrix (X)	Dry Sheet	Soft tissue repair of damaged, ruptured tissue, i.e. muscle flap, hernia	No
Oasis *Cook biotech*	Porcine SIS (X)	Dry Sheet	Wound healing, ulcers	No
Xenoform *Boston Scientific*	Calf bovine dermis (X)	Dry Sheet	Strengthen damaged, weak, rupture soft tissue membrane i.e. pelvic organ prolapse	No
PriMatrix *Integra life Sciences*	Foetal Bovine dermis (X)	Dry Sheet	Wound management i.e. surgical wounds, trauma wounds, draining wounds, burns	No
Epiflex *Deutsches Institut für Zell- und Gewebeersatz*	Human dermis (A)	Dry Sheet	Soft tissue regeneration, burns, wound healing	Yes

(DIZG)				
Allomax *Bard Davol*	Human dermis (A)	Dry Sheet	Soft tissue repair, replacement, reconstruction	Yes
Flex HD *Ethicon*	Human dermis (A)	Hydrated Sheet	Breast reconstruction, abdominal defects	Yes
OrthADAPT *Pegasus Biologics*	Equine pericardium (X)	Dry Sheet	Orthopaedic soft tissue repair and reinforcement	Yes

2.2. DECELLULARIZATION TECHNOLOGIES

Decellularization defines the process by which all cellular and genetic components are removed while ECM components remain detectable and functional (Hinderer, Layland and Schenke-Layland, 2016). Some methods of decellularization can also retain the ECMs natural three-dimensional (3D) structure. This permits use of the ECM architecture and structure to support reseeding or repurposing of the ECM bioscaffold. The optimal decellularization method is dependent on the tissue or organ used as well as the intended downstream application (Thomas W. Gilbert, Sellaro and Badylak, 2006).

To verify the completeness of the decellularization process, Crapo, Gilbert and Badylak, 2011 defined three factors to measure successful decellularization:

- <50 ng double-stranded DNA (dsDNA) per mg of dry weight.
- Remaining DNA fragments are <200 base-pairs (bp). In comparison, intact genomic DNA is greater than 10 kilo-bases (kb) when separated by gel electrophoresis.
- Acellularity (no visible nuclei via haematoxylin and eosin staining and/or DAPI staining).

Agents commonly used for decellularization are listed in tables 2 & 3 below.

Table 2 Commonly used decellularization agents for cell removal

Category	**Agent**	**Impact (Positive +, Negative -)**	**Reference**
Detergents *(Destroys cells by breaking phospholipid membrane)*	**Sodium dodecyl sulphate (SDS)** *(Ionic)*	+ DNA content less than 50ng/mg of ECM dry weight	(Karina H Nakayama *et al.*, 2010; Du *et al.*, 2011; Shafiq *et al.*, 2012; O'Neill *et al.*, 2013; Gardin *et al.*, 2015)
		+ Absence of cell nuclei	(Ott *et al.*, 2008; Karina H Nakayama *et al.*, 2010; Du *et al.*, 2011; O'Neill *et al.*, 2013; Song *et al.*, 2013; H. Wang *et al.*, 2015; Zhang *et al.*, 2016)
		+ Level of total collagen amount not significantly changed	(Du *et al.*, 2011; O'Neill *et al.*, 2013)
		+ Glycosaminoglycan content unchanged	(Ott *et al.*, 2008)
		+ ECM proteins detectable	(Ott *et al.*, 2008; Du *et al.*, 2011; O'Neill *et al.*, 2013; Song *et al.*, 2013; H. Wang *et al.*, 2015)
		+ Minimal changes in morphology	(Karina H. Nakayama *et al.*, 2010; Du *et al.*, 2011)
		+ Architecture intact	(Ott *et al.*, 2008, 2010; Song *et al.*, 2013; Gilpin *et al.*, 2014)
		+ Strength of the tissue remained	(Lumpkins, Pierre and Mcfetridge, 2008)
		- Significantly reduced amount of sGAG and	(O'Neill *et al.*, 2013; Faulk *et al.*, 2014)

		laminin and fibronectin not well retained	
		- Residual detergent has been detected	(White *et al.*, 2016)
		- ECM proteins damaged	(Faulk *et al.*, 2014)
	Sodium deoxycholate (SDC) *(Ionic)*	+ Complete cell removal	(Poornejad *et al.*, 2016)
		+ ECM proteins detectable	(Shupe *et al.*, 2010a)
		+ Architecture intact	(Gilpin *et al.*, 2014; Poornejad *et al.*, 2016)
		- Cells not completely removed	(H. Wang *et al.*, 2015)
		- Disrupted architecture/structure	(H. Wang *et al.*, 2015)
		- Residual detergent has been detected	(White *et al.*, 2016)
	Triton-X-100 *(Non-ionic)*	+ Intact ECM basement membrane	(Shafiq *et al.*, 2012)
		- Cells not completely removed	(Karina H Nakayama *et al.*, 2010; Shupe *et al.*, 2010b; Du *et al.*, 2011; Shafiq *et al.*, 2012; Caralt *et al.*, 2015; Y. Wang *et al.*, 2015b; Poornejad *et al.*, 2016)
		- Disrupted architecture/structure	(H. Wang *et al.*, 2015)
		- Damage of ECM proteins	(Karina H. Nakayama *et al.*, 2010)
		- Softening of the tissue	(Lumpkins, Pierre and Mcfetridge, 2008)
		- Residual detergent has been detected	(White *et al.*, 2016)
	CHAPS	+ DNA content less	(Petersen *et al.*, 2010;

	(Zwitterionic)	than 50ng/mg of ECM dry weight	O'Neill *et al.*, 2013)
		+ Level of total collagen amount not significantly changed	(O'Neill *et al.*, 2013)
		+ ECM proteins detectable	(Petersen *et al.*, 2010; O'Neill *et al.*, 2013; Tsuchiya *et al.*, 2014)
		+ Architecture intact	(Petersen *et al.*, 2010; Gilpin *et al.*, 2014; Tsuchiya *et al.*, 2014)
		- Significantly reduced amount of sGAG	(Petersen *et al.*, 2010; O'Neill *et al.*, 2013; Tsuchiya *et al.*, 2014)
		- ECM proteins not well retained	(O'Neill *et al.*, 2013; Faulk *et al.*, 2014)
		-Destroyed tissue structure	(Du *et al.*, 2011; Tsuchiya *et al.*, 2014)
		- Cells not completely removed	(Gilpin *et al.*, 2014)
Acid *(Facilitate hydrolysis and degeneration of proteins)*	**Peracetic acid (PAA)**	+ Morphology characteristic	(Gilbert *et al.*, 2008; Syed *et al.*, 2014; Poornejad *et al.*, 2016)
		- Cells not completely removed	(Syed *et al.*, 2014; Y. Wang *et al.*, 2015a; Poornejad *et al.*, 2016)
Solvents *(Removal of lipids; dehydration of cells to facilitate lysis)*	**Ethanol/ Acetone**	+ Facilitates cell removal	(Sawada *et al.*, 2008; Wang *et al.*, 2017)
		- Stiffens the ECM structure	(Lumpkins, Pierre and Mcfetridge, 2008)

Table 3 Commonly used biological agents for cell removal

Category	Agent	Impact (Positive+/Negative -)	Reference
Enzymes *(Facilitate cell disassociation from the ECM and degradation of nucleic acids)*	**Trypsin-EDTA**	+ Complete decellularization	(Meyer *et al.*, 2006)
		- Cells not completely removed	(Prasertsung *et al.*, 2008; Poornejad *et al.*, 2016)
		- Degeneration and fragmentation of ECM	(Meyer *et al.*, 2006; Caralt *et al.*, 2015; Poornejad *et al.*, 2016)
		- Growth factor loss	(Caralt *et al.*, 2015)
		- Loss of mechanical strength	(Poornejad *et al.*, 2016)
	Nucleases: Deoxyribonuclease (DNase)/ Ribonuclease (RNase)	+ Promote cell removal/detachment	(Brown *et al.*, 2002; Ketchedjian *et al.*, 2005; H. Wang *et al.*, 2015; Mazza *et al.*, 2015)
		- Typically needs the combination with other agents	(Brown *et al.*, 2002; Ketchedjian *et al.*, 2005; H. Wang *et al.*, 2015; Mazza *et al.*, 2015)

Chemical and biological agents used for decellularization can be applied to the organ/tissue in several different ways, each having their own advantages and disadvantages. The most commonly used techniques are listed below:

- **Agitation:** Mechanical agitation is usually applied while the sample is immersed in a liquid agent (Macchiarini *et al.*, 2008; Montoya and McFetridge, 2009; Yang *et al.*, 2010). Agitation would help to lyse cells by facilitating greater exposure to the agent and enhancing removal of cellular

debris (Thomas W Gilbert, Sellaro and Badylak, 2006). Mechanical agitation is useful when little/no vascular network is present in the tissue and/or the tissue is very thin i.e. blood vessels (Montoya and McFetridge, 2009), trachea (Macchiarini *et al.*, 2008) or urinary bladder (Yang *et al.*, 2010) can be decellularized quickly, with very little equipment (in comparison to other decellularization technologies). Macchiarini *et al.*, 2008 successfully decellularized a donor tracheal segment with an agitation and immersion procedure, which was followed by repopulation of the graft with the patient's own chondrocytes as well as epithelial cells and a successful implantation into the recipient. After 1 month, the recipient's native tissue and the graft were indistinguishable (Macchiarini *et al.*, 2008). The disadvantages of mechanical agitation is that complete decellularization of dense or thick samples is not possible due to the large diffusion distance; hence mechanical agitation is limited to thin tissue with a high surface area.

- **Perfusion:** Organ perfusion utilizes the existing vascular network of the organ or tissue to assist distribution of the decellularization agent within the entire three-dimensional structure of the organ/tissue (Baptista *et al.*, 2009). It also facilitates the removal of cell debris and residual detergent by utilizing the physiological flow/pressure of arteries and veins (Guyette *et al.*, 2014). Advantages of perfusion decellularization are the preservation of the 3-dimensional structure and no limit in size or type of the organ (Keane *et al.*, 2015). Whole-organ decellularization by perfusion has been successfully demonstrated on heart (Ott *et al.*, 2008), liver (Uygun and Yarmush, 2013), lung (Ott *et al.*, 2010) and kidney (Song *et al.*, 2013). Disadvantages of perfusion decellularization include a high degree of technical skill to cannulate the organ; the low- throughput nature of the procedure (i.e. only one organ at a time), and the need of specialized equipment (such as tubing, pumps, support rings, etc.).
- **Pressure:** The application of pressure is used to force the decellularization agent into the organ/tissue and burst cell membranes (Gilpin and Yang, 2017). This facilitates increased exposure to the enzymatic or chemical agents. During depressurization, residual cellular debris is removed from the organ/tissue (Prasertsung *et al.*, 2008). The benefit of using pressure

application as a decellularization technology is a decrease in processing time, thereby reducing exposure time to harmful detergents and subsequently preserving more of the ECM structure. Sasaki *et al.*, 2009 claimed that high pressure (147,000 psi for 10 minutes) had a more effective result in cell removal than using 1% (w/v) Triton-X-100 or SDS alone. A limitation of the application of pressure is the need for specialized apparatus and the limited volume capacity of the pressure vessel.

The time to achieve complete removal of cellular and genomic material with any of the aforementioned techniques is dependent on several factors, such as the type and concentration of the agent used, the density/thickness of the tissue and the intensity and duration of the agitation (if used; Crapo, Gilbert and Badylak, 2011).

2.2.1. Optimal decellularization technology

Any decellularization technique and/or agent that impacts cell removal also has an undesirable/damaging effect on the ECM bioscaffold (Crapo, Gilbert and Badylak, 2011). However, despite these issues, ECM derived scaffolds remain a promising option for tissue engineering applications. The ideal architecture, functional complexity and suitability of the ECM as a biocompatible scaffold for tissue or organ fabrication present an ideal alternative to traditional organ or tissue transplantation.

For the optimal decellularization technology, complete decellularization is required. Being able to source unlimited tissue from allogeneic as well as xenogeneic origin requires complete decellularization. Londono *et al.*, 2017 showed that ECM bioscaffolds spiked with increasing amounts of residual cellular content (DNA, cell membrane or mitochondria) caused a dose-dependent immune response *in vitro*. Only ECM bioscaffolds containing low levels of genomic or cellular material can be considered a suitable transplantation alternative, which will result in the desired natural host response by degrading the biomaterial and naturally replacing it with functional tissue (Badylak 2014). The maximum level of retained genomic or cellular material that is considered suitable for transplantation is yet to be established. As previously discussed, such decellularization and complete removal of cellular content usually requires chemical and/or biological agents as well as physical force. This

causes unavoidable damage such as disruption of the ECM architecture and/or loss of ECM surface structure (Meyer *et al.*, 2006; Karina H Nakayama *et al.*, 2010; Du *et al.*, 2011), alteration of the ECM properties (Klaas *et al.*, 2016) or removal/degeneration of ECM proteins (Meyer *et al.*, 2006; O'Neill *et al.*, 2013). Another problem with using decellularization agents is the difficulty to fully remove the chemical post decellularization, resulting in residual toxicity. White *et al.*, 2016 showed with a ToF-SIMS study that fragments of various decellularization agents (Triton-X-100, SDC, SDS) remained detectable in an acellular urinary bladder scaffold. Collectively, these problems determine and/or influence the success of *in vitro* and *in vivo* reseeding (Tapias and Ott, 2014).

Ideally, the most suitable decellularization technology should be rationally picked based on the tissue or organ used and the proposed purpose of use after decellularization.

2.2.2. Supercritical fluid based decellularization technology

A relatively new decellularization technology is the use of supercritical carbon dioxide ($scCO_2$) for cell removal (Sawada *et al.*, 2008).

2.2.3. Supercritical fluids

A fluid is described as supercritical fluid (SCF) if the temperature and pressure are above the critical point. The critical point describes the highest point where the gas and liquid phase can coexist in equilibrium (Figure 2.1). SCFs are in a state where liquid and gas phases are indistinguishable due to the same density. SCFs benefit from liquid like density and solvation capability as well as gas like viscosity and thus diffusivity (Quirk *et al.*, 2004). Common SCFs with their critical points are listed below (Benner, Ricardo and Carrigan, 2004).

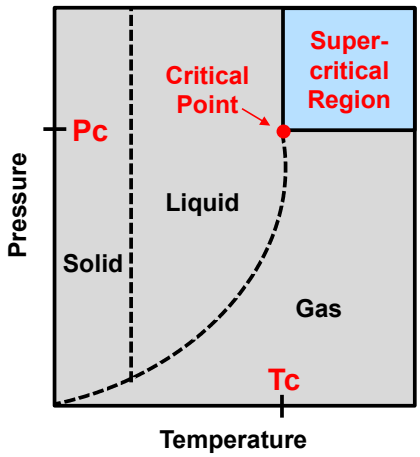


Figure 2.1 Phase Diagram SCF

Table 4 Critical temperature and critical pressure of common supercritical fluids. Information attained from (Benner, Ricardo and Carrigan, 2004)

Liquid	Temperature (°C)	Pressure (psi)
Nitrogen (N)	-147.15	492.31
Methane (CH_4)	-82.15	673.08
Ethane (C_2H_6)	31.85	708.35
Carbon dioxide (CO_2)	31.85	1071.34
Ammonia (NH_3)	132.85	1645.95
Water (H_2O)	373.85	3203.72

Supercritical fluids have many applications; the food industry uses SCF extraction for the removal of fat from certain food products, purification of spices, and elimination of alcohol or caffeine content (Brunner, 2005). The pharmaceutical industry also applies SCFs for particle generation and sizing, encapsulation, controlled delivery and sterilization purposes (Perrut, 2003).

2.2.4. Supercritical carbon dioxide

The most commonly used SCF is supercritical carbon dioxide ($scCO_2$; Quirk *et al.*, 2004; Duarte, Mano and Reis, 2009). $ScCO_2$ has mild operating conditions (31.85°C at 1071.335 psi) and is therefore very suitable for thermo-sensitive reactions (Benner et al. 2004). Further advantages of $scCO_2$ are stability, low toxicity, non-flammable,

non-corrosive, environmentally friendly, inexpensive and it is readily available (Quirk *et al.*, 2004; Duarte, Mano and Reis, 2009).

$ScCO_2$ is extensively used in the food industry for the removal of lipids/oils from animal derived products such as beef (Chao *et al.*, 1991), salmon (Tanaka, Sakaki and Ohkubo, 2004), trout (Hardardottir and Kinsella, 1988) and antarctic krill (Yamaguchi *et al.*, 1986). This is particularly interesting because it highlights the potential utility of $scCO_2$ as a decellularization technology. For instance, $scCO_2$ potentially has the diffusivity capability to penetrate throughout mammalian tissue and it has the ability to disrupt lipids in mammalian tissue (i.e. the phospholipid bilayer in the cell membrane, which would facilitate cell removal).

$ScCO_2$ has also been used in the biomedical field for sterilization of tissues such as cortical bone (Russell *et al.*, 2013, 2015), tendon (Nichols, Niles and Cortiella, 2009), amniotic membrane (Wehmeyer, Natesan and Christy, 2015), lung matrices (Jenna L. Balestrini *et al.*, 2016) and heart valves (Hennessy *et al.*, 2017). It is therefore speculated that this aspect could also be very interesting for a potential $scCO_2$ decellularization technology because further sterilization treatment of the produced ECM scaffold may not be required, which could therefore streamline production processes.

In comparison to decellularization agent based technologies, a $scCO_2$ based technique could offer reduced processing time (Sawada *et al.*, 2008; Wang *et al.*, 2017), a solvent-free procedure ($scCO_2$ will be eliminated through depressurization), no use of animal derived products for processing (such as trypsin), and could be performed on tissues without a vascular structure. This could potentially overcome challenges associated with currently used decellularization techniques such as denaturation of the ECM proteins by harsh decellularization agents, residual decellularization agents such as detergents, and technical challenges associated with perfusion equipment. However, the size of the high pressure autoclave will determine the maximum size of the tissue to be decellularized.

To date, there are a total of 8 publications (December 2019) using $scCO_2$ for decellularization of mammalian tissue (see table 5). In the initial publication by Sawada and colleagues (2008), the authors developed a decellularization methodology by utilizing $scCO_2$ in combination with ethanol and described successful removal of cell nuclei (determined by qualitative histological staining) from porcine aorta. Several publications have since followed, demonstrating the use of $scCO_2$ and ethanol for successful decellularization of human lipoaspirate tissue (Wang *et al.*, 2017), porcine and bovine cornea (Guler *et al.*, 2017; Huang *et al.*, 2017) as well as rat heart tissue (Seo, Jung and Kim, 2017). More recent papers have further developed hybrid $scCO_2$ decellularization methods by combining the use of $scCO_2$ and detergents, such as LS-54 (Antons *et al.*, 2018) and SDS (Casali *et al.*, 2018). Both studies utilized prolonged exposure (24 - 48 h) to decellularization agents prior to a brief $scCO_2$ exposure (1 h) to achieve decellularization of bovine cartilage, horse tendon, human skin (Antons *et al.*, 2018) and porcine aorta (Casali *et al.*, 2018). However, experiments that utilize multiple treatment steps require appropriate controls to determine how each variable contributed to the end result, which unfortunately was not described in the aforementioned studies. Hence, the exact role/effect of $scCO_2$ on tissue decellularization remains unknown.

Table 5 $scCO_2$ based decellularization technologies

Tissue	Method	Impact	Reference
Human lipoaspirate tissue	Extraction Temp.: 37 °C Pressure: 2610 psi Modifier: Ethanol Extraction time: 3 h	• Absence of cell nuclei & lipids (SEM, Histology) • Preservation of ECM components such as collagen Type I/III/IV, elastin, fibronectin, laminin (Immunohistochemistry) • Glycosaminoglycans retained (Blyscan sulfated GAG assay kit) • Fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) retained (Quantikine immunoassay kit)	**(Wang *et al.*, 2017)**

		• ECM coating was tested for biocompatibility with mesenchymal stem cells (ASCs), human umbilical vein endothelial cells (HUVECs), immortalized human keratinocyte (HaCaT) cells (Presto Blue, LIVE/DEAD viability)	
Porcine aorta	Extraction Temp.: 37 °C Pressure: 2900 psi Modifier: Ethanol Extraction time: 1 h	• Absence of cell nuclei (Histology) • Reduced level of phospholipids dependent on extraction • Preserved mechanical strength (Tensile tester)	**(Sawada *et al.*, 2008)**
Rat heart	Extraction Temp.: 37 °C Pressure: 5076 psi Modifier: Ethanol Extraction time: 6 h *Note: washed with DNase after $scCO_2$ treatment (5 days)*	• Absence of cell nuclei (Histology, DNA quantification via Dneasy Blood and Tissue kit) • Preservation of ECM components such as collagen laminin, fibronectin and myosin heavy chain (Immunohistochemistry) • Glycosaminoglycans unchanged (DMMB) • Collagen retained (Sircol collagen assay kit) • No immune response *in vivo* (Immunohistochemistry of macrophage infiltration)	**(Seo, Jung and Kim, 2017)**
Porcine aorta	Extraction Temp.: 37 °C Pressure: 4000 psi Modifier: None	• Absence of cell nuclei (Histology, DNA quantification via DNAzol)	**(Casali *et al.*, 2018)**

	Extraction time: 1 h *Note: **Hybrid method**, pre-soaked with 0.2% (w/v) EDTA (1h) followed by 0.1% (v/v) SDS, DNase, RNase (48h) prior pre-saturated $scCO_2$ exposure*	• Preserved hydration status (weight change) • Preserved mechanical strength (uniaxial ring testing) • No residual SDS toxicity (SDS Detection and Estimation Kit)	
Porcine cornea	Extraction Temp.: 45 °C Pressure: 5076 psi Modifier: Ethanol Extraction time: 1.5 h *Note: pre-soaked in water and 2M NaCl 30min (3x)*	• Absence of cell nuclei (Histology, DNA quantification with Qubit 2.0 Fluorometer) • Reduced Glycosaminoglycan content (Blyscan Gag assay kit) • Maintained Collagen (SDS Page gel) • Decreased mechanical strength (Materials Testing System) • No immune response *in vivo*	**(Huang *et al.*, 2017)**
Ovine aorta and bovine cornea	Extraction Temp.: 37 °C Pressure: 2500 psi Modifier: Ethanol Extraction time: 1 h	• Absence of cell nuclei (Histology, DNA quantification via Nanodrop) • ECM structure retained (Histology, SEM)	**(Guler *et al.*, 2017)**

Bovine cartilage	Extraction Temp.: 37 °C Pressure: 3625 psi Modifier: None Extraction time: 1 h *Note:* ***Hybrid method****, pre-scCO₂ treatment 2% LS-54 (24h), Preparation of tissue - Cartilage has been freeze-thawed (6x), Trypsin, Osmotic shock (24h)*	• Absence of cell nuclei (Histology, DNA quantification via Nanodrop) • Preservation of ECM components such as laminin, fibronectin (Immunohistochemistry) • Preservation of collagen (Histology) • Reduced glycosaminoglycan content (Histology, DMMB) • Mechanical strength reduced (uniaxial test system) • Biocompatibility (Presto Blue, Zone of inhibition assay, qualitative MTT)	**(Antons *et al.*, 2018)**
Horse tendon	Extraction Temp.: 37 °C Pressure: 3625 psi Modifier: None Extraction time: 1 h *Note:* ***Hybrid method****, pre-scCO₂ treatment 2% LS-54 (24h)*		
Human skin	Extraction Temp.: 37 °C Pressure: 3625 psi Modifier: None Extraction time: 1 h *Note:* ***Hybrid method****, pre-scCO₂ treatment 2% LS-54 (24h) Preparation of tissue - remove epidermis (24h)*		
Porcine and bovine pericardium	Extraction Temp.: 35°C Pressure: 1450 psi Modifier: 25 wt% hydrogen peroxide, 1.25 M sodium hydroxide	• Mechanical strength retained (uniaxial tensile testing, ultimate tensile stress, fracture toughness) • Preserved hydration status (water uptake and swelling)	**(Halfwerk *et al.*, 2018)**

	and 0.1 M phosphoric acid Extraction time: 1 h *Note:* ***Nova 2200 (Novasterilis, USA)*** *was used for decellularization*		

Taken together, there is promising potential for the development of a $scCO_2$ decellularization technology based on the successful use of $scCO_2$ in the food industry to extract oil/lipids as well as the use of $scCO_2$ for sterilization purposes of mammalian tissue. The emerging field of $scCO_2$ decellularization is relatively new and the technology as well as the mechanism is yet to be fully established. It is noteworthy that there has been no successful report using $scCO_2$ alone to decellularize mammalian tissue. The limited but available literature in the field has mainly utilized a combination of $scCO_2$ with a decellularization agent but is lacking appropriate controls/experimental design to conclusively characterise the developed hybrid/$scCO_2$ decellularization method. Therefore, a comprehensive characterization of the effect of $scCO_2$ on mammalian tissue was performed in this thesis to enhance the knowledge gap in the field and to facilitate an insight into the underlying mechanism of $scCO_2$ decellularization.

3. Aims & Objectives

3.1 AIMS

This PhD project was centred on investigating the use of $scCO_2$ for the decellularization of mammalian tissue, whereby the resultant ECM bioscaffold would be suitable for tissue engineering applications. The following over arching hypotheses were developed and tested:

- ***Hypothesis (1): $scCO_2$ removes cellular material from mammalian tissue***

  This hypothesis was developed because $scCO_2$ has been commonly used within the food industry for the extraction of lipids from animal derived products. Therefore, it is possible that $scCO_2$ could also be utilised to disrupt the phospholipid component of mammalian cell membranes, therefore enabling decellularization. In support of this, a number of publications have demonstrated the promising potential for $scCO_2$ to be used as part of a decellularization method (Sawada *et al.*, 2008; Wang *et al.*, 2017). However, these studies presented only subjective analysis of decellularization efficacy, which did not fulfil the successful decellularization criteria published by Crapo, Gilbert and Badylak, 2011.

- ***Hypothesis (2): Decellularization by $scCO_2$ is improved by combination with decellularization agents (i.e. detergents, solvents etc.)***

  This hypothesis was developed because detergents are well known to break the phospholipid membranes of cells and facilitate the removal of cellular materials from biological tissue. Therefore, it is possible that the addition of a detergent might further facilitate the removal of any residual genomic material not already removed by the $scCO_2$ decellularization method. There are a number of publications that have attempted to improve the decellularization process by combining detergents with $scCO_2$ (Antons *et al.*, 2018; Casali *et al.*, 2018). However, all of these studies use several decellularization agents at high

concentrations or long durations, which made it difficult to elucidate the impact of the $scCO_2$ component of the methodology.

- ***Hypothesis (3): Tissues exposed to $scCO_2$ are non-cytotoxic in vitro***

This hypothesis was developed because $scCO_2$ is commonly used for sterilization purposes in the biomedical industry. Therefore, it was considered likely that tissue or ECM scaffolds that were exposed to $scCO_2$ would not be cytotoxic to cells cultured *in vitro*.

3.2 OBJECTIVES

- **Objective (1): Develop and optimize an effective $scCO_2$ extraction process for tissue decellularization**
  - Investigate the effect of surface area, moisture content and exposure time to fully understand the effect of $scCO_2$ alone on mammalian tissue
  - Assess extent of decellularization using quantitative methods such as residual DNA content
  - Evaluate the impact of $scCO_2$ exposure on the ECM structure by using quantitative methods such as hydroxyproline content
  - Assess the gross morphological changes within the ECM scaffold after $scCO_2$ exposure using Histology
  - Compare the developed $scCO_2$ decellularization method to a "gold standard" detergent-based decellularization method
  - Validate the developed $scCO_2$ decellularization method on a structurally different second tissue type

- **Objective (2): Examine the effect of single decellularization agents to facilitate the removal of cellular and extracellular components from the $scCO_2$ decellularized ECM bioscaffold**
  - Investigate the effect of Trypsin/EDTA, Triton-x-100, Sodium deoxycholate (SDC) and LS-54 in combination with $scCO_2$ on cell removal of mammalian tissue

- Compare the impact of sequence of $scCO_2$ exposure when used in combination with decellularization agents (i.e. detergents, solvents etc.)
- Assess extent of decellularization using quantitative methods such as residual DNA content
- Evaluate the impact of the decellularization agents in combination with $scCO_2$ exposure on the ECM structure by using quantitative methods such as hydroxyproline content

❖ **Objective (3): Determine the cytotoxicity of the ECM scaffold decellularized by $scCO_2$ alone as well as in combination with a single decellularization agent on HepG2 and 3T3 cells *in vitro***

- Investigate the biocompatibility of tissue treated with Trypsin/EDTA, Triton-x-100, Sodium deoxycholate (SDC) and LS-54 in combination with $scCO_2$ on HepG2 and 3T3 cells
  - Assess indirect biocompatibility by exposing cells to media that has been incubated with the tissue scaffolds for 30 h and 90 h
  - Assess direct biocompatibility by exposing cells directly to the ECM tissue scaffold

4. Materials & Methods

4.1. OVERVIEW

This chapter will detail all materials and methods used throughout this thesis. All high-pressure related work was conducted in the School of Chemistry and all biomolecular and biochemical analyses were performed in the Centre for Biomolecular Sciences (CBS), University of Nottingham, UK.

4.2. MATERIALS

4.2.1. Chemicals & Equipment

Materials & Equipment	Supplier
1,9-dimethylmethylene blue	Sigma Aldrich, 341088, UK
100bp DNA ladder	New England Biolabls, NO467, UK
1kb DNA ladder	New England Biolabls, NO552, UK
6x loading dye	Promega, G1881, UK
Agarose powder	Sigma Aldrich A9539, UK
Carbon dioxide	SCF grade 4.0 CO_2, BOC special gases, UK
Chondroitin sulphate	Sigma Aldrich, C4384, UK
Dehypon LS-54	Conservation Resources, UK
Disodium phosphate	Sigma Aldrich, 71649, UK
Dry ethanol	Sigma Aldrich, 676829, UK
Ethanol	Fisher Scientific, E/0650DF/17, UK
Ethidium bromide	Sigma Aldrich, 1239-45-8, UK
Ethylenediaminetetraacetic acid	Fischer Scientific, D/0700/53, UK
Foetal bovine serum	Sigma Aldrich, F9665, UK
Genomic mouse DNA	Promega, G3091, UK
Gentamicin	Sigma Aldrich, G1397, UK
Glycine	Sigma Aldrich, G6600, UK
Heating jacket	made in-house, School of Chemistry, University of Nottingham
Hepatocellular carcinoma cell line (HepG2)	HepG2, ATCC, HB8065
High pressure pump PM-101	New Ways of Analytics, NWA, Germany

HiP needle valve (two-way)	High Pressure Equipment, HiP, UK
Histoclear	National Diagnostics, HS-202, USA
Hydrochloric acid	Sigma Aldrich, 435570, UK
Hydroxyproline Assay	Sigma Aldrich, MAK008, UK
L-cysteine	Sigma Aldrich, C1276-50G, UK
L-Glutamine	Sigma Aldrich, G7513,UK
Minimum Essential Medium Eagle	MEM, Sigma Aldrich, M4526, UK
Mouse embryonic fibroblast (NIH/3T3)	Kindly donated by RMCT Division
MTT assay	Roche, 11465007001, UK
Non-essential amino acids	NEAA, Sigma Aldrich, M7145, UK
Orbital shaker	OHAUS, SHHD1619DG, USA
O-Ring	ethylene propylene diene monomer (EPDM), Brammer, UK
Papain	Sigma Aldrich, P3375-25G, UK
Paraffin wax	Sigma Aldrich, P3683, UK
Paraformaldehyde	Sigma Aldrich, P6148, UK
Penicillin and Streptomycin	Fisher Scientific, 11528876, UK
Peroxyacetic acid	Fischer Scientific, 257755000, UK
Phenol/Chloroform/Isoamyl	Sigma Aldrich, 77617, UK
Phosphate buffered saline	Fisher Scientific, 1282-1680, UK
Phospholipid assay	Sigma Aldrich, MAK122, UK
Pico green assay	Life technologies, P11496, UK
Pressure monitor	made in-house, School of Chemistry, University of Nottingham
Pressure transducer	RDP Electronics, UK
Proteinase K	Invitrogen, 25530-049, UK
Snoop	Swagelok, UK
Sodium chloride (NaCl)	Fisher Scientific, S/3160/63, UK
Sodium Deoxycholate	Sigma Aldrich, D2510-100G, UK
Sodium dodecyl sulphate	Sigma Aldrich, L3771-500G, UK
Stainless steel clamp	made in-house, School of Chemistry, University of Nottingham
Stainless steel pipe	SS316, Swagelok, UK
TAE buffer	Fisher Scientific, B49, UK
TE buffer	PanReac AppliChem, 7l012281, UK
Temperature monitor	made in-house, School of Chemistry, University of Nottingham
Thermocouples	Type K, RS Instruments, UK
Tris-HCL	Fisher Scientific, 77-86-1, USA
Triton-x-100	Sigma Aldrich, T-8787, UK
Trypsin	Thermo Fisher Scientific, 15090-046, UK
Trypsin/EDTA	Sigma Aldrich, T4049, UK

4.2.2. Biological tissue

Porcine liver and aorta was obtained from a EU-certified local butcher (Nottingham, UK). Tissues were harvested immediately after slaughter, transferred on ice to The University of Nottingham and stored at approximately -20°C until used.

4.2.3. High-pressure equipment

4.2.3.1. High-pressure pump and autoclave system

A 34kg industrial grade carbon dioxide cylinder was used to supply the high-pressure laboratory with CO_2. The CO_2 was stored in the vapour phase and a CO_2 regulator was set to release CO_2 at a pressure of approximately 795 psi (~ 55 bar) to a PM-101 high-pressure pump where the gaseous CO_2 was liquefied via a condenser and a refrigerated pump.

The high-pressure pump was connected to a stainless steel pipe line that connected the whole laboratory to the CO_2 supply. Each high-pressure autoclave was individually connected to the CO_2 line in an allocated fume hood via a two-way HiP needle valve that enabled the use or cut off from the CO_2 supply. When opened, the liquid CO_2 flooded into the system via a pressure transducer that was connected to a pressure monitor to give real time readings of the pressure level in the system. The system was set to not exceed 4000 psi.

The high-pressure autoclave was also equipped with a heating jacket that allowed controlled heating of the system. Two thermocouples were connected to the autoclave and a temperature monitor for real time temperature readings of the system. In case of exceeding the maximum pressure levels, the pressure and temperature monitors were connected to a safety trip box that automatically switches off the heating to not further increase the pressure of the system. The autoclave had two exit taps (two way HiP needle valve) for the pressure to be released (Figure 4.1 Figure 4.2).

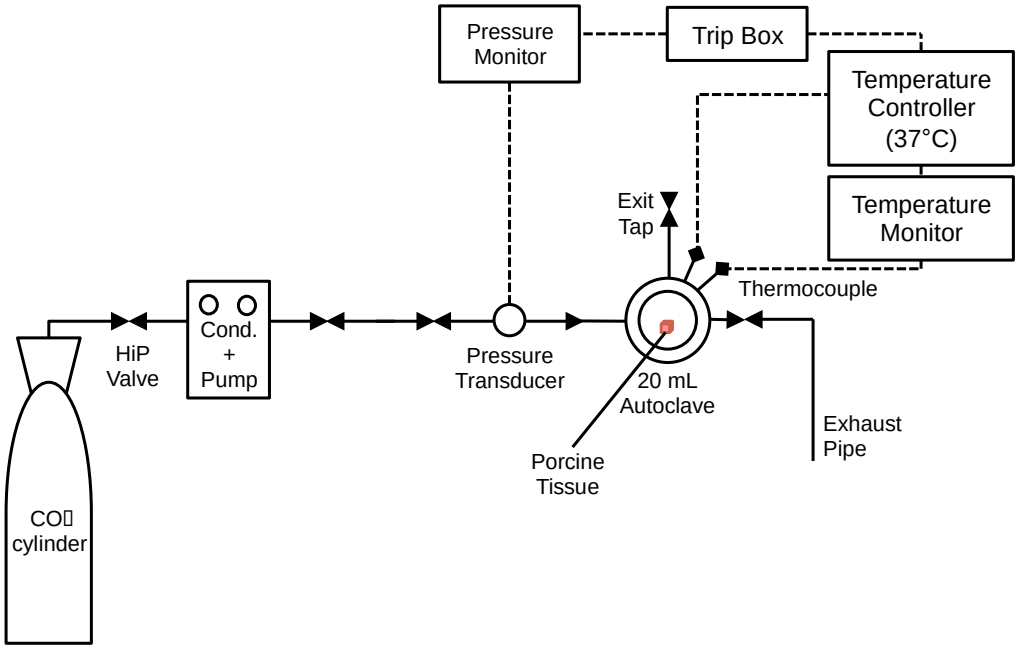


Figure 4.1 Schematic of high-pressure pump and autoclave system of the supercritical carbon dioxide rig.

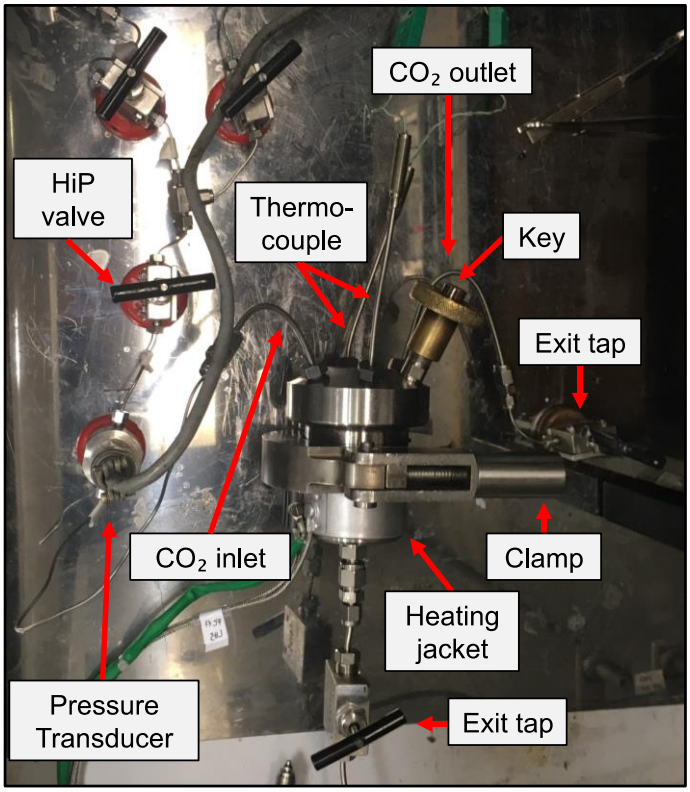


Figure 4.2 Representative overhead image of high-pressure autoclave set up.

4.2.3.2. High-pressure autoclave

The high-pressure autoclave used for all experiments consisted of a 20 mL stainless steel (316) high-pressure vessel, that was designed, developed and built in-house at the School of Chemistry, University of Nottingham, UK. The autoclave was made up of two parts: an autoclave head and an autoclave base (Figure 4.3). The autoclave head was connected to a CO_2 inlet and a CO_2 outlet, two thermocouples and an autoclave key (explained further below).

The autoclave base contained a 20 mL void and was connected to a two way HiP needle valve via a frits filter (to avoid any tissue blocking the stainless steel pipes, Swagelok, UK) that was further connected to a stainless steel pipe that lead directly into the outlet of the fume hood. This was a safety element purposely put in place for working with biological tissue in chemistry and to avoid any biological contamination within the chemistry building.

The autoclave head and base were sealed with a 42 x 2 mm O-Ring and interlocked before a stainless steel clamp was placed around it and secured the interlock. The clamp could only be tightened and secured with a unique pattern on the top of the autoclave key that was individual to each autoclave (Figure 4.4). This was a safety element purposely put in place for the work with high-pressure, as to undo the clamp the autoclave key had to be removed first which automatically releases the pressure of the system.

All experiments were carried out and recorded using pound-force per square inch (psi) as a pressure unit measurement. As a reference, ambient pressure was 14.7 psi.

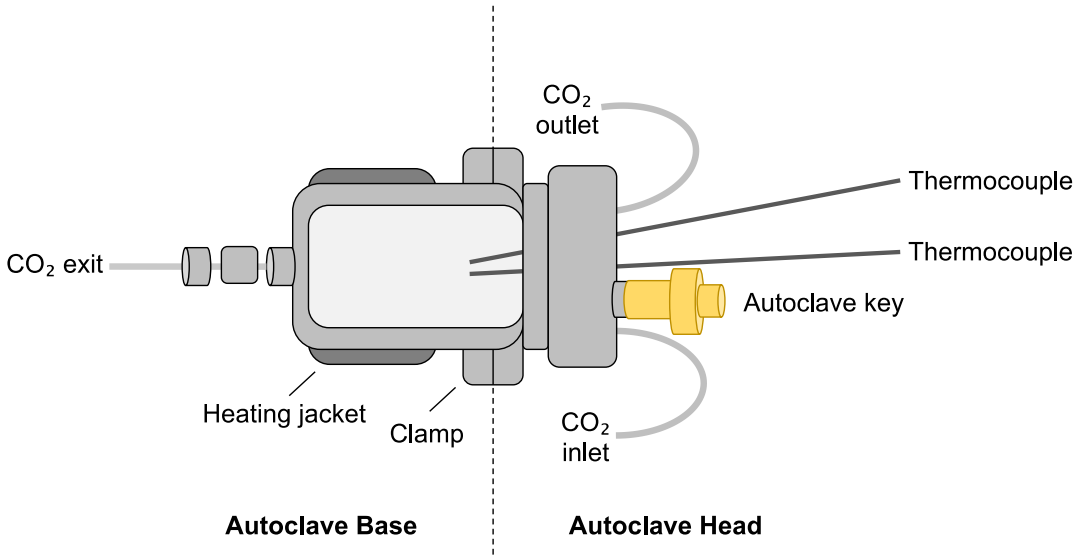


Figure 4.3 Schematic of high-pressure autoclave.

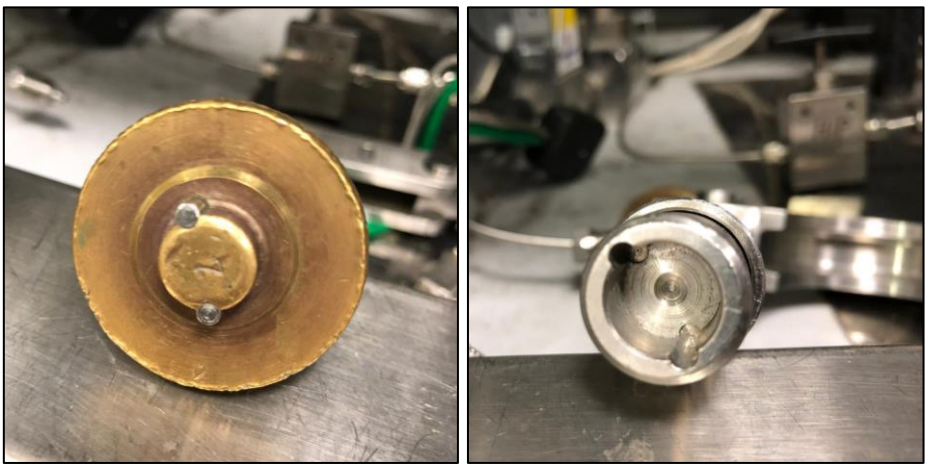


Figure 4.4 Representative images of the unique locking mechanism of key and clamp.

4.3. METHODS

4.3.1. High-pressure technology

4.3.1.1. Leak test of high-pressure equipment

Before an experiment was carried out using the high-pressure equipment, a leak test at working conditions was performed. For this purpose, the high-pressure autoclave was pressurised and heated to working conditions (37°C, ~2900 psi; see section 4.2.3.1. High-pressure pump and autoclave system).

All fittings and joints were then covered in Snoop, a liquid leak detector that creates bubbles if a gas leaks out, which enables the location of the leak to be detected. In the case of a leak, the system was completely depressurised and the leaking joint was tightened. The system was then re-tested for leaks again until no more leaks were found. In the absence of a leak, the system was depressurised and used immediately.

4.3.1.2. Solubility testing in $scCO_2$

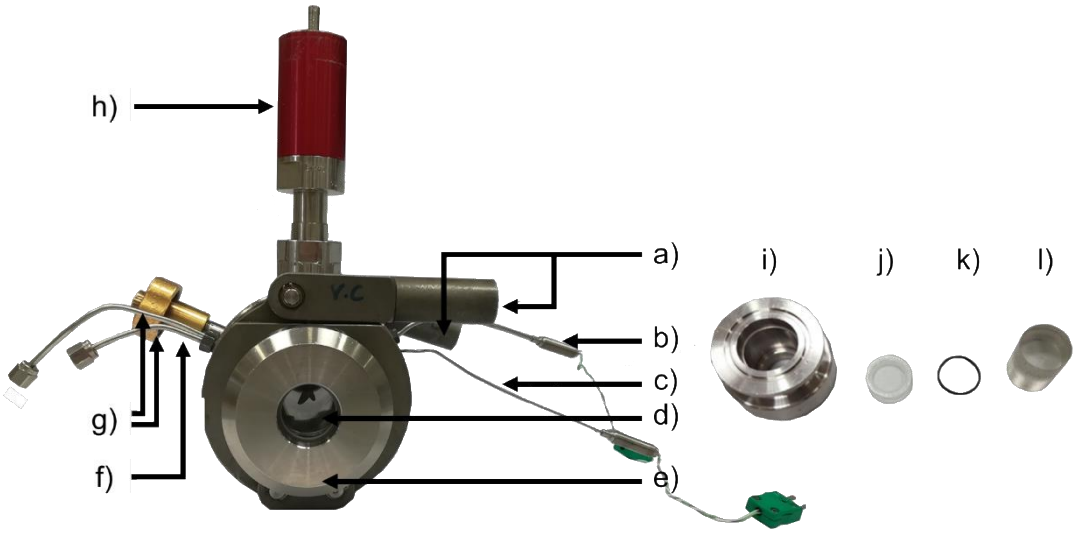
All modifiers that were added in any way to the $scCO_2$ phase were first tested in a view cell for their solubility in $scCO_2$. The view cell had the same set-up as a standard high-pressure autoclave but was further equipped with two autoclave heads instead of one (front and back) which each had a 36 x 40 mm sapphire glass window that enabled visualisation of the process, as well as a magnetic overhead stirrer, which was not used (Figure 4.5, Figure 4.6).

The following modifiers were tested in the high-pressure view cell: Ethanol, 0.02% Trypsin (v/v)/0.05% Ethylenediaminetetraacetic acid (EDTA, w/v); 3% Triton-x-100 (v/v); 4% Sodium Deoxycholate (w/v), Dehypon LS-54. A leak test of the equipment at working condition (37°C, ~2900 psi) was performed before any experiments were carried out (see section 4.3.1.1.).

Five hundred microliters of liquid modifier was added into a 1 x 1 cm round glass dish that was placed in the centre of the view cell. The operating protocol was

followed as described in more detail (see section 4.3.2.1. Supercritical carbon dioxide decellularization 4.3.2).

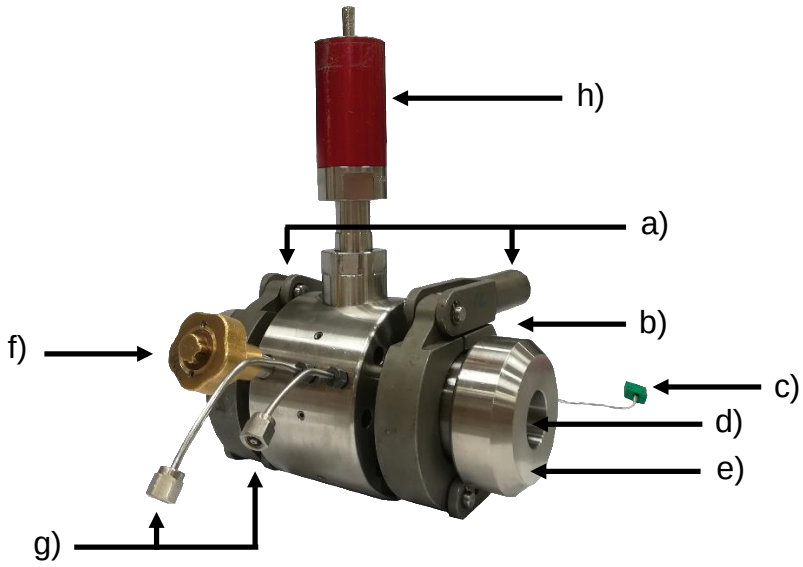
Briefly, the high-pressure view cell was pressurised to ~1000 psi and heated to 30°C, before the pressure was further increased to ~2600 psi and a temperature of 37°C. Once the temperature of 37°C was reached, the modifier was left to sit in scCO_2 for 15 minutes and was visually assessed for solubility. The heating jacket was then set to 0°C and the high-pressure system fully vented via the CO_2 exit tap within 1 minute. Representative images were taken throughout the process.



Legend

a) Back/front clamps	e) Head	i) Head
b) Internal thermocouple	f) Safety key	j) *Teflon* spacer
c) External thermocouple	g) Inlet/outlet pipes	k) O-Ring
d) Sapphire window	h) Magnetically coupled overhead stirrer	l) Sapphire window

Figure 4.5 Representative image of all view cell components.



Legend

a) Back/front clamps	e) Head
b) Internal thermocouple	f) Safety key
c) External thermocouple	g) Inlet/outlet pipes
d) Sapphire window	h) Magnetically coupled overhead stirrer

Figure 4.6 Representative image of the assembled view cell.

4.3.2. Decellularization methodologies

4.3.2.1. Supercritical carbon dioxide decellularization

Supercritical carbon dioxide ($scCO_2$)-based decellularization of porcine liver/aorta was performed using the 20 mL stainless steel (316) pressure vessel equipped with a controlled heating jacket, as described above (see section 4.2.3.1. High-pressure pump and autoclave system).

Liver/aorta was placed into the autoclave, a new O-Ring (described in section 4.2.3.2 High-pressure autoclave) was placed between the autoclave head and base and the system was interlocked. The clamp was placed around the joint connecting the autoclave head and base and tightened using the unique pattern on the top of the autoclave key. When ethanol was used, 6 mL of dry ethanol was injected into the autoclave via the autoclave keyhole using a syringe with a constant pressure of ~75 psi to avoid hydration of the ethanol. The autoclave key was screwed in the autoclave head to fully seal and lock the autoclave system. All two way HiP needle valves were closed to not let any CO_2 escape the system.

A leak test (see section 4.3.1.1.) was carried out at working conditions (37°C, ~2900 psi) before any experiments could be executed. A gradual pressurisation process was performed for the leak test as well as the experiment; the high-pressure autoclave system was first pressurised to ~1000 psi and heated to 30°C, once pressure and temperature were stable the pressure was further increased to ~2600 psi and a temperature of 37°C. Once the temperature of 37°C was reached, the duration of $scCO_2$ exposure was counted. The temperature was maintained at 37°C +/- 3°C and never exceeded 40°C. After the desired $scCO_2$ exposure time had elapsed, the heating jacket was set to 0°C to stop the heating and the system vented to ambient pressure via the two way HiP needle valve within 1 minute as the vessel was cooling (Figure 4.7). The $scCO_2$ exposed tissue was removed from the high-pressure autoclave and stored at approximately -20°C until further use.

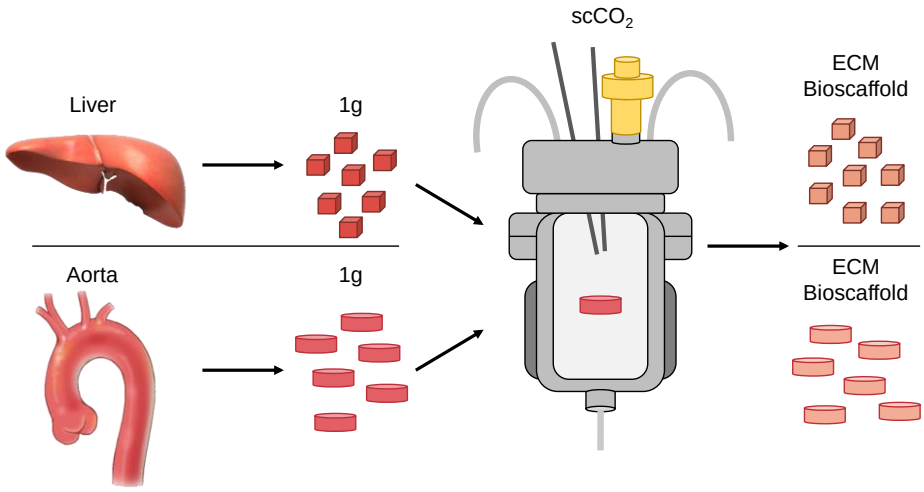


Figure 4.7 Experimental design of supercritical carbon dioxide decellularization.

4.3.2.2. Detergent decellularization

Detergent based decellularization of the porcine tissue was performed following an adapted protocol by Loneker *et al.*, 2016.

Briefly, frozen liver or aorta was thawed and thinly sliced using a mandolin slicer (liver) or cut into rings with a scalpel (aorta) before washing 3 times for 30 minutes using an orbital shaker in dH_2O at 300 rpm (1 L conical flask = 750 mL dH_2O, 2 L conical flask = 1500 mL dH_2O) to remove excess blood.

Tissue slices/rings were gently compressed between sheets of greaseproof paper in an attempt to aid penetration of the detergent before each of the following detergent treatments were applied at room temperature for 1h at 300 rpm: 0.02% Trypsin (v/v)/0.05% Ethylenediaminetetraacetic acid (EDTA, w/v) to aid cell disassociation; 3% Triton-x-100 (v/v) to facilitate cell permeabilisation and 4% Sodium Deoxycholate (w/v) to emulsify remaining fats. After each detergent treatment, tissue slices/rings were washed at room temperature for 3 x 15 minutes in dH_2O at 300 rpm. The extracellular matrix (ECM) materials were stored in the fridge overnight.

The following day, liver/aorta ECM material was washed in 0.1% Peroxyacetic acid diluted in 4% ethanol (v/v in dH_2O) at room temperature for 2 h at 300 rpm. The

ratio of 0.1% Peroxyacetic acid to ECM material (wet weight) was 20:1 (v/w). This was followed by four washes: 1x phosphate buffered saline (PBS), 1x dH_2O, 1x PBS, 1x dH_2O at room temperature for 15 minutes at 300 rpm before the liver/aorta ECM material was lyophilised for 48 h and stored at approximately -20°C until used.

4.3.3. Biomolecular and biochemical analytical techniques

4.3.3.1. DNA Extraction

Liver and aorta tissues were digested using two different enzymatic methods to lyse the cell membranes and release the DNA (details below).

Liver: Briefly, 100 mg of lyophilised liver tissue was powdered (using a pestle and mortar cooled in liquid nitrogen) and digested in 2 mL of proteinase K buffer (20 mg/mL Proteinase K, 1 M Tris-HCL (pH 8), 5 M NaCl, 0.5M EDTA (pH 8) and 10% Sodium dodecyl sulphate (SDS) for 24 h at 60°C or until no visible tissue remained.

Aorta: Briefly, 100 mg of lyophilised aorta tissue was powdered (using a pestle and mortar cooled in liquid nitrogen) and digested in 2 mL of Papain buffer (125 µg/mL), 10 mM L-cysteine, 10 mM EDTA, 100 mM Disodium phosphate for 24 h at 60°C or until no visible tissue remained.

Once fully digested (regardless of the digestion method), DNA was extracted using a phenol-chloroform extraction method. Two millilitres of 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol mixture was added to each sample, before it was vortexed and centrifuged for 10 minutes (10,000 rcf at 4°C). This caused a phase separation, with the organic/lower phase containing the phenol/chloroform/isoamyl as well as lipids and cellular debris, while the aqueous/upper phase contained the DNA. The aqueous/upper phase (containing the DNA) was transferred to a separate tube and the process repeated twice (or until no more white precipitate (protein) was visible). Three Molar sodium acetate (pH 5.2) (1/10th of the sample volume; i.e. 200 µL in 2 mL) and 4 mL of 100% Ethanol were added to the purified upper phase

(containing the DNA), and then frozen on dry ice (approximately -80°C; ~45 minutes) to aid precipitation of the DNA.

Samples were then centrifuged (10,000 rcf, 4°C, 10 minutes) to pellet the DNA. The supernatant was carefully removed and discarded. The DNA pellet was washed using 7 mL of 70% ethanol followed by another centrifugation (10,000 rcf, 4°C, 10 minutes). The supernatant was again removed and discarded. The DNA pellet was air-dried at room temperature for 10 minutes before being re-suspended in 1 mL of 1x TE buffer.

4.3.3.2. Pico green assay (DNA quantification)

The Pico Green Assay was used to quantify DNA content. DNA content was used as a marker for decellularization as successful decellularization is associated with markedly reduced DNA content.

A DNA standard curve was generated by serial dilution of a calf thymus DNA stock solution (dsDNA 2 μg/mL, in 1x TE buffer). Standard curve concentrations were: 2000, 1000, 500, 250, 125, 62.5, 31.25, and 0 ng/mL). Samples were diluted in 1x TE buffer to ensure they were within the standard curve range (typical dilution used for liver/aorta was 1:1000).

Quant-It PicoGreen reagent (1:200 in 1x TE buffer), a fluorescent nucleic acid dye, was prepared immediately prior to use and wrapped in aluminium foil to protect from light. One hundred microliters of each standard and sample were loaded onto a black 96-well plate, followed by 100 μL of Quant-It PicoGreen reagent. The plate was then incubated in the dark for 5 minutes at room temperature. The concentration of DNA (ng/mL) was estimated using a TECAN Infinite M200 PRO UV plate reader (Excitation 480 nm, Emission 520 nm) and normalised to dry tissue weight (post-decellularization). Each sample and standard was assayed in either duplicate or triplicate, which were then averaged to obtain a robust single representative value for each sample.

4.3.3.3. Rehydration capacity

A rehydration study was performed to assess the capacity of the tissue (liver/aorta) to rehydrate. This was performed to indicate whether the weight lost during the decellularization process was due to the loss of water content or other cellular components. Therefore, tissue (~1 g) was incubated in 5 mL of dH_2O for 24 h under gentle shaking. Tissue weight was taken before and after this incubation step.

4.3.3.4. Phospholipid assay

The Phospholipid assay was used to quantify choline-containing phospholipids as major components of the cell membrane.

Briefly, 10 mg of dry tissue was homogenized in 50 µL of ultrapure water and 50 µL of the assay buffer. The samples were vortexed before centrifugation for 10 minutes at 10,000 rcf, 4°C. Twenty microliters of the supernatant was used for the assay. A standard was generated from a 2 mM Phosphatidylcholine standard stock solution in ultrapure water. The following standard curve concentrations were used: 0, 30, 60, 120 and 200 µM.

A reaction mix of the following ratio was prepared per reaction well: 1µL enzyme mix, 1 µL PLD enzyme and 1 µL of dye reagent to 85 µL of assay buffer. The reaction mix enzymatically hydrolysed the phospholipids contained in the sample and released choline, that was detected via choline oxidase and a H_2O_2 binding dye. The phospholipid concentration (µM) was estimated using a TECAN Infinite M200 PRO UV plate reader (Absorbance 570 nm) and normalised to dry tissue weight (post-decellularization). Each sample and standard were assayed in duplicate, which were then averaged to obtain a single representative value for each sample.

4.3.3.5. DMMB (1,9-dimethylmethylene blue) assay

The 1,9-dimethylmethylene blue (DMMB) Assay was used to quantify sulphated glycosaminoglycan (sGAG) content. sGAG content was used in combination with collagen content (see section 4.3.3.6. Hydroxyproline assay) as a marker for retained ECM scaffold components.

Briefly, 10 mg (aorta) or 20 mg (liver) were enzymatically digested to break down protein substrates in 1 mL of 125 μg/mL papain buffer (125 μg/mL), 10 mM L-cysteine, 10 mM EDTA, 100 mM Disodium phosphate at 65°C overnight or until no visible tissue remained. DMMB reagent, a light-sensitive cationic dye that specifically binds to sGAG, was generated by dissolving 16 mg/L of 1,9-dimethylmethylene blue in 5 mL of 100% ethanol before adding 40 mM glycine 40 mM NaCl and dH_2O to make up the desired quantity of DMMB reagent solution. The pH of the DMMB reagent solution was adjusted to 1.5 pH using hydrochloric acid. The DMMB reagent was wrapped in aluminium foil (to protect it from light) and kept at room temperature for up to 3 months.

A standard curve was produced by serial dilution of a 1 mg/mL chondroitin sulphate solution in papain buffer (see above). The final standard curve concentrations were: 100, 75, 50, 37.5, 25, 18.75, 12.5 and 0 μg/mL. Twenty microliters of each standard and sample were loaded onto a clear 96-well plate, followed by 200 μL of DMMB reagent. The plate was read within 10 minutes after the addition of the DMMB reagent. The sGAG concentration (μg/mL) was estimated using a TECAN Infinite M200 PRO UV plate reader (Absorbance 525 nm – 595 nm) and normalised to dry tissue weight (post-decellularization). Each sample and standard was assayed in duplicate, which were then averaged to obtain a single representative value for each sample.

4.3.3.6. Hydroxyproline assay

The Hydroxyproline Assay was used to quantify hydroxyproline, which is a major component of collagen (helix structure stabiliser), therefore serving as an indicator of collagen content. Collagen content was used in combination with sGAG content (see section 4.3.3.5. DMMB (1,9-dimethylmethylene blue) assay) as a marker for retained ECM scaffold components.

Briefly, 15 mg of dry tissue was homogenised in 100 μL of dH_2O and hydrolysed with 100 μL of 12 M hydrochloric acid at 120°C (using a heating block with 1.5 mL inserts) for 3 hours to break down all dry tissue components. Discolouration of the

samples occurred. Samples were mixed by vortexing and then centrifuged at 10,000 rcf for 3 minutes before being diluted in dH_2O to ensure they were within the standard curve range (typical dilution used for liver and aorta was 1:100). Ten microliters of each sample was transferred in duplicate to a clear 96-well plate. Hydroxyproline standards were generated from a 1 mg/mL Hydroxyproline standard stock solution and transferred directly to a clear 96-well plate. The following standard curve concentrations were used: 1, 0.8, 0.6, 0.4, 0.2 and 0 μg/well. All samples/standards were plated in duplicate. Once plated, the clear 96-well plate was placed in a laboratory oven at 60°C to dry samples (60 minutes).

Chloramine T/Oxidation Buffer (6:94 ratio) mixture was prepared immediately prior to use. Chloramine T oxidises the available hydroxyproline and reacts to form a pyrrole. One hundred microliters of Chloramine T/Oxidation Buffer was added per sample/standard and incubated for 5 minutes at room temperature.

Diluted DMAB reagent consisting of a 1:1 ration of DMAB Concentrate and perchloric acid/isopropanol solution was then prepared for immediate use. One hundred microliters of DMAB concentrate and perchloric acid/isopropanol solution was added to the samples/standards before incubating at 60°C for 90 minutes. This causes a colorimetric reaction of the oxidised hydroxyproline. The concentration of collagen (μg/mL) was estimated using a TECAN Infinite M200 PRO UV plate reader (absorbance 560 nm) and normalised to dry tissue weight (post-decellularization). Each sample and standard was assayed in duplicate, which was then averaged to obtain a single representative value for each sample.

4.3.3.7. DNA gel electrophoresis

Gel electrophoresis was used to visualise the integrity of the extracted DNA.

A 1.5% (w/v) agarose gel was prepared from agarose powder and 1x TAE buffer. A gel cast was prepared; the sides were taped off with masking tape and a gel comb was inserted in the pre-allocated place.

The agarose solution was heated in a microwave until fully dissolved and left to cool for 5-10 minutes (hand-warm). Ethidium bromide (3 µL per 100 mL) was then added and gently mixed. Ethidium bromide intercalates with DNA and fluoresces under UV light, enabling macroscopic visualisation of the DNA. The gel solution was poured into the prepared gel cast and left to set at room temperature for about 30-45 minutes or until set.

Once set, the masking tape and gel comb were removed from the gel and the gel was submersed in the running tank containing 1x TAE buffer. Samples containing extracted DNA (see section 4.3.3.1 DNA Extraction) were mixed with 6x loading dye to enable loading and visualisation of DNA migration through the agarose gel. Each lane was either loaded with ~500 ng DNA (or as close as possible) or with 15 µL of extracted DNA.

A 100 base pair (bp) and/or 1 kilo base pair (kb) DNA ladder was loaded as an indicator of DNA size. Genomic mouse DNA was used as a positive control. The gel was run at 100 volts for approximately 45 minutes or until DNA had migrated ¾ of the length of the agarose gel. DNA is negatively charged and therefore migrates through the gel towards the positive (anode) electrode. Short DNA strands will migrate quicker through the agarose gel than long DNA strands, hence separating DNA fragments by size. This separation can then be visualised under ultraviolet light and imaged using an ImageQuant LAS-4000 Fujifilm.

4.3.3.8. Histology

Histology was performed to enable microscopic visualisation of both cellular and extracellular structures.

Tissue was fixed in 3.7% (w/v) paraformaldehyde (PFA), using a 20:1 ratio (PFA:Tissue), straight after each experiment. The tissue was incubated in PFA for 48 hours before rinsing with the same volume PBS. An automated tissue processor (Leica TP1020) was used to penetrate the tissue with melted paraffin wax. Briefly, the tissue was dehydrated using increasing concentrations of ethanol (25%; 50%; 75%; 95%; 100%) to fully remove water content from the tissue. Once the tissue was

fully penetrated by ethanol, the ethanol was replaced by Histoclear, a clearing agent that is miscible in ethanol and paraffin wax. In the final step, Histoclear was replaced with melted paraffin wax (~60°C). The whole process took 16 hours.

Following this process, the tissue was wax embedded using a wax embedder (Leica EG1160). Briefly, the tissue was placed in a stainless steel base mould, orientated as required and then covered with melted wax before left to set on a cooled plate (-20°C) until fully hardened. The histopathology unit at Queens Medical Centre (QMC) Nottingham (UK) conducted sectioning, staining and imaging. Tissue sections were cut into 5 mm sections. Three sections were taken per sample. A Hamamatsu Nanozoomer NDP slide scanner was used to scan all slides.

The following stains were chosen for histological analysis:

- Haematoxylin; (H) (basic dye) binds to the acidic components/structures (negatively charged) of the cell such as the nucleic acids. It shows up as purple/blue.
- Eosin; (E) (acidic dye) will bind to the cationic (positively charged) or basic components/structures of the cell such as the amino groups in proteins in the cytoplasm. It shows up red/pink.
- Alcian Blue; (AB) (basic dye) that binds to acidic polysaccharides (negatively charged) such as sulphated and non-sulphated glycosaminoglycans. It shows up turquois/blue.
- Picro Sirius Red; (PSR) is a combination of Yellow Picric (acid dye), Sirius Red (acid dye) and a metal complex (basic dye). The Yellow Picric and Sirius dye stain the positively charged cell components such collagen fibres and connective tissue red as well as protein-enriched areas such as the cytoplasm various shades of yellow. The metal complex stains the amniotic structures (negatively charged) of the cells, such as the nucleic acids in grey/brown.

4.3.4. *In vitro* cytotoxicity

4.3.4.1. Culture conditions for HepG2 & NIH/3T3 cells

The human liver hepatocellular carcinoma cell line (HepG2) and mouse embryonic fibroblast cell line (3T3) were used for the *in vitro* studies. Both cell lines were cultured in alpha-Minimum Essential Medium Eagle (MEM) that was supplemented with 1% non-essential amino acids (NEAA); 10% Foetal bovine serum (FBS); 1% L-Glutamine; 1% Penicillin (100 units per mL) and Streptomycin (100 μg/mL) to create a favourable artificial environment for HepG2 and NIH/3T3 cells. In addition 0.1% Gentamicin (50 mg/mL), an antibiotic, was added to reduce the risk of infection to the cells when adding animal derived tissue directly into the culture media (see section 4.3.4.6. Direct contact assay). Both cell lines were incubated within standard culture conditions: 37°C, 21% O_2, 5% CO_2 and 85% relative humidity. The culture media was renewed every 2-3 days with fresh culture media to provide the necessary nutrients for growth and also to remove waste metabolites that accumulate over time.

4.3.4.2. Passaging HepG2 & NIH/3T3 immortalised cell lines

Both cell lines (HepG2 and NIH/3T3) were grown in T75 cell culture flasks using alpha-MEM culture medium (described above), which was changed every other day until cells reached approximately 85-90% confluence. To maintain the cells in a proliferative state, the cells were sub-cultured (passaged) into a new flask at a lower cell-density to continue cell proliferation and to expand the cell population.

Briefly, medium was removed from near confluent HepG2 and NIH/3T3 cells before gently washed with 10 mL of warm (37°C) PBS in the culture flask to ensure that all culture medium components were removed (in particular, foetal bovine serum because this can inactivate Trypsin). The cells were dissociated from the bottom of the T75 flask by incubating the cells in 2 mL of warm Trypsin/EDTA (37°C). Trypsin is a proteolytic enzyme that breaks down the protein bonds from the adhesion of the cells to the culture flask and works optimally at a temperature of 37°C in combination with EDTA. The flask was gently tapped to detach the cells

(confirmed by the presence of floating cells when viewed under the microscope). Trypsin-EDTA was neutralised and inactivated by the addition of 8 mL MEM culture media that contains 10% FBS. The cell suspension was centrifuged for 5 minutes at 120 x g and the cell pellet was re-suspended in 10 mL of fresh, pre-warmed MEM cell culture medium, followed by a 1:5 dilution before seeding onto a new T75 flask(s).

4.3.4.3 Counting cells

The HepG2 and NIH/3T3 cells were counted using a haemocytometer. A haemocytometer is a modified microscopic slide that enables visualisation of a counting chamber (Figure 4.8) designed to count the numbers of cells within a fixed area/volume and therefore estimate the concentration of cells in the cell suspension.

A coverslip is attached on top of the counting chamber (depth between the coverslip and counting chamber are 0.1 mm) and 10 μL of cell suspension are inserted into the gap between coverslip and counting chamber.

Using an inverted microscope a square is visible that is divided into nine big squares that represent 1 mm^2 each which are further divided into several squares (Figure 4.8). All cells within the 4 x 1 mm^2 corner squares are counted as well as cells that are on two of the adjoining borders (Figure 4.8). The average cell number recorded in the 4 x 1 mm^2 squares (total number of counted cells/counted squares = average number of cells per 0.1 mm^3 or 0.1 μL).

To convert microliters into millilitres the average number of cells per 0.1 μL is multiplied by 10^4 to give an estimate of total number of cells per mL and further multiplied by the volume of the cell-suspension to give the total number of cells. The cell suspension is then diluted accordingly to give the appropriate number of cells per well or plate.

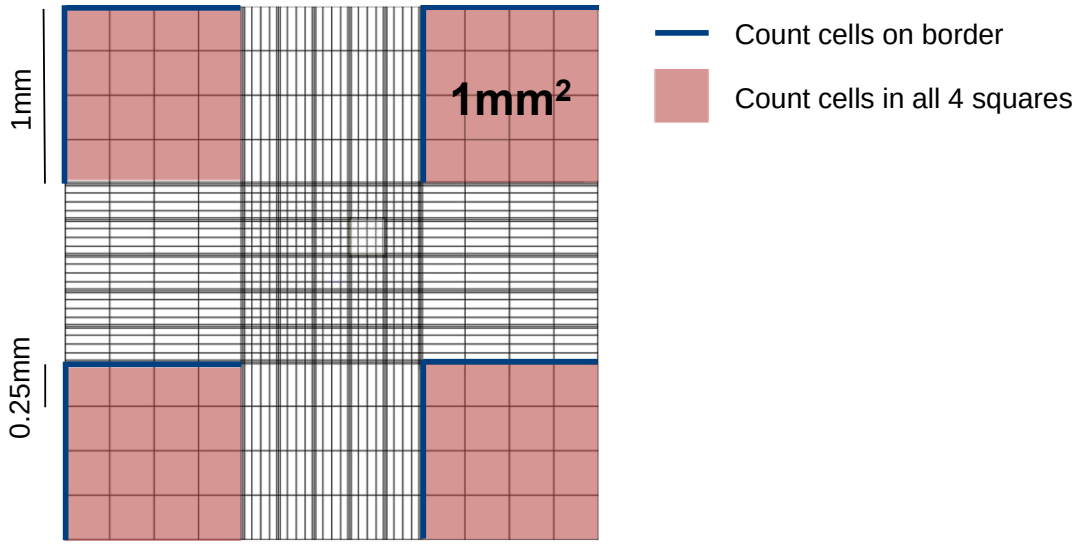


Figure 4.8 Principle of haemocytomter and cell counting.

4.3.4.4. Seeding 24-well plates with a known density cells

For the direct contact and extract assay (see section 4.3.4.5. Extract assay ; 4.3.4.6. Direct contact assay) tissue culture treated 24- well plates were seeded with a known concentration of cells to have the same starting point for all the *in vitro* experiments.

The total number of cells in the cell suspension was estimated as described above in section 4.3.4.3 Counting cells. The volume of cell suspension required to provide the desired number of cells was calculated by:

$$(total\ amount\ of\ cells\ required\ \div\ total\ cell\ number) \times\ total\ volume\ of\ cell\ suspension = volume\ of\ cell\ suspension\ required$$

A cell density of 200,000 HepG2 and 150,000 NIH/3T3 cells were seeded in 0.5 mL per well of a tissue culture treated 24-well plate.

4.3.4.5. Extract assay

An extract assay was performed to assess the *in vitro* cytotoxicity of the materials developed within this thesis.

The HepG2 and NIH/3T3 cells were used to test cytotoxicity of materials derived from liver and aorta respectively. The extraction vehicle for all samples was alpha-MEM culture media (see section. Culture conditions for HepG2 & NIH/3T3 cells, 4.3.4 above), which is required for cell growth but enables extraction of polar and non-polar substances of the tissue samples.

The tissue samples (liver/aorta) were biopsied with a 5 mm skin biopsy punch straight after the treatment and frozen at -20°C until further processed. Tissue samples were thawed and washed 3 x 5 minutes in PBS. The tissue samples were placed in 5 mL alpha-MEM for 30 h (37°C) at 300 rpm using aseptic techniques. After 30 h, half the volume of extraction media (2.5 mL) was removed and the tissue sample was left in the remaining media (2.5 mL) for a further 60 h (i.e. 90 h in total) to have a more concentrated solution of the eluate.

A confluent cell monolayer (HepG2 for liver tissue samples, NIH/3T3 for aorta tissue samples) was cultured on tissue culture treated 24-well plates. Once confluent, the MEM culture media was removed and replaced with filter sterilised eluate MEM culture media for 24 h. Each eluate was tested on three separate cell monolayers (wells). As a positive control that was known to cause a cytotoxic response, 5% Triton-x-100 made up in alpha MEM culture media was used. The blank was alpha MEM culture media only without a cell monolayer and the negative control, which did not cause any cytotoxic response, was alpha MEM culture media on the cell monolayer. Representative images were taken after 24 h before analysing cellular metabolic activity (see section 4.3.4.7. MTT cell viability assay).

4.3.4.6. Direct contact assay

A direct contact assay was performed to assess the direct impact of the materials developed in this thesis on HepG2 (for liver samples) and NIH/3T3 (for aorta

samples) cells. The tissue samples (liver/aorta) were biopsied (5 mm) straight after the treatment and frozen at -20°C until used. Tissue samples were thawed and washed 3 x 5 minutes in PBS. A confluent cell monolayer (HepG2 for liver tissue samples, NIH/3T3 for aorta tissue samples) was cultured on tissue culture treated 24-well plates. The alpha-MEM media was removed and a single piece of tissue was carefully placed in the middle of each well before fresh culture media was added for 24 h. Each condition was tested with one piece of tissue on a single cell monolayer. The positive and negative controls were the same as in section 4.3.4.5 Extract assay, above). Representative images were taken after 24 h before analysing cellular metabolic activity (see section 4.3.4.7. MTT cell viability assay).

4.3.4.7. MTT cell viability assay

The MTT assay was used to assess cell viability and proliferation after cells have been exposed to external factors such as eluate media (see section 4.3.4.5. Extract assay) or tissue samples (see section 4.3.4.6. Direct contact assay).

A confluent cell monolayer was seeded with HepG2 (for liver) and NIH/3T3 cells (for aorta) in a tissue culture coated 24-well plate. Once confluent, the MEM culture media was removed and either replaced with 500 µL eluate MEM media (extract assay) or a tissue sample and 500 µL of fresh MEM culture media (direct contact assay) and incubated for 24 h (37°C, 21% O_2, 5% CO_2 and 85% relative humidity). Fifty microliters of MTT Reagent, a yellow tetrazolium that is soluble in media and cell permeable, is taken up by all metabolically active cells and reduced to a purple insoluble formazan crystal salt within a 4-hour incubation. The purple formazan salt crystals are then solubilised overnight (37°C, 21% O_2, 5% CO_2 and 85% relative humidity) by the addition of the provided detergent reagent (500 µL per well). The concentration was determined by measurement of the colorimetric absorbance at 570 nm and a reference wavelength of 680 nm.

4.3.5. Statistical analysis

4.3.5.1. Statistical analyses

Statistical analyses were performed using the statistical software PRISM (Graph Pad). All statistical analyses were conducted using a One-Way ANOVA (post hoc Dunnett's multiple comparison test) or Two-Way ANOVA (post hoc Turkey's multiple comparison test), depending on the number of variables. For instance, a One-Way ANOVA followed by Dunnett's multiple comparison test (post hoc) was used when independent factors were compared to each other i.e. different time point of exposure to $scCO_2$. A Two-Way ANOVA, followed by Turkey's multiple comparison test (post hoc), was used when two independent factors are compared to a dependent factor i.e. different time point of exposure to $scCO_2$ with or without the addition of ethanol. All data were normally distributed. Results were deemed significant if $p < 0.05$. In all figures, * indicates level of significance to native tissue control (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p > 0.0001$) and ^ indicates significance level to $scCO_2$ alone (^$p < 0.05$, ^^$p < 0.005$, ^^^$p < 0.0005$, ^^^^$p > 0.0001$). All data are mean +/- SEM.

5. Development and characterization of a $scCO_2$ decellularization methodology

5.1 INTRODUCTION

The first publication demonstrating the utility of $scCO_2$ to decellularize mammalian tissue came from Sawada *et al.*, 2008, which has become the most cited paper in the $scCO_2$ decellularization field (61 citations, Google Scholar, December 2019). Sawada *et al.*, 2008 reported on a quick (60 minutes) method for decellularization that used ethanol in combination with $scCO_2$ as an extraction medium to generate an acellular scaffold. The general concept behind this novel decellularization technique was that $scCO_2$ would enhance the removal of residual genetic material and also remove residual toxicity of decellularization agents that were used in conjunction with $scCO_2$ to enhance the decellularization process (i.e. ethanol). Such features could offer potential improvements to the safety profile of acellular scaffolds compared to those generated using detergent based methods. Sawada *et al.*, 2008 reported that their novel decellularization technology using $scCO_2$ effectively removed cell nuclei (assessed by qualitative H&E staining), decreased phospholipid concentration (a biomarker for residual cellular membranes) and retained structural properties (using stress-strain analysis and rehydration capacity). The work of Sawada *et al.*, 2008 was a milestone in the area of decellularization, however, the group have not published further validation or application of their method since 2008.

The aim of this chapter was therefore to further explore the methodology published by Sawada *et al.*, 2008. In particular, the efficacy of using $scCO_2$ in combination with ethanol as a hybrid decellularization methodology was tested using quantitative endpoints (DNA content). The effect of exposure time, surface area and tissue moisture content were also investigated in an attempt to further enhance and

understand the decellularizing effects of this hybrid $scCO_2$ and ethanol methodology. Variables such as moisture content, surface area and exposure time have previously been show to impact removal of lipids from animal derived tissues in the food industry (Yamaguchi *et al.*, 1986; King, Johnson and Friedrich, 1989; Boselli and Caboni, 2000; Tanaka, Sakaki and Ohkubo, 2004). Finally, the method reported by Sawada *et al.*, 2008 was investigated on a second tissue type (liver) to determine whether structurally distinct tissue types are differentially affected by $scCO_2$ and ethanol.

5.2 MATERIALS & METHODS

A detailed and comprehensive explanation of the materials and methods used here can be found in the materials and methods chapter (see Chapter 4). The following experimental set up and techniques were used in brief:

5.2.1 Solubility testing in $scCO_2$

The solubility of liquid carbon dioxide as well as ethanol (500 µl) was observed in a high-pressure autoclave at working conditions (~2900 psi, 37°C) for 15 minutes to assess phase behaviour of CO_2 and solubility of ethanol in $scCO_2$, respectively.

5.2.2 Supercritical carbon dioxide decellularization

To assess the impact of liver tissue moisture content and surface area on decellularization using $scCO_2$, liver tissue was either freeze dried (24 h), snap frozen in liquid nitrogen and milled using a coffee grinder or sliced with a scalpel. Three liver tissue samples (n=3) were prepared per condition tested and each liver tissue sample weighed ~ 1 g. For the $scCO_2$ exposure, each biological sample (liver or aorta) was placed in the high-pressure autoclave and the system interlocked. Dry ethanol (6 mL), when used, was injected via the autoclave keyhole, before the high-pressure system was fully sealed and locked. The high-pressure autoclave system was pressurised to ~1000 psi and heated to 30°C, before increasing to ~2600 psi and a temperature of 37°C. The temperature was maintained at 37°C +/- 3°C. After the desired $scCO_2$ exposure duration, the system was vented to ambient pressure within 1 minute.

The $scCO_2$ exposed tissue (liver or aorta) was removed from the high-pressure autoclave, weighed if needed, and either used immediately (Rehydration capacity, Histology) or stored at approximately -20°C until further use (DNA extraction, Phospholipid assay, DMMB assay, Hydroxyproline assay).

5.2.3 Detergent Decellularization

Detergent based decellularization of biological tissue (liver or aorta) was performed following an adapted protocol by Loneker *et al.,* 2016. Following detergent based decellularization, ECM material was either used immediately (Histology) or freeze

dried (48 h) and stored at approximately -20°C until further use (DNA extraction, Phospholipid assay, DMMB assay, Hydroxyproline assay). Detergent decellularization was performed in batches (i.e. up to 40 g of tissue pieces per 2 L conical flask); three samples were used for analysis (n=3).

5.2.4 DNA extraction

DNA was extracted using a phenol-chloroform extraction method to enable DNA quantification (Pico green assay) and DNA fragmentation analysis (DNA Gel electrophoresis). One hundred micrograms of lyophilised tissue (n=3 per condition) was used for DNA extraction and resuspended in 1 mL of TE buffer before being stored at approximately -20°C until further use (Pico green assay, DNA gel electrophoresis).

5.2.5 Pico green assay

Following DNA extraction, a Pico green assay was used to quantify DNA content as a marker for decellularization. DNA extracts were thawed at room temperature and vortexed prior to use. The Pico green assay was performed according to the manufacturers instructions. Each standard and sample was assayed in either duplicate or triplicate, which were averaged to obtain a robust single representative value for each sample.

5.2.6 DNA gel electrophoresis

DNA gel electrophoresis was used to visualise the fragmentation of the extracted DNA. Briefly, a 1.5% (w/v) agarose gel, containing ethidium bromide (3 µL per 100 mL), was prepared. DNA extracts (n=3 per condition) were thawed at room temperature, vortexed and mixed with 6x loading dye. Each lane was either loaded with ~500 ng DNA (or as close as possible) or with 15 µL of extracted DNA per tissue sample. A DNA ladder (100 bp or 1 kb) was loaded as an indicator of DNA size. Genomic mouse DNA was used as a positive control.

5.2.7 Phospholipid assay

A phospholipid assay was used to quantify choline-containing phospholipids as a biomarker of residual cell membrane components. Ten micrograms of lyophilised

liver tissue samples (n=3 per condistion) were assayed. The phospholipid assay was performed according to manufacturers instructions. Each sample and standard were assayed in duplicate, which were averaged to obtain a single representative value for each sample.

5.2.8 DMMB assay

The DMMB assay was used to quantify sulphated glycosaminoglycan (sGAG) content as a marker for retained ECM scaffold components. Ten micrograms of lyophilised aorta or twenty micrograms of lyophilised liver tissue were assayed (n=3 per condition). The DMMB assay was performed according to manufacturers instructions. Each sample and standard was assayed in duplicate, which were averaged to obtain a single representative value for each sample.

5.2.9 Hydroxyproline assay

The hydroxyproline assay was used to quantify hydroxyproline as a marker of retained ECM scaffold components. Fifteen micrograms of lyophilised tissue (liver or aorta, n=3 per condition) was assayed. The hydroxyproline assay was performed according to manufacturers instructions. Each sample and standard was assayed in duplicate, which was averaged to obtain a single representative value for each sample.

5.2.10 Rehydration capacity

A rehydration study was performed to assess the capacity of the liver tissue to rehydrate immediately after scCO_2 decellularization. Liver tissue (n=3 per condition, ~1 g each) was incubated in 5 mL of dH_2O for 24 h under gentle shaking (300 rpm).

The same experimental setup was also used to investigate the effects of a dH_2O washing step (up to 24 h) alone on liver tissue (n=3 per condition, ~1 g each).

For the rehydration study, tissue weight was taken before and after the incubation step whereas for the extended dH_2O washing of liver tissue, DNA was extracted and quantified using the Pico green assay.

5.2.11 Histology

Histology was performed to enable microscopic visualisation of both cellular and extracellular structures. One tissue sample per condition tested (n=1; liver and aorta) was fixed in 3.7% (w/v) paraformaldehyde (PFA) immediately after the experiment (48 h). Briefly, each sample was automatically processed and wax embedded in-house before sectioning, staining and scanning by the histopathology unit at Queens Medical Centre (QMC) Nottingham (UK). Liver and aorta tissue sections were cut into 5 mm sections. Three sections were taken per sample. One section per sample was stained and scanned.

5.2.12 Statistical analysis

Statistical analyses were performed using the statistical software PRISM (Graph Pad). All statistical analyses were conducted using a One-Way ANOVA (post hoc Dunnett's multiple comparison test) or Two-Way ANOVA (post hoc Turkey's multiple comparison test), depending on the number of variables. Results were deemed significant if $p < 0.05$. All data are displayed as mean +/- SEM.

5.3 RESULTS: COMBINED EFFECT OF SCCO$_2$ AND ETHANOL

The aim was to investigate the utility of supercritical carbon dioxide (scCO_2) to decellularize aorta. To do so, the methodology published by Sawada *et al.*, 2008, who reported combined use of scCO_2 with ethanol to decellularize aorta, was investigated. Subsequently, the method was also investigated to decellularize liver tissue, which differs in texture and structure compared to that of aorta.

5.3.1. Solubility of liquid CO_2

The solubility of liquid CO_2 can be seen in the representative view cell images above (Figure 5.1, below, images a - f). Liquid CO_2 was pumped into the view cell (Figure 5.1, image b). As pressure and temperature were increased, the liquid CO_2 transitioned into the supercritical phase at the critical point (P: 1071 psi, T: 32°C, (Figure 5.1, image c). At this point, the liquid and gas phase became indistinguishable due to having the same density. For experimental working conditions (~2900 psi, 37°C) the CO_2 was maintained in the supercritical phase (Figure 5.1, image d). During depressurization, scCO_2 reversed back into liquid CO_2 at the critical point (P: 1071 psi, T: 32°C, Figure 5.1, image e), before being vented from the system (Figure 5.1, image f).

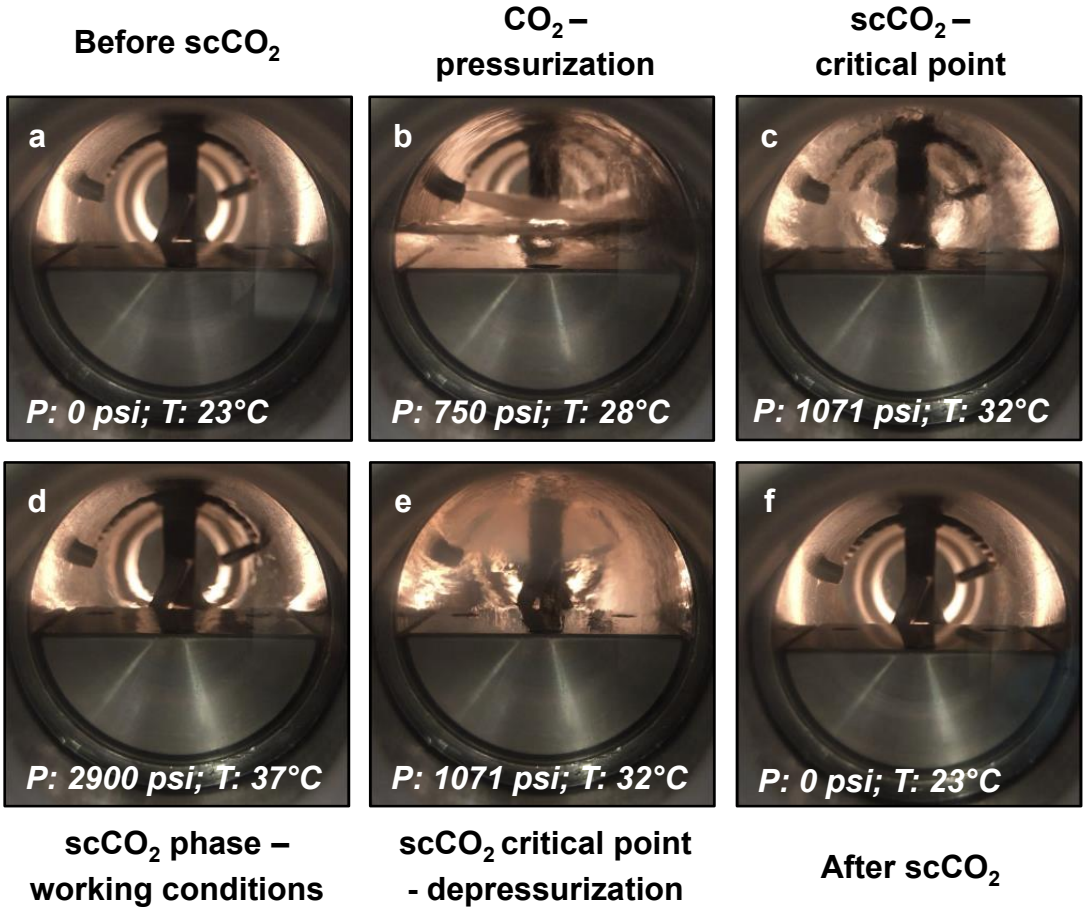


Figure 5.1 Representative view cell images (sequential a - f) of liquid carbon dioxide transitioning to the supercritical carbon dioxide phase followed by a depressurization. P = Pressure, T = Temperature

5.3.2. Solubility of ethanol in $scCO_2$

The solubility of ethanol (contained in glass vessel) in liquid CO_2 can be seen in the representative view cell images (Figure 5.2, images a - f). Liquid CO_2 was pumped into the view cell (Figure 5.2, image b). As pressure and temperature were increased, the liquid CO_2 rises above the level of ethanol within the glass vessel, before it transitioned into the supercritical phase at the critical point (P: 1071 psi, T: 32°C, Figure 5.2, image c). At this point, ethanol was solubilized in the $scCO_2$ and liquid and gas phase became indistinguishable due to having the same density. For experimental working conditions (~2900 psi, 37°C) the CO_2 and ethanol were maintained as a single phase in the supercritical region (Figure 5.2, image d). During depressurization, $scCO_2$ and ethanol reverted back into liquid CO_2 and ethanol at the critical point (P: 1071 psi, T: 32°C Figure 5.2, image e), before being vented from the system. A small proportion of ethanol was retained Figure 5.2, image f).

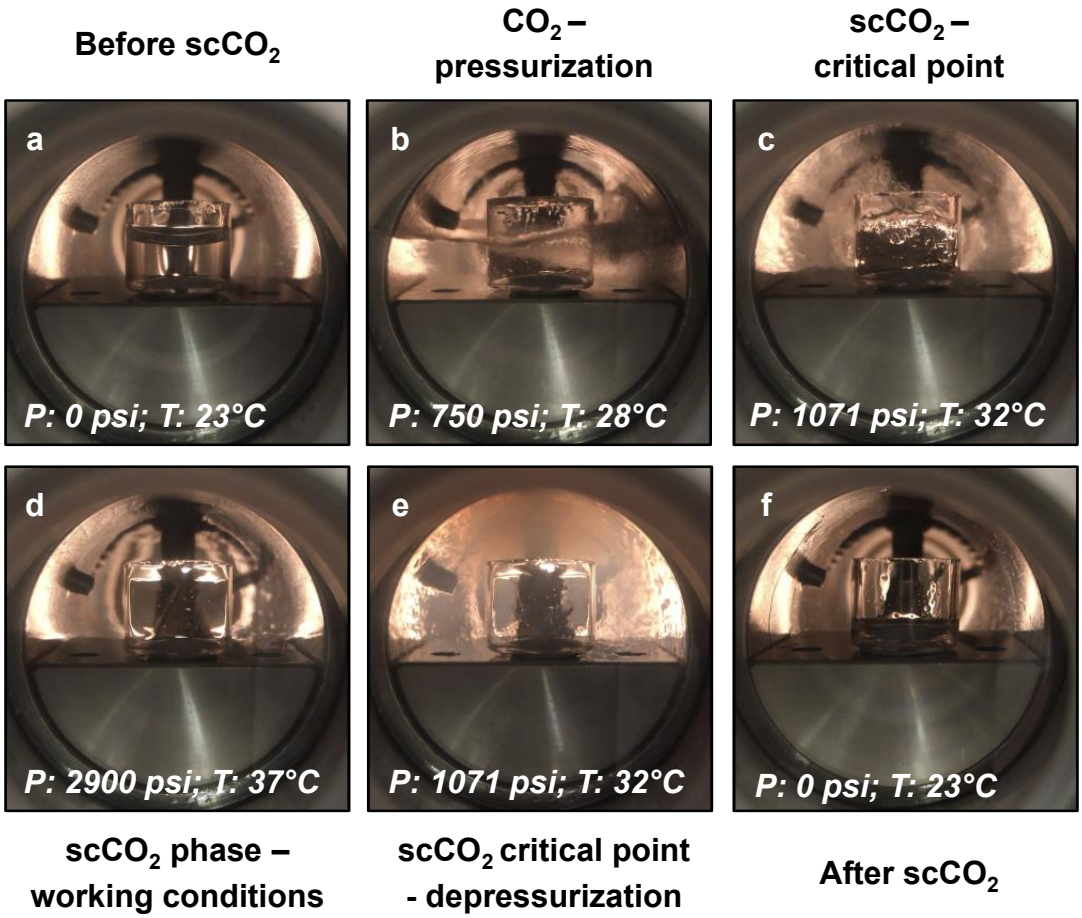


Figure 5.2 Representative view cell images (sequential a - f) of the solubility of ethanol in $scCO_2$, followed by depressurization of the high-pressure system. P = Pressure, T = Temperature

5.3.4. Effect of $scCO_2$ +/- ethanol on aorta tissue decellularization

Following confirmation that ethanol is soluble in $scCO_2$, the combined effects of ethanol and $scCO_2$ on aorta tissue were investigated.

The exposure of aorta tissue to $scCO_2$ for 15 or 60 minutes did not affect the DNA content compared to a native aorta tissue control (Figure 5.3 A). The addition of ethanol to the $scCO_2$ phase resulted in a slightly higher DNA content after 15 (p = 0.0384), but not 60 minutes exposure compared to that of native aorta tissue (Figure 5.3 A).

Relative to the native tissue (aorta) control, phospholipid concentration remained unchanged after 15 or 60 minutes of $scCO_2$ exposure in combination with or without ethanol (Figure 5.3 B). Furthermore, there was no significant difference in the rehydration capacity of aorta tissue after $scCO_2$ exposure, with or without ethanol (Figure 5.3 C).

Tissue weight pre- and post-$scCO_2$ exposure was not significantly changed. In contrast, exposure to the combined ethanol-$scCO_2$ mixture for 15 or 60 minutes reduced tissue weight by approximately 25% compared to native aorta tissue (p < 0.0001). In comparison to $scCO_2$ alone, the combined ethanol-$scCO_2$ mixture reduced tissue weight by 21% (15 minutes, p < 0.0001) and 16% (60 minutes, p = 0.0018; Figure 5.3 D).

Representative images show that aorta exposed to $scCO_2$ or ethanol in combination with $scCO_2$ (15 and 60 minutes) appeared whiter in colour compared to the pale pink colour of the native aorta tissue (Figure 5.3 E). Aorta tissue exposed to ethanol also appeared more dry, which is in agreement with the marked reduction in tissue weight following exposure to ethanol.

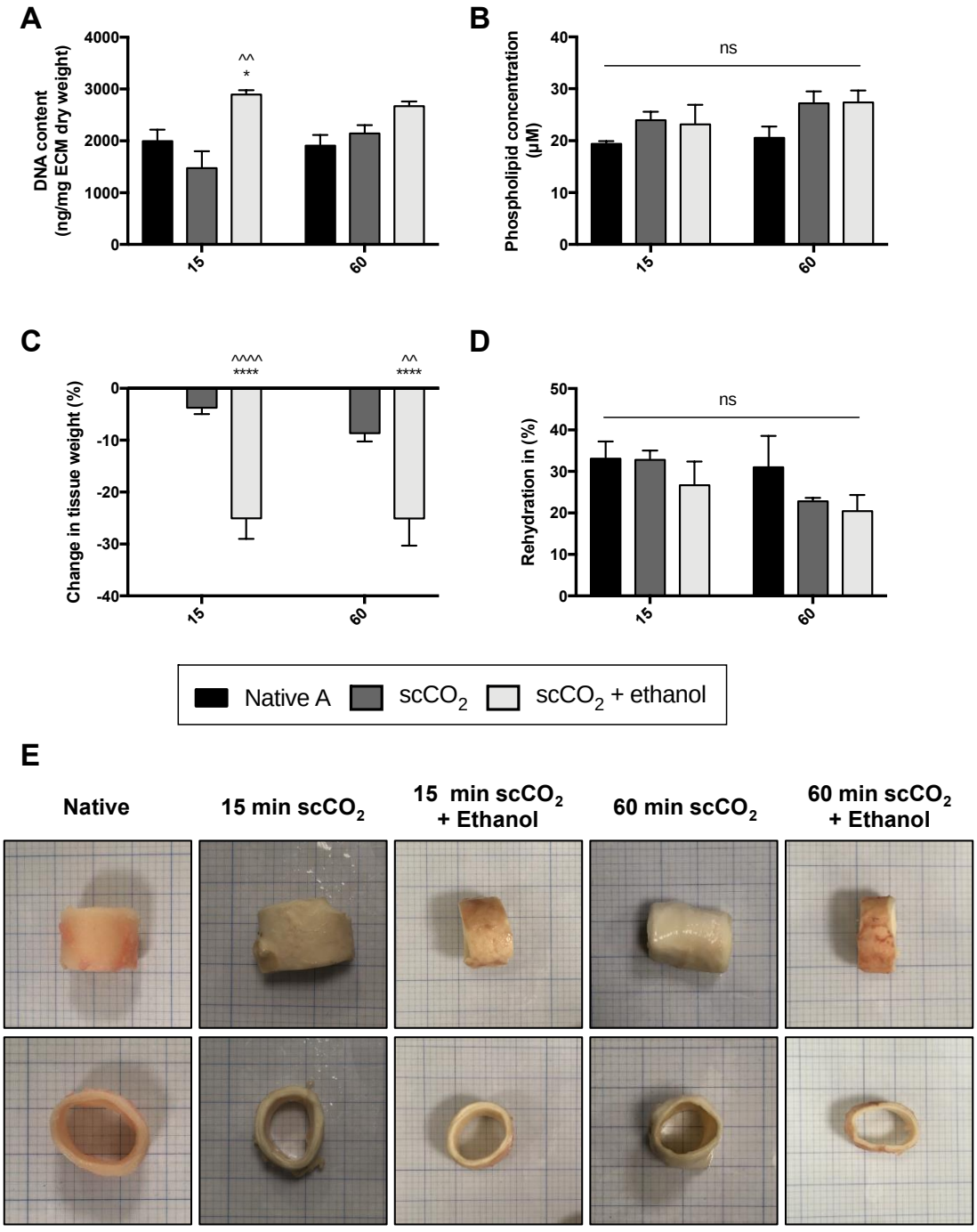


Figure 5.3 Aorta tissue (~1g) exposed to 15 or 60 minutes of $scCO_2$ +/- ethanol (A) DNA content. (B) Phospholipid concentration. (C) Tissue weight change. (D) Rehydration. (E) Representative images (scale: $1cm^2$). All data were normalized to tissue weight and analysed using a two-way ANOVA, n=3. All data are mean +/- SEM. * indicates significance level to the native tissue control (* $p < 0.05$, **** $p < 0.0001$). ^ indicates significance level to $scCO_2$ alone (^^ $p < 0.005$, ^^^^ $p < 0.0001$)

5.3.5. Histological assessment of aorta tissue exposed to $scCO_2$ +/- ethanol

To confirm the lack of decellularization by combined $scCO_2$ and ethanol, histology was performed to assess if microscopic changes were present.

Cell nuclei (purple; stained by H&E) were present in all conditions tested, with no visually obvious difference compared to that of native aorta tissue (Figure 5.4). This is largely in agreement with the absence of a measurable change in DNA content (Figure 5.3 A).

The strong yellow staining by PSR and blue staining by AB in all conditions indicates the presence of protein and glycosaminoglycans, respectively. Aorta tissue exposed to ethanol in combination with $scCO_2$ appeared perforated, with numerous white spaces evident in sections stained with H&E and PSR (Figure 5.4).

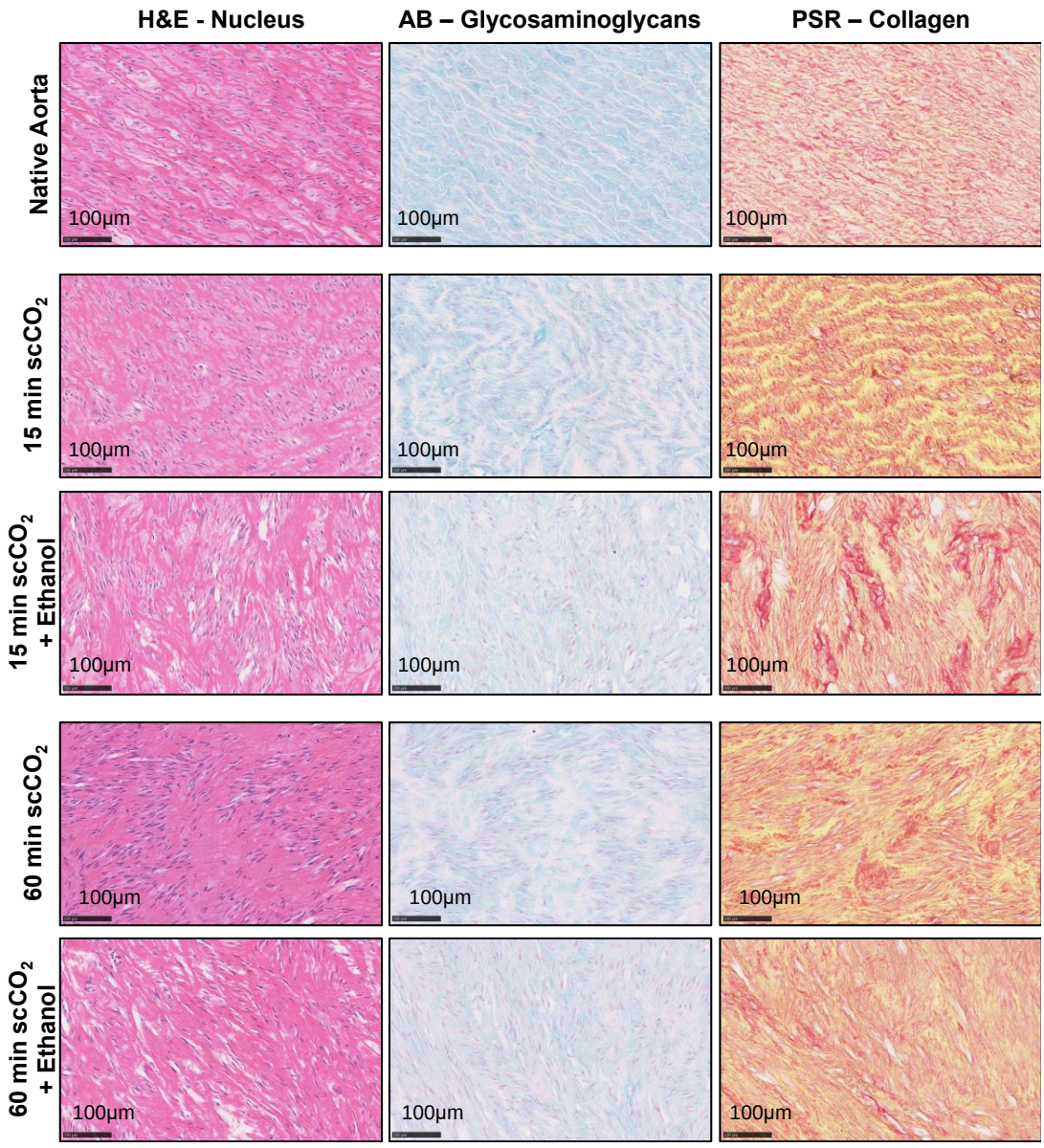


Figure 5.4 Representative histological images of native aorta exposed to 15 or 60 minutes of $scCO_2$ +/- ethanol. Staining intensity for cell nuclei (H&E, purple), collagen (PSR, red), protein-enriched areas (PSR, yellow) and glycosaminoglycans (AB, blue) was similar for all conditions tested.

5.3.6. Effect of $scCO_2$ +/- ethanol on liver tissue decellularization

It was next investigated if the combination of $scCO_2$ and ethanol produced a greater decellularizing effect on liver compared to aorta.

The exposure of liver tissue to 15 or 60 minutes of $scCO_2$ with or without the addition of ethanol showed no significant change in DNA content (Figure 5.5 A). There was a significant decrease of 14% (15 minutes) and 17% (60 minutes) in liver tissue weight after $scCO_2$ exposure only and 19% (15 minutes) and 16% (60 minutes) after $scCO_2$ in combination with ethanol ($p < 0.0001$, Figure 5.5 B).

Representative images show that liver tissue exposed to $scCO_2$ only appeared more red-brownish in colour after 15 minutes and dark brown in colour after 60 minutes, compared to the dark brown colour of native liver tissue (Figure 5.5 C). Regardless of exposure time (15 or 60 minutes), the liver tissue exposed to $scCO_2$ in combination with ethanol appeared paler in colour compared to native liver tissue. Exposure to $scCO_2$ +/- ethanol removed the wet shine that was present in the native liver tissue control, indicating that the liver tissue is drier after exposure to $scCO_2$ +/- ethanol (Figure 5.5 C).

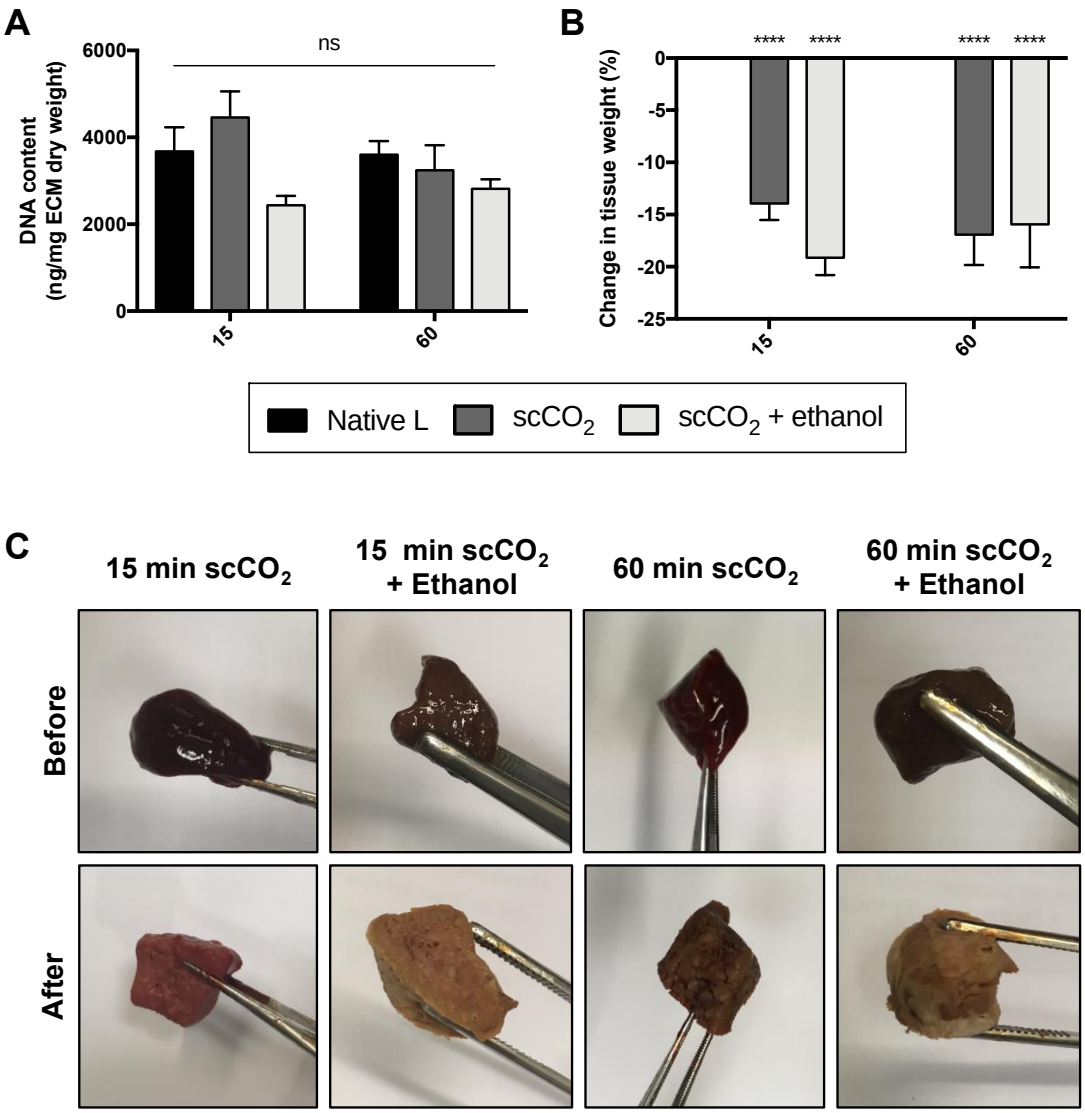


Figure 5.5 Liver (L) tissue (~1g) exposed to 15 or 60 minutes of $scCO_2$ +/- ethanol (A) DNA content. (B) Tissue weight change. (C) Representative images. All data were normalized to tissue weight and analysed using a two-way ANOVA, n=3. All data are mean +/- SEM. (*) Star indicates significance level to the native tissue control (**** $p <$ 0.0001)

5.3.7. Summary

In summary, the results shown in this section showed that exposure to 15 or 60 minutes of $scCO_2$ +/- ethanol did not produce a measurable removal of cellular material from aorta tissue, which is contradictory to the work published by Sawada *et al.*, 2008. In addition, application of the same methodology to liver tissue did not reduce DNA content (a marker of decellularization).

5.4 RESULTS: OPTIMIZATION OF A $SCCO_2$ DECELLULARIZATION METHOD

The aim was to develop an alternative $scCO_2$ based decellularization method for use on mammalian tissue. Hence, the following studies investigated whether $scCO_2$ can be utilised as a decellularization agent. It was hypothesised that variables such as moisture content, surface area and exposure time would enhance the deceullarization process report. Such variables are commonly manipulated during method development by the food industry to facilitate the extraction of fats and lipids by $scCO_2$ (Yamaguchi *et al.*, 1986; King, Johnson and Friedrich, 1989; Boselli and Caboni, 2000; Tanaka, Sakaki and Ohkubo, 2004).

5.4.1. Effect of liver tissue moisture content and $scCO_2$ on residual DNA content

The effect of tissue water content was investigated as a variable to enhance cell removal from liver tissue by $scCO_2$.

Removing moisture content, by pre-lyophilizing the liver tissue, had no effect on DNA content following 15 or 60 minutes $scCO_2$ exposure (Figure 5.6 A). Whilst DNA content was unchanged, residual DNA was fragmented following $scCO_2$ exposure, as demonstrated by electrophoretic separation of DNA on an agarose gel (Figure 5.6. B). Therefore, removal of water content prior to 15 or 60 minutes $scCO_2$ exposure did not enhance or impair the efficacy of $scCO_2$ to remove DNA content.

There was no difference in gross morphology, including shape or colour, between pre-lyophilized native liver tissue (control) compared to pre-lyophilized liver tissue that had been exposed to $scCO_2$ for either 15 or 60 minutes (Figure 5.6 C).

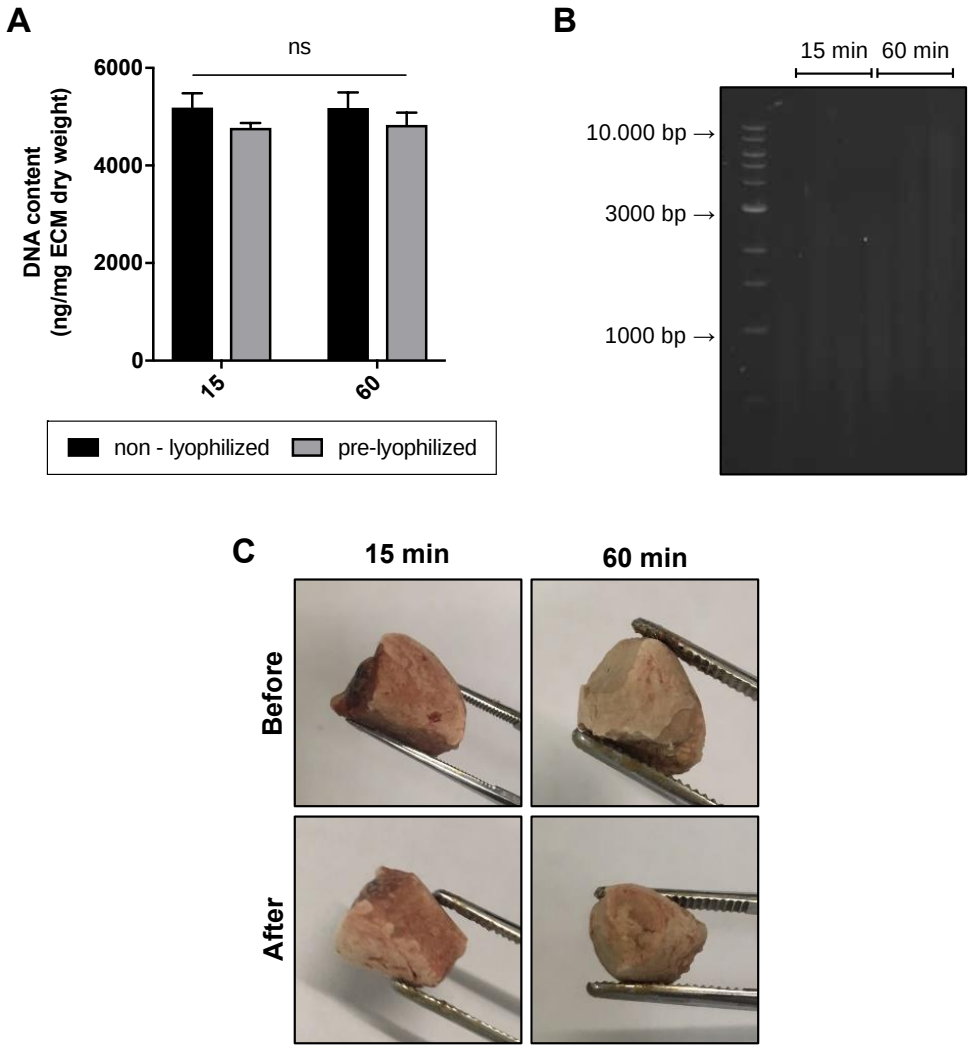


Figure 5.6 Pre-lyophilised liver tissue (~1 g) exposed to 15 or 60 minutes of $scCO_2$ (A) DNA content. (B) DNA Gel electrophoresis (note: faint DNA smear was identified for all samples). (C) Representative images of lyophilized liver. All data are normalized to tissue weight and analysed using a two-way ANOVA, n=3. All data are mean +/- SEM

5.4.2. Effect of increased surface area of liver tissue in combination with $scCO_2$ on residual DNA content

It was next assessed if an increased surface area would facilitate cell removal from liver tissue by $scCO_2$.

DNA content of pre-milled, but not square or thinly sliced, liver tissue was significantly reduced following exposure to 15 and 60 minutes $scCO_2$ (p = 0.0384 and p = 0.0055, respectively compared to square tissue; Figure 5.7 A).

Representative images show that liver tissue, irrespective of shape/size, turned a dark brown colour following exposure to $scCO_2$ (15 and 60 minutes), compared to the bright red colour of native liver tissue (Figure 5.7 B).

Following exposure to $scCO_2$, irrespective of size/shape, residual liver DNA was fragmented, as seen by faint smears rather than distinct bands (Figure 5.7 C).

Taken together, increased tissue surface area improved the efficacy of $scCO_2$ to reduce DNA content of liver tissue.

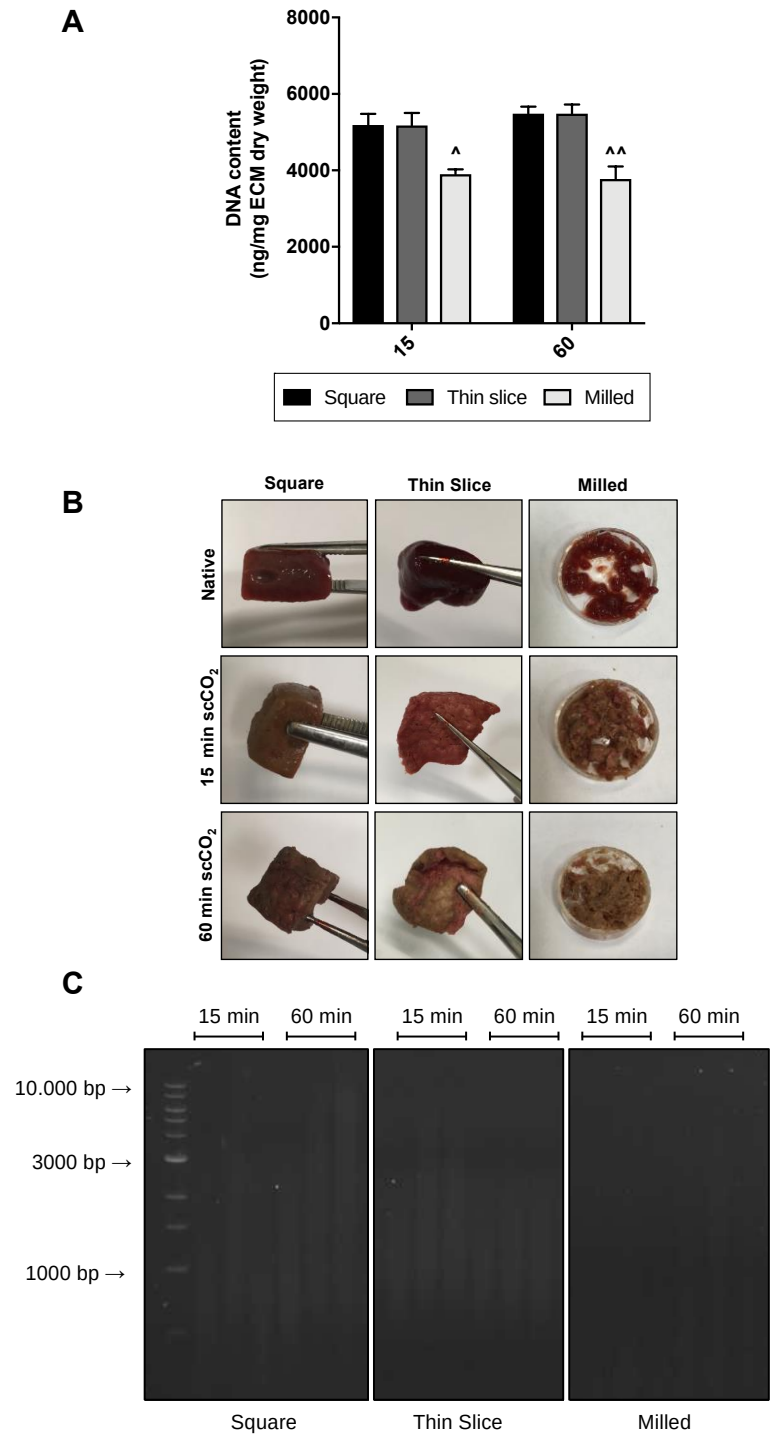


Figure 5.7 Pre-cut (square, thin slice, milled) liver tissue (~1g) exposed to 15 or 60 minutes of scCO2 (A) DNA content. (B) Representatives images. (C) DNA Gel electrophoresis (Note: faint DNA smears were identified for all samples). All data are normalized to tissue weight and analysed using a two-way ANOVA, n=3. All data are mean +/- SEM. ^ indicates significance level to the square tissue (^ $p < 0.05$, ^^ $p < 0.005$)

5.4.3. Effect of an agitated wash after $scCO_2$ exposure on liver tissue DNA content

The addition of a wash step (300 rpm, 24 h) following $scCO_2$ exposure (15 and 60 minutes) was then investigated to evaluate the combined effect of washing and $scCO_2$ exposure for reducing in DNA content.

Incubating liver tissue in water whilst shaking at 300 rpm produced a time-dependent reduction in DNA content. A significant decrease in DNA content was observed following a wash duration of 2, 4, 8, 16 and 24 h ($p = 0.013$, $p = 0.0001$, $p = 0.0002$, $p = 0.0001$, $p = 0.0001$, respectively; Figure 5.8 A). This result indicated that washing alone produces a marked decrease in DNA content.

Whilst the exposure of liver tissue to 15 or 60 minutes of $scCO_2$ did not change the DNA content, exposure to a 24 h agitated wash, resulted in a significantly reduced DNA content compared to the (non-washed) native liver tissue control ($p = 0.0207$ at 15 minutes, $p = 0.0177$ at 60 minutes) and $scCO_2$ alone treated liver tissue control ($p = 0.0096$ at 15 minutes, $p = 0.0041$ at 60 minutes). The addition of a washing step after 15 or 60 minutes $scCO_2$ exposure significantly decreased the DNA content ($p = 0.0064$ and $p = 0.0067$, respectively) compared to the native liver tissue control and compared to the $scCO_2$ only control ($p = 0.0032$ and $p = 0.0016$ respectively). There was no significant difference between $scCO_2$ in combination with an agitated wash compared to the washed native liver tissue at both time points tested (Figure 5.8 B).

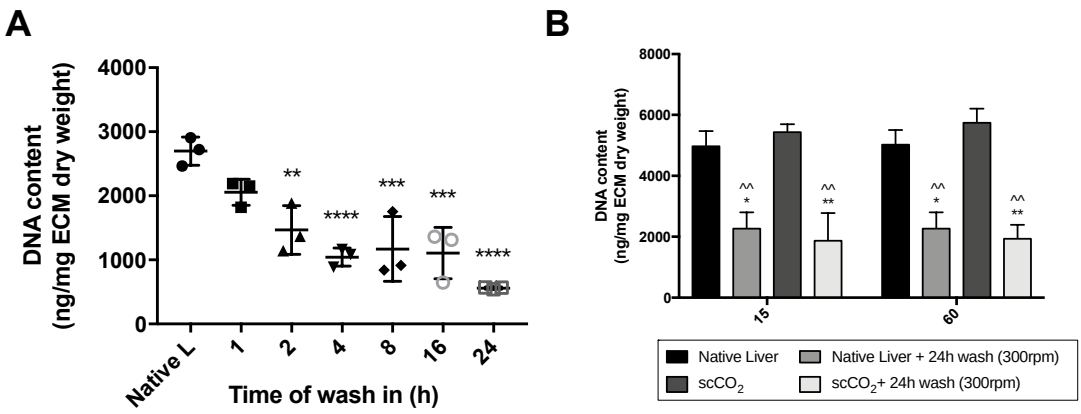


Figure 5.8 Agitated dH_2O wash of native liver tissue (Native L, ~1 g) alone or after $scCO_2$ exposure (A) DNA content following agitated dH_2O wash. Data were analysed using a one-way ANOVA, n=3. (B) DNA content following sCO_2 +/- 24 h 300 rpm dH_2O wash. Data were analysed using a two-way ANOVA, n=3. All data are normalized to tissue weight. All data are mean +/- SEM. * indicates significance level to the native tissue control (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p > 0.0001$). ^ indicates significance level to $scCO_2$ alone (^ $p < 0.05$, ^^ $p < 0.005$)

5.4.4. Effect of $scCO_2$ exposure time on liver tissue decellularization

Previous experiments (see above) demonstrated little effect of up to 1 h $scCO_2$ exposure on cell removal. It was therefore investigated if longer exposures to $scCO_2$ improved cell removal from liver tissue.

Increased exposure to $scCO_2$ (0.25 h, 1 h, 2 h, 5 h, 72 hours) reduced the residual DNA content of liver tissue in a time-dependent manner. The longer the exposure the less DNA could be detected, with a significant reduction after 5 h ($p < 0.0001$) and 72 h ($p < 0.0001$;Figure 5.9 A).

The increased exposure to $scCO_2$ (0.25 h, 1 h, 2 h, 5 h, 72 hours) reduced liver tissue weight by 14% (0.25h, $p = 0.0052$), 17% (1 h, $p = 0.0012$), 16% (2 h, $p = 0.0021$), 16% (5 h, $p = 0.0016$) and 21% (72 h, $p = 0.0002$; Figure 5.9 B). Residual DNA content of liver tissue was fragmented, irrespective of $scCO_2$ exposure duration (Figure 5.9 C).

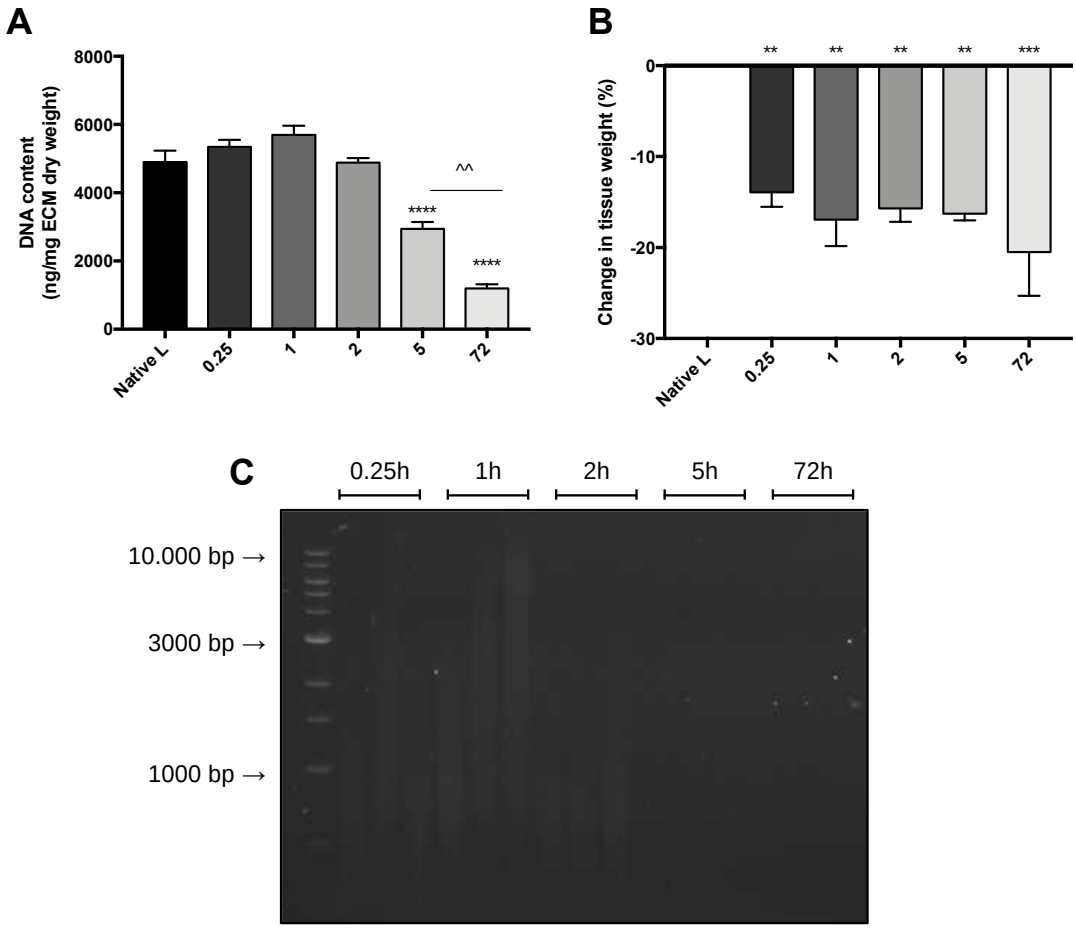


Figure 5.9 Liver tissue (~1g) exposed to 0.25 h, 1 h, 2 h, 5 h and 72 h of $scCO_2$ (A) DNA content; (B) Tissue weight change; (C) DNA Gel electrophoresis (Note: faint DNA smears were identified for samples exposed to $scCO_2$ for 0.25-2 hours only). All data were normalized to tissue weight and are analysed using a one-way ANOVA, n=3. All data are mean +/- SEM. * indicates significance level to the native tissue control (** $p < 0.005$, *** $p < 0.0005$, **** $p > 0.0001$). ^ indicates significance level to $scCO_2$ alone (^^ $p < 0.005$)

5.4.5. Effect of an agitated wash, moisture content and surface area after extended exposure time in $scCO_2$ on liver tissue decellularization

Following the successful effect of prolonged $scCO_2$ exposure (5 h, 72 h), previously tested variables such as washing, moisture content and surface area were re-investigated in combination with prolonged $scCO_2$ exposure.

When liver tissue was incubated in water whilst shaking at 300 rpm, DNA content was reduced in a time dependent manner, with the lowest DNA content achieved at 24 h $p < 0.0001$ (Figure 5.8). Whilst $scCO_2$ alone (for 5 h or 72 h) moderately reduced DNA content compared to native liver tissue, this did not reach statistical significance. The impact of an agitating wash step was investigated following exposure to $scCO_2$ for 5 h and 72 h. At both time points tested (5 h and 72 h), a 24 h agitating wash step did not result in a significant reduction in DNA content compared to the native liver tissue or $scCO_2$ only control (Figure 5.10 A).

In a subsequent experiment, there was a significant reduction in DNA content after 5 h ($p = 0.0024$) and 72 h ($p = 0.0011$) of $scCO_2$ exposure compared to the native liver tissue control. However, when the liver tissue was pre-lyophilized, $scCO_2$ exposure for 5 h or 72 h did not alter the DNA content.

Pre-milled liver tissue showed a significant reduction of DNA content after 5 h and 72 h compared to the native liver tissue, $p = 0.0183$ and $p = 0.0016$ (respectively). In comparison to 5 h and 72 h $scCO_2$ exposure only (square tissue control) the DNA content of pre-milled liver tissue was not significantly reduced (Figure 5.10 B).

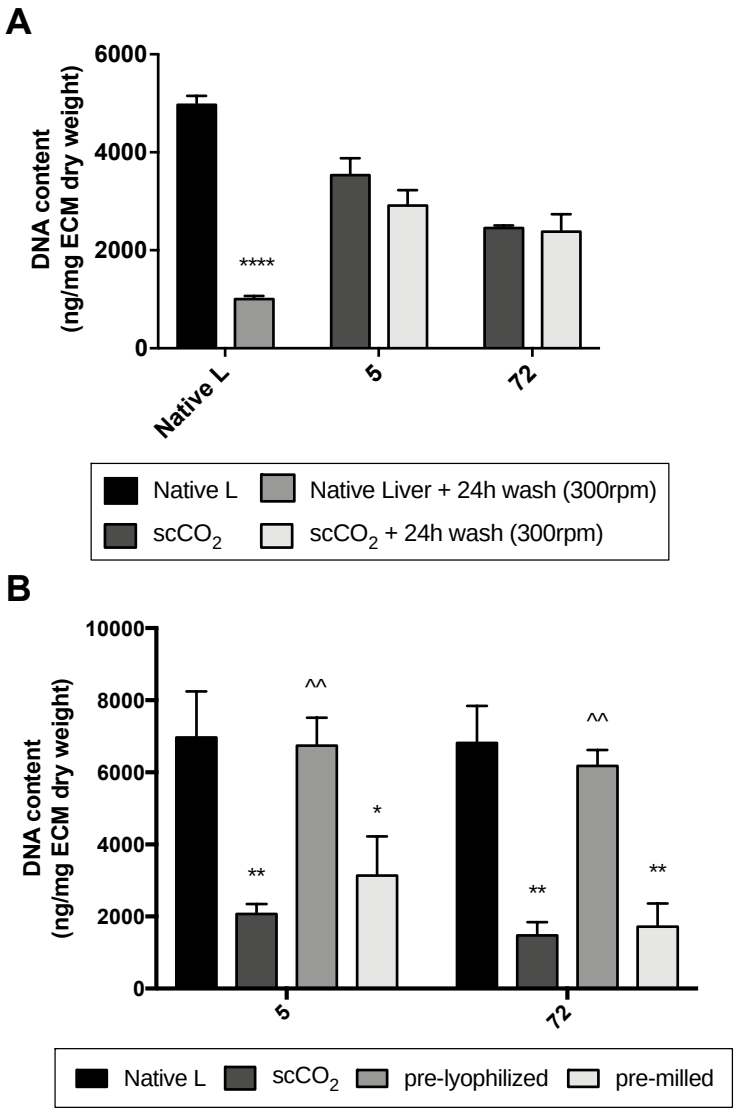


Figure 5.10 Native liver tissue (Native L, ~1 g) exposed to 5 h and 72 h of $scCO_2$ (A) DNA content, $scCO_2$ followed by a 24 h agitated wash; (B) DNA content of pre-lyophilised and pre-milled tissue. All data are normalized to tissue weight and analysed using a two-way ANOVA, n=3. All data are mean +/- SEM. * indicates significance level to the native tissue control (* $p < 0.05$, ** $p < 0.005$). ^ indicates significance level to $scCO_2$ alone (^^ $p < 0.005$, ^^^^ $p > 0.0001$)

5.4.6. Summary

In summary, the results showed that increasing the surface area of liver tissue facilitated cell removal during 15 and 60 minutes of $scCO_2$ exposure. Furthermore, prolonged exposure to $scCO_2$ resulted in a significantly enhanced, time-dependent reduction of DNA content, particularly between 5 and 72 hours. Finally, removal of liver tissue water content prior to prolonged $scCO_2$ exposure (5 h and 72 h) completely inhibited the reduction in DNA content that was otherwise observed in liver tissue containing the natural quantity of water. These results indicate that exposure time, surface area and water content are instrumental in facilitating decellularization of liver tissue by $scCO_2$.

5.5 RESULTS: VALIDATION OF A $SCCO_2$ DECELLULARIZATION METHOD

The aim was to identify the minimum exposure time to $scCO_2$ whilst retaining the decellularization effect observed following 72 h exposure. In addition, the developed $scCO_2$ decellularization method was validated on a second tissue, aorta.

5.5.1. Effect of prolonged exposure time in $scCO_2$ on liver tissue decellularization

It was previously shown that 72 h exposure to $scCO_2$ reduced the DNA content of native liver tissue (Figure 5.9), therefore the aim here was to determine whether the same level of DNA reduction could be achieved after a shorter duration.

Increasing exposure to $scCO_2$ (5 h, 24 h, 48 h and 72 hours) significantly reduced the residual DNA content of liver tissue in a time-dependent manner. The longer the exposure, the less DNA could be detected, with a significant reduction after 5 h (p = 0.0083), 24 h (p = 0.0002), 48 h (p < 0.0001) and 72 h (p < 0.0001) compared to a native liver tissue control (Figure 5.11 A). Residual DNA content of liver tissue was increasingly fragmented with increasing duration of exposure $scCO_2$ (Figure 5.11 E).

The tissue weight was significantly reduced by 21% (5 h), 26% (24 h), 26%, (48 h) and 25% (72 h, Figure 5.11 B), compared to the starting weight. There was no significant change in sulphated glycosaminoglycan or collagen content after 5 h, 24 h, 48 h and 72 h of $scCO_2$ exposure compared to the native liver tissue control (Figure 5.11 C+D).

Representative images of liver exposed to $scCO_2$ (5-72 hours) are shown in the Appendix (A 1.1).

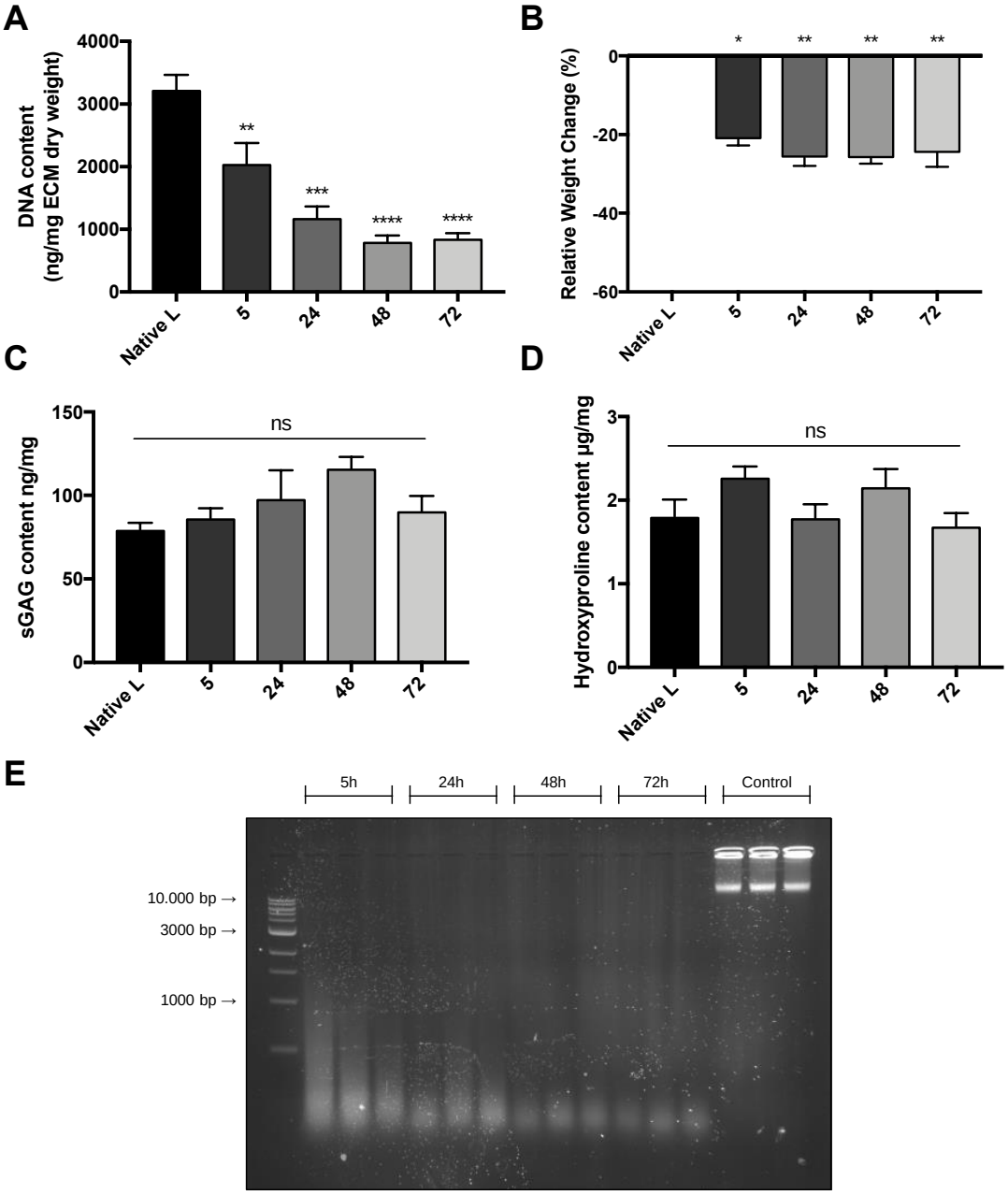


Figure 5.11 Liver tissue (~1g) exposed to 5 h, 24 h, 48 h and 72 h of $scCO_2$ (A) DNA content; (B) Tissue weight change; (C) sGAG concentration; (D) Hydroxyproline content; (E) DNA Gel electrophoresis. All data are normalized to tissue weight and analysed using a one-way ANOVA, n=3. All data are mean +/- SEM. * indicates significance level to the native tissue control (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p > 0.0001$)

5.5.2. Histological analysis of liver tissue following prolonged $scCO_2$ exposure

Histology was used to determine the effect of prolonged $scCO_2$ exposure on microscopic changes within liver tissue.

Histological analysis of liver visually confirmed the presence of cell nuclei (purple) with H&E staining in native liver as well as 5 h $scCO_2$ treated liver (H&E images, Figure 5.12). The 72 h $scCO_2$ exposed liver, in contrast, did not contain any detectable cell nuclei.

The alcian blue (AB) stain showed overall uniformity in the presence of glycosaminoglycans (blue) throughout all time points tested, which was comparable to that of the native liver tissue control (AB images, Figure 5.12).

The presence of collagen (red) and protein-enriched areas (shades of yellow), as measured by picro sirius red staining (PSR images, Figure 5.12), was similar in density across all groups. However, liver tissue exposed to $scCO_2$ (5 h and 72 h) displayed considerable white spaces within the tissue, which differed to that of the native control tissue, indicating a less dense structure.

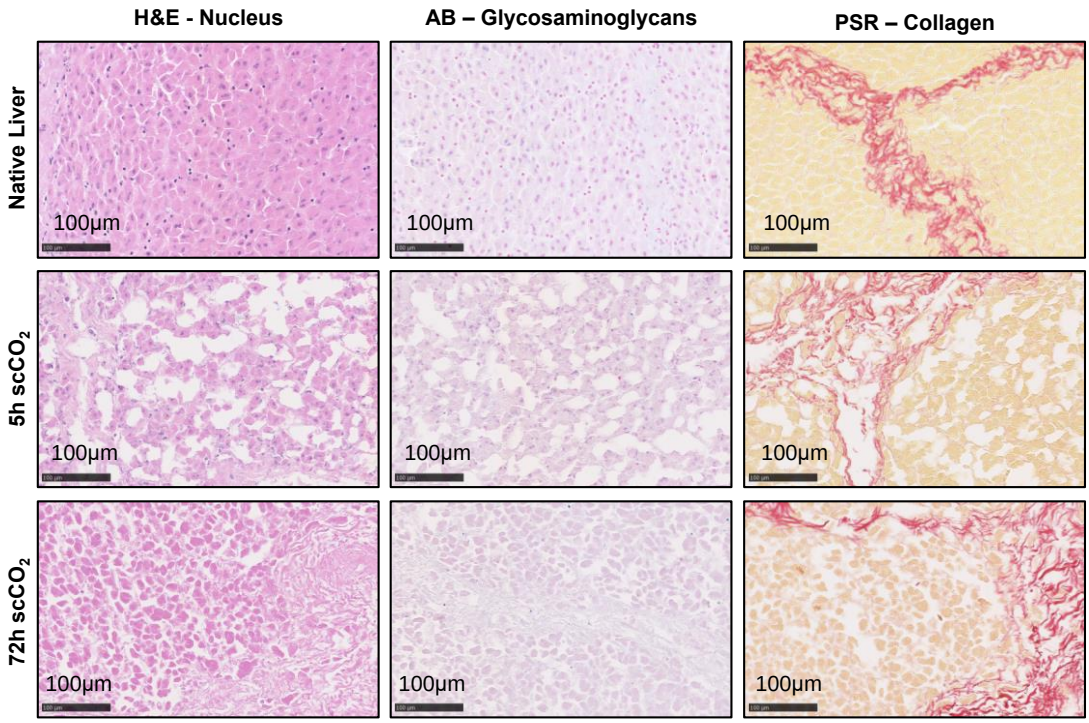


Figure 5.12 Representative histological images of liver tissue exposed to 5 h and 72 h of $scCO_2$. Cell nuclei (H&E, purple) were not present after 72 h of $scCO_2$ exposure. Staining intensity for collagen (PSR, red), protein-enriched areas (PSR, yellow) and glycosaminoglycans (AB, blue) was similar across all conditions. $scCO_2$ exposure (5 h and 72 h) increased the prevalence of white spaces/gaps within the tissue compared to the native liver tissue.

5.5.3. Effect of $scCO_2$ decellularization compared to detergent decellularization

Given that 48 h exposure to $scCO_2$ produced the greatest reduction in DNA content (see above), it was next compared to the gold-standard detergent based decellularization method to determine relative efficacy.

Detergent based decellularization of liver tissue reduced the DNA content by 95% (residual DNA content: 251 ng/mg of ECM dry weight, $p < 0.0001$) whereas $scCO_2$ decellularization reduced DNA content by 75% (residual DNA content: 803 ng/mg of ECM dry weight, $p < 0.0001$ compared to native; $p = 0.005$ compared to detergent (Figure 5.13 A).

Representative images show that native liver tissue is bright red in colour, whereas $scCO_2$ decellularized liver tissue has changed to a brown colour and the detergent decellularized liver tissue is white in colour. The gross structure/morphology was maintained following $scCO_2$ exposure whereas the detergent decellularized liver tissue did not resemble the original structure of the liver tissue (Figure 5.13 B).

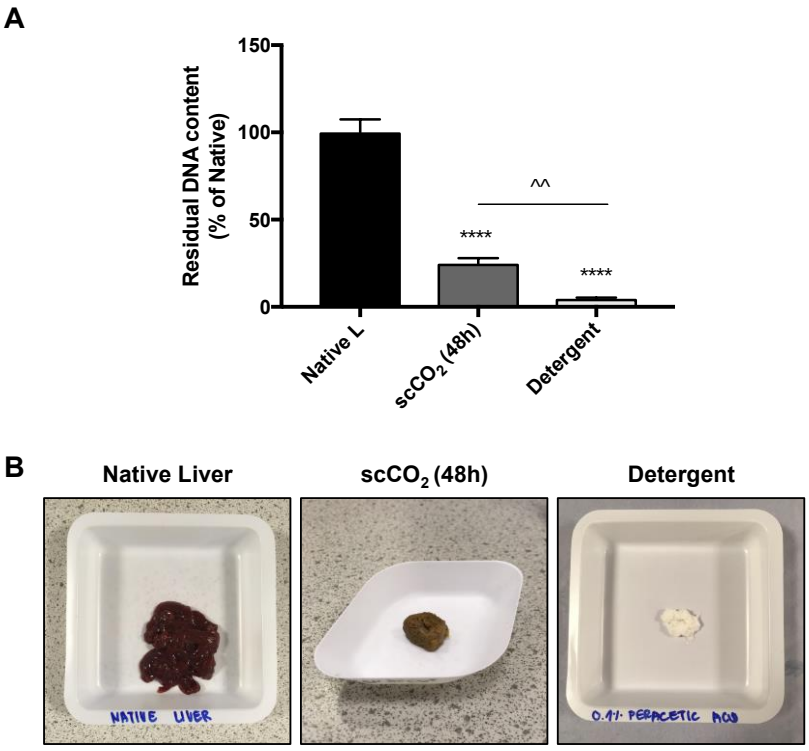


Figure 5.13 Liver detergent decellularization compared to $scCO_2$ decellularization (A) Relative change in DNA content. (B) Representative images. Data are normalized to dry tissue weight and analysed using a one-way ANOVA, n=3. All data are mean +/- SEM. All data are mean +/- SEM. * indicates significance level to the native liver tissue (Native L) control (****$p > 0.0001$). ^ indicates significance level to $scCO_2$ alone (^^$p < 0.005$)

5.5.4. Histological analysis of $scCO_2$ decellularization compared to detergent decellularization

Histology was next used to determine the effect of $scCO_2$ and detergent decellularization and on microscopic changes within liver tissue.

Histological analysis of native liver visually confirmed the presence of cell nuclei (purple) with H&E staining. There were no cell nuclei (purple) visible in the detergent decellularized or $scCO_2$ decellularized liver tissue. The detergent decellularized liver had an overall fragmented structure without any visible coordination of the remaining structures. In contrast, the $scCO_2$ decellularized liver tissue showed a more structured network (H&E images, Figure 5.14). The alcian blue (AB) stain showed overall uniformity in the presence of glycosaminoglycans (blue) throughout the native liver tissue control and $scCO_2$ decellularized liver tissue, whereas the detergent decellularized liver tissue had reduced glycosaminoglycans (AB images, Figure 5.14). The presence of collagen (red) and protein-enriched areas (shades of yellow) was qualitatively confirmed by picro sirius red staining. The native liver tissue and the $scCO_2$ decellularized liver tissue showed the intact collagen structures (red) and protein-enriched areas (yellow), whereas the detergent decellularized liver tissue did not show any protein-enriched areas (yellow).

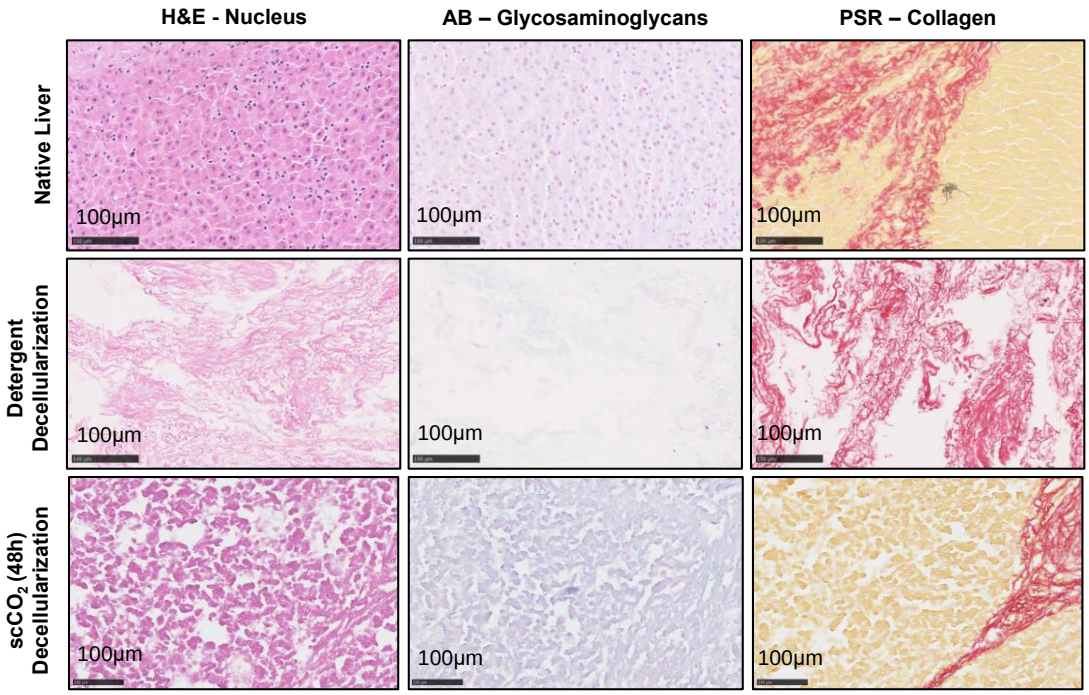


Figure 5.14 Representative histological images of liver that has been decellularized using detergents compared to $scCO_2$. Cell nuclei (H&E, purple) were not visible following detergent decellularization or $scCO_2$ decellularization. Detergent decellularized liver showed a fragmented mesh-like structure whereas $scCO_2$ decellularized tissue was more similarly structured to the native liver control. Staining intensity for glycosaminoglycans (AB, blue) and protein-enriched areas (PSR, yellow) was reduced following detergent decellularization compared to the native liver control and the $scCO_2$ decellularized liver tissue.

5.5.5. Effect of prolonged exposure to $scCO_2$ on aorta tissue

Next, the same processing conditions that were used on liver were investigated on a second tissue (aorta) to confirm the methodology was applicable across different tissue types.

Exposure of aorta tissue to $scCO_2$ significantly reduced the DNA content of aorta tissue after 48 h ($p = 0.0043$) and 72 h ($p = 0.0027$), but not 5 h or 24 h, compared to a native aorta tissue control (Figure 5.15 A). The exposure to 5 h, 24 h, 48 h and 72 h of $scCO_2$ significantly reduced the aorta tissue weight in a time-dependent manner by 23% ($p = 0.0355$), 41% ($p = 0.0004$), 45% ($p < 0.0001$) and 48% ($p < 0.0001$), respectively, Figure 5.15 B)

The sulphated glycosaminoglycan and hydroxyproline content remained unchanged throughout all time points tested (Figure 5.15 D).

Residual DNA content of aorta tissue was increasingly fragmented with increasing duration of exposure $scCO_2$ (Figure 5.15 E).

Representative images of aorta exposed to $scCO_2$ (5-72 hours) are shown in the Appendix (A 1.2).

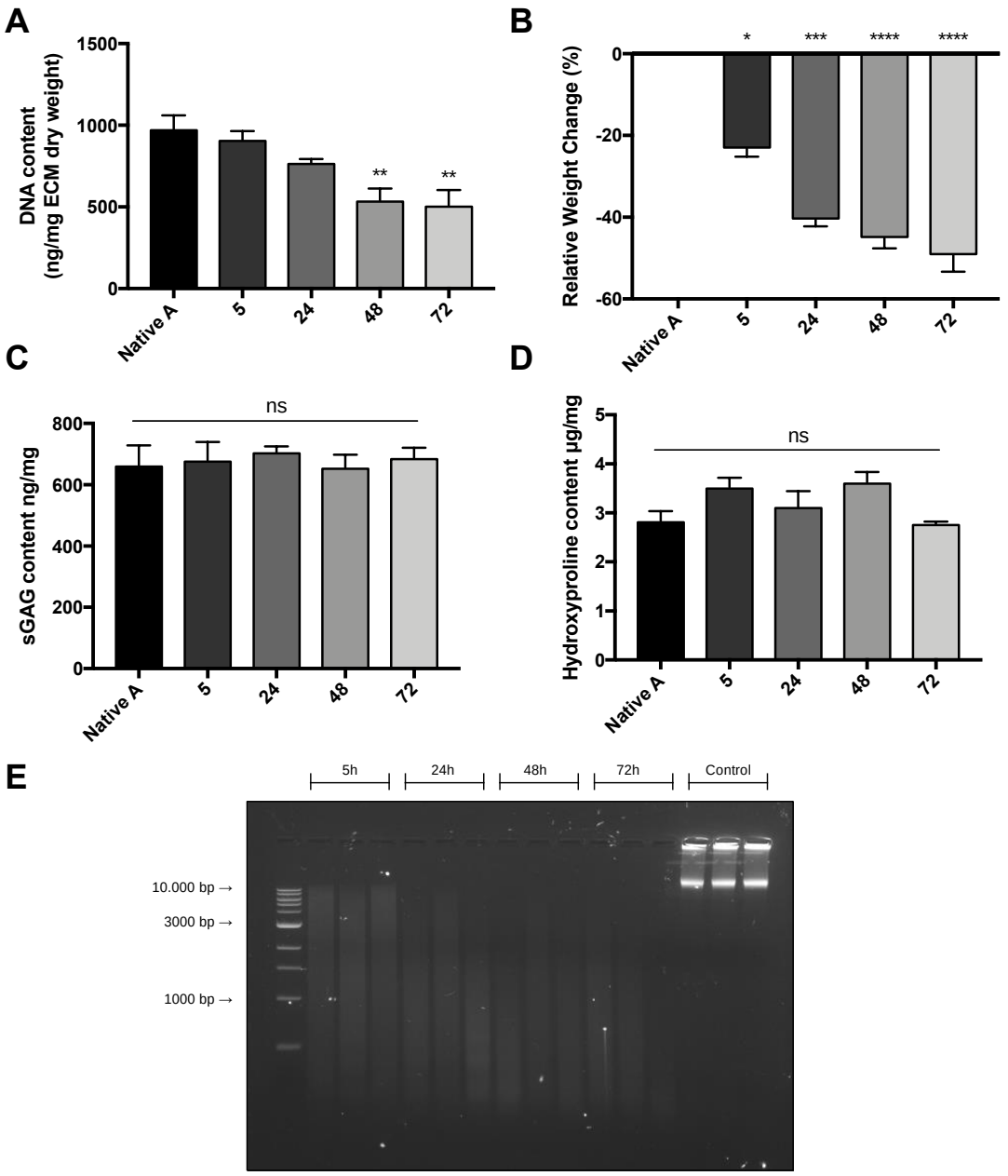


Figure 5.15 Aorta tissue (~1 g) exposed to 5 h, 24 h, 48 h and 72 h of $scCO_2$ (A) DNA content. (B) Tissue weight change. (C) sGAG concentration. (D) Hydroxyproline content. (E) DNA Gel electrophoresis. (F) Representative images. All data are normalized to tissue weight and analysed using a one-way ANOVA, n=3. All data are mean +/- SEM. * indicates significance level to the native tissue control (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p > 0.0001$)

5.5.6. Histological analysis of aorta tissue after prolonged $scCO_2$ exposure

Histology was then performed to assess changes to the microscopic structure after $scCO_2$ decellularization.

Histological analysis of aorta visually confirmed the presence of cell nuclei (purple) with H&E staining in native aorta as well as 5 h $scCO_2$ exposed aorta. In contrast, the 72 h $scCO_2$ exposed aorta tissue did not show any detectable cell nuclei (purple; H&E images, Figure 5.16).

The alcian blue (AB) stain showed overall uniformity in the presence of glycosaminoglycans (blue) throughout all time points tested (AB images, Figure 5.16).

The presence of collagen (red) and protein-enriched areas (shades of yellow) was qualitatively confirmed by picro sirius red staining (PSR images, Figure 5.16). It is noticeable that both $scCO_2$ time points (5 h and 72 h) revealed a few structured white spaces within the aorta tissue.

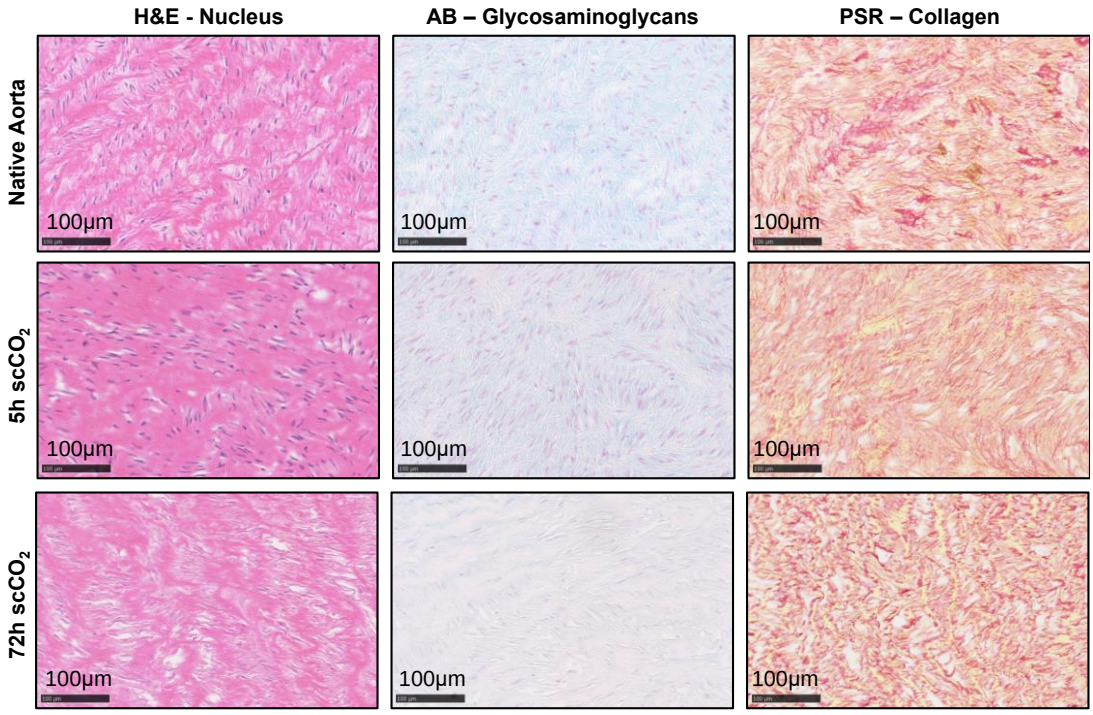


Figure 5.16 Representative histological images of native aorta exposed to 5 h and 72 h of $scCO_2$. Cell nuclei (H&E, purple) were present following 5 h but not 72 h of $scCO_2$ exposure. Staining intensity for glycosaminoglycans (AB, blue), collagen (PSR, red) and protein-enriched areas (PSR, yellow) was similar for all conditions. $scCO_2$ exposure (5 h, 72 h) increased white spaces within the aorta tissue.

5.5.7. Effect of $scCO_2$ decellularization compared to detergent decellularization

Given that 72 h exposure to $scCO_2$ produced the greatest reduction in DNA content (see above), it was next compared to the gold-standard detergent based decellularization method to determine relative efficacy.

Detergent based decellularization of aorta tissue reduced the DNA content by 68% (residual DNA content: 1038 ng/mg of ECM dry weight $p < 0.0001$) whereas $scCO_2$ decellularization achieved a DNA content reduction of 48% (residual DNA content: 509 ng/mg of ECM dry weight, $p < 0.0010$). The difference in DNA content between detergent and 72 h $scCO_2$ decellularized aorta was not significant (20%; $p = 0.6907$; Figure 5.17 A).

Representative images show that native aorta tissue is a light pink colour, whereas $scCO_2$ decellularized aorta tissue and detergent decellularized aorta tissue changed to a more white/opaque colour. The gross structure/morphology was maintained in the $scCO_2$ decellularized aorta tissue and the detergent decellularized aorta tissue (Figure 5.17 B).

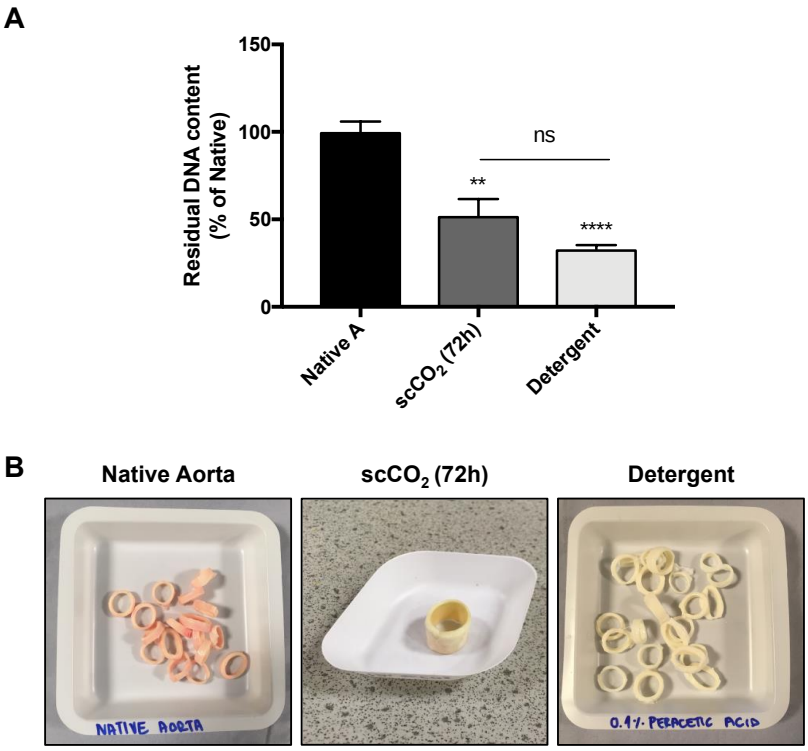


Figure 5.17 Aorta detergent decellularization compared to $scCO_2$ decellularization (A) Relative change in DNA content. (B) Representative images. Data are normalized to dry tissue weight and analysed using a one-way ANOVA, n=3. All data are mean +/- SEM. All data are mean +/- SEM. * indicates significance level to the native aorta tissue (Native A) control (****p > 0.0001)

5.5.8. Histological analysis of $scCO_2$ decellularization compared to detergent decellularization

Histology was then performed to assess changes to the microscopic structure after $scCO_2$ and detergent decellularization.

Histological analysis of native aorta visually confirmed the presence of cell nuclei (purple) with H&E staining. In contrast, there were no cell nuclei (purple) visible in the detergent decellularized or $scCO_2$ decellularized aorta tissue. The detergent decellularized aorta showed numerous large gaps between a wavy network of filament-like bands (pink), whereas $scCO_2$ decellularized aorta tissue had a more compact network of filament-like bands with only very small gaps in between (H&E images, Figure 5.18). The alcian blue (AB) stain showed overall uniformity in the presence of glycosaminoglycans (blue) throughout the native aorta tissue control and the detergent decellularized aorta tissue, whereas the $scCO_2$ decellularized aorta tissue showed only very weak blue staining (AB images, Figure 5.18). The presence of collagen (red) and protein-enriched areas (shades of yellow) was qualitatively confirmed by picro sirius red staining (PSR images, Figure 5.18). The native aorta tissue as well as the $scCO_2$ decellularized aorta tissue showed the intact collagen structures and protein-enriched areas, whereas the detergent decellularized aorta tissue did not show any protein-enriched areas (yellow).

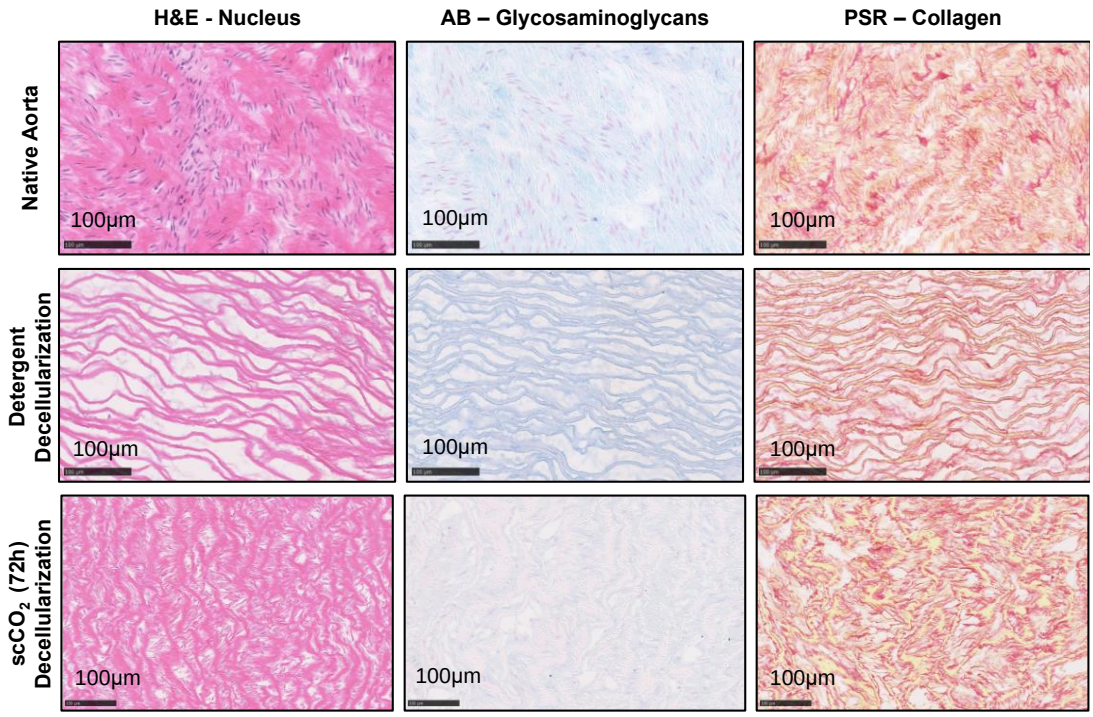


Figure 5.18 Representative histological images of aorta that has been decellularized using detergents compared to $scCO_2$. Cell nuclei (H&E, purple) were not visible in detergent decellularized or $scCO_2$ decellularized aorta tissue. Detergent decellularized aorta showed large gaps between a wavy network of filament-like bands (H&E, pink). In contrast, $scCO_2$ decellularized tissue had a more compact network of filament-like bands (H&E, pink) with smaller gaps. Staining intensity for glycosaminoglycans (AB, blue) was reduced by $scCO_2$ exposure but not detergent decellularization. Protein-enriched areas (PSR, yellow) were not present following detergent decellularization of aorta tissue.

5.5.9. Summary

In summary, 48 hours was the minimum time required to achieve the maximum reduction in DNA content of liver by $scCO_2$ alone. However, the reduction in DNA content was more marked using a detergent based method. The same $scCO_2$ based decellularization technique was applied to aorta tissue however 72 hours of exposure was required to produce a reduction in DNA content that was comparable to that produced by a detergent based decellularization method.

5.6 DISCUSSION

5.6.1. Combined effect of $scCO_2$ and ethanol

The prior paper from Sawada *et al.*, 2008 showed the utility of $scCO_2$ in combination with ethanol as an extraction medium for decellularizing aorta tissue. Using the same experimental conditions, the method was repeated here to determine the utility of this technique to decellularize aorta tissue. Moreover, the same method was then applied to liver tissue to determine the robustness of the methodology to decellularize other, structurally diverse, tissue types in a reproducible manner.

In contrast to the effects reported by Sawada *et al.*, 2008, a combined $scCO_2$ and ethanol mixture did not produce a significant decrease in DNA or phospholipid content of aorta or liver tissue herein. Moreover, the presence of cell nuclei following exposure to $scCO_2$ and ethanol affirmed these findings. Therefore, these results indicate that short exposures to $scCO_2$ in combination with ethanol did not provide an effective hybrid decellularization methodology.

The reason for the disparity in findings compared to those reported previously is difficult to determine. The temperature (37°C) and pressure level (~2900 psi) were maintained the same as those described by Sawada *et al.*, 2008. It was also confirmed herein that the pressure and temperature levels were suitable to produce the expected $scCO_2$ phase and were capable of solubilizing ethanol in $scCO_2$. This study used the same quantity (~1 g) and type of tissue (porcine aorta) as previously reported. The high-pressure autoclave by Sawada *et al.*, 2008 had a greater volume (50 cm^3) compared to that used in this work (20 cm^3). However, to account for this, the ratio of ethanol to autoclave volume was maintained the same between studies (i.e. 15 mL dry ethanol: 50cm^3, 6 mL dry ethanol: 20cm^3). Sawada *et al.*, 2008 regulated the temperature (37°C) of the system using a water bath, whereas the current study used a heating jacket attached to the outside of the autoclave base, which was unlikely to account for the difference in findings between studies. Another aspect that was different between the two studies was that the experiments reported previously used a stirrer to facilitate mixing. Unfortunately, the high-pressure autoclave used for the studies herein was not equipped with a stirrer bar. A

stirrer might have facilitated mixing and solubilisation of ethanol into $scCO_2$, however, the solubilisation test confirmed successful solubilisation of ethanol into $scCO_2$ without a stirrer.

Whilst the study presented herein tried to replicate the physical conditions/materials used by Sawada *et al.*, 2008 as closely as possible, several aspects related to experimental design and statistical analysis were not reported in the earlier paper. For instance, no statistical analyses were reported and no information on group sizes were included. The lack of error bars on the figures presented by Sawada *et al.*, 2008, indicate that the results were possibly obtained from a single piece of aorta tissue. Composition of animal tissue varies widely, so replicates of at least three distinct tissues pieces per condition were used herein as biological replicates to account for biological variation. Furthermore, the absence of statistical analyses on the data reported by Sawada *et al.*, 2008 make it difficult to determine the probability that the effects seen were not simply obtained by chance. A p value threshold of < 0.05 (i.e. less than 5%) was used herein to demonstrate the probability that a result occurred by chance.

To date, there are 4 additional publications that have employed $scCO_2$ in combination with ethanol for decellularization of mammalian tissue (Guler *et al.*, 2017; Huang *et al.*, 2017; Seo, Jung and Kim, 2017; Wang *et al.*, 2017). Two of these publications described the successful removal of DNA from human lipoaspirate and porcine cornea tissue by $scCO_2$, as measured by histological staining (H&E; Huang *et al.*, 2017; Wang *et al.*, 2017), which is not a quantitative method. The other two publications showed a reduction in DNA content of 96% and 68% on rat heart and ovine aorta tissue, respectively (Guler *et al.*, 2017; Seo, Jung and Kim, 2017). However, it is difficult to compare the results presented herein with those of the aforementioned published studies, as the only comparable factor between the studies is the use of ethanol in combination with $scCO_2$. The temperature used in all the aforementioned studies varied from 37°C - 45°C, the pressure ranged from 2500 psi – 5067 psi, the exposure time from 1 h – 6 h and the pre-treatment from freeze-drying to pre-soaking (Guler *et al.*, 2017; Huang *et al.*, 2017; Seo, Jung and Kim, 2017; Wang *et al.*, 2017). Herein, a temperature of 37°C, pressure of ~2900psi and

exposure time of up to 60 minutes were used, in accordance with the publication by Sawada *et al.*, 2008, which might have caused the difference in outcome. The temperature used in this study was purposely chosen based on human physiological temperature (Nilsson, Molokwu and Olsson, 2016), and did not exceed >40°C to avoid denaturation of proteins (Lepock, Frey and Ritchie, 1993) and subsequent damage to the 3D structure of the ECM scaffold. The pressure level was sufficient enough to achieve the supercritical state with carbon dioxide (≥ 31.85°C and ≥ 1071.335 psi; Benner, Ricardo and Carrigan, 2004). The results presented in this thesis were in agreement with published work by Casali *et al.*, 2018 and Antons *et al.*, 2018. Casali *et al.*, 2018 replicated the work by Sawada *et al.*, 2008 using the same experimental conditions (tissue, pressure, temperature, exposure time) and revealed retained cell nuclei with H&E staining. In the published discussion with reviewers, Antons *et al.*, 2018 reported no success in decellularization of cartilage (bovine), tendon (horse) and skin (human) tissue using $scCO_2$ and ethanol. In addition, the use of ethanol in combination with $scCO_2$ caused increased tissue stiffness (unpublished observations; Casali *et al.*, 2018), which is detrimental to downstream applications of the resultant ECM scaffold.

To summarise, $scCO_2$ (up to 60 minutes) in combination with ethanol did not facilitate cell removal from aorta or liver tissue, which contrasts that reported by Sawada *et al.*, 2008. However, the utility of $scCO_2$ in combination with ethanol as an extraction medium for decellularization might still be a feasible option when optimizing aspects such as temperature, pressure, exposure time and pre-treatment, as demonstrated by others (Guler *et al.*, 2017; Huang *et al.*, 2017; Seo, Jung and Kim, 2017; Wang *et al.*, 2017).

5.6.2. Optimization of a $scCO_2$ decellularization method

$ScCO_2$ is commonly used in the food and pharmaceutical industry for extraction of fat/oil, purification of spices and sterilization purposes (Perrut, 2003; Brunner, 2005). It was hypothesised that $scCO_2$ can facilitate decellularization by penetration of the tissue and disruption of the phospholipid bilayer of the cell membrane, based on the ability of $scCO_2$ to extract fat/oil from animal derived tissues (King, Johnson and Friedrich, 1989; Boselli and Caboni, 2000; Tanaka, Sakaki and Ohkubo, 2004).

Optimization of the $scCO_2$ decellularization method developed herein involved manipulation of tissue moisture content, surface area, exposure time and agitation that has previously been shown to be beneficial in the food and pharmaceutical industry (Yamaguchi *et al.*, 1986; King, Johnson and Friedrich, 1989; Chao *et al.*, 1991; King *et al.*, 1996; Scott L Taylor, Eller and King, 1997; Montanari *et al.*, 1998; Boselli and Caboni, 2000; Gopalan *et al.*, 2000; Tanaka, Sakaki and Ohkubo, 2004). $ScCO_2$ exposure reduced tissue DNA content in a time-dependent manner, with a significant reduction in DNA content with exposures longer than 5 hours. This effect was dependent on tissue moisture content because pre-lyophilization of the tissue prevented DNA removal. Increasing the tissue surface area (by pre-milling the tissue) improved the ability of $scCO_2$ to reduce tissue DNA content.

It is difficult to compare the results presented herein with those of previously published experiments using $scCO_2$. To date, 5 publications have employed $scCO_2$ for decellularization of mammalian tissue using $scCO_2$ in combination with ethanol (discussed above, Sawada *et al.*, 2008; Guler *et al.*, 2017; Huang *et al.*, 2017; Seo, Jung and Kim, 2017; Wang *et al.*, 2017) whereas 2 publications utilised $scCO_2$ in combination with detergents (Antons *et al.*, 2018; Casali *et al.*, 2018). None of these publications described the successful removal of DNA by $scCO_2$ alone. Therefore, the results presented here are, to the best of our knowledge, the first to report the decellularization potential of $scCO_2$ alone.

An unexpected finding in this study was that the removal of liver tissue water content (by freeze drying) prior to $scCO_2$ exposure inhibited the ability of $scCO_2$ to reduced DNA content (as a marker of decellularization). This suggests that water content is important for successful decellularization using $scCO_2$. Interestingly, this finding was in contrast to published literature that showed reduced moisture content is generally beneficial for the removal of fat/oil of animal derived tissues or products using $scCO_2$ (Yamaguchi *et al.*, 1986; King, Johnson and Friedrich, 1989; Boselli and Caboni, 2000; Tanaka, Sakaki and Ohkubo, 2004). King, Johnson and Friedrich, 1989 were able to facilitate quicker fat content removal of several meat matrices by dehydrating the meat matrices prior to $scCO_2$ exposure (King, Johnson and Friedrich,

1989) while Yamaguchi *et al.*, 1986 was able to show a higher yield of oil extracted from pre-lyophilised antarctic krill compared to non-dried antartic krill (Yamaguchi *et al.*, 1986). Boselli and Caboni, 2000 and Tanaka, Sakaki and Ohkubo, 2004 showed a successful phospholipid removal of egg yolk and salmon roe (respectively) that was pre-lyophilised (Boselli and Caboni, 2000; Tanaka, Sakaki and Ohkubo, 2004). The temperature used in all the aforementioned studies varied from 33°C - 80°C and the pressure ranged from 2567 psi – 10008 psi (Yamaguchi *et al.*, 1986; Clarke, 1991; Boselli and Caboni, 2000; Tanaka, Sakaki and Ohkubo, 2004). Herein, a temperature of 37°C and ~2900psi were used, which might have caused the difference in outcome. Oils are generally more soluble in $scCO_2$ at higher temperatures (Friedrich, 1982). However, as discussed previously, the temperature used in this study was purposely chosen based on human physiological temperature (Nilsson, Molokwu and Olsson, 2016), and did not exceed 40°C to avoid denaturation of proteins (Lepock, Frey and Ritchie, 1993) and subsequent damage to the 3D structure of the ECM scaffold. The pressure level selected was sufficient to achieve the supercritical state with carbon dioxide (≥ 31.85°C and ≥ 1071.335psi; Benner, Ricardo and Carrigan, 2004). In addition, as described in the material and methods chapter, the high-pressure system at the University of Nottingham is set to not exceed 4000 psi as a safety feature.

Increasing the surface area of the liver tissue (by milling) prior to $scCO_2$ exposure facilitated decellularization, as measured by a reduction in DNA content compared to a native (non-milled, square) liver tissue. In contrast, thinly slicing the liver tissue to increase surface area was insufficient to enhance the decellularization capabilities of $scCO_2$. The benefits of an increased surface area for $scCO_2$ exposure are well documented in the literature (McGhee, Black and Brekke, 1974). McGhee, Black and Brekke, 1974 highlighted the benefit of small sample size to aid fat extraction regardless of the extraction method applied. Various studies have since followed showing a successful oil/fat extraction with supercritical carbon dioxide using smaller particle sizes such as ground meat products (Chao *et al.*, 1991; King *et al.*, 1996; Taylor, Eller and King, 1997) and oil seeds (Taylor, Eller and King, 1997; Montanari *et al.*, 1999; Gopalan *et al.*, 2000). Whilst milling of the tissue prior to decellularization might be acceptable when generating ECMs for the use of ECM

hydrogels or coatings, this approach would not facilitate the production of larger ECM products for tissue grafts or organ regeneration.

A further finding in this study was that prolonged washing of liver tissue in water resulted in a time-dependent reduction in DNA content. This suggests that water alone can be used as an extraction medium, which is likely due to a change in osmotic pressure thereby facilitating cell lysis.

When increasing the duration of $scCO_2$ exposure of the liver tissue from 15 minutes up to 3 days, DNA content was only significantly reduced from 5 hours onwards compared to a native liver tissue control. This is in agreement with previously published literature on oil extraction using $scCO_2$ on beef (Chao *et al.*, 1991), fish (Hardardottir and Kinsella, 1988; Tanaka, Sakaki and Ohkubo, 2004) and sheep bone (Fages *et al.*, 1998; Frayssinet *et al.*, 1998). The duration of $scCO_2$ exposure in the aforementioned literature varied from 4 h to 12 h (Hardardottir and Kinsella, 1988; Chao *et al.*, 1991; Frayssinet *et al.*, 1998; Tanaka, Sakaki and Ohkubo, 2004), indicating that prolonged exposure to $scCO_2$ is required when using mammalian tissue. This is further supported by Wang *et al.*, 2017 and Seo, Jung and Kim, 2017 who both showed cell removal using $scCO_2$ in combination with ethanol after 3 h and 6 h, respectively. Based on the findings presented herein (as well as that of others), all subsequent experiments were carried out with a minimum of 5 hours $scCO_2$ exposure.

Exposure of liver tissue to $scCO_2$ caused fragmentation of DNA. Although the mechanism by which $scCO_2$ causes DNA fragmentation is not known, it is possibly due to shear stress caused by extraction. Previous publications on the use of $scCO_2$ for decellularization have not reported effects on DNA fragmentation. However, this is a crucial aspect when evaluating decellularized biomaterials for *in vivo* use as it can trigger an immunogenic response when transplanted into a recipient (Crapo, Gilbert and Badylak, 2011). The less fragmented the DNA the more it represents the original genomic DNA causing an immune response of the body (Nagata, Hanayama and Kawane, 2010) and rejection of the foreign material (Zheng *et al.*, 2005). Badylak and Gilbert, 2009 showed that a variation of commercially available ECM

products contained small DNA fragments (< 300 bp) that are unlikely to cause any immune response and safe to use in clinic. Hence, fragmentation of the DNA was considered and assessed in the studies presented here.

5.6.3. Validation of a $scCO_2$ decellularization method

The $scCO_2$ decellularization method developed above was further validated on a second tissue, aorta. Exposure of liver and aorta to 5 h, 24 h, 48 h and 72 h of $scCO_2$ produced a time-dependent reduction in DNA content compared to native tissue. This was confirmed by histological staining, which highlighted a reduced number of nuclei after 5 h exposure to $scCO_2$ and no visible nuclei at all after 72 h exposure. To date, there are no reports using only $scCO_2$ exposure for decellularization purposes without the addition of modifiers or pre-/post-treatments. A head-to-head comparison against a typical detergent based decellularization method was performed in this study; the detergent based method produced a 95% and 68% reduction in DNA content for liver and aorta, respectively, whereas the $scCO_2$ decellularization method achieved a 75% (48 h) and 47% (72 h) reduction, respectively for liver and aorta. Therefore, the $scCO_2$ method described here produced comparable/similar effects on DNA content compared to that of a typical detergent based method. It should be noted that the duration of 72 h of $scCO_2$ exposure for aorta tissue was chosen because 72 h produced the greatest reduction in DNA content. In addition, aorta tissue was particularly difficult to decellularize and therefore the longest possible duration was used.

The $scCO_2$ decellularization method developed reduced DNA content of liver and aorta tissue by 75% and 47%, respectively. Aorta tissue required a 24 h longer exposure to $scCO_2$ than liver tissue to achieve the similar level reduction of DNA content. Detergent decellularization caused a 95% and 68% reduction in DNA content on liver and aorta respectively, indicating that aorta tissue is more difficult to decellularize. This can potentially be explained by the difference in tissue structure. While both tissues contain mainly Type I and III collagen, aorta contains much more elastin (Rojkind, Giambrone and Biempica, 1979; Vouyouka *et al.*, 2001). The high amount of elastin makes the structure of aorta much more stiff (Sherratt, 2003) which

might alter the penetration capability of the tissue and arguably make decellularization more difficult.

It is noteworthy that the reduction in DNA content with prolonged $scCO_2$ exposure reached a plateau at approximately a 75% decrease. For industrial application/utilisation of this $scCO_2$ method, it might be important to further reduce the processing time to be able to produce high quality ECM scaffolds for clinical applications as quickly as possible. The detergent decellularization was quicker to perform and ultimately provided a more efficient removal of DNA from liver tissue. However, histological images of detergent based decellularized liver and aorta revealed a more fragmented structure compared to that of the $scCO_2$ decellularization method, indicating that detergents might cause detrimental changes to the ECM structure, making them unsuitable for scaffold applications (Faulk *et al.*, 2014; White *et al.*, 2016). Similar changes to the ECM structure were visible in the aorta tissue treated with detergents. The biochemical and biophysical properties of the ECM structure are strongly influencing the cell microenvironment (Rozario and Desimone, 2010), making an undamaged ECM structure a key aspect for a successful recellularization and degradation of the ECM scaffold *in vivo*. The damage associated with detergent decellularization has been shown to be caused by disruption of the ECM basement membrane, organization, architecture as well as surface structure, and to leave residual harmful detergents behind (Nakayama *et al.*, 2010; White *et al.*, 2016). The effect of $scCO_2$ on the ECM scaffold has not been properly assessed yet. However, the H&E images presented herein revealed a compact and organized structure indicating no damage to the ECM structure which is in agreement with published literature (Sawada *et al.*, 2008; Huang *et al.*, 2017; Casali *et al.*, 2018).

5.7 CONCLUSION

To conclude, the use of 15 or 60 minutes of a combined $scCO_2$ and ethanol mixture to facilitate cell removal was not successful, which was in contrast to the findings reported by Sawada *et al.*, 2008. However, the use of $scCO_2$ as an alternative to current detergent based decellularization methodologies showed promising results when exposure exceeded 5 hours. Moreover, maximal reductions in DNA content were achieved following 48-72 hours. Interestingly, these promising results were inhibited when the moisture content of the tissue was removed prior to $scCO_2$ exposure, which revealed the importance of water content within the tissue. A 75% and 48% reduction in DNA content was achieved when liver and aorta tissue were exposed to 48 h and 72 h of $scCO_2$, respectively. This is the first successful report of a reduction in DNA content using a $scCO_2$ only based decellularization method, without the addition of modifying chemicals.

6. Optimization and characterization of a hybrid $scCO_2$ decellularization methodology

6.1. OVERVIEW

In this chapter, the aim was to optimize the $scCO_2$ decellularization method by the addition of decellularization agents (i.e. a hybrid methodology). It was hypothesised that the addition of commonly used decellularization agents (Trypsin/EDTA, Triton-x-100, SDC) or a solvent with high solubility in $scCO_2$ (LS-54) in combination with $scCO_2$ would facilitate further cell removal from tissue.

6.2. INTRODUCTION

The two most recent publications in the area of $scCO_2$ decellularization demonstrate the utility of $scCO_2$ in combination with decellularization agents to decellularize mammalian tissue (Antons *et al.*, 2018; Casali *et al.*, 2018). Casali *et al.*, 2018 developed a 2-day hybrid Sodium Dodecyl Sulfate (SDS) /$scCO_2$ decellularization methodology to generate an acellular scaffold from porcine aorta. The authors used pre-saturated $scCO_2$ in an attempt to remove residual SDS toxicity. Furthermore, they tested if $scCO_2$ in combination with a detergent would facilitate cell removal while maintaining tissue hydration and mechanical properties of the scaffold (Casali *et al.*, 2018). The authors reported that their method effectively removed cells, decreased residual SDS concentration and retained structural properties of the ECM. The publication by Antons *et al.*, 2018 employed LS-54 in combination with $scCO_2$ to create an acellular bioscaffold from horse tendon, bovine cartilage and human skin for tissue engineering applications. The approach behind this method was to pre-treat the tissue with a physical (freeze-thawing, osmotic shock) or biological (trypsin) treatment followed by saturation of the tissue in a CO_2-philic detergent (to add polarity to the CO_2) prior to $scCO_2$ exposure (Antons *et al.*, 2018). Antons *et al.*,

2018 demonstrated that their method effectively removed cells from three different tissues, retained cell adhesion molecules and reduced sulphated glycosaminoglycan (sGAG) content, while the resultant scaffold was biocompatible in an *in vitro* cell-based assay (Antons *et al.*, 2018). Both studies demonstrated an innovative and novel approach to using $scCO_2$ for decellularization by utilizing modifying agents to facilitate cell removal. In addition, the authors showed that $scCO_2$ could also be used to remove residual toxicity from other decellularization agents whilst also achieving an additive/synergistic decellularization effect on the scaffold. Such technology could therefore alleviate toxicity issues caused by residual detergents in acellular scaffolds compared to those generated using a detergent based methods alone (White *et al.*, 2016).

The current study explored the combined effects of decellularization agents prior to or following $scCO_2$ exposure to determine whether a new combination approach could provide additive or synergistic effects on decellularization efficacy. Different additives were selected based on their mode of impact in decellularization: 0.02% Trypsin/0.05% EDTA to aid cell disassociation (Meyer *et al.*, 2006); 3% Triton-x-100 to facilitate cell permeabilization (Shafiq *et al.*, 2012) and 4% Sodium Deoxycholate to emulsify fats (Poornejad *et al.*, 2016). Each of these commonly used for decellularization (Meyer *et al.*, 2006; Shupe *et al.*, 2010; Shafiq *et al.*, 2012; Gilpin *et al.*, 2014) and form part of the comparator decellularization protocol used in this thesis. In addition, 2% LS-54 was investigated to negatively charge the CO_2 and aid $scCO_2$ extraction of negatively charged cellular components i.e. DNA.

Another aspect explored in this chapter was the sequence of adding the decellularization agents before or after $scCO_2$ exposure.

a) Decellularization agent <u>prior</u> to $scCO_2$: The rationale behind this approach was that residual decellularization agent, when added prior to $scCO_2$, will remain on the outside of the tissue. When pressurized, the tissue is penetrated by $scCO_2$ and the residual decellularization agent and thereby facilitates cell removal. In addition the decellularization agent (if soluble in $scCO_2$) will be removed through the venting process via the $scCO_2$.

b) Decellularization agent post $scCO_2$: If the decellularization agent is applied after $scCO_2$ exposure, the $scCO_2$ will have penetrated/perforated the tissue first, enabling the decellularization agent to more easily infiltrate the tissue to remove cellular material.

6.3. MATERIALS & METHODS

A detailed and comprehensive explanation of the materials and methods used here can be found in the materials and methods chapter (see Chapter 4). The following experimental set up and techniques were used in brief:

6.3.1 Solubility testing in $scCO_2$

The solubility of 0.02% Trypsin/0.05% EDTA, 3% Triton-x-100, 4% Sodium Deoxycholate and 2% LS-54 (500 µl each) was observed in a high-pressure autoclave at working conditions (~2900 psi, 37°C) for 15 minutes.

6.3.2 Supercritical carbon dioxide decellularization

Pre-treatment of liver and aorta tissue with a range of decellularization agents was performed prior to or following $scCO_2$ exposure to assess the combined impact on cell removal. Briefly, biological tissue (liver or aorta, ~1 g) was placed in 20 mL of either 0.02% Trypsin/0.05% EDTA, 3% Triton-x-100, 4% Sodium Deoxycholate and 2% LS-54 for a duration of 1 h at 300 rpm. Three tissue samples were used per condition (liver, aorta; n=3). For the $scCO_2$ exposure, each biological sample (liver or aorta) was placed in the high-pressure autoclave and the system interlocked. The high-pressure autoclave system was pressurised to ~1000 psi and heated to 30°C, before increasing to ~2600 psi and a temperature of 37°C. The temperature was maintained at 37°C +/- 3°C. After the desired $scCO_2$ exposure duration, the system was vented to ambient pressure within 1 minute.

Following decellularization, tissue samples were stored at approximately -20°C until further use.

6.3.3 Detergent Decellularization

Detergent based decellularization of liver or aorta tissue was performed using an adapted protocol published by Loneker *et al.,* 2016. This approach utilises sequential incubations in multiple decellularization agents. The effect of the individual decellularization agents as well as the combined effect of multiple decellularization agents was examined. Three tissue samples (n=3) were removed after exposure to each individual decellularization agent followed by 3 x 15 minutes washes at 300

rpm. In addition, samples were removed after each step of the sequential decellularization protocol. ECM material was freeze dried (48 h) and stored at approximately -20°C until further use (i.e. DNA extraction, DMMB assay, Hydroxyproline assay). Detergent decellularization was performed in batches (max. 40 g per 2 L conical flask); three samples were used for analysis (n=3 per condition).

5.3.4 DNA extraction

DNA was extracted using a phenol-chloroform extraction method to enable DNA quantification (Pico green assay) and DNA fragmentation analysis (DNA Gel electrophoresis). One hundred micrograms of lyophilised tissue (n=3 per condition) was used for DNA extraction and resuspended in 1 mL of TE buffer before being stored at approximately -20°C until further use (Pico green assay, DNA gel electrophoresis).

6.3.5 Pico green assay

Following DNA extraction, a Pico green assay was used to quantify DNA content as a marker for decellularization. DNA extracts were thawed at room temperature and vortexed prior to use. The Pico green assay was performed according to the manufacturers instructions. Each standard and sample was assayed in either duplicate or triplicate, which were averaged to obtain a robust single representative value for each sample.

6.3.6 DMMB assay

The DMMB assay was used to quantify sulphated glycosaminoglycan (sGAG) content as a marker for retained ECM scaffold components. Ten micrograms of lyophilised aorta or twenty micrograms of lyophilised liver tissue were assayed (n=3 per condition). The DMMB assay was performed according to manufacturers instructions. Each sample and standard was assayed in duplicate, which were averaged to obtain a single representative value for each sample.

6.3.7 Hydroxyproline assay

The hydroxyproline assay was used to quantify hydroxyproline as a marker of retained ECM scaffold components. Fifteen micrograms of lyophilised tissue (liver or aorta, n=3 per condition) was assayed. The hydroxyproline assay was performed according to manufacturers instructions. Each sample and standard was assayed in duplicate, which was averaged to obtain a single representative value for each sample.

6.3.8 Statistical analysis

Statistical analyses were performed using the statistical software PRISM (Graph Pad). All statistical analyses were conducted using a One-Way ANOVA (post hoc Dunnett's multiple comparison test) or Two-Way ANOVA (post hoc Turkey's multiple comparison test), depending on the number of variables. Results were deemed significant if $p < 0.05$. All data are displayed as mean +/- SEM.

6.4. RESULTS

6.4.1. Effect of single and combined decellularization agents on liver and aorta tissue

Commonly used decellularization protocols involve sequential incubations in decellularization agents. The effects of these agents were therefore assessed both individually (Figure 6.1 A-B) and in combination (sequential incubations, left to right on x-axis; Fig. 5.1 C-D).

Incubating liver tissue in 0.02% Trypsin/ 0.05% EDTA, but not Triton-x-100 (3%), Sodium deoxycholate (SDC, 4%) or peracetic acid, for 1 hour resulted in a significantly reduced DNA content by 40% relative to a native liver control (60%, $p = 0.0142$, Figure 6.1 A). In contrast, the DNA content of aorta remained unchanged after a 1-hour incubation in all individually tested decellularization agents (Figure 6.1 B). When tissue was incubated sequentially in each decellularization agent, the DNA content of liver and aorta was reduced in a synergistic manner. The further along the decellularization process (left to right on the x-axis) and hence also the longer exposure to decellularization agents, the greater the reduction in DNA resulting in a 94% ($p = 0.0001$) and 51% ($p = 0.0022$) reduction for liver and aorta, respectively (Figure 6.1 C and Figure 6.1D). Taken together, none of the tested decellularization agents, with the exception of Trypsin/EDTA for liver, were sufficient enough alone to significantly reduce the DNA content of either liver or aorta. However, combining multiple decellularization agents sequentially resulted in a synergetic effect to reduce DNA content.

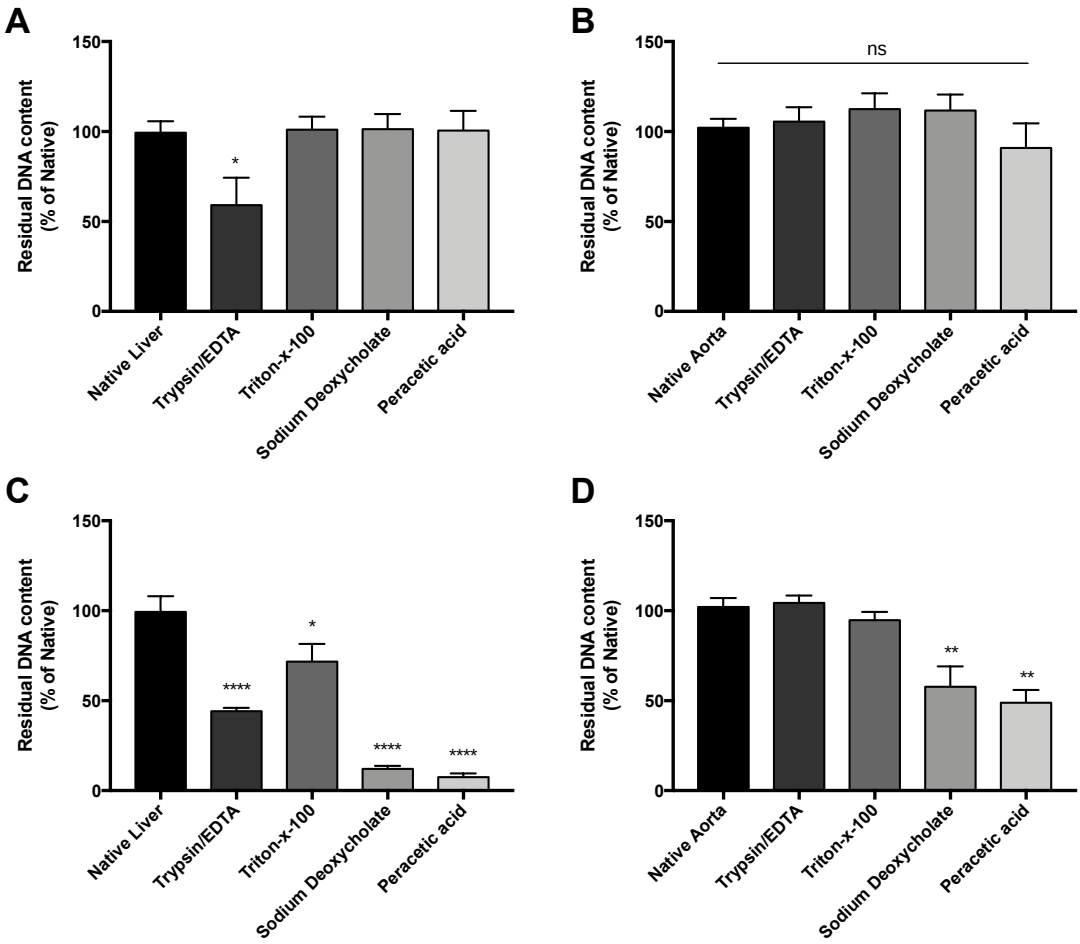


Figure 6.1 Residual DNA content of liver (A, C) and Aorta (B, D) tissue exposed to Trypsin/EDTA, Triton-x-100, Sodium Deoxycholate and Peracetic acid individually (A, B) or when added sequentially (left to right on x-axis; C, D). All data are normalized to tissue weight and analysed using a one-way ANOVA, n=3. All data are mean +/- SEM. * indicates significance level to the native tissue control (* $p < 0.05$, ** $p < 0.005$, **** $p < 0.0001$)

6.4.2. Solubility testing in $scCO_2$

The solubility of Trypsin/EDTA, Triton-x-100, SDC and LS-54 in CO_2 can be seen in the representative view cell images above (left to right, Figure 6.2). As pressure and temperature were increased, the liquid CO_2 rises above the level of decellularization agent within the glass vessel (Figure 6.2). At supercritical conditions (~1071 psi, 32°C; 2nd row in Figure 6.2) SDC, but none of the other modifiers, transitioned into white, dense foam. When left at working conditions (~2900 psi, 37°C; 3rd row in Figure 6.2), SDC remained in the foam state and LS-54 went slightly cloudy. The consistency of Trypsin/EDTA and Triton-x-100 remained unchanged, however, a concaved surface tension was apparent (3rd row in Figure 6.2). At the critical point during depressurization, two-phases were visible with all tested decellularization agents, indicating partial solubility as the CO_2 escapes from the liquid decellularization agent. The lower phase was the residual decellularization agent and the upper phase likely consisted of CO_2 that transitioned out of the supercritical phase (4th row in Figure 6.2). Once fully depressurized Triton-x-100 and SDC were in the foam state whereas Trypsin/EDTA and LS-54 were a cloudy liquid. The penetration of $scCO_2$ throughout the decellularization agent carbonated all modifiers resulting in a formation of bubbles of all decellularization agents tested, confirming partial solubility (5th row in Figure 6.2 above).

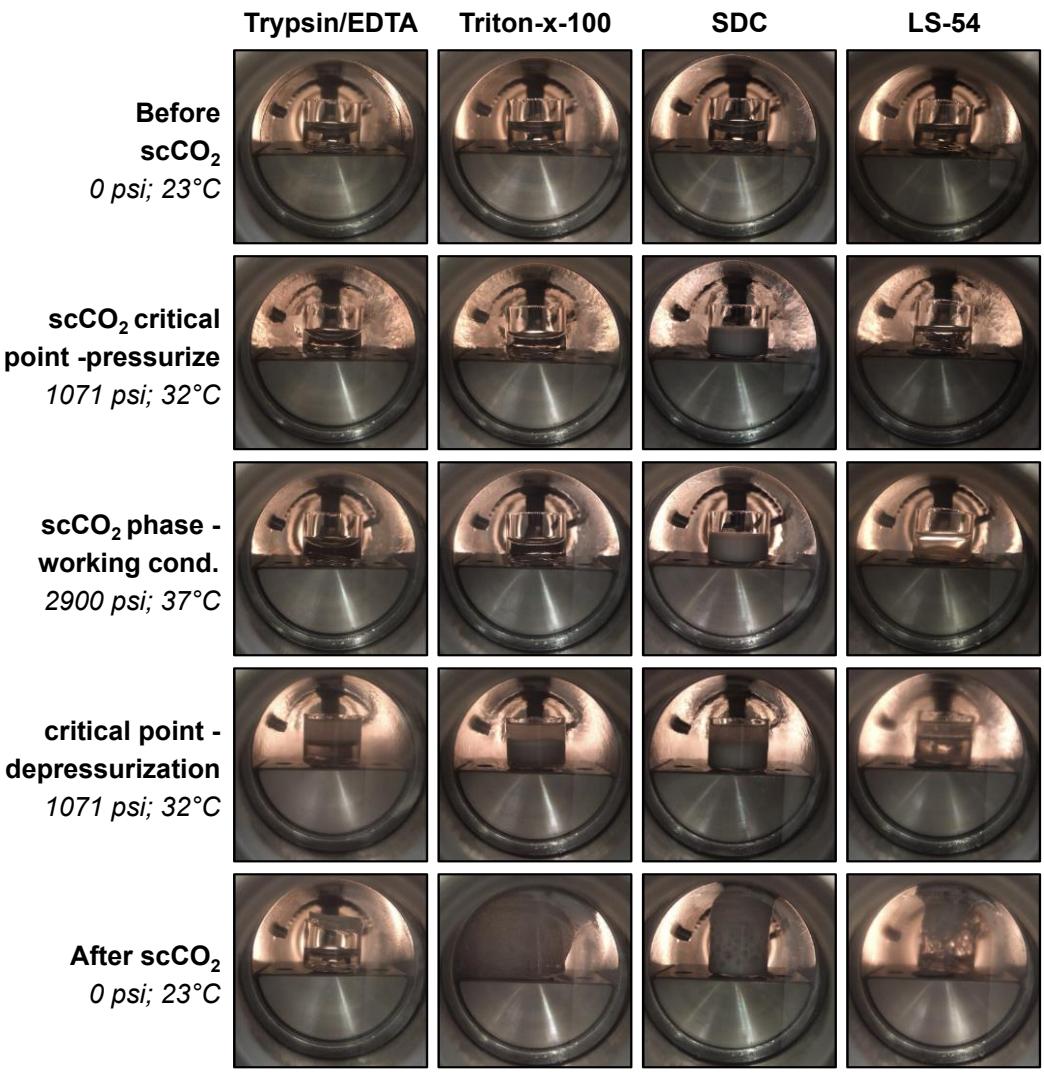


Figure 6.2 Representative view cell images of the solubility of Trypsin/EDTA, Triton-x-100, SDC, LS-54 (left to right) in $scCO_2$, followed by depressurization of the high-pressure system.

6.4.3. Effect of $scCO_2$ in combination with decellularization agents on liver tissue decellularization

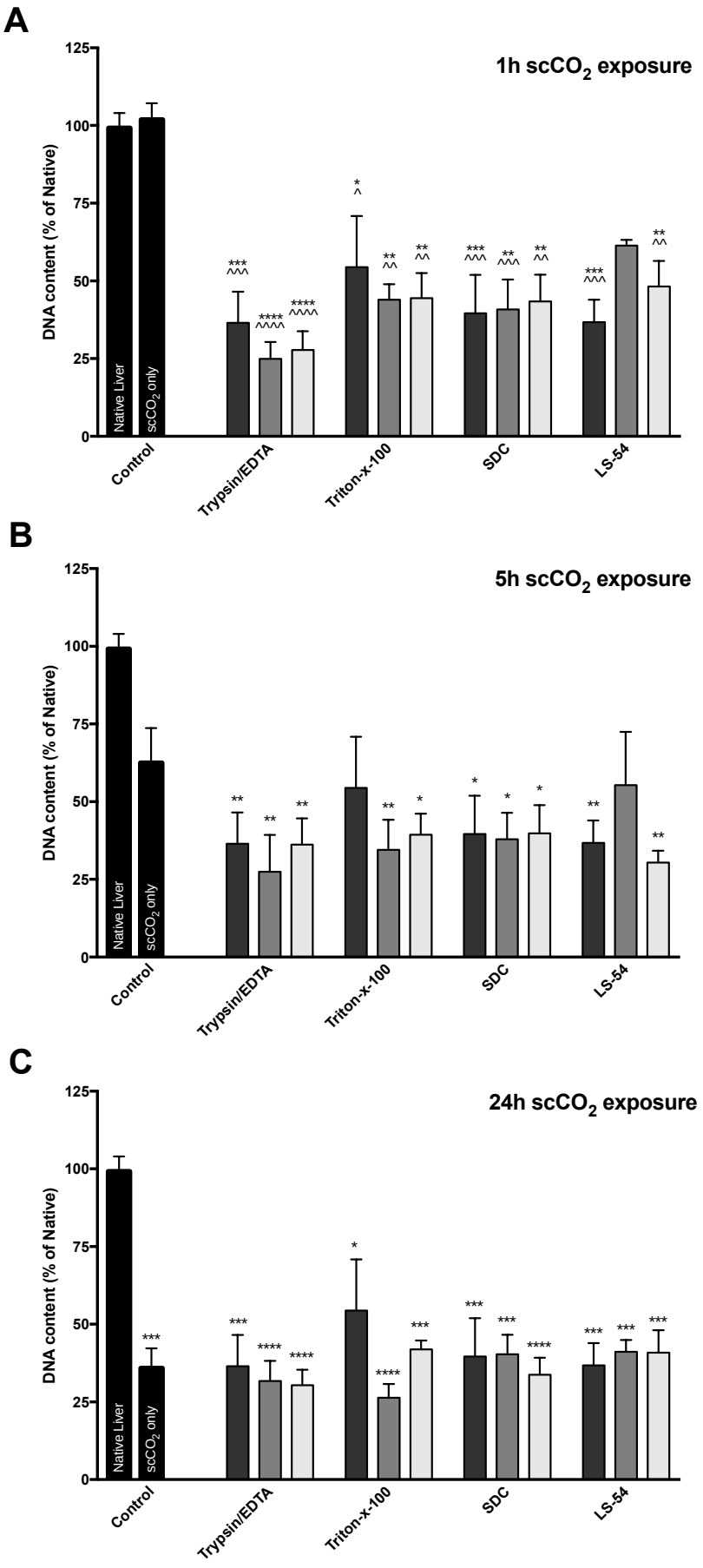
It was investigated if a hybrid approach that combined $scCO_2$ decellularization with a short duration to a modifying agents (Trypsin/EDTA, Triton-X-100, SDC or LS-54) facilitated cell removal on liver tissue compared to $scCO_2$ alone.

Liver tissue exposed to $scCO_2$ displayed a significant reduction in DNA content after 24 h (37%, $p = 0.0001$), 48 h (25%, $p < 0.0001$) and 72 h (26%, $p < 0.0001$), but not 1 h or 5 h, compared to a native liver tissue control (Figure 6.3 A-E).

Incubation of liver tissues in decellularization agents (Trypsin/EDTA, Triton-X-100, SDC or LS-54) for 1 hour reduced DNA content on average by 45-60%. This reduction in DNA content by incubating in decellularization agents was therefore comparable to that produced by 24 hours exposure to $scCO_2$ alone (63% reduction; Figure 6.3 C).

When tissue was exposed to increasing durations of $scCO_2$ exposure prior to incubation in decellularization agents for 1 hour, there was no additive effect on DNA content reduction compared to the same duration of $scCO_2$ exposure only for all time points (1-72 hours) and decellularization agents tested (Trypsin/EDTA, Triton-X-100, SDC or LS-54; Figure 6.3 A-E). Similarly, when the order was reversed and liver tissue was first incubated in decellularization agents prior to $scCO_2$ exposure, there was no improvement in DNA reduction compared to $scCO_2$ only (Figure 6.3 A-E). However, it is noteworthy that pre-incubation of the liver tissue in decellularization agents tended to impair the magnitude of DNA reduction when exposed to $scCO_2$ for 48 and 72 hours (Figure 6.3 D-E).

Representative images of liver incubated in each of the decellularization agents for 1 hour and exposed to $scCO_2$ (1-72 hours) are shown in Appendix (A 1.3 & A 1.4).


A
1h scCO2 exposure
DNA content (% of Native)
125
100
75
50
25
0
Native Liver
scCO2 only
Control
Trypsin/EDTA
Triton-x-100
SDC
LS-54
B
5h scCO2 exposure
C
24h scCO2 exposure

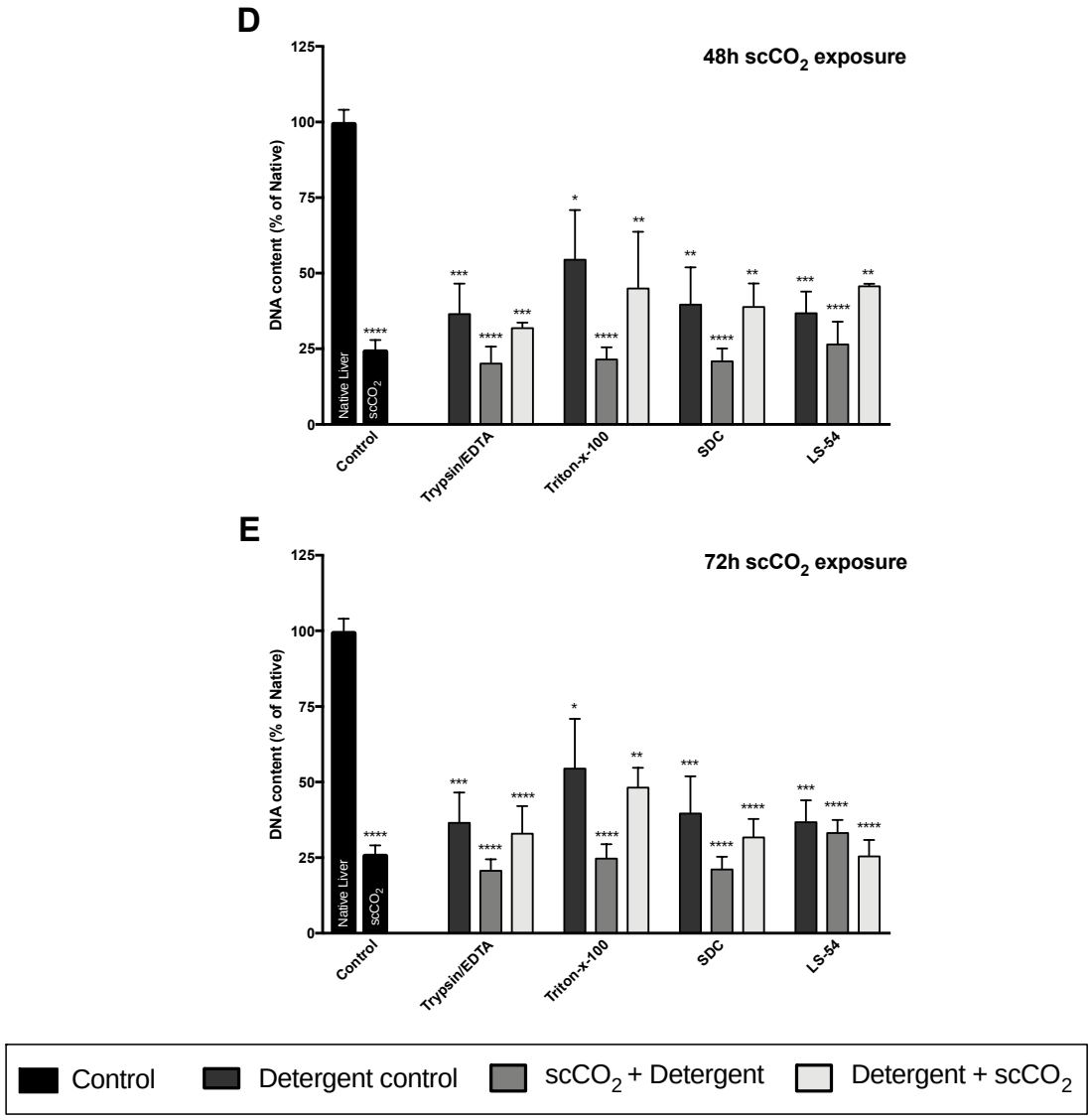


Figure 6.3 Liver tissue exposed to $scCO_2$ (1-72 hours) prior to or following incubation in decellularization agents (A) 1h; (B) 5h; (C) 24h; (D) 48h; (E) 72h. All data are normalized to tissue weight and analysed using a two-way ANOVA, n=3. All data are mean +/- SEM. * indicates significance level to the native tissue control (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p > 0.0001$). ^ Indicates significance level to $scCO_2$ alone (^ $p < 0.05$, ^^ $p < 0.005$, ^^^ $p < 0.0005$, ^^^^ $p > 0.0001$)

6.4.4. Effect of 48 h $scCO_2$ in combination with decellularization agents on liver tissue sGAG and Hydroxyproline content

Glycosaminoglycan (sGAG) and hydroxyproline content was measured as a marker of residual/retained extracellular matrix.

The sGAG content remained unchanged between native liver, $scCO_2$ control (48 h) as well as all decellularization agent-only controls tested (Figure 6.4 A). Interestingly, the combination of decellularization agent pre- or post- $scCO_2$ exposure resulted in a significantly increased sGAG content (Figure 6.4 A). The increase in sGAG content was similar regardless of whether $scCO_2$ exposure was performed pre- or post- decellularization agent treatment (Figure 6.4 A). Because these data were normalised to tissue dry weight, it is likely that the relative proportion of sGAG was increased rather than the total amount. In contrast to the effects on sGAG, the hydroxyproline content remained unchanged throughout all decellularization agent and $scCO_2$ conditions tested (Figure 6.4 B).

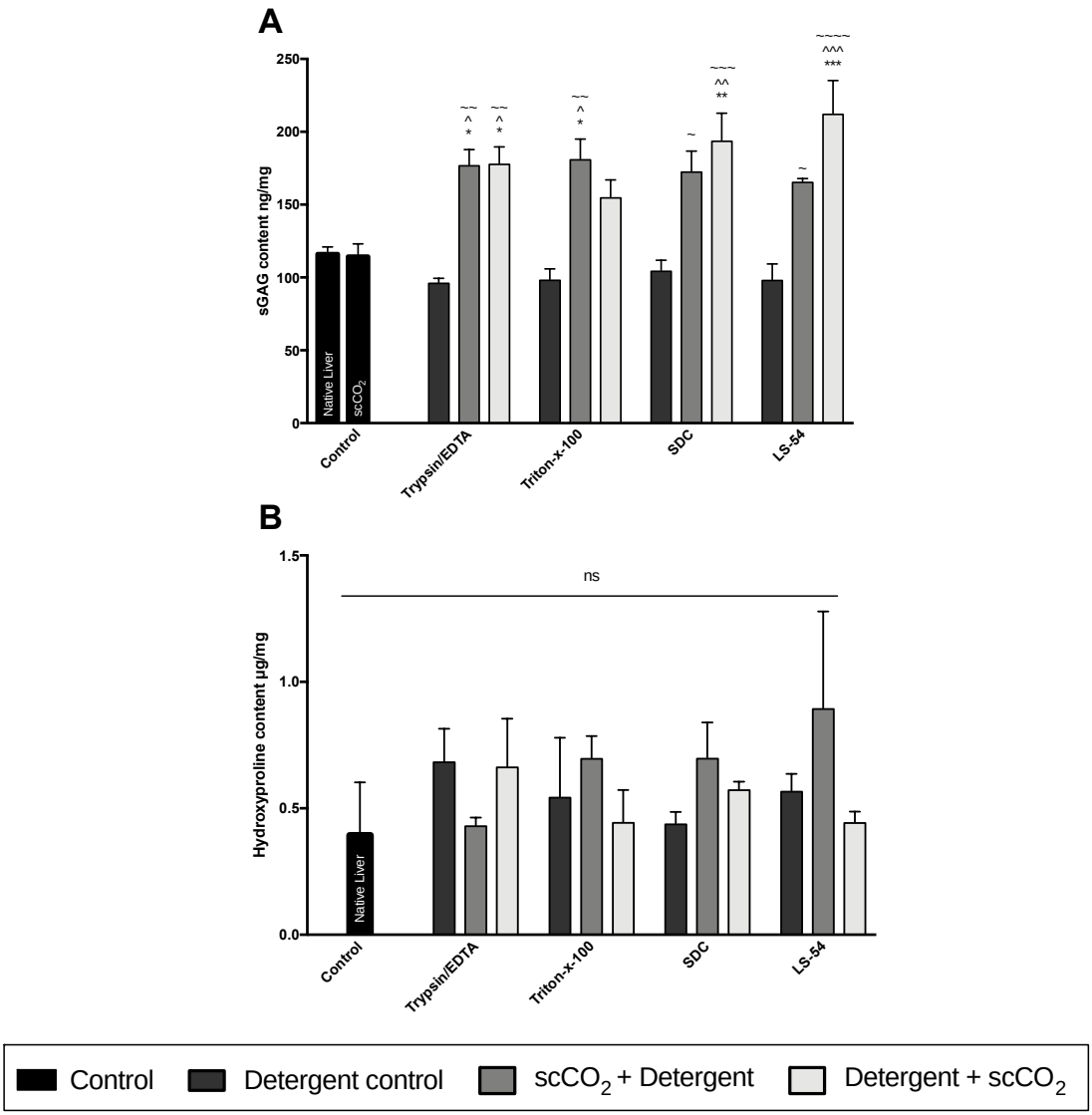


Figure 6.4 Liver tissue exposed to decellularization agents prior to or following $scCO_2$ for 48 hours (A) sGAG content; (B) Hydroxyproline content. No $scCO_2$ only control. All data are normalized to tissue weight and analysed using a two-way ANOVA, n=3. All data are mean +/- SEM. * indicates significance level to the native tissue control (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$). ^ Indicates significance level to $scCO_2$ alone (^ $p < 0.05$, ^^ $p < 0.005$, ^^^ $p < 0.0005$) ~ indicates significance level to decellularization agent control (~ $p < 0.05$, ~~ $p < 0.005$, ~~~ $p < 0.0005$).

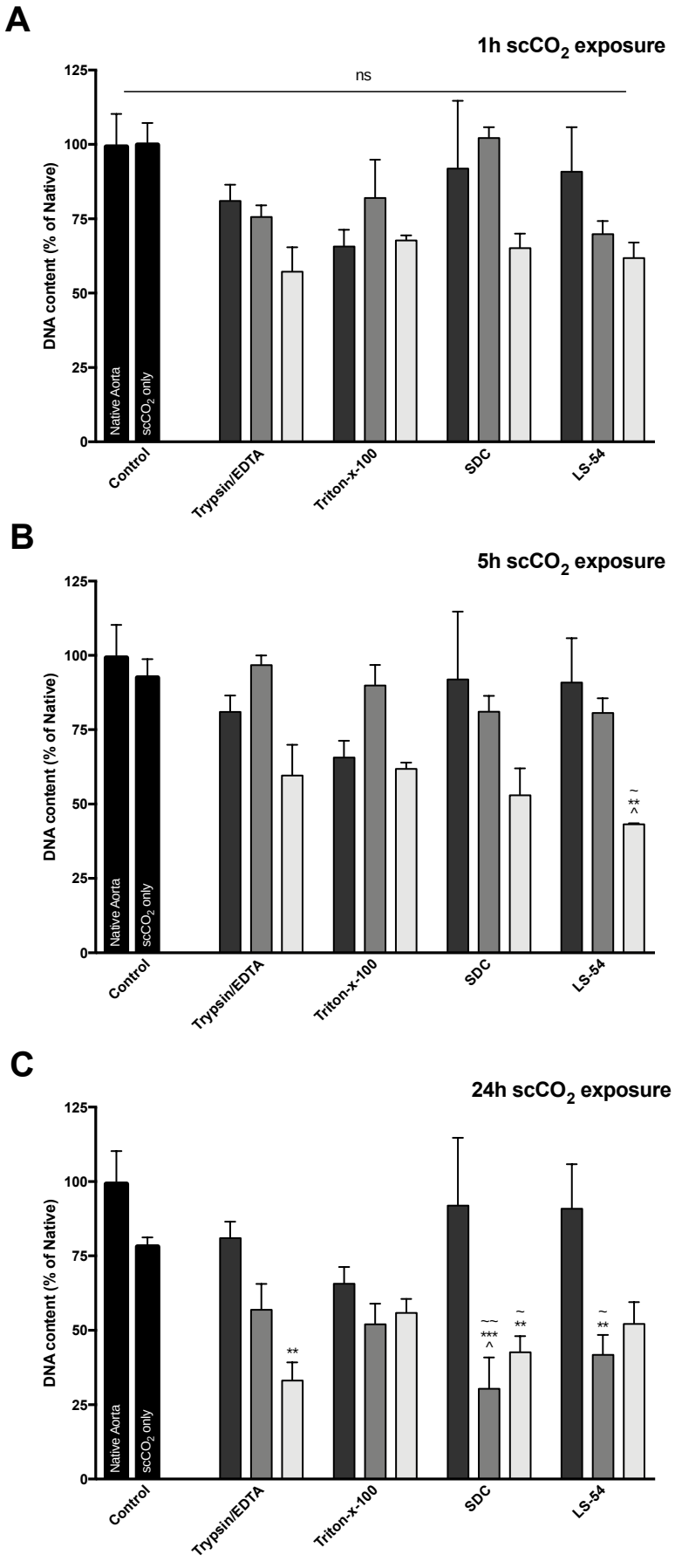
6.4.5. Effect of $scCO_2$ exposure on aorta in combination with decellularization agents

A hybrid approach that combined $scCO_2$ decellularization with a short duration to a modifying agents (Trypsin/EDTA, Triton-X-100, SDC or LS-54) was tested on aorta tissue to enhance cell removal compared to $scCO_2$ alone.

Increasing durations of $scCO_2$ exposure (1-72 hours) reduced DNA content of aorta tissue in a time dependent manner, although this did not reach statistical significance. The average reduction in DNA content was 45% and 48% was reached after 48 h and 72 h $scCO_2$ exposure (respectively, Figure 6.5).

Incubation of aorta tissue in decellularization agents (Trypsin/EDTA, Triton-X-100, SDC or LS-54) for 1 hour did not significantly alter DNA content (Figure 6.5).
When aorta tissue was exposed to increasing durations of $scCO_2$ exposure prior to incubation in decellularization agents for 1 hour, there was an additive effect on DNA content reduction compared to the same duration of $scCO_2$ exposure only (24 – 72 hours) for each decellularization agents tested (Trypsin/EDTA, Triton-X-100, SDC or LS-54; Figure 6.5). Similarly, when the order was reversed and tissue was first incubated in decellularization agents prior to $scCO_2$ exposure, there was a similar improvement in DNA reduction compared to $scCO_2$ only (5 – 72 hours, Figure 6.5). These effects were most pronounced with 72 hours $scCO_2$ exposure, suggesting that a combined hybrid $scCO_2$ method was more effective at decellularizing aorta compared to $scCO_2$ only. This is in contrast to the effects seen with liver tissue, whereby $scCO_2$ decellularization was not improved by the addition of decellularization agents.

Representative images of aorta incubated in each of the decellularization agents for 1 hour and exposed to $scCO_2$ (1-72 hours) are shown in Appendix (A 1.5 & A 1.6).


A
1h scCO2 exposure
ns
DNA content (% of Native)
125
100
75
50
25
0
Native Aorta
scCO2 only
Control
Trypsin/EDTA
Triton-x-100
SDC
LS-54
B
5h scCO2 exposure
DNA content (% of Native)
~
**
^
C
24h scCO2 exposure
DNA content (% of Native)
**
~~
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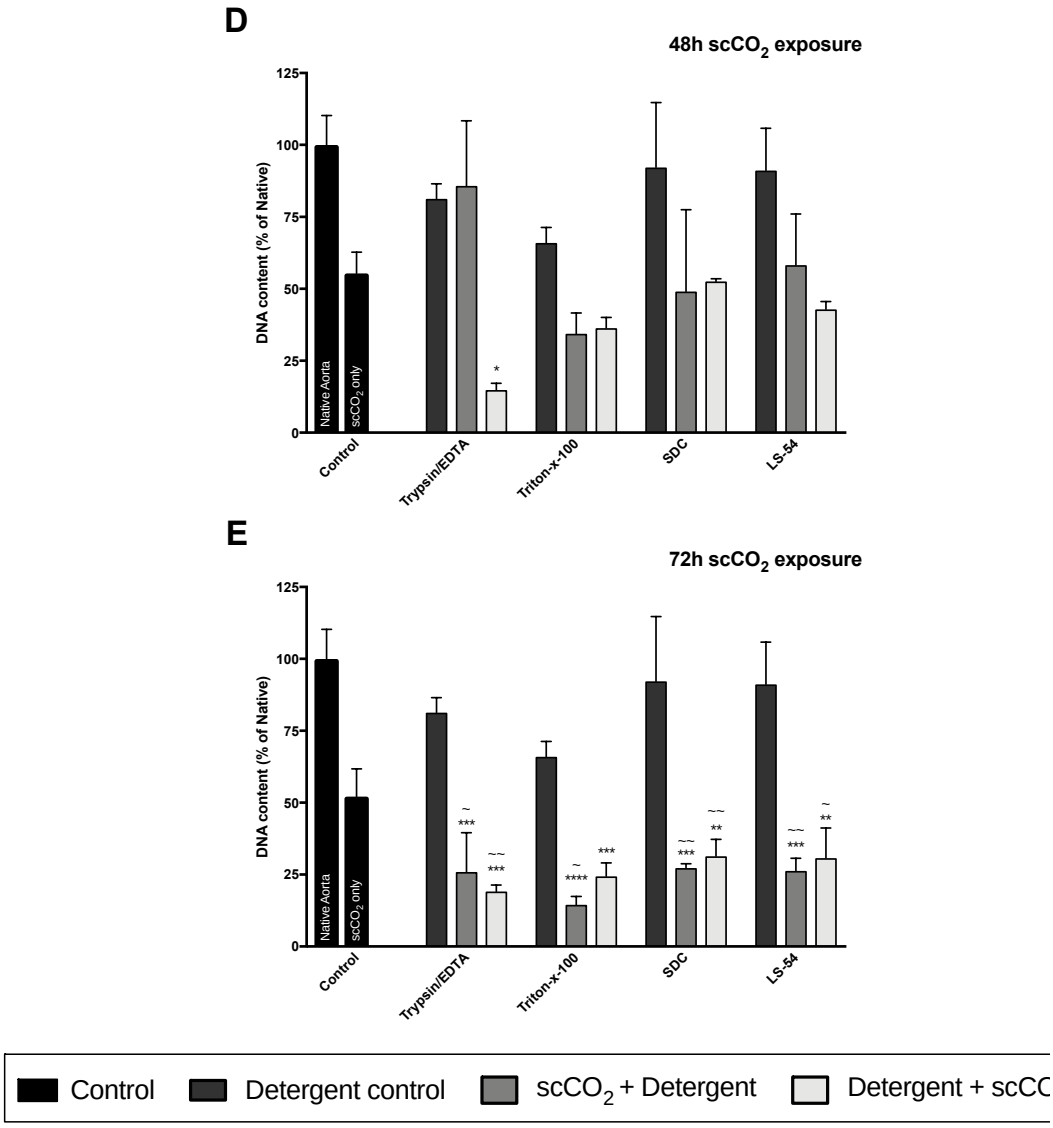

Figure 6.5 Aorta tissue exposed to decellularization agents for 1 hour prior to or following $scCO_2$ (1-72 hours) (A) 1h; (B) 5h; (C) 24h; (D) 48h; (E) 72h. All data are normalized to tissue weight and analysed using a two-way ANOVA, n=3. All data are mean +/- SEM. * indicates significance level to the native tissue control (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p > 0.0001$). ^ Indicates significance level to $scCO_2$ alone (^ $p < 0.05$). ~ Indicates significance level to decellularization agent only control (~ $p < 0.05$, ~~ $p < 0.005$)

6.4.6. Effect of 72 h $scCO_2$ in combination with decellularization agents on aorta tissue sGAG and Hydroxyproline content

Glycosaminoglycan and hydroxyproline content were measured as markers for retained ECM components.

Glycosaminoglycan (sGAG, except for $scCO_2$ + Trypsin/EDTA) and hydroxyproline content was not different between native aorta and aorta exposed to $scCO_2$ for 72 h or in combination with decellularization agents (Figure 6.6 A).

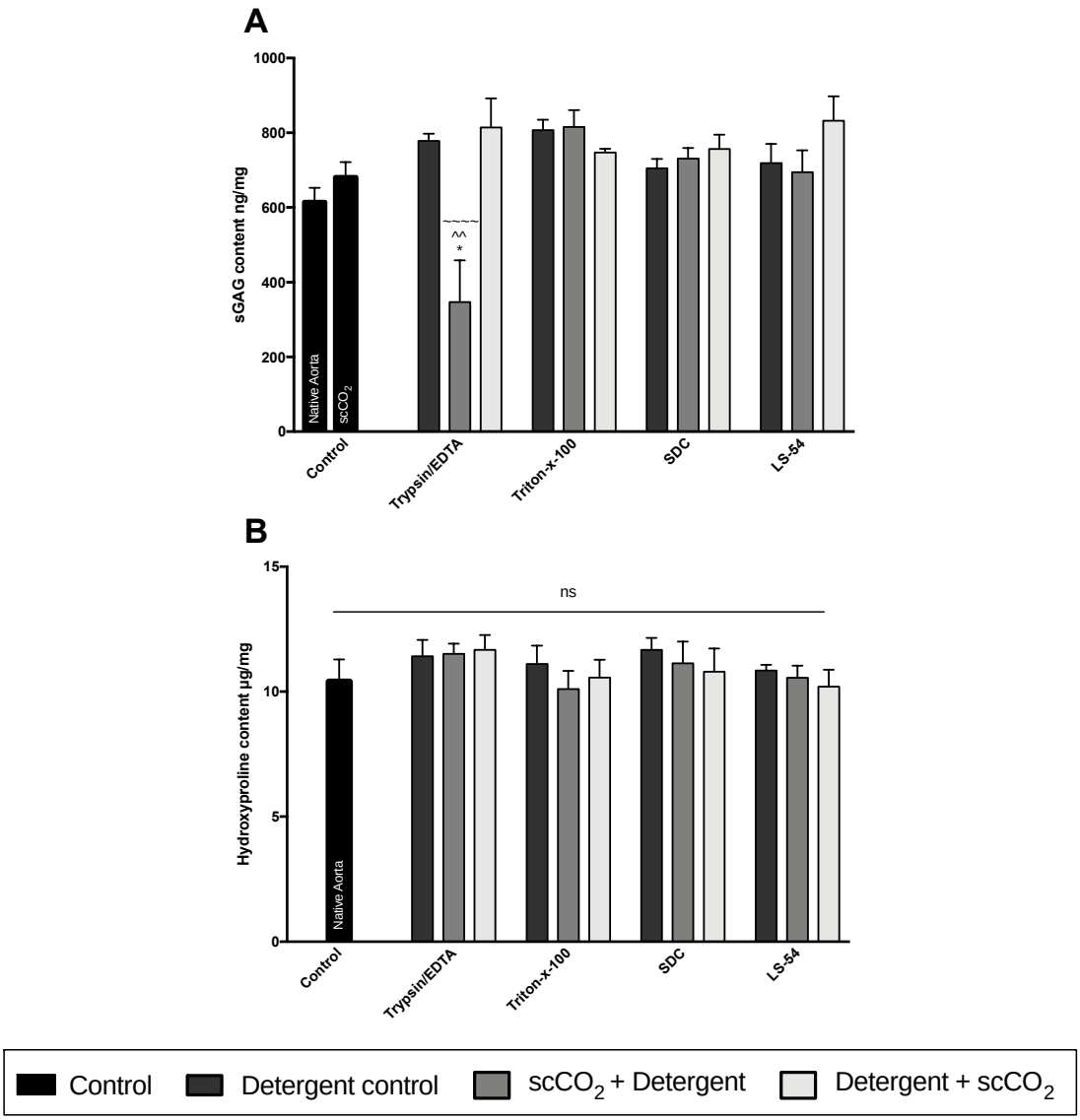

Figure 6.6 Aorta tissue exposed to decellularization agents for 1-hour prior to or following $scCO_2$ for 72 hours (A) sGAG content; (B) Hydroxyproline content. All data are normalized to tissue weight and analysed using a two-way ANOVA, n=3. All data are mean +/- SEM. * indicates significance level to the native tissue control (* $p < 0.05$). ^ Indicates significance level to $scCO_2$ alone (^^ $p < 0.005$) ~ indicates significance level to decellularization agent control (~~~~ $p < 0.0005$).

6.5. DISCUSSION

This study investigated the level of DNA reduction produced by a detergent based decellularization technique using multiple decellularization agents in combination as well as individually, which will be discussed in 6.5.1 below. Furthermore, a novel hybrid decellularization agent/ $scCO_2$ methodology was developed and will be discussed in 6.5.2 below.

6.5.1. Selection of decellularization agents

The use of decellularization agents to develop 3D ECM bioscaffolds from donor tissues/organs for biomedical applications is recognised worldwide (Smart *et al.*, 2014). Herein, a detergent based decellularization technique (used as a comparator throughout this thesis) utilized a combination of 3 decellularization agents (Trypsin/EDTA, Trition-x-100, SDC) under constant agitation to reduce DNA content. This effect was dependent on the additive/synergistic effect of each of the decellularization agents used sequentially, as none of the decellularization agents were able to significantly reduce DNA content alone. This is in agreement with published data by Prasertsung *et al.*, 2008; Nakayama *et al.*, 2010; Wang *et al.*, 2015 who all confirmed the incomplete removal of cells using Trypsin/EDTA, Trition-x-100 and SDC (respectively) individually. However, other studies have shown complete removal utilising Trypsin/EDTA (Meyer *et al.*, 2006), Triton-x-100 (Shafiq *et al.*, 2012) and SDC (Poornejad *et al.*, 2016). This is likely due to the decellularization agent concentration and exposure time used, which varied from 0.05% - 1% and 24 h – 48 h depending the on decellularization agent used.

In this thesis, a 1 hour exposure was chosen to minimize exposure to the decellularization agents and rather support/facilitate decellularization by $scCO_2$ (discussed below). Hence, the duration used here was relatively short in comparison to those used by others and could explain the difference in results. One hour of exposure was specifically chosen based on the previously established decellularization protocol (Loneker *et al.*, 2016) that combined the exposure to 3 decellularization agents for 1 hour each. In this thesis, the combination of the three decellularization agents (1 hour each) achieved a 95% and 67% reduction in DNA content on liver and aorta (respectively). In addition to liver, this protocol has been

shown to be successful for the decellularization of heart (Wainwright *et al.*, 2010), spinal cord and brain tissue (Crapo *et al.*, 2012) and the resulting decellularized ECM scaffold has been used for various clinical applications such as acute vocal fold repair (Gilbert *et al.*, 2009) and neurological recovery after trauma (Wu *et al.*, 2016).

Despite excellent efficacy, many decellularization agents cause unavoidable damage to the ECM structure and architecture (Meyer *et al.*, 2006; Nakayama *et al.*, 2010; Du *et al.*, 2011) and leave residual toxicity of the decellularization agents within the tissue (White *et al.*, 2016). The presence of residual decellularization agents and any associated cytoxicity within ECM scaffolds might make reseeding and integration of the scaffold for clinical application challenging. It is crucial to find the optimal balance between cell removal and damage to the ECM to ease *in vivo* reseeding and facilitate cell migration and differentiation to repair tissues and eventually fully replace the tissue scaffold with functional tissue (Rickey *et al.*, 2000).

Overall, this study suggests that a detergent based decellularization method requires sequential short exposures to a combination of decellularization agents to achieve complete cell removal. It was therefore hypothesised that the addition of a short exposure to a decellularization agent prior to or post $scCO_2$ would have an additive/synergistic effect on cell removal compared to $scCO_2$ only.

6.5.2. Decellularization agents in combination with $scCO_2$

$ScCO_2$ has previously been utilised in combination with decellularization agents to decellularize mammalian tissue (Antons *et al.*, 2018; Casali *et al.*, 2018). It was therefore hypothesised that the addition of decellularization agents to $scCO_2$ would enhance the decellularization efficacy compared to $scCO_2$ alone, based on the effectiveness of the decellularization agents to remove cellular material (Meyer *et al.*, 2006; Shafiq *et al.*, 2012; Poornejad *et al.*, 2016). Moreover, it was investigated whether the sequence in which decellularization agents were added to the $scCO_2$ (i.e. pre- or post $scCO_2$ exposure) affected the extent of cell removal.

The exposure of liver to $scCO_2$ (48 h or 72 h) followed by a decellularization agent (Trypsin/EDTA, Trition-x-100, SDC) had the same effect on cell removal as $scCO_2$

only. However, when a decellularization agent (Trypsin/EDTA, Trition-x-100, SDC) was added prior to $scCO_2$ (48 h, 72 h) the reduction in DNA content was inhibited compared to the effect of $scCO_2$ only. The mechanism behind this is not clearly defined. One potential explanation is that the addition of decellularization agents prior to $scCO_2$ exposure disrupted the cell membranes in the tissue and released water content, leading to reduced moisture content within the tissue. This could be important because in Chapter 5 it was shown that removal of water content by lyophilisation prior to $scCO_2$ exposure inhibited the effectiveness of $scCO_2$ to reduce DNA content. In addition, prior exposure to detergents might have lysed cells open, releasing DNA, which when uncoiled would takes up a large relative area and could coat the outside of the tissue making it harder for $scCO_2$ to penetrate or further cellular material to be released. Interestingly, this effect did not occur on aorta tissue. This would be in agreement with both mechanisms suggested before, as decellularization of aorta overall was less efficient, indicating that less cell membranes have been disrupted by the decellularization agent prior $scCO_2$ exposure.

In contrast to the effects seen on liver, the addition of decellularization agents to aorta, independent of the sequence added, tended to reduce DNA content compared to $scCO_2$ only. It is difficult to compare the results presented herein with those of previously published experiments using $scCO_2$ in combination with decellularization agents because the experimental set up and design of the studies differs. To date, 2 publications have employed $scCO_2$ in combination with decellularization agents for decellularization of tissue (Antons *et al.*, 2018; Casali *et al.*, 2018). Casali *et al.*, 2018 combined a pre-treatment (0.2% EDTA, 1h) with a detergent decellularization treatment (0.1% SDS, 48h) followed by 1 h pre-saturated (water and ethanol) $scCO_2$ exposure (2000 psi, 37°C) using similar temperature and pressure conditions to this study (2900 psi, 37°C) but also completed the detergent treatment with a DNase incubation treatment. DNase is an enzyme that breaks down DNA (Gilpin and Yang, 2017). Whilst the tissue DNA content was reduced, the experimental design did not permit interpretation of whether the reduced DNA content was due to $scCO_2$, the detergent treatment or the DNase *per se*.

Antons *et al.*, 2018 developed a hybrid decellularization technology for cartilage, tendon and skin utilizing a physical or biological pre-treatment (freeze-thaw cycles, trypsin-mediated digestion, osmotic shock) and a pre-soak with a CO_2-philic detergent treatment (2% LS-54, 24 h) followed by 1 h $scCO_2$ treatment (Antons *et al.*, 2018). Whilst Antons *et al.*, 2018 showed a reduced tissue DNA content on all tissues tested (bovine cartilage, human skin, horse tendon) the experimental design did not permit interpretation of whether the reduction in DNA content was due to the CO_2-philic detergent or the physical or biological pre-treatment of the tissue as the study design did not include appropriate controls. In addition, both studies (Antons *et al.*, 2018; Casali *et al.*, 2018) applied their 24 h – 48 h decellularization agent treatment under constant agitation but again, did not account for it in their experimental design with an appropriate control.

In this study, the application of decellularization agents for 1 hour under constant agitation did not impact the DNA content. However, as shown in Chapter 5, constant agitation even in water caused a significant time-dependent reduction on DNA content from 2 hours onwards. Hence, the reduction in DNA content described by Antons *et al.*, 2018 and Casali *et al.*, 2018 could be at least in part caused by the agitation applied to the tissue. The experimental design of both, Casali *et al.*, 2018 and Antons *et al.*, 2018 do not permit conclusive comparisons with the data presented here. Overall, this study suggested that $scCO_2$ decellularization of aorta, but not liver tissue, was improved by the addition of decellularization agents pre or post $scCO_2$ exposure.

6.6. CONCLUSION

A novel decellularization method for aorta was developed utilising a short incubation in decellularization agent (Trypsin/EDTA, Triton-x-100, SDC, LS-54) followed by a 72 h $scCO_2$ exposure. This hybrid decellularization agent/ $scCO_2$ method reduced DNA content by 75%, which was superior to $scCO_2$ or detergent decellularization alone (48% and 51%, respectively). Interestingly, the developed hybrid method was tissue specific (i.e. it was not successful with liver). This highlights the need to test new decellularization methods on multiple tissue types and that tissue specific methods should be developed to maximise the quality and utility of the resultant ECM scaffold. This study also raised some important methodological development questions such as the importance of sequence when combining decellularization agents prior to or following $scCO_2$, because in this study the addition of the decellularization agent to liver tissue prior to 48 – 72 h $scCO_2$ exposure inhibited the effect of $scCO_2$ alone, potentially indicating a decreased permeability of the tissue. Overall, this study suggested that there is scope to improve $scCO_2$ decellularization by the addition of decellularization agents depending on the tissue type.

7. *In vitro* cytotoxicity of decellularized ECM bioscaffolds

7.1. OVERVIEW

In this chapter, the aim was to evaluate the cytotoxicity of the previously produced ECM scaffolds. It was hypothesised that the ECM bioscaffolds produced by exposure to $scCO_2$ would be less cytotoxic to HepG2 and 3T3 cells than those produced by exposure to decellularization agents. Furthermore, it was also hypothesised that subsequent $scCO_2$ exposure following treatment with commonly used decellularization agents would reduce the cytotoxicity of ECM bioscaffolds compared to decellularization agents alone.

7.2. INTRODUCTION

To date, there are 3 publications in the area of $scCO_2$ decellularization, which have tested the biocompatibility of the developed scaffolds *in vitro* (Huang *et al.*, 2017; Seo, Jung and Kim, 2017; Wang *et al.*, 2017; Antons *et al.*, 2018). Antons *et al.*, 2018) demonstrated that their novel $scCO_2$ based decellularization methodology (in combination with other agents, enzymatic digestions or physical disruption) produced a biocompatible scaffold (made from bovine cartilage, horse tendon and human skin) that had no impact on cell viability or metabolic activity *in vitro*. Similarly, Wang *et al.*, 2017 also demonstrated the biocompatibility of a decellularized human adipose tissue scaffold produced using $scCO_2$ in combination with ethanol. The authors generated an ECM coating material from the scaffold and demonstrated that cells grown on this material displayed increased proliferation and viability compared to standard tissue culture plates. Neither Huang *et al.*, 2017 or Seo, Jung and Kim, 2017 reported *in vitro* cytotoxicity data for their ECM scaffolds produced using $scCO_2$ based methods but confirmed biocompatibility of rat heart and porcine corneal scaffolds (respectively) by implantation *in vivo* without complications or infections.

The experiments in this chapter aimed to evaluate the cytotoxicity of the previously developed ECM scaffolds (Chapters 4 and 5) generated from porcine liver and porcine aorta tissue that had been exposed to $scCO_2$ alone or in combination with decellularization agents. Direct contact and extract assays were performed using HepG2 (hepatocytes) and 3T3 (fibroblast) cells for liver and aorta, respectively. Direct contact assays involved placing the ECM scaffold directly in contact with the cell monolayer. Extract assays involved incubating the ECM scaffold in culture media (without cells) for 30 or 90 hours before transferring the eluate onto a monolayer of cells. In accordance with the ISO-Guidelines 10993 but not in an ISO accredited manner, an MTT assay for metabolic activity was used to evaluate cytotoxicity of the ECM scaffolds (see methods chapter for details).

Subsequently, the effect of applying $scCO_2$ to liver and aorta tissue following incubation in decellularization agents was investigated, in order to determine whether this improved/reduced cytotoxicity associated with those decellularization agents. The rationale underpinning this research question was that the solvation capability of $scCO_2$ would promote clearance of CO_2 soluble decellularization agents, such as LS-54. This has previously been demonstrated by Antons *et al.*, 2018 and Casali *et al.*, 2018 who both utilized a 1h $scCO_2$ exposure within their decellularization methodology as a rinsing/washing step to remove residual toxicity of the decellularization agent used rather than utilizing $scCO_2$ for the decellularization process *per se*.

7.3. MATERIALS & METHODS

A detailed and comprehensive explanation of the materials and methods used here can be found in the materials and methods chapter (see Chapter 4). The following experimental design and techniques were used:

7.3.1 Supercritical carbon dioxide decellularization

Pre-treatment of liver and aorta tissue with a range of decellularization agents was performed prior to or following $scCO_2$ exposure to assess the combined impact on biocompatibility. Briefly, biological tissue (liver or aorta, ~1 g) was placed in 20 mL of either 0.02% Trypsin/0.05% EDTA, 3% Triton-x-100, 4% Sodium Deoxycholate and 2% LS-54 for a duration of 1 h at 300 rpm. For the $scCO_2$ exposure, each biological sample (liver or aorta) was placed in the high-pressure autoclave and the system interlocked. The high-pressure autoclave system was pressurised to ~1000 psi and heated to 30°C, before increasing to ~2600 psi and a temperature of 37°C. The temperature was maintained at 37°C +/- 3°C. After the desired $scCO_2$ exposure duration, the system was vented to ambient pressure within 1 minute.

Following decellularization, tissue samples were stored at approximately -20°C until further use (i.e. direct contact assay, extract assay).

7.3.2 Detergent Decellularization

Detergent based decellularization of biological tissue (liver or aorta) was performed following an adapted protocol by Loneker *et al.,* 2016 to gain an understanding of the biocompatibility of ECM scaffolds produced by detergent decellularization. ECM material was stored at approximately -20°C until use. A single sample was used for an extract assay (n=1).

7.3.3 Culture conditions for HepG2 & NIH/3T3 cells

HepG2 and 3T3 cells were used for *in vitro* studies of liver and aorta ECM bioscaffolds, respectively. Cell lines were cultured in alpha-MEM supplemented with 1% NEAA; 10% FBS; 1% L-Glutamine; 1% Penicillin/Streptomycin and 0.1% Gentamicin within standard culture conditions (37°C, 21% O_2, 5% CO_2 and 85% relative humidity). Culture media was renewed every 2-3 days.

7.3.4 Passaging HepG2 & NIH/3T3 immortalised cell lines

Cell lines (HepG2 and NIH/3T3) were grown in T75 using alpha-MEM (described above) until 85-90% confluence. Culture medium was then removed and cells gently washed with 10 mL of warm (37°C) PBS. Cells were dissociated from the bottom of the T75 flask by 3 minutes incubation with 2 mL of warm Trypsin/EDTA (37°C). The flask was then gently tapped to detach cells. Trypsin-EDTA was inactivated by the addition of 8 mL MEM. The cell suspension was centrifuged for 5 minutes at 120 x g and the cell pellet was re-suspended in 10 mL of fresh, pre-warmed MEM medium, followed by a 1:5 dilution before seeding onto a new T75 flask(s).

7.3.5 Seeding 24-well plates with a known density cells

HepG2 and NIH/3T3 cells were seeded at 200,000 and 150,000 cells, respectively, per well of a 24-well tissue culture treated plate (0.5 mL media per well).

7.3.6 Extract assay

An extract assay was performed to assess the *in vitro* cytotoxicity of the materials developed within this thesis. HepG2 and NIH/3T3 cells were used to test cytotoxicity of liver ECM and aorta ECM, respectively. Extraction vehicle was alpha-MEM. Briefly, tissue samples (liver/aorta) were biopsied (5 mm) immediately after $scCO_2$ +/- decellularization agent treatment and frozen at -20°C until further processed. Tissue samples (one per condition, n=1) were thawed and washed 3 x 5 minutes in PBS before being placed in 5 mL alpha-MEM for 30 h (37°C) at 300 rpm. After 30 h, half the extraction media (i.e. 2.5 mL) was removed and the tissue sample left in the remaining media (2.5 mL) for a further 60 h (i.e. 90 h in total).

A cell monolayer (HepG2 for liver, NIH/3T3 for aorta) was cultured on treated 24-well plates. Once confluent, MEM culture media was removed and replaced with filter-sterilised eluate MEM for 24 h. Each eluate was tested on three separate cell monolayers (i.e. n=3 wells). The positive control was 5% Triton-x-100. A blank was included containing only alpha MEM (i.e. no cells) and a negative control was used containing standard alpha MEM media on a cell monolayer. Representative images were taken after 24 h exposure to the eluate.

7.3.7 Direct contact assay

A direct contact assay was performed to assess the direct impact of the materials developed in this thesis on HepG2 (liver) and NIH/3T3 (aorta) cells. Briefly, tissue samples (liver/aorta) were biopsied (5 mm) immediately after $scCO_2$ +/- decellularization agent treatment and frozen at -20°C until further processed. Tissue samples (one per condition, n=1) were thawed and washed 3 x 5 minutes in PBS. A confluent cell monolayer (HepG2 liver, NIH/3T3 aorta) was cultured on 24-well plates. Alpha-MEM was removed and a single piece of tissue was placed in each well before fresh culture media was added for 24 h. Each condition was tested with one piece of tissue on a single cell monolayer. Positive and negative controls were the same as in section 4.3.4.5 Extract assay (see above). Representative images were taken after 24 h exposure to the eluate.

7.3.8 MTT cell viability assay

An MTT assay was used to assess cell viability and proliferation after cells were exposed to external factors such as eluate media (see section 4.3.4.5 Extract assay) or tissue samples (see section 4.3.4.6 Direct contact assay). The MTT assay was performed according to manufacturer instructions. Each standard and sample was assayed in duplicate, which were averaged to obtain a robust single representative value for each sample.

7.3.9 Statistical analysis

Statistical analyses were performed using the statistical software PRISM (Graph Pad). All statistical analyses were conducted using a One-Way ANOVA (post hoc Dunnett's multiple comparison test) or Two-Way ANOVA (post hoc Turkey's multiple comparison test), depending on the number of variables. Results were deemed significant if $p < 0.05$. All data are mean +/- SEM.

7.4. RESULTS

7.4.1. Indirect and direct effect of $scCO_2$ exposed liver tissue on HepG2 cells

HepG2 cells were cultured in eluate from $scCO_2$ decellularized liver tissue to investigate cytotoxicity. The positive control (5% Triton-x-100) reduced HepG2 cell survival by approximately 96% in all assays relative to the negative (media only) control (Figure 7.1 A-C; $p < 0.0001$). HepG2 survival (%) was reduced but did not reach statistical significance when cultured in 30 or 90 hour eluate from liver tissue exposed to $scCO_2$ only (5 h, 24 h, 48 h, 72 h) compared to the negative control (media only; Figure 7.1 A-B). In contrast, the 30 hour, but not 90 hour, eluate from native liver tissue significantly reduced cell survival by 45% compared to the negative control ($p = 0.0388$). The 90 hour, but not 30 hour, eluate from detergent decellularized liver tissue also significantly reduced cell survival by 20% compared to the negative control ($p = 0.0280$).

Subsequently, the HepG2 cells were cultured in the presence of $scCO_2$ decellularized liver tissue to determine direct cytotoxicity. HepG2 cells cultured in direct contact with a sample of liver tissue that had been exposed to $scCO_2$ for 5 h, 24 h, 48 h, but not 72 h, showed reduced survival by approximately 50% compared to the negative control (media only; 5 h $scCO_2$ ($p = 0.0131$), 24 h $scCO_2$ ($p = 0.0437$), 48 h $scCO_2$ ($p = 0.0210$; Figure 7.1 C). This effect was similar to that produced by direct contact with the native liver control tissue ($p = 0.0051$ compared to the negative control).

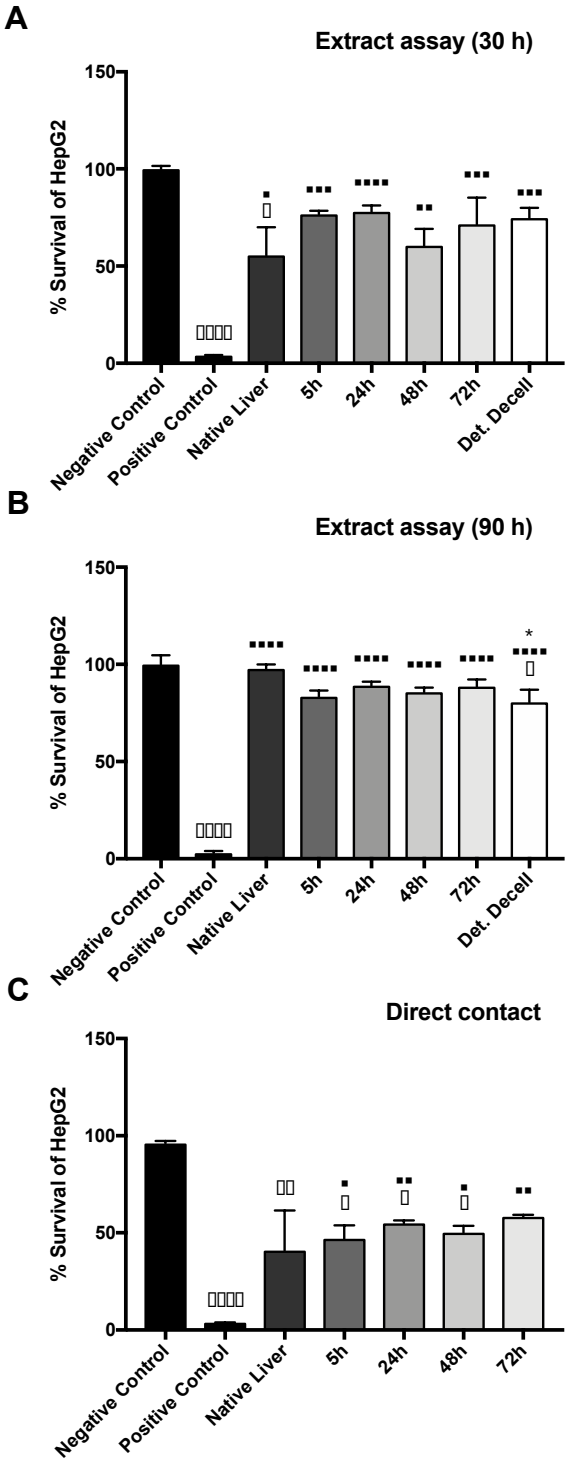

Figure 7.1 Percentage survival of HepG2 cells after exposure to 5 h, 24 h, 48 h and 72 h scCO₂ exposed liver tissue. (A) Extract assay 30 hour eluate; (B) extract assay 90 hour eluate and (C) direct contact assay 30 hours. Data were analysed using a one-way ANOVA, n=3. All data are mean +/- SEM. □ indicates significance level to the negative control (□ $p < 0.05$, □□ $p < 0.005$, □□□□ $p < 0.0001$). ▪ indicates significance level to the positive control (▪ $p < 0.05$, ▪▪ $p < 0.005$, ▪▪▪▪ $p < 0.0001$).* indicates significance level to the native tissue control (* $p < 0.05$)

In standard culture media HepG2 cells grew to form a fully confluent cell monolayer and remained as such throughout the experiment (negative control, Figure 7.2). In contrast, media containing 5% Trition-x-100 (positive control, Figure 7.2) caused cell death represented by circular, yet adherent cells. When native liver tissue was placed onto the HepG2 cell monolayer for 24 h, floating cells were observed. Similarly, liver tissue exposed to $scCO_2$, independent of duration, caused cell detachment when placed onto HepG2 cell monolayer for 24 h (Figure 7.2). These observations were in agreement with the changes observed in cell viability described above (Figure 7.1).

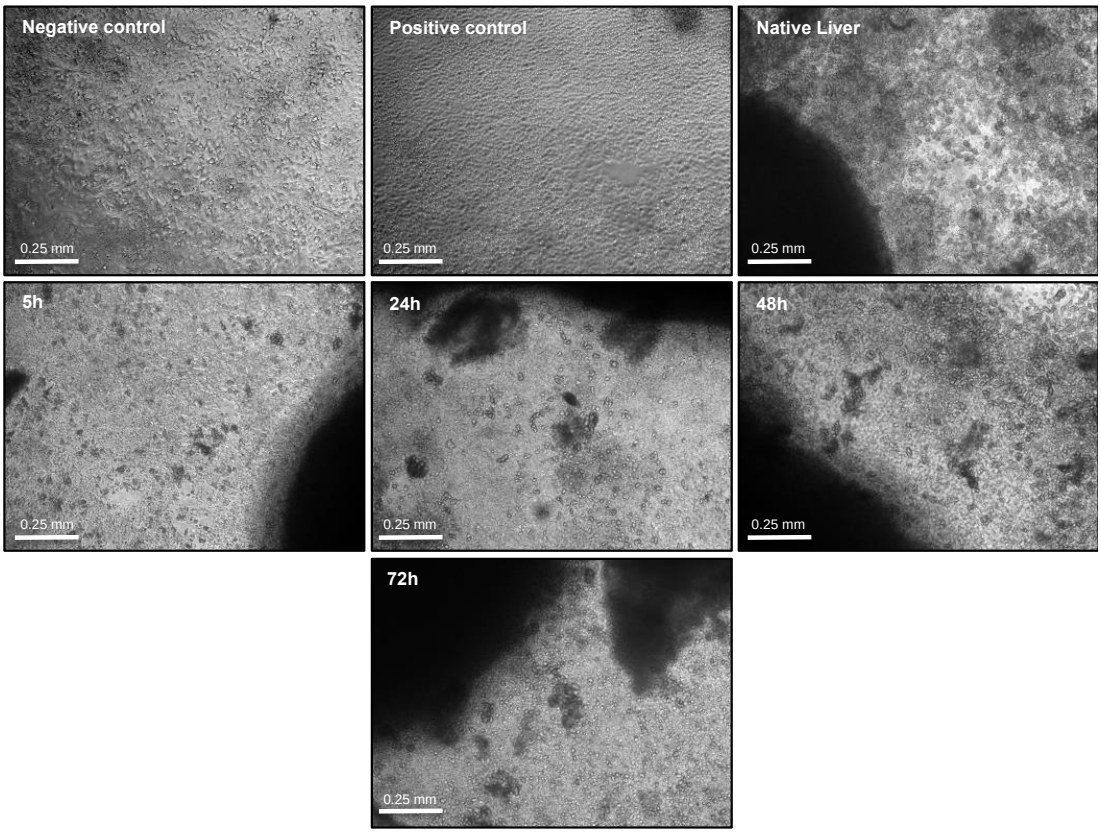

Figure 7.2 Representative phase contrast image of *in vitro* HepG2 cell response to $scCO_2$ decellularized ECM bioscaffold

7.4.2. Indirect and direct cytotoxic effect of liver tissue exposed to $scCO_2$ in combination with decellularization agents

HepG2 cells were cultured in eluate from hybrid decellularized liver tissue (i.e. $scCO_2$ in combination with decellularization agents) to investigate cytotoxicity. The positive control (5% Triton-x-100) reduced HepG2 cell survival by approximately 95% in all assays relative to the negative (media only) control (Figure 7.3 A-C; $p < 0.0001$). A 30 hour eluate from liver tissue exposed to $scCO_2$ + Triton-x-100 ($p < 0.0001$), $scCO_2$ + SDC ($p = 0.0182$), LS-54 only ($p = 0.0095$) and LS-54 + $scCO_2$ ($p < 0.0001$) reduced HepG2 survival (%) by 96%, 21%, 51% and 88%, respectively compared to the negative control (media only), Figure 7.3 A). In contrast, 90 hour eluate from liver tissue exposed to $scCO_2$ + SDC ($p = 0.004$) and LS-54 only ($p = 0.0049$), but none of the other groups tested, reduced HepG2 survival (%) by 26% and 25%, respectively (Figure 7.3 B). Interestingly, the only decellularization agent that caused a cytotoxic response by itself after both 30 h and 90 h was eluate from liver tissue exposed to LS-54 only (Figure 7.3 A, B). The 90 hour, but not 30 hour, eluate from native liver tissue significantly reduced cell survival by 24% compared to the negative control ($p = 0.01$, Figure 7.3 B).

Next, the HepG2 cells were cultured in the presence of hybrid decellularized liver tissue to determine the impact of direct contact. The direct contact assay of HepG2 cells with samples of liver tissue exposed to $scCO_2$ in combination with decellularization agents was conducted with an N=1 (due to time restraints), hence no statistical analysis of these samples was possible. Overall, the data showed a HepG2 cytotoxic effect of all liver samples that had been exposed to $scCO_2$ in combination with decellularization agents. In particular, $scCO_2$ + Trypsin/EDTA, SDC only, LS-54 only and LS-54 + $scCO_2$ (Figure 7.3 C), markedly reduced cell survival. Moreover, Triton-x-100, SDC and LS-54 followed by $scCO_2$ exposure showed a lower survival (%) of HepG2 cells compared to the decellularization agents pre-treated with $scCO_2$ (Figure 7.3 C). The negative, positive as well as native liver tissue control were performed in N=3. Interestingly, the native liver control resulted in a cytotoxic response with a 90% reduced survival of HepG2 cells compared to the negative control (Figure 7.3 C). This is in contrast to the findings of the extract assay (Figure 7.3 A, B).

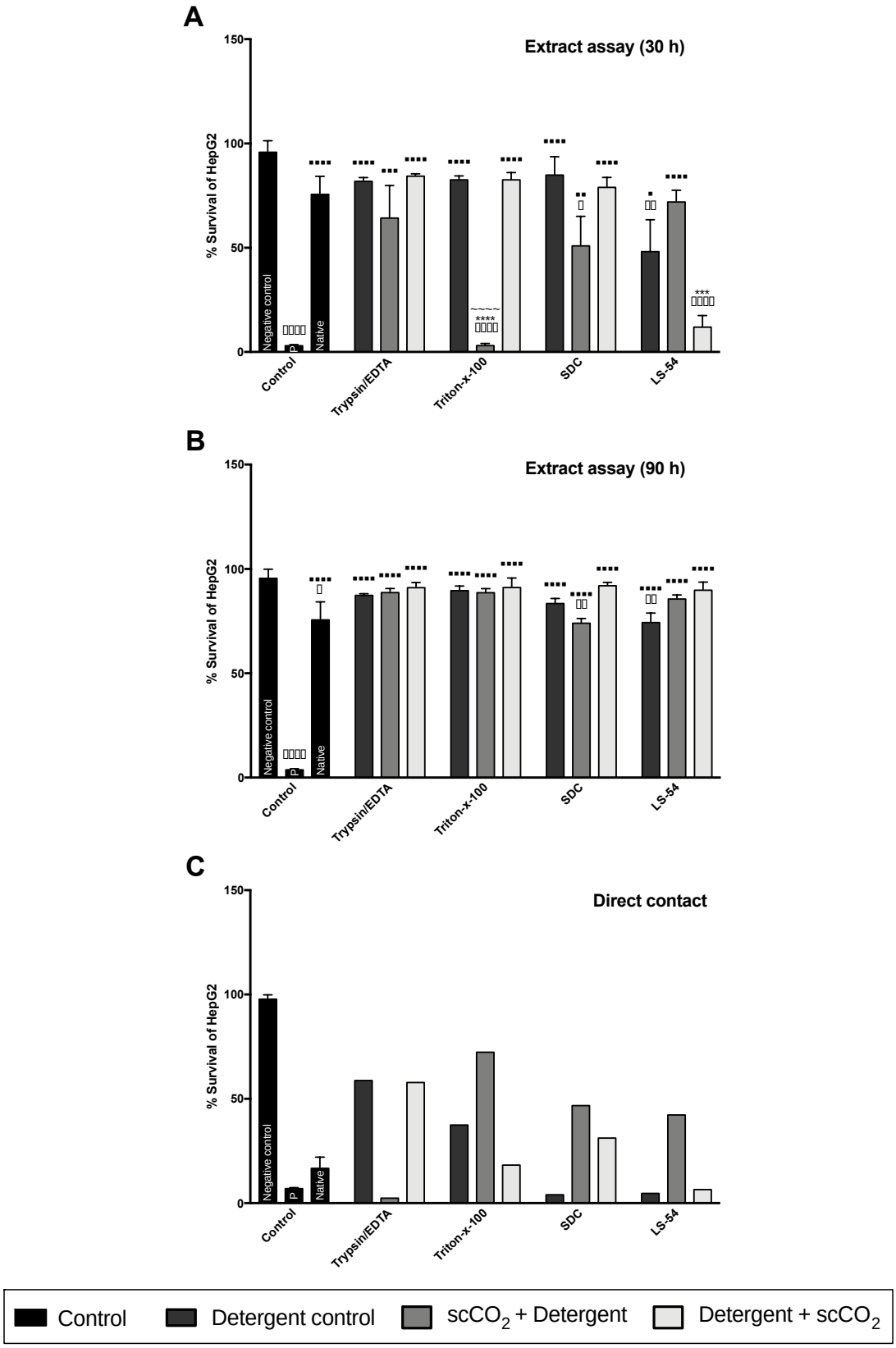

Figure 7.3 Percentage survival of HepG2 cells after 1 hour exposure to Trypsin/EDTA, Triton-x-100, SDC, LS-54 prior to or following 48 hour $scCO_2$ exposed liver tissue. (A) Extract assay 30 hour eluate, (B) extract assay 90 hour eluate and (C) direct contact assay 30 hours. Data

were analysed using a two-way ANOVA, n=3 (except for direct contact assay, n=1; controls n=3). All data are mean +/- SEM. □ indicates significance level to the negative control (□ $p < 0.05$, □□ $p < 0.005$, □□□□ $p > 0.0001$). ▪ indicates significance level to the positive control (▪ $p < 0.05$, ▪▪ $p < 0.005$, ▪▪▪ $p < 0.0005$, ▪▪▪▪ $p > 0.0001$).* indicates significance level to the native tissue control (*** $p < 0.0005$).~ indicates significance level to the detergent control (~~~~ $p > 0.0001$). P = positive control

In standard culture media, HepG2 cells grew to form a fully confluent cell monolayer and remained as such throughout the experiment (negative control, Figure 7.4). In contrast, media containing 5% Trition-x-100 (positive control, Figure 7.4) caused cell death represented by circular, yet adherent cells. Relative to the negative control, which was a confluent cell monolayer, all HepG2 cell monolayers exposed to 24 h of decellularization agent-treated liver tissue showed signs of cell death including areas of cell detachment and floating cells. These effects were most evident in the Trypsin/EDTA + $scCO_2$, $scCO_2$ + Triton-x-100 and SDC only (Figure 7.4).

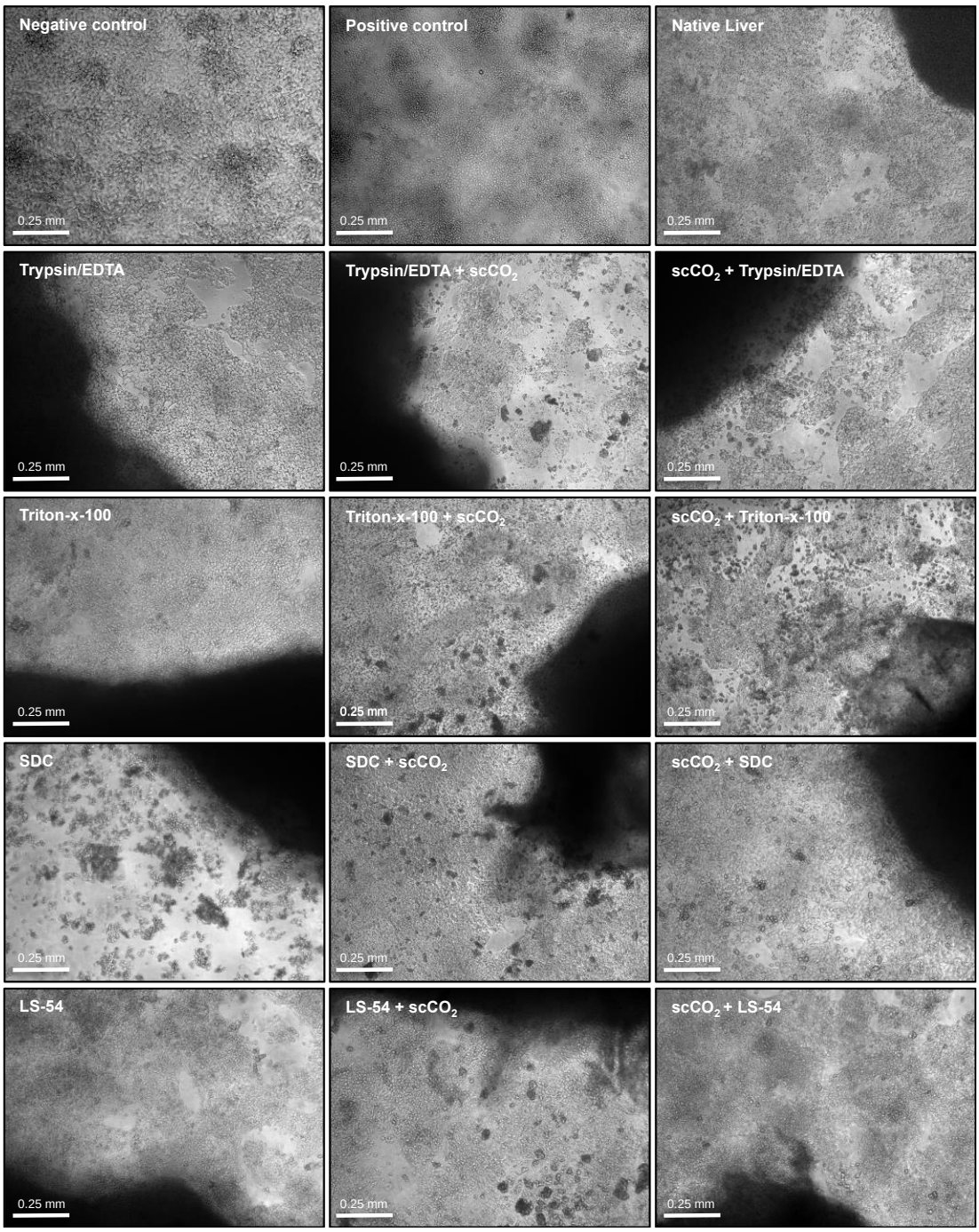

Figure 7.4 Representative phase contrast images of *in vitro* HepG2 cell cytotoxic response to direct contact with decellularized ECM bioscaffold produced by exposure to $scCO_2$ in combination with decellularization agents

7.4.3. Indirect and direct cytotoxic effect of $scCO_2$ and detergent decellularization on 3T3 cells

3T3 cells were cultured in eluate from $scCO_2$ decellularized aorta tissue to investigate cytotoxicity. The positive control (5% Triton-x-100) reduced 3T3 cell survival by approximately 96% in all assays relative to the negative (media only) control (Figure 7.5 A-C; $p < 0.0001$). 3T3 cell survival (%) was unchanged when cultured in 30 or 90 hour eluate from aorta tissue exposed to $scCO_2$ (5 h, 24 h, 48 h, 72 h) compared to the negative control (media only; Figure 7.5 A-B). In contrast, the 30 and 90 hour eluate from detergent decellularized aorta tissue reduced cell survival by 45% and 40% (respectively) compared to the negative control (30 h: $p = 0.0009$), 90 h: $p = 0.0019$)).

Subsequently, the 3T3 cells were cultured in the presence of $scCO_2$ decellularized aorta tissue to determine direct cytotoxicity. 3T3 cells in direct contact with a sample of aorta tissue that had been exposed to $scCO_2$ for 5 h and 24 h, but not 48 h and 72 h, show reduced survival by approximately 60% compared to the negative control (media only; 5 h $scCO_2$ ($p = 0.0177$), 24 h $scCO_2$ ($p = 0.0107$); Figure 7.5 C).

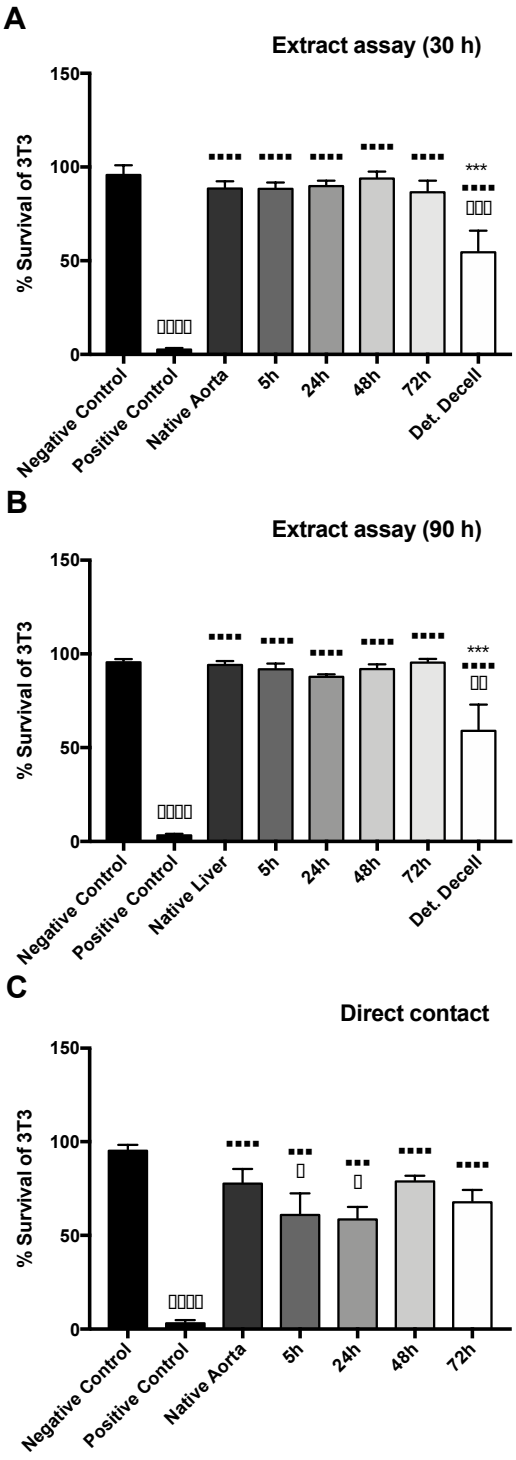

Figure 7.5 Percentage survival of 3T3 cells after exposure to 5 h, 24 h, 48 h and 72 h scCO₂ exposed aorta tissue. (A) Extract assay 30 hour eluate (B) extract assay 90 hour eluate and (C) direct contact assay 30 hours. Data were analysed using a one-way ANOVA, n=3. All data are mean +/- SEM. □ indicates significance level to the negative control (□ $p < 0.05$, □□ $p < 0.005$, □□□ $p < 0.0005$, □□□□ $p > 0.0001$). ▪ indicates significance level to the positive control (▪▪▪ $p < 0.0005$, ▪▪▪▪ $p > 0.0001$).* indicates significance level to the native tissue control (*** $p < 0.0005$)

In standard culture media, 3T3 cells grew to form a fully confluent cell monolayer and remained as such throughout the experiment (negative control, Figure 7.6). In contrast, media containing 5% Trition-x-100 (positive control, Figure 7.6) caused cell death represented by circular, yet adherent cells. When native aorta was placed onto the 3T3 cell monolayer for 30 h, a few circular floating cells were observed and overall the morphology of the 3T3 cells was more spindle like, possibly due to a less dense monolayer or slowed proliferation (native aorta, Figure 7.6). The same observation applied when $scCO_2$ exposed aorta tissue was placed onto a 3T3 cell monolayer for a duration of 30 h. However, culturing 3T3 cells with 5 h and 48 h $scCO_2$ exposed aorta tissue also resulted in areas with no cell attachment. Regardless of the duration of $scCO_2$ exposure, a few circular floating cells were observed when placed onto a 3T3 cell monolayer. (Figure 7.6). These observations were in agreement with the changes observed in cell viability described above (Figure 7.5).

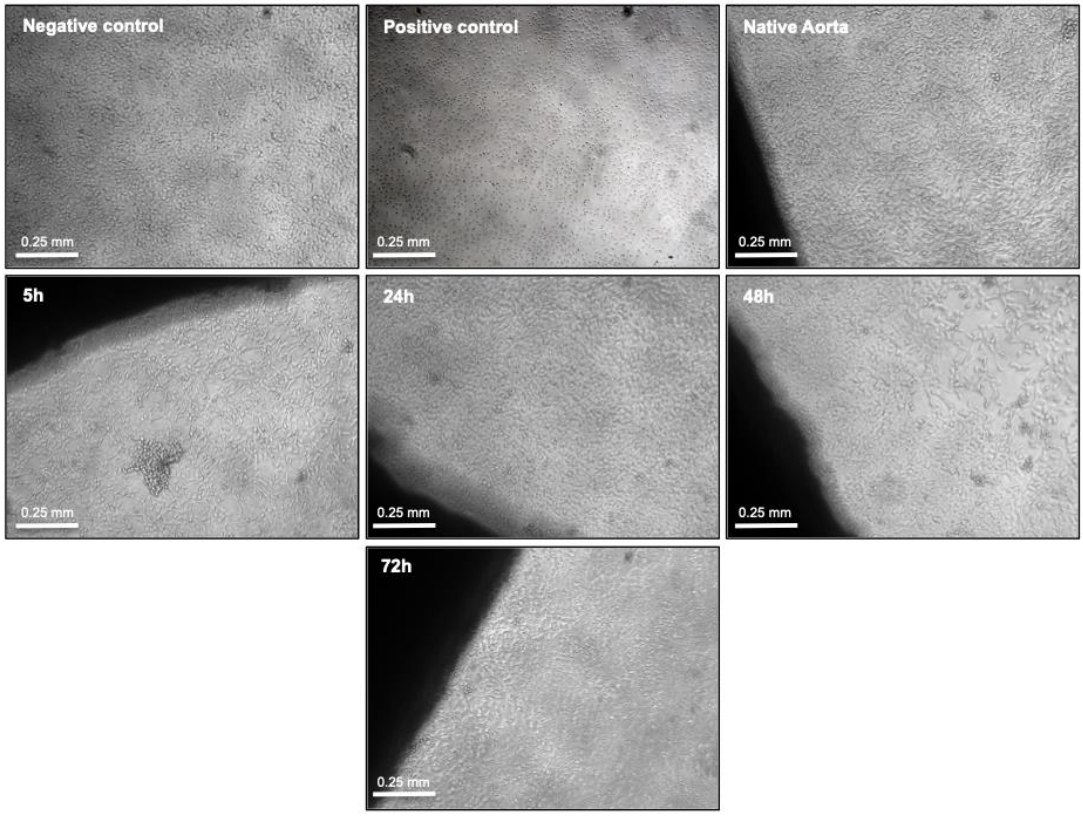

Figure 7.6 Representative phase contrast images of *in vitro* 3T3 cell cytotoxic response to $scCO_2$ decellularized ECM bioscaffold

7.4.4. Indirect and direct cytotoxic effect of $scCO_2$ in combination with decellularization agents on 3T3 cells

3T3 cells were cultured in eluate from hybrid decellularized aorta tissue (i.e. $scCO_2$ in combination with decellularization agents) to investigate cytotoxicity. The positive control (5% Triton-x-100) reduced 3T3 cell survival by approximately 97% in all assays relative to the negative (media only) control (Figure 7.7 A-C; $p < 0.0001$). A 30 hour eluate from aorta tissue exposed to $scCO_2$ + Triton-x-100 ($p = 0.0065$), Triton + $scCO_2$ ($p = 0.0441$), SDC + $scCO_2$ ($p = 0.0032$), LS-54 only ($p = 0.0039$) as well as $scCO_2$ + LS-54 ($p = 0.0083$) and LS-54 + $scCO_2$ ($p = 0.0002$) reduced 3T3 cell survival (%) compared to the negative control (media only; Figure 7.7 A). Aorta tissue exposed to $scCO_2$ prior to and following treatment with Triton-x-100 and SDC reduced 3T3 cell survival (%; Figure 7.7 A). In contrast, 90 hour eluate from aorta tissue exposed to LS-54 (LS-54 only ($p = 0.0339$), LS-54 + $scCO_2$ ($p = 0.0366$) and $scCO_2$ + LS-54 ($p = 0.0002$), but none of the other tested groups, reduced survival on average by 65% compared to the negative control (media only; Figure 7.7 B).

Next, the 3T3 cells were cultured in the presence of hybrid decellularized aorta tissue to determine the impact of direct contact. The direct contact assay of 3T3 cells with samples of aorta tissue exposed to $scCO_2$ in combination with decellularization agents was conducted with an N=1, hence no statistical analysis of these samples was possible. Overall, the data showed a strong 3T3 cell cytotoxic effect of all aorta samples that were exposed to $scCO_2$ in combination with decellularization agents. In particular, the Triton-x-100 groups (Triton-x-100 only, Triton-x-100 + $scCO_2$, $scCO_2$ + Triton-x-100) and LS-54 treated groups (LS-54 only, LS-54 + $scCO_2$, $scCO_2$ + LS-54 Figure 7.7 C) produced a marked decrease in cell survival of on average 88% and 96%, respectively. The negative, positive and native aorta tissue controls were performed as N=3. The native aorta control resulted in a cytotoxic response with a 22% reduced survival (%) of 3T3 cells compared to the negative control ($p = 0.0419$, Figure 7.7 C).

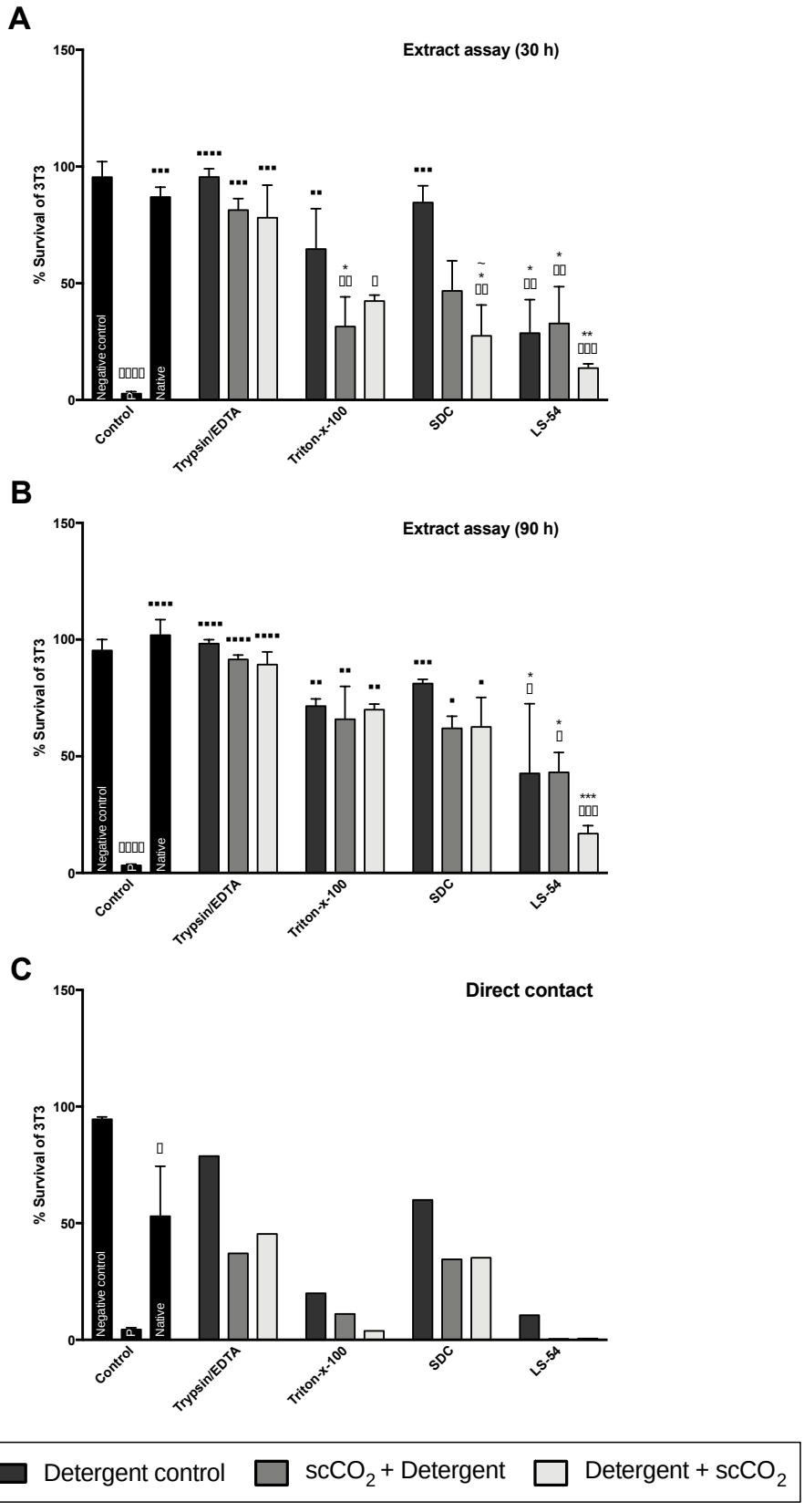

Figure 7.7 Percentage survival of 3T3 cells exposed to decellularized aorta tissue. (A) Extract assay 30 hour eluate (B) extract assay 90 hour eluate (C) direct contact assay 30 hours. Data were analysed using a two-

way ANOVA, n=3 (except for direct contact assay, n=1; controls n=3). All data are mean +/- SEM. □ indicates significance level to the negative control (□ $p < 0.05$, □□ $p < 0.005$, □□□ $p < 0.0005$, □□□□ $p > 0.0001$). ▪ indicates significance level to the positive control (▪ $p < 0.05$, ▪▪ $p < 0.005$, ▪▪▪ $p < 0.0005$, ▪▪▪▪ $p > 0.0001$).* indicates significance level to the native tissue control (*$p < 0.05$, **$p < 0.005$, *** $p < 0.0005$).~ indicates significance level to the detergent control (~ $p > 0.05$)

In standard culture media 3T3 cells grew to form a fully confluent cell monolayer and remained as such throughout the experiment (negative control, Figure 7.8). In contrast, media containing 5% Trition-x-100 (positive control, Figure 7.8) caused cell death represented by circular, yet adherent cells. Overall, there was no visual difference between the pre- or post- decellularization agent $scCO_2$ exposure of 3T3 cells; the level of observed cell density was similar. Relative to the negative control, which was a confluent cell monolayer, all 3T3 cell monolayers exposed to 24 h of decellularization agent treated aorta tissue showed signs of cell death including areas of cell detachment and floating cells. These effects were most evident in the LS-54 treated group (LS-54 only, LS-54 + $scCO_2$, $scCO_2$ + LS-54) (Figure 7.8). These observations were broadly in agreement with the changes observed in cell viability described above (Figure 7.7).

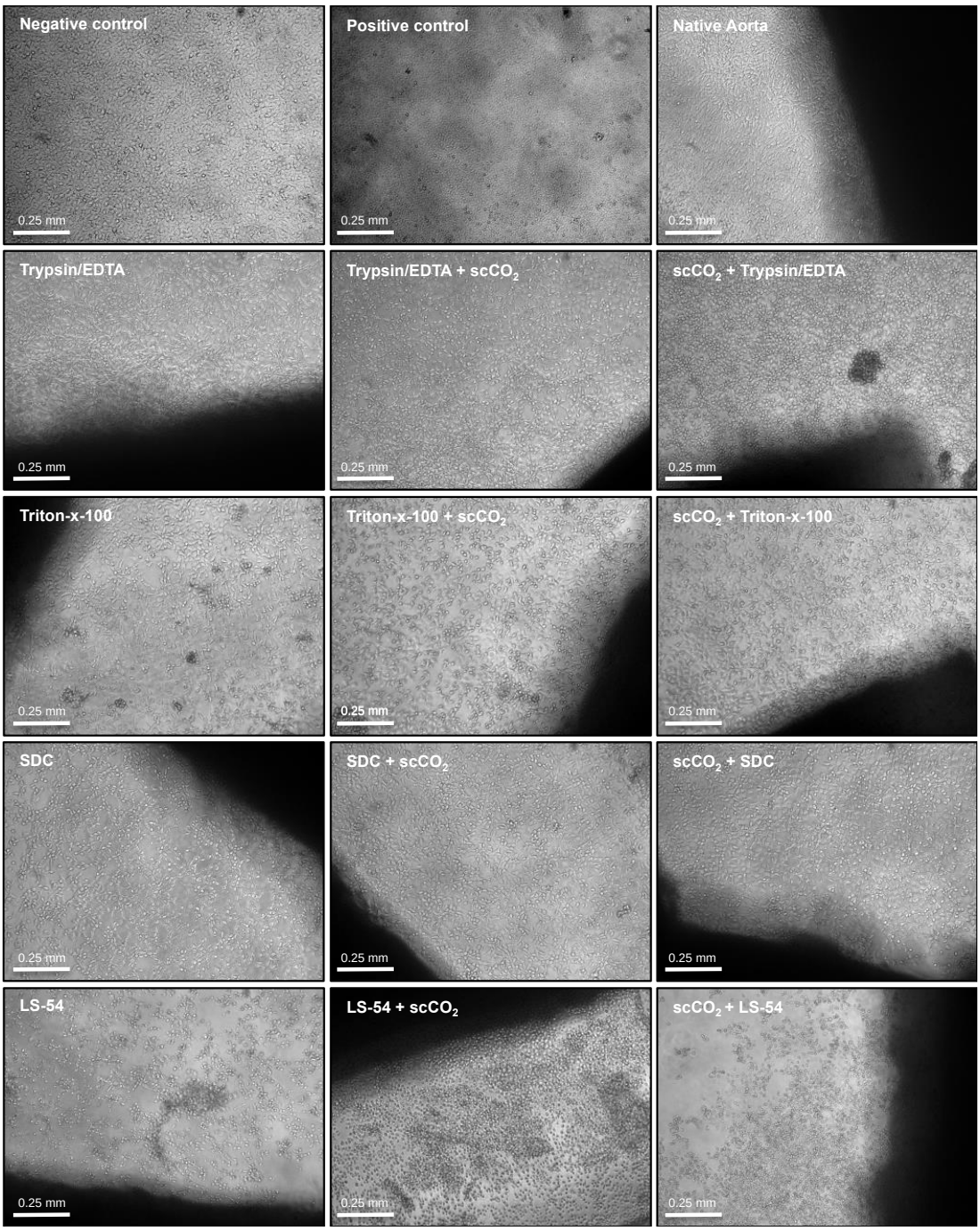

Figure 7.8 Representative phase contrast images of *in vitro* 3T3 cytotoxic response to $scCO_2$ in combination with decellularization agent decellularized ECM bioscaffold

7.5. DISCUSSION

7.5.1. Biocompatibility of decellularized ECM bioscaffolds

Biocompatibility is one of the major aspects to consider for downstream applications of decellularized ECM scaffolds, whether *in vitro* or *in vivo*. Detergent decellularization methods require chemical and/or biological agents that remove cellular material, however, these same chemical/biological agents also damage the ECM structure (Meyer *et al.*, 2006; Karina H. Nakayama *et al.*, 2010; Du *et al.*, 2011) and can leave residual toxic materials within the scaffold (White *et al.*, 2016). Residual chemical/biological agents can cause a cytotoxic response making the scaffold unsuitable for downstream applications. Therefore, the assessment of ECM scaffold biocompatibility *in vitro* is an important step to evaluate the suitability for the scaffold to progress into *in vivo* testing.

The direct and extract assays used herein investigated two different aspects of biocompatibility; the direct assay assessed the cytotoxicity of the scaffold itself by placing it directly onto the cell monolayer, whereas the extract assay tested whether any toxic components are leaching out of the scaffold. In general, there was an obvious difference between cell viability using extract versus direct exposure assays, with the direct assays tending to cause more cell death than the extract assays. However, it is noteworthy that the direct contact assays were performed as an N=1 (i.e. no replicates), compared to N=3 for the extract assay, so it is difficult to draw meaningful and robust conclusions from the direct assays. Hence, results of the direct assays will not be discussed in detail here.

The difference in cell viability between direct and extract assay might be due to several other reasons. For example, the eluate used for the extract assays was sterile filtered prior to use, which would have reduced the risk of infection and removed any large particles (> 0.2 μm) that might have otherwise caused a cytotoxic response. In contrast, when the intact scaffold was placed onto the cell monolayer in its entirety, a more concentrated presence of toxicity (if present) was locally applied, which might have caused a more marked decrease in cell survival. Overall, the use of native tissue rarely resulted in a drastic cytotoxic response, however if it occurred it is likely due

to the lack of sterility of the tissue. This will need further investigation. Another unintended aspect that might have caused the reduced cell survival and high variability is the physical interaction of the tissue scaffold (liver and aorta) directly on the cell monolayer. In addition, with the scaffold placed directly onto the cell monolayer, the cells below the scaffold might have been deprived of oxygen, which would likely have reduced cell viability. An alternative approach for future experiments of this type would be to secure the tissue/ECM onto the centre of the plate prior to seeding cells to assess whether the tissue/ECM causes a zone of inhibited cell growth.

The $scCO_2$ decellularized ECM scaffolds (liver and aorta) developed within this project did not cause an overtly cytotoxic response. For instance, when cells were indirectly exposed to eluate from ECM scaffolds or directly exposed to the ECM scaffold itself, cell viability was only slightly reduced. This suggested that exposing tissue to $scCO_2$ *per se* was unlikely to cause a cytotoxic response. As previously discussed in Chapter 5, this thesis presents the first report on successful decellularization utilizing $scCO_2$ only, hence there is no literature on the biocompatibility of $scCO_2$ only treated tissue in combination with cells. However, the use of $scCO_2$ for sterilization of tissue scaffolds has gained a lot of attention in recent years (Nichols, Burns and Christopher, 2009; Russell *et al.*, 2013, 2015; Wehmeyer, Natesan and Christy, 2015; Balestrini *et al.*, 2016; Hennessy *et al.*, 2017). $ScCO_2$ has been used for successful sterilization (SAL 6) of rabbit cortical bone (Russell *et al.*, 2013, 2015), human tendon (Nichols, Burns and Christopher, 2009), human amniotic membrane (Wehmeyer, Natesan and Christy, 2015a), rat lung matrices (Balestrini *et al.*, 2016) and porcine heart valves (Hennessy *et al.*, 2017), indicating that whilst there are no reports on cytotoxicity, the sterilizing effects of $scCO_2$ would most likely result in a beneficial effect on biocompatibility, compared to untreated native tissue at least. This might explain why tissues (liver and aorta) exposed to $scCO_2$ did not produce a cytotoxic response in extract or direct biocompatibility assays used herein.

Cells cultured in eluate from detergent decellularized aorta, but interestingly not liver, caused a cell-based cytotoxic response. The reason for the difference between

tissue types is unclear because the same decellularization protocol was used for both tissues. This was possibly due to greater residual DNA content retained in the aorta tissue compared to liver because residual DNA has previously been shown to cause impaired biocompatibility (Londono *et al.*, 2017). In addition, residual decellularization agents that remain in the tissue following decellularization have been reported as a potential cause of cytotoxicity (White *et al.*, 2016). Whether certain tissue types are more liable to retain residual detergents or solvents than others is not known and is a potential reason for the differences observed here between liver and aorta. Furthermore, differences in the sensitivity of HepG2 and 3T3 cells to residual decellularization agents might also have affected the outcome reported here. The cytotoxic response to aorta was somewhat in disagreement with previous data reported by Crapo *et al.*, 2012 and Wainwright *et al.*, 2010 who both showed no cell-based cytotoxicity when utilizing the same decellularization technology for porcine optic nerve, spinal cord brain tissue and heart. Again, this could be related to differences in tissue-specific retention of residual decellularization agents and/or differential sensitivity of the different cell types used. It is also noteworthy that the authors used a different quantification method for the cytotoxicity, which might have caused the disparity in outcome. Herein, the MTT assay was used to evaluate cytotoxicity in accordance with the ISO Guidelines 10993 (note: experiments were not conducted in a ISO accredited manner).

The extract exposure of a Trypsin/EDTA treated ECM scaffold (+/- $scCO_2$, liver and aorta) to a HepG2 or 3T3 cell monolayer, did not cause a cytotoxic response compared to the negative control (media treated). This is not surprising considering Trypsin/EDTA is a combination of an enzyme and a chelating agent that is frequently used to gently isolate and release cells during tissue digestion and from tissue culture treated plastic (i.e. without killing the cells). Furthermore, any residual Trypsin/EDTA remaining in the tissue was unlikely to have a detrimental effect on the cultured cells due to a large dilution effect within the eluate or culture media. In addition, any residual Trypsin/EDTA would likely have been inactivated by the high serum concentration (10%) used in the culture media herein. Trypsin is also known to digest itself over time (Vandermarliere, Mueller and Martens, 2013), which could be another reason for the non-cytotoxic response. It is noteworthy, that the exposure

to $scCO_2$ prior or following the Trypsin/EDTA treatment did not impact cell viability.

The extract exposure of a Triton-x-100 treated ECM scaffold (+/- $scCO_2$, liver and aorta) to a HepG2 or 3T3 cell monolayer caused the greatest cytotoxic response with HepG2 cells, in particular when Triton-X-100 followed $scCO_2$ exposure. White *et al.*, 2016 showed in a ToF-SIMS study that residual detergent fragments of Triton-x-100 remained in the ECM scaffold after decellularization, which might have caused the cell-based cytotoxicity observed here. Indeed, the study of White *et al.*, 2016 demonstrated that Triton-x-100 was particularly susceptible to being retained in tissues following the decellularization process, which is in agreement with biocompatibility issues reported herein. Cartmell and Dunn, 2000; Nakayama *et al.*, 2010 as well as Woods and Gratzer, 2005 all reported inefficient decellularization using Triton-x-100 and damage to the ECM structure on kidney (monkey), tendon (rat) and ligaments (porcine) (respectively), indicating the adverse effects of Triton-x-100. Moreover, Borner *et al.*, 1994 used 2% Triton-x-100 in a model of necrotic cell death to intentionally cause cell death of PC-3, SW-620 and HT-29 cells demonstrating the cytotoxicity of Triton-X-100, which is less than the concentration used herein (3%). It is noteworthy, that the exposure to $scCO_2$ following the Triton-x-100 treatment improved cell viability of both cell lines tested, indicating that $scCO_2$ might be able to remove some of the residual Triton-x-100 and thereby limit its toxicity.

The extract exposure of a SDC treated ECM scaffold (+/- $scCO_2$, liver and aorta) caused cytotoxicity to a HepG2 and 3T3 cell monolayer. This is in disagreement with previously published literature whereby SDC is claimed to be a weak detergent (Wang *et al.*, 2015). Dutton Sackett *et al.*, 2018 showed no cytotoxic response of HUVEC and INS-1 832 cell lines to SDC decellularized pancreas ECM hydrogel. Wang *et al.*, 2015 showed improved hHpSCs cell viability when cultured on SDC decellularized liver. Hence, both studies indicate SDC to be non-cytotoxic. However, Wang *et al.*, 2015 reported that SDC decellularization resulted in an unusual ECM ultrastructure when applied to porcine liver and kidney, with retained residual cellular components instead, which might have caused a cytotoxic response in the

experiment presented herein. Interestingly, cell viability of HepG2 was generally unaffected by the addition of tissue treated with SDC in combination with $scCO_2$, whereas the viability of 3T3 cells was reduced. This disparity between cell lines might be explained due to different sensitivities of the different cell lines.

The most cytotoxic decellularization agent tested herein was LS-54. The extract exposure of a LS-54 treated ECM scaffold (+/- $scCO_2$, liver and aorta) to a HepG2 or 3T3 cell monolayer caused cell-based toxicity with little or no survival of the cells. This is not in agreement with previously published literature by Antons *et al.*, 2018 who utilized a 24 h LS-54 soak prior to $scCO_2$ exposure as part of the decellularization methodology and showed no inhibition of cell growth/viability when directly exposed to the scaffold. However, Antons *et al.*, 2018 applied a 24 h PBS wash after the decellularization methodology to remove any residual toxicity, whereas in this study only a 3 x 5 minutes PBS wash was applied, which might have caused the differences in outcome. LS-54 is a commonly used household surfactant that has gained attention due to its solubility in $scCO_2$ and the effectiveness to remove bacterial endotoxins from biomaterials in combination with $scCO_2$ (Tarafa *et al.*, 2010). However, LS-54 is reported as highly hazardous to aquatic life in the MSDS Data Sheet, which supports the observation of poor cellular biocompatibility in this study. Hence, extended or advanced washing protocols will likely be required if using LS-54 to decellularize tissue. It was hypothesised that an alternative to the extended washes to remove residual toxicity could be the use of $scCO_2$. However, the exposure to $scCO_2$ post the LS-54 treatment decreased cell survival on both cell lines tested. This was unexpected because the addition of $scCO_2$ alone did not cause a cytotoxic response.

One of the aims of this chapter was to investigate the utility of $scCO_2$ to reduce cytotoxicity when using detergents. Therefore, the study presented herein tested the effect of $scCO_2$ prior to or following detergent treatment. There was a trend that the addition of $scCO_2$ following detergent treatment (Trypsin/EDTA, Triton-x-100, SDC) might facilitate cell survival in subsequent biocompatibility assays, which could potentially be explained by the hypothesis that $scCO_2$ - soluble detergents will be removed from the scaffold via $scCO_2$. However, the data for LS-54, a well known

$scCO_2$-soluble detergent, showed a reduction in cell survival when exposed to $scCO_2$ following the detergent treatment. Therefore the data are inconclusive and need further investigations with a higher number of replicates to make a conclusive decision.

It is noteworthy, that the 30 h eluate reduced cell viability to a greater extent than that of the 90 h eluate for all conditions tested (liver and aorta). This is surprising because the effect of the tested decellularization agents was therefore less harmful to the cells when the eluate was generated by longer exposure to the tissue. This might be due a loss of activity/degradation of the decellularization agents after prolonged incubation in media. Therefore, a 30 h eluate produced from detergent treated tissue is likely sufficient for extract assays, rather than 90 h.

7.6. CONCLUSION

To conclude, $scCO_2$ decellularized ECM scaffolds (liver and aorta) are generally biocompatible and non-cytotoxic on HepG2 and 3T3 cells. However, detergent decellularized ECM scaffolds (liver and aorta) caused cytotoxicity that was not removed when $scCO_2$ was added prior to or following the detergent treatment. It is therefore likely that additional washing steps are required to improve downstream biocompatibility, however the strength and duration of wash could detrimentally impact the resultant ECM scaffold. The use of $scCO_2$ in combination with detergent decellularization methods needs further exploration to better understand the interaction of $scCO_2$ with decellularization agents and in particular, how $scCO_2$ can be used to remove residual toxicity.

8. General Discussion, Conclusions & Future work

8.1 OVERVIEW

This PhD project was centred on investigating the use of $scCO_2$ for the decellularization of mammalian tissue, whereby the resultant ECM bioscaffold would be suitable for tissue engineering applications.

The following over arching hypotheses were tested and will be discussed below:

- ***Hypothesis (1):*** *$scCO_2$ removes cellular material from mammalian tissue*
- ***Hypothesis (2):*** *Decellularization by $scCO_2$ is improved by combination with decellularization agents*
- ***Hypothesis (3):*** *Tissues exposed to $scCO_2$ are biocompatible in vitro*

8.1.1 $scCO_2$ removes cellular material from mammalian tissue

The work described in Chapter 5 tested the hypothesis that $scCO_2$ removes cellular material from mammalian tissue without the need for addition of a decellularization agents. To test this hypothesis, the effect of tissue moisture content and surface area, as well as $scCO_2$ exposure time, were assessed for their ability to facilitate $scCO_2$ to reduce DNA content.

The results of Chapter 5 were in agreement with the hypothesis that $scCO_2$ removes cellular material from mammalian tissue. In particular, data presented in this thesis revealed, for the first time, that prolonged exposure to $scCO_2$ (5 – 72 hours) resulted in a time-dependent reduction of DNA content. For liver tissue, this time dependent effect began to plateau beyond 48 hours, indicating that this was the minimum duration of exposure required to achieve maximum DNA reduction (75% decrease). For aorta tissue, which is structurally different from liver, the maximum DNA reduction was achieved at the longest duration tested (i.e. 72 hours, 48% decrease),

suggesting that the degree of decellularization by $scCO_2$ may vary between tissue types.

To date, there are no regulatory guidelines from medical device authorities as to the definition of a successfully decellularized ECM product. However, Crapo, Gilbert and Badylak, 2011 defined 3 factors to verify successful decellularization that are commonly accepted and used in the area of decellularization:

1) Less than 50 ng of dsDNA per mg of ECM dry weight
2) Remaining DNA fragments are < 200 base pairs (bp)
3) Acellularity

It is noteworthy that the accepted level of residual DNA (50 ng/mg) is unrealistically low and highly dependent on the type of tissue being used. Furthermore, numerous commercially available ECM products on the market do not meet this strict threshold and yet have received clinical approval for use in humans (Gilbert, Freund and Badylak, 2010). Hence, recent publications tend to report the relative percentage decrease in DNA content compared to that of the native tissue. To put the results obtained by $scCO_2$ in this thesis into context, the detergent decellularization methodology used as a positive comparator herein reduced the DNA content on average by 95% for liver tissue. Therefore, whilst the 75% reduction in DNA for liver tissue by $scCO_2$ was promising, there is still scope for further optimisation to achieve the level of decellularization obtained by "gold-standard" detergent based methods.

Only a handful of studies have reported the utilization of $scCO_2$ in the context of tissue decellularization (Sawada *et al.*, 2008; Guler *et al.*, 2017; Huang *et al.*, 2017; Seo, Jung and Kim, 2017; Wang *et al.*, 2017; Antons *et al.*, 2018; Casali *et al.*, 2018). None of the aforementioned studies have shown successful cell removal using $scCO_2$ alone. For example, Sawada *et al.*, 2008 showed no measurable removal of cell nuclei from aorta tissue when exposed to 1 hour of $scCO_2$ only (assessed by H&E staining) at the same pressure and temperature levels as used herein. Antons *et al.*, 2018 also showed that DNA content of cartilage, tendon and skin was not

changed by 1 hour exposure. Whilst the authors used the same temperature as that used in this thesis, they used a slightly higher pressure level (~ 3600 psi) compared to the study herein (~2900 psi). These data are in agreement with those produced in this thesis, which showed that 1 hour exposure to $scCO_2$ alone was not sufficient to remove cellular material from mammalian tissue. Importantly however, the work described herein extended the understanding of how $scCO_2$ can be utilised for decellularization by revealing that prolonged exposures to $scCO_2$ were required to remove cellular material form mammalian tissue. For instance, a minimum of 5 hours exposure to $scCO_2$ was required to achieve a significant reduction in DNA content, indicating that the 1 hour exposure to $scCO_2$ reported by others (Sawada *et al.*, 2008; Antons *et al.*, 2018) might not have been sufficient to achieve measurable cell removal.

In contrast to the results reported here and by others (Sawada *et al.*, 2008; Antons *et al.*, 2018), Casali *et al.*, 2018 showed that 1 hour $scCO_2$ exposure produced an approximately 50% reduction in DNA content compared to native aorta tissue. This was surprising considering Casali *et al.*, 2018 used only 1 hour of $scCO_2$ exposure at the same temperature as used herein and a slightly higher pressure (~ 4000 psi compared to ~ 2900 psi used here). However, Casali *et al.*, 2018 utilised a CO_2 flow system/extraction, which means that whilst the pressure was constantly kept at the same level (via a backpressure regulator), the CO_2 was pumped through the autoclave at a speed of 1 mL/min. All experiments within this thesis were performed as batch extraction rather than flow extraction. Hence, the high-pressure autoclave was pressurised to the desired pressure and temperature before being left for the required $scCO_2$ exposure time. This difference in methodology could potentially facilitate cell removal as the constant flow and movement through the autoclave system could serve to wash/agitate the tissue and therefore promote cell removal. Given the data presented by Casali *et al.*, 2018, flow extraction might further facilitate $scCO_2$ based cell removal and therefore warrants further exploration in future experiments.

To conclude, prolonged exposure (5 - 72 hours) to $scCO_2$ in a batch extraction system reduced DNA content of liver and aorta tissue herein. The recent report from

Casali *et al.*, 2018 suggests that these exposure times could be reduced by using a flow extraction system, rather than batch, which might help reduce the time required to achieve decellularization by $scCO_2$.

8.1.1.1 Impact of pressure level on $scCO_2$ decellularization:

The mechanism underlying $scCO_2$ decellularization could also be simply due to high pressure physically breaking the cells within the tissue.

Hashimoto *et al.*, 2010 and Funamoto *et al.*, 2010 utilized high-hydrostatic pressure (HHP) to decellularize cornea and aorta. The technique developed utilized very high hydrostatic pressure of 142,137 psi for 10 minutes, followed by a 3 day agitated wash in DNase for cornea and a 14 day wash in PBS for aorta. The group reported that the high-hydrostatic pressure disrupted the cells and the washing steps in DNase or PBS removed cellular debris, indicating both parts of the methodology are required for a successful decellularization. However, the experimental design did not include a high-hydrostatic pressure only group so it was not possible to determine whether the high hydrostatic pressure alone was capable of decellularizing tissue. The work in this thesis demonstrated that an agitated washing step alone was capable of substantially reducing DNA content in a time-dependent manner, suggesting that effects reported by Hashimoto *et al.*, 2010 and Funamoto *et al.*, 2010 were likely at least in part due to the prolonged washing steps.

Therefore, the exact impact of increased pressure alone to physically break the cells of the tissue and facilitate cell removal requires further exploration.

8.1.1.2 Decellularization by $scCO_2$ was time dependent and tissue specific:

This thesis revealed that the duration of $scCO_2$ exposure is instrumental to the successful removal of DNA from mammalian tissue.

One of the main reasons for using $scCO_2$ is that it benefits from the unique properties of liquid like density and solvation capability as well as gas like viscosity and thus

diffusivity (Quirk *et al.*, 2004). Interestingly, the diffusivity coefficient of $scCO_2$ decreases when density increases (Sassiat *et al.*, 1987). It is therefore speculated, that $scCO_2$ has the ability to diffuse through tissue but the speed of diffusivity is dependent on the density and surface area of the tissue. Hence, increased exposure time might have allowed for greater diffusivity and therefore greater decellularizing effects with increasing exposure times.

The greater reduction in DNA content of liver versus aorta might also be explained by the same concept. For instance, liver tissue (~1g) has a calculated density of 1079 kg/m^3 and required 48 h $scCO_2$ exposure to significantly remove DNA content, whereas aorta tissue (~1g) has a slightly higher density of 1102 kg/m^3 and therefore required longer $scCO_2$ exposure (72 h) (IT'IS Foundation 2019). In addition, the improved decellularizing effects of $scCO_2$ when surface area was increased might have occurred because the distance for diffusivity was decreased.

Therefore, $scCO_2$ exposure duration is likely to facilitate cell removal due to the time required by $scCO_2$ to diffuse through the tissue. Previous publications that showed no effect on cell removal utilized only 1 hour exposure to $scCO_2$ (Sawada *et al.*, 2008; Antons *et al.*, 2018), which potentially was not of a sufficient duration to fully penetrate throughout tissue.

8.1.1.3 Decellularization by $scCO_2$ was dependent on tissue moisture content:

In an attempt to aid cell removal, tissue moisture content was removed prior to $scCO_2$ exposure based on methods used by the food industry to extract oils and lipids (Yamaguchi *et al.*, 1986; King, Johnson and Friedrich, 1989; Boselli and Caboni, 2000).

Opposite to the expected outcome, it was revealed that when water content was removed from the tissue (via lyophilisation) prior to $scCO_2$ exposure (5 and 72 h), the reduction in DNA content was completely inhibited compared to that of tissue containing the natural quantity of water. This was very surprising considering that the food industry has previously reported enhanced fat, phospholipid and lipid removal when moisture content of meat, egg yolk and antarctic krill was reduced (respectively; Yamaguchi *et al.*, 1986; King, Johnson and Friedrich, 1989; Boselli and Caboni, 2000).

The addition, rather than removal, of water has previously been used during $scCO_2$ decellularization of cornea (Huang *et al.*, 2017). The authors used the addition of water to promote swelling of cells prior to decellularization by $scCO_2$. Taken together with the results presented in this thesis, it is interesting to speculate that tissue water content might be essential for decellularization.

The mechanism for why water content facilitates decellularization by $scCO_2$ could be due to the reaction of CO_2 with water resulting in formation of carbonic acid (H_2CO_3):

$$CO_2 + H_2O = H_2CO_3$$

The increase in carbonic acid within the tissue would subsequently decrease the intracellular pH. A study by Spilimbergo *et al.*, 2005 claimed that the change in pH caused by $scCO_2$ was the most likely reason for inactivation of bacteria during pasteurization by $scCO_2$. The authors showed that the higher the pressure and subsequently the more dissolved CO_2, the greater the reduction in internal pH and the lower the viability of bacteria. They speculated that the acidity potentially changed

permeability of the bacterial cell wall. The same mechanism could in theory be applied to the utility of $scCO_2$ for mammalian cell decellularization described here in that an intracellular decrease in pH potentially facilitated cell membrane disruption to aid $scCO_2$ decellularization.

To sum up, $scCO_2$ likely diffuses through the tissue and the pressure forces the CO_2 into the H_2O containing tissue/cells and reacts to form carbonic acid. Once fully saturated, the tissue/cells would retain the carbonic acid until depressurization. It is speculated that when depressurized quickly, the gas build up and pressure of the carbon dioxide release "explosively" breaks open the cell membrane and releases the cellular material.

An alternative approach for future studies might be to pre-saturate the $scCO_2$ with water in an attempt to better hydrate the tissue and promote swelling of the cells. This might then enable greater decellularization due to either the pressure of the $scCO_2$ to burst the cells more easily or to allow a greater build up of carbonic acid. Hence, future experiments should investigate the addition of water to $scCO_2$ to further improve cell removal but also to improve our understanding on the mechanism of $scCO_2$ decellularization.

Taken together, the data presented in Chapter 5 are the most comprehensive characterisation of the effects of $scCO_2$ alone on mammalian tissue decellularization. It is the first successful report showing a reduction in DNA using a $scCO_2$ based decellularization method (batch-system) without the addition of modifying agents. Finally it is speculated here that the main underlying mechanism of $scCO_2$ decellularization is a combination of diffusivity of CO_2, high pressure and decreased internal pH.

8.1.2 Decellularization by $scCO_2$ is improved by combination with decellularization agents

The work described in Chapter 6 assessed the hypothesis that the addition of a short (1 hour) exposure to commonly utilized decellularization agents in combination with $scCO_2$ would enhance the removal of cellular material compared to a $scCO_2$ only method. These studies also determined whether the sequence that decellularization agents were added, i.e. prior to or post $scCO_2$ exposure, were crucial to the overall decellularization efficacy. Trypsin/EDTA, Triton-x-100, SDC and LS-54 were assessed for their effectiveness to facilitate $scCO_2$ decellularization.

Results of Chapter 6 were only in partial agreement with the hypothesis because utilising a short incubation with a decellularization agent (Trypsin/EDTA, Triton-x-100, SDC, LS-54) prior to or following $scCO_2$ exposure on aorta, but not liver tissue, successfully reduced DNA. For instance, this hybrid decellularization agent/$scCO_2$ method reduced aorta DNA content by 75%, which was superior to $scCO_2$ or detergent based decellularization alone on aorta tissue (48% and 67%, respectively). In contrast, none of the decellularization agents tested (Trypsin/EDTA, Triton-x-100, SDC, LS-54) enhanced cell removal from liver tissue compared to that of $scCO_2$ alone. In addition, this study (Chapter 6) also highlighted that the addition of the decellularization agent to liver tissue prior to 48 – 72 h $scCO_2$ exposure actually impaired the subsequent decellularizing effect of $scCO_2$ alone, which interestingly did not occur on aorta tissue. Hence, these results indicate that a combined/hybrid method that utilizes both $scCO_2$ and decellularization agents has tissue specific actions on aorta versus liver, and should be considered when developing new methods on other tissues.

The decellularization of aorta tissue by $scCO_2$ was only improved by the addition of decellularization agents prior to or following prolonged (48 - 72 hours) $scCO_2$ exposure but not short $scCO_2$ exposures (1-24 hours). This is in contrast to recent reports of successful cell removal using $scCO_2$ in combination with decellularization agents (Antons *et al.*, 2018; Casali *et al.*, 2018). The notable difference between these studies and those performed here was the duration of treatments. For instance, the authors utilised the opposite approach to that tested here, in that short exposures

to $scCO_2$ were combined with long incubations in decellularization agents, whereas this thesis sought to reduce incubation in decellularization agents by using longer exposures to $scCO_2$ in combination with short decellularization agents treatments. Both aforementioned studies utilized $scCO_2$ as a washing step, to aid removal of harmful detergents, rather than for decellularization *per se*. Antons *et al.*, 2018 developed a hybrid method around the "CO_2-philic" decellularization agent LS-54 that is known to be soluble in $scCO_2$ and hence might be removed through the venting process of CO_2. Moreover, Casali *et al.*, 2018 showed that 1 hour $scCO_2$ exposure had the same effect on removal of SDS toxicity as a 24 h PBS wash. Therefore, it is difficult to compare the results obtained herein due to the different objectives of these papers (i.e. removal of toxicity versus removal of cells). Furthermore, the multiple treatment steps reported by Antons *et al.*, 2018; Casali *et al.*, 2018 were not controlled for and therefore did not allow determination of how each variable, in particular the decellularization agents, contributed to the effects of $scCO_2$.

The mechanism as to how commonly used decellularization agents work to facilitate cell removal from mammalian tissue is widely understood. Ionic and non-ionic detergents such as Sodium deoxycholate and Triton-X-100 (respectively) lyse cells by breaking the phospholipid membrane (Gilbert, Sellaro and Badylak, 2006; Crapo, Gilbert and Badylak, 2011; Gilpin and Yang, 2017). Trypsin/EDTA cleaves proteins involved in cell attachment and therefore facilitates cell disassociation from the ECM (Gilbert, Sellaro and Badylak, 2006; Crapo, Gilbert and Badylak, 2011; Gilpin and Yang, 2017). LS-54 is not commonly used in the area of decellularization but more so as a solvent in combination with $scCO_2$. It was speculated that LS-54 was able to change the polarity of $scCO_2$ and therefore facilitate $scCO_2$ extraction of cellular components, which will be further discussed below (Antons *et al.*, 2018; Casali *et al.*, 2018).

Previous publications by Guler *et al.*, 2017; Seo, Jung and Kim, 2017; Antons *et al.*, 2018; Casali *et al.*, 2018 speculated that the addition of decellularization agents add polarity to the non-polar $scCO_2$, which subsequently facilitates the extraction of soluble polar tissue components such as DNA and phospholipids. This concept is

similar to that presented by the food industry whereby $scCO_2$ extraction is used to remove lipids/oils from animal derived products with the same polarity (Yamaguchi *et al.*, 1986; Hardardottir and Kinsella, 1988; Chao *et al.*, 1991; Tanaka, Sakaki and Ohkubo, 2004). However, this thesis reports successful cell removal utilizing $scCO_2$ alone (i.e. without altering the polarity with modifying agents). This is interesting because it does not comply with the polarity speculations presented in previous publications (Guler *et al.*, 2017; Seo, Jung and Kim, 2017; Antons *et al.*, 2018; Casali *et al.*, 2018). Therefore, the successful cell removal by $scCO_2$ alone seen within this thesis demonstrates that modifying the polarity of $scCO_2$ is not necessarily required for successful decellularization by $scCO_2$ and that other mechanisms are likely involved.

The mechanism by which individual decellularization agents impact the tissue prior to or following exposure to $scCO_2$ is likely to be similar to the use of decellularization agents on their own, however the order in which they are added (i.e. pre or post $scCO_2$) may influence their efficacy. It was speculated, that if the decellularization agent used was soluble in $scCO_2$ then it would subsequently be diffused throughout the tissue, enabling penetration of the decellularization agent into the tissue/cells and therefore facilitating enhanced cell removal. In addition, when depressurized, CO_2 naturally diffuses out of the tissue, removing the solubilized decellularization agent and residual toxicity. However, the enhanced removal of cellular material from aorta by the addition of decellularization agents prior to as well as post $scCO_2$ does not conclusively support this speculated mechanism. Further experiments that better test this mode of action might involve pre-saturating the $scCO_2$ with a CO_2 soluble decellularization agents in an attempt to fully utilise the diffusivity of $scCO_2$ and penetrate the tissue by $scCO_2$ and decellularization agent. This might then enable greater decellularization due to effectiveness of the decellularization agent that was transported via $scCO_2$ throughout the tissue. In addition, this might benefit the removal of residual toxicity due to the solubilized decellularization agent being simultaneously vented with $scCO_2$ and reduce the cytotoxic response caused by the scaffold.

Taken together, the data presented in Chapter 6 showed a successful improvement of $scCO_2$ decellularization of aorta, but not liver, by incorporation of a 1 hour incubation in decellularization agents prior to or following $scCO_2$ exposure. It is the first successful report showing a reduction in DNA using a short incubation in a decellularization agent (i.e. 1 hour) in combination with prolonged $scCO_2$ exposure.

8.1.3 Tissues exposed to $scCO_2$ are biocompatible in vitro

The work in Chapter 7 tested the hypothesis that tissues exposed to $scCO_2$ are non-cytotoxic *in vitro*. To test this hypothesis, cultured hepatocytes (HepG2 cells) and fibroblasts (3T3 cells) were exposed to ECM scaffolds that were developed by $scCO_2$ decellularization (for liver and aorta respectively). The results showed that the cultured cells remained metabolically active in the presence of the ECM scaffolds and therefore confirmed the hypothesis that $scCO_2$ decellularized ECM scaffolds are non-cytotoxic.

The lack of a cytotoxic response to tissue exposed to $scCO_2$ is perhaps not surprising considering $scCO_2$ has been used for sterilization of cortical bone (Russell *et al.*, 2013, 2015), tendon (Nichols, Burns and Christopher, 2009), amniotic membrane (Wehmeyer, Natesan and Christy, 2015a), lung matrices (Balestrini *et al.*, 2016) and heart valves (Hennessy *et al.*, 2017). The mechanism underlying the sterilisation by $scCO_2$ involves inactivation of bacteria by lowering pH and inactivating intracellular enzymes (Spilimbergo *et al.*, 2005). It is therefore likely that $scCO_2$ decellularization could also serve to inactivate bacteria within the tissue/ECM scaffold and therefore prevent/limit adverse biocompatibility issues when implanted with cells *in vitro* or *in vivo*.

To date, this thesis presents the only report on biocompatibility of $scCO_2$ decellularized tissue. However, it was also discovered in this thesis that a hybrid $scCO_2$ method utilizing $scCO_2$ and decellularization agents, in particular LS-54, caused mild-moderate cytotoxic effects *in vitro*. Therefore residual decellularization agents in the tissue were probably not removed when subsequently exposed to $scCO_2$, even when $scCO_2$ exposure was as long as 48/72 hours. This resulted in a greater cytotoxic response compared to cells exposed to $scCO_2$ only treated tissue. This was somewhat unexpected and likely requires further investigation with

increased sample sizes because others have not reported impaired biocompatibility using similar hybrid decellularization methods prior to *in vitro* (Wang *et al.*, 2017; Antons *et al.*, 2018) or *in vivo* testing (Huang *et al.*, 2017). Antons *et al.*, 2018 showed that tendon, cartilage or skin that was decellularized by a hybrid LS-54/scCO$_2$ methodology did not cause a cytotoxic response when placed in direct contact with cells *in vitro*. Wang *et al.*, 2017 reported no biocompatibility problems when exposing cells to a coating material developed from ethanol/scCO$_2$ decellularized lipoaspirate tissue. Huang *et al.*, 2017 implanted ethanol/scCO$_2$ decellularized corneas *in vivo* (rabbits) and did not report any biocompatibility issues. These data are therefore in contrast to the findings presented here and are likely due to differences in the protocols used. For example, Antons *et al.*, 2018 used an additional 24 hour PBS wash step to remove residual LS-54. However, they did not control for this wash step in their experimental design. This means that LS-54 might not have been removed by scCO$_2$, and instead might have been removed by the additional wash step in PBS. This also indicates that the LS-54 was potentially not solubilized and did not penetrate throughout the tissue.

Further studies could also investigate a pre-saturation of scCO$_2$ with scCO$_2$ soluble detergents, such as LS-54, to determine whether scCO$_2$ could serve as a carrier and also facilitate the removal of residual decellularization agents once solubilised in CO$_2$. Another aspect to consider is the addition of washing steps post hybrid scCO$_2$ decellularization to further assist in removing residual decellularization agents from the tissue. However, it is noteworthy that physical agitation of additional wash steps might also negatively impact the ECM scaffold.

Finally future studies could also investigate the biocompatibility and functionality of scCO$_2$ decellularized tissue scaffolds *in vivo*. It would be useful to determine whether any residual cellular material not removed by scCO$_2$ decellularization causes an immune response *in vivo*. Whilst residual DNA in ECM scaffolds has been demonstrated to cause adverse immune responses when implanted *in vitro* (Londono, Gorantla and Badylak, 2016), no assessments have been performed *in vivo* using ECM scaffolds with varying quantities of other residual cellular materials.

It is important that decellularization agents used, maintain an appropriate balance between cell removal and cytotoxicity. As previously discussed, there are no guidelines/ regulations regarding an accepted criteria/quality of decellularized ECM scaffolds for use in clinic. Interestingly, Crapo, Gilbert and Badylak, 2011 defined 3 factors for successful decellularization but did not account for biocompatibility of the scaffold. This should potentially be added as 4th component for successful decellularization, as a biocompatible, i.e. non-cytotoxic scaffold is required to facilitate cell survival and/or attachment.

8.3 CONCLUSIONS

The work presented in this thesis has advanced the field of decellularization by the development of a $scCO_2$ based decellularization technology for liver and aorta tissue. The novelty of this work lies in the comprehensive characterisation of the response of mammalian tissue to the application of $scCO_2$. In contrast to previous publications, which are limited and have mainly focussed on $scCO_2$ in combination with decellularization agents/modifiers, this work demonstrated the ability of $scCO_2$ alone to remove cellular material from mammalian tissue. Furthermore, this work revealed that the chronological sequence of combining short incubations in decellularization agents prior to or following $scCO_2$ exposure can further enhance $scCO_2$ cell removal, which has not been shown in previous publications. In addition, the results here highlight that tissue specific decellularization methodologies are required to produce an ECM scaffold with optimal properties for the intended downstream application.

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Appendix

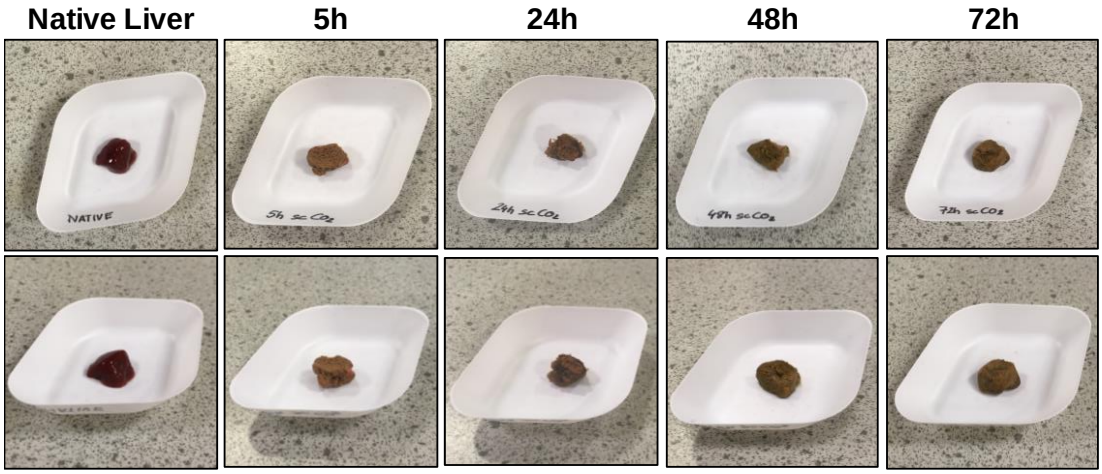

Figure A1.1 Representative images of liver tissue exposed to $scCO_2$ (5 – 72 h)

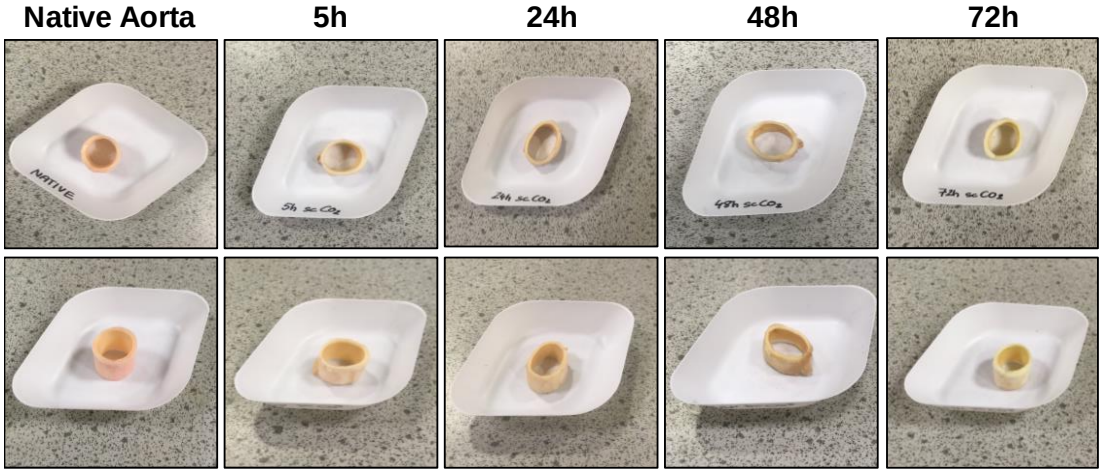

Figure A1.2 Representative images of aorta tissue exposed to $scCO_2$ (5 – 72 h)

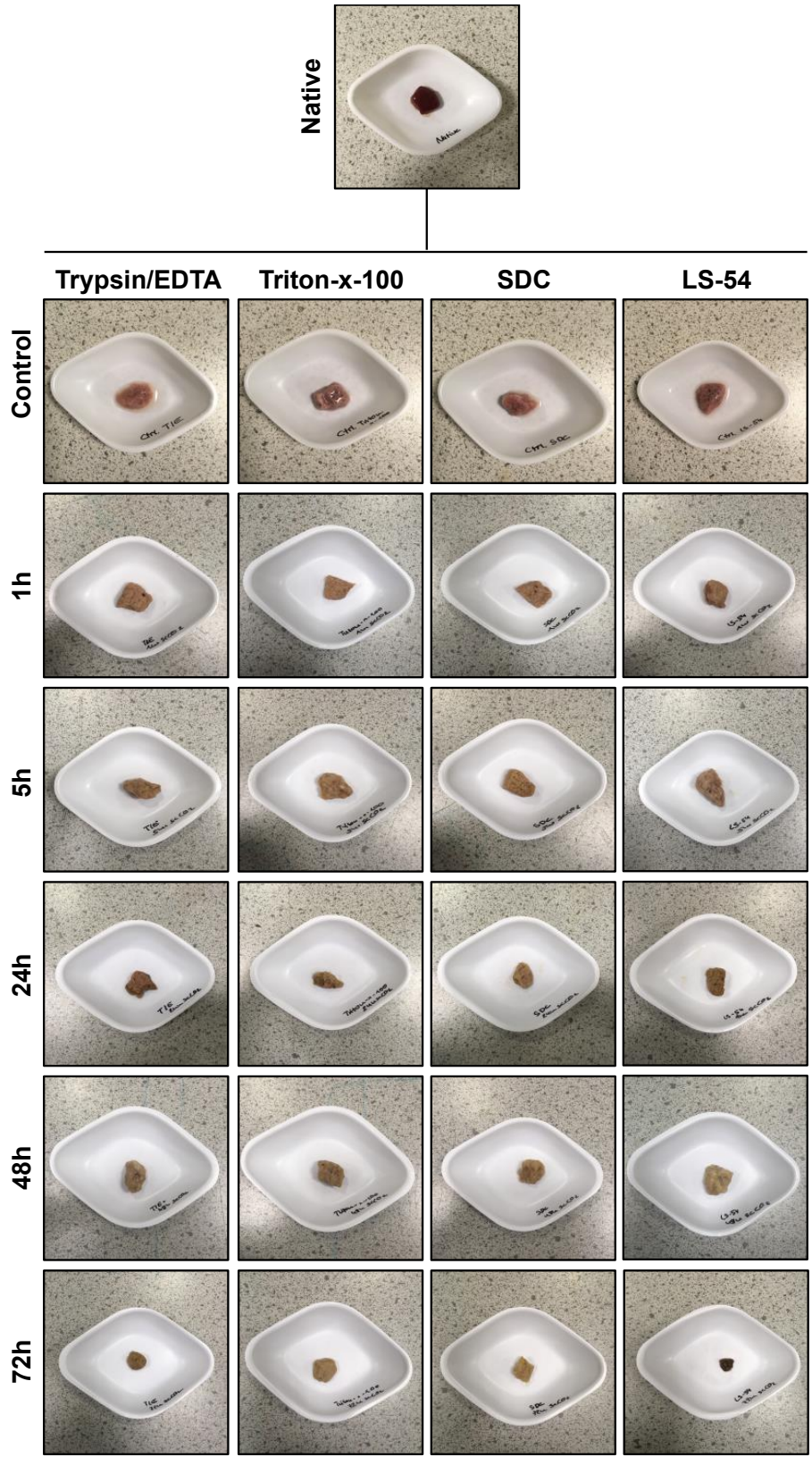

Figure A1.3 Representative images of liver tissue incubated in decellularization agent (1 h) followed by $scCO_2$ exposure (1 – 72 h)

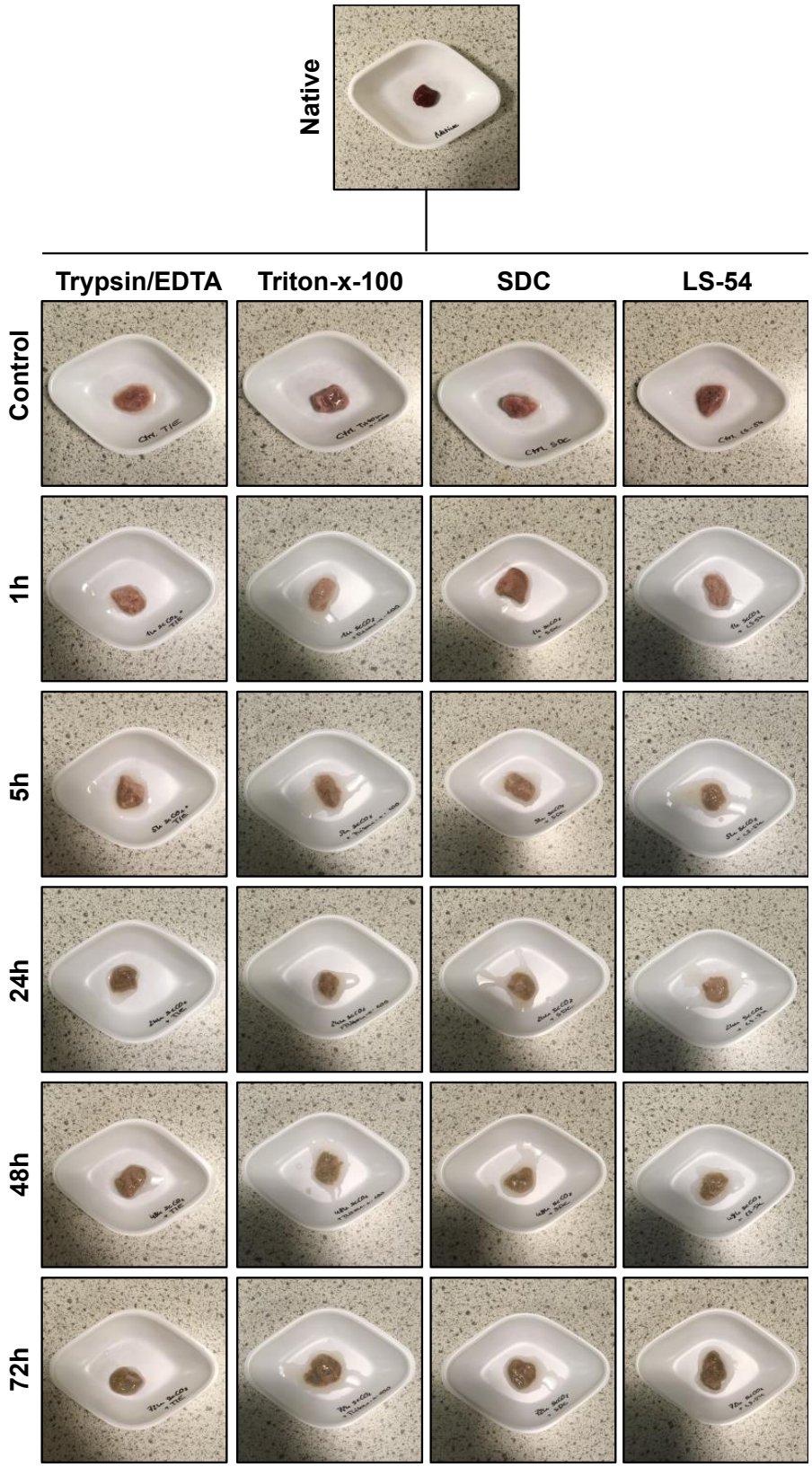

Figure A1.4 Representative images of liver tissue exposed to $scCO_2$ (1 – 72 h) followed by incubation in decellularization agents (1 h)

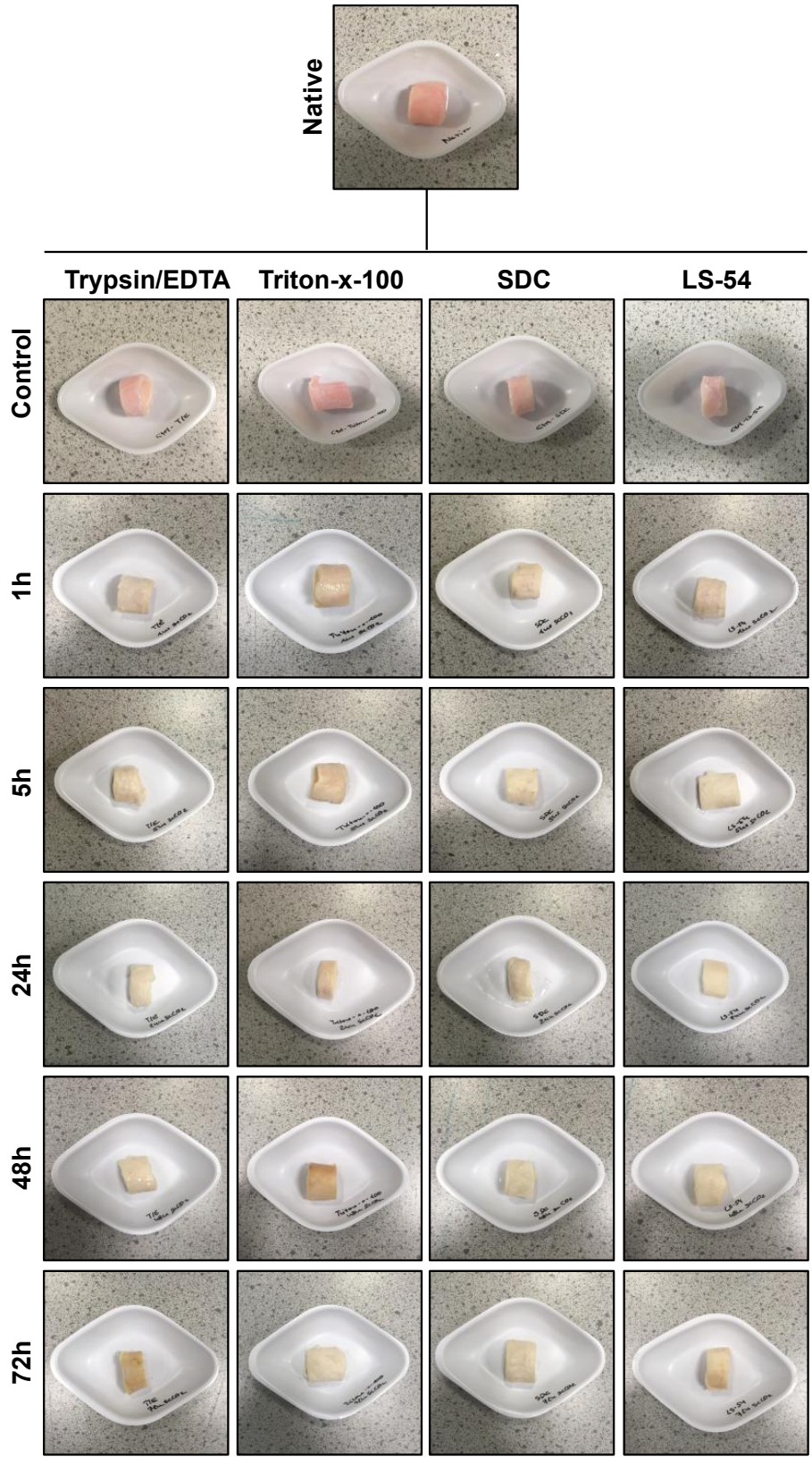

Figure A1.5 Representative images of aorta tissue incubated in decellularization agent (1 h) followed by $scCO_2$ exposure (1 – 72 h)

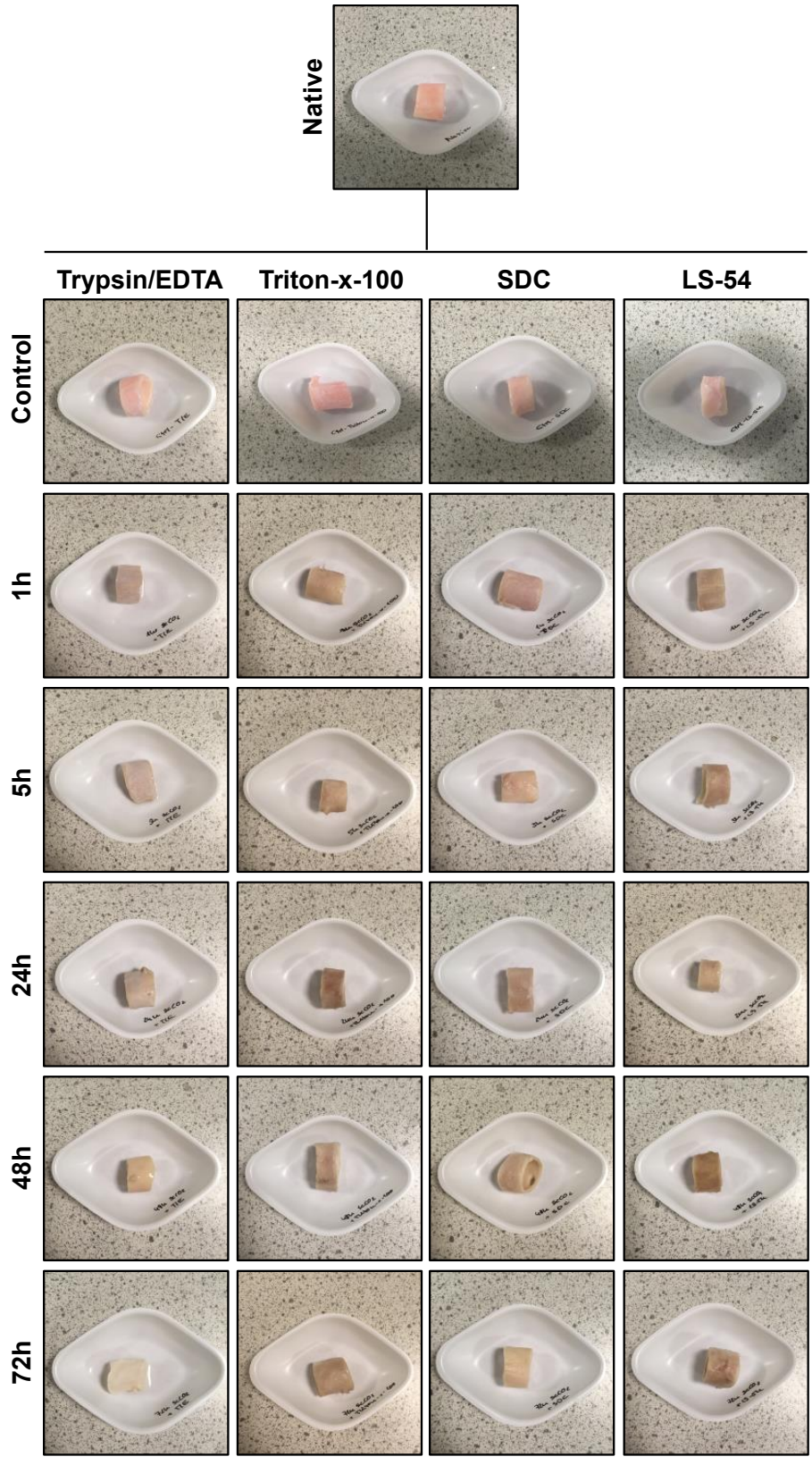

Figure A1.6 Representative images of aorta tissue exposed to $scCO_2$ (1 – 72 h) followed by incubation in decellularization agents (1 h)