Development of a safety system and contaminant quantification methods for use within a bioartificial liver device

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Declaration

I, Stephanie Annie Gibbons confirm that the work presented in this thesis is my own. Where information has been obtained from other sources, I can confirm that this has been indicated in the thesis.

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Abstract

The prognosis for patients with acute liver failure is poor, as such a novel method for the treatment of these patients is urgently required. The Liver Group bioartificial liver (BAL) has been developed to meet this need. The BAL consists of an extracorporeal circuit, in which the patient's plasma is processed through an alginate-encapsulated HepG2 cellular biomass. These cells synthesise proteins and perform a detoxification function, replacing that of the patient's own liver.

The aim of this PhD thesis was to characterise a filtration system for incorporation within the BAL, enabling regulatory requirements for use in patients to be met. Specifically, the system was to remove potential contaminants originating from the biomass, such as cell debris and DNA. The filtration system was to also be assessed for the removal of endotoxin, originating from the patient as a cofactor of their liver failure, to aid the patient's recovery.

This thesis led to the development of a protocol for the incorporation of a filtration system into the BAL. Assays for the detection of DNA and endotoxin within human plasma samples were successfully optimised for use both *in vitro* and *in vivo*. These assays enabled the detection of DNA and endotoxin at a lower level concentration of 0.1 ng/µl and 1 EU/ml, respectively, facilitating characterisation of the safety system to sufficient sensitivity limits required to meet regulatory guidelines.

DNA, endotoxin and particles were consistently removed from plasma samples by the filtration system, whilst beneficial components of the plasma such as albumin and fibrinogen, native to the patient or produced by the biomass, were maintained.

To conclude, this thesis demonstrates that the filtration system was able to remove potential BAL-originating contaminants, meeting regulatory guidelines to enable its use in patients. The filtration system also demonstrated endotoxin removal capacity, providing an additional functional element of this system.

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Abbreviations

3D	3-dimensional
AFP	α-fetoprotein
α -MEM	α-minimal essential medium
ANOVA	Analysis of variance
BAL	Bioartificial liver
BSA	Bovine serum albumin
CV	Coefficient of variance
EDTA	Tetrasodium ethylenediaminetetraacetic acid
ELAD	Extracorporeal Liver Assist Device
ELISA	Enzyme-linked immuno-sorbent assay
ELS	Encapsulated liver cell spheroids
EMA	European Medicines Agency
FBB	Fluidised bed bioreactor
FCS	Foetal calf serum
FDA	Fluorescein diacetate
FDA	Food and Drugs Administration
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
LAL	Limulus amebocyte lysate
LoD	Limit of detection
MHRA	Medicines and Health Regulatory Agency
mtDNA	Mitochondrial DNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PERV	Porcine endogenous retrovirus
PI	Propidium iodide
PK	Proteinase K
QC	Quality control
qPCR	Quantitative PCR
UV	Ultra violet

Chapter 1

Introduction

1. Introduction

To introduce the reader and lay down the context of this project, background information relating to this PhD thesis will be discussed. Further information on the function of the liver itself including its structure, key cells relevant to patients with liver failure and their vital role in the activity of the liver will be discussed. A background on liver failure and the current treatment options available will also be reviewed, with a focus on both currently available and work-in-progress extracorporeal liver support devices. I will then discuss in further detail as to why a quality system is of such importance in this instance, with a focus on current guidelines to enable this technology to progress to the next stage, what needs to be performed to meet these, and how this can be accomplished.

1.1 The liver

1.1.2 An introduction to the liver and its function

The liver is the largest organ in the body, representing between 2% and 3% of an average individual's body weight. It performs numerous functions including detoxification, transformation of biological molecules, excretion of waste products, synthesis of proteins and the production of hormones.[1]

The liver is divided into distinct lobes, each of which are made up of individual lobules containing millions of hepatocytes surrounding a capillary. The liver is connected to the circulatory system via the hepatic artery and the portal vein, which carry oxygenated blood from the aorta and nutrient rich blood from the digestive system, respectively.[1] These main blood networks subdivide into capillary networks named liver sinusoids which lead to individual functional units of the liver, lobules. Hepatocytes are responsible for carrying out the functional aspects associated with the liver.[2] The liver is a remarkable organ which has the ability to

regenerate itself. It was previously demonstrated that the liver is able to return to its original mass after a 65% hepatectomy,[3] with complete regeneration occurring 1 year following surgery. Patients presenting with cirrhosis demonstrate worse regeneration than those without. Although the liver is unable to restructure each of the four lobes, it can reform lobules and minor anatomical structures.[3]

1.1.3 Key cells of the liver and their processes

The liver contains two subsets of cells, parenchymal and non-parenchymal cells. Hepatocytes make up the parenchymal cells of the liver and are the most abundant cell type within the organ, making up between 70% and 80% of the liver volume.[1,4] Hepatocytes are responsible for carrying out the liver's functional processes, including the synthesis and storage of proteins and detoxification. Hepatocyte numbers within the average human liver vary between 1×10^{11} and 2×10^{11}, although the liver can continue to function adequately in terms of detoxification and synthesis of novel agents at just 35% of this number. Non-parenchymal cells represent 40% of the total cell number of the liver, but only 6.5% of liver volume and consist of cholangiocytes (epithelial cells), Kupffer cells, liver sinusoidal endothelial cells and stellate cells.[4]

Biotransformation of molecules occurs within the liver. The cytochrome P450 family are the most important drug-metabolising enzymes present in the liver, catalysing the oxidation, reduction or hydrolysis of substances for elimination via the kidneys. Cytochrome P450 is found primarily within the membrane of the endoplasmic reticulum present within hepatocytes. In addition to metabolising drugs, cytochrome P450 is involved in the synthesis and subsequent breakdown of hormones, the synthesis of cholesterol and the metabolism of vitamin D. These enzymes also contribute to the conjugation and subsequent clearance of bilirubin, a by-product of the breakdown of red blood cells and haemoglobin.[5]

As stated above, the liver is involved in the conjugation of bilirubin. Unconjugated bilirubin is conjugated with a molecule of glucuronic acid, an example of

glucuronidation, via the enzyme glucuronyltransferase; this changes bilirubin's properties to make it soluble in water enabling excretion. Conjugated bilirubin is excreted into the bile, passes through the gall bladder and into the intestines where it is further transformed and either excreted in the faeces or absorbed back into the hepatic circulatory system, here it is either recirculated via the gallbladder or transported to the kidneys where it is excreted in the urine.[1]

1.2 Liver failure

Liver failure arises when the liver is unable to perform its metabolic and synthetic functions to an adequate level. Liver failure is a potentially life-threatening condition, particularly in its acute form.

1.2.1 Types of liver failure

Liver failure can be categorised as acute or chronic, with a third form, acute-on-chronic, resulting when patients with chronic liver failure develop the features of acute liver failure. Cirrhosis is the primary cause of chronic liver failure, with long term damage resulting from multiple potential causes including alcohol consumption, hepatitis B or C infection and non-alcoholic fatty liver disease which can lead to scar tissue formation. Scar tissue can replace the normal parenchyma, blocking blood flow to the organ and disrupting its function. Acute liver failure differs from chronic liver failure and is the result of loss of function of 80–90% of liver cells. It is defined as rapid onset severe liver injury resulting in hepatic encephalopathy and coagulopathy in individuals without pre-existing liver disease.[6] This injury can be the result of a reaction to a medication, excessive alcohol consumption, or viral hepatitis. Acute liver failure can lead to death via hepatic encephalopathy, cerebral oedema, haemorrhage and/or sepsis which, subsequently, can lead to multiorgan failure.[6]

1.2.2 Complications associated with liver failure

The liver is involved in the clearance of bacteria and toxins, therefore, in patients with liver failure, this clearance mechanism is suppressed, leading to the build-up of bacteria and toxins in the patient. Sepsis arises when the body severely reacts to infection, leading to injury of its own tissues as a result of inflammatory pathogenesis which can subsequently lead to potentially life-threatening organ injury.[7]

1.2.3 Treatment options for patients with liver failure

Chronic liver failure is primarily treated by removing the source of the initial problem, for example, treating hepatitis with anti-viral medication or terminating alcohol consumption in those with alcohol-induced fibrosis. In the case of acute liver failure, treatment options are limited. Treatment in intensive care for patients with acute liver failure is supportive, including plasma and blood transfusions, antibiotics and attempts to reduce intracranial pressure. Frequently, the only long-term treatment pathway to consider is that of transplantation.[6] Due to the rapid progressive nature of acute liver failure, identification of a suitable organ for transplantation is rare. A report on survival rates for patients undergoing liver transplantation, published in September 2015 by NHS Blood and Transplant, states adult patients have a 90% chance of survival 1-year following, and 80% 5-years following, transplant.[8] A total of 31 liver disease and transplant centres in the United States collected data between 1998 and 2013, these data demonstrated that only 22.3% of patients with acute liver failure received a transplant and, of individuals listed for a transplant who did not receive this, 42.6% did not survive.[6]

Hepatocyte transplant has been used both as a bridge for patients with acute liver failure, to buy time until transplantation or, to promote regeneration of this acute injury.[9] Liver assist devices exist and can be used either to bridge the gap between liver failure and transplantation of a suitable organ, or to allow time for the patient's own liver to recover. These devices can be categorised into artificial and bioartificial devices. Artificial liver devices depend upon adsorption based on

physical and chemical gradients; these perform a purely detoxification function, Bioartificial liver devices, on the other hand, utilise cells to attempt to replicate, as closely as possible, the function of the native liver.[10] The bioartificial approach, therefore, provides both detoxification and synthetic functions, and may well be preferable.

1.2.3.1 Artificial liver support devices

The majority of artificial liver devices are based on replacing the detoxification process of the patient's own liver through the removal of both water-soluble and protein-bound substances. This can be performed in a number of ways, for example, using albumin in a dialysis set-up, performing separation and subsequent filtration of the patient's blood plasma, or by using a plasmapheresis machine to undergo therapeutic plasma exchange.

An example of an albumin-based approach is the molecular adsorbents recirculating system (MARS); on treatment with this system, the patient's whole blood is circulated in a cross-flow system across a 50–60 kDa albumin impermeable membrane. A solution of 20% albumin is flowed in parallel enabling the exchange of materials between the patient's blood and the albumin filtrate, the albumin solution is passed through a secondary circuit where toxins are removed using a charcoal column and anion exchange technology. This technique removes the build-up of bilirubin and bile acids from the patient's blood. A meta-analysis of data gained through the use of the MARS has demonstrated no survival benefit compared with standard medical treatment.[11]

Therapeutic plasma exchange has been used clinically since the 1960s. In this method the patient's blood plasma is separated from their whole blood, this plasma is either replaced with plasma donated from a healthy individual (fresh frozen plasma; FFP) or replaced with a solution of human serum albumin. This process, in effect, removes any plasma-based toxins and inflammatory mediators that have built up in the patient's circulatory system, replacing the patient's lost plasma with

healthy plasma or albumin. A randomised controlled trial of patients undergoing therapeutic plasma exchange demonstrated a survival advantage and an increase in patients discharged from hospital compared with standard medical therapy.[12]

The liver has many functions aside from detoxification. These include the synthesis and subsequent release of abundant plasma proteins such as albumin. Therefore, the ideal therapy for patients with acute liver failure would replicate all functions of the native human liver, including this synthesis of proteins, detoxification of ammonia, and metabolism of drugs, carbohydrates and lipids. Systems which move closer to this ideal incorporate a live biological aspect in the form of liver cells. These bioartificial liver devices will now be discussed to view their current status and future prospects.

1.2.3.2 Extracorporeal bioartificial liver support devices

Bioartificial liver support devices can use a range of cell types including, human hepatocytes, xenogeneic cells and hepatocyte-derived cell lines. Human primary hepatocytes present with functionality closer to that of the native human liver compared with various cell lines. These primary cells are difficult to obtain due to the lack of available organs, they also have a low *in vitro* survival rate, meaning that they are difficult to culture to a sufficient quantity.[13] Xenogeneic cells pose a potential risk in terms of transmission of endogenous viruses and can possibly lead to the patient experiencing immunogenicity reactions against these cells. Hepatocyte-derived cell lines have been shown to lack the functionality of native hepatocytes,[13] although, this functionality has been improved through utilising different culture conditions. A three-dimensional cell culture has been shown to more closely replicate the conditions that are seen in the native organ, and has been shown to produce cells with a greater functionality when compared with cells grown in a monolayer culture.[14]

Currently, there are many bioartificial liver systems in development which use a range of cell sources. The Extracorporeal Liver Assist Device (ELAD), for which

clinical trials are currently underway, is one example of these. The ELAD system uses C3A cells derived from the HepG2 human hepatoblastoma cell line. These cells are suspended within a hollow fibre dialysis cartridge. As yet, early clinical trials do not demonstrate a survival advantage compared with standard medical treatment.[15,16]

The Academisch Medisch Centrum Amsterdam (AMC-BAL) is another such example of an extracorporeal bioartificial liver system. This system originally consisted of primary porcine hepatocytes suspended in a polyester fibre matrix. In this system, the patient's plasma is separated from the whole blood and the plasma component is passed through the matrix, where it comes into direct contact with the porcine cells. The effectiveness of this system was assessed in twelve patients with acute liver failure, all patients treated demonstrated haemodynamic stabilisation and improvement in intracranial pressure. Out of the twelve patients treated eleven made it through to transplantation and one, following two sessions of treatment with the AMC-BAL, required no transplant.[17,18] An AMC-BAL has now been produced which incorporates HepaRG cells, a human liver cell line, as many European countries would not allow treatment with a bioartificial liver containing a porcine cell line, due to xenotransplantation-related risk including potential transmission of porcine endogenous retrovirus (PERV).[19] This new AMC-BAL was assessed in a rat model, demonstrating increasing survival time compared with an acellular model and demonstrating improvement in clinical parameters of liver failure including decreasing hepatic encephalopathy progression and ammonia accumulation.[19]

1.3 The Liver Group Bioartificial Liver support device

The UCL Liver Group Bioartificial Liver device, which for the remainder of this thesis will be referred to as the BAL, uses cells encapsulated in alginate, a hydrogel which is both biocompatible and semi-permeable. By encapsulating cells in this manner, the cells may be exposed to the patient's plasma. The BAL utilises HepG2 cells, a hepatoblastoma cell line, as its biological component, which perform many

functions of the liver. One HepG2 clone has previously been used in an FDA-approved clinical trial of ELAD in patients with acute liver failure deemed to have a 50% survival rate, no biocompatibility issues were observed.[15] Utilising a human cell line bypasses potential problems arising from xenogeneic systems, such as immunogenicity and transmission of endogenous viruses such as PERV. The BAL has been assessed *in vitro* and *in vivo* in animal models including rabbit and pig.[20,21]

It was shown that the proliferative capacity of HepG2 cells maintained consistency *in vitro* for 20 days. To enable this, cells were encapsulated into alginate microspheres, referred to as encapsulated liver cells (spheroids; ELS) from this point forward, and cultured in static culture conditions. Although their proliferative capacity was reduced compared with monolayer cultures, the protein production of these HepG2 cells was also maintained, with a maximum concentration produced between days 8 and 10. Proteins produced by these HepG2 cells in 3D culture include albumin, fibrinogen, prothrombin, α-1-acid glycoprotein (AGP) and alpha-1-antitrypsin (α1AT). Additionally, cells cultured in this fashion demonstrated an increased detoxification function compared with monolayer cells.[22,23] The research group then went on to study the effect of a micro-gravity culture condition on the proliferative capacity and function of these cells. When these cells were cultured as ELS under rotating conditions, their viability and structure, including integrity of the alginate spheroids in which they were encapsulated, were maintained over a 10-day period. Additionally, cell number demonstrated a 4.5-fold increase when compared with HepG2 cells grown in static culture conditions. A similar increase in protein production was seen with cells cultured under these micro-gravity conditions.[23] The next stage for the group was to further attempt to mimic the microgravity conditions that would be experienced by cells in the native liver environment. The ELS were cultured within a fluidised bed bioreactor where they experienced a microgravity environment for an 8-day period. These culture conditions have previously shown to increase the mass transfer and biological function of cells. Once cultured, these ELS were exposed to either plasma from a healthy human or plasma from a patient with liver failure for an 8-hour period under microgravity conditions to replicate the environment in which they would act within the BAL. After culture in these conditions, cell viability was maintained

with minimum cell damage incurred. Cells were shown to conjugate bilirubin, synthesise urea and demonstrated increased cytochrome P450 1A activity.[14]

1.3.1 Previous work on the BAL in vivo

Initially, a small-scale BAL was tested within an acetaminophen-induced acute liver failure model in rabbits. Rabbits were used in this scenario as they are the larger of the 'small' animals and they are easily available for experimentation. In these experiments both healthy and liver-failure rabbits were treated with either empty alginate spheres or alginate encapsulated HepG2 cells (ELS) to ascertain whether any effect of the treatment was due to the alginate or system itself or due to the HepG2 cell biological component.[21]

Following this trial and further enhancement of the biomass, the Liver Group BAL was assessed within a porcine model. This model used the surgically induced method to produce acute ischaemic liver failure. It is hypothesised that the BAL would run for an 8-hour period in the treatment of patients with acute liver failure, therefore, an 8-hour treatment time was used for this experiment.[20] In this experiment, a control was performed using a BAL containing alginate spheres with no HepG2 cell component, this was again to understand whether the beneficial effect of the BAL was due to the cells themselves, or something else in the system. This experiment demonstrated that, when compared with the control artificial liver containing no cellular component, an improvement in multiple areas specific to acute liver failure was seen. These included a decrease in intracranial pressure, an increase in the ability of the blood to form a clot, an increase in the conjugation of bilirubin, a decrease in acidosis and an increase liver-specific protein synthesis.[20]

1.3.2 Meeting regulatory requirements for the BAL

Regulatory requirements and the registration process for new medical products vary dependent on whether the treatment in question is a 'medicine' (drug) or a 'medical device'. The MHRA define a medicine as:

"Any substance or combination of substances presented as having properties of preventing or treating disease in human beings. Or, any substance or combination of substances that may be used by or administered to human beings with a view to restoring, correcting or modifying a physiological function by exerting a pharmacological, immunological or metabolic action, or making a medical diagnosis".[24]

By this definition, the Liver Group BAL could be a medicinal product. Although, when the Medical Devices Directive definition of a medical device is observed, this view may change. The Medical Devices Directive define a medical device as:

"Any instrument, apparatus, appliance, software, material or other article, whether used alone or in combination, including the software intended by its manufacturer to be used specifically for diagnostic and/or therapeutic purposes and necessary for its proper application, intended by the manufacturer to be used for human beings for the purpose of: diagnosis, prevention, monitoring, treatment or alleviation of disease, or, diagnosis, monitoring, treatment, alleviation of or compensation for an injury or handicap, or, investigation, replacement or modification of the anatomy or of a physiological process, control of conception, and which does not achieve its principal intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its function by such means."[25]

When these two statements are observed closely, it would seem that the Liver Group BAL would better fit under the definition of a 'medical device'. As the Bioartifical liver, once set up and fully functional, is comprised of multiple parts; for example, the biomass, the filtration circuit and the COBE Spectra plasmapheresis machine used to separate patients plasma from their whole blood. It is intended to be used for the '*treatment or alleviation of disease',* with the disease being acute liver failure, it is intended to temporarily replace the physiological processes usually performed by the liver, which fits under the following statement '*replacement or modification of the anatomy or of a physiological process'.* The only section of this definition which doesn't quite fit

in with the medical device setting is the following '*which does not achieve its principal intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its function by such means',* as the BAL maintains its function through metabolic processes undergone in the HepG2 cell biomass, although the last segment of this definition '*may be assisted in its function by such means'* leaves this open to interpretation.

To gain Food and Drug Administration (FDA) approval, a medical device should demonstrate relevant clinical experience. This clinical experience is used to generate a benefit-risk profile for the medical device. This profile is then used to determine whether the medical device requires further testing and characterisation prior to approval. Long-term endpoints in terms of biocompatibility need to be assessed, for example potential carcinogenic effect of the device or chronic toxicity. These tests are not required in cases where the life expectancy of the patient in which the therapy is aimed is limited. This is due to the decreased likelihood of their survival to the onset of these potential effects. When designing *in vivo* experiments, biocompatibility endpoints should be identified for measurement, and designed in such a way as to view any adverse biological response to the device. Potential endpoints include toxicity and thrombogenecity.[26]

To apply for a first-in-man study, a device must present previous *in vivo* animal study data, including relevant toxicity screening. In addition, complete information relating to the manufacture of the device is required, including origin certificates for all biologicals and consumables. Detailed protocols for its development and use and a full plan including protocols for its use within the clinical trial situation.[13] It is for this reason that rigorous testing of the BAL, as per its intended use in patients, be performed prior to application for first-in-man studies.

1.3.3 The need for a filtration system in the Liver Group BAL

The development of an extracorporeal BAL to treat patients with acute liver failure by the Liver Group has led to the requirement of a fully optimised filtration protocol

to enable this device to be used in a clinical setting. To summarise, the BAL currently comprises fully functioning HepG2 liver cells contained within a 3D environment consisting of alginate beads stabilised via a crosslinking with calcium ions. Once cultured, these ELS are contained within an external chamber. The patient's plasma is passed through this chamber where the ELS act to replace the functions that the patient's own liver cannot perform. This allows time for regeneration of the patient's own liver, or, if this is no longer a possibility, provides the patient with sufficient liver function to see them through until a donor organ becomes available for transplantation.[14] A key element of this device is the removal of deoxyribonucleic acid (DNA), endotoxin and particulates from the patient's plasma after it has been processed by the BAL. For this technology to be medicines and health products regulatory agency (MHRA) compliant safety measures need to be addressed prior to return of the treated plasma to the patient.[27,28]

1.3.4 Potential contaminants within the patient-BAL circuit

There are several potential components of the biomass which could yield contaminants during BAL perfusion. Additionally, as a result of their liver failure, the patient's own plasma will accumulate harmful substances. In order for the BAL to meet regulatory requirements, only the removal of contaminants originating from the BAL itself are required; removal of any additional contaminants originating from the patient would provide an additional benefit, but is not a requirement of these safety regulations.

1.3.4.1 DNA

Cellular apoptosis, programmed cell death, occurs throughout the body as a normal physiological process. This process is highly regulated and precisely balances cell division in adult tissues, ensuring that adult tissues remain a constant size. During apoptosis, the cell decreases in size and nuclear DNA is degraded into fragments, the cell changes structures upon its surface which signal that the cell should be

ingested though phagocytosis. Phagocytosis of apoptotic cells ensures that the cell's contents are contained, and the organic matter recycled.[29] It has been found that although highly controlled, small fragments of DNA generated through apoptosis may be present in the circulatory system as cell-free circulating DNA (cfDNA).[30–32] In contrast to apoptosis, necrosis is the process of premature cell death due to injury. This process is not well controlled, with external influences such as trauma, infection and toxins leading to unregulated cell digestion. During necrosis the integrity of the cell membrane is diminished, releasing the products of cellular digestion into the extracellular space. The processes of apoptosis and necrosis both lead to the degradation of DNA, although the tightly controlled and random events within these processes, respectively, lead to the presence of different forms of DNA within the circulatory system. During apoptosis, DNA is degraded into 185–200 base pair fragments, whereas for necrosis, DNA degradation is not controlled, therefore, the fragments released vary in size, and are typically longer than this.[31] The difference in length of cfDNA can be used as a measure of DNA integrity, defined as a ratio of small to large fragments of DNA. The measurement of this ratio can enable conclusions to be drawn as to the general cellular health of a sample, by providing an estimate of the ratio at which cells are undergoing apoptosis or necrosis.[31]

Many cells will undergo necrosis as a result of acute liver failure, which leads to a higher than normal level of cfDNA.[1,33] In addition to cfDNA native to the patient, any cell death that may occur in the biomass over the course of patient treatment could potentially lead to the release of DNA from the HepG2 cells into the patient's plasma. As this DNA is not native to the patient, it is advised that it be removed. At present, there is debate as to any risk posed by cfDNA, but as the World Health Organisation declared DNA as a contaminant, it is essential for it to be removed from the patient's plasma after treatment with the BAL in order for this system to meet current and potential future regulatory guidelines.[3435] The actual quantity of cfDNA can vary in healthy individuals from 2.5 ng/ml (0.0025 ng/µl) up to 27.0 ng/ml (0.027 ng/µl), with an average of 15 ng/ml (0.015 ng/µl).[36,37]

1.3.4.2 Endotoxin

Lipopolysaccharides (LPS) and lipooligosaccharides (LOS) make up the endotoxin molecule which is present within the outer cell membrane of gram-negative bacteria (Figure 1). It is an amphiphilic molecule with a size of around 10 kDa, exhibiting an overall negative charge which is a result of phosphorylation of the core oligosaccharide region (Figure 1).[38,39] The immune response stimulated upon bacteria introduction into the body can cause the disassociation of endotoxin from the bacterial membrane via activation of the complement pathway and phagocytic responses.[39,40]

In a healthy individual, the liver acts as the primary site of endotoxin clearance. Small quantities of endotoxin may cross from the intestine into the circulatory system, where it is removed by monocytes including Kupffer cells within the liver. During acute liver failure the body is in an immunosuppressed state, due to the disruption of monocytes, neutrophils, Kuppfer cells and the complement system. As such, approximately 30% of patients with acute liver failure also present with fungal infections as the body is lacking in the mechanisms that would clear introduced pathogens in a healthy individual.[41] During liver disease, the permeability of the gut membrane is increased, this can lead to leaching of endotoxin into the portal vein, and therefore, spread of endotoxin into the circulatory system.[33] It has been seen that endotoxin levels are increased in patients with non-alcoholic fatty liver disease, compared with the healthy population. The marked-increases in endotoxin levels in early stage fibrosis have even been suggested to be used as an early indicator of liver damage, although these levels fluctuate, and no definitive measure of endotoxin concentration has been related to various stages of liver disease.[42,43] In acute liver failure, both the availability and ability of albumin to bind and remove endotoxin from the patient's circulation is irreversibly reduced, leading to a build-up of endotoxin in the patient's circulation and subsequent endotoxaemia, potentially leading to sepsis. Additionally, the presence of high endotoxin levels within patients with acute liver failure has been linked to an increased chance of complications during treatment. Therefore, the removal of endotoxin from the patient's plasma may provide a survival benefit..[33,44]

It is worth noting that it is unlikely that any endotoxin contamination would arise from the BAL biomass, and, therefore, any potential endotoxin present within the system would have originated from the patient themselves.[27] For this reason, as touched upon earlier, a specific mechanism for the removal of endotoxin is not a regulatory requirement of this system, but is rather something that may enhance the patient's survival benefit.[33,44]

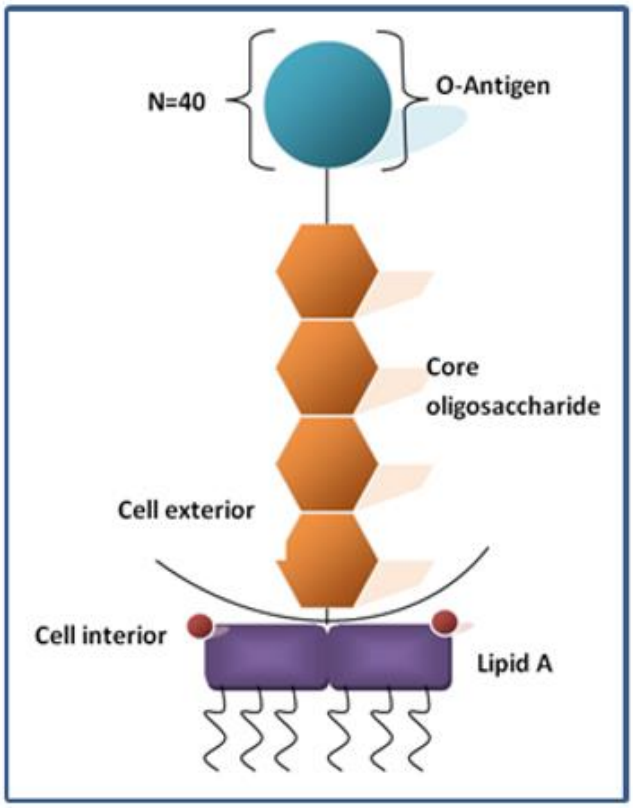

Figure 1. Molecular structure of lipopolysaccharide (LPS). *The O antigen comprises the hydrophilic component of the molecule, consisting of a repeating unit specific to bacterial serotype. The core oligosaccharide region contains a short chain of sugar molecules, displaying diversity across bacterial species. The lipid A region comprises the hydrophobic internal structure and is responsible for the toxic effects of LPS.*

1.3.4.3 Particles

Due to the nature of the BAL biomass, particles in the form of cell debris and alginate from the ELS may be present in small quantities within the plasma after

passing through the system. In order to prevent transmission of these to the patient during therapy, a method for their safe removal is required.[28]

1.4 Detection of contaminants

1.4.1 Detection of DNA in plasma

DNA can be detected using a range of systems, from PCR and electrophoresis to using optical systems such as spectrophotometers. One such spectrophotometer is the NanoDrop system manufactured by Thermo Fisher, this system uses surface tension in combination with fibre optic technology to gain a measure of nucleic acid content with a sample. The technology is based the specific pattern of UV light absorption by nucleic acids, which is at 260 nm. The measure of optical density (log of incident over transmitted light) is used to calculate a specific concentration. The benefit of this system is that it can also provide a measure of sample purity, the disadvantage is that nucleic samples are recommended to be at a concentration above 0.4 ng/µl (400 ng/ml).

Electrophoresis analysis systems are now available such as the Agilent TapeStation which uses a gel matrix, integral dyes and a ladder to separate samples using molecular weight and gain outputs in terms of nucleic acid fragment size and total concentration. The high sensitivity system requires a minimum sample concentration of 10 pg/µl (10 ng/ml), and is, therefore, more sensitive in a saline sample than the NanoDrop system.

The most sensitive method for the detection of DNA is quantitative real time polymerise chain reaction (qPCR). This technique monitors the amplification of DNA via the fluorescence either of dyes which interweave the DNA double strand, or through fluorescent sequence-specific probes. In brief, a sequence-specific primer is used as a template for DNA amplification. The PCR reaction proceeds through a series of cycles, each of which is broken down into three distinct stages.

These are initial denaturation and separation of the DNA double helix, which occurs at a high temperature; lowering of the temperature to allow for binding of the primers to the template DNA; subsequent polymerisation and extension of the primers carried out by DNA polymerase at a slightly increased temperature. This cycle is repeated a number of times, with each repeat producing a doubling of the DNA product. Fluorescence is detected at each cycle allowing for real-time detection of DNA amplification. Using a curve of known standards, an accurate measure of DNA concentration can be deduced, with a limit of detection of 0.02 pg/µl (0.02 ng/ml) in saline.

The Alu repeat sequence of DNA is abundant in the genome and represents more than 10% of human genetic material.[31] It has previously been targeted using 115 base pair primers to gain a measure of DNA released from cells undergoing apoptosis.[31]

Various components present in human plasma are inhibitory to mechanisms for the detection of DNA, in particular PCR reactions. Al-Soud et al 2000[45] used a process of elimination to characterise plasma components with the aim of revealing specific factors that contribute to inhibition of DNA detection, so that this could be further understood and overcome. Their studies focused on inhibitors specific to PCR reactions. Their results demonstrated that the main component of plasma interfering with the PCR reaction was IgG, an abundant immunoglobulin comprising approximately 75% of the antibodies found in human serum.[45] It was found that the inhibitory effect of IgG was removed upon heating the sample to 95°C prior to addition to the PCR reaction. Although, when this was performed in the presence of DNA, the opposite effect occurred, blocking amplification of the target due to the interaction of template DNA and IgG at high temperatures.[45] It was further found that the inhibitory effect of IgG could be removed if the plasma was treated using DNA-agarose beads prior to addition to the PCR reaction mixture, this was due to inhibitors in the plasma binding the DNA-agarose beads. Upon observing the inhibitory effect of whole blood on the PCR reaction, the same group demonstrated that the addition of 0.4% wt./vol bovine serum albumin (BSA) enhanced the efficiency of PCR amplification in blood samples where haemoglobin was present, counteracting the negative effect of iron which at concentrations of

greater than 25 µM reduces DNA synthesis to <10%.[46] The addition of BSA to the PCR reaction in Light Cycler instruments is occasionally recommended. It acts to coat the wall of the capillary tube, therefore, reducing binding of the reaction mixture to the tube itself, relieving the inhibition of DNA amplification.[45]

In order to work with plasma, samples in question need to be treated with anticoagulation factors, these factors, along with the inhibitory factors already present in native plasma, may incur an additional inhibitory effect on the PCR reaction as is seen with Heparin, which competes with the target DNA, providing yet another obstacle to overcome.[47,48]

Various methods exist to isolate cfDNA including systems based on magnetic beads such as those manufactured by KingFisher or Roche. The Nucleospin blood kit, manufactured by Machery-Nagel, is suitable for use on tissues/blood samples. It isolates DNA or RNA by lysing cells before using a silica membrane and salt concentration gradient to bind DNA whilst washing away inhibitory components. The DNA is eluted from the column using a low salt gradient. This technique is ideal if the study aim is to observe whether or not DNA is present in the sample, but it is not suitable for use within the scope of this thesis as DNA is lost in the process, meaning an exact quantification of the starting concentration cannot be gained.[30]

Phenol-chloroform extraction is another technique used. Here, samples are lysed, mixed with a phenol-chloroform mixture and centrifuged. Proteins and lipids will separate to the bottom of the container and DNA will remain in the supernatant. As with the Nucleospin blood kit, there is sample loss in the process meaning that the exact concentration of DNA present in the initial sample cannot be accurately deduced.[49]

Due to the limitations of pre-existing technologies, it was decided to proceed with an excising method of DNA detection but use a novel method of DNA isolation, to ensure minimal sample loss in the process. To select a pre-existing method for the detection of DNA, and ensure this method provides a suitable sensitivity for DNA detection, a range of methods will be observed using known quantities of DNA in

saline samples to obtain the method with the most potential for further characterisation.

1.4.2 Detection of endotoxin in plasma

The presence of endotoxin within substances can be quantified using the Limulus amebocyte lysate (LAL) assay. This originated from a discovery by Frederik Bang in 1956 that the blood of a horseshoe crab would coagulate in the presence of gram-negative bacteria.[50,51] This discovery led to the development of the LAL assay which originally used amebocytes isolated from the blood of the horseshoe crab to test for the presence of endotoxin. Currently, there are four main types of LAL assay, these can either be kinetic, whereby the time taken for the reaction to occur is used as measure of endotoxin or endpoint, where the absolute endpoint reading after a pre-defined time limit is used to infer endotoxin concentration. These are the gel clot assay, the chromogenic assay, the fluorescent assay and the turbidimetric assay, all of which are based upon this clotting reaction of amebocytes isolated from *Limulus polyphemus.*[52,53]

Methods for the detection of endotoxin have progressed from using reaction factors isolated directly from the horseshoe crab into using more sustainable recombinant factors.[54] One such assay which uses this recombinant technology is the PyroGene recombinant Factor C assay manufactured by Lonza. This is an endpoint fluorescent assay approved by the FDA and considered comparable to traditional LAL methods; fluorescence is measured at time zero and after one hour of incubation, with endotoxin concentration derived as the log of fluorescence intensity generated from a known standard curve. In this assay, the hydrophobic lipid A region of the endotoxin molecule reacts with recombinant Factor C, a protease zymogen, which subsequently cleaves a fluorescent substrate leading to activation of a coagulation cascade. This lipid A region activates Factor C causing it to act on a fluorogenic substrate, producing a fluorescent signal, see Figure 2.[52,53] An alternative method to this, which provides an additional 10-fold sensitivity is the PyroGent gel clot LAL assay, also manufactured by Lonza. In the PyroGent

assay, endotoxin acts as a catalyst for the activation of a proenzyme, coagulase. Initially, activation rate is directly determined by the concentration of endotoxin present in the sample. Once activated, coagulase goes on to hydrolyse coagulogon bonds which, once hydrolysed, form a compound called coagulin which generates a gelatinous clot. Development of this clot is monitored by viewing the increase in turbidity (optical density) over time, which can be used to calculate endotoxin concentration referencing a known standard curve.

Inhibitors in plasma have been shown to interfere with the LAL assay. There are different treatment processes available in the literature that can be used to overcome this, including diluting and heating, chloroform extraction and trifluoroacetic extraction. The process of diluting the sample followed by heat treating for various times and temperatures has been used widely and shows the most promise in terms of sample recovery and sensitivity. This is in part due to the lack of sample treatment with harsh chemicals or the transfer of sample between various tubes.[55,56] Variations of this method are discussed within the literature with DuBose et al 1980[57] diluting plasma in endotoxin free water to a concentration of 1 in 3, followed by heating to 100^{o}C for 10 minutes; Pearson et al in 1985[55] used a 1 in 10 dilution followed by a 10 minute heat treatment at 70^{o}C and more recently Bailey et al 2009[58] performed dilution to 1 in 10 followed by heat treatment at 75^{o}C for 30 minutes.[58]

An additional complication associated with use of the LAL assay is false activation by β-glucans, which are soluble glucose polysaccharides, produced by many prokaryotic and eukaryotic organisms known as a pathogen-associated molecular pattern, which activate the immune system. The presence of β-glucans in substances being processed using the LAL assay can cause a false positive reading as these compounds can activate the LAL enzyme cascade. This is through the activation of Factor G, whereas endotoxin activation occurs via Factor C. Both the PyroGene and PyroGent systems from Lonza bypass this Factor G activation step and so are not sensitive to β-Glucan contamination and will not elicit a β-Glucan-related false positive result. It is for this reason that these two assays were selected for further characterisation for use within testing of the BAL. Both EDTA and

heparin are known to inhibit the LAL reaction, which poses an additional complication when using plasma taken from the BAL.[54]

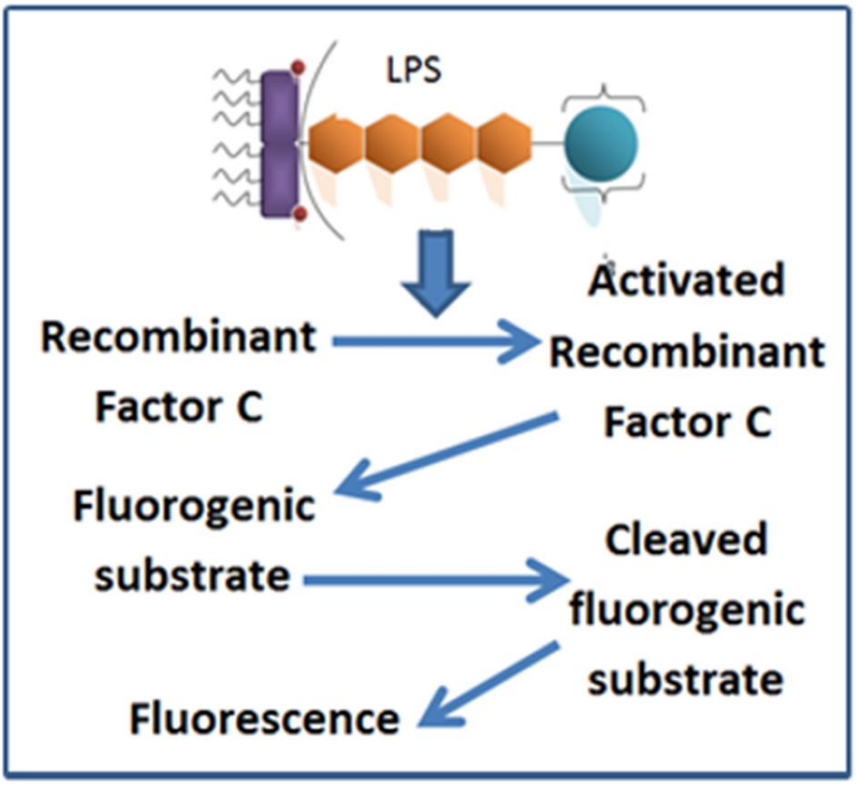

Figure 2. Principle of endotoxin (lipopolysaccharide; LPS) detection using the recombinant Factor C (rFC) assay. *Upon activation by LPS, RFc, a protease, cleaves a zymogen substrate to release a product containing a fluorescent moiety. Fluorescence is quantified by detection at 380 nm excitation and 440 nm emission; signal is proportional to LPS concentration.*

1.4.3 Detection of particles

In order to characterise any leaching of materials from the BAL and their subsequent removal, techniques for the measurement of particles are required, of which there are several available. Regulatory requirements set out by the United States Pharmacopeia provide a maximum range in terms of particle size and number

that is acceptable to be returned to the patient. For small volume injections this is 3000 particles of a size greater than 10 µm and 300 particles per container greater than 25 µm, for large volume injectables this is 12 particles greater than 10 µm and 2 particles greater than 25 µm/ml.[28,59] Requirements set out by the European Medicines Agency for the development of monoclonal antibodies for injection are less specific, stating that solutions for injection should be "practically free from particles".[59–61] Certain techniques can be excluded for the analysis of alginate particles, cell debris and other factors which may be released from the BAL; these techniques include size analysis via sieving/ aerodynamic dispersion.[46] Selection of optimal particulate analysis techniques will depend upon size distribution. Laser diffraction provides an effective way of observing particles in the micron range whereas dynamic light scattering or optical microscopy techniques such as NanoSight give a smaller size range in the nanometre area.[62,63] Techniques such as small angle X-ray scattering and small angle neutron scattering provide a further smaller size range, these techniques use the same principle as laser diffraction but with a shorter light path and so generate a greater resolution, enabling the observation of particulates in the lower end of the Nano-scale. These techniques are highly specialised and require large amounts of training, sophisticated equipment and expertise for results analysis.[63]

Particle size analysis is complicated for irregular-shaped particles, as the majority of particulate size analysis techniques are based on the assumption that each particle is spherical in nature. A spherical particle can be described in one dimension, providing a single numerical result in terms of particle size. Microscopy and automated image analysis are the only techniques currently available that can describe particle size using a range of values, further complicating analysis.[64] Thus the most suitable techniques for this application are laser diffraction, dynamic light scattering and optical microscopy imaging. A summary of these techniques can be found in Chapter 2, Table 2.

1.4.3.1 Optical microscopy

Conventional microscopy can be used to make manual measurements of particle size. The accuracy of this can be enhanced with the use of analysis techniques such as NanoSight; which uses a laser to illuminate particles which are observed using a conventional light microscope. A camera records the sample and software tracks particle movement, analysing particle size and density via tracking Brownian motion.[65,66,67]

1.4.3.2 Laser diffraction

Laser diffraction provides a short analysis time and a highly accurate, reproducible method to gain particle size distribution from either wet or dry samples. Most systems use a Helium-Neon light source and have an optical system for Fourier transformation of diffracted light onto the detector, using light refraction as the real component and the absorption as the imaginary component of the Fourier equation. The forward diffraction of light in this instance is purely dependent upon particle size.[68]

This technique requires particles in suspension to be of an appropriate concentration; too few particles would provide an inaccurate result whereas too high a particle burden would introduce error via over-estimating the scattering produced by fine particles. This method provides an equivalent diameter which is not directly related to particle volume or surface. It may be necessary to corroborate results gained here with particle size distribution data from other sources such as microscopy to ensure accuracy.[62]

1.4.3.3 Dynamic light scattering

Dynamic light scattering (photo-correlation spectroscopy) looks at changes in intensity of light passed through a sample containing liquid due to Brownian motion, enabling a smaller size distribution of particles to be observed than laser

diffraction. The hydrodynamic diameter, which provides a value for particle size is gained from this; providing an absolute measurement with a lower limit of ~2 nm depending upon the laser intensity and power.[68] Algorithms used in this process provide information in the form of mean size, widths and peaks. Using this technique particles over 3 µm in size may distort measurements, therefore, samples containing particles over this size require analysis via laser diffraction to corroborate results.[69]

A range of different particle sizing techniques are required to gain a broad insight into the types of particles expected within the system and to ensure their adequate removal.

1.5 Contaminant removal methods

Contaminants, both biological and physical, can most often be removed by filtration. The process of filtration can be defined as the act of removing unwanted substances from any fluid.[70] The four key methods for the removal of contaminants from fluids using filtration consist of sieving, interception, impaction and diffusion.[71,72] Sieving refers to the process of capturing contaminants within the filter pores based on an absolute porosity and retention capacity, interception differs to this, in that particles coming into contact with filter fibres are adsorbed onto their surface as they pass. Impaction occurs when a large particle, due to its inertia, is unable to change course within the liquid in response to obstruction presented by filter fibres, the particle is subsequently captured by the filter fibre. Diffusion refers to the retention of small particles as a result of Brownian motion, these small particles are in the 0.1 µm size range, this motion leads to an increased chance of the particle coming into contact with, and being adsorbed onto, the filter fibre.[73–75] Various methods for the filtration of substances exist, the correct technique should be selected depending on the sample properties requiring treatment.

1.5.1 Dead-end filtration

In this process the substance to be filtered passes directly through the filter leaving behind the fraction too large to pass through. This form of filtration is used for both macro (2-50 μm) and micro (<2 μm) contaminants. This subset can be further categorised into 2 distinct methods namely surface filtration and depth filtration.[70]

1.5.1.1 Surface filtration

Surface filtration (cake filtration) is a method of dead-end filtration whereby a thin filter medium is used to capture particles upon the filter surface. As the particles load, cake formation occurs at the filter surface reducing the flow rate of the filtrate (see Figure 3a, page 27). This form of filtration is used mainly when the solution for processing contains a high particulate burden exhibiting a wide variety of size and shape. When solutions containing particles of a small uniform size are used, filter fouling is observed at a faster rate due to complete filter blockage caused by compact filter cake formation[76]. This process is not observed until a much later stage, if at all, when the filtrate contains particulates with a greater variation in shape and size as these build up to form a less compact filter cake with interconnecting channels enabling the solution to pass through, further increasing the filter efficiency. This method of filtration is typically less expensive than others as the filter media manufacturing technique is simpler[77].

1.5.1.2 Depth filtration

Depth filtration (deep bed filtration) is a sub-form of dead-end filtration. This method differs from surface filtration as particles become embedded throughout the depth of the filter media as opposed to purely on the surface.[78] Depth filters consist of thick cartridges which often display a gradient in porosity through the filter with larger pore sizes at the outer edge, decreasing towards the centre. This maximises mechanical retention properties by creating tortuous paths consisting of channels of filter media (see Figure 3b, page 27).[71] Depth filtration can be used to process solutions containing a high particulate burden as their thicker and varying porosity

nature enables retention of a greater volume of particulates before filter fouling occurs.[79] This form of filtration is only feasible when the particle:pore size relationship is conducive to the process for which the cartridge was designed. Due to the broad range of pore sizes seen within these filters they require a reliance on adsorptive retention within the filter media for a proportion of their capacity. Basic principles of particulate adsorption observed within these filters consist of sieving, impaction and interception. Particles of approximately 0.5 µm in size are removed via a sieving mechanism within the larger porous structures towards the top of the filter in line with flow.[80] Particles below this size are removed either by impaction or interception.[81] During this process the filtrate only requires one filtration cycle to ensure unwanted contaminants are removed. Fouling of depth filters can occur due to particulate deposition deep within the depth of the filter fibres.[78] Depth filters are often characterised in terms of their nominal pore size instead of an absolute pore size due to the interconnective and gradient nature of the media, this nominal pore size is based on empirically measured retention characteristics.[73,74]

1.5.1.3 Cross-flow filtration

In cross-flow filtration the filter membrane is in a position parallel to the flow. Liquid travels tangentially across the filter surface rather than into and through the filter as seen in dead-end filtration. Two output streams are generated, the filtrate stream containing the portion of fluid passed through the filter and the retentate stream which is recycled through the system. This process reduces the caking effect and prolongs the filter's operational time. Particles that come into contact with this membrane are adsorbed onto the surface (see Figure 3c). This method of filtration is commonly used within blood purification where a pore size of 0.2–0.8 µm enables the retention of platelets and blood cells whilst allowing the onward passage of proteins and plasma.[82] In contrast to depth and surface filtration, the filtrate is often passed over the filter multiple times in a recirculating system to ensure the removal of unwanted substances.[76]

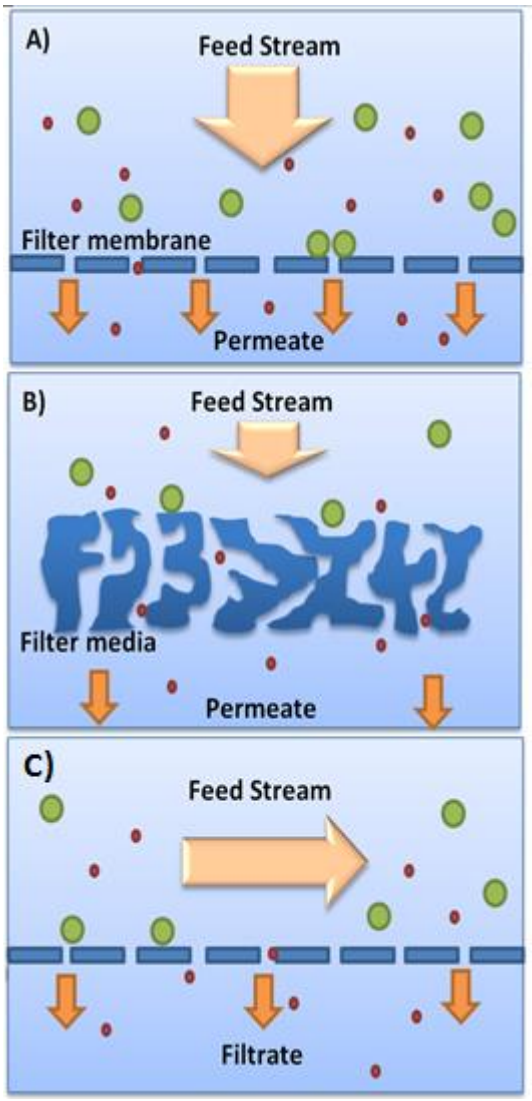

Figure 3. Methods for the filtration of liquid media*. A) Surface filtration, the fluid is passed directly over a porous membrane which retains particles greater than the nominal pore size. B) Depth filtration, a thick gradient of filter media containing channels creating tortuous paths and asperities traps particulates within the media as opposed to purely on the surface. C) Cross flow filtration, the filter membrane is situated parallel to the flow, liquid travels transiently across the surface creating two flows, a filtrate stream and retentate stream, particles above a nominal pore size are adsorbed onto the filter surface. The retentate stream is continuously recycled through the filter.*

1.5.2 Filter capacity

The retention capacity describes the capacity of the filter either before failure or as the pressure drop exceeds a defined level. Capacity is limited by filter fouling, causing the filtrate flux to fall below an acceptable threshold,[83] thus limiting the volume passed through a given area. Fouling can occur through a number of mechanisms such as complete pore blockage and internal pore blockage.[84] Sufficient filter media is required to ensure capacity is not exceeded during use. Capacity is dictated by the surface area of the filter media.[84–86] This is important to ascertain to ensure robustness of the filter system within the desired setting. It is also important to ensure that the filter can stand up to the volumetric load subjected to it over the full course of treatment with the BAL.

1.5.2.1 Cake formation

Filter cake is formed via the accumulation of substances on the filter surface, retained by the media as a solution is processed. As particle burden in the filter cake increases, the thickness of the cake itself follows suit leading to an increase in flow resistance.[87] Cake formation is dependent on the ratio of filter media pore size and particulate size within the filtrate.[85] The empirical 1/3 law states that cake formation occurs when the particle size within the solution is greater than 1/3 that of the media pore size.[34] The manner in which particles are deposited can be divided into 4 sub categories: complete blocking; intermediate blocking; bridging and standard blocking.[70,76] The exact mode of cake formation occurring upon a filter media can be calculated using the following equation:

$$d^2t / dV^2 = k_1 (dt/dv)^{k2}$$

Where: v= cumulative filtrate volume

t = time

k_1 and K_2 = empirical constants

The value of K_2 can be used to characterise the type of cake formation observed. Where k=0 displaying bridging, 1= intermediate blocking, 1.5= standard blocking and 2=complete blocking.[88]

1.5.3 Filter aids

Filter aids are substances incorporated into the filter media, used to aid function and prolong filter life. They consist of inert materials used in pre-treatment of the filter. There are two modes of function of filter-aids: firstly, a pre-coat which forms a layer of second medium protecting basic filter media; the second being an admix, which functions to improve flow rate through the filter by decreasing cake compressibility and increasing cake permeability. Common filter aids used are diatomaceous earth, perlite (a silica based substance), cellulose, asbestos, agricultural fibres, sawdust and rice hull. A selection of different grades of diatomaceous earth changes the characteristics of filtration. Finer grades increase clarity of the filtrate, with smaller particle sizes leading to lower process particulate removal, although this also decreases the flow rate able to pass through the filter at any given time.[89,90] Charge modification chemistries can be performed on filter media to retain a positive charge, enhancing adsorption of negative molecules which would otherwise pass through the porous framework. Electrokinetic adsorption via the use of positively charged filter media removes bacterial cell wall, fungi, viruses and other negatively charged contaminants such as DNA.[91,92] This net positive charge is maintained throughout the filter provided use within a specific pH range.[78,81]

1.5.4 Contaminant removal methods in use in biological purification

Blood filters are used during kidney dialysis, these are nominal pore sized filters (40 µM) and are used ensure that no products of clot formation are transferred to the patient. These processes do not require a more complex component such as depth filtration as these fluids do not contain foreign particles. Blood filters are

also used during surgery and blood transfusions, to ensure removal of any clots prior to transfusion to the patient.[93] Depth charge filters are typically used within the purification of therapeutic antibodies, such as in the processing of recombinant FVIII for use in patients with haemophilia, and the processing of monoclonal antibodies directed against specific interleukin molecules for the treatment of autoinflammatory diseases.[91] These filters are used to remove cells and cell debris, and they have also been reported to be trialled in the removal of host cell protein contaminants through electrostatic and hydrophobic adsorptive interactions.[94] The incorporation of filters into bioartificial livers to remove blood cells prior to and post treatment have been described briefly,[16,95] and, although not widely publicised, details of the filter system used within the AMC-BAL can be observed within their patent application; here, the use of two filters to process the patient's plasma following treatment with the bioartificial liver is described, the function of this is to remove cells and liver cell debris.[96] I have been unable to find description of a more advanced filtration system, such as those also utilising a charged component, to protect the patient from the biomass itself.

1.5.5 Optimal filter method to fit the BAL requirements and filtrate

Recirculating systems required for cross-flow filtration are not practical for the BAL set up. The process of surface filtration is not specific enough to meet requirements allowing certain substances through whilst inhibiting others. Additionally, a recirculating system would increase the dead-volume required for use within the BAL circuit. Dead volume is important to consider as in order to fill this, additional plasma supplies, in the form of fresh frozen plasma would be required, increasing the volume as such also has the potential to dilute the beneficial effects of the BAL, as proteins synthesised and excreted from the biomass would be diluted further in the additional volume. With dead-end filtration, the filter can be connected directly into the circuit, removing the need for a dual stream of liquid and reducing dead-volume. Therefore, the optimal method of filtration for incorporation within the BAL circuit is depth filtration. This will enable the maximum volumetric throughput, providing a higher chance of meeting

requirements of BAL treatment. Addition of filter aids to the principle filter media as well as charge modification chemistries will also enable removal of other contaminants without inhibiting the thoroughfare of biomass synthesised beneficial proteins to the patient, although this balance will require full characterisation.

1.6 Filters for use within the BAL

To ensure that the BAL has a robust safety mechanism, both an upstream and downstream filter will be used. The upstream filter will be the primary filter of focus, this will be used to purify the plasma in terms of particles, and charged contaminants such as DNA and endotoxin. The downstream filter, in contrast, will be used as a back-up providing an absolute particle sieving capacity, ensuring any debris are cleared prior to plasma return to the patient.

1.6.1 The 3M Cuno depth charge 60ZB05A filter series

Depth charge filters with anion exchange media, carbon and other affinity resins will be characterised. The use of graded density media increases the capability for contaminant loading, prolonging filter life during the treatment phase. These filters possess two distinct layers - the upstream having a more open porous structure than the downstream layer. These layers are designed to optimise filter performance for different applications and can be selected independently. In addition to these distinct layers, they also contain a filter aid in the form of diatomaceous earth, this functions to enhance the filter's retention capacity for sub-micron particles. Diatomaceous earth is FDA approved and used in the production of many foods and biologicals.[97] The filter media of the 60ZB05A filter series has additionally been optimised using charge modification chemistries causing this media to retain a net positive charge. This translates to an enhanced adsorption profile of the filter for negatively charged molecules. As previously discussed, this translates as an ability of the filter to remove negatively charged substances such as endotoxin and DNA.[91,92] The net positive charge of this filter is maintained provided use within a

specific pH range, which encompasses that of human plasma.[78,81] These filters are available in a range of sizes and filter surface area, from 25 cm^2 to 1040 cm^2, with a nominal pore size of 3–0.2 µM, making them ideal for use within a scale model system.[98] A description of these filters and their characteristics can be found in Table 1.

1.6.2 The 3M Betafine™ DP filter series

The 3M Betafine™ DP filter series are comprised of pleated polypropylene filter media, these filters are designed with absolute pore size ratings ranging from 0.2–70 µm. The pleated nature of these filters ensures a high surface area to provide a great mechanical sieve retention capacity; this also enables the filter to maintain a consistently low pressure drop, an important component in a biological system. These filters are also available in a variety of sizes, including a 3 inch filter and a 6 inch filter, enabling these to also be used within a scale model system.[99] A description of these filters and their characteristics can be found in Table 1.

Table 1. Overview of available upstream and downstream filters suitable for use within the bioartificial liver.[100,101]

	Filter type	Surface area (cm^2)	Pore size nominal/absolute	Hold-up volume (L)	Maximum pressure drop (mmHg)
Upstream (Depth Plus™ 60ZB05A)	E0025FSA	25	3–0.2 µm Nominal	0.014	1,800
	E0170FSA	170	3–0.2 µm Nominal	0.48	
	E0340FSA	340	3–0.2 µm Nominal	0.4	
	E1020FSA	1020	3–0.2 µm Nominal	0.74	
Downstream (Betafine™ DP series)	PPG 060 B 01	~600	0.6 µm Absolute	0.3	
	PPG 060 B 01	~600	1.2 µm Absolute		

1.6.3 Considerations for scale filtration models

Due to the lack of available plasma of a sufficient quantity, it will be important that a scale filtration protocol can be used to analyse the capacity and efficacy of the various filters. When assessing filters using a scaled model, it is important to assess these using a consistent flux to accurately replicate the exposure of the filter media to the plasma.[72] Flux is defined as the rate of mass flow per unit area. The calculation of flux is complex if the orientation of the flow of the solution is not perpendicular to the filter surface as the equation for calculating flux is as follows:

$$Q = A \times v \times \cos\Theta$$

Q = flux

A = surface area

V = velocity

cosΘ = angle of flow relative to surface

In the case of a solution flow which is perpendicular to the filter surface, Θ is equal to zero, meaning that cosΘ is equal to one, meaning that this equation can be simplified to

Flux = flow rate/ unit area[72]

This equation will be used to assess scale models at an equivalent flux to that seen in the full-scale filter, ensuring consistent filter media exposure to plasma samples as the model is up-scaled. 3M provide a guide as to the differential pressure to expect with increasing flux, this guide is within a water sample, and therefore we expect a greater flux to be seen when plasma samples are used, although the flux used within the filtration system is well below that used in bioprocessing and therefore, the additional viscosity presented by the plasma should not present a problem here (Figure 4).

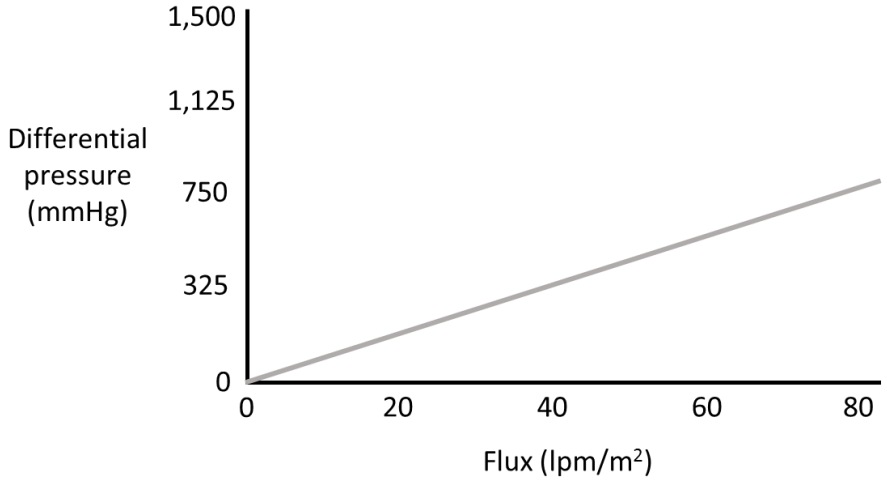

Figure 4. Flux and corresponding differential pressure expectations for the 60ZB05A filter series*. The expected differential pressure increases linearly with increase in flux. Edited from 3M Cuno 60ZB05A product information.*[101]

1.7 Hypothesis and aims

The overall aim of this PhD project is to characterise a filtration protocol, for incorporation within the extracorporeal BAL circuit that is suitable and robust enough to protect the patient from any potential contaminants originating from biomass, such as HepG2 DNA and particles such as cell debris. This filtration protocol will also be assessed for the removal of endotoxin originating from the patient as a means to aid the patient's recovery. To enable the testing of the functionality of this filtration protocol, I aim to develop assays for the detection of DNA in human plasma samples at a level sufficient enough to meet regulatory requirements, along with developing assays for the detection of endotoxin within human plasma samples sufficient to characterise its removal from the circuit. As such, I propose the following hypotheses:

Hypotheses

1. A filtration system, proven to be able to remove any potential contaminants which may arise from the BAL biomass, including both physical particle contaminants and biological agents such as DNA, will meet regulatory requirements, thus enabling its use in patients
2. A filtration system will also have the capacity to remove endotoxin contamination, thus providing an additional functional element of this system. This would further protect patients suffering from sepsis where bacterial contamination of the blood and its subsequent effects is a common final cause of death in patients with liver failure

Together with these hypotheses, I have formulated the aims below, which I will address within this thesis. These aims are specific to each chapter, with the exclusion of Chapter 2, which dictates the materials and methods, and Chapter 7, which contains the overall discussion.

Aims:

- Chapter 1 and 3: To identify potential contaminants which would be necessary to remove from the BAL circuit so that plasma return to the patient is therapeutic, without potential harmful effects arising from the treatment
- Chapter 3: To define and develop suitable methodologies to assess relevant contaminants within both simple (e.g. saline) and complex (e.g. plasma) samples
- Chapter 4: To determine both the capability and the capacity required of the filtration system in order for it to meet regulatory requirements for use within patients
- Chapter 5: To test the filtration system on small and large scales *in vitro* to establish the parameters of its efficacy
- Chapter 6: To prepare for, and analyse, contaminant removal from porcine plasma when the safety circuit is used in a porcine pre-clinical model of acute liver failure

Chapter 2

Materials and methods

2. Materials and Methods

2.1 Monolayer HepG2 culture

HepG2 cells (ECACC Wiltshire) were used for cell culture work, unless otherwise stated. These cells comprise the biomass of the Liver Group BAL.

Materials

Complete culture media (values relate to the final concentration):

α- Minimum Essential Medium (MEM; Gibco #32571-028)
Foetal calf serum (FCS; PAA #A15-101)
Insulin (0.27 IU/mL)
Penicillin/streptomycin (45 U/ml [penicillin], 45 µg/ml [streptomycin])
Bovine serum albumin (BSA)/linoleic acid (0.05 mg/ml; Sigma #L9530)
Sodium selenite (0.017 µg/ml; Sigma #S5261)
Hydrocortisone (0.364 µg/ml; Sigma #H0888)
Thyrotropin-releasing hormone (TRH; 0.364 µg/ml; Sigma #P1319)
Fungizone (1.1 µg/ml; Gibco #15290-026)

Fresh frozen plasma media:

Fresh frozen plasma (FFP) was used instead of FCS in the complete culture media to produce FFP media. Heparin (40 IU/ml; Multiparin, CP Pharmaceuticals) was added to the FFP media to prevent clotting.

HepG2 Cells (passage 40–60)
Nunc™ cell culture flasks
Trypan blue (2% suspended in phosphate buffered saline [PBS]; Sigma #T-6146)
PBS (Gibco)

TrypLE™ Select (x1 concentration; Thermo Fischer #12563011)
Hank's balanced salt solution without calcium (HBSS; PAA #H15-010)

2.1.1 Cell seeding and passaging

HepG2 cells were seeded into cell culture flasks at a density of $1x10^6$ in 15 ml of supplemented media per T80 flask, $1.5x10^6$ in 30 ml media per T175 flask and $2x10^6$ in 100 ml media per TripleFlask. Flasks were laid flat in an incubator at 37°C in an environment of 95% air and 5% CO_2. Every 48 h the media was changed for pre-warmed complete media and cells were grown to 80% confluency. Cells were passaged, beginning with a 3x wash with HBSS to remove unattached cells (8 ml/T80; 15 ml/T170; 50 ml/TripleFlask). Pre-warmed (37°C) TrypLE select was filter sterilised into the flask (6 ml/T80; 10 ml/T175; 25 ml/Triple) covering the entire monolayer; the flask was incubated at 37°C for 10 minutes allowing monolayer detachment. Cell suspension was transferred to a 50 ml Nunc™ tube and an equal volume of pre-warmed complete culture media was used to rinse the flask removing any remaining cells. The solution was centrifuged at 272 RCF for 4 minutes at room temperature to pellet the cells. After removal and disposal of the supernatant the pellet was re-suspended in pre-warmed culture media (37°C; 2 ml/T80; 5 ml/T175; 10 ml/Triple) and passed slowly through a 21 G needle three times to disperse the cells.

2.1.2 Cell counts and viability

Trypan Blue was used to stain cells and analyse viability, with viable cells remaining colourless and non-viable cells taking up the Trypan Blue stain. Cell counts were performed using a haemocytometer and a light microscope under a magnification of x10. 20 µl 2% Trypan blue of was added to a solution containing 20 µl cell suspension and 160 µl HBSS. 9 µl of this solution was loaded into each side of a haemocytometer and cell counts were performed for each of the two sides, this was repeated three times. The readings were multiplied by $x10^5$ to estimate the

number of cells per ml, to take into account the multiplication factor of the cytometer (10^4) and the dilution factor of the cell suspension (1 in 10). Cell number and percentage viability were calculated from the average number of live and dead cells over the six total readings.

2.2 Production of the alginate encapsulated liver cells

The encapsulation of HepG2 cells within alginate spheres (ELS) was performed by members of the Liver Group, the fluidised bed bioreactor phase was also performed by other members of the Liver Group. In brief, HepG2 cells were cultured in a monolayer as described above until 80% confluency was reached. Cells were detached and encapsulated within 1% alginate spheres of approximately 500 µM diameter using a GeniaLab® Jetcutter™. This encapsulation was enabled by taking advantage of a crosslinking reaction in which the sodium ions within the alginate polymer chains are replaced with calcium ions enabling the joining of multiple polymer chains. Volumes of encapsulated cells in the range of 100 ml to 2 L were produced. These encapsulated cells were either cultured in a bioreactor, in which case the spheres were modified to contain 2% glass beads of 10–50 µm in size, to optimise movement of the beads within the fluidised bed bioreactor (FBB), or in static culture in T175 flasks. Under both culture conditions complete media was further enhanced by the addition of glucose (4.4 ml/ 500 ml media; Sigma #G8769).

2.2.1 Cell counts of cells in 3D culture

Materials

NucleoCounter® spectrophotometer (Chemometec, Sartorius UK)
NucleoCounter® software version 1.0
NucleoCasette™ (Chemometec)
Reagent A100 (Chemometec; #910-003)
Reagent B (Chemometec; #910-002)
16 mM EDTA/0.15M NaCl (pH 7.4; AppliChem #A1105)

HBSS without calcium (PAA #H15-010)

A NucleoCounter® spectrophotometer was used to estimate counts of cells within 3D culture by measuring nuclei number. ELS (250 µl) were placed into a 1.5 ml microfuge tube, washed twice with 1 ml HBSS and dissolved in 1 ml 16 mM EDTA/0.15 M NaCl (pH 7.4) for 5 minutes at room temperature. Once dissolved the cells were pelleted at 13000 x g for 5 minutes and the supernatant was discarded. The pellet was re-suspended in 500 µl phosphate-buffered saline and vortexed to re-suspend the individual cells. 500 µl of cell solution was transferred to a fresh microfuge tube to which 0.5 ml of reagent A100 was added, this was vortexed for 5 seconds followed by addition of 0.5 ml Reagent B and vortexing for a further 5 seconds prior to loading into a NucleoCasette™ and insertion into the NucleoCounter®. The dilution factor of the ELS in PBS was taken into account when calculating cell numbers. This cell count was performed 5 times and the mean and SD were calculated.

2.2.2 Calculating viability of cells in 3D culture

Materials

Fluorescein diacetate (FDA; 1 mg/mL; Sigma #F7378)
Propidium iodide (PI; 1 mg/mL Sigma #70335)
PBS supplemented with calcium (Sigma #D8662)

A total of 200 µl of ELS were placed in a 1.5 ml microfuge tube and washed with 1 ml of PBS. Following washing, ELS were suspended in 500 µl PBS to which 20 µl of PI and 10 µl of FDA were added, the solution was agitated and left for 90 seconds to allow cellular uptake of the stains. PBS was siphoned off using a pipette and cells were washed with 1 ml PBS to remove any excess stain, 500 µl PBS was used to re-suspend the cells. Stained ELS were transferred onto a microscope slide and observed using a Nikon® Eclipse microscope and DX1200 camera with NIS Elements analysis software. Excitation and emission filters of 465 and 515 nm, respectively, were used for FDA, and 510 and 590 nm, respectively,

for PI. For each repeat, two photographs were taken of the cells under the microscope, one using the FDA settings and one using the PI settings. By taking these two images of the same set of cells the pixel density obtained from each of these images could be used to calculate the percentage viability of the cells. The total FDA integral density was divided by the combined integral density of FDA and PI multiplied by 100. This was performed 5 times on separate images, with the mean and SD calculated from these repeats.

2.3 Methods involving the extraction and quantification of DNA

2.3.1 Mammalian crude DNA extraction for use in experiments

Materials

Lysis buffer pH 8.4: 200 mM NaCl
100 mM TrisHCL
5 mM EDTA
0.2% SDS

100% Propan-2-ol

Proteinase K (100 µg/5 ml lysis buffer)

Cells were cultured as described previously to 80%–100% confluency in a Nunc™ Triple, T175 or T80 flask. Culture media was removed and cells were washed 3x with HBSS (8 ml/T80; 15 ml/T175; 50 ml/Triple). An equal ratio of lysis buffer for each flask size was added along with 100 µg lyophilised proteinase K /5 ml lysis buffer. The solution was incubated for 4 hours at 37°C. Alternatively, excess cells obtained from passaging were centrifuged at 1200 RPM for 4 minutes and the supernatant was discarded, 10 ml of lysis buffer containing 20 µg lyophilised proteinase K/1 ml cell pellet was added, incubating for 4 hours at 37°C. An equal volume of propan-2-ol to lysis buffer was added and mixed gently for 5 minutes, the solution was transferred to a 50 ml Nunc™ tube where the resulting DNA aggregate was lifted above the liquid level using a pipette and allowed to dry for 15

mins. The aggregate was transferred to a fresh PCR grade microfuge tube and re-suspended in PCR grade water via pipetting and vortexing. A heated block at 37ºC was used for 15 minutes if necessary to encourage re-suspension of DNA. Concentration and total quantity of DNA obtained was assessed using NanoDrop analysis.

2.3.2 NanoDrop analysis of DNA concentration

Materials

PCR grade water

NanoDrop 1000 spectrophotometer

NanoDrop analysis software version 3.8.1

A NanoDrop spectrophotometer was used to assess the concentration of DNA in samples re-suspended in water, observing absorbance ratios at 260/280 nm and 260/230 nm. A 1.5 µl sample was used for each analysis and repeats of N=5 were performed unless a wide variance was observed in which case this number was increased. The purity of the sample was also assessed for protein and chemical contamination via the 260/280 nm and 260/230 nm ratios, respectively, with ratios of 1.8 and 2.0 deemed pure. NanoDrop software was used to collect results. The mean and SD was used to gauge total concentration.

2.3.3 Quantitative Polymerase Chain Reaction (qPCR)

2.3.3.1 Preparation of proteinase K aliquots

Materials

Proteinase K (Sigma Aldrich #P2308)

TTE buffer:

50 mM Tris base

1 mM EDTA

2.5% Tween 20

Lyophilised proteinase K was re-suspended in TTE buffer at a concentration of 1 mg/ml. Aliquots containing 32 µl of the proteinase K suspension were transferred to a lyophiliser for 4 hours, or until the sample was fully dehydrated. Proteinase K aliquots were stored at –20 ^{o}C until further use. Upon use, the powder was re-suspended in an appropriate volume of TTE buffer and mixed using a pipette.

2.3.3.2 Plasma pre-treatment

Materials

PCR grade water

TTE buffer:

50 mM Tris Base

1 mM EDTA

2.5% Tween 20

Proteinase K

Samples for qPCR analysis were diluted 1 in 500 in PCR grade water. 20 µl of sample was added to 20 µl of TTE buffer containing 3.2 µg of proteinase K. Samples were heated at 50°C for 2 hours. TTE buffer (160 µl) was added to the sample and transferred to a block set at 95°C for 15 minutes to denature the proteinase K. The sample was briefly centrifuged to collect droplets of condensation (in samples where protein contamination was low this pre-treatment step was omitted and the sample was diluted directly to 1 in 5,000).

2.3.3.3 qPCR analysis of DNA concentration

Materials

<u>PCR mix (Per 20 µl tube)</u>

10 µl Hot start Taq (Qiagen 203205)

1 µl 115 base pair forward primer 0.0025 µM
(5'CCTGAGGTCAGGAGTTCGAG 3')
1 µl 115 base pair reverse primer 0.0025 µM
(5'CCGGAGTAGCTGGGATTACA 3')
0.5 µl Sybr Green 1 in 20,000 dilution (Biogene 1765)
2.5 µl 0.025 M $MgCL_2$ (Sigma M1028-1M)

QPCR with 1 set of Alu repeat primers, engineered to produce 115 base pair Alu repeat amplicons, was used to assess DNA quantity in water, saline, plasma or culture media samples. The qPCR reaction was performed in duplicate or triplicate in 20 µl PCR tubes, each tube contained 15 µl of PCR mix and 5 µl of sample. Samples were run against a 5 point standard curve containing HepG2 DNA, isolated using the protocol above, logarithmically diluted in the range of 0.02 to 200 pg/µl to determine the absolute DNA concentration in each sample. Samples were analysed on the Rotor Gene™ 3000 PCR machine using the following cycle:

95°C 15 minutes hold
95°C 15 seconds
64°C 30 seconds
72°C 30 seconds
} 40 Cycles
72°C 10 minutes*

*A melt curve was performed where the temperature increased from 45°C to 95°C at a rate of 1°C per minute.
As each plasma sample was diluted 1 in 5,000 this technique provided a limit of detection of 0.1 ng/µl within plasma samples.

2.4 Methods involving the extraction and quantification of endotoxin

2.4.1 Extraction of endotoxin from bacterial culture

Materials

E.coli stock

LB Broth

SDS buffer pH 6.8

100 mM TrisHCL

5 mm EDTA

4% SDS

Proteinase K (10 mg/ml, Sigma Aldrich)

DNAse (10 mg/ml; Sigma Aldrich)

RNAse (10 mg/ml; Sigma Aldrich)

Propan-1-ol (Sigma Aldrich)

On overnight culture of E.coli 5 ml of Luria Broth (LB) was started by inoculating 5 ml of LB with a single E. coli colony grown on LB media on a petri dish, this inoculation was incubated in a shaking incubator at 37 °C and 200 rpm. The process was repeated, upscaling to gain 4 litres of E.coli stock. The bacteria were pelleted in a micro centrifuge at 10,600 x g for 10 minutes, followed by lyophilisation of the pellet. The lyophilised pellet was ground to a fine powder with a pestle and mortar, to this SDS buffer and proteinase K were added. This solution was heated at 60^{o}C for 3 hours. Propan-1-ol was added to the solution, vortexed and samples were sonicated in a 37^{o}C water bath for a further 15 minutes at 65^{o}C. Samples were centrifuged at 20,600 x g for 10 minutes, the pellet was further processed twice with propan-1-ol, repeating the centrifuge steps to remove contamination. Endotoxin was re-suspended in saline and the concentration was measured using the PyroGene recombinant Factor C assay.

2.4.2 PyroGene recombinant Factor C assay of endotoxin concentration

Materials

PyroGene® recombinant Factor C assay (Lonza 50-658U)

- Recombinant Factor C enzyme solution (R50-658)
- Fluorogenic substrate (S50-658)
- Assay buffer (B50-658)
- E. coli endotoxin standard (O55:B5, E50-643)
- LAL reagent water (W50-640)

0.1 M NaOH in 60% ethanol

10ml endotoxin free glass tubes, baked for 3 hours at 250°C

Endotoxin free, sterile filtered pipette tips (Starlabs®)

Endotoxin free, sterile clear 96 well plate (Nunc®)

Hyclone tissue culture water endotoxin free

Plate sealer

Fluorescence microplate reader, FLUOstar Omega® (BMG Labtech)

MARS data analysis software (BMG Labtech) version v3.01 R2

A Class 2 microbiological safety cabinet was used to perform all endotoxin assays to ensure minimal contamination. All glassware was baked at 250°C for 3 hours before use. Prior to use the tissue hood was sterilised using UV-light and trigene following which surfaces were exposed to 0.1M NaOH in 60% ethanol for 6 hours after which surfaces were wiped with endotoxin-free water. This was performed to break down any LPS molecules and ensure all surfaces were free from endotoxin contamination.

Endotoxin standards were prepared using the reconstituted lyophilised Lonza endotoxin standard to gain logarithmic dilutions of 10, 1, 0.1, 0.01 and 0.001 EU/ml. Plasma samples were prepared for analysis by dilution of the sample 1 in 200 in endotoxin-free water followed by heat treating at 70°C for 20 minutes. Culture media samples were diluted 1 in 10 followed by heat treating at 70°C. Saline samples were analysed neat. Samples for assessment were snap frozen upon

collection and stored at –80ºC until required for analysis, at which point they were defrosted at room temperature within the tissue cabinet. The PyroGene® recombinant Factor C assay, including endotoxin standards, was cold-stored, and used within four weeks of opening, as per the manufacturer's instructions, excluding the fluorescence buffer which, once prepared, was used immediately.

To ensure separation of endotoxin and accurate sample reading, each sample and standard were vortexed for 1 minute prior to addition to a 96-well plate. 100 µl of samples, standards and blank were added to the 96-well place in triplicate and incubated for 10 minutes at 37°C. Whilst the plate was incubating the fluorescence buffer was prepared in the following ratio:

1x fluorescence enzyme
4x Assay buffer
5x Fluorogenic substrate

At timed intervals 100 µl of fluorescence buffer was added to each well and fluorescence was measured immediately at 37°C using 380 nm excitation and 440 nm emission wavelength using the Omega Labtech Fluorostar plate reader to gain a reading for blank fluorescence. The plate was incubated within the plate reader for one hour at 37°C after which the fluorescence was reanalysed. Mars data analysis software was used to export the data. The initial blank values were subtracted from the one-hour reading values to normalise results. Endotoxin concentration was determined by plotting a standard curve. The dilution effect for plasma and culture media samples was taken into account when calculating the final endotoxin concentration in EU/ml.

2.5 Particulate analysis

Particle analysis was performed using one of three methods, NanoSight, Mastersizer or Zetasizer depending on the particulate burden and size distribution

of the sample in question. A detailed breakdown of the measurement limits and characteristic for each measurement method can be seen in Table 2.

2.5.1 Sample preparation

For some particulate analysis techniques particle density within a sample was required to meet a certain threshold, therefore, sample preparation was performed to reach this density. Samples were pooled via aliquoting 2 ml into a microfuge tube and centrifuging at 16,100 RCF (13,200 RPM) in an Eppendorf centrifuge 5415R. Supernatant was carefully removed with a pipette and a further 2 ml of sample was added to the same tube, the process was repeated until the desired concentration was obtained. Depending on sample type, a wash step was performed. For media samples this consisted of adding 2 ml MilliQ water to the tube, re-centrifuging and removing the supernatant. A saline wash in the same manner preceded this for plasma samples. Alginate samples were not washed. Samples were re-suspended in a volume of 0.5–1 ml sterile filtered water. The original sample volume and volume of water used to re-suspend were recorded allowing the original sample concentration to be determined.

2.5.2 NanoSight nanoparticle tracking analysis

Materials

NanoSight Optical system

NanoSight nanoparticle tracking analysis (NTA) analysis software version 2.3

Liquid samples of 0.2 ml were analysed using the NanoSight particle sizing optical system. To gain accurate particle concentration, a particle density above $1x10^7$ particles/ml was required. Particles were observed under Brownian motion to distinguish individual particles from background noise. The particle size range for this technique is 0.01–2 μm (Table 2)

2.5.3 Mastersizer laser diffraction

Materials

Mastersizer 2000 laser system

Mastersizer 2000 operating software version 5.60

Liquid samples of 80 ml were analysed directly, without concentration, using the Malvern Mastersizer particle sizer. This technique uses laser diffraction and obscuration to calculate the size of particulates within a sample in a range of 0.02 to 2000 µm (Table 2).

2.5.4 Zetasizer dynamic light scattering

Materials

Zetasizer Nano S series

Zetasizer analysis software version 7

Liquid samples of 0.5 ml were assessed for particle distribution using the Zetasizer Nano series particulate sizer. This technique uses dynamic light scattering to gain an average particle size in terms of the Z-average (overall average size) for the sample and can detect particles in the range of 0.003–10 µm (Table 2).

Table 2. An overview of methods for the analysis of particles in liquid media.

Method	Technique	Quantitative vs. Qualitative	Volume required	Particulate size	Particulate concentration required for analysis	Sample pre-treatment
NanoSight	Optical microscopy	Quantitative	0.3 ml	0.01–2 µm	$>1x10^3$	Multiple centrifuge
Mastersizer	Laser diffraction	Qualitative	70 ml	0.02–2000 µm	Defined as obscuration threshold	Neat sample
Zetasizer	Dynamic light scattering	Qualitative	0.5 ml	0.003–10 µm	$\sim>1x10^5$	Multiple centrifuge

2.6 Biochemical assays

2.6.1 Enzyme-linked immunosorbent assays

A sandwich enzyme-linked immunosorbent assay (ELISA) was used to assess the albumin, α-1-antitrypsin, α-1-glycoprotein and fibrinogen concentration pre- and post-filtration.

Materials

Primary antibody (Albumin: Dako cat# A0001, polyclonal rabbit)

Secondary HRP-linked antibody (Albumin: Abcam cat # ab24458-200, mouse anti-human serum albumin)

96 well Nunc Immuno coated plates (Fisher cat. # DIS-971-030J)

Coating buffer pH 9.7

0.318 g Na_2CO_3

0.586 g $NaHCO_3$

200 ml distilled water

PBS-Tween pH 7.4

40 g NaCL

1 g KCl

7.2 g $Na_2HPO_4.2H_2O$

1 g KH_2PO_4

5000 ml distilled water

500 µl Tween 20

Blocking buffer

1.25 g powdered milk

25 ml PBS tween

OPD solution

2 OPD tablets (Dako #s204530)

12 ml distilled water

6 µl H_2O_2

Stopping reagent

1 M H_2SO_4

The primary antibody was diluted in coating buffer to give a final concentration of 10 µg/ml, 100 µl was added to each well of an Immuno 96 microwell plate. The plate was wrapped in cling film and left refrigerated overnight at 4^{o}C.

The plate was washed 3 times with PBS Tween and left dry briefly using a plate washer. Binding sites were blocked by adding 100 µl blocking buffer and incubating at room temperature for 1 hour. 100 µl of each sample (diluted in PBS), standard (200 ng/ml; 100 ng/ml; 50 ng/ml; 25 ng/ml; 12.5 ng/µl; 6.25 ng/µl) and quality control were added in triplicate, followed by incubation at 37^{o}C for 90 minutes. Quality control was produced internally using 24 hour conditioned media from 70% confluent HepG2 cells.

The plate was washed 3 times with PBS Tween and left dry using the plate washer. The secondary antibody was diluted in blocking buffer to give a final concentration of 0.5 µg/ml. 100 µl of this solution was added to each well and the plate incubated on the bench for a further hour. The plate was washed 5 times with PBS tween and left dry.

A total of 100 µl OPD solution was added to each well at timed intervals to begin the colour development reaction. The plate was incubated at room temperature under tin foil until sufficient colour had developed. 50 µl of 2 M sulphuric acid was added at timed intervals to stop the reaction, inhibiting further colour development. The absorbance intensity was read at 492 nm using a Manta plate reader. A standard curve was generated and used to determine the concentration of each sample.

2.6.2 Biochemistry analysis of plasma samples

Samples collected from experiments were analysed for the concentration of specific biological molecules (proteins, lipids and ions), in addition to the ELISAs described above.

These additional analyses included: proteins, aspartate transaminase; alanine transaminase; IgG; IgM; IgA; ions, calcium, phosphate and sodium; lipids, HDL, LDL and total triglycerides; glucose; bilirubin. These were measured using biochemistry analyses. These assays were kindly performed by individuals at the Clinical Biochemistry department at the Royal Free Hospital, London.

2.7 Plasma Preparation for use in experiments

To prevent plasma clotting during filtration experiments heparin sodium was added to plasma at a concentration of 20 units (U)/ml. In plasma collected from the Cobe Spectra plasmapheresis machine (apheresis machine) or FFP, a citrate assay was performed to analyse the quantity of citrate present in the plasma sample. A sufficient quantity of $CaCl_2$ was added to the plasma to counteract the effect of the citrate. Heparin was added prior to this to ensure the plasma did not clot. In the case of plasma collected from patients with acute liver failure, these procedures were followed along with the use of full personal protective equipment to ensure safety.

2.7.1 Citrate assay

Plasma used for these experiments was either from patients undergoing total exchange using either an apheresis machine for clinical purposes or through donations of FFP. For the majority of experiments, plasma was assessed for clarity and visually cloudy plasma was rejected as this was indicative of high cell death typically following a specific treatments that may have been undergone by the patient prior to plasmapheresis. Plasma obtained from these instances caused the

filters to prematurely block, which is why this plasma was rejected. Each batch of plasma obtained was analysed for citrate levels. Citrate was neutralised prior to use with alginate ELS, as citrate chelates calcium ions, incubating the ELS in plasma containing citrate would cause the migration of calcium ions from the ELS into the plasma, causing the ELS to dissolve.

Materials:

Biosentec citrate assay Kit

1 M Perchloric acid

UV Spectronic UNICAM Spectrophotometer

Vision 32 software

Protein was removed from plasma samples prior to performing the citrate assay, 1 volume of sample was mixed with 1 volume 1M Perchloric acid and centrifuged for 5 minutes at 10,000 x g at room temperature. The supernatant was diluted to a range of 0.03-0.4 g/L citrate. Disposable cuvettes were used to analyse the sample, reagents were added to the cuvette in volumes following the table below.

Citrate assay reagent volumes, all volumes are per 1.5 ml cuvette.

	Blank	**Sample**
R1 (30 ml – Buffer pH 7.8)	0.5 ml	0.5 ml
R2 (6 ml – NADH 10 mg)	0.1 ml	0.1 ml
R3 (0.6 ml - L-MDH 270U / L-LDH 565U)	0.01 ml	0.01 ml
Water	0.5 ml	0.45 ml
Sample	0	0.05 ml
Mix and read* at 340 nm	$OD1_{Blank}$	$OD1_{Sample}$
R4 (3.2 ml – CL 10 U)	0.01 ml	0.01 ml
Mix, wait 10 min and read* 340 nm	$OD2_{Blank}$	$OD2_{Sample}$

*Before the addition of R4, absorption was measured at 340 nm by performing a scan from 300–400 nm, this was taken as the blank value. Following this the sample was mixed and incubated at room temperature for 10 minutes followed by a second scan (sample scan). This was performed in triplicate for each sample.

The values of optical density OD1 and OD2 for the blank and each sample were used to calculate citrate concentration via the formula below providing a concentration in g/L:

$$\text{Citrate g/L} = ((OD1_{Sample}\text{-}OD2_{Sample})\text{-}(OD1_{Blank}\text{-}OD2_{Blank}) \times 0.68302) \times 2$$

The dilution factor of the sample was accounted for in the end calculation. The mM of citrate present in the sample were calculated (1 mM citrate = 192.1 mg/l).

2.7.2 Calcium requirement for citrated plasma

Materials

1 M Calcium chloride (CaCl) sterilised

3 moles of calcium bind 2 moles of citrate, therefore, a sample containing 2 mM of citrate must be neutralised with 3 mM of calcium. In addition to this a further 1.56 mM CaCl was added to maintain ELS integrity.

2.7.3 Heparin addition to plasma

Materials

Heparin sodium 5000 IU/ml (Wockhardt UK Ltd)

To prevent plasma clotting during filtration experiments, heparin sodium was added to plasma at a concentration of 20 U/ml prior to addition of CaCl.

2.7.4 Heparin analysis using activated partial thromboplastin time (APTT) in plasma samples

The concentration of heparin present in saline and plasma samples was analysed using the APTT assay. This was kindly performed by members of the Haematology department at the Royal Free Hospital, London. The plasma used for these experiments was FFP (plasma from healthy patients to ensure normal clotting activity), further processing was performed to deplete this plasma of platelets by centrifugation twice at 2000 x g for 12 minutes.

Briefly, plasma samples for analysis using APTT were collected in citrated blood collection tubes. Plasma was incubated at 37°C with cephalin, a phospholipid and kaolin, a contact activator, calcium was added to initiate the clotting cascade. The time between the addition of calcium and the formation of a fibrin clot is recorded and a measure of heparin concentration is achieved using a reference database specific for each laboratory.

2.7.5 Spiking of plasma samples with DNA

Materials

FFP
Apheresis System isolated plasma
Bioline human genomic DNA (#BIO-35025)
Isolated DNA as per protocol above

Neat plasma samples, either FFP or obtained from the Apheresis System were spiked with known quantities of DNA and vortexed briefly to mix. Samples were incubated for 1 hour prior to use to allow time for any inhibitory effect of the plasma to manifest.

2.7.6 Spiking of plasma samples with endotoxin

Materials

FFP

Apheresis System isolated plasma

E. coli O55:B5 endotoxin (Lonza)

Isolated endotoxin as per protocol above

Neat plasma samples, either FFP or from the Apheresis System were spiked with known quantities of endotoxin and vortexed for 1 minute to mix. Samples were incubated for 1 hour prior to use to allow time for any inhibitory effect of the plasma to manifest.

2.8 Assembly of Filtration circuit

2.8.1 Pre-use treatment of filters

Prior to use the upstream capsule filters were autoclaved for 30 minutes at 126°C and rinsed with sterile filtered water. The volume of water required for rinse is proportional to filter size at 54 $L/m^{2.}$ A breakdown of quantities required can be seen in Table 3. The filter must not contain air during use, therefore, on addition of water, the output was clamped off and a syringe placed into the outlet port whilst the upper void of the filter was filled using a flow rate of 90 ml/min. Once air was clear of the top side of the filter, the pump was paused and syringe removed and replaced with a male cap; the clamp downstream of the filter was removed and the pump restarted, allowing the water to pass through the filter media.

The downstream filter did not require a pre-use rinse; it was supported via a clamp stand. Both filter types were rinsed with sterile saline prior to addition of plasma, ensuring the media in both filters was fully wetted prior to use, and no air entered the system. Both models of filter were used in a single direction of flow.

2.8.2 Filter circuit set-up

The circuit was set up with either filter individually or with both filters in series in a sterile environment (Class 2 safety cabinet). 4.8 mm internal diameter 1.6 mm wall thickness tubing was used to connect pressure transducers and filters to each other and the outlet. This tubing was connected to 2.4 mm wall thickness Santoprene tubing used in the pump head itself. The filters were connected via a sanitary fitting flange, fitted with a silicone O ring, and held together using a reusable nylon flange clamp. Pressure transducers were attached to the circuit via male and female luer fittings. The set-up of the filters and circuit varied depending upon the purpose of the experiment (see Figure 5, Figure 6, Figure 7 and Figure 8).

2.8.3 Running the filter system

Plasma or culture media was pumped through the filters at a flux of 0.176 ml/min/m^2, pressure and flow rate were observed at regular intervals throughout the experiment. T-piece connectors were used in various places for sample collection.

Table 3. Filter size, relevant pre-use rinse and maximum usage parameters:

Downstream 60ZB05A scalable capsule filter surface area size	**Volume of sterile filtered water required for rinse (ml)**	**Maximum flow rate (ml/min)**	**Maximum differential Pressure (mmHg)**
25 cm^2	135	115	1,012
170 cm^2	918	-	1,800
340 cm^2	1,836	-	1,800
1020 cm^2	5300	-	1,800

2.8.4 Small-scale filter circuit set-up

Plasma flowed from a reservoir through the Watson Marlow peristaltic pump, the upstream 25 cm^2 depth charge filter and mini downstream 0.6µm porosity filter in series and was subsequently collected via within an outlet reservoir. Pressure was recorded prior to the upstream filter and at the output of the downstream filter to calculate pressure drop across the two filters (Figure 5).

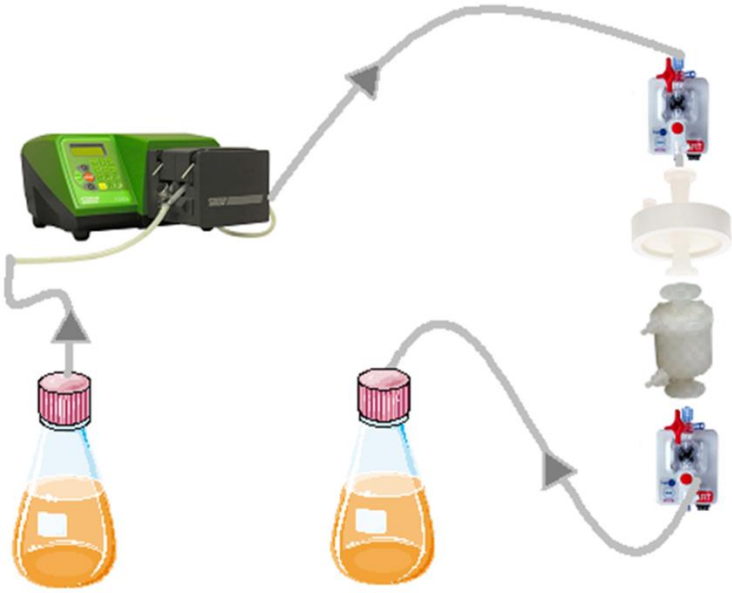

Figure 5. Set-up of the small scale filtration circuit. *A small scale circuit was used to assess filter efficacy using small quantities of plasma.*

2.8.5 Set up of the mini columns and scaled-down bioartificial liver circuit

Materials

2 Watson Marlow multi-channel pumps
ELS or empty alginate beads (control)
Plasma (FFP; liver failure), culture media or saline

The mini columns were set up as depicted in Figure 6 and Figure 7. Briefly, a reservoir containing plasma was placed beneath the mini columns, plasma was pumped from the reservoir up through the ELS-containing mini column, fluidising the cells, the mini column output was split into two circuits, one returning to the reservoir at high speed, the other a low flow rate filtration circuit, for this circuit the plasma passed through a pressure transducer prior to flowing through the upstream 25 cm^2 filter, a second pressure transducer was placed before the downstream 0.6 µm filter. A second sample port was present after the downstream 0.6 µm filter before the plasma returned to the reservoir.
A multi-channel Watson Marlow pump was used to run the mini column circuit. A linear flow rate of 0.6 mm/second was calibrated prior to each use to enable sufficient fluidisation of the ELS within plasma.

A second Watson Marlow pump was used to power the filtration circuit. This circuit was calibrated to ensure that a flux of 0.176 $ml/min/m^2$ over the filter was used. The upstream 25 cm^2 and mini downstream 0.6 µm filters were used for these mini column experiments.

Each column was primed with media or plasma prior to addition of the ELS. The ELS were washed in the same solution as was in the column. Time 0 samples were taken after incubation of the ELS in plasma for 10 minutes to allow equilibration of ELS with the plasma. Throughout the experiment, samples were taken through the septum at sample port 1 and sample port 2 (pre and post filter; Figure 6) using a needle, samples were snap frozen in liquid nitrogen using cryovials for analysis of biological components (proteins, ions and lipids) and introduced contaminants (DNA and endotoxin) at a later date.

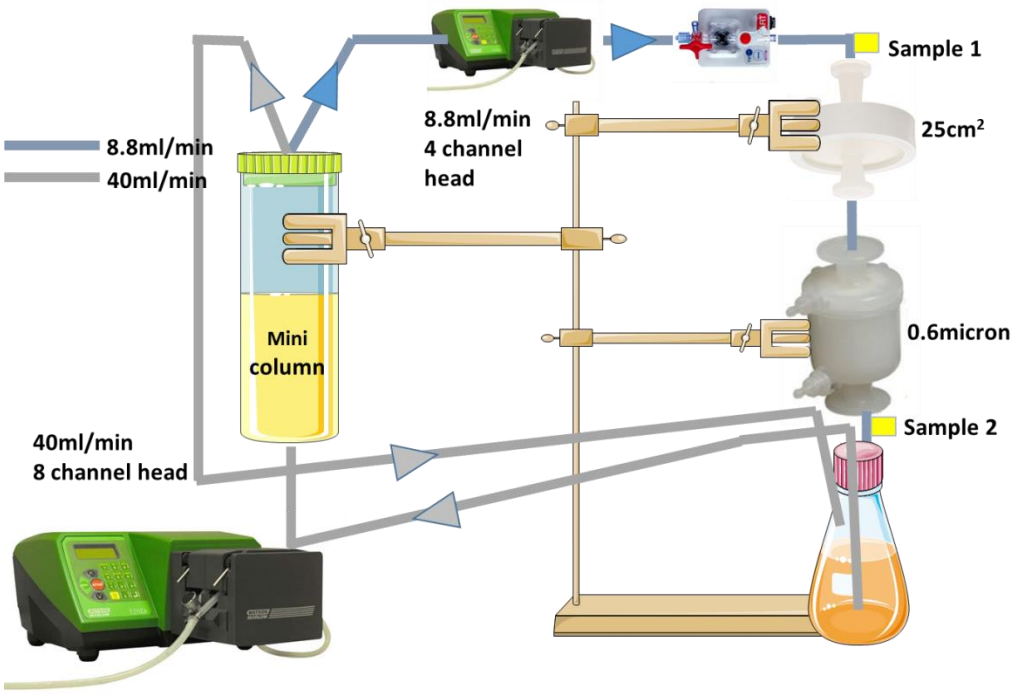

Figure 6. Small scale bioartificial liver mini column filter circuit. *A scale model of the filtration circuit was used to assess filter efficacy. Plasma was pumped through the mini columns containing encapsulated liver cells, mini column output was split into two streams, one directly linked to the reservoir and the other passed through the filter circuit, containing the upstream 25 cm^2 and downstream 0.6µm filters.*

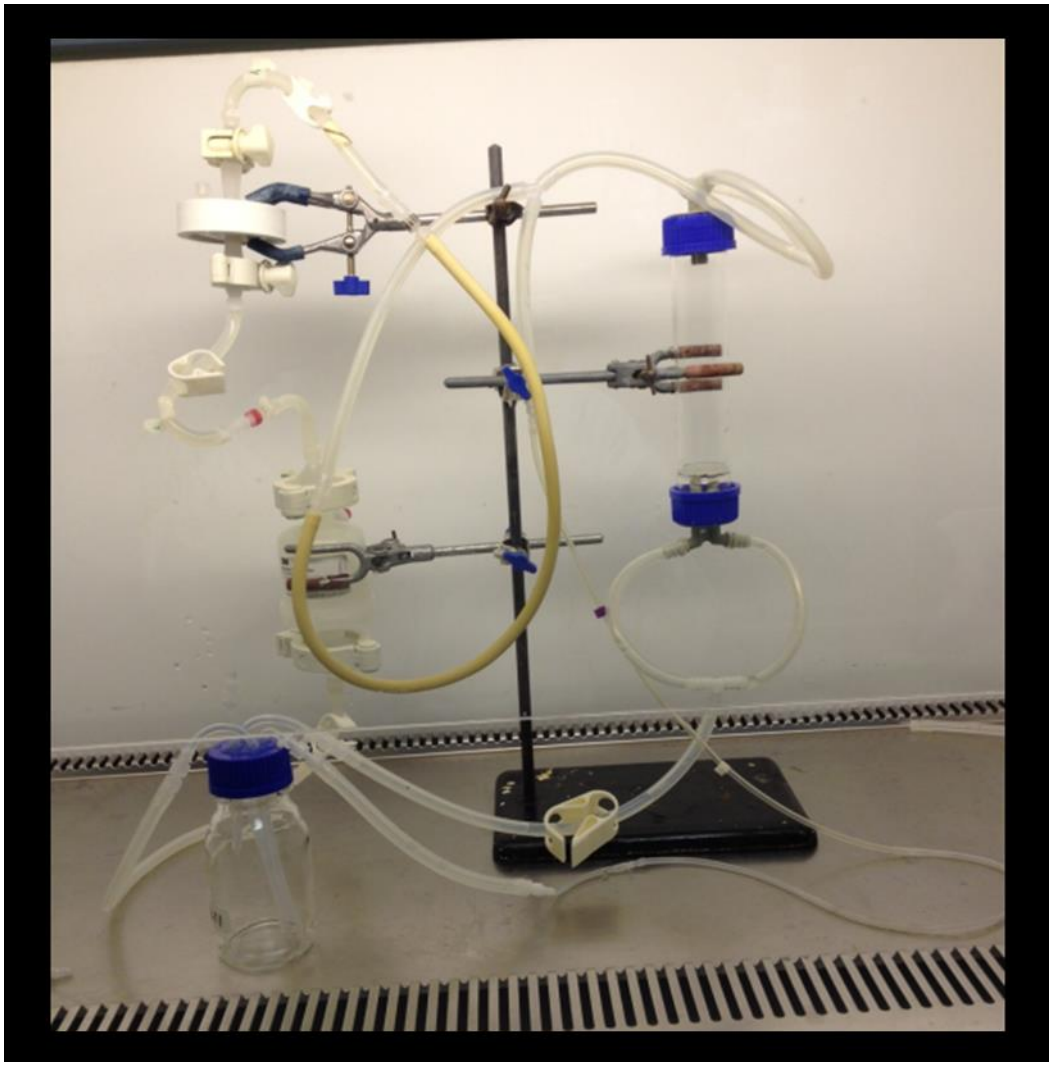

Figure 7. Mini column experiment set-up. *ELS were fluidised in the mini column using plasma, prior to the plasma being transferred to the filter circuit containing the upstream 25 cm^2 and downstream 0.6µm filters.*

2.8.6 Filtration circuit set-up in line with the bioreactor

The filter circuit was set up with the bioreactor in place (see Figure 8). A Y-piece was used in the top of the bioreactor to separate the output into two, one to recirculate back to the reservoir at a high flow rate and the other through the filtration circuit at 90 ml/minute prior to returning to the reservoir (patient). Blood filters were used in the top of the bioreactor to prevent any ELS entering the filter system. Sample ports in the form of T pieces were added with sterile septa connectors to enable sample collection.

A bioreactor infected with bacteria was used to test the capacity of the upstream depth charge and downstream filters. The biomass was acidified to pH 2 for 1 hour with HCl to kill any bacteria followed by neutralisation with NaOH. Plasma was pumped through the chamber and around both circuits at an initial rate of 90 ml/min. Samples were taken from the initial plasma before contact with the chamber, post-chamber pre-filters, post-upstream filter, post-upstream and downstream filters and post-both filters downstream for both DNA and endotoxin analysis.

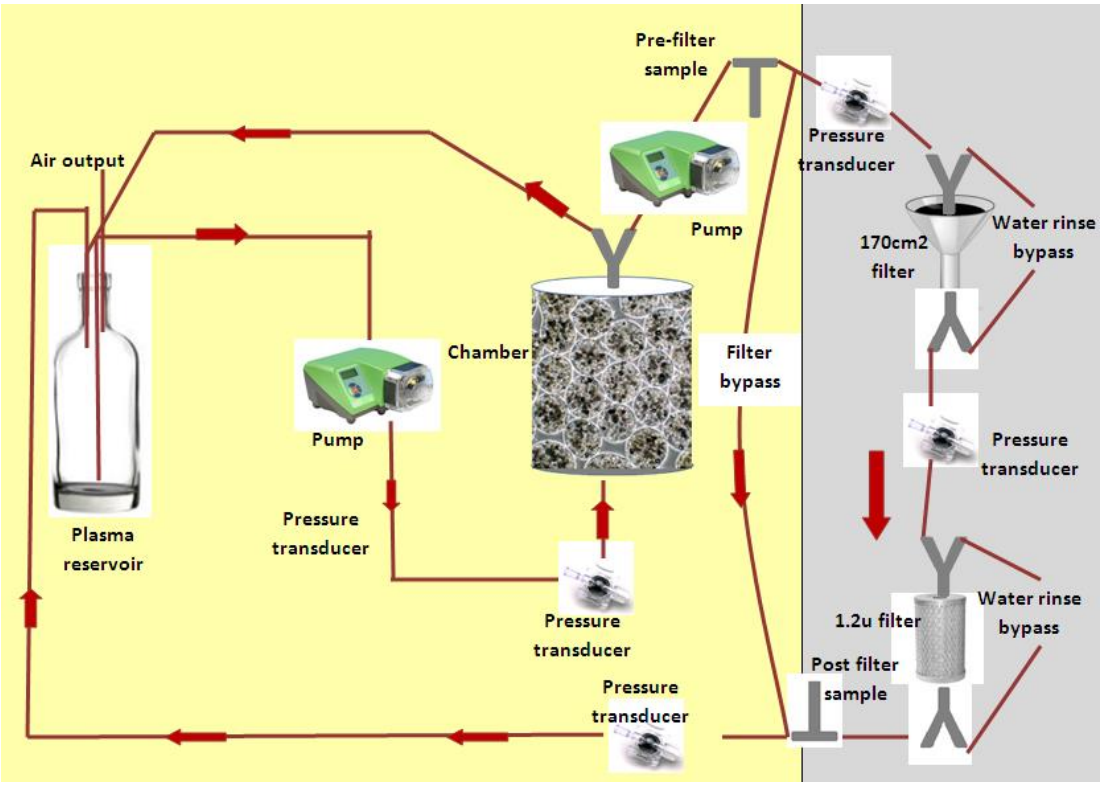

Figure 8. Set up of the filter series within the bioartificial liver (BAL) circuit. *The BAL chamber is fluidised via a pump inlet; two outlet ports are generated at the top of the chamber- one returning to the reservoir, one passing through the filter circuit where pressure transducers are connected to continuously measure the pressure drop across the filters. Sample ports are present to sample at different points in the circuit. The yellow half of the image depicts the BAL set-up and the grey depicts the filter safety circuit set-up.*

2.9 Statistical analysis

Sample replicates are stated within the figure legends throughout this thesis. The majority of data are presented as mean +/- SD unless stated otherwise. Where results refer to the absolute concentration of samples, these data have taken into account the dilution factor of said samples and present the results as would be seen in the neat sample.

Microsoft Excel was used to collect data, in the case of calculating concentrations of substances based on a standard curve, this was also performed in Excel; data were exported to IMB SPSS, which was used to perform statistical analysis.

In the case of comparing data for two variables, these data were analysed for statistical significance using either the paired two-tailed Student's T-test or the unpaired two-tailed Student's T-test, depending on the form of the data. Where more than two variables were to be compared, a one-way analysis of variance (ANOVA) was performed to assess statistical significance between and within variables, this was followed, as needed, by *post hoc* analyses using the Bonferroni procedure to determine where the significance, if any, lay. The choice of statistical analyses is specified along with the P-value in figure legends, where appropriate.

In Tables and Figures, statistically significant P-values are expressed using an asterisk (*), this refers to significance at a level of $P<0.05$. Where there were not enough materials available to enable multiple repeats of experiments to be performed, for example the lack of available liver failure plasma samples, this has been stated; analysis for statistical significance in this instance was not calculated, this has been made clear in any conclusions drawn from these data.

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Chapter 3

Development of assays for the detection of DNA and endotoxin in plasma

3. Development of assays for the detection of DNA and endotoxin in plasma

3.1 Introduction

To enable the characterisation of a filtration protocol for incorporation within the Liver Group extracorporeal BAL circuit, assays for the detection of any potential contaminants in the form of HepG2 DNA originating from biomass must be developed.[20] As this filtration protocol will also be assessed for the removal of endotoxin originating from the patient, an efficacious assay is also required to detect endotoxin.[33,102]

These DNA and endotoxin assays need to be suitable for use in both simple (e.g. saline) and complex (e.g. plasma) samples. Additionally, these assays are required to have a detection level sufficient to meet regulatory requirements for the BAL to be considered for use in humans.[103,104,105]

Although assays for the detection of both DNA and endotoxin exist, and currently many different options for analysing these substances are available, mixed results are reported when these are used in human plasma samples. Human plasma, being a highly diverse mix of various proteins, cytokines and salts, contains many inhibitors for various reactions; as such, true detection of contaminants in this media is a difficult task.[46,57]

This chapter will discuss the characterisation of DNA and endotoxin assays to determine their suitability for use in the assessment of the filtration system. It will discuss the identification of optimal assays, their further characterisation and enhancement through the use of sample pre-treatment. The overall aim is to gain the most sensitive limit of detection possible, whilst maintaining the integrity and consistency of the selected assays.

3.2 Materials and Methods

3.2.1 Detection of DNA using the Agilent TapeStation system

Materials

Agilent genomic DNA screen tape
Agilent 2200 TapeStation
Agilent 2200 TapeStation software version A.02.02
PCR grade water
Sample buffer

The Agilent 2200 TapeStation was used to assess the quantity and fragment size of DNA in diluted plasma samples. This assay separates DNA by molecular weight using electrophoresis, the software compares the bands produced against a sample of known concentration and size, from 10 pg/µl–1000 pg/µl. Doubling dilutions of plasma in PCR grade water from 1 in 2 down to 1 in 8192 were assessed. A total of 1 µl of sample and 10 µl of sample buffer were added to 0.1 ml PCR tubes, centrifuged briefly and vortexed for 5 seconds followed by a further centrifuge to pellet any droplets. These samples were processed using the Agilent genomic DNA screen tape. Results were analysed and DNA concentration calculated from the DNA ladder using the Agilent 2200 TapeStation analysis software.

3.2.2 Detection of endotoxin using the PyroGent assay

Materials

PyroGent-5000 kinetic turbidimetric LAL assay (Lonza, N283-06)
- PyroGent -5000 LAL reagent
- PyroGent-5000 LAL reconstitution buffer
- E. coli O55:B5 endotoxin

0.1 M NaOH in 60% ethanol
10 ml endotoxin-free glass tubes, baked for 3 hours at 250°C

Endotoxin free, sterile filtered pipette tips (Starlabs®)
Endotoxin free, sterile clear 96 well plate (Nunc®)
Hyclone tissue culture water, endotoxin free
Plate sealer
Fluorescence microplate reader, FLUOstar Omega (BMG Labtech)
MARS data analysis software (BMG Labtech; version 2.10)

The PyroGent assay was used to assess the quantity of endotoxin present in plasma samples. In the presence of endotoxin, reagents within the PyroGent assay clot, causing the sample to turn cloudy. The time taken for this to occur is used to calculate endotoxin concentration in samples using a known standard curve. A Class 2 biological safety tissue cabinet was used to perform all endotoxin assays, and was decontaminated as described in Chapter 2. Endotoxin standards were prepared using the Lonza endotoxin standard to gain concentrations of 10, 1, 0.1, 0.01 and 0.001 EU/ml. Samples to be analysed were diluted to an appropriate concentration in endotoxin-free water. To ensure even dispersion of endotoxin in the sample, each sample and standard were vortexed for 1 minute prior to addition to a 96-well plate.

The plate reader was set to specific measurement parameters:

Change in time (seconds): 60
Measurement filter (nm): 340
Change in measure of optical density: 30
Number of reads: 100

100 µl of samples, standards and blank were added to the 96-well place in triplicate, avoiding the introduction of bubbles as this would disrupt the assay. The plate was incubated for at least 10 minutes at 37ºC. Whilst the plate was incubating, the reagent buffer was re-suspended, 100 µl was added to each well. Measurements were started and the plate was left in the plate reader for 1 hour and 40 minutes until each of the 100 measurements had completed. Endotoxin concentration was determined from the standard curve, with time taken for the solution to become turbid inversely proportional to endotoxin concentration.

3.2.3 Perchloric acid treatment of plasma samples

Materials

0.2 M perchloric acid (Sigma Aldrich #311421)

0.2 M NaOH (Sigma Aldrich #795429)

15 mM tris-HCL (Sigma Aldrich #T5941) pH 7.5

Samples were treated with an equal volume of 0.2 M perchloric acid and incubated at 37^{o}C for 20 minutes. Samples were centrifuged to remove denatured proteins and the supernatant was transferred into a fresh sterile Nunc tube. The solution was neutralised using an equal volume of NaOH to the original volume of perchloric acid and was further diluted in a double quantity of tris-HCL.

3.3 Results

3.3.1 DNA assay development

FFP or plasma collected from the apheresis machine was used in these experiments; spiked with known quantities of DNA for each of the three methods observed. The breakdown of each technique assessed can be seen in Table 4.

3.3.1.1 NanoDrop

Using the NanoDrop spectrophotometer, a dilution of 1 in 10,000 plasma in water was required to inhibit plasma protein interference, as clarified using the 260 nm and 280 nm ratios to calculate purity. Plasma samples spiked with known quantities of DNA were analysed and provided a limit of detection, taking into account dilution factor of the original plasma sample, of 20,000 ng/µl (Table 4).

3.3.1.2 Agilent TapeStation

Using the Agilent TapeStation system, a 1 in 256 dilution of plasma in water was required to prevent interference. Taking the original sensitivity of the assay into consideration this provided an endpoint limit of detection of 2560 ng/µl DNA (Table 4).

3.3.1.3 qPCR

qPCR was performed on known concentrations of DNA in plasma to generate a standard curve, Bioline human genomic DNA was amplified using ALU repeat primers targeting 115 base pair sequences in the human genome. Initial analysis of qPCR in plasma samples showed a 1 in 10,000 dilution necessary to fully prevent

inhibitory effects of plasma proteins. With an assay sensitivity of 0.02 pg/µl this provided an endpoint limit of detection of 0.2 ng/µl, surpassing other methods observed. A further range of dilutions were assessed, spiked with concentrations of DNA decreasing logarithmically from 200 pg/µl to 0.02 pg/µl to generate various standard curves, these were plotted against a water standard, with a 1 in 5,000 dilution demonstrating the lowest dilution factor that corresponded with the water standard curve (Figure 9).

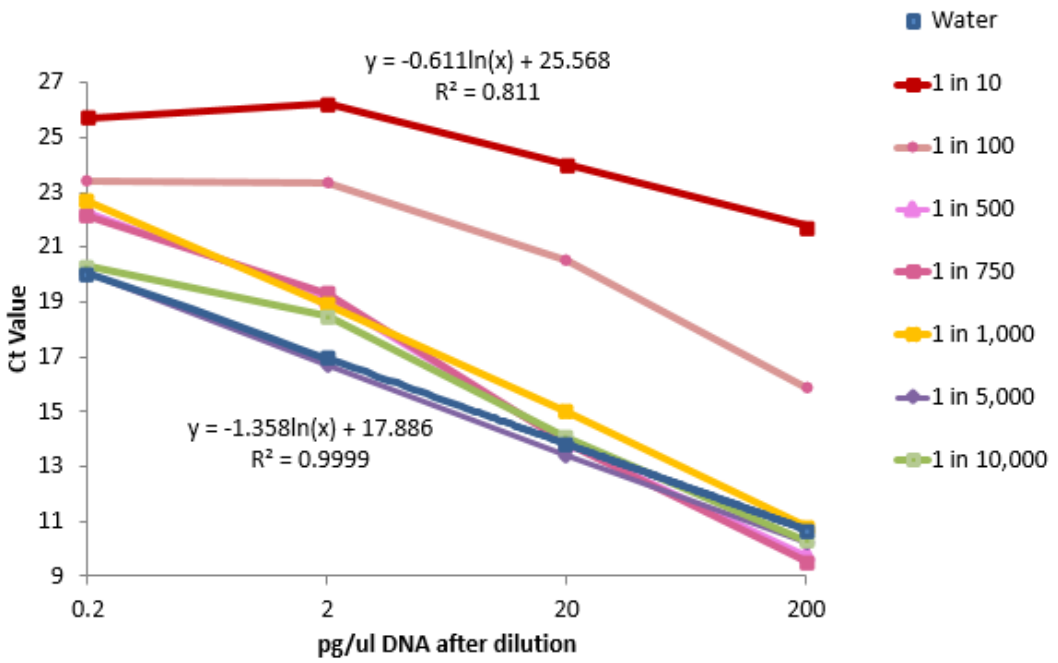

Figure 9. qPCR analysis of DNA standard curves in a range of diluted plasma samples. *Plasma samples were spiked with known concentrations of DNA in a linear fashion, samples were diluted to varying degrees and analysed using qPCR compared with a water control. A dilution of 1 in 5,000 demonstrated the closest linearity compared with water standard curve. N=1. R^2 represents the coefficient of correlation for the standard curves.*

Table 4. Assays for the detection of DNA and their corresponding sensitivity in water and plasma samples. *Limit of detection (LoD) refers to the lowest concentration at which the reaction can detect DNA, taking into account dilution factor of the sample.*

Method	Sensitivity in assay mix (pg/µl)	Dilution factor in plasma	End LoD (ng/µl)
NanoDrop	2,000	1 in 10,000	20,000
Agilent TapeStation	10,000	1 in 256	2560
qPCR	0.02	1 in 5,000	0.1

3.3.2 Developing the qPCR assay further

To ensure that the assay developed would be robust in detecting HepG2 DNA, a crude lysis was performed on confluent monolayer HepG2 cells to release DNA in such a way as to best represent what could occur in the biomass. DNA isolated via this method was analysed against highly purified human genomic DNA from Bioline. Twenty base pair primers were used to target 115 base pair Alu repeat sequences within the human genome. Hotstart taq polymerase was used to catalyse the reaction and Sybr Green double strand detection to detect DNA amplification in real time. It was seen that DNA extracted from HepG2 cells provided consistent results when analysed using qPCR to that of highly pure human DNA from Bioline (Figure 10). Standards were produced using HepG2 DNA and were used for further characterisation of the qPCR assay.

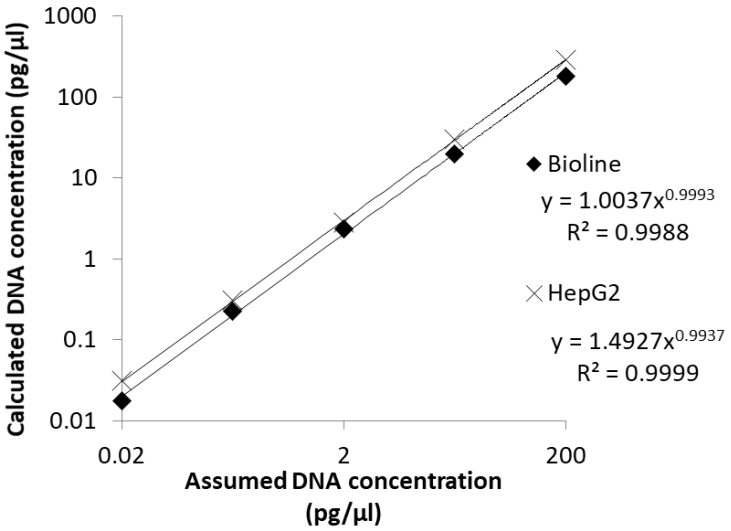

Figure 10. qPCR analysis of Bioline and HepG2 DNA standard curves in water. *Bioline and HepG2 DNA standards were produced in water in a linear fashion and analysed using qPCR. N=1. R^2 represents the coefficient of correlation for the standard curves.*

3.3.2.1 Plasma pre-treatment

Proteinase K was used as a pre-treatment method for plasma to reduce any inhibition from residual protein following sample dilution. Plasma samples were spiked with HepG2 DNA to provide an endpoint concentration of 20 pg/µl. Plasma samples were diluted 1 in 5,000 in PCR-grade water. DNA spiked samples were subjected to different treatments: either dilution alone; dilution with a proteinase K treatment of one of two concentrations (32 or 3.2 µg/20 µl sample); or dilution, proteinase K treatment and a centrifuge step. Samples were diluted prior to proteinase K treatment as heat treatment of the proteinase K reaction mix caused agglomeration of the plasma samples. It was seen that although the difference was not significant, samples without a proteinase K treatment and the samples that contained a centrifuge step produced a lower % recovery of DNA than those in which a proteinase K step was used in absence of a centrifuge step, Figure 11.

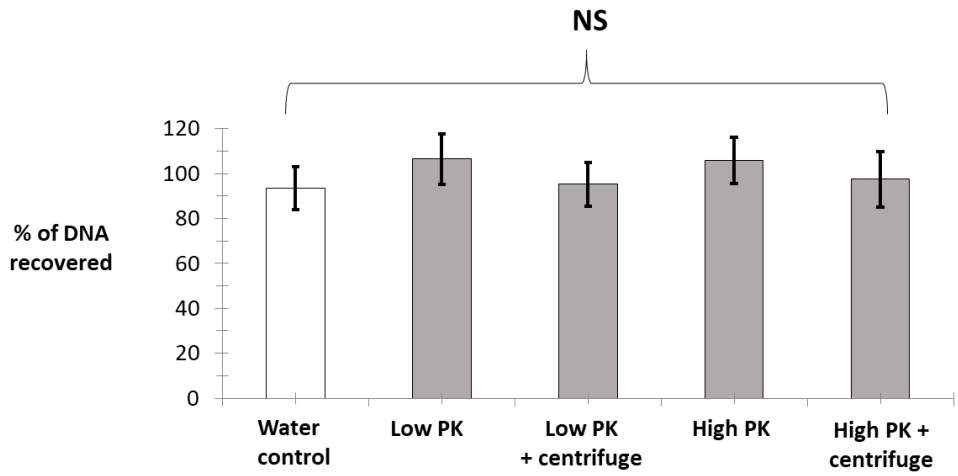

Figure 11. Effect of different proteinase K (PK) treatment methods on DNA recovery from plasma samples. *Neat plasma samples containing known quantities of DNA were treated with either 3.2 µg or 32 µg of PK with (low PK + centrifuge; high PK + centrifuge, respectively) or without (low PK; High PK, respectively) centrifuging at 13,000 x g for 10 minutes, samples were analysed for DNA concentration using qPCR. There was no significant difference in recovery of DNA between treatments. Water control contains DNA and no PK. N=4, average +/- standard deviation. Significance was assessed using a one-way ANOVA and a 95% level of confidence, P= 0.64.*

3.3.2.2 Plasma dilution

Using the proteinase K pre-treatment, a repeat of the previous experiment was performed, whereby a range of plasma dilutions were spiked with a logarithmic range of DNA concentrations and assayed using qPCR, comparing the standard curves produced against that of DNA in water. Starting at a 1 in 10 dilution, inhibition of plasma on the qPCR reaction was still evident; the dilution factor was increased to 1 in 100 reducing inhibition slightly, but still without displaying linearity. The dilution of plasma was further increased until an acceptable r^2 value, and a standard curve produced matching that of water was achieved. This dilution was 1 in 5,000, as previously demonstrated without the proteinase K pre-treatment method (results not shown). The standard curve produced following proteinase K treatment provided a better match to that of DNA in water than the curve of the same dilution without this pre-treatment. We tested samples spiked with 2 pg/µl DNA with and without proteinase K treatment. It was shown that recovery of DNA following proteinase K treatment was significantly greater compared with samples undergoing no pre-treatment, Figure 12.

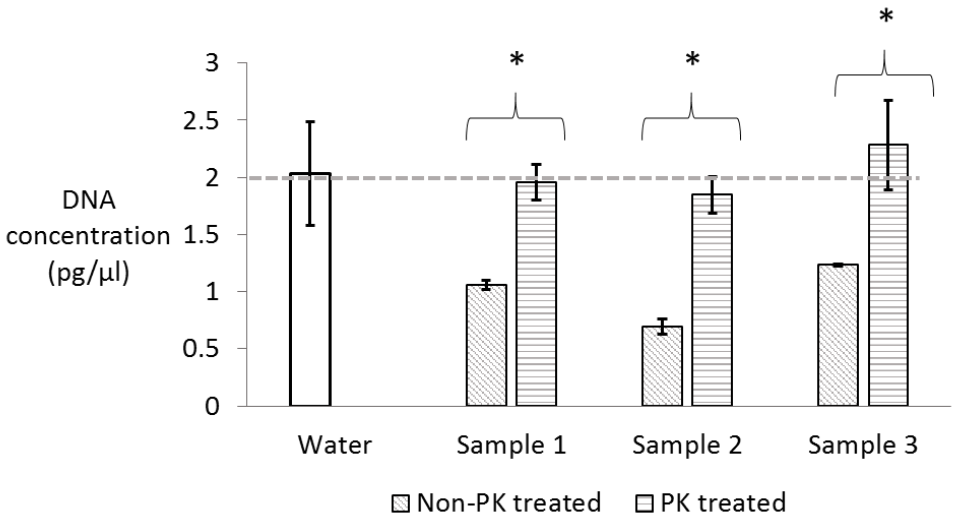

Figure 12. Effect of proteinase K (PK) treatment on revealing levels of DNA present in plasma samples. *Neat plasma samples containing known quantities of DNA were diluted in water with or without PK treatment to assess whether PK treatment enhanced DNA recovery from the sample. A significant difference was seen in the recovery of DNA from PK pre-treated samples compared with those without. N=4, average +/- standard deviation. Significance was assessed using a paired Students T Test with a 95% level of confidence; *P<0.05.*

To view whether the detection limits of the qPCR reaction in plasma could be extended any further, the qPCR reaction was tested down a further 10-fold to a lower limit of 0.002 pg/µl in the diluted sample. It was seen that there was no significant difference between the non-template control (NTC) and the 0.002 pg/µl sample, therefore, the limit at which the standard curve would be run to, and the lower limit of detection remained 0.02 pg/µl (see Figure 13).

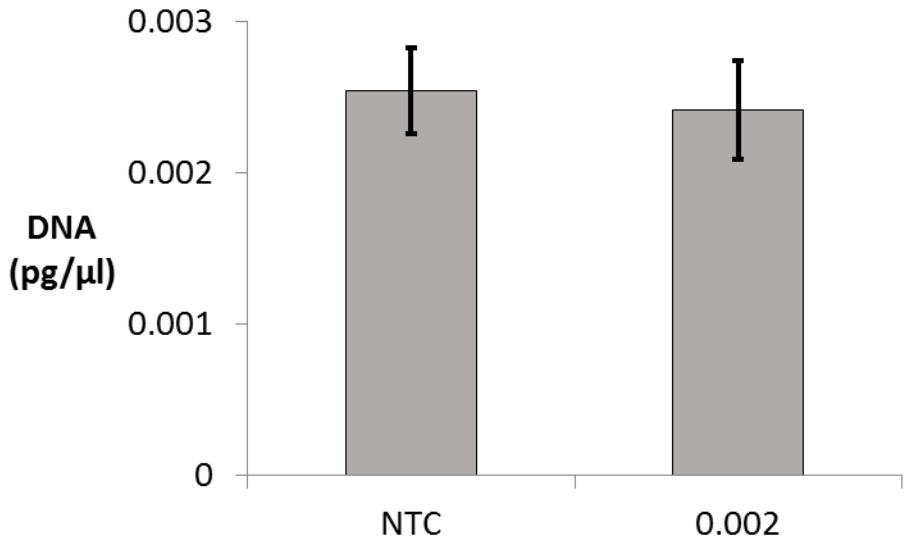

Figure 13. Efficacy of qPCR at detecting low quantities of DNA in plasma. *qPCR was performed on diluted plasma samples containing 0.002 pg/µl DNA. There was no difference between the amplification of the non-template control (NTC) and DNA samples meaning that qPCR could not accurately assess this low level of DNA in plasma. N=4, average +/- standard deviation. Significance was assessed using an unpaired Students T Test with a 95% level of confidence; P= 0.15.*

3.3.2.3 Consistency of the DNA assay across plasma samples

Once an optimal plasma dilution was obtained, a demonstration of assay consistency using both fatty and non-fatty plasma obtained from different donors and blood groups was required. Standard curves were produced from four patient samples; qPCR and plasma treatment were performed on different days for each sample to ensure repeatability of the results. These four standard curves produced were consistent with each other and with the water comparison, all samples provided an r^2 value of >0.99, see Figure 14.

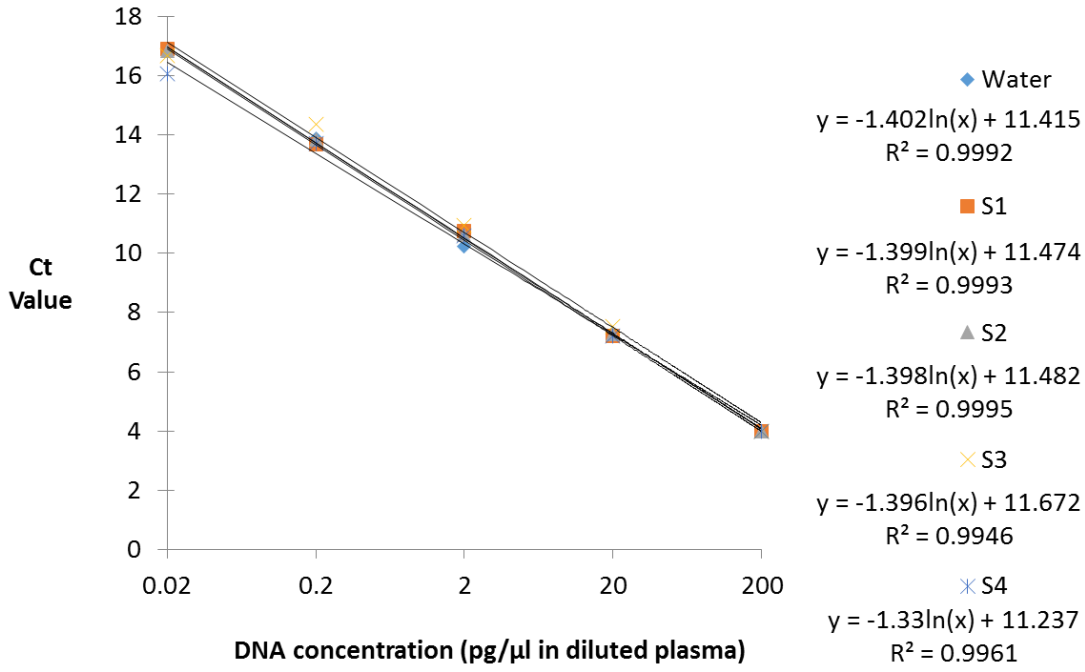

Figure 14. DNA assay consistency across different plasma samples. *Plasma taken from four healthy volunteers was linearly spiked with known quantities of DNA from 0.02–200 pg/µl, treated with proteinase K and analysed using qPCR to generate a standard curve for each sample. Consistent qPCR amplification was seen between individual plasma samples and the water control. N=2 within sample replicates; S1–S4 represent plasma samples obtained from different volunteers. R^2 represents the coefficient of correlation for the standard curves.*

3.3.2.4 Consistency of the DNA assay at detecting low levels of DNA

The next stage of assay characterisation was to ensure consistency of the assay at detecting low levels of DNA. 8 plasma samples were diluted and spiked with 2 pg/µl of DNA; treated with proteinase K and analysed by qPCR against a water standard curve. All samples demonstrated a good, consistent recovery of DNA at this low concentration with no significant difference in DNA quantity recovered by the qPCR reaction, Figure 15.

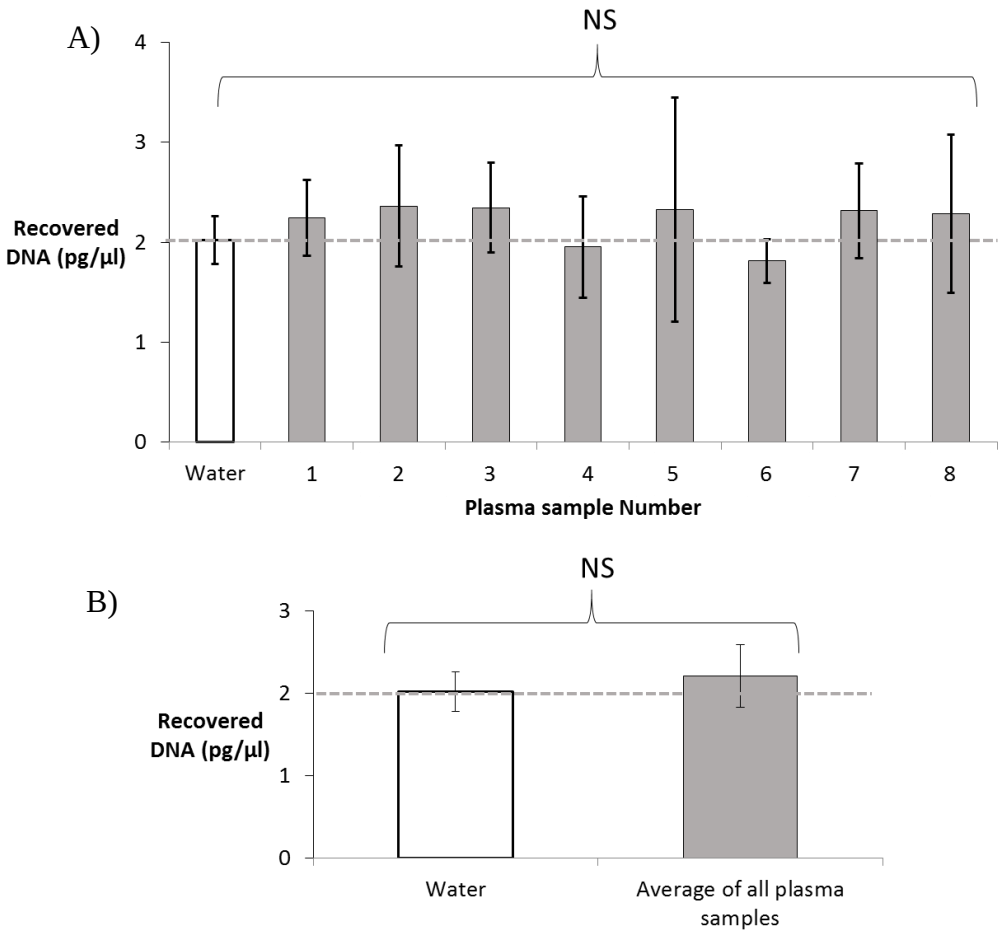

Figure 15. Assessing the efficacy of the DNA assay for detecting low levels of DNA. *A) Plasma samples obtained from 8 donors were spiked with DNA, diluted and proteinase K treated, DNA recovery from the samples was calculated using qPCR to assess inter-plasma variability of the assay. No significant difference was seen between any of the samples or the individual samples and the water control. Significance was assessed using a one-way ANOVA and a 95% level of confidence, P= 0.65 between groups; B) DNA recovery results from the 8 plasma samples were combined and compared with the water control. Significance was assessed using an unpaired Student's T-test and a 95% level of confidence, P=0.52. All values are average +/- standard deviation.*

3.3.2.5 Consistency of the DNA assay in the presence of anticoagulants

To ensure the qPCR assay was not inhibited by the use of anticoagulants which will be used in future chapters to prevent plasma samples from clotting, qPCR was performed in the presence of these factors to estimate any inhibitory effect on DNA recovery. Vacutainers containing either heparin, citrate or EDTA were filled with DNA-free water, DNA was added to give a final concentration of 250 ng/µl. The samples were diluted 1 in 5,000 and analysed using qPCR. Neat DNA-containing anticoagulant samples were additionally frozen at -20^{o}C for 24 hours and reanalysed to view any effect of cold-storage on DNA recovery. It was seen that no negative effects were produced with use of the anticoagulants on qPCR reaction. This result was true for both fresh and frozen samples, Figure 16.

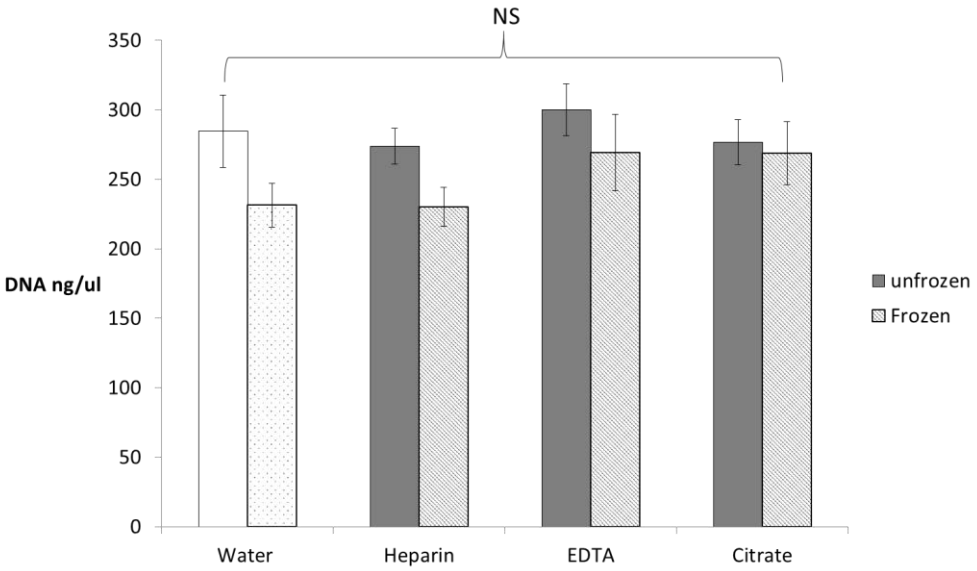

Figure 16. Efficacy of the DNA assay in the presence of anticoagulants. *Anticoagulated plasma was spiked with known quantities of DNA, samples were either frozen or assessed directly. Samples were diluted and proteinase K treated, DNA concentration was analysed using qPCR to assess whether the presence of anticoagulant, or the process of sample freezing, impacted the efficacy of the DNA assay. No significant difference was detected between anticoagulant or unfrozen/ frozen samples. N=4, average +/- standard deviation. Significance was assessed using a one-way ANOVA and a 95% level of confidence, P= 0.24.*

3.3.3 Endotoxin assay for plasma samples

The PyroGent and PyroGene endotoxin assays were used to quantify endotoxin in plasma samples. These assays detect endotoxin using two different methods, kinetic and endpoint, respectively.

3.3.3.1 The PyroGent assay

3.3.3.2 Dilution and heat treatment methods to enhance assay efficacy in plasma samples

To view if the dilution and subsequent heat treatment of plasma samples had an effect on endotoxin recovery, samples were diluted to either 1 in 10, 1 in 20, 1 in 50 or 1 in 100 followed by heat treatment at 70^{o}C for 20 minutes. Recovery of endotoxin increased with increasing dilutions, with an approximately 50% recovery seen with a 1 in 100 dilution, see Figure 17.

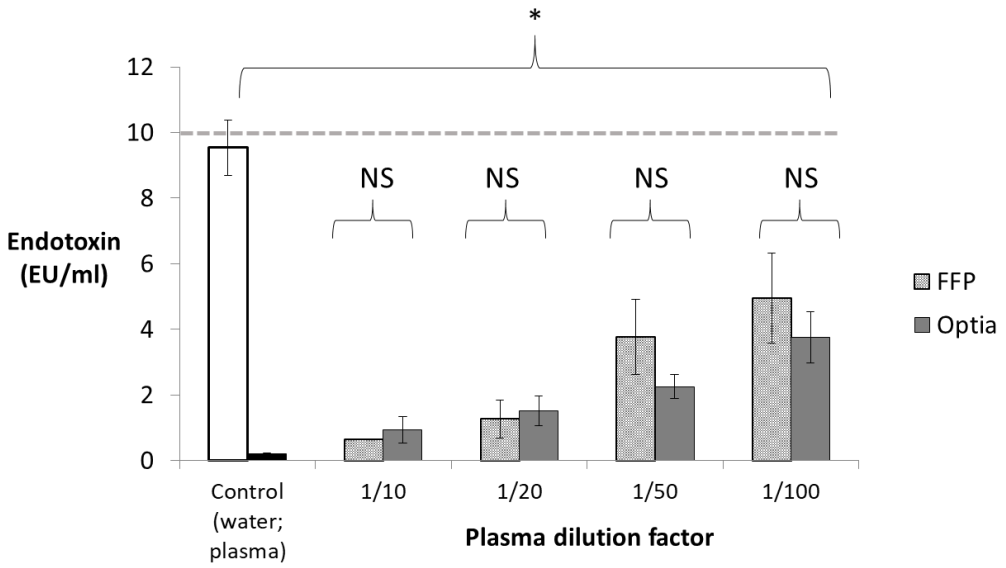

Figure 17. Efficacy of the PyroGent endotoxin assay in spiked plasma collected from two extraction methods. *Fresh frozen plasma (FFP) or plasma collected from Optia plasmapheresis (Optia) were spiked with 10 EU/ml of endotoxin before being diluted to varying degrees and analysed for the presence of endotoxin using the PyroGent endotoxin assay, to estimate the variance in efficacy of this assay over a range of plasma dilutions and to assess whether plasma source impacted endotoxin detection. A significant reduction in endotoxin recovery between each of the plasma dilutions and the water sample was seen (*P<0.05). No significant difference was seen in endotoxin recovery between FFP and Optia plasma (1 in 10 P=0.42; 1 in 20 P=0.69; 1 in 50 P=0.22; 1 in 100 P=0.39). Control values were as expected. N=4, average +/- standard deviation. Significance between plasma dilutions and water samples were assessed using a one-way ANOVA and a 95% level of confidence. Significance between endotoxin recovery in FFP and Optia samples at each dilution were assessed using an unpaired Students T test and a 95% level of confidence.*

To establish whether the plasma would still present its inhibitory effect following dilution and heat treatment, and to denature plasma proteins, prior to endotoxin addition, plasma was diluted either 1 in 10, 1 in 20, 1 in 50 or 1 in 100 and heat treated at 70^{o}C for 20 minutes prior to addition of 10 EU/ml of endotoxin and incubation for 1 hour at 37^{o}C. The samples were analysed with the PyroGent endotoxin assay and endotoxin recovery ranged from 70–120% with no false

positive results in the blank plasma controls. At the lower dilutions of plasma from plasmaphereses more accurate recovery of endotoxin was demonstrated than with FFP, see Figure 18.

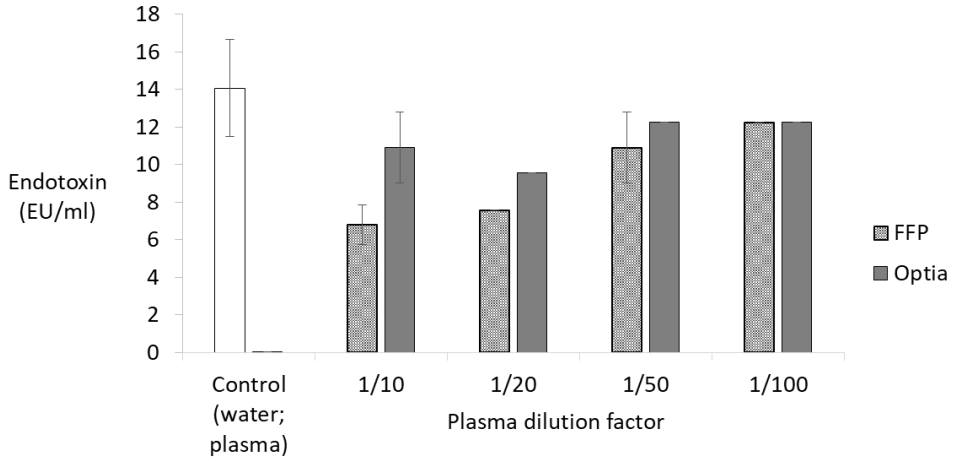

Figure 18. Efficacy of endotoxin detection using the PyroGent endotoxin assay in denatured plasma spiked with endotoxin. *Fresh frozen (FFP) or plasma collected from Optia plasmapheresis (Optia) was diluted to varying degrees and heat treated at 70^{o}C for 20 minutes to denature plasma proteins, prior to the addition of 10 EU/ml endotoxin. Endotoxin recovery from the samples was assessed using the PyroGent endotoxin assay to view whether denaturation of plasma proteins improved efficacy of endotoxin detection. At 1/50 and 1/100 dilutions endotoxin recovery was similar to control. N=2, average +/- high low values.*

3.3.3.3 Perchloric acid treatment of plasma: effect on endotoxin assay efficacy

Plasma samples were spiked with 10 EU/ml of endotoxin and subjected to perchloric acid treatment as detailed above. It was seen that less than 10% of recovery of endotoxin occurred using this method see Figure 19.

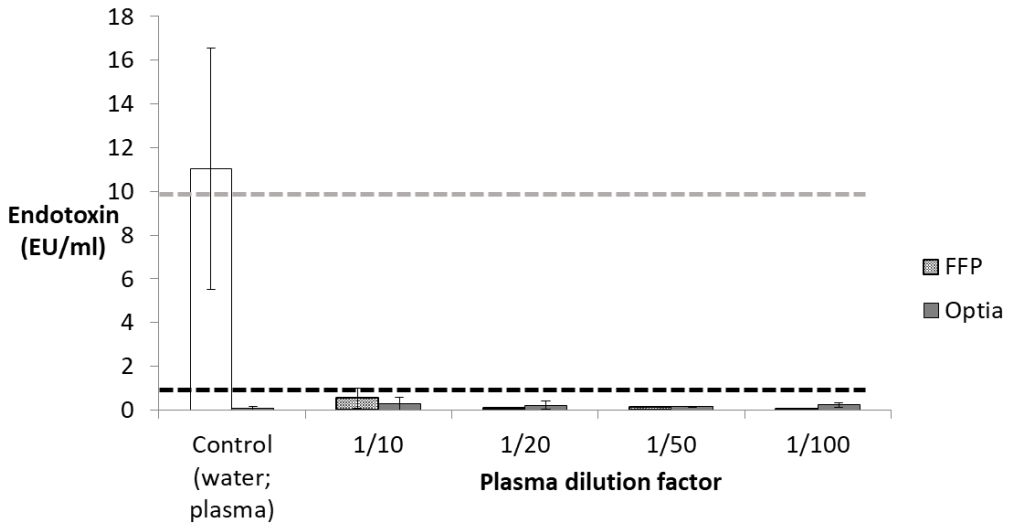

Figure 19. Efficacy of endotoxin detection using the PyroGent endotoxin assay in endotoxin-spiked plasma pre-treated with perchloric acid. *Fresh-frozen (FFP) or Optia plasmapheresis-obtained (Optia) plasma samples were spiked with 10 EU/ml endotoxin (hashed horizontal grey line), treated with perchloric acid and neutralised using NaOH in an attempt to enhance detection of endotoxin. Endotoxin could not be detected above the limit of detection of the assay (1 EU/ml; black hashed horizontal line) following this treatment. N=2, average +/- high low values.*

3.3.3.4 Proteinase K treatment of plasma samples: effect on endotoxin assay efficacy

Plasma samples were spiked with 10 EU/ml of endotoxin and subjected to proteinase K treatment as detailed above. Recovery of endotoxin was low with a considerable sample to sample variability, see Figure 20.

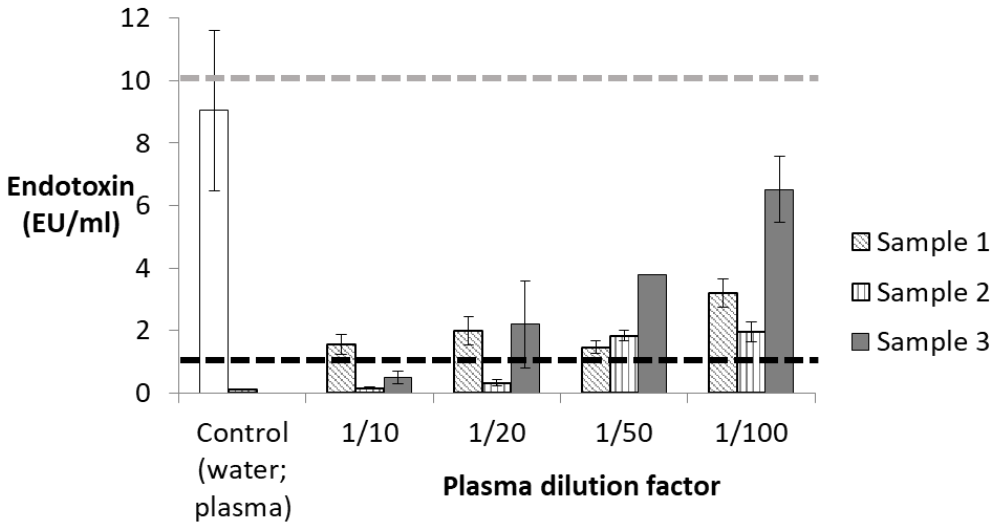

Figure 20. Efficacy of endotoxin detection using the PyroGent endotoxin assay in endotoxin-spiked plasma pre-treated with proteinase K (PK). *Plasma samples were spiked with 10 EU/ml endotoxin (hashed horizontal grey line) and treated with PK in an attempt to enhance detection of endotoxin using the PyroGent endotoxin assay. Recovery was considerably lower than expected, compared with the water control; recovery was slightly improved with plasma dilution. Black horizontal hashed line represents lower limit of detection. N=2 within-sample repeats, average +/- high low values.*

3.3.4 The PyroGene assay

3.3.4.1 Albumin interference

Albumin is present in human plasma at a high concentration, and is demonstrated to interfere with assays for the detection of DNA and endotoxin. To investigate the extent to which albumin had an inhibitory effect on the PyroGene assay, endotoxin was incubated in saline with and without albumin at a concentration of 40 g/L. Samples were taken every hour over a 6 hour period to determine the extent of inhibition at different time points. The inhibitory effect of albumin occurred consistently after a 20 minute incubation time, see Figure 21 .

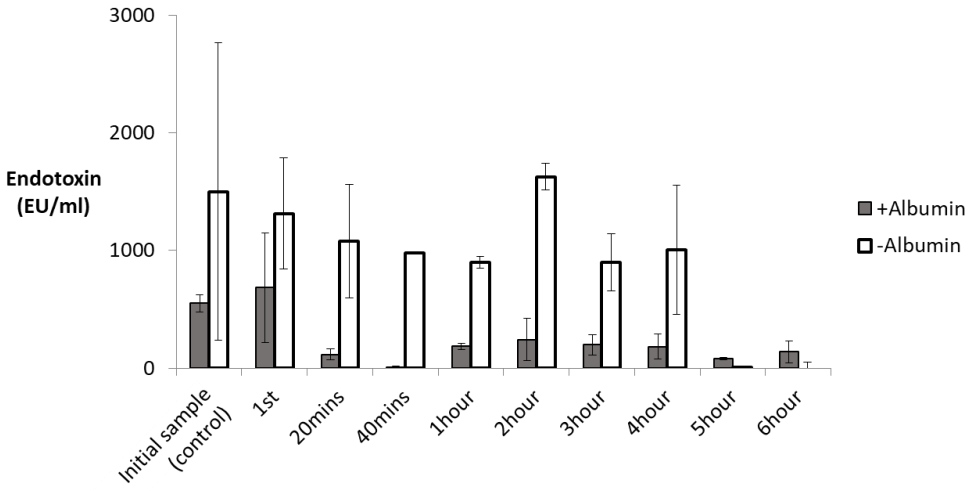

Figure 21. Efficacy of endotoxin detection using the PyroGene endotoxin assay in the presence and absence of albumin. *Endotoxin was incubated with saline +/- 40 g/L albumin, hourly samples were analysed for endotoxin concentration using the PyroGene endotoxin assay to view whether physiological levels of albumin interfered with detection of endotoxin. Presence of albumin considerably decreased recovery of endotoxin compared with the saline sample. N=2, average =/- high low values.*

3.3.4.2 Proteinase K treatment using the PyroGene assay

Saline containing albumin at a concentration of 40 g/L was spiked with endotoxin and subjected to proteinase K treatment as detailed above. The use of proteinase K treatment had a positive effect on the recovery of endotoxin, although recovery did not match that of the saline control, see Figure 22.

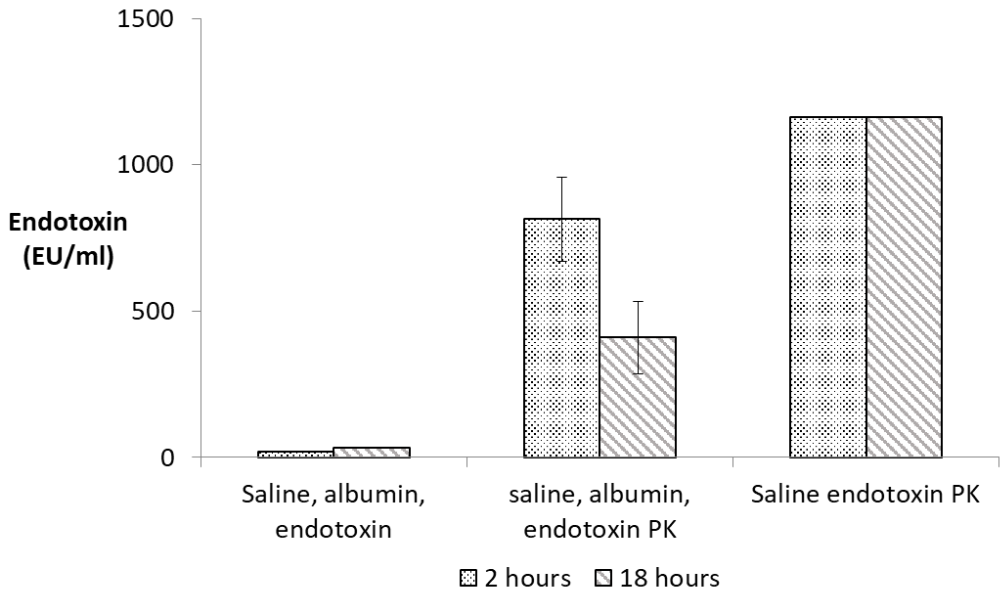

Figure 22. Effect of proteinase K (PK) treatment on the efficacy of detection of endotoxin in albumin samples using the PyroGene endotoxin assay. *40 g/L albumin saline samples were spiked with endotoxin. Samples were pre-treated with PK for a period of 2 or 18 hours in an attempt to enhance detection of endotoxin. Recovery of endotoxin was considerably higher in samples treated with PK for 2 hours compared with those treated for 18 hours and those without PK treatment. PK treatment did not negatively impact on endotoxin recovery from saline only samples. N=2; average +/- high low values.*

3.3.4.3 Refining the PyroGene assay

The LAL assay was applied to analyse consistency across patient samples. 3 different FFP samples were diluted 1 in 200, spiked with endotoxin (1 EU/ml) and heat treated at 70°C for 20 minutes. The recovery from FFP samples was shown to be lower than that for water, but no significant difference was observed between the plasma samples, see Figure 23.

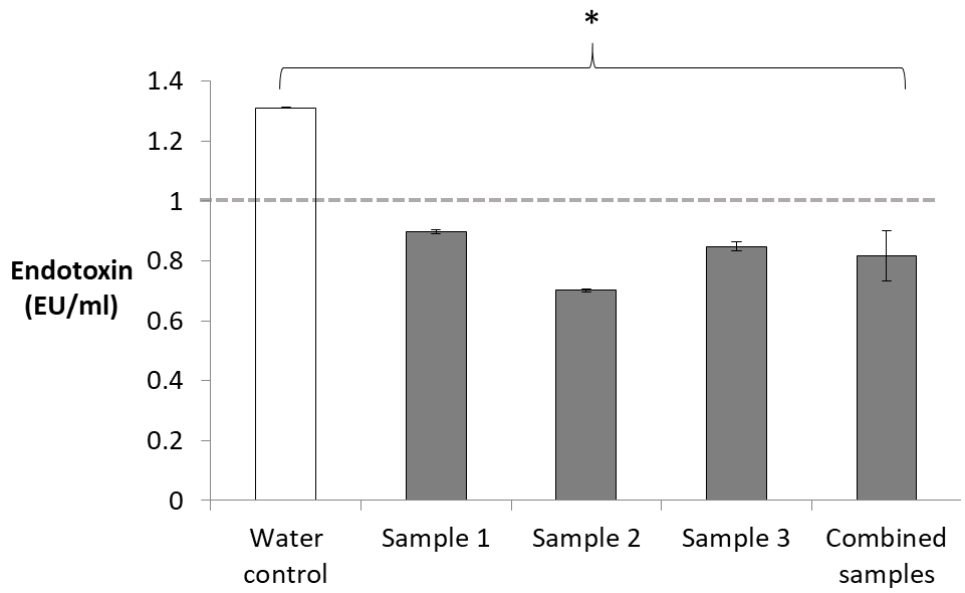

Figure 23. Efficacy of the PyroGene endotoxin assay in diluted and heat treated endotoxin-spiked plasma. *Plasma obtained from 3 donors was diluted 1 in 200 in endotoxin-free water, spiked with 1 EU/ml of endotoxin, heat treated at 70°C for 20 minutes and analysed using the PyroGene endotoxin assay, compared with water samples treated in the same way to view whether plasma pre-treatment enhanced endotoxin recovery. Endotoxin recovery from plasma was significantly lower than water (P<0.05), no difference in plasma sample-to-sample recovery was observed. N=2 within sample replicates, average +/- high low for individual samples and average +/- standard deviation for combined samples. Significance between combined samples and water was assessed using the unpaired Student's T test and a 95% level of confidence.*

3.4 Discussion

3.4.1 DNA assay

3.4.1.1 An assay for the detection of DNA in plasma

The use of qPCR to assess DNA quantity in plasma samples enabled the development of an assay protocol suitable for use in plasma down to a lower limit of detection of 0.1 ng/µl. This lower limit of detection was calculated from the qPCR reaction having a sensitivity of 0.02 pg/µl, and plasma samples requiring a dilution factor of 1 in 5,000 for the reaction to be reliable in this media. Dilution of plasma in this manner provided the smallest dilution at which plasma components ceased interfering with the qPCR reaction. It was clear at dilutions above this factor that various plasma components were inhibiting the reaction, seen as consistently higher Ct values, translating to a decrease in template DNA seen by the reaction mix, and a failure to produce an acceptable standard curve. The inhibitory effect of plasma has been widely observed with IgG, the primary source of this interference.[45,55,56] This assay was rigorously tested using plasma samples obtained from different donors, through different methods, on different days and at both high and low concentrations ensuring an effective and robust assay that will be continuously used throughout this project.

Citrate chelates calcium ions required to maintain ELS integrity. For this reason, heparin is used as the anticoagulant for any plasma that comes into contact with the BAL. It is known that heparin has an inhibitory effect on qPCR reactions, we have demonstrated here that there was no significant difference in DNA recovery in the presence of heparin compared with DNA control at a 1/5,000 dilution.[45,48] One further issue the BAL may pose to the qPCR reaction is the increase in calcium ions present in the plasma required to maintain integrity of the ELS. Calcium ions are known to inhibit qPCR at a concentration double that of magnesium in the reaction mix, this is due to the calcium ions competing with magnesium chloride as a co-factor for the DNA polymerase.[46] Magnesium chloride is present in the qPCR

reaction mix at a final concentration of 0.0031 M, following addition of sample; physiological levels of calcium chloride are approximately 0.002 M, lower than the value at which it would inhibit a qPCR reaction.[106] Due to the high dilution factor of the plasma performed in order to gain accurate results, this may not present an issue, but it is important to note and will be explored further in future chapters, with the use of a consistent control, to ensure the analysis of DNA in human plasma samples via qPCR is robust, particularly in the presence of the BAL-treated plasma, in which calcium is used to maintain integrity of the biomass.

3.4.1.2 Application of the DNA assay sensitivity to BAL requirements

As discussed in Chapter 1, the exact concentrations of DNA considered acceptable within medical devices and drugs are unclear. Based on *in vivo* studies of decellularised scaffolds, the maximum level of DNA contamination to avoid immunogenicity in terms of an adverse host cell response was less than 50 ng of double stranded DNA per mg of extracellular matrix dry weight.[104] It has been reported that average human plasma samples are 80 mg dry weight per ml of liquid. The DNA detection method described above was assessed to view whether it would theoretically meet sensitivity limits required to detect 50 ng DNA per 80 mg dry plasma. The DNA detection limit achieved using the proteinase K treatment protocol is 0.1 ng/µl, equal to 100 ng/ml, which equates to 100 ng of DNA in 80 mg dry plasma weight. Therefore, 100 ng of DNA per 80 mg of plasma dry weight provides a value of 1.2 ng of DNA per mg of dry weight plasma. This is well below the limit for provoking an immune response described above (50 ng/mg of dry weight for decellularised scaffolds[104]), suggesting that the limit of detection for this qPCR method is more than sensitive enough detect DNA at the minimal concentration at which it might provoke an immune response.

In addition to the sensitivity limits discussed above, to ensure that this assay is sensitive enough for use within the BAL setting, it is important to gain an understanding of the concentration of DNA expected to be released from the system. To do this the DNA concentration of HepG2 cells is used to calculate the

total quantity of DNA found within the BAL biomass, this is related to the total volume of liquid in which this DNA could potentially release into. Goepfert et al 2011[107] analysed DNA concentration in HepG2 cells, stating that the modal number of HepG2 cells is 55, equating to a DNA level per cell of 7.83 pg.[107] At high cell density the BAL will contain up to $1x10^{11}$ cells, equating to $7.83x10^{11}$ pg of DNA per 7 litres of plasma (as would be the quantity within the BAL-patient system) or $1.1x10^{5}$ pg/µl of DNA. Using our qPCR assay with a limit of detection of 0.1 ng/µl, this system can detect as little as 0.08% cell death occurring within the BAL biomass at a high cell concentration in a circulating system containing 7 litres of fluid. At a low cell density the BAL will contain $7x10^{10}$ cells in 7 litres of plasma, or $5.48x10^{11}$ pg of DNA. This equates to $7.83x10^{4}$ pg/µl of DNA, meaning detection of 0.13% cell death at low cell densities. The upper limit of qPCR is 1000 ng/µl, so by further diluting the sample DNA can be detected in plasma passed through the BAL at cell deaths from 100% to 0.08%. This demonstrates the suitability of this assay for use in future work, as presented in Chapters 4–6.

3.4.2 An assay for the detection of endotoxin in plasma

3.4.2.1 The PyroGent assay

The PyroGent assay recommends a 1 in 10 dilution for plasma samples, with a lower limit of detection of 0.001 EU/ml in testing situations only. We found that by diluting and heating plasma, approximately 50% recovery of endotoxin could be produced using this assay, although results were highly variable with different patient plasma samples, as can be seen from the large error bars. Upon treatment with perchloric acid, less than 50% recovery of endotoxin was produced. The pH of the samples were analysed prior to use with the PyroGent assay and a Tris-HCL buffer was used to ensure the pH remained stable, therefore, inhibition was unlikely to be due to the pH of the sample being incompatible with the assay reagents. An alternative reason as to why the assay may not work could be the high concentration of salt produced from the neutralisation of the perchloric acid with the NaOH, this may disrupt the enzyme within the PyroGent system.[108] Results demonstrated that

proteinase K treatment of samples for use with this detection assay proved ineffective at producing desirable results. As sufficient results were not generated with this assay, a literature search was performed for alternative methods, the PyroGene recombinant Factor C assay was selected for characterisation.[52]

3.4.2.2 The PyroGene assay

The PyroGene assay is a recombinant Factor C assay, selected for further characterisation after reviewing current literature.[52] Using the endotoxin protocol described in the methods section, the limit of detection for endotoxin in plasma taking into account the dilution factor is 2 EU/ml. When this assay was used in media samples containing 10% FFP, the sensitivity of this assay was increased to 0.2 EU/ml, due to there being 10-fold less dilution. The defined acceptable endotoxin concentration for a medical device as per the FDA guidelines is device-dependent but either, 0.5 EU/ml or 20 EU/device.[109] For the BAL to meet regulatory requirements, its components must adhere to this regulation prior to treatment of the patient. This means that the filters themselves, the biomass and any associated tubing, chambers and connectors must be lower than this limit. As the filters can be rinsed and analysed for endotoxin concentration using saline or water, the lower limit of detection here would be 0.01 EU/ml, providing sufficient coverage for regulatory testing. Within the biomass itself, the testing would be performed in the growth medium in which the cells were maintained prior to transfer to the patient's plasma, therefore, the lower limit of detection for this is 0.2 EU/ml, lower than the required limit of detection for regulatory testing. This assay will be used for the detection of endotoxin in all future work.

Chapter 4

Characterising a small-scale filtration system for use within a bioartificial liver

4. Characterising a small scale filtration system for use within a bioartificial liver

4.1 Introduction

As its functional component, the liver group BAL uses HepG2 cells encapsulated in alginate, a hydrogel which is both biocompatible and semi-permeable.[20] By encapsulating cells in this manner, the cellular biomass may be directly exposed to the patient's plasma during treatment. The process of BAL treatment may lead to leaching of contaminants such as cell-free DNA, cell debris, and alginate particles from the biomass into the patient's plasma. It is essential that these contaminants are removed from the patient's plasma before it is returned to their circulatory system. For this reason, a method for the removal of DNA and particles from the patient's plasma after it has been processed by the BAL and prior to return to the patient is required for this technology to meet regulatory guidelines for use in patients, as described in Chapter 1.[27,59,28]

The presence of high endotoxin levels within the plasma of patients with acute liver failure has been linked to an increased chance of complications during treatment.[33,44] As such, in addition to contaminants originating from the BAL biomass, the filtration system will be assessed for the removal of endotoxin originating from the patient's own plasma, providing an additional functional element of this system, with the aim of further protecting these patients from bacterial contamination of the blood and its subsequent effects, which are a common final cause of death in patients with liver failure.[33]

Inhibitors in plasma have been shown to interfere with assays for the detection of DNA and endotoxin. DNA and endotoxin measurements will be used in this chapter to assess the efficacy of the filtration system on a small scale.

The use of a depth filter, as identified in Chapter 1, within the BAL circuit will enable a high volumetric throughput, providing a greater chance of this filtration meeting requirements of a BAL treatment cycle. The 3M Cuno depth charge

60ZB05A filter series contain anion exchange media, carbon and other affinity resins and a net positive charge to enable removal of negatively charged biological components such as DNA and endotoxin. This filter series is available in a variety of scales, the smaller scales of which will be characterised in this chapter.[97]

The aims of this chapter are to determine both the capability and the capacity required of the filtration system in order for it to meet regulatory requirements and be suitable for use within patients, and to assess the filtration system on a small-scale *in vitro* to establish the parameters of its efficacy.

4.2 Methods

4.2.1 Characterising DNA release from HepG2 cells in plasma

The number of cells per volume of plasma that may observed under BAL conditions was calculated as $1.4x10^7$ cells/ml (as a high cell density BAL contains $1x10^{11}$ cells and the circuit would contain 7 L of plasma: $1x10^{11}/7000=1.2x10^7$). From this, cell seeding densities for cell death within the BAL were calculated including situations in which: 100%, 50%, 30% 10% and 0.1% of cell death occur. Using these numbers the expected concentration of DNA released into the BAL under each condition was calculated. Cells were seeded at these densities into 1 ml pooled plasma samples which were subjected to a number of freeze-thaw cycles to ensure cell death. Each sample was analysed using qPCR, as described in Chapter 2, and DNA release from cells was quantified.

4.2.2 Characterising DNA release from ELS in plasma and media to calculate worst case and expected scenarios

Materials

ELS
Complete FFP alpha-MEM media
Plasma
Cell major mixer in 37°C incubator

ELS were placed into 50 ml Nunc tubes containing either cell growth media or plasma at known volumes. Cells were incubated at 37°C, mixing gently to simulate expected conditions within the FBB. Samples were taken from the initial media prior to addition of ELS, after 10 minutes allowing equilibration between alginate and media and after 8 and 24 hours of incubation. These samples were analysed for DNA concentration using qPCR. A worst case scenario was also performed where ELS were freeze/thawed prior to addition to FFP media to provide a 100% cell mortality comparison.

4.3 Results

4.3.1 DNA release

4.3.1.1 DNA release from monolayer HepG2 cells

The release of DNA from monolayer cells seeded into plasma were compared against expected values calculated using cell concentration and chromosome number (C number) of the HepG2 cell line. The observed DNA concentration was greater than that expected (Figure 24), perhaps representing a proportion of cells in S or G2 phase of the cell cycle. The increase in observed DNA concentration would unlikely be due to existing DNA levels in the plasma, as the control sample tested negative for the presence of DNA.

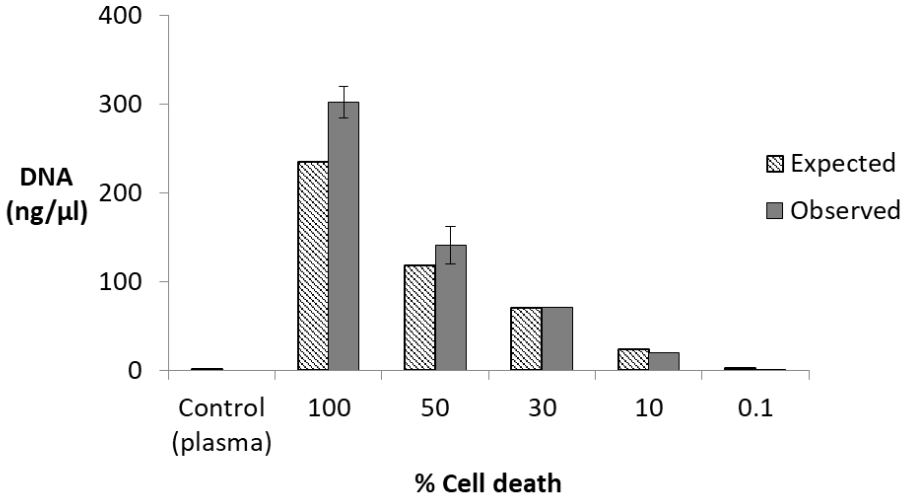

Figure 24. Quantification of DNA release from monolayer HepG2 cells subjected to freeze-thaw cycles. *HepG2 cells were seeded into plasma at densities representing different scenarios of cell death within the bioartificial liver. DNA release from cells was quantified using qPCR and compared with calculated expected values. An increase in observed DNA released was seen compared expected values. N=4 samples, average +/- standard deviation.*

4.3.1.2 DNA release from ELS in plasma

Day 12 ELS (i.e. ready to use in the BAL) incubated in plasma and subjected to a gentle rotation within a 37°C incubator 24 hours produced samples at 0, 8, and 24 hours. A 100% mortality comparison was gained using freeze/thaw cycles. Samples incubated in plasma show a release of DNA into plasma, with the worst case scenario producing a DNA level equal to that of the calculated expected for this scenario (Figure 25). After 8 and 24 hours of treatment, cell death was calculated in the samples working back from the 100% cell death control.

8 hour plasma samples: 100% cell death control: 39.5 ng/µl DNA released

8 hour plasma sample: 0.22 ng/µl DNA released

(0.22/39.5)*100 = 0.56% cell death

24 hour plasma samples: 100% cell death control: 39.5 ng/µl DNA released

8 hour plasma sample: 0.57 ng/µl DNA released

(0.57/39.5)*100 = 1.44% cell death

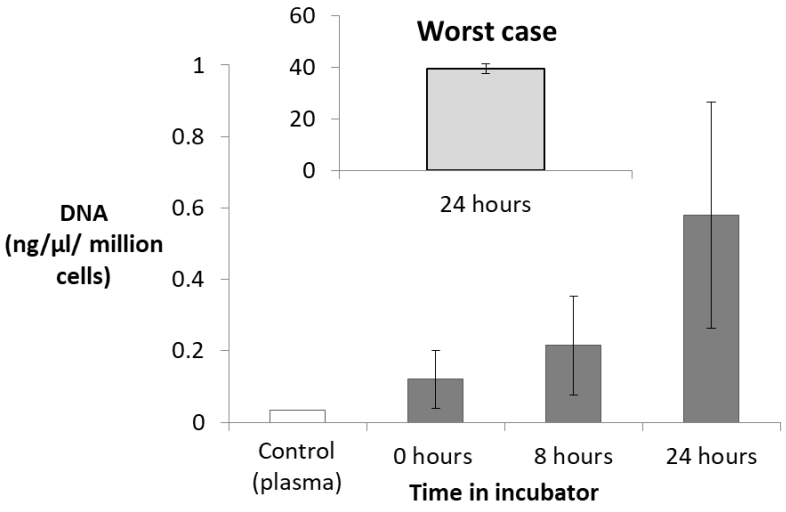

Figure 25. Quantification of DNA release from encapsulated liver cells (ELS) under gentle incubated rotation for 24 hours. *Day 12 ELS were incubated in plasma and subjected to a gentle rotation within a 37°C incubator for 24 hours to quantify expected DNA release from ELS under bioartificial liver treatment conditions. DNA release occurred linearly over the 24 hour period. Inset: cells incubated in plasma were freeze/thawed to induce cell death in order to calculate a reference value for DNA release from a 100% cell mortality control. N=4, average +/- standard deviation.*

4.3.2 Particle release from empty alginate spheres in serum-free cell culture media using the upstream 170 cm2 and downstream 1.2 µm filters in series

A total of 18 litres of culture media from a Day 0–5 media change performed on a culture of empty alginate spheres was passed through the upstream 170 cm^2 and downstream 1.2 µm filters in series. This media change was used as it is likely to represent the greatest particle burden. Samples were taken following filtration with the upstream 170 cm^2 filter alone, the downstream 1.2 µm filter alone and both filters in series. Samples were analysed using NanoSight, Zetasizer and Mastersizer particulate sizing technologies.

4.3.2.1 Mastersizer analysis

Samples from each condition were analysed using the Mastersizer analysis software. MilliQ water was used as a comparison. The results demonstrated removal of particulates in the 10 µm–600 µm range (this was within the measurement range of the Mastersizer [0.02–2000 µm]). Data shown here are a percentage of sample volume. A peak of particles at the higher end of the spectrum was seen, this was also observed in the MilliQ water control (Figure 26).

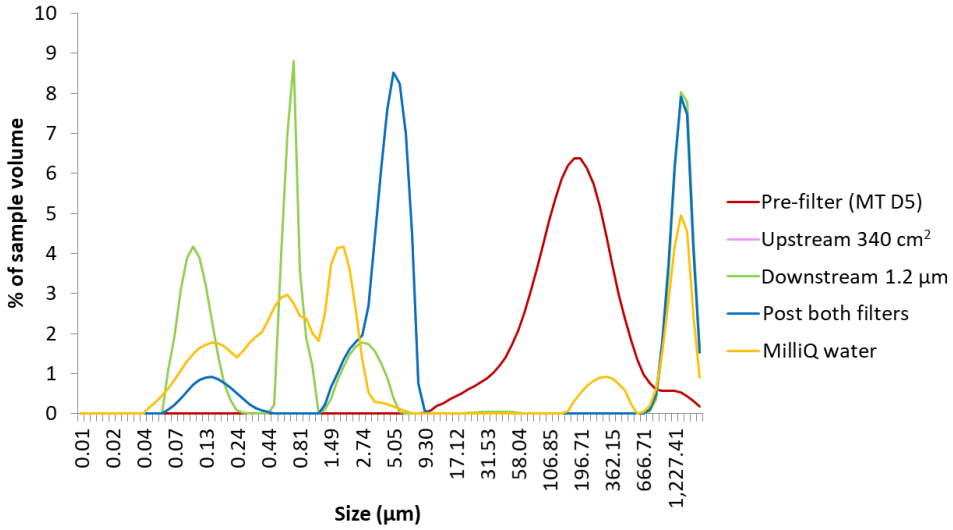

Figure 26. Particle size analysis of Day 5 empty alginate bead conditioned culture media using a Mastersizer. *Culture media (18 litres) from a Day 5 media change of empty alginate beads was passed through the upstream 170 cm^2 and downstream 1.2 µm filters in series. Samples were taken following the 170 cm^2 filter alone, the 1.2 µm filter alone and both filters in series to assess alginate particle removal by the filters. Removal of particles in the 10–600 µm region was observed. N=1.*

4.3.2.2 Zetasizer analysis

Results produced from Zetasizer analysis corroborate the Mastersizer data, showing a reduction in average particle size from the pre- and post-filtered samples (Figure 27). It is worth noting that the measurement range of this system is 0.003–10 µm.

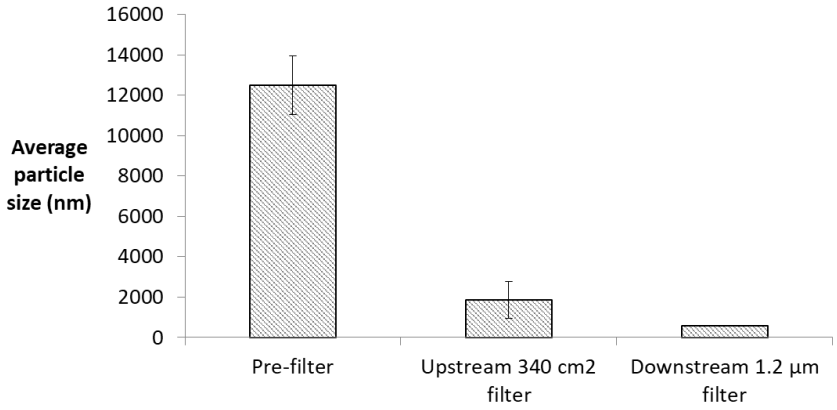

Figure 27. Particle size analysis of Day 5 empty alginate bead conditioned culture media using a Zetasizer. *Culture media (18 litres) from a Day 5 media change of empty alginate beads was passed through the upstream 170 cm^2 and downstream 1.2 µm filters in series to assess alginate particle removal by the filters. Samples were compared with the pre-filter sample. A decrease in average particle size was observed seen between the pre- and post-filter samples. N=1, average +/- standard deviation of N=4 within-sample replicates.*

4.3.2.3 NanoSight analysis

A decrease in particle size was seen from the pre-filter samples to both post-filter samples, with the upstream 170 cm^2 filter displaying reduction of particles down to <0.3 µm, and the downstream 1.2 µm filter to <1 µm (Figure 28). The measurement range of this system is 0.01–2 µm (10–2,000 nm).

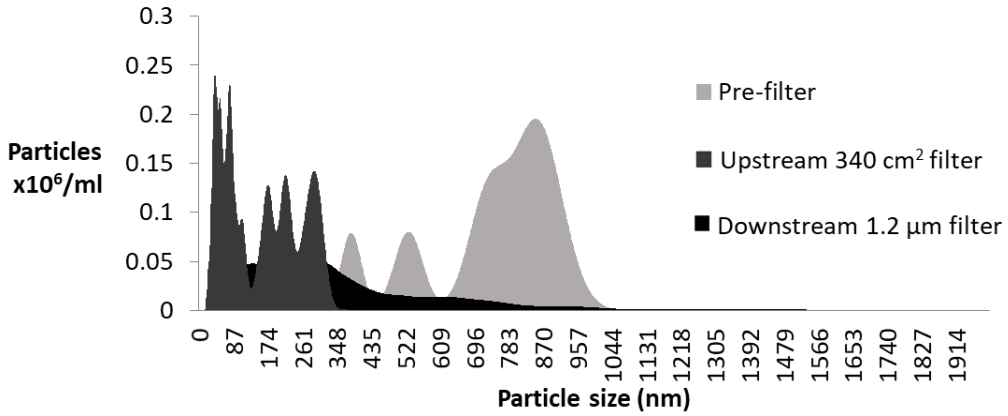

Figure 28. Particle size analysis of Day 5 empty alginate bead conditioned culture media using a NanoSight particle sizer. *Culture media (18 litres) from a Day 5 media change of empty alginate beads was passed through the upstream 170 cm^2 and downstream 1.2 µm filters in series to assess alginate particle removal by the filters. Samples were assessed using a NanoSight particle sizer. A decrease in number of larger (>400 nm) particles was observed seen between the pre- and post-filter samples. N=1 experiment, results presented are average of N=10 within-sample replicates.*

4.3.3 Volumetric capacity of the filtration system

The volumetric capacity of the upstream 170 cm^2 filter and the downstream 1.2 µm filter were analysed in series. This was performed by gaining a large quantity of human plasma and using a 'brute force' approach to challenge the filters until the maximum volumetric capacity was reached. 40 litres of plasma were passed through the filters in series, with a considerable pressure increase seen at the last 5 litres (Figure 29**)**.

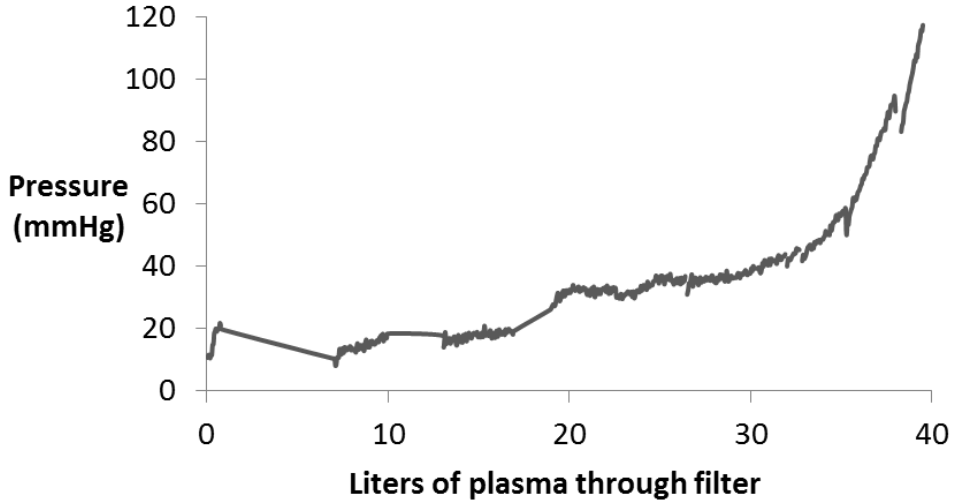

Figure 29. Volumetric capacity of the filter system as assessed using pressure drop across the upstream 170 cm^2 and downstream 1.2 µm filters in series. *The upstream 170 cm^2 and downstream 1.2 µm filters were challenged with 40 L of plasma. Pressure drop was measured across the filters to assess filter blockage and ascertain total volumetric capacity. Pressure drop began to increase after the filters had processed 30 L of plasma. N=1.*

Following this, to further enhance the safety of this device, a smaller nominal pore sized filter was selected for further characterisation, the 0.6 µm downstream filter.

4.3.4 Simultaneous DNA and endotoxin from plasma removal by the filtration system

During initial experiments into filter capacity, plasma was collected and used to test the volumetric capacity and DNA and endotoxin removal capabilities. The DNA capacity of a 25 cm^2 filter was assessed with 3 DNA spikes in plasma. A breakthrough in filter efficiency was observed after filtration of 1186 ml plasma, containing 0.312 mg DNA. Additionally, filter blockage as assessed by an increase

in pressure and total reduction of flow after 2 litres of plasma had been processed. Retrospectively it was realised plasma used in this experiment was obtained from donors undergoing a specific clinical treatment leading to the death and lysis of red blood cells at large volume in the plasma donated, as would not be seen for patients presenting with acute liver failure – for all experiments performed onwards plasma donated from these patients was omitted.

DNA and endotoxin were introduced to the 170 cm^2 filter in 3 separate spikes scaled up from the 25 cm^2 experiment, all of which were introduced during the filtration of 3.5 litres of plasma from a single patient. DNA totalled 4.243 mg (equivalent to 5.4 x 10^8 HepG2 cells). Endotoxin was introduced at 1.2 EU/ml, just under the quantity thought to be present in patients with liver failure, which is approximately 1.84 EU/ml.[43,110] It was seen that the 170 cm^2 removed DNA and endotoxin consistently from the plasma to below the limit of detection (Figure 30).

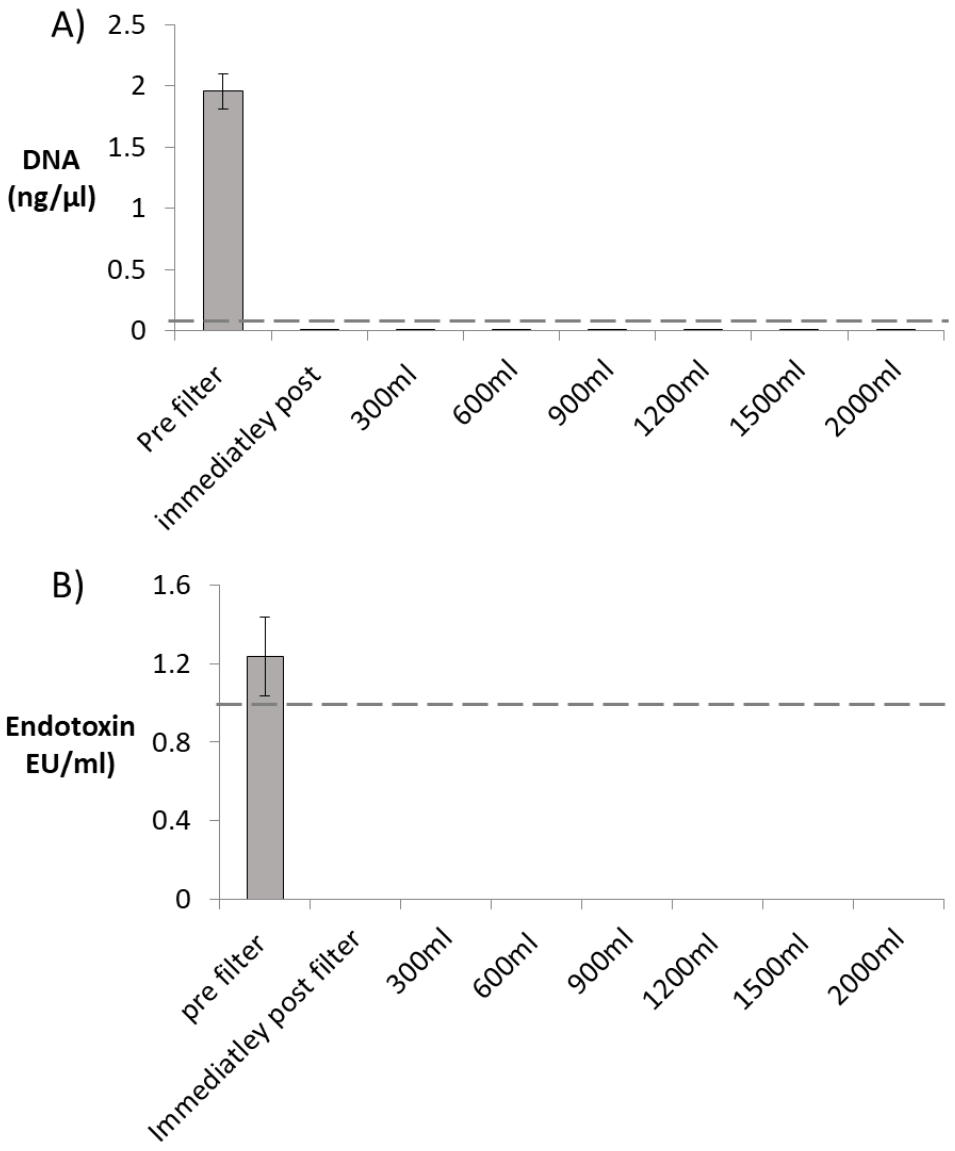

Figure 30. Simultaneous removal of DNA and endotoxin from plasma by the upstream 25 cm^2 scale filter. *2 L of fresh frozen plasma spiked with HepG2 DNA and endotoxin was passed through the upstream 25 cm^2 filter to assess its capacity for the simultaneous removal of A) DNA and B) endotoxin. DNA and endotoxin were consistently removed to below the limit of detection (hashed horizontal line) for the full 2 L sample. N=2, average +/- high low values.*

4.3.5 Mini column experiments in healthy plasma

A series of experiments were performed using mini columns and healthy human plasma collected from FFP donations. These experiments were designed to provide a scaled-down model of the BAL, as it would be in a clinical setting, incorporating the filtration circuit. The upstream 25 cm^2 and downstream 0.6 µm filters were used for these experiments. This was repeated using four different filtration sets, four mini columns and four plasma samples.

The number of cells per ml of ELS was reduced during the course of the experiment, as expected, but the percentage viability was maintained (Figure 31). AFP production significantly increased over the course of the 8 hour experiment demonstrating production of AFP by the HepG2 cells. Glucose levels decreased over the 8 hour time period, demonstrating metabolism by the cells (Figure 32, page 110).

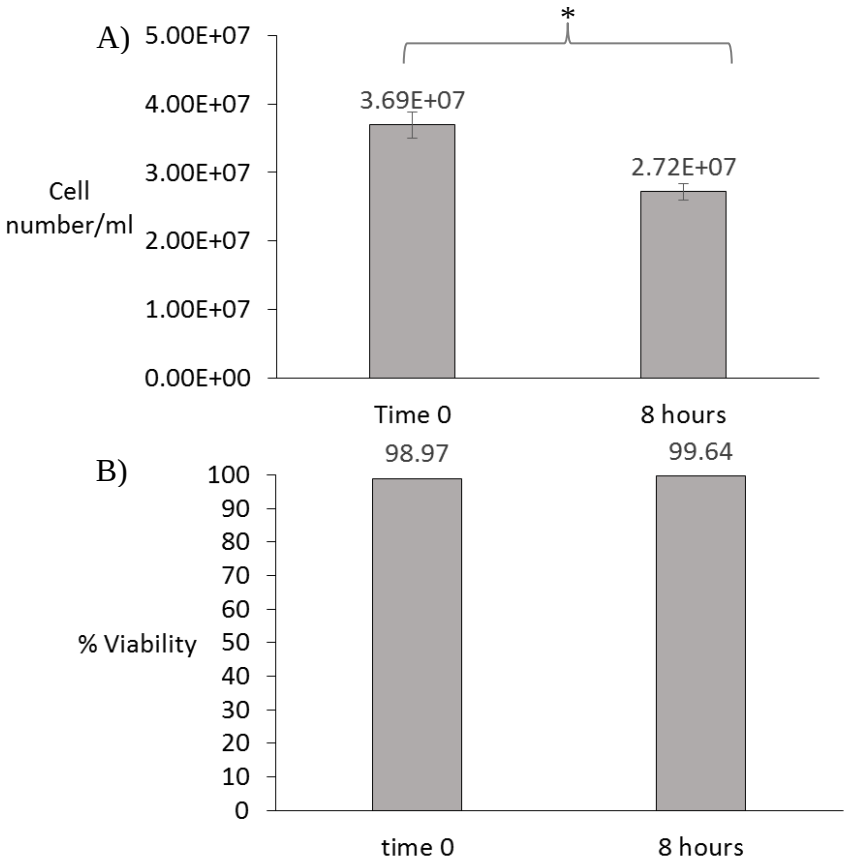

Figure 31. Cell number and viability of encapsulated liver cells (ELS) subjected to a healthy plasma scale bioartificial liver (BAL) treatment incorporating the filtration system. *ELS were fluidised within mini columns connected to the filtration system using healthy plasma for 8 hours. Cell number and viability were assessed prior to and post-8 hour treatment to view whether the BAL set-up negatively impacted cellular health. Cell number was significantly reduced during the course of the experiment (top) but viability was maintained (bottom). N=4, average +/- standard deviation; A) *P<0.05; B) P=0.65. Significance was assessed using the paired Student's T test and a 95% level of confidence.*

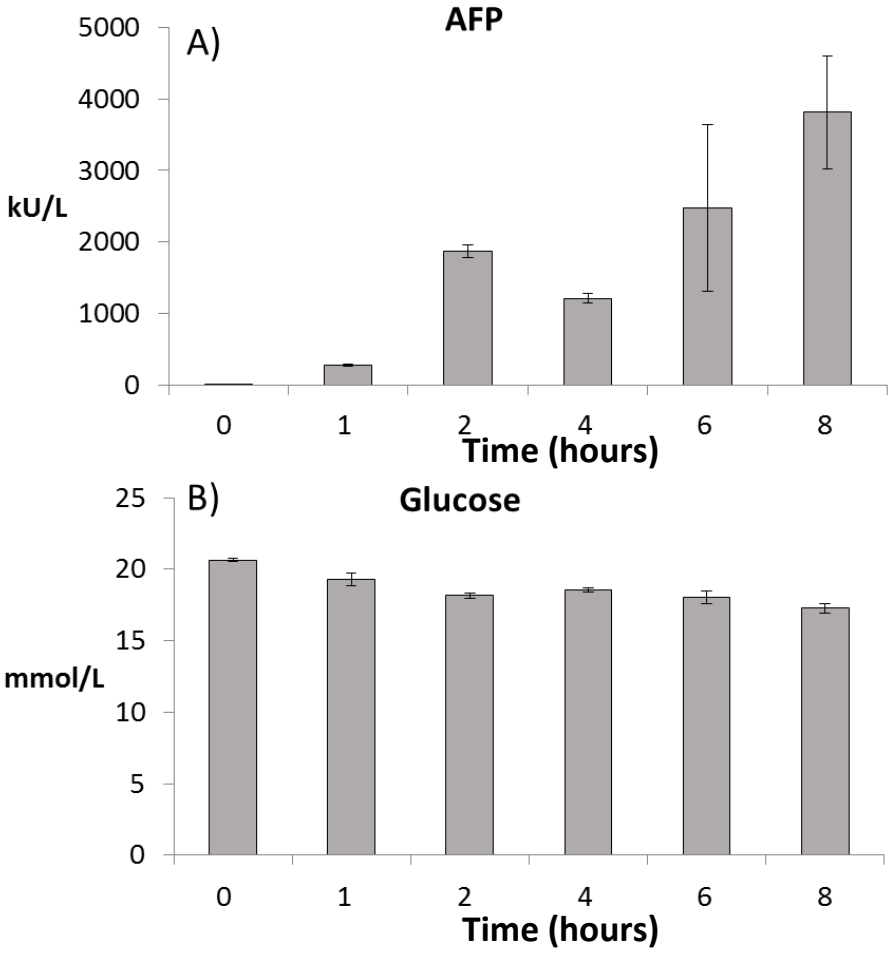

Figure 32. α-fetoprotein (AFP) production and glucose consumption by encapsulated liver cells (ELS) subjected to a healthy plasma scale bioartificial liver (BAL) treatment incorporating the filtration system. *ELS were fluidised within mini columns connected to the filtration system using healthy plasma for 8 hours. Hourly samples were taken to estimate AFP production and glucose consumption by the ELS. A) AFP production significantly increased from baseline to 8 hours ($P<0.05$). B) Glucose levels significantly decreased from baseline to 8 hours ($P<0.05$). N=4, average +/- standard deviation. Significance between the baseline (0 hour) and 8-hour timepoints were analysed using a Paired Student's T test and a 95% level of confidence.*

Large protein molecules were selected for assaying over the 8 hour treatment period to view whether the filter had any effect on their levels present in plasma. IgM, IgG and IgA were analysed by the clinical biochemistry team at the Royal Free Hospital.

It was seen that there was no significant difference in the quantity of any of these molecules at any point over the 8 hour treatment period (Figure 33).

Additional proteins were also assayed, including albumin, alpha-1-antitrypsin, alpha-1-acidglycoprotein and fibrinogen. There was no significant difference in the presence of albumin, or alpha-1-acidglycoprotein over the 8 hour period. Alpha-1-antitrypsin demonstrated an initial decrease from baseline pre-experiment levels to the first hour of recirculation, but no further reduction following. The concentration of fibrinogen initially peaked at the one-hour sample, and proceeded to steadily decrease over the 8 hour treatment period (Figure 34, page 113).

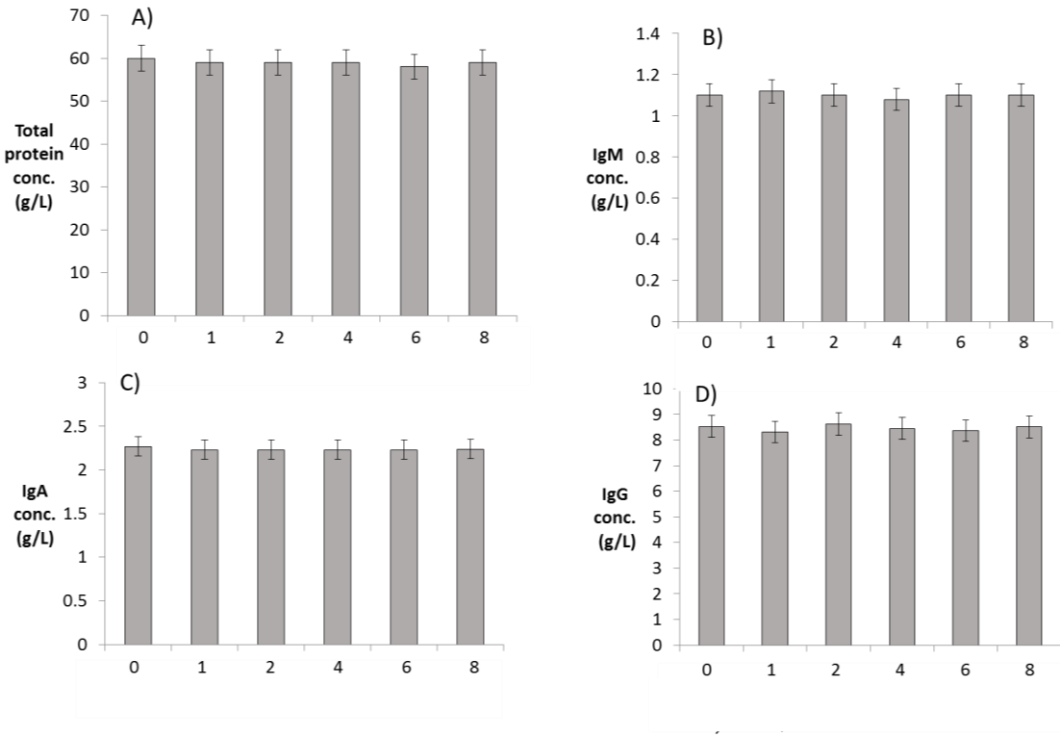

Figure 33. Maintenance of immunoglobulin levels through an 8-hour healthy plasma scale bioartificial liver (BAL) treatment incorporating the filtration system. *ELS were fluidised within mini columns connected to the filtration system using healthy plasma for 8 hours. Hourly samples were taken to assess maintenance of immunoglobulin levels. A) No significant difference was seen in total protein concentration from baseline to 8 hours (P=0.13). B) No significant difference was seen in IgM concentration from baseline to 8 hours (P=0.98). C) No significant difference was seen in IgA concentration from baseline to 8 hours (P=0.98). D) No significant difference was seen in IgG concentration from baseline to 8 hours (P=0.98). N=4, average +/- standard deviation. Significance was analysed using a paired Student's T test (baseline and 8 hour values) with a 95% level of confidence.*

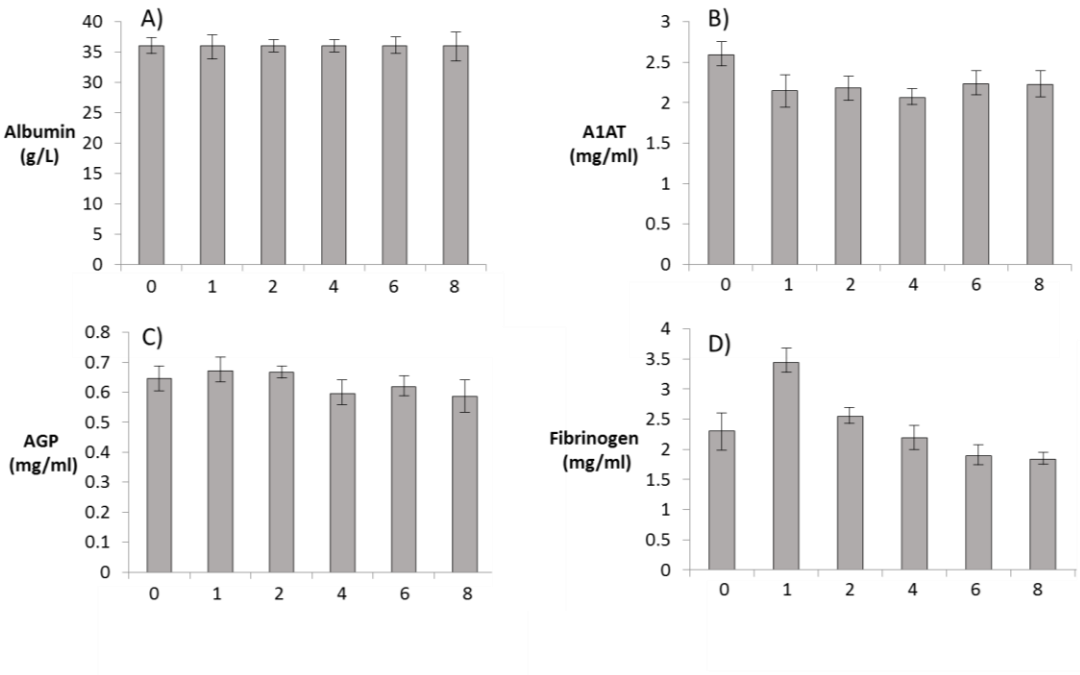

Figure 34. Plasma protein levels through an 8-hour healthy plasma scale bioartificial liver (BAL) treatment incorporating the filtration system. *ELS were fluidised within mini columns connected to the filtration system using healthy plasma for 8 hours. Hourly samples were taken to estimate plasma protein levels using ELISAs specific to human antigen. A) No significant difference was seen in albumin concentration from baseline to 8 hours (P=0.60 baseline vs. 8 hours). B) An initial decrease in apha-1-antitrypsin (A1At) concentration from baseline to 1 hour was seen, remaining consistent over the remainder of the 8 hour treatment period (P=0.59). C) No significant difference was seen in alpha-1-acid glycoprotein (AGP) concentration from baseline to 8 hours (P=0.10). D) Fibrinogen initially increased from baseline to the one hour sample, it steadily declined over the remainder of the 8 hour treatment period (P=0.07 baseline vs. 8 hours). N=4, average +/- standard deviation. Significance was analysed using a paired Student's T test with a 95% level of confidence for baseline vs. 8 hour values.*

Further biochemical parameters were measured at baseline and at hourly intervals over the eight hour treatment period. It was seen that there was a significant increase in the concentration of sodium ions and a significant decrease in the concentration of calcium ions present in the plasma from baseline to the one hour sample (Figure 35A and C). No additional change was seen between 1 and 8 hours. Additionally, potassium demonstrated a steady decrease, with a significant difference seen when

comparing the pre-filtration value with the 8 hour time point (Figure 35B). The concentration of phosphate ions showed a significant increase from baseline to the one hour time point, following this the level slowly declined over the remainder of the eight hour period (Figure 35D).

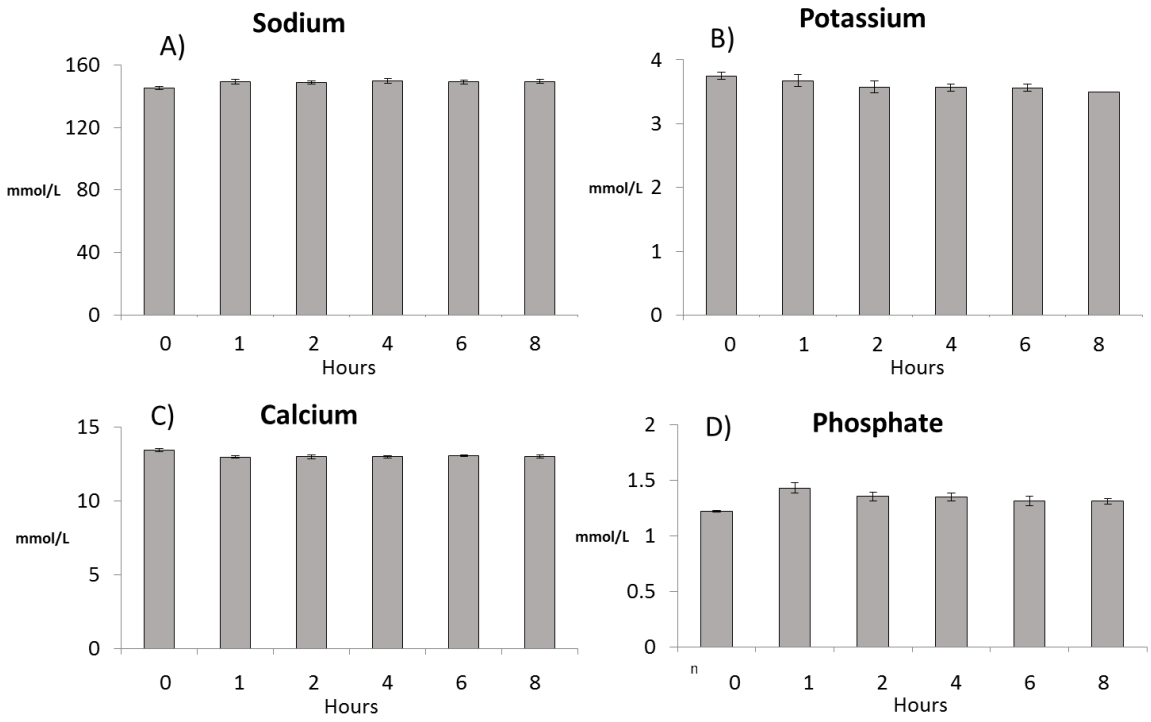

Figure 35. Plasma ion levels through an 8-hour healthy plasma bioartificial liver (BAL) scale treatment incorporating the filtration system. *Encapsulated liver cells were fluidised within mini columns connected to the filtration system using healthy plasma for 8 hours. Hourly samples were taken to estimate any change in plasma ion levels over the treatment period. A) Sodium ion concentration significantly increased from baseline to 8 hours ($P<0.05$). B) Potassium concentration significantly decreased from baseline to 8 hours ($P<0.05$). C) Calcium concentration significantly decreased from baseline to 8 hours ($P<0.05$). D) Phosphate concentration increased from baseline one hour, there was no significant difference between baseline and 8 hours ($P=0.75$). N=4, average +/- standard deviation. Significance was analysed for baseline vs. 8 hours using a Student's T test with a 95% level of confidence.*

Plasma lipid levels were also assessed for the samples over the 8 hour treatment period. Assays were performed for the detection of high density lipoprotein, low density lipoprotein and total triglyceride concentration. Total triglyceride

concentration remained stable throughout the eight hour treatment period, with no change from the baseline value seen until the 8 hour time point. Here a lower concentration of triglyceride was observed (Figure 36C). No significant change from baseline was seen in either high density lipoprotein or low density lipoprotein (Figure 36A and B). The change in urea concentration was also assessed. Urea concentration steadily increased over the 8 hour period with a significant increase seen between baseline and 8 hour time points (Figure 37).

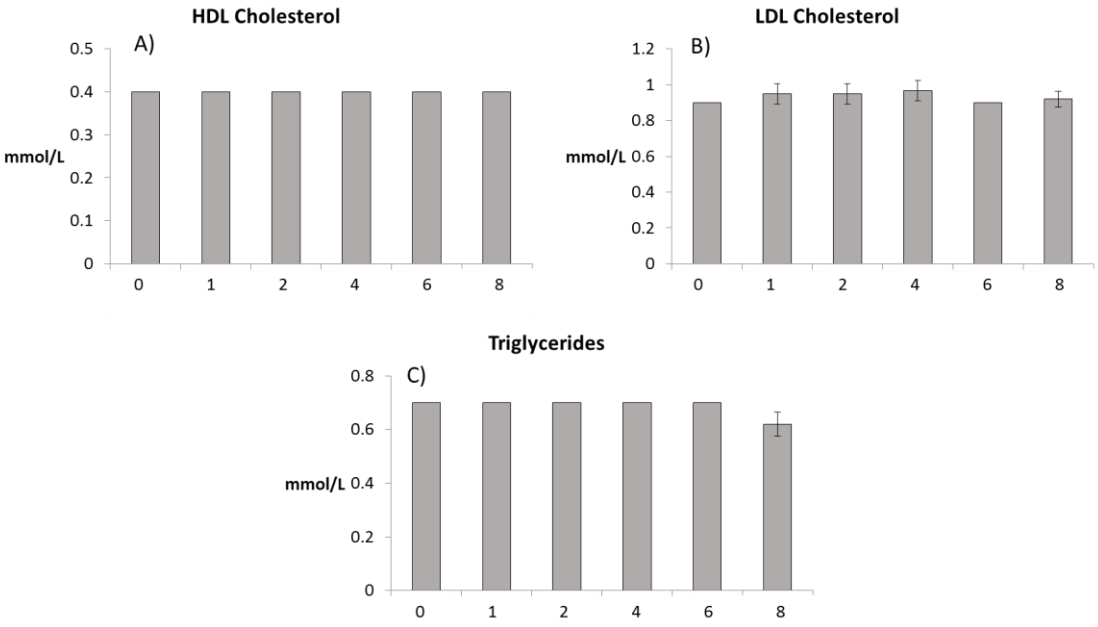

Figure 36. Plasma lipid levels through an 8-hour healthy plasma scale bioartificial liver (BAL) treatment incorporating the filtration system. *ELS were fluidised within mini columns connected to the filtration system using healthy plasma for 8 hours. Hourly samples were taken to estimate any change in plasma lipid levels over the treatment period. A) No significant difference was seen in high density lipoprotein (HDL) concentration from baseline to 8 hours (P=0.06). B) No significant difference was seen in low density lipoprotein (LDL) concentration from baseline to 8 hours (P=0.29). C) No significant difference was seen in total triglyceride concentration from baseline to 8 hours (P=0.09). N=4, average +/- standard deviation. Significance was analysed for baseline vs. 8 hours using a Student's T test with a 95% level of confidence.*

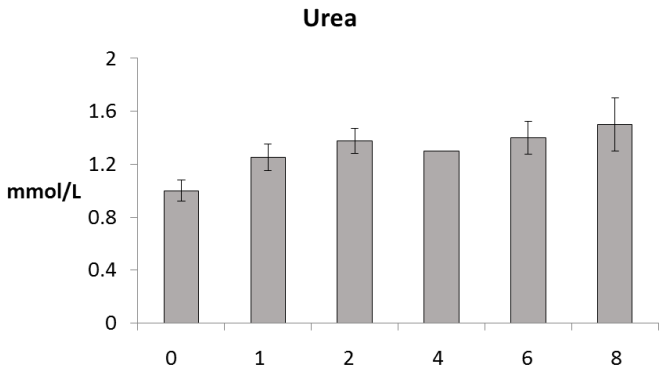

Figure 37. Plasma urea levels through an 8-hour healthy plasma bioartificial liver (BAL) scale treatment incorporating the filtration system. *ELS were fluidised within mini columns connected to the filtration system using healthy plasma for 8 hours. Hourly samples were taken to estimate any change in urea levels over the treatment period. Urea levels increased steadily with a significant increase seen between baseline and 8 hours (P=0.02). N=4, average +/- standard deviation. Significance was analysed for baseline vs. 8 hours using a Student's T test with a 95% level of confidence.*

4.3.6 Mini column experiment in liver failure plasma

The mini column experiment performed in healthy human plasma was repeated with plasma from a patient presenting with liver failure who had undergone therapeutic plasma exchange using a plasma apheresis system. The experiment using this liver failure plasma was again designed to provide a scaled-down model of the BAL, as it would be in a clinical setting, incorporating the filtration circuit. Upstream 25 cm^2 and downstream 0.6 µm filters were used.

As was seen in the healthy plasma mini column experiments, a decrease in cell number was seen from the beginning to the end of the experiment, with no change in viability (Figure 38). The encapsulated cell morphology was maintained throughout the 8 hour treatment period, as assessed by microscopy, see Figure 39 (page 119). Conjugated, unconjugated and total bilirubin were measured, with samples taken pre- and post- filters to view whether bilirubin present in the liver

failure plasma was removed by the filters. There did not seem to be a difference in the levels of bilirubin pre- and post- filtration (Figure 40; page 120). Additionally, in samples taken over the 8 hours, no DNA was present in any sample (including the time 0), suggesting continual removal by the filter system (Figure 41; page 121).

The levels of alanine transaminase (ALT) and aspartate aminotransferase (AST), markers of liver injury, were also assayed. For both proteins, there was a reduction seen between baseline and one hour of treatment. No further decrease was seen following this (Figure 42; page 122).

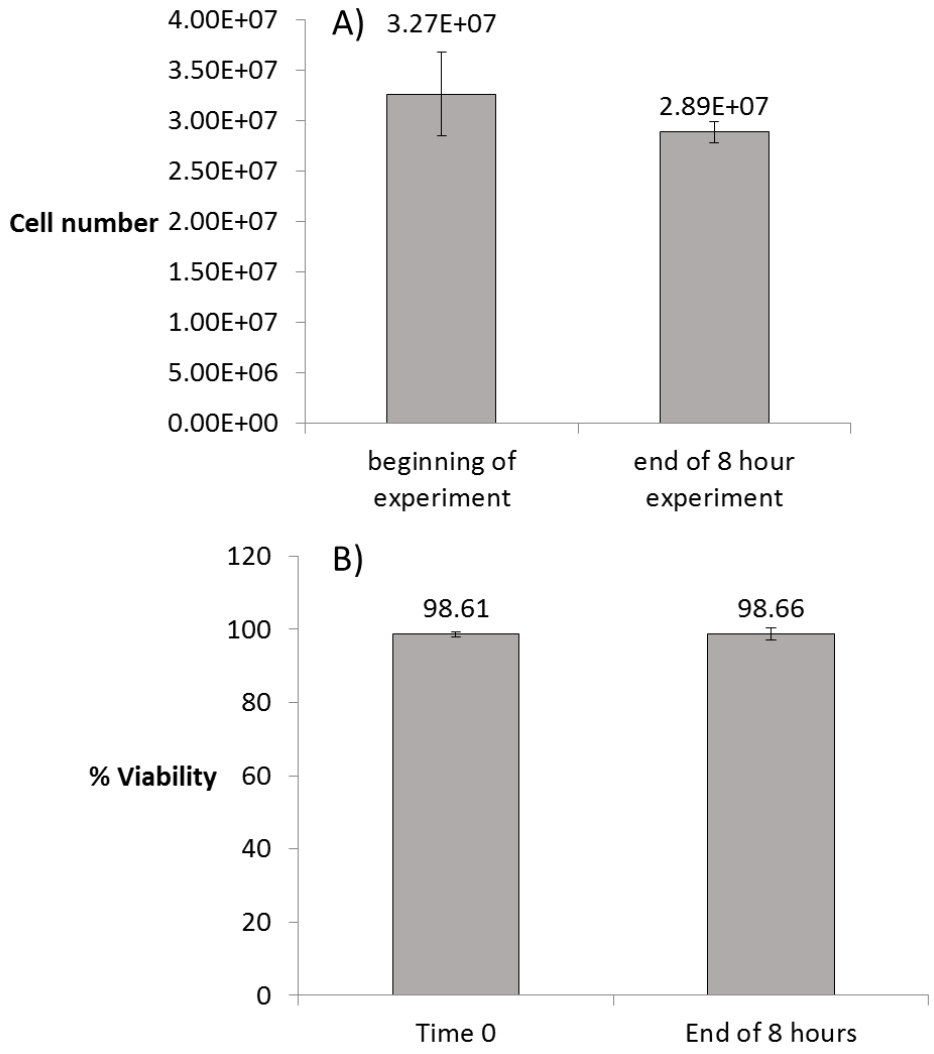

Figure 38. Cell number and viability of encapsulated liver cells (ELS) subjected to a liver failure plasma scale bioartificial liver (BAL) treatment cycle including filtration circuit. *ELS were fluidised within mini columns connected to the filtration system using liver failure plasma for 8 hours. Cell number and viability were assessed prior to and post-8 hour treatment to view how liver failure plasma impacted cellular health. A) Cell number was significantly reduced during the course of the experiment (P<0.05). B) Cell viability was maintained from 0 to 8 hours (P=0.83). N=4, average +/- standard deviation. Significance was assessed using the paired Student's T test and a 95% level of confidence.*

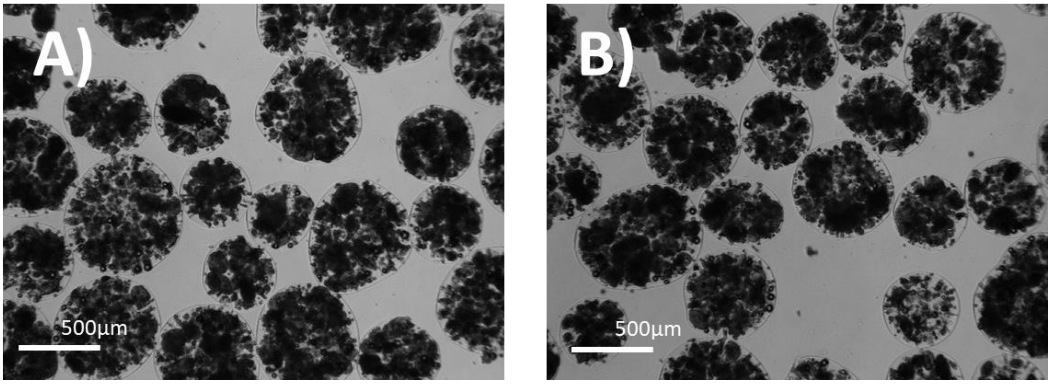

Figure 39. Morphology of encapsulated liver cells (ELS) subjected to a liver failure plasma bioartificial liver (BAL) scale treatment cycle including filtration circuit. *ELS were fluidised within mini columns connected to the filtration system using liver failure plasma for 8 hours, ELS morphology was viewed pre- (A) and post- (B) 8 hours using optical microscopy (x10) to estimate any detrimental effect of BAL treatment on ELS integrity. ELS integrity was maintained through 8 hours of treatment.*

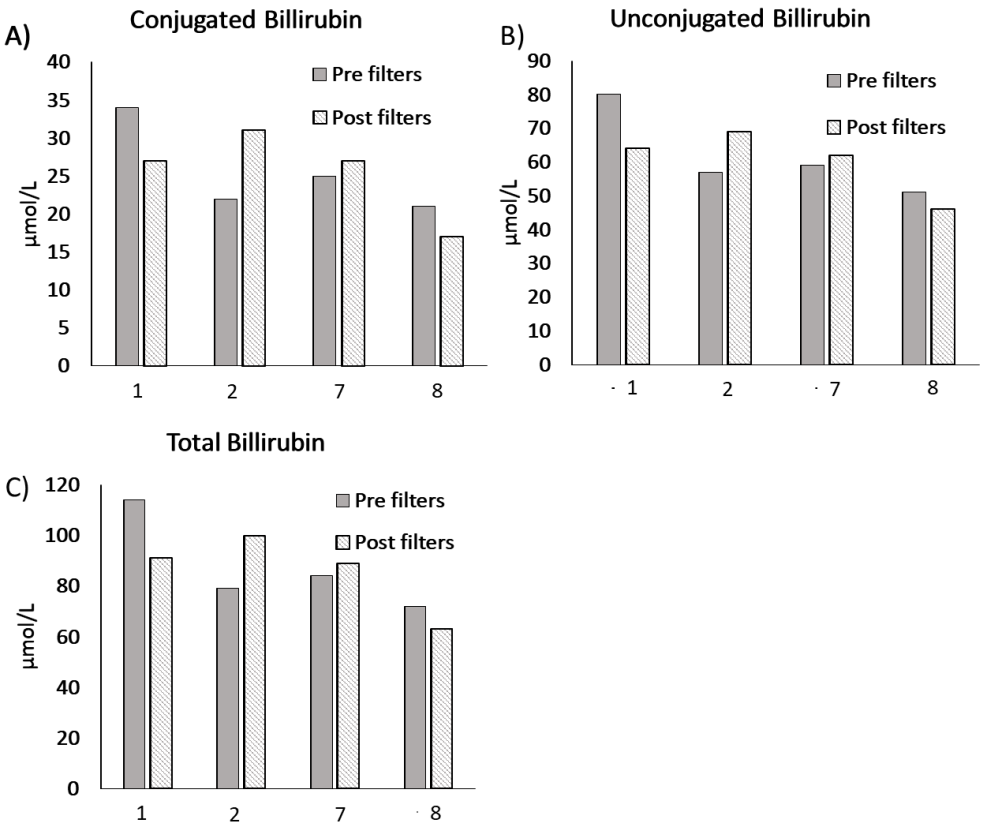

Figure 40. Plasma bilirubin levels through an 8-hour liver failure plasma bioartificial liver (BAL) scale treatment incorporating the filtration system. *Encapsulated liver cells were fluidised within mini columns connected to the filtration system using liver failure plasma for 8 hours. Bilirubin levels were assessed prior to and during the 8 hour treatment to estimate any change. A) Levels of conjugated bilirubin decreased over the course of the experiment. B), Levels of unconjugated bilirubin decreased over the 8 hours. C) Total bilirubin decreased over the 8 hours. N=2, average values.*

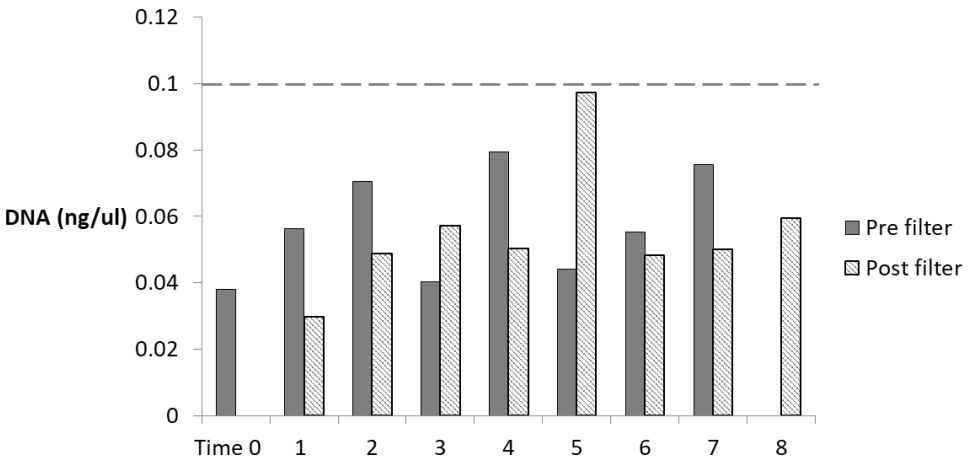

Figure 41. Plasma DNA levels through an 8-hour liver failure plasma bioartificial liver (BAL) scale treatment incorporating the filtration system. *Encapsulated liver cells were fluidised within mini columns connected to the filtration system using liver failure plasma for 8 hours. DNA levels were assessed during the 8 hour treatment to estimate any change using qPCR. DNA was consistently below the limit of detection (hashed horizontal line). N=4.*

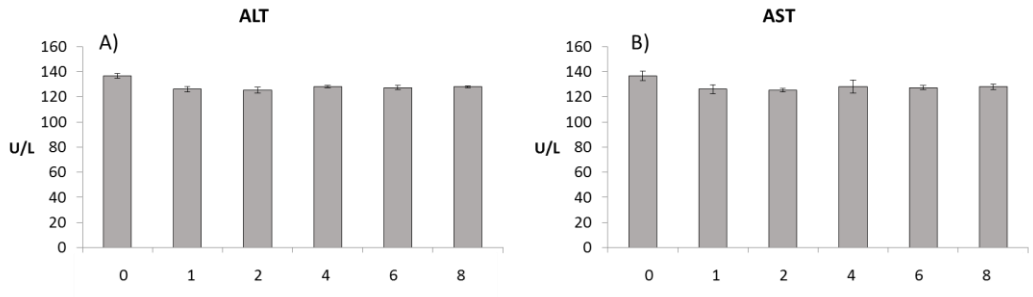

Figure 42. Plasma alanine transaminase (ALT) and aspartate transaminase (AST) levels through an 8-hour liver failure plasma bioartificial liver (BAL) scale treatment incorporating the filtration system. *Encapsulated liver cells were fluidised within mini columns connected to the filtration system using liver failure plasma for 8 hours. AST and ALT levels were assessed prior to and during the 8 hour treatment to estimate any change. A) ALT concentration decreased from baseline to 1 hour, with no further decrease between 1–8 hours. B) AST concentration decreased from baseline to 1 hour, with no further decrease between 1–8 hours. N=4, average +/- standard deviation. Significance was assessed using the paired Student's T test and a 95% level of confidence.*

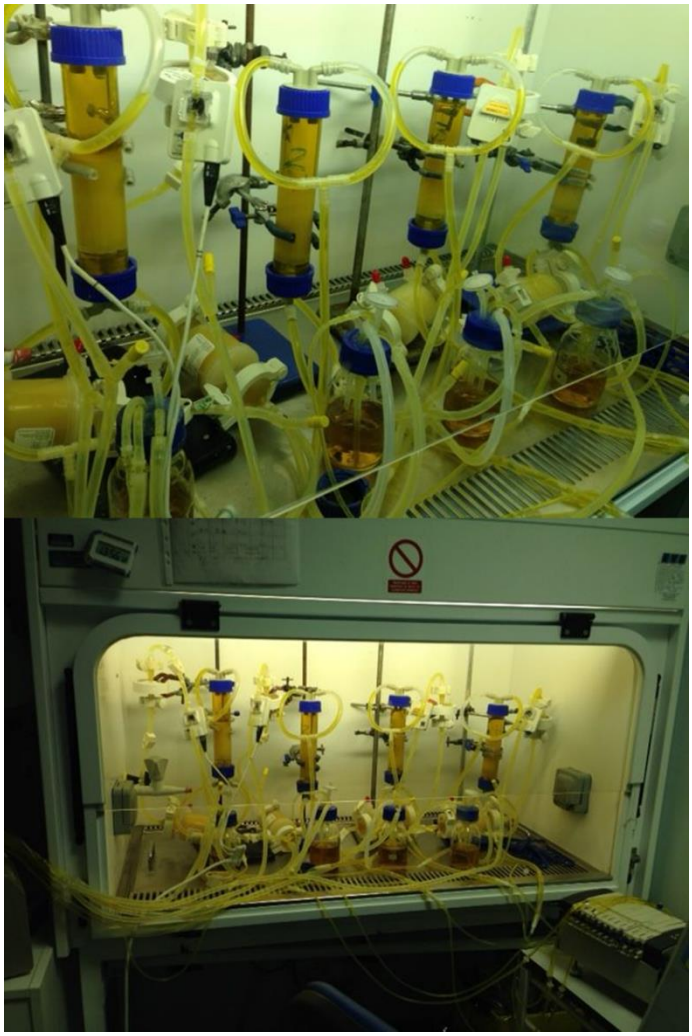

Figure 43. Photographic detail of the set-up of an N=4 mini column experiment within a Class 2 biological safety cabinet. *Encapsulated liver cells were fluidised within mini columns containing liver failure plasma and connected to the filtration system for 8 hours.*

4.4 Discussion

4.4.1 The required capacity of the filtration system for DNA

qPCR proved effective at detecting both DNA released from individual and encapsulated HepG2 cells in plasma. Observation of HepG2 cells alone in plasma showed a higher DNA concentration than was expected. After a literature search it was seen that this increased DNA quantity may be due to cells currently undergoing S or G2 phase of the cell cycle, at which point the quantity of DNA present in the nucleus would be double the expected value.[111] DNA release from ELS under gentle rotation with an incubator for 24 hours was only 1.5% of that observed from encapsulated cells in which 100% cell death was analysed. We can use this value to gain an idea of DNA release from cells within the BAL under treatment conditions, and in a scenario of 100% cell death using the quantity of DNA present in a HepG2 cell, which as determined using the model chromosome number of 55 provides 7.83 pg/cell.[111]

If cell death occurred at 1.5% during an 8 hour treatment cycle, the quantity of DNA to be removed by the filters would be:

At high cell densities: ($1x10^{11}$ x 7.83 pg/DNA in a cell[111] x 0.015) = **11.7 mg DNA**

At low cell densities: ($7x10^{10}$ x 0.015) = **8.22 mg DNA**

Compared with:

100% cell death at high densities = 783 mg DNA

100% cell death at low densities = 548 mg DNA

To ensure the safety of this device, the filter system needs to have the capacity to remove 783 mg of DNA to represent the worst case scenario of 100% cell death and subsequent total DNA release into the system. As the volume of cells required to analyse this is so high, analysis of this will be performed in subsequent chapters using a 13.6 x smaller scale model, whereby the filter will be challenged with 58 mg of DNA.

4.4.2 Removal of alginate particles by the filtration system

The use of three separate particulate analysis techniques could, potentially, provide a thorough way to analyse and corroborate data. Unfortunately, due to the multiple components found in plasma samples, and the tendency of plasma to clot when stored frozen, as is required to use the particle analysis machines, it is difficult to accurately analyse plasma samples for particle concentration. Although samples can be assessed to view the relative reduction in particle size and burden, results do not provide enough detail to enable full conclusions to be drawn. Culture media from a Day 5 media change treated with the filtration system demonstrated consistent reduction in particle size and number. Mastersizer analysis specifically demonstrated removal of particulates in the 10 µm–600 µm range. A peak of particles around 1,000 µm was seen, which was also observed in the MilliQ water control, this was likely caused by microbubbles within the system, the manufactures recommend using a MilliQ water control, as was done here, to define this.[112] The Mastersizer analysis process has drawbacks in that the absolute number of particles cannot be quantified as this technique uses obscuration of light to determine the percentage composition of a sample within specified size brackets, rather than gathering data regarding individual particles.[62,112] Zetasizer and NanoSight software were also used to analyse samples, Zetasizer analysis provided a similar disadvantage in that a distribution of the concentration of particles by size could not be obtained, although this analysis did demonstrate a reduction in the average particle size from 12 µm in the initial sample to <2 µm post-upstream and <1 µm post-downstream filter. NanoSight analysis provides an ideal in terms of particle size and the concentration of particles within that size bracket, although it can only measure particles up to 2 µm in diameter and therefore, would not detect those larger particles shown to be present in the pre-filter sample by Mastersizer analysis.[66,113]

4.4.3 Capacity of the filtration system for plasma, DNA and endotoxin

It is essential to know the total volumetric capacity of the filters to calculate the optimal filter size and the number of filters which might be required for a full BAL treatment cycle. At a flow rate of 60 ml/min, as would be seen in the BAL circuit returning to the patient, 3.6 litres of plasma would need to pass through the filters per hour, equating to 28.8 litres of plasma over an 8 hour treatment cycle.[20] The total volumetric capacity of the filter must, therefore, exceed this; alternatively a number of filters could be used in parallel during the treatment cycle to meet this requirement.

After initial plasma-filter experiments it was evident there may be a problem with the plasma we were using, due to samples being particularly cloudy prior to filtration and becoming clear post-filtration. Using this plasma, the capacity of the filter was highly reduced. Retrospectively the cloudy plasma was identified as a result of dead blood cells due to therapeutic treatment of the patient prior to total plasma exchange.[114] For future work these patients were excluded from the study, as this plasma does not represent anything that would come into contact with the BAL in clinical practice.

The volumetric capacity of the 170 cm^2 upstream filter and the 1.2 µm downstream filter were analysed in series using a 'brute force' approach. 40 litres of plasma were passed through the filters in series, with a pressure increase seen at the last 5 litres, demonstrating that the volumetric capacity of the filters can exceed the 28.8 litres required of an 8 hour BAL treatment cycle. It is difficult to assess the total volumetric capacity on more than one occasion, due to the lack of availability of sufficient quantities of plasma, therefore, this experiment was only performed once. As the 1.2 µm downstream filter demonstrated a very low pressure drop on multiple occasions, to further enhance the safety of the filtration system, a smaller pore sized filter, 0.6 µm, was selected for further use.

The 25 cm^2 filters were initially analysed using FFP spiked with both endotoxin and HepG2 DNA, to gain an indication as to the removal capacity of these filters. This was repeated to view whether the equivalent occurred in the 170 cm^2 filter

scaling up the quantities of DNA and endotoxin used accordingly, to assess whether simultaneous challenge with these two substances impacted the filter's efficacy for their removal. It was seen that the 170 cm^2 removed all DNA and endotoxin from the plasma to below the limit of detection. This removal of 4.234 mg DNA (equivalent of DNA released from the BAL during an 8-hour treatment, scaled down for the 170 cm^2 filter) and 4,200 EU (just below the approximate total of a patient with liver failure, which is approximately 5,520 EU in a 70 kg human with 3 litres of plasm[115]) demonstrated the potential of the filter system to remove DNA and endotoxin contamination simultaneously. Low levels of DNA and endotoxin were used here for this initial experiment. The efficacy of the filter system at removing higher levels of these contaminants will be discussed in the next chapter.

4.4.4 Maintenance of plasma proteins by the filtration system in a scale BAL model

The 25 cm^2 upstream filter and mini 0.6 µm downstream filters were used in scale BAL experiments. The 25 cm^2 upstream filter has 13.6x less surface area than the full-scale 340 cm^2 upstream filter, therefore to ensure the correct flux was used in these experiments as would be seen in the full-scale, the flow rate of the large-scale filters was re-calculated using for use in the mini-column experiments. This was as follows:

60 ml/min divided by 13.6 = 4.4 ml/min

4.4 ml/min = 264 ml/hour passing through the filter system

Four replicates of the mini column experiments were performed, involving four different filter four different plasma samples. Over the course of the experiment the number of cells per ml of ELS was reduced, but the percentage viability was maintained, suggesting the plasma and filtration circuit did not have a detrimental effect on cell health. This decrease in number of cells/ml is not seen *in vivo* experiments,[20,116] this is unlikely due to leaching of cells from the ELS, as this was not observed using light microscopy. The decrease in number may be due to swelling of the ELS when moving from culture media to plasma, which would

cause a decrease in cells/ml of alginate, or due to sampling error at the end of the experiment, although the former appears more likely as this effect was also observed in the liver failure plasma experiment.

Increase in AFP over the 8 hours demonstrated the ELS functionality, additionally, glucose levels decreased over the suggesting active cell metabolism.

The concentration of large protein molecules assayed, (IgM, IgG and IgA) over the 8 hour treatment period did not change. Additionally, there was no significant difference in the presence of albumin, AGP or A1AT over the 8 hour period. Fibrinogen concentration initially peaked at the one-hour sample, and proceeded to steadily decrease over the 8 hour treatment period. This initial peak may be due to the leaching of fibrinogen from the ELS into the plasma, as these cells synthesise fibrinogen.[22] Fibrinogen is a smaller molecule than IgM, 340 kDa compared with 950 kDa in size[117] with an isoelectric point of, 5.1-6.3 compared with IGM's 5.5–7.4.[117] Due to this, it seems unlikely that the subsequent decrease in fibrinogen concentration was due to its removal from the plasma by the filtration system, as there was no effect on the concentration of IgM. It is possible that between the collection of samples and the analysis of these proteins, some clotting occurred within the plasma samples which could have altered the level of fibrinogen present for analysis.[118]

Further biochemical parameters demonstrated a significant increase in the concentration of sodium ions and a significant decrease in the concentration of calcium ions present in the plasma from baseline to the one hour sample with no additional change seen between 1 and 8 hours. This is likely due to sodium ions present in the alginate leaching into the plasma and calcium ions present in the plasma being cross-linked with the alginate monomers.[23] The change in concentrations of these ions were minor, and all except calcium fell within the reference for healthy samples: 135–147 mmol/L for sodium; 3.5–5.5 mmol/L for potassium; 0.90–1.45 for phosphate. The reference value for calcium is 2.25–2.75 mmol/L, this is considerably lower than the 13.5 mmol/L seen in our experiment. This is likely due to the addition of calcium to plasma to counteract the effect of citrate prior to addition to the ELS, and is something to be aware of for future

experiments.[119] Urea concentration significantly increased from baseline to 8 hours, the maintenance of urea synthesis by HepG2 cells has been previously reported so this result is as expected and demonstrates the functionality of the biomass.[14,22,120]

4.4.5 Liver failure mini column experiments

As was seen in the healthy plasma mini column experiments, a decrease in cell number was seen from the beginning to the end of the experiment, with no change in viability or bead morphology, demonstrating that the liver failure plasma had no detrimental effect on the stability of the encapsulated HepG2 cells. It was interesting to see that the decrease in cell number over the 8 hour period was similar to that observed in healthy plasma, suggesting that incubation in this liver failure plasma did not cause additional cell death over the treatment period. Conjugated, unconjugated and total bilirubin were measured, there did not appear to be a change in levels of bilirubin pre- and post- filtration it is difficult to see from these results whether the biomass itself had any effect on the concentration of any of these forms of bilirubin, as has been previously demonstrated.[14,20] Although relatively large at 584 kDa, unconjugated bilirubin is an uncharged molecule, whereas conjugated bilirubin is charged, a result of its carboxyl group, making it soluble in water,[117,118] no difference was observed in the levels of either of these compounds pre- and post-filtration. The levels of ALT and AST, markers of liver injury, were also assayed. As expected the levels were high, as is seen in liver injury.[1]

The next chapter will look at the full-scale filters, along with additional experiments required before this filtration system can proceed to *in vivo* use.

Chapter 5

Characterising a full-scale filtration protocol for use within a bioartificial liver

5. Full-scale filters

5.1 Introduction

This chapter discusses the use of the full-scale filters, namely the upstream 340 cm^2 60ZB05A depth charge filter and the downstream 0.6 µm filter. The main aim of this chapter was to assess the full-scale filter combination for suitability of use within the full-scale BAL circuit. This was performed to ensure that these full-scale filters were fit for purpose to continue into *in vivo* experiments.

5.1.1 Requirements of the full-scale filter

It is essential to know the total volumetric capacity of the filters to calculate optimal filter size and filter number required in the bioartificial liver circuit. At a flow rate of 60 ml/min 3.6 litres of plasma would pass through the filters every hour of treatment. It is hypothesised to treat a human patient with acute liver failure, an 8 hour treatment cycle with the BAL would be required.[20]

In terms of treatment times:

> 8 hours of treatment would mean a total of 28.8 litres of plasma passing through the filters over this period.

The actual quantity of the plasma within the BAL-patient circuit in a treatment-scenario is 7 litres. This is broken down into 3 litres of plasma within the biomass and associated BAL circuit, 3 litres of plasma within the patient[115] and 1 litre of plasma comprising the dead volume of the filtration circuit. This 7 litres will be within a recirculating system, where the filters will be constantly challenged with plasma that has flowed through the biomass and patient. Therefore, the total volumetric capacity of the filter must exceed 28.8 litres for use *in vivo*. In previous

chapters, a 40 litre volume of plasma was passed through the 170 cm^2 filter, surpassing this quantity.

5.1.2 Additional safety testing in these filters

The reduction of tetrazolium salts found in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT (pale yellow), to coloured (dark blue) formazan compounds can be used to determine the metabolic activity of cells in culture. The production of this coloured compound can be measured using a wavelength of 570 nm, the greater the metabolic activity of the cells, the greater the production of formazan compounds from the tetrazolium salts.[121] This leads to an increase in absorbance at 570 nm, conversely, when cells are less metabolically active, less blue compound is produced leading to a decreased absorbance. In this way, the metabolic activity can be inferred by relating the values achieved to a positive control. This method can be used to gain an understanding of cytotoxicity, as when cells are exposed to a cytotoxic environment their metabolic activity decreases..[122] This method would provide a simple way to gain an indication as to whether filtration of plasma has any detrimental effect on HepG2 cells encapsulated in alginate.

5.2 Methods

5.2.1 MTT toxicity assay

Patient plasma samples were processed using the upstream 340 cm^2 and downstream 0.6 µm filters in series, as described in detail in Chapter 2. Samples of plasma were obtained prior to, and post, filtration for use in the assay.

Day 12 ELS were obtained, cells were washed briefly with plasma and BSA-free culture media to remove traces of plasma originating from the cell culture process. Cell counts were performed and an appropriate volume of cells were seeded into 96 well plates. An appropriate volume of either filtered plasma, unfiltered plasma or a culture media control was added to each of the cell samples. Cells were incubated in the solution at 37^{o}C for a total of either 8 or 24 hours. At this point an MTT assay was performed on the cells as described below.

MTT assay

Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
Sterile PBS
4mM HCl in isopropanol
96 well clear flat bottomed culture plate
Spectrophotometer at 450nm and analysis software (Omega Readers spectrophotometer, Manta analysis software)

0.75 mg/ml stocks of MTT were prepared in PBS and stored in single use aliquots at -20°C until required.

Liquid was aspirated from the HepG2 cells which were previously incubated in either culture media (control), filtered plasma or unfiltered plasma for either 8 or 24 hours, 100 µl of each sample was added in triplicate to a 96 well plate. 100 µl of MTT (diluted 0.75 mg/ml in PBS) was added to each well and incubated at 37ºC for 3 hours, until the formation of blue crystals at the base of the 96 well place could be seen with the naked eye. Liquid was aspirated from the wells with caution taken not to dislodge the crystals, 100 µl of acidified isopropanol was added to each well, and the plate was sealed with a plate sealer and placed on an orbital shaker for 30 minutes to dissolve the crystals. The absorbance was read using the spectrophotometer at 570 nm. The value produced is inversely proportional to the metabolic activity occurring, with high values representing low metabolic activity. Readouts were normalised using cell number and transformed to a percentage of the control (cells incubated in culture media) to gain a reference of metabolic activity.

5.3 Results

5.3.1 Toxicity testing of the full-scale filters

5.3.1.1 Testing filters for the presence of pre-existing endotoxin

The upstream 340 cm^2 and downstream 0.6 µm filter were analysed for the presence of pre-existing endotoxin contamination. Samples were collected from the initial filter rinse and snap frozen in liquid nitrogen for subsequent analysis during the preparation of six of each type of filter.

Samples taken from the first 50 ml of water flushed through the filter, the last 50 ml of the water wash and 1 litre into the saline rinse were analysed using the PyroGene endotoxin assay. All samples collected were below the limit of detection, confirming that the filters did not contain any endotoxin contamination (Figure 44).

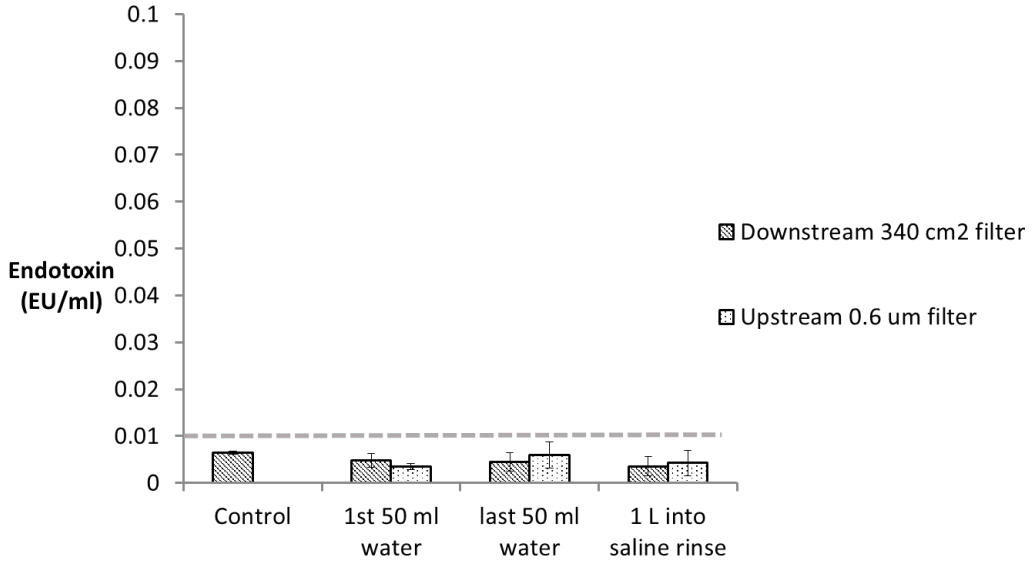

Figure 44. Assessing the upstream 340 cm^2 and downstream 0.6 µm for the presence of pre-existing endotoxin. *Samples were taken from the initial water and saline flush and analysed using the PyroGene Endotoxin assay. Both filters tested negative for endotoxin contamination. Hashed horizontal grey line represents the lower limit of detection. N=6, values shown are average +/- standard deviation.*

5.3.1.2 Testing empty alginate beads for the presence of endotoxin

To ensure that no endotoxin contamination within the BAL would originate from the alginate itself, empty alginate beads were used to ascertain this. Empty beads were set up using the mini columns, and were recirculated in saline for 8 hours. A sample was taken every hour to measure the level of endotoxin using the PyroGene endotoxin assay. The endotoxin level recovered was 5–10 fold below limit of detection (0.01 EU/ml) consistently over the 8 hour period (Figure 45). This demonstrates that the alginate spheres themselves should not present a contamination issue.

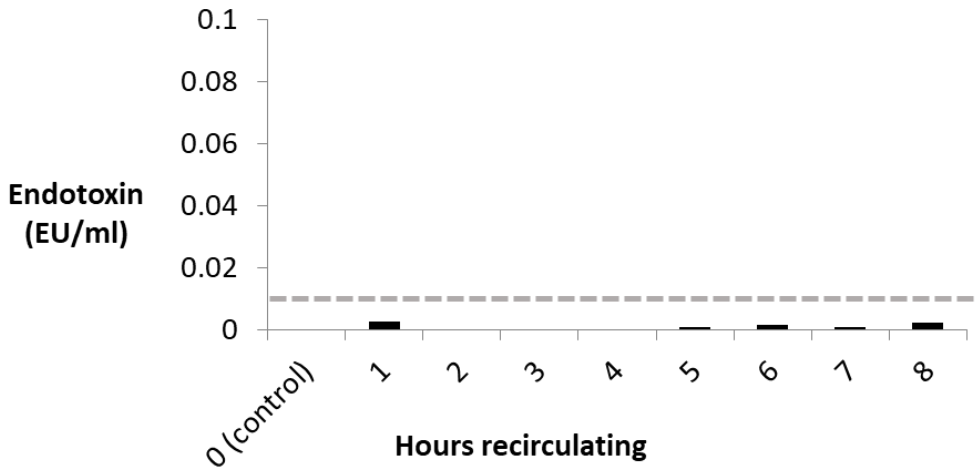

Figure 45. Assessing alginate for the presence of pre-existing endotoxin contamination. *Empty alginate spheres recirculated in saline for 8 hours tested negative for the presence of endotoxin at each hourly interval. Hashed horizontal grey line represents the lower limit of detection. N=4, values shown are average +/- standard deviation.*

5.3.1.3 Testing media samples taken from Day 12 of the ELS biomass culture for the presence of endotoxin

Media samples were taken from four of the full-scale ELS FBB biomass cultures at Day 12. Samples were diluted 1 in 10 and analysed for the presence of endotoxin using the PyroGene endotoxin assay. Three out of the four samples tested provided negative results, these were FBB A, B and C (below the limit of detection, 0.1 EU/ml; Figure 46) and one provided a positive result, FBB D.

It was later seen that FBB D, which had tested positive for the presence of endotoxin at Day 12, had succumbed to a bacterial infection. This bacterial infection was likely to have been pre-existing at the time of sample collection, and therefore, the cause of this positive endotoxin result. The positive product control for each of these experiments provided an as expected return of endotoxin.

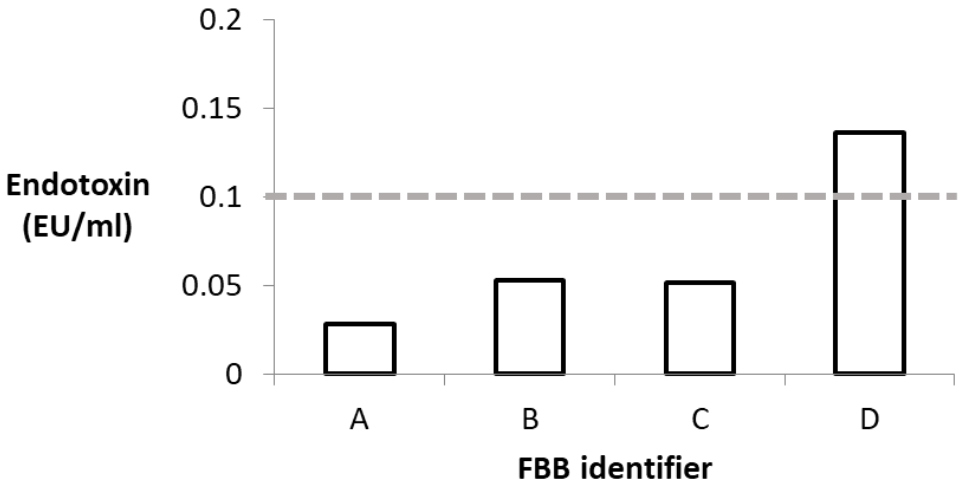

Figure 46. Levels of endotoxin in Day 12 FBB media samples. *Media samples were taken at Day 12 from four full-scale FBB experiments and analysed for the presence of endotoxin. FBB A, B and C tested negative, FBB D tested positive. FBB D's positive result was likely due to bacterial infection. Hashed horizontal grey line represents the lower limit of detection. Results presented are the average of three media samples taken from each FBB on Day 12.*

5.3.1.4 Testing filtered plasma for metabolic effects on HepG2 cells

To view whether filtration of the plasma using the filter series had any detrimental effect on the HepG2 cells encapsulated in alginate, an MTT metabolic assay was performed. Samples were obtained from plasma prior to filtration and after filtration with both the upstream 340 cm^2 and downstream 0.6 µm filters for analysis. This was performed using four different patient plasma samples and four different sets of filters. It was seen that following incubation of the encapsulated HepG2 cells for a period of either 8 hours (as is the treatment time in the BAL) or 24 hours, no significant difference was produced with respect to change in metabolic activity of cells incubated in non-filtered plasma compared with filtered plasma, Figure 47.

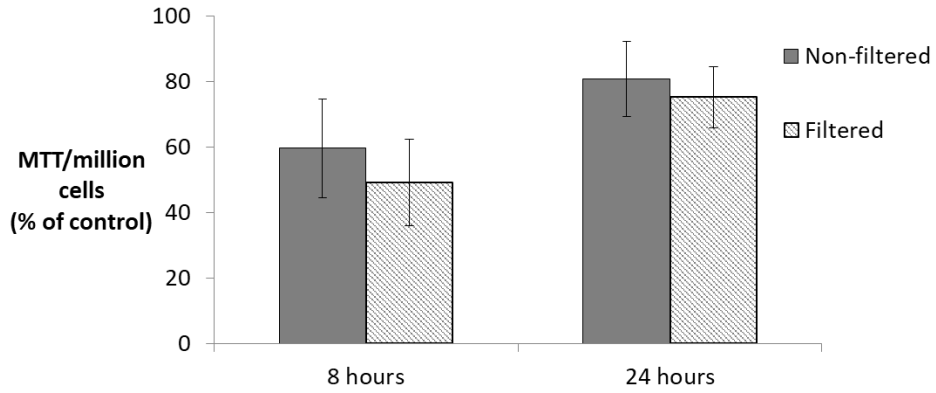

Figure 47. Metabolic activity of encapsulated liver cells (ELS) following incubation in filtered plasma. *. ELS were incubated in filtered or non-filtered plasma for a period of 8 or 24 hours to view whether plasma filtration had any detrimental effect on metabolic activity of the ELS. There was no significant difference in ELS metabolic activity between filtered or non-filtered plasma at either 8 (P=0.19) or 24 hours (P=0.15).* ***N=4****, results are average +/- standard deviation, Significance was assessed using the Paired Students T-test at a 95% level of confidence.*

5.3.2 Heparin removal using the full-scale filters

The full-scale filters were primed with 10 IU/ml heparin in saline using a recirculating system. A significant decrease was seen in the concentration of heparin in the sample prior to filtration and after one hour of recirculation, no further decrease was seen between one and three hours of recirculation (Figure 48A). Following priming of the filters, heparinised plasma was recirculated through the same filters enabling analysis of any additional removal of heparin. It was seen that there was no change in heparin concentration from the pre-filtration sample compared with the filtered sample after 8 hours of recirculation (Figure 48B).

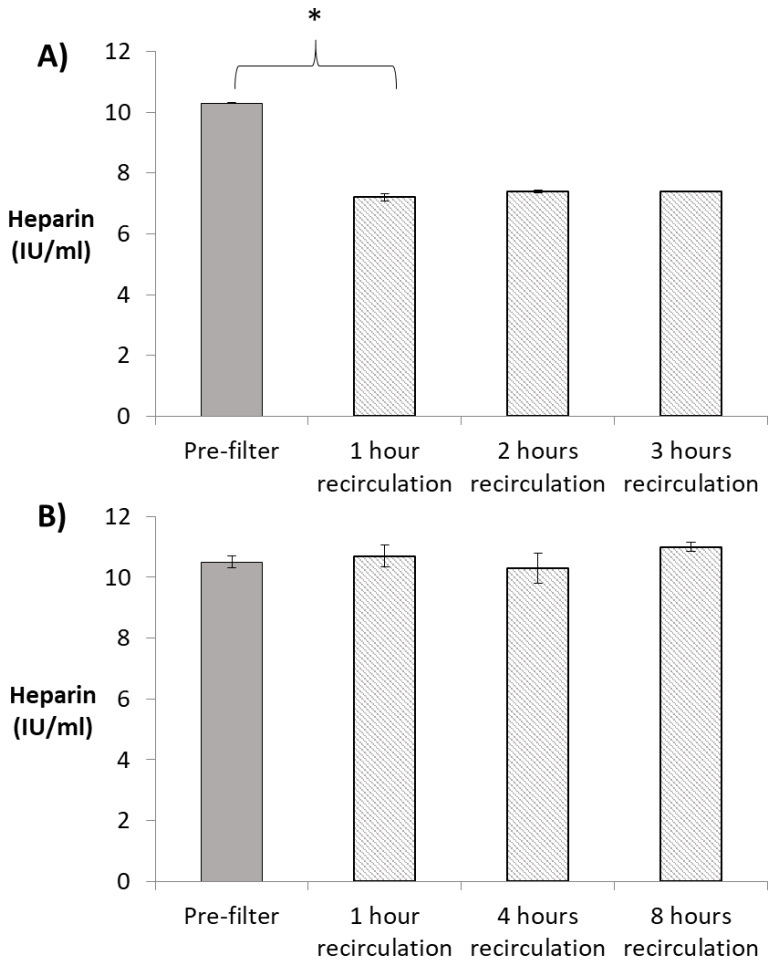

Figure 48.Heparin priming of the upstream 340 cm^2 filter. *A) Filter priming. 10 IU/ml heparin-saline was recirculated through the upstream 340 cm^2 filter, a significant reduction in heparin concentration was seen pre- and post-filtration ($p<0.05$). B) Heparin concentration was maintained over 8 hours of recirculation in filtered plasma following priming of the filters with heparin-saline. Values presented are average +/- standard deviation (n=4, A) and average +/- high/low value (n=2, B). Significance for A was assessed using ANOVA with a 95% level of confidence; *$p<0.05$.*

5.4 Discussion

5.4.1 Examining filter output for the presence of pre-existing endotoxin

To enable the filtration system to pass MHRA and FDA regulatory standards as a medical device, it needs to adhere to the requirements for a maximum endotoxin concentration. The defined acceptable endotoxin concentration for a medical device, as per the FDA guidelines is 0.5 EU/ml or 20 EU/device,[109] dependent upon the nature of said device, therefore, the output of the filters, prior to coming into contact with patient plasma will need to be below this value.

Five filters of each type, upstream 340 cm^2 and downstream 0.6 µm, were analysed for the presence of pre-existing endotoxin. Samples were taken during the initial 50 ml of the recommended pre-use water rinse and the initial first 1 L of the saline rinse, which were performed prior to use of the filters with plasma. All samples taken from all 5 filters returned negative results for the presence of endotoxin. As 3M market their 60AB05A filter series for the purification of biologicals this was as expected.[101]

5.4.2 Endotoxin contamination of empty alginate beads

Raw materials used in the production of the biomass need to be cleared for potential endotoxin contamination to enable the BAL to meet MHRA and FDA guidelines.[27,103,109] It has been previously reported that standard laboratory alginate presents a source of endotoxin contamination, as high as 10 EU/ml.[123] Methods are available for the purification of alginate, which can reduce its bioburden to the level of the solvent in which it is dispersed.[124] Alginate used to produce cells within the BAL has undergone previous purification prior to use, but, to ensure that no endotoxin contamination within the BAL would originate from the alginate itself, empty alginate spheres were tested for endotoxin content. Empty alginate spheres were recirculated in sterile saline for 8 hours, samples were taken hourly to measure

the level of endotoxin. The endotoxin level recovered was 5–10 fold below limit of detection (0.01 EU/ml) consistently over the 8 hour period, demonstrating that the alginate beads themselves should not present a contamination issue.

5.4.3 Endotoxin within the BAL biomass

The biomass also required assessment for potential endotoxin contamination to enable it to meet the regulatory requirements outlined above. All cell culture media and disposables used in the culture of the biomass are endotoxin free, therefore, there should be no endotoxin present within the biomass, unless a bacterial infection is observed. To assess this, media samples taken from various FBB experiments on Day 12 of the culture process were tested for endotoxin. Three out of the four samples analysed, FBB A, B and C, tested negative (below the limit of detection, 0.1 EU/ml) and one, FBB D, tested positive. It was later seen that FBB D had succumbed to a bacterial infection (this infection was not apparent when sample D was collected) which was likely the cause of this positive result. This proved a useful positive control, demonstrating the assay was capable of detecting endotoxin in the early stages of a bacterial infection in the BAL.

5.4.4 The effect of filtered plasma on the metabolic activity of ELS

To view as to whether filtering of plasma had any detrimental effect on the metabolic activity of encapsulated HepG2 cells, an MTT assay was performed. ELS were used in these experiments, as opposed to monolayer HepG2 cells, to better represent the conditions that would be seen within the BAL circuit. When cells are exposed to a cytotoxic environment their metabolic activity decreases, this decrease in metabolic activity can be observed using the MTT assay.[122] This method has been described previously as an alternative to the thymidine assay to measure cellular toxicity.[121,122,125] Samples were obtained from plasma prior to filtration and after filtration with both filters. Following incubation of the encapsulated HepG2 cells for a period of either 8 hours (as is the treatment time in the BAL[20]) or 24

hours, no significant difference was observed with respect to change in metabolic activity of cells incubated in non-filtered plasma compared with filtered plasma (Figure 47, page 139). The values produced were as expected following the incubation of cells with plasma.[20] These results suggest that the process of filtration should not have any detrimental effect on the health of the HepG2 cells within the BAL biomass.

5.4.5 Process for heparin priming

It is extremely important in the setting of the BAL *in vivo* that heparin concentration is maintained at an optimal level, to prevent any clot formation during *in vivo* experiments.[20] Heparin must be used as the anticoagulant of choice for use with the BAL due to alternative methods chelating calcium ions from the alginate ELS, causing them to break down and release the individual HepG2 cells within the BAL system.[14]

Upon upscaling from the 170 cm^2 surface area filters characterised in previous experiments to the 340 cm^2 surface area filters to be used *in vivo*, a doubling in filter surface is seen. This translates as a greater surface area for the removal of particles, and charged substances. As heparin has a strong negative charge,[117] and previous chapters demonstrate that it is removed initially by the filters, it was important to ascertain whether the heparin priming protocol used in previous chapters will continue to be effective with the increased surface area seen here.

The great negative charge of heparin is the basis behind its anticoagulant activity, contributing to its powerful electrostatic interaction with thrombin.[117] Most commercially available heparin has a molecular weight of around 12–15 kDa, which is relatively low compared with a molecule of endotoxin which is generally above 100 kDa,[27] but is similar to the size of a 200 base pair DNA fragment, as is produced when cells undergo death by apoptosis, which is 12 kDa.[31]

It was seen that initially ‘priming’ the filters with saline containing heparin at a concentration of 10 IU/ml lead to no further heparin removal when plasma-containing heparin was recirculated. After searching the literature I am yet to find an exact reason as to why this may be, as it was previously demonstrated that priming these filters with heparin produced no detrimental effect on their capacity to remove DNA and endotoxin, using this charge chemistry.

The next chapter, Chapter 6, will investigate the use of the upstream 340 cm^2 and downstream 0.6 µm filters, described here, in the translational setting, *in vivo*. Additional complications associated with moving into the *in vivo* setting will be explored along with further testing of these full-scale filters in a pre-clinical trial.

Chapter 6

Translating the filtration circuit for use within an *in vivo* setting

6. Translating the filtration circuit for use within an in vivo setting

6.1 Introduction

This chapter describes the use of the filtration circuit within the bioartificial liver device *in vivo* in a porcine model, along with the challenges which were met moving the system into this environment.

6.1.1 The process of in vivo testing

In vivo testing of medical devices and drugs is a requirement prior to their use in a human population. It is used to gain information primarily on the safety and efficacy of the product. Various models are available for testing of the medical device, including healthy models, to ensure the device presents no toxicity to the patients, and liver-failure models.

Animal models of acute liver failure can be divided into two distinct areas: surgery, such as devascularisation of the liver, thereby inducing ischaemic acute liver failure and partial hepatectomy; induced pharmacological damage using toxins such as paracetamol and thioacetamide.[126,127] The choice of a suitable method for inducing acute liver failure is difficult as all methods have drawbacks, such as individual animals having varying tolerability to hepatotoxins, and the effectiveness of surgery being operator-dependant. The induction of ischaemic acute liver failure by devascularisation of the liver presents a predictable model that can be relevant for the instability of patients presenting with acute liver failure.[126,127]

With regard to the choice of animal model suitable for experimentation and induction of acute liver failure, this is dependent upon the current status of the technology as to whether a large or small animal model should be used.

Porcine models present an ideal environment for testing the BAL as they have a large liver volume relative to their size. The size of a porcine liver can range between 0.9 to 1.3 kg, which is close to the 1.2 to 1.5 kg seen in humans.[127]

6.1.2 Additional complications faced when moving from the bench to the in vivo setting

Additional complications are faced when moving technology from the laboratory bench to the *in vivo* setting. Due to the pre-existing complex nature of the Liver Group BAL it is important to keep the filtration circuit as simple as possible whilst still maintaining full functionality. The filtration circuit explored in this thesis has been designed with this in mind.

In vivo testing currently being performed on the Liver Group BAL is within a surgically-induced acute ischaemic liver failure porcine model. Modifications to existing contaminant-testing protocols need to be performed for use within this setting. As the qPCR reaction described in previous chapters is specific for human DNA, a porcine-specific equivalent was required to ensure that full quantification of DNA could be observed, as opposed to only quantifying DNA originating from the biomass. Previously, Martin *et al* 2009 demonstrated the use of porcine-specific primers to analyse the quantity of porcine DNA present in feedstuffs. They elected to target a mitochondrial rRNA gene due its increased sensitivity compared with single or low copy nuclear DNA targets.[128] The primers they developed are specific to the mitochondrial 12S rRNA gene and are designed to amplify a 75 base pair fragment and will only amplify porcine DNA.[128] These primers will be explored for efficacy of use within porcine plasma in the *in vivo* setting in this chapter.

An additional complication of moving from lab bench to *in vivo* studies may be the presence of residual PFC within the ELS biomass, which will be used within the BAL.[20] As PFC is a large molecule of approximately 0.25 µm in size,[129] and the nominal porosity of the upstream filter (the 60ZB05A filter series) is in the range of 0.2–3 µm[72,100] it may present a problem if not all is removed prior to use of the

BAL with the filtration circuit. Therefore, PFC will also need to be assessed to ensure it produces no detrimental effect on the filters.

6.1.3 Current Liver Group Bioartificial liver experiments

The current animal study being performed by the Liver Group to assess the BAL is within a surgically induced acute ischaemic liver failure model. These experiments are being performed in pigs, and the filtration system is being utilised as part of the extracorporeal BAL circuit. The use of the filter set-up in a remote venue, as would occur in human patients, meant that certain protocols were required to prepare the filters for transportation and subsequent use within the closed BAL system in the *in vivo* setting. This chapter discusses the addition of these protocols, and the further characterisation of assays optimised in Chapter 3 to ensure their effectiveness in porcine plasma and this translational setting.

As such, the aim for this chapter was to assess the suitability of the filter system for use within an *in vivo* model and to develop protocols for the preparation of these filters, and the subsequent quality checks following use in such a scenario.

6.2 Materials and Methods

6.2.1 Extraction and quantification of porcine DNA for use in experiments

Materials:

Lysis buffer pH 8.4: 200 mM NaCl
100 mM TrisHCL
5 mm EDTA
0.2% SDS

100% Propan-2-ol

Proteinase K (100 µg/5 ml lysis buffer)

Sections of pork were obtained from a local butcher and cut into 1 g segments. Segments were mixed with DNA-free saline, crushed to a fine paste using a pestle and mortar and transferred to a microfuge tube. Lysis buffer was added to the microfuge tube at a volume two times that of the cell mixture, with 100 µg lyophilised proteinase K per 5 ml lysis buffer. The solution was incubated for 4 hours at 37°C. Following incubation, an equal volume of propan-2-ol was added to the lysed tissue solution and mixed gently for 5 minutes to precipitate the porcine DNA. The solution was transferred to a 50 ml Nunc™ tube where the resulting DNA aggregate was lifted above the liquid level using a pipette and allowed to dry for 15 mins. The aggregate was transferred to a fresh PCR grade microfuge tube and re-suspended in PCR grade water via pipetting and vortexing, where DNA proved difficult to dissolve, the solution was placed within a heated block at 37ºC for 15 minutes after which pipetting and vortexing was repeated. The concentration and total quantity of DNA obtained was assessed using NanoDrop analysis, as described in Chapter 2, Materials and Methods.

6.2.2 Analysis of porcine and human DNA concentration within porcine plasma using qPCR

Plasma for assessment using qPCR was pre-treated with proteinase K, as detailed in Chapter 2, Materials and Methods.

qPCR using porcine or human primers

Materials

PCR mix (Per 20 µl tube)

10 µl Hot start Taq (Qiagen #203205)

Porcine primers:

1 µl 75 base pair amplicon forward primer 0.0025 µM
(5' CCTCCTCAAGCATGTAGT 3')

1 µl 75 base pair amplicon reverse primer 0.0025 µM
(5' GTTACGACTTGTCTCTTCGTGCA 3')

Human primers:

1 µl 115 base pair amplicon forward primer 0.0025 µM
(5' CCTGAGGTCAGGAGTTCGAG 3')

1 µl 115 base pair amplicon reverse primer 0.0025 µM
(5 CCGGAGTAGCTGGGATTACA 3')

0.5 µl Sybr Green 1 in 20,000 (Biogene #1765)

2.5 µl 0.025 M $MgCL_2$ (Sigma #M1028-1M)

QPCR was performed specific for the detection of either human or porcine DNA. For the detection of human DNA, 1 set of Alu repeat primers, engineered to produce 115 base pair Alu repeat amplicons, were used to assess DNA quantity. For the detection of porcine DNA, primers previously designed by Martin *et al* 2009[128] specific for the amplification of a 75 base pair fragment of the porcine-specific 12S rRNA gene were used. The qPCR reaction was performed in duplicate or triplicate in 20 µl PCR tubes, each tube containing 15 µl of PCR mix and 5 µl of sample. Samples were run against a 5 point standard curve containing either HepG2 DNA

or porcine DNA, isolated using the protocol above, logarithmically diluted in the range of 0.02 to 200 pg/µl to determine the absolute DNA concentration in each sample. Samples were analysed on the Rotor Gene™ 3000 PCR machine using the following cycle:

95°C	15 minutes hold	
95°C	15 seconds	40 Cycles
64°C	30 seconds	
72°C	30 seconds	
72°C	10 minutes*	

*A melt curve was performed where the temperature increased from 45°C to 95°C at a rate of 1°C per minute.

As each sample was diluted 1 in 5,000 this technique provided a limit of detection of 0.1 ng/µl.

6.2.3 Small scale testing of filter system as per use in the in vivo setting

Components were assessed using a scale-filter circuit. Reservoir bottles were used in place of the Cobe Spectra plasmapheresis (apheresis) machine and animal model. Plasma was passed from the input reservoir using the peristaltic pump, through the upstream 25 cm^2 filter and downstream mini 0.6 µm filter. Once filtered, liquid was subsequently collected via the outlet. Pressure was recorded pre-upstream and post-downstream filter to calculate pressure drop across the two filters.

6.2.3.1 Calculating scale of the 25 cm2 filter for use of appropriate quantities of testing material

The 25 cm^2 filter presents with 13.6 times less surface area than the full-scale 340 cm^2 filter; to ensure the correct flux is used in these experiments, the flow rate

of the large-scale filters is re-calculated using this scale to find the correct flow rate for use in the small-scale experiments. This is as follows:

60 ml/min divided by 13.6 = 4.4 ml/min to be used
4.4 ml/min is the equivalent of 264 ml/hour

To mimic conditions of an 8 hour treatment period, 2.117 litres of pig plasma is required if we only want the plasma to be seen by the filter once. The filter is primed with heparin prior to use, as previously described in Chapter 2. Due to the short half-life of heparin, an additional heparin bolus was required 4 hours into the experiment.

6.2.3.2 Using porcine plasma to test the scaled-down filter circuit

Pig blood was obtained from an abattoir, this was passed through the apheresis machine to separate the plasma component from the whole blood.

This porcine plasma was spiked with human DNA and endotoxin at high concentrations. The upstream 25 cm^2 scale-filter and downstream mini 0.6 µm filter were used for this experiment.

The DNA concentration required for spiking the plasma was calculated as the total DNA quantity present in the BAL scaled down by 13.6 (as per the scale model above). In total, there are $1x10^{11}$ cells present within a high-cell BAL; each HepG2 cell contains 7.83 pg of DNA, leading to 7.83×10^{11} pg DNA in total in the biomass.[111] Scaling this down by 13.6 leads to a total of $5.8x10^{10}$ pg/DNA in the small-scale system which is equal to 58 mg/DNA in the 2.11 litres of plasma, providing an end concentration of 22 ng/µl.

An endotoxin concentration of 2,000 EU/ml which is equivalent to 4.24 million endotoxin units in the 2.11 litres of plasma was used to challenge this scale system. This is considered a high quantity of endotoxin and presents a worst-case scenario.

6.2.3.3 Testing of PFC effect on the filter in a small scale

A total of 480 ml saline and 20 ml PFC were mixed using magnetic stirrer at fast speed. The PFC-saline solution was recirculated through the filter for 3 hours at 4.4 ml/min providing an equivalent flux to a 60 ml/min flow rate through the 340 cm^2 filters as would be seen in the BAL. This allows for the solution to pass through the filters at least 1.5 times.

6.2.4 Preparing the filters for delivery to the in vivo experiments

To prepare the filters for shipping to South Africa, where the *in vivo* experiments were being performed all filters were autoclaved for 15 minutes at 121°C. Following this the 0.6 µm filters required an initial rinse with 1 litre of sterile tissue culture water, and the 340 cm^2 filters required a 2 litre rinse. This was performed by flowing sterile water through the filters using a Watson Marlow peristaltic pump, ensuring that all air was removed from the filters and displaced with liquid. This water rinse was followed by an equivalent volume of saline, to replace the water in the filter with a sterile saline solution.

After washing the filters and rinsing with saline, heparin was used to prime the 60ZB05A filters, as described in Chapter 2. Filters for shipping were stored in heparinised saline containing Pen Strep and Fungizone to ensure no contaminant growth during transit.

6.2.5 The filtration circuit for use within an in vivo setting

The filtration circuit was modified for use within an *in vivo* setting. This circuit was designed to be incorporated within the existing apheresis machine and BAL set-up.

In the BAL, whole blood is taken from the animal model and processed via the apheresis machine, the blood cells are separated from the plasma and the plasma is passed into the biomass chamber of the BAL. This plasma passes into the bottom of the chamber, acting to fluidise the ELS, maintaining them within a microgravity environment. The output from the biomass chamber is split in two, one side is recirculated at a high flow rate back through the chamber, so the plasma comes into contact with the biomass multiple times. The second stream passes through the slow-circuit filter system. In the filter system the plasma passes through the initial 340 cm^2 filter and then through the downstream back-up 0.6 µm filter before being returned back to the apheresis machine. Once returned to the machine the BAL-processed, filtered plasma is reintroduced to the whole blood and fed back to the animal model at a controlled flow rate (see Figure 50).

The controlled flow rate used within the porcine liver failure model is 60 ml/min. The plasma must pass through both the 340 cm^2 filter and the downstream 0.6 µm filter before being passed back to the animal. Modifications of this circuit from previous *in vitro* analyses include the addition of three bypass circuits. The first of these (blue, see Figure 49) bypasses only the 340 cm^2 filter, in case of any blockage as determined by an increase in pressure build up at pressure transducer 1. The second is a bypass circuit for only the 0.6 µm filter. This is in place in case the 340 cm^2 filter is fully functional but there is a build-up of pressure in this downstream filter, as determined by pressure transducer 2. The last bypass circuit bypasses the full filter system, in case of a problem with both filters. Two reservoirs are in place in this circuit to ease the flow of liquid through the system, these provide an excess drainage point in case of a decrease in flow rate through the filters and additionally a buffer providing extra fluid to maintain flow through thee circuit in case of a lack of fluid input (see Figure 49).

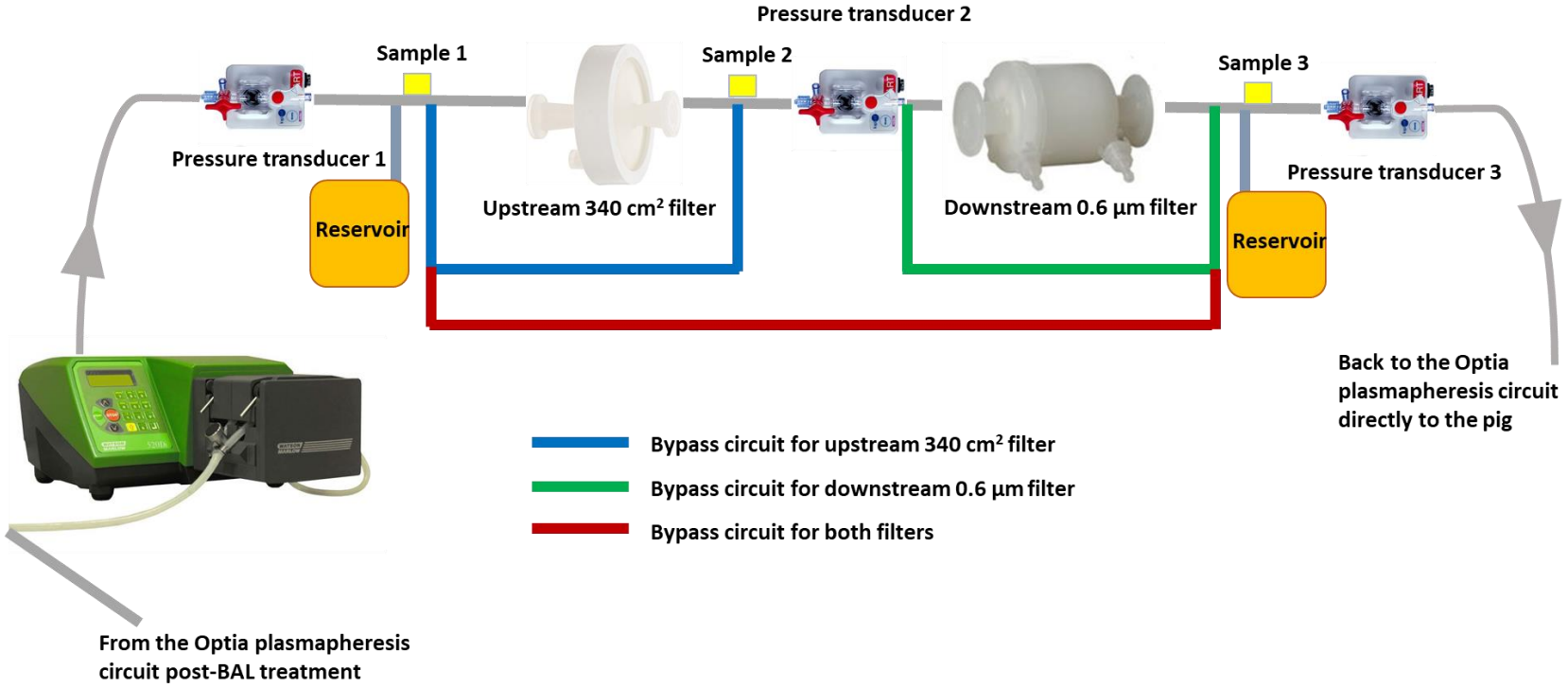

Figure 49. Filtration system model for incorporation into the in vivo bioartificial liver circuit. *Plasma is pumped through the circuit using a controlled flow rate of 60 ml/min, passing through both the upstream 340 cm^2 filter and downstream 0.6 µm filter before return to the animal.*

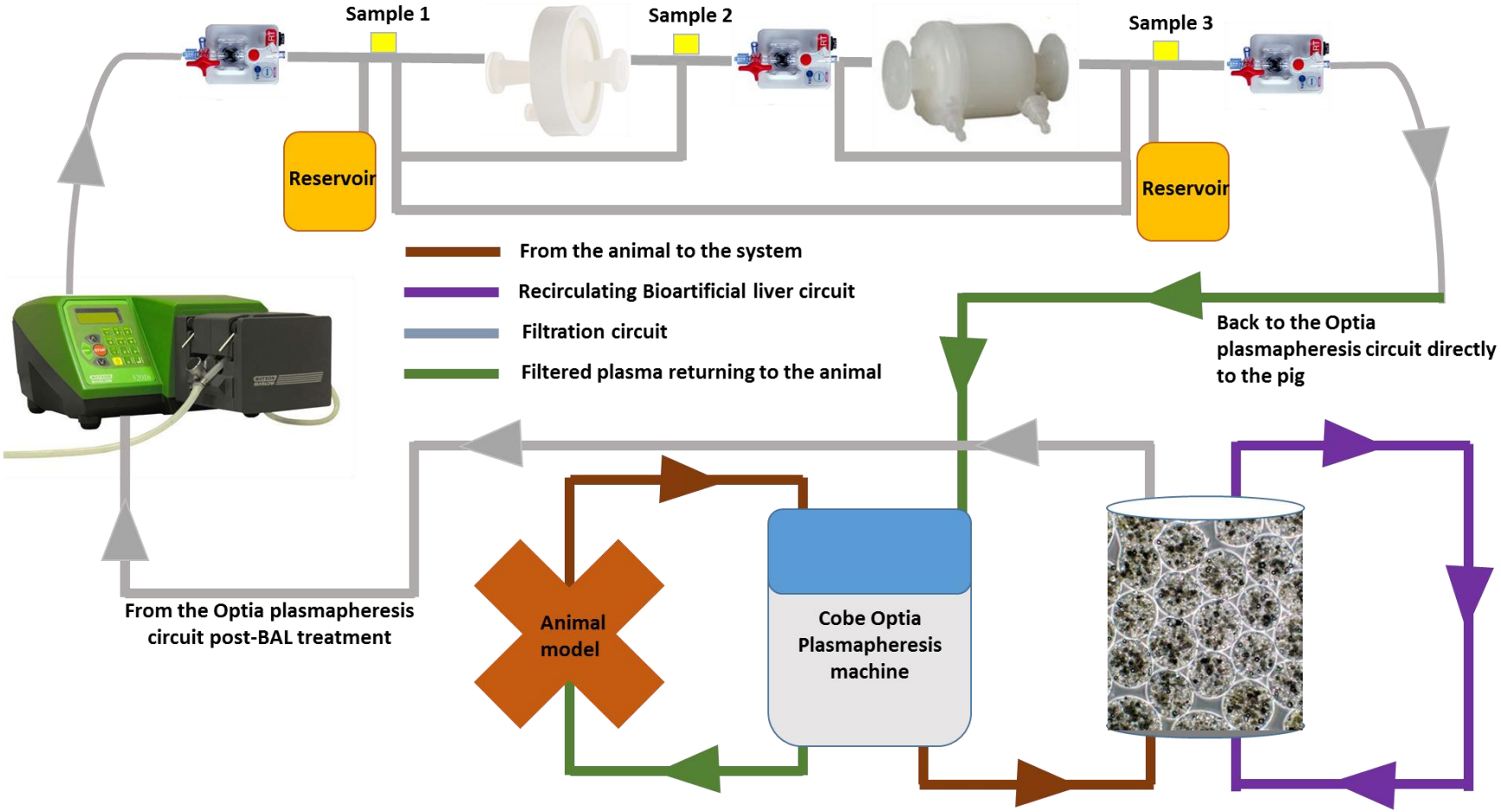

Figure 50. In vivo bioartificial liver (BAL) model including filtration system. *Plasma is extracted from whole blood using the Cobe Spectra and flowed through the BAL. The BAL output is split in two, one stream is recirculated at a high flow rate back through the BAL, the second passes through the filter system, filtered plasma is reintroduced to the whole blood and fed back to the animal model at a controlled flow rate.*

6.3 Results

6.3.1 Characterising the qPCR assay for use in porcine plasma

To enable identification of both DNA originating from the biomass and DNA originating from the porcine animal model during *in vivo* experiments a qPCR assay specific to porcine DNA was characterised. Two qPCR reactions were performed using porcine primers specific to the mitochondrial 12S rRNA gene.[128] One reaction was performed in a sample containing human DNA extracted from HepG2 cells and the other in a sample containing porcine DNA extracted from pork. A standard curve from 0.02 to 200 pg/µl was produced and no cross reactivity was seen between the human DNA and porcine primers. The porcine primers successfully amplified the porcine DNA maintaining linearity throughout the standard curve. The cross-reactivity of human primers with porcine DNA was also tested, again, two qPCR reactions were performed using human primers, one in a sample containing human DNA extracted from HepG2 cells and the other in a sample containing porcine DNA extracted from pork. As with the porcine-specific primers, no cross reactivity was seen between the human primers and porcine DNA (Figure 51).

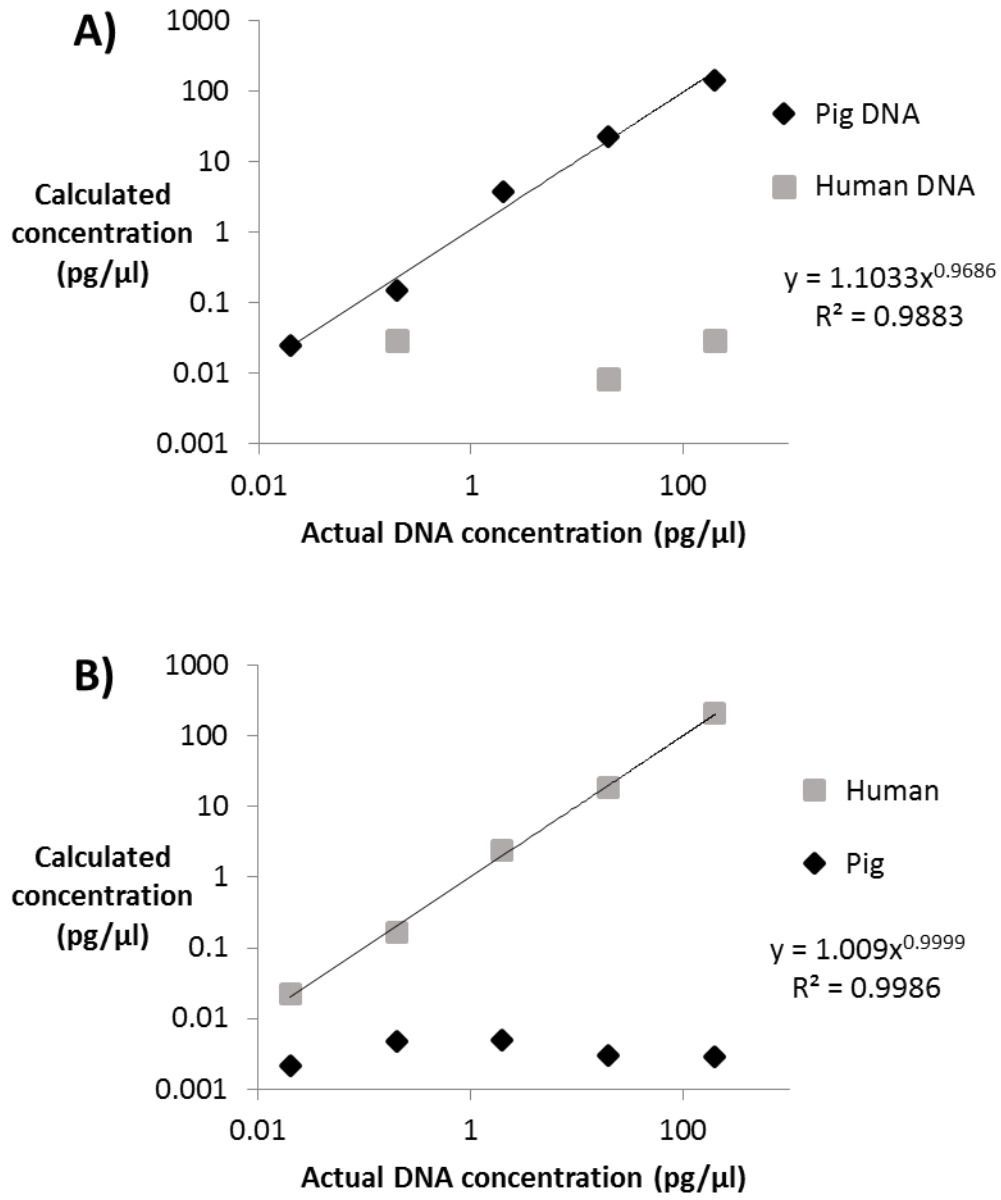

Figure 51. Cross-reactivity of the porcine DNA-specific qPCR assay. *A) Porcine primers successfully amplified porcine DNA, no cross-reactivity was seen between human DNA and porcine primers. B) No cross reactivity was seen between the human primers and porcine DNA.*

6.3.2 Testing of PFC effect on the scale-filter

To view whether any PFC contamination from the transport of the ELS would have any detrimental effect on the pressure of the filters, PFC in saline was recirculated through the filter for 3 hours at 4.4 ml/min. A slight increase in pressure was seen

in the upstream filter following the water wash, as expected, due to the difference in net charge between water and saline. Following this pressure remained consistent and well below the recommended maximum limit for this filter series of 1800 mmHg. The volume of PFC seen by the filter equates to 472 ml going through the large filter when this is scaled up, this demonstrated that the presence of PFC in the solution presented no negative effect with regard to pressure of the filters (see Figure 52).

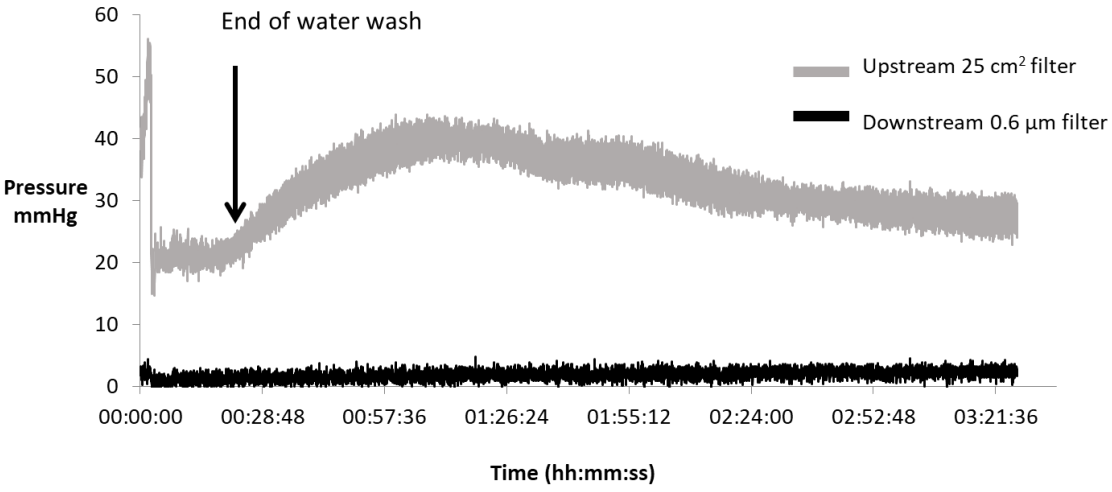

Figure 52. Effect of PFC in saline on filtration pressure. *A PFC-saline solution was recirculated through the filtration circuit for 3 hours. Presence of PFC in the filtrate did not result in an increase in pressure of either the upstream 25 cm^2 filter or downstream 0.6 µm filter. N=1.*

6.3.3 Testing the filtration of pig plasma spiked with DNA and Endotoxin using a scale-model

Using a scaled-down model for the filtration of porcine plasma the removal of DNA, endotoxin and the capacity of the filter to fulfil a full 8 hour simulated treatment phase was assessed.

Porcine plasma was spiked with human DNA at a concentration equivalent to what would be released if 100% cell death occurred within the bioartificial liver, scaled down to suit this scale experiment. No human DNA was observed in the porcine plasma prior to addition of the HepG2 DNA spike, as expected, and the correct quantity of DNA was recovered from the porcine plasma post-DNA spike and pre-filtration (Figure 53). Samples were collected hourly and no DNA was present in any of these filtered samples, demonstrating that the filter had removed all traces of DNA This equates to a total of 58 mg/DNA in 2.11 litres of plasma which is equivalent to 100% DNA which could be present in the BAL if all DNA was released from the cellular biomass, scaled down for the 25 cm^2 filters (Figure 53).

Endotoxin was also used to spike the porcine plasma at a high concentration of 2000 EU/ml. This equates to a total of 4.22 million EU in the 2.11 litres of plasma. This was to ensure that the filter was robust enough to perform in a worst-case scenario. A total of 80% of endotoxin was shown to be recovered from the initial spiked plasma sample. Following filtration, no endotoxin was detected in any of the samples taken at hourly intervals demonstrating that the filter successfully removed the full endotoxin load (Figure 54).

Pressure drop for both the upstream and downstream filters was maintained throughout the eight hour experiment at less than 35 mmHg, with no build-up in pressure observed that could indicate fouling of the filters (Figure 55; page 163).

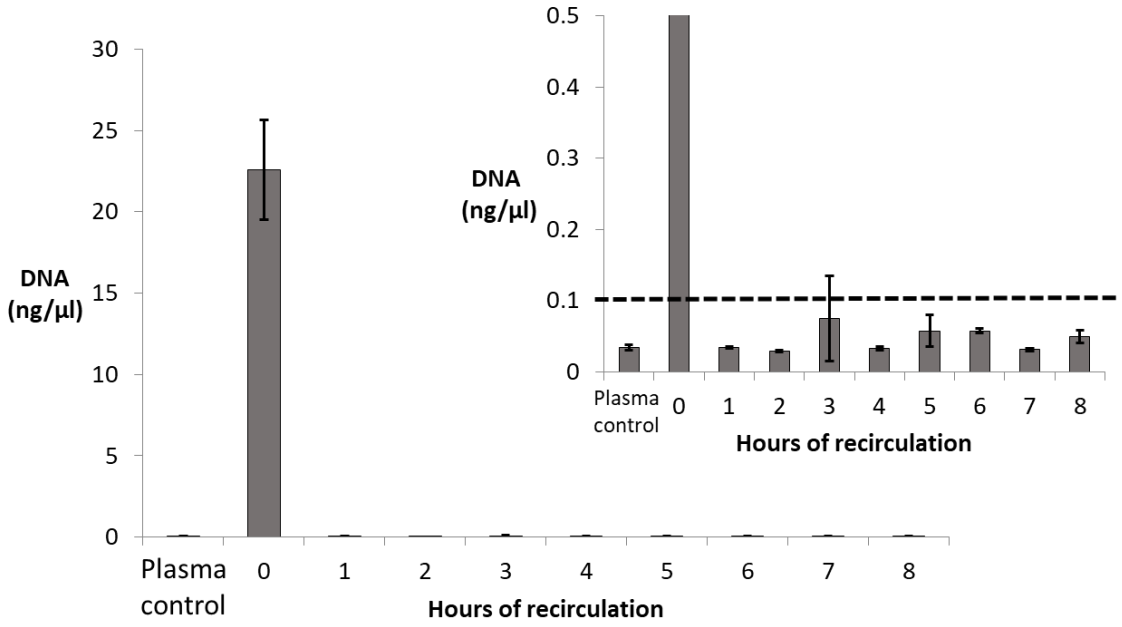

Figure 53. Effective capacity of the filtration system for the removal of DNA from plasma. *The filtration system was challenged with porcine DNA-spiked plasma in a recirculating set-up for 8 hours. Samples were taken hourly over the 8-hour period. The scale filter system successfully removed 58 mg of porcine DNA from 2.11 litres of plasma. Plasma control relates to plasma prior to introduction of the DNA spike. Time 0 represents the DNA-spiked plasma before filtration. The figure inset focuses on the lower end of the detection limit, the horizontal hashed line represents the lower limit of detection. N=4; data are average values +/- standard deviation.*

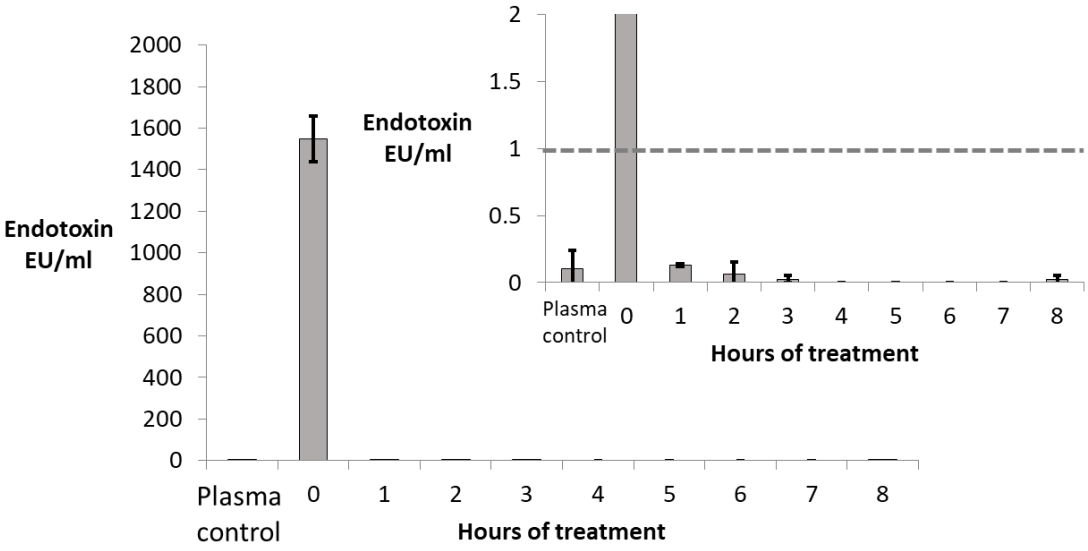

Figure 54. Effective capacity of the filtration system for the removal of endotoxin from plasma. *The filtration system was challenged with porcine DNA-spiked plasma in a recirculating set-up for 8 hours. Samples were taken hourly over the 8-hour period. The scale filter system successfully removed 4.22 EU from 2.11 litres of plasma. Plasma control relates to plasma prior to introduction of the endotoxin spike. Time 0 represents the endotoxin-spiked plasma before filtration. The figure inset focuses on the lower end of the detection limit, the hashed horizontal line represents the lower limit of detection. N=4; data are average values +/- standard deviation.*

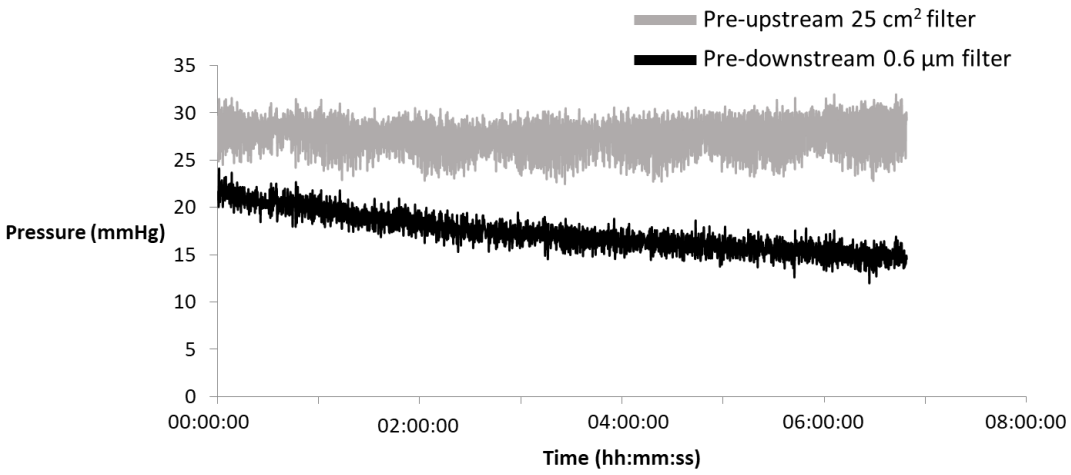

Figure 55. Effect of 8 hours of plasma treatment on physical parameters of the filtration system; pressure drop. *Pressure drop across the upstream 25 cm^2 and downstream 0.6 µm scale filters did not increase over 8-hours of plasma re-circulation. N=1.*

6.3.4 DNA levels in the BAL-treated pig without the filtration circuit

An *in vivo* experiment was performed in a porcine model of the BAL without the filtration circuit present. Samples taken from this experiment were provided for analysis. Samples were taken of the porcine plasma prior to treatment, mid-way through treatment and at the end of the treatment cycle. Using a porcine-specific qPCR assay it was seen that porcine DNA was present in all three samples. A slight reduction in concentration of the porcine DNA was seen at the end of the experiment, potentially representing dilution of the porcine DNA with the liquid within the BAL system (Figure 56a*).* The samples were analysed using qPCR specific for human DNA. As expected, no human DNA was present in the samples pre-BAL treatment. The concentration of human DNA increased slightly between mid-way through the treatment and end of treatment, this may be due to cell death within the BAL leading to the release of DNA into the porcine plasma (Figure 56b).

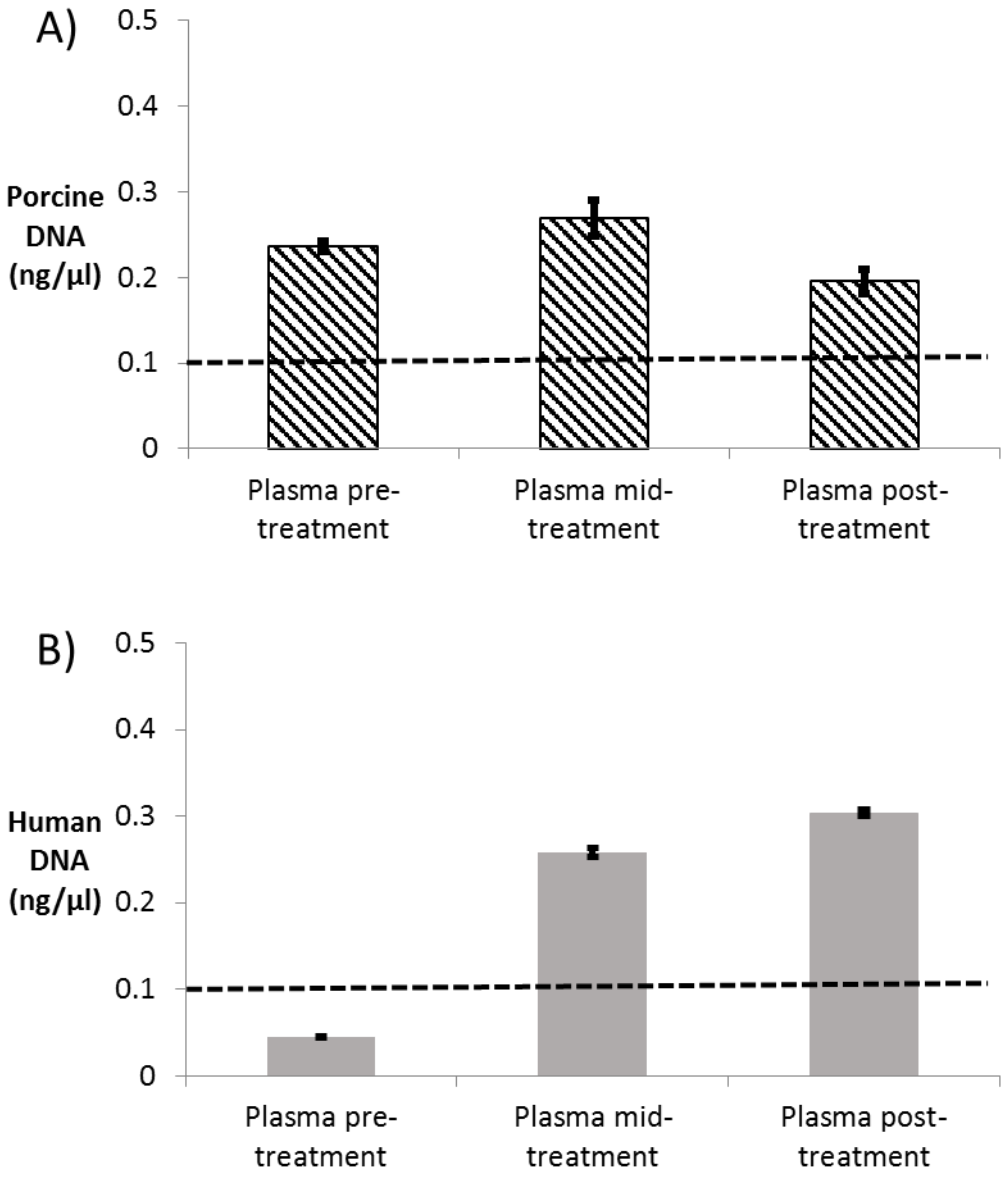

Figure 56. Porcine and human DNA levels in BAL-treated porcine plasma without the filtration circuit. *Plasma samples prior to, mid-way through and following in vivo treatment with the BAL were assessed for the presence of porcine and human DNA. A) Porcine DNA was detected consistently in all 3 samples, as expected. B) Human DNA was detected in the BAL-treated porcine plasma sample, as expected. N=1 experiment, values are average of N=4 within-sample replicates +/- standard deviation. Hashed horizontal line represents the lower limit of detection.*

6.3.5 Analysis of samples from the BAL-treated pig including the filtration circuit

Samples were brought back from an *in vivo* experiment for analysis. This *in vivo* experiment was performed in a porcine acute liver failure model, treated with the BAL containing the filtration circuit. Samples were taken at intervals over the treatment period with 'time 0' representing the sample taken prior to filtration at the experiment start. A consistent reduction in particle number was seen, although a reduction in total particle size could not be ascertained (Figure 57).

Pressure data were also collected for subsequent analysis during this experiment. Pressure transducers, as depicted in Figure 49 (page 155), were used to collect pressure from before the upstream 340 cm^2 filter (pressure transducer 1), between the filters (pressure transducer 2) and after the downstream 0.6 µm filter (pressure transducer 3; Figure 58; page 167). Pressure remained constant throughout the treatment duration and well below the recommended maximum limit (1,800 mmHg[101]).

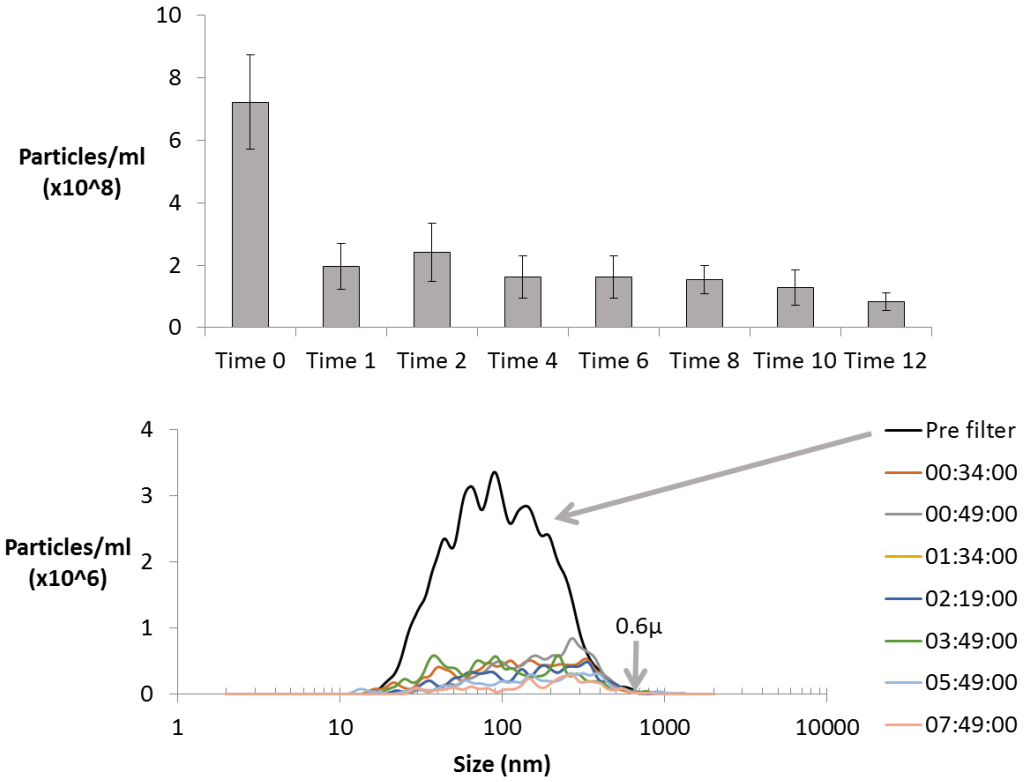

Figure 57. Particle reduction by the filtration system in vivo. *Particle analysis was performed using NanoSight analysis in porcine plasma obtained from an in vivo experiment. There was a consistent decrease in particle number after treatment with the filtration system. N=1; data are average of N=5 within-sample replicates ± standard deviation.*

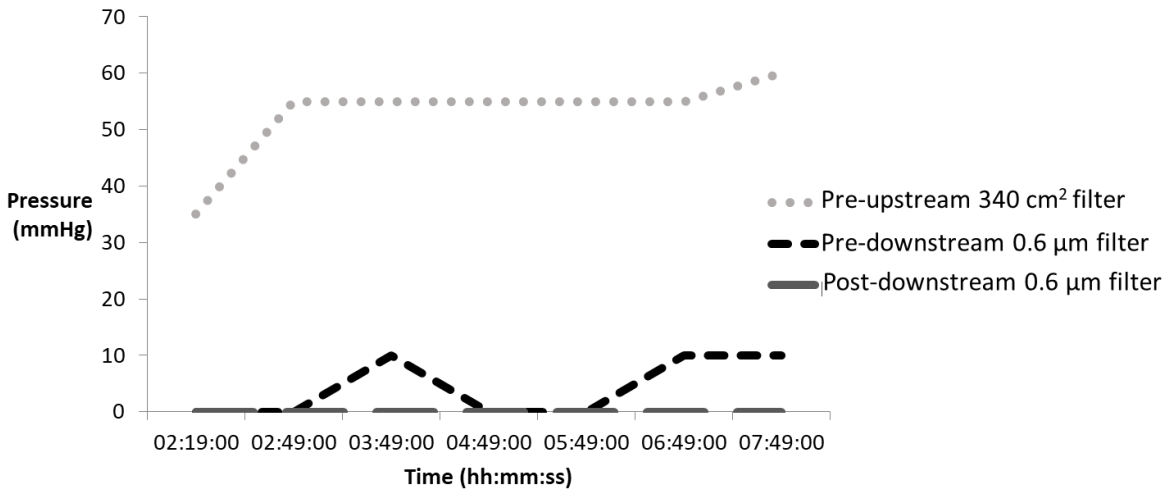

Figure 58. Effect of 8-hours of in vivo BAL treatment on the pressure drop of the filtration system. *Pressure drop across the upstream 340 cm^2 filter and downstream 0.6 µm filter was continually assessed over 8-hours of in vivo treatment with the BAL. Pressure drop remained consistently low (<70 mmHg). N=1.*

6.4. Discussion

6.4.1 Developing a new qPCR assay for use in pig plasma

To enable the detection of DNA originating from the pig in the *in vivo* BAL experiments, the use of primers specific to porcine DNA was required. Martin *et al* 2009 demonstrated the use of just such primers to analyse the quantity of porcine-specific DNA present in feedstuffs. The primers they developed target the mitochondrial 12S rRNA gene.[128] These primers were designed to amplify a 75 base pair fragment of this 12S rRNA gene. A mitochondrial rRNA gene was chosen due to this demonstrating improved sensitivity compared with single or low copy nuclear DNA targets.[128]

To test the cross-reactivity of human primers with porcine DNA, two qPCR reactions were performed using human primers, one in a sample containing human DNA extracted from HepG2 cells and the other in a sample containing porcine DNA extracted from pork. No cross reactivity was seen between the human primers and porcine DNA. This enabled plasma obtained from the *in vivo* experiments to be analysed separately for DNA originating from the pig and DNA originating from the BAL biomass itself. Due to the nature of the liver failure model, we expected large quantities of porcine DNA to be present within any plasma samples taken throughout the *in vivo* experiments.

6.4.2 Additional checks on pressure levels in the filtration system

It is unlikely that anything more than a few parts-per-million of PFC would be present in the biomass following transport and preparation for treatment, but due to the properties of PFC (non-miscible in water[130]), it was important to check its effect on performance of the filtration system. The effect of PFC on pressure-drop of the filters was assessed to ensure that any residual PFC left over from the transport of the ELS did not have a detrimental effect on the filter.[129,72] Both upstream and downstream filters were recirculated with saline containing PFC over a three hour

period. Pressure pre- both the upstream and downstream filters remained constant over the three hour period, no detrimental effect on the filters with regard to pressure was seen (Figure 52; page 159).

6.4.3 A scale model of the BAL filtration circuit using pig plasma spiked with DNA and Endotoxin

To ensure that the filters were effective in porcine plasma, a scaled-down model of the filtration circuit used within the BAL, as used described in Chapter 4. This model was used to assess the sufficient removal of DNA, endotoxin and the capacity of the filter to fulfil a full 8 hour simulated treatment phase without a detrimental effect on pressure drop.

This model was intended to replicate a worst-case scenario of 100% cell death within the bioartificial liver, to ensure that the full filtration system would be able to cope with this in the setting of porcine plasma in the *in vivo* experiments. The correct quantity of DNA was recovered from the initial DNA-spiked plasma sample, demonstrating the efficiency of the qPCR assay (Figure 53; page 161). No human DNA was detected in any of the eight post-filter plasma samples taken at hourly intervals during the treatment cycle. This demonstrates that the filter was able to remove the total of 58 mg of human DNA used as challenge in the 2.11 litres of porcine plasma. When this value is up scaled to gain a scale value for the 340 cm^2 filter, this represents removal of 100% of DNA present in the BAL itself.

As well as challenging the scale filtration circuit with the addition of DNA, a large quantity of endotoxin was also used to spike the porcine plasma. The concentration of endotoxin used was 2000 EU/ml in a total volume of 2.1 litres of plasma, equating 4.24 million EU. This high quantity was to ensure that the filter is robust enough to perform in a worst-case scenario. Recovery of endotoxin from the original spiked plasma sample was 80%. Although this is not perfect, it is as expected from an endotoxin assay as they are known for providing erratic results and being difficult to use to quantify the endotoxin content in plasma samples, with

studies quoting a 2-fold margin of error.[52,54,131] Following filtration, samples collected at hourly intervals over the 8 hour treatment period demonstrated no presence of endotoxin after flow through the filter. These results demonstrate the filter's capacity for sufficient endotoxin removal over the treatment period (Figure 54; page 162). Endotoxin levels in patients with liver failure, although consistently higher than healthy controls, are highly variable and mixed results have been published in the literature.[42,132] In one paper, in patients with alcoholic hepatitis, plasma endotoxin levels have been reported to be 184.4 +/- 159.4 pg/ml, which is approximately 1.84 EU/ml.[43,110] This equates to a total of 5,520 EU in a 70 kg human with 3 litres of plasma.[115] Therefore, these results suggest that the capacity of the scale-model of the filtration system for endotoxin removal is sufficient to remove that originating from the patient and in a worst-case scenario, from an external contaminating source.

It is worth noting that the filters were primed with heparin prior to use, this 'priming' was seen to have no detrimental effect on the filter's capacity for DNA and endotoxin removal, despite maintaining the level of heparin within the system, a highly negatively charged molecule.[117] One potential reason for this may be the way in which heparin was utilised as an anticoagulant. Heparin mediates inactivation of activated factor X and thrombin by binding antithrombin via a high-affinity pentasaccharide, it is the high charge density present in this region that mediates the electrostatic reaction with thrombin.[117,118] This binding could potentially mask heparin's negative charge from detection by the filters.

Pressure drop for both the upstream and downstream filters was maintained at a constant level throughout the eight hour experiment, with no build up in pressure representing fouling of the filters observed (Figure 55; page 163). This suggests that the volumetric capacity of both filters for plasma is sufficient to see through a full eight hour BAL treatment cycle.

6.4.4 Testing the quantity of DNA present during treatment with the BAL without the filtration system in place

An *in vivo* experiment was performed in a porcine model utilising the BAL without the filtration circuit in place. Samples were taken from this experiment and transported back to the UK for analysis. Samples of porcine plasma prior contact with the BAL, mid-way through the treatment cycle and at the end of the treatment cycle were obtained. These samples were analysed using porcine-specific qPCR and human-specific qPCR to detect the quantity of porcine DNA and human DNA, respectively, present in these samples.

As was expected, porcine plasma prior to treatment with the BAL contained no human DNA. Upon comparing the porcine-DNA-specific samples both pre-treatment, mid-way through treatment and at the end of treatment it was seen that the quantity of porcine DNA present decreased slightly, a dilution effect of the porcine plasma during the experiment for a combination of reasons including from the saline that is constantly fed into the system, providing a set level of heparin throughout the surgical procedure, and the healthy plasma used to prime the BAL circuit prior to connection with the liver-failure porcine model. In regard to human DNA present within the samples, following treatment with the biomass, human DNA was detected in the two plasma samples that were taken mid-way through treatment and at the end of treatment. The quantity of human DNA detected in the porcine plasma increased from the beginning to the end of the treatment period. This increase potentially represents cell death and subsequent DNA release from HepG2 cells within the BAL.

By viewing the concentration of human DNA present in the porcine plasma at the end of the treatment period, this can be worked back to determine a theoretical percentage of cell death that may have occurred within the biomass over the treatment period. This quantity was calculated using the modal number, namely the number of chromosomes actually present in the cell type, for HepG2 cells, which is 55.[111] Using this number, and the value for the quantity of DNA present in a human 46-chromosome cell, 6.55 pg[30,111], the total quantity of DNA present within

the biomass can be calculated using the number of cells present within this particular BAL run, 1.46 x 10^7.

> **DNA in a HepG2 cell:** (6.55 pg of DNA per cell / 46) * 55 provides 7.83 pg of DNA present in a single HepG2 cell
>
> **Quantity of HepG2 DNA in the BAL experiment:**
>
> 7.83 * 1.46 x 10^7 = 1.14x10^8 pg of HepG2 DNA
>
> **Concentration of HepG2 DNA in the full *in vivo* BAL system:** 1.14x10^8 / 7000 (ml of plasma in system) = 1.6 x 10^4 pg/ml DNA
>
> **Percentage of cell death within the system:** 16 pg/µl of DNA would be present in total if 100% cell death occurred within the bioartificial liver. A concentration of 0.25 pg /µl of DNA was seen in the samples, working back this equates to **1.56% cell death** within the biomass

This result should be interpreted with caution as it does not account for DNA that may have been degraded already in the BAL system, for DNA that may adhere to tubing, and it does not provide an analysis of cell viability. This result could be further corroborated using viability data and a cell count to gain percentage viability of cells and an absolute cell number at the end of the experiment. In an ideal scenario, samples would be taken throughout the treatment phase and analysed to assess the viability of the biomass to ensure sufficient cell viability for use within the treatment phase. During the pre-clinical trials, these measurements were taken prior to treatment initiation, mid-treatment and post-treatment, cell viability was reduced by only 6% following 8 hours of BAL treatment in liver failure models.[116] The next chapter will go on to explore this and further questions raised in the preceding chapters.

Chapter 7

General discussion and future work

7. General discussion and future work

This PhD project set out to characterise a filtration protocol which could be incorporated within the Liver Group extracorporeal BAL circuit. It was required to be pre-prepared for use in a remote location, as would be the case when treating patients. The filtration protocol was to protect the patient from any potential contaminants originating from the BAL, which included cell debris, alginate particles and HepG2 DNA, whilst maintaining beneficial plasma components that are both native to the patient and produced by the BAL, such as albumin and fibrinogen.[14] As an additional functional aspect, the filtration system was to be assessed for endotoxin removal capability, which may originate from the patient, to enhance the therapeutic potential of the BAL. To enable this, assays for the detection of the contaminants, DNA and endotoxin, within plasma samples were successfully characterised and used to assess the capacity of the filtration system *in vitro* using small- and full-scale models, before progressing to pre-clinical trials. Here we will discuss the pros and cons of the data presented in this thesis, along with suggestions for future work.

7.1 The detection of contaminants in plasma

In order to characterise a variety of contaminants, several assays were required to be developed that would be functional in plasma samples. These were successfully quantified in Chapter 3, and will be discussed in further detail below.

7.1.1 DNA

Due to the nature of BAL treatment, there is the potential for DNA released from the HepG2 cell biomass to come into contact with the patient's plasma; this is required to be removed by the filtration system prior to plasma return to the patient.[35] A method for the detection of human DNA in plasma samples was

required to assess efficacy of the filtration system for the removal of this contamination. A DNA assay was developed using a combination of plasma pre-treatment with proteinase K and a 1/5,000 dilution, prior to quantification using qPCR. This assay used DNA isolated from HepG2 cells as a standard and was shown to be consistent in various plasma samples, and in the presence of anticoagulants; the assay had a limit of detection of 0.1 ng DNA\µl plasma (Chapter 3).

A literature search of regulatory guidelines, including those set by the MHRA, EMA and WHO, was performed to identify an absolute limit for the presence of extracellular DNA contamination in medical devices. These guidelines for the quantity of DNA allowed to be present within medical devices and drugs are not specific, although DNA is listed as a contaminant and the quantity is required to be reduced.[35,130] With the advent of new cell-based therapeutics, it will be of importance to keep up to date with any subsequent guidelines to ensure that the BAL continues to fulfil these as they evolve. As discussed in Chapter 3, the detection limit of the DNA assay is 100 ng DNA/ml plasma, equating to 1.2 ng DNA/mg dry plasma weight, demonstrating that the DNA assay is efficacious at detecting DNA to approximately 50-fold lower than the limit of provoking an immune response, which has been reported to be 50 ng/mg.[104,134]

It was calculated that the DNA assay would be able to detect a lower range of 0.08–0.13% of cell death occurring within the BAL in a circulating system containing 7 litres of fluid, as would be seen during treatment.[115] A theoretical estimate as to the health of the BAL during patient treatment could be gained from this, although, due to the large volume of plasma in the system, and as cell death during treatment is likely to occur gradually, it is unlikely that DNA would disperse evenly in the system, as would be required for an accurate measure.

7.1.2 Endotoxin

Patients presenting with acute liver failure are in an immunosuppressed state, with a decreased ability to remove endotoxin, leading to endotoxaemia and potentially subsequent sepsis.[41] Sepsis is a key cause of death in patients with end-stage liver failure, and is established as a result of the patient's immune system reacting to these increased levels of endotoxin in the circulation.[7,135] For this reason, the filtration system was assessed for the removal of endotoxin originating from the patient to enhance their recovery. As such, a method for the detection of endotoxin in plasma samples was required.

Using the PyroGene assay, with pre-treatment of plasma consisting of 1/200 dilution in endotoxin-free water and heat treatment at 70ºC for 20 minutes, a limit of detection of 2 EU/ml plasma, or 0.2 EU/ml of media samples containing 10% FFP was achieved.[52] Although endotoxin recovery from plasma samples was approximately 20% lower than that achieved in the positive product control, this is as is seen in the literature. One group has published a method for the detection of endotoxin detection in hyaluronic acid (HLA)-based medical devices. Here, they demonstrated that recovery of endotoxin from HLA was 2-fold less than in water prior to digestion of HLA, a lower recovery than was seen in our assay, showcasing the difficulty of accurately quantifying endotoxin.[136] Additionally, the PyroGene assay characterised in this thesis is now approved for use in regulatory testing by the United States Pharmacopeia, providing that suitable validation is performed. The validation performed in this thesis may well be sufficient for this purpose.[137]

7.1.3 Particles

Unfortunately, due to the multiple components found in plasma samples, and the tendency of plasma to clot when stored frozen,[118] it was difficult to accurately analyse plasma samples for particle concentration. A consistent reduction in particle size and total number was observed using both Mastersizer and NanoSight analysis techniques, but results do not provide enough detail to enable full

conclusions to be drawn and these techniques were used with caution. Using Mastersizer analysis, large particles were seen to be present in the plasma samples, thought potentially to be plasma clots or agglomeration of alginate.[138] These particles may interfere with results produced by the Mastersizer, as they would obscure detection of smaller particles. These larger particles were not seen with NanoSight analysis, as the maximum particle size suitable for this detection method was 2 µm (as described in Table 2, page 52). For future analyses, these samples could be filtered using a 2 µm filter, the filtrate analysed using the NanoSight and the retentate re-suspended and analysed with the Mastersizer, ensuring no interference from either end of the median particle distribution.

7.2 Capacity of the filtration system for DNA, endotoxin, particles and plasma

Aims of this thesis included the identification of potential contaminants necessary to remove from the BAL circuit, to ensure treatment is therapeutic, without potential harmful effects arising; determining both the capability and the capacity required of the filtration system in order for it to meet regulatory requirements for use within patients, and the testing of this system on small and large scales *in vitro* to establish the parameters of its efficacy. These were assessed in Chapters 4 and 5, where potential contaminants were identified and the suitability of the filtration system to remove these in order to meet regulatory guidelines was demonstrated, as determined using small- and large-scale models, discussed below.

The quantity of DNA that could potentially be released from the BAL under 8-hour treatment conditions was calculated to be 8.22–11.7 mg, compared with 548–783 mg DNA in the whole biomass. In Chapter 6 the filters were challenged with an equivalent quantity of full BAL-DNA content using a 13.6 x scaled down model, demonstrating 100% removal of DNA contamination. This supports the suitability of the filters for use in a full-scale BAL setting, and suggests that they would be efficacious at protecting the patient in a worst-case scenario.

In patients with alcoholic hepatitis, there is reported to be in the around 5,520 EU present in an average 65 kg patient with 3 L of plasma.[43,110] The upstream filter achieved successful removal of 4.24 million endotoxin units from plasma, demonstrating robustness of the filtration system to support endotoxin removal originating from the patient and in a worst-case scenario from an external contaminating source. Multiple systems are in development for the removal of endotoxin from patients with liver failure. The ADVanced Organ Support system, which is based on albumin dialysis, has been assessed in a two-hit porcine model of endotoxaemia, here they use change in pH and temperature to remove endotoxin from albumin in the recirculating system.[139] Although systems such as this could theoretically be used in combination with the BAL, they would result in an additional complex circuit, which would exhibit a further hold-up volume and require greater quantity of FFP to treat the patient. An advantage of the filtration system presented here, is that this is used in line with the existing BAL circuit, with no need for additional resources.

The filtration system demonstrated consistent reduction in both particle size and total number, although, as discussed earlier, these results should be viewed with caution as the methods for particle analysis had substantial drawbacks, with regard to sample storage prior to analysis, and the limits of the techniques themselves. Additional methods to characterise particles in plasma would be beneficial to corroborate results gained in this thesis, these are discussed below, along with future work that could be performed here.

The volumetric capacity of the filters, with regard to a suitable measure of pressure drop, was also demonstrated, showing that the volumetric capacity of both filters in series is sufficient to see through an 8-hour BAL treatment cycle.[116] Due to the availability of sufficient quantities of plasma, this full-scale volumetric capacity experiment could unfortunately not be repeated. Pressure analysis from the filtration system *in vivo* would provide additional support to corroborate this result, hopefully demonstrating the efficacy of the filters in an 8-hour *in vivo* BAL treatment.

7.3 Examining the filtration system for any detrimental effect on plasma

The upstream 25 cm^2 filter and mini downstream 0.6 µm filter were used in scaled-down mini column experiments replicating the BAL in healthy and liver failure plasma. Cell functionality in the form of AFP production, glucose metabolism and urea production was confirmed in healthy plasma, as has been described previously.[14,22] These experiments demonstrated consistent maintenance of plasma proteins following processing for 8 hours with the filtration system, whilst DNA released from the ELS was consistently removed over the 8-hour period, as could be seen when compared to ELS incubation in plasma without the filtration system. This suggests that the filter's affinity for DNA does not negatively impact the concentration of beneficial plasma proteins, either produced by the biomass or the patient themselves.

An MTT assay demonstrated that the processing of plasma using the filtration system did not negatively impact ELS metabolism over an 8- or 24-hour incubation period. This suggests that the process of filtering plasma does not negatively interfere with the useful biomass products. It would be beneficial in future work to assess this further, for example using cytokine analysis.[20] Examining any potential toxic effects of these filters, or of plasma processed using these filters, could be performed to further support regulatory applications, including cytokine assays and lymphocyte proliferation studies to estimate any detrimental effect of the filters themselves.[43]

7.4 Regulatory requirements of the filtration system

As previously discussed, to enable the BAL to meet MHRA and FDA regulatory guidelines as a medical device, it needs to adhere to maximum endotoxin and particles quantities, along with demonstrating DNA removal capabilities.[24,27,28,105,109]

The filtration system demonstrated consistent removal of DNA to below the limit of detection of the qPCR assay. An upper limit in terms of capacity of the filtration system for DNA was not reached, including testing the system with DNA quantity equivalent to 100% of DNA present in the BAL biomass. Human DNA concentration from the end of an *in vivo* BAL treatment without the filtration circuit in place was used to determine approximate cell death within the biomass over the treatment period. If used in future, this could be corroborated using viability analysis and cell counts at the end of the experiment to gain an overview of cell health within the BAL over the course of treatment. Although, this does not account for DNA that may have been degraded already in the BAL and assumes an equal distribution of DNA within the plasma. In the pre-clinical trials, samples are taken at beginning, mid-point and end of the treatment phase and analysed for viability, this information could be used in future to predict BAL health, enabling early termination of treatment in case of any mass cell death.[116]

All BAL components are required to have a maximum endotoxin concentration which is below the limits for a medical device stipulated by the FDA (0.5 EU/ml or 20 EU/device).[109] Both the upstream and downstream filters, and the empty alginate spheres tested negative for the presence of endotoxin, meeting these regulatory requirements.[101] The biomass was also assayed for endotoxin contamination at Day 12 of culture. One BAL experiment tested positive for endotoxin, and following this was seen to be contaminated with bacteria. This result demonstrates that there may be potential to use the endotoxin assay as a test for bacterial contamination throughout the biomass culture, as will be discussed later.

Regulatory requirements set out by the United States Pharmacopeia state that solutions for injection should not exceed 12 particles greater than 10 µm/ml, or 2 particles greater than 25 µm/ml.[105] The filtration system reduced particle number and size in an *in vivo* BAL experiment to <0.6 µm, with a 4-fold reduction in total particle number. Additionally, *in vitro,* the filtration system was seen to reduce alginate particle size from 12 µm to <1 µm using Zetasizer analysis. Although these results should be observed with caution due to the limitations of the particle analysis systems, this suggests that the filtration system should enable the BAL to meet these regulatory requirements.

7.5 Future work

The results presented here have demonstrated the suitability of this filtration system for incorporation within the Liver Group BAL. The filtration system removed contaminants from plasma to a level sufficient for the system to meet regulatory guidelines, and progressed to *in vivo* experimentation; however, further questions could be answered if additional time were available. These are discussed below.

Assays for the detection of HepG2 and porcine DNA were characterised, enabling identification of DNA origin in BAL-treated plasma samples *in vivo*. An estimate of cell death within the BAL was gained using HepG2 DNA concentration, this could be performed *in vivo* in a porcine model as there was no assay cross-reactivity between HepG2 DNA and porcine DNA; however, when this device is used in humans, the qPCR reaction would detect all human DNA, not only that originating from the BAL. Therefore, the characterisation of a quantitative assay specific to the HepG2 cell line would be beneficial. This could be normalised by corroborating data with absolute cell numbers to provide a method of characterising cell death within the BAL in humans. Cell-free DNA assays can be used as cancer biomarkers, to detect both the presence of cancer and its characteristics, which can guide treatment choice, demonstrating the feasibility for detecting specific cell characteristics within human plasma samples.[140] Short tandem repeat (STR) analysis could be looked into for detecting any HepG2 DNA, whereby probes are targeted to the STR regions specific to these cells, and PCR is performed to detect their presence.[141] Although, this technique is more complex than the DNA assay described in this thesis, requires looking at multiple loci, and may not be transferable to cell-free DNA.

The endotoxin assay presented here detected bacterial contamination of the BAL biomass, before this was apparent visually, this assay could be used as an early biomarker for the presence of infection within the BAL, enabling termination of the cell culture process in the case of an early stage infection. This would save valuable resources in this instance and would offer an additional safety guard to

ensure no contamination from BAL to patient. A similar method has been reported in the literature, whereby a biomarker for early diagnosis of sepsis was observed; here, it has been shown that endotoxin activity levels in patients with sepsis are positively associated with disease severity.[142,143]

Due to the limits of both NanoSight and Mastersizer analyses, and the potential of alginate and plasma to agglomerate during storage and transport accurate particle quantification proved difficult. Further methods to characterise particles present in plasma samples would be beneficial to provide additional data on the efficacy of the BAL for their removal. In particular, a method for the detection of alginate particles within plasma samples would support the efficacy of this system for removing BAL-originating contamination. Mass spectrometry has been used to identify alginate particles in complex samples, specifically pigs faeces, and could offer potential for the detection of alginate in plasma here, although samples required complex pre-treatment prior to analysis.[138]

The final aim of this thesis was to prepare for, and analyse, contaminant removal from porcine plasma when the safety circuit was used in a porcine pre-clinical model of acute liver failure. This was performed on N=1, demonstrating reduction in particle size and quantity. It would be interesting and valuable to collate the remaining data from the filtration system *in vivo*, including pressure analysis and samples from pre- and post-filtration for DNA, endotoxin and particle quantification. This would be beneficial to support the results gained in this thesis and would provide further insight into the efficacy of the BAL *in vivo*, along with information on any complications encountered in this setting. Although, as already discussed, results of particle analysis should be interpreted with caution, due to the nature of sample transport prior to performing particle detection assays, and due to limitations of the assays themselves. Due to the affinity of the filter for endotoxin, it would also be interesting to observe whether the filtration system alone would demonstrate any therapeutic activity in a model of sepsis. Currently, National Institute for Health and Care Excellence guidelines recommend antibiotic treatment and intravenous fluid resuscitation in this instance.[135] As endotoxin is implicated in sepsis, and has been shown to be a positive predictor of sepsis severity, its removal

using the filtration system provides a therapeutic benefit, as has been demonstrated with albumin dialysis systems.[139]

7.6 Conclusion

The data presented in this PhD thesis support the hypothesis that a filtration system, proven to be able to remove any potential contaminants which may arise from the BAL biomass, will meet regulatory requirements, thus enabling its use in patients; additionally, data presented here also support the second hypothesis that this filtration system will also have the capacity to remove endotoxin contamination, providing an additional functional element of this system.

Assays for the detection of DNA and endotoxin in human plasma were successfully identified, providing a limit of detection sensitive enough to assess the BAL for compliance with regulatory standards. These assays were used to assess the capacity of the filtration system for removal of contaminants throughout this thesis. *In vitro* testing of the filters demonstrated removal of significant quantities of DNA and endotoxin, whilst leaving the concentration of beneficial plasma proteins unchanged. Additionally, a combination of the two filters also provided a consistent reduction in particle size and number. The removal of these contaminants, DNA and particles, may be sufficient for the BAL to meet regulatory guidelines for particle and DNA concentration, although further investigation using different particle analysis techniques would be beneficial to support this. Additionally, individual components of the BAL and filtration system tested negative for the presence of endotoxin, providing further evidence of their suitability to progress to clinical trials. This thesis described additional barriers to overcome whilst progressing to *in vivo* use, such as the development of a porcine-specific DNA assay, along with defining standard operating procedures to enable the use of the filtration system in a remote location, as would be the case in humans.

To conclude, a filtration system was proven to reduce potential contaminants, including both physical particle contaminants and biological agents such as DNA, which may arise from the BAL biomass, to the extent at which the BAL can meet

regulatory requirements, thus enabling its use in patients. The filtration system also demonstrated the capacity to remove endotoxin contamination, providing an additional functional element of this system which would further protect patients suffering from sepsis.

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