Development of a portable adsorbent technology for the treatment of systemic inflammation in a range of clinical environments.

By

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September 2012

This thesis is submitted in partial fulfilment for the degree of

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Declaration:

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed:

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Acknowledgements:

Firstly, I would like to thank my supervisor Professor Terence Gourlay for his advice, support and encouragement throughout this EngD project. His wealth of knowledge in the field of cardiovascular devices has been invaluable in undertaking this research. Without his vision and experience, this project could not have begun.

I'd also like to make particular mention of Mr. Stephen Murray, to whom I owe a great deal. His expertise in the workshop has led to the creation of many ideas that may not have become a reality otherwise. His patience and willingness to take the time to discuss concepts and designs have been invaluable throughout my time here.

I am also indebted to the various workshop and laboratory technicians throughout the department who have aided me over the years, from biology lab techniques, to electronic devices.

I'd like to thank Brightwake Ltd. for their technical advice and support throughout the design phase of this research.

Cheers to the boys in the EngD study room. Without you guys, I would never have learned to juggle, or play cricket in an office; soft skills that I hope will make me popular in my next workplace.

Thanks to Moeed in particular, you brought me up to speed with my, initially dubious, experimental biology techniques, and were a huge part in making this thesis happen. The constant abuse, however, does make this appreciation much less heartfelt. I'm sure that's what you'd want though.

Thanks also to my parents. Your constant questioning as to when the thesis would be complete was a regular reminder to get on with my work.

I would like to acknowledge the financial support from the EPSRC. Without the finding providers, this EngD project could not have begun.

Above all, I’d like to thank Julia for her support throughout these last few years.

Abstract:

Background:

Systemic inflammation, whether it is the result of infectious or traumatic insult, typically has a high mortality rate, often with the exact cause of death remaining elusive. Observations in recent years have led to specific definitions for infectious and non-infectious systemic inflammation, as well as a greater understanding of the pathophysiology on the molecular and cellular levels.

Despite the multitude of insults that can lead to systemic inflammation, the symptoms and pathophysiology of the condition remain similar, and the development of pharmaceutical or technological treatment typically focuses on the targeting of the intrinsic molecular mechanisms associated with the progression of the condition.

Objectives:

In this research project, the use of an extracorporeal adsorbent technology is proposed to remove inflammatory mediators from circulating blood. The main objective of this study is to develop a miniaturised portable adsorbent technology. This can be achieved by completing a series of smaller objectives, these include;

The development of a miniaturised portable blood pump.

The immobilisation of adsorbent microbeads in a manner which allows continuous blood flow across the beads without causing an embolism risk or high resistance to flow.

The effective integration of these extracorporeal technologies in a manner that leads to effective cytokine removal from circulating blood to a clinically relevant degree with a technology that lends itself to a broad range of applications.

Approach:

Designs were developed for a series of blood pump concepts, some of which were modelled computationally; others were developed into prototypes and tested in the laboratory. Subsequent to the development of these concepts, laboratory testing of the adsorbent material was performed to further inform the design of the overall device. Following this, a clinical study was undertaken in which the device was deployed in one of the situations in which we envisage its use, post cardiopulmonary bypass cytokine filtration.

Outcomes:

Upon completion of this research project, multiple concepts had been proposed and explored for the development of a portable integrated cytokine adsorption technology. Many remained in a conceptual phase, as a result of various limiting factors. Others were developed into prototypes, and integration was achieved with relatively little complication.

In the testing of the adsorbent device, a series of complications was discovered in relation to the use of interleukin proteins *in vitro.* Limitations were found in using these proteins in the laboratory setting, which were critical to this research as they have a significant impact on the assumption of cytokine stability in control solutions.

The subsequent testing of the device in near-clinical studies, reinforced these findings, but also showed the considerable ability of the cytokine adsorption device to perform well under these conditions. Through this research, we were able to develop a truly portable cytokine adsorption technology and confirm its efficacy under clinical conditions.

List of Abbreviations:

ACCP – American College of Chest Physicians

ALI – Acute Lung Injury

ARDS – Acute Respiratory Distress Syndrome

BLI – Blast Lung Injury

BSA – Bovine Serum Albumin

CAD – Computer Aided Design

CARS – Compensatory Anti-inflammatory Response Syndrome

CFD – Computational Fluid Dynamics

CHD – Continuous Haemodialysis

CHF – Continuous Haemofiltration

CHFD – Continuous High-Flux Dialysis

CRRT – Continuous Renal Replacement Therapy

DAMP – Damage Associated Molecular Pattern

DC – Direct Current

DIC – Disseminated Intravascular Coagulation

ECMO – Extracorporeal Membrane Oxygenation

EMEA – European Medicine Agency

FBS – Foetal Bovine Serum

FDA – Federal Drug Administration

HIV – Human Immunodeficiency Virus

HVH – High-Volume Haemofiltration

ICU – Intensive Care Unit

IL-6 – Interleukin 6

IL-1β – Interleukin - 1β

IL-8 – Interleukin – 8

IED – Improvised Explosive Device

LPS – Lipopolysaccharide

LTA – Lipoteichoic Acid

MDS – Microspheres Detoxification System

MODS – Multiple Organ Dysfunction Syndrome

MRSA – Methicillin Resistant Staphylococcus Aureus

NHS – National Health Service

PAMP – Pathogen Associated Molecular Pattern

PBI – Primary Blast Injury

PBS – Phosphate Buffered Saline

PE – Plasma Exchange

PMF – Polymyxin Fibre

PRR – Pattern Recognition Receptor

PVC – Polyvinylchloride

RBC – Red Blood Cell

SARS – Severe Acute Respiratory Syndrome

SCCM – Society of Critical Care Medicine

SEM – Scanning Electron Microscope

SFM – Severe Falciparum Malaria

SIRS – Systemic Inflammatory Response Syndrome

SNISIRS – Severe Non-infectious SIRS

SOAP – Sepsis Occurrence in Acutely ill Patients.

TNF – α – Tumour Necrosis Factor – α

TFPI – Tissue Factor Inhibiting Pathway

TLR – Toll-Like Receptor

VAD – Ventricular Assist Device

Thesis Outline:

In chapter 1, the subject of systemic inflammation is introduced from a clinical perspective. Initially, the focus is on the burden systemic inflammation places on healthcare providers. In addition, the many insults that can lead to inflammation, both infectious and non-infectious, are discussed.
Following this, is a discussion of the pathophysiology of systemic inflammation, and the various pharmaceutical and technological treatments that have been developed in an attempt to treat it. This discussion leads on to the objectives and proposed methodologies of this research project.

In chapter 2, we discuss the development of extracorporeal technologies from an engineering perspective, initially reviewing available extracorporeal technologies, and then manufacturing methods, computational modelling and integration methods, required to develop a portable adsorbent device. This leads to the final design of the device and the decisions on which it was based.

In chapter 3, we present the data from a series of laboratory experiments that were designed to test the technology developed. This led to unexpected complexities in laboratory methodologies that call into question the reliability of *in vitro* methods for the investigation of cytokine adsorption.

Chapter 4 details the methods and findings of a clinical study, in which the technology developed in this project was deployed. In this chapter, we present cytokine adsorption profiles for our 3 target cytokines. This was performed in whole human blood, which was residual in a post-surgery extracorporeal circuit.

In chapter 5, the results from this thesis are discussed with reference to the current status in this field.

Chapter 6 provides overall conclusions from the research, together with a projected direction for the future work.

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1. Introduction:

1.1 Definition of SIRS/sepsis.

The term sepsis has existed for many centuries along with many other terms, such as; infection, blood poisoning, bacteraemia, septicaemia, septic syndrome and septic shock. However, until a consensus conference by the American college of chest physicians (ACCP) and the Society of critical care medicine (SCCM) was held in 1991, these terms had no specified definition. The use of multiple terms, each having unclear definitions, resulted in a great deal of data being distorted or misrepresented in research literature until this time. Further confusion has resulted from the use of terms sepsis, septic syndrome or septicaemia when referring to non-infectious systemic inflammation, potentially having arisen from trauma rather than infection. The ACCP/SCCM consensus conference provided a framework to define the various clinical conditions that result in systemic inflammation. Systemic inflammation can be the result of a multitude of clinical insults, ranging across many types of injury or insult, and though they have similar clinical manifestations and treatments, they should not be considered to be the same condition (Bone et al. 1992).

Systemic inflammatory response syndrome (SIRS) was the umbrella term originally developed at this conference to broadly define the complex clinical syndrome arising from a non-specific insult or injury. SIRS is characterised by a number of clinical symptoms and was defined as more than one of the following;

1. Body temperature <36°C or >38°C
2. Heart rate > 90 beats/min
3. Respiratory rate > 20 breaths/min or hyperventilation with $PaCO_2$ less than 32mmHg.
4. White blood cell count >12000/mm^3, <4000/mm^3 or with > 10% immature neutrophils.

Sepsis was further defined as SIRS in the presence of a known infection. The terms severe sepsis or severe SIRS, and septic or sterile/aseptic shock are indicative of the severity of the condition (septic or aseptic) and are respectively associated with; multi organ dysfunction, including haemodynamic abnormality, and end stage multi organ failure in which homeostasis cannot be maintained without clinical intervention. (Davies & Hagen 1997).

Though the terminology defined at this conference was employed by many medical professionals, by the year 2000, a vast majority of intensive care clinicians felt that the term SIRS was too sensitive and non specific, and the use of the term sepsis lacked a common definition as the majority of intensive care clinicians failed to provide the consensus definition of sepsis when asked to define it. (Poeze et al. 2004).

A further definitions conference was held in 2001 to update these definitions. Figure 1 summarises the consensus reached at this conference and demonstrates the distinction between sepsis and non infectious SIRS.

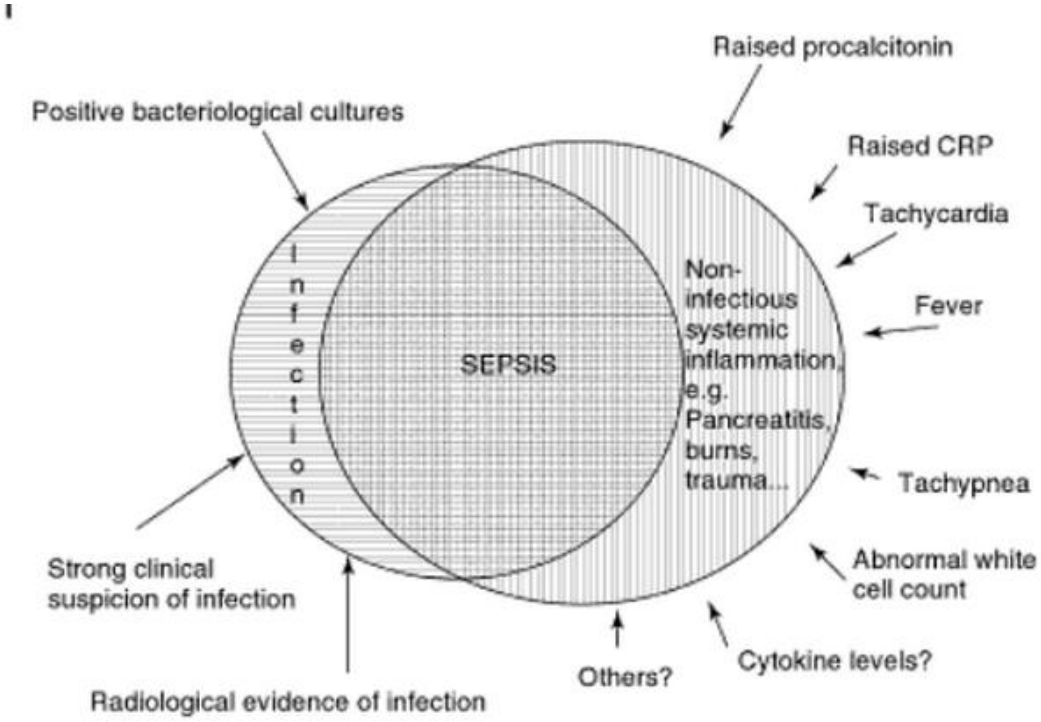

Figure 1 - Venn Diagram displaying the definition of SIRS and sepsis. Taken from "Sepsis and Non-infectious Systemic Inflammation" by Cavaillon and Adrie, 2009.

When dealing specifically with the cardiothoracic surgery field, the definition of SIRS, requires further markers (Landis et al. 2010), these are;

- The measurement of at least 1 inflammation marker.
- Reporting at least 1 clinical end point.
- Reporting a core set of CPB and perfusion criteria that may be linked to outcomes.

1.2 Burden of Sepsis:

1.2.1 Epidemiology and Mortality:

Much of the literature of recent years on systemic inflammation has had a particular focus toward sepsis, with non-infectious SIRS being left considerably underrepresented. A great deal of research and statistics has been published relating specifically to sepsis. Though sepsis is essentially a sub-category of SIRS, it is often the case nowadays to find sepsis and non-infectious SIRS being placed into two distinct categories, despite their very similar clinical characterisation.

Despite the similarities in the clinical aspects of these conditions, an overrepresentation of sepsis in the literature still exists, with non-infectious SIRS not being taken fully into account (Hotchkiss & Karl 2003; Lever & Mackenzie 2007; Venkataraman et al. 2003; Gao et al. 2008). It is felt by many that there is a lack of proper identification of SIRS in the clinical setting. It is likely that this is due, in part, to the fact that in the sepsis scenario, the molecular mechanism behind microbial infection leading to inflammation is fairly well understood, whereas the mechanisms leading to inflammation resulting from traumatic tissue injury are less so. Another factor in the under representation of non-infectious SIRS is that in many cases systemic inflammation is recorded as sepsis (with an associated "culture negative" finding when checking for infection) (Rangel-Frasuto et al. 1995). In accordance with the definitions set out at the consensus conference, this terminology is inaccurate and "culture-negative sepsis" should not be considered sepsis, but (non-septic) SIRS. The later 2001 conference addressed the issue of cases in which infection was suspected but unconfirmed and broadened the definition of sepsis to include infection that is "strongly suspected, without being microbiologically confirmed". Suspicion of infection, however, is extremely subjective and varies enormously from centre to centre and indeed clinician to clinician.

The ambiguity and misrepresentation of data referring to both septic and non-septic SIRS have lead to the skewing of epidemiological studies and in turn an underestimation of the burden of SIRS overall on healthcare providers. This was addressed by Dulhunty et al. in a study in which the differences between severe sepsis and severe non infectious SIRS (SNISIRS) were characterised (Dulhunty et al. 2008). In this study, it was found that severe sepsis had a prevalence of 20%

(707/3,543) in the intensive care unit (ICU) with a mortality of 27%, whereas SNISIRS accounted for 28% (980/3,543) of ICU cases, with a mortality of 25%. This study also showed that SNISIRS was more common than severe sepsis on admission to the ICU. However, patients remaining in ICU for 4 days or more were more likely to have severe sepsis. Another interesting finding in this study was that the most common cause of death in severe sepsis is multi-organ failure, whereas SNISIRS, despite its similar prevalence and mortality, tended not to progress to multi-organ failure in as many cases, but far more often resulted in death as a result of neurological failure or cardiac arrest. This is partly, but not entirely, associated with the nature of the initiating insult e.g. traumatic head injury or cardiac arrest. These findings identified the gap in the literature relating to SIRS and also showed results, relating to severe sepsis, that were comparable with numerous other studies focusing on severe sepsis, of particular note is the European "SOAP (sepsis occurrence in acutely ill patients) study" (Sprung et al. 2006).

Despite the underestimation of the combined burden of SIRS/sepsis overall, the burden of sepsis alone is enormous, with figures from European studies conservatively suggesting an incidence of approximately 90 cases per 100,000 population and a mortality rate of 35%. Other studies in the USA, however, have suggested that the incidence of sepsis has increased 3-fold between 1979 and 2000 from 83 to 240 cases per 100,000 population. This increasing trend has also appeared in the United Kingdom and France (Mascia et al. 2008; Harrison et al. 2006) It has been suggested that this may be due to; an increasingly elderly population, increased recognition of disease, increased performance of invasive procedures and organ transplant, increased use of immunosuppressive agents and chemotherapy, increased use of indwelling lines and devices, and an increase in chronic diseases, such as end-stage renal disease and HIV (Filbin 2010).

It is now well accepted that SIRS/sepsis affects 50% of all ICU patients as well as remaining the leading cause of infant death worldwide (Fortenberry & Paden 2006; Sandeman et al. 2008).

SIRS/sepsis should be considered the second biggest killer in the UK, after cardiovascular disease, with a huge impact on the heath economy; 50% of ICU cases

with unacceptably high rates of mortality, comparative to the mortality rates of myocardial infarction in the 1960s.

To date, there is no known cure for SIRS/sepsis and treatment is only supportive.

1.2.2 Cost:

The cost of severe sepsis alone has been estimated in multiple studies. In a European study, a typical episode of severe sepsis was estimated to cost a healthcare organisation approximately €25,000 and this has been extrapolated to suggest that the annual cost to Europe as a whole is approximately €7.6billion. Studies in the USA have cited a similar cost for a single episode of sepsis as being $25,000 per case and state an overall healthcare cost of approximately $17billion. Based on the assumption that there are 100,000 cases in the UK annually, the direct cost to the NHS has been suggested to be £2.3billion. However, sepsis is a condition that affects individuals indiscriminate of age or overall health. It has been anticipated that the real cost to the community and economy as a whole is much larger than this figure, perhaps 4 or 5 times this cost (Daniels 2009).

These estimations may be further under representative of the disease as they are based on the assumptions that; all patients with SIRS are in an ICU, and that dying patients are receiving aggressive rather than palliative treatment or that the disease has been defined and recorded accurately in such cases.

It is difficult to get a true understanding of the cost of SIRS/sepsis. But, despite the fact that studies and estimations that currently exist should be considered conservative estimations, the prevalence, mortality and cost of SIRS/sepsis remain extremely crude.

1.3 Aetiology:

Though SIRS and sepsis share many clinical aspects, both presenting with fever, tachycardia, tachypnoea, and leukocytosis, the aetiology of septic and aseptic SIRS differ.

Sepsis can be the result of many different infections such as; viral, bacterial, fungal or parasitic. SIRS, however, can be associated with non infectious insults such as; trauma, burns, pancreatitis, blood exposure to artificial bypass circuits, or ischemia-reperfusion injuries, to name but a few (Sandeman et al. 2008).

Of all the aetiologies, and their associated molecular patterns i.e. damage associated molecular pathways (DAMPs), the triggering of toll-like receptors (TLRs) and the subsequent release of cytokines is widely considered to be the convergence point of the molecular patterns associated with the initiation of a systemic inflammatory response. The inflammatory cascade following the triggering of TLRs presents limited differences in the pathophysiology, molecular pathways or clinical events, regardless of the nature of the initiating insult (Brunn & Platt 2006; Gao et al. 2008).

Despite this fact, it is still necessary to distinguish between cases of SIRS and sepsis and their initiating insults. This is particularly important when considering a clinical strategy of resuscitation and for the suitable enrolment of patients into a clinical trial.

1.3.1 Trauma:

Tissue injury can result from many insults, such as; trauma, major surgery, pancreatitis, or severe heart failure. Such tissue injury is often the cause of systemic inflammation.

Severe trauma causes activation of nearly all aspects of the immune system and rapidly leads to SIRS. The initial pro-inflammatory response is followed by a compensatory anti-inflammatory response syndrome; cited in some medical literature as “CARS”. Though use of this term has not been universal, it is widely accepted that a later stage anti-inflammatory phase exists during a case of systemic inflammatory response. During this anti-inflammatory phase, immunosuppression occurs and the risk of infection and subsequent “secondary sepsis” is common (Lenz et al. 2007).

After traumatic injury, three distinct time periods are typically recognised. The first period is immediately after trauma. Death as a direct result of trauma will usually

occur within this period, typically less than 1 hour after traumatic insult. The second period is within the first 24hours, death usually being the result of hypovolaemia, hypoxia, or severe head trauma. In the event that a patient survives these first two time periods, there is a high risk of SIRS/sepsis developing in the third time period.

As with other forms of systemic inflammation, trauma is associated with DAMPs that are recognised by TLRs. This molecular pathway leads to the production of local inflammatory mediators, the activation of systemic inflammatory and complement cascades, and abnormal haemodynamics, including coagulopathy (Lenz et al. 2007). The progression of the pathophysiology of systemic inflammation will be discussed later.

1.3.2 Infection:

The pathogens associated with sepsis can vary enormously. Infectious insults can take the form of bacteria (both gram-positive and gram-negative), viruses, parasites, or fungi.

Common examples of such insults could be antibiotic resistance bacteria (such as MRSA), influenza, malaria or *Candida*.

Gram-negative bacteria:

The European "SOAP study" (sepsis occurrence in acutely ill patients) found that 38% of sepsis cases were the result of gram-negative bacteria. 30% of these cases showed the presence of an antibiotic resistant strain of the bacteria, and these cases presented a significantly higher mortality and treatment cost (Neuhauser et al. 2003).

A common example of a gram-negative bacterium is *pseudomonas aeruginosa*, which is commonly implicated in hospital cross infection.

Breakdown of the gram-negative bacteria cell wall results in the release of lipopolysaccharide (LPS), also known as endotoxin. LPS/endotoxin is heavily implicated in the initiation of sepsis as it is recognised as a toxin by TLR-4 and begins the inflammatory cascade. Injection of gram-negative bacteria has traditionally been the most popular method by which to induce sepsis in an animal model, though the suitability of this method is increasingly under question.

Gram-positive bacteria:

Again, the European SOAP study found that gram-positive bacteria were responsible for 40% of sepsis cases. As well as their prevalence in sepsis, cases of gram-positive bacteria represent the most common class of pathogens associated with bacterial biofilm build-up on the surfaces of medical devices (Costerton 1999; Stewart & Costerton 2001).

Since the existence of antibiotics, gram-positive bacteria appear to have gained a considerable degree of resistance and this has provoked considerable fears as these antibiotic resistant strains of bacteria are increasing in frequency, especially in ICUs.

A notable example of such a bacterium is methicillin-resistant *Staphylococcus aureus* (MRSA), regularly cited in the media as a "hospital superbug" (Koontz 2000).

Parasites:

Severe falciparum malaria (SFM) is considered by the World Health Organisation to be one of the most serious infectious emergencies, often requiring treatment in the ICU. *Plasmodium falciparum* malaria is the cause of 1.5 – 2.7 million deaths a year. Approximately 1million of these are children and roughly 90% of these deaths occur in endemic areas, notably tropical Africa. The pathophysiology associated with SFM is extremely complex but shares many specific features with sepsis (Clark 2007; Hunter 2007). *P.falciparum* causes activation of macrophages and T-lymphocytes. Monocytes are also activated by malarial toxins. These activations subsequently result in a systemic inflammatory response, SIRS, and the secretion of inflammatory mediators. Of these cytokines, tumour necrosis factor (TNF-α), in particular, leads to a positive feedback loop of inflammation.

Fungi:

Recent surgery, acute renal failure, antibiotics, corticosteroids, parenteral nutrition, neutropaenia, burns, mechanical ventilation, indwelling central venous catheter, and haemodialysis; these are all risk factors associated with fungal colonisation and systemic infection. The most common form of bloodstream fungal infections are *Candida* (Spellberg & Edwards 2002). *Candidas* account for approximately 9% of bloodstream infections, most commonly presenting in those patients immunosuppressed. Mortality among surgical patients that develop *Candida*-associated bloodstream infections are particularly high however, at 41%. Like many other pathogens in the initiation of sepsis, *Candidas* are considered pathogens by the immune system and are recognised by TLRs (different species of *Candidas* are recognised by different TLRs); as with other cases, this subsequently results in the release of inflammatory mediators and the initiation of the inflammatory cascade.

Viruses:

The discussion of cases of severe influenza in patients has traditionally cited terminology such as; “cytokine storm”, “sepsis-like syndrome” or “sepsis with acute lung injury” (Patel et al. 2009). Proper use of the term sepsis has largely been omitted from the literature with respect to viruses, such as influenza; though it is widely accepted that severe influenza manifests itself very similarly to bacterial or

malarial sepsis and the systemic inflammatory response is a major cause of morbidity and mortality associated with influenza (Clark 2007; Rainsford 2006).

1.3.3 Clinical Injuries: Post Cardiotomy and Reperfusion Injuries:

Post-cardiotomy systemic inflammation and ischemic-reperfusion injury are commonly cited in the literature as forms of SIRS arising from clinical injury.

Ischemic-Reperfusion Injury:

Ischemic-reperfusion injury is a complex insult that comprises intracellular injury as well as inflammatory response. Typically, it is during the reperfusion phase of ischemic-reperfusion procedures that an inflammatory response occurs. This is true for both warm and cold ischemia. It remains contentious as to what exactly triggers the inflammatory response but it is believed that it may be triggered by broken cell debris, activation of the complement cascade, macrophages, endothelial or dentritic cells, or the binding of TLRs. Furthermore, recent research has shown substantive coagulation abnormalities in ischemic-reperfusion injury (Gailani & Renné 2007) and it may be the coagulation-inflammation molecular cross-talk that is responsible for the inflammatory response. Regardless of the responsible molecular pathway, ultimately pro- and anti-inflammatory mediators are released systemically leading to SIRS, organ damage, and multiple organ dysfunction syndrome (MODS) (de Groot & Rauen 2007).

Post-Surgical Injury:

Cardiac surgery has been shown to be associated with high concentrations of serum cytokines levels, activation of the complement cascade and the secretion of IL-6, IL-8, and TNF-α. It is, as yet, unclear as to what exactly causes the post-operative SIRS, but it is believed that the cause is the combination ischemic-reperfusion injury from the peripheral vascular system, direct tissue damage from the surgery, or blood contact with an extracorporeal device (Asimakopoulos & Gourlay 2003).

1.3.4 Military/Emergency Situations:

The proposal of this project began with a particular focus toward military, emergency or critical care situations. As such, discussed here are examples of the context in which we anticipate this specific portable technology being employed.

Blast injuries:

Explosive blasts have become the most common and destructive terrorist weapon around the world, as well the most common form of combat injury in modern warfare. This is largely due to the ease of manufacture and cost-effectiveness of developing improvised explosive devices (IEDs) (Ciraulo & Frykberg 2006; Moulton 2009). Primary blast injury (PBI) is caused by the overpressure in the atmosphere, following a blast, leading to trauma in air filled organs. It has been noted that the lungs are particularly susceptible to blast trauma as there is a large air/tissue surface area. Blasts often lead to acute lung injury (ALI), occasionally known as blast lung injury (BLI) when in this context. Pulmonary dysfunction, acute respiratory distress syndrome (ARDS), and SIRS are common sequelae to blast trauma, and BLI is the most common cause of death following a blast injury (Marti et al. 2006; Mayo & Kluger 2006). Gastro-intestinal injury is also a very common cause of death with blast injuries as rupture of the gastro-intestinal tract often leads to death by sepsis (Born 2005).

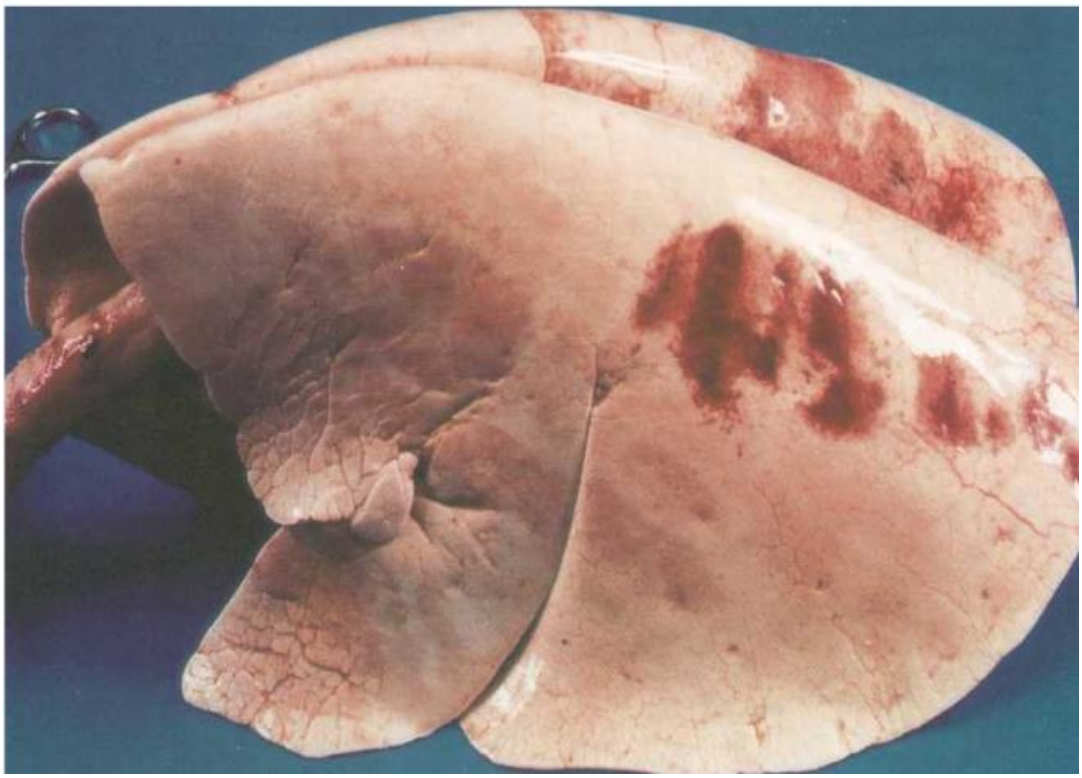

Figure 2 - Image of lung bruising from a blast injury.

It is very common for a blast injury to be further complicated by secondary infections. These can be caused by initial wound contamination or by nosocomial infections associated with long term care. Worth particular mention are multiple-drug resistance organisms (Hospenthal et al. 2008). Mayo and Kluger observed that some patients who fell victim to a terrorist attack in Tel Aviv presented with a "hyperinflammatory state that did not correlate with the complexity of the sustained injury". It was also noticed that this did not occur with patients who were far from the centre of the explosion or patients who had no skin injury. It is believed that this was the cause of toxic substances that were released upon explosion of the device, penetrating a patient's skin and causing systemic inflammation by both trauma (mechanical and chemical), as well as secondary infection (Mayo & Kluger 2006).

Pandemics and Biodefence:

Health pandemics, the threat of biowarfare/bioterrorism, and the emergence antibiotic or antiviral resistant biological agents have led to public health, military and defence establishments taking an interest in the concept of developing medical responses to such threats (London et al. 2010).

It is believed that death by the systemic release of cytokines, a "cytokine storm", has been the result of a number of pandemics in recent time. The mechanism of death from such a cytokine storm is either the result of the virus killing so many cells that it is directly responsible for MODS, or indirectly responsible for MODS, from either the pro-inflammatory phase of sepsis, allowing cytokines to inflame and damage organs, or the anti-inflammatory phase of sepsis, allowing the infecting organism to thrive and damage organs.

Often in the cases of the immunocompromised e.g. elderly patients, secondary infection/secondary sepsis can lead to acute lung injury (ALI) and pneumonia.

The 1918 Spanish flu is an example of a viral pandemic causing the cytokine storm (SIRS). It infected approximately 25% of the world population, with a mortality of 10%. Within 18months of the outbreak of the virus, approximately 40million people had died.

Pandemics are not restricted to viruses however. Bacterial pandemic can also exist, as in the case of *Yersinia pestis*, otherwise known as "The Black Death". This plague is believed to have reduced Europe's population by one third. Though modern medicine can respond to bacterial infection by antibiotics, antibiotic-resistant strains of bacteria are on the rise; MRSA is a well-known example of this, regularly portrayed in the media as a superbug.

In the 21st century, fears of pandemic outbreak have been seen on numerous occasions. In 2002-2003, severe acute respiratory syndrome (SARS) raised fears among many, as it caused over 800 deaths worldwide. SARS failed to become a global pandemic however. Though the mortality rate was crude and the virus had the ability to spread from human to human with ease, those infected developed a rapid onset of symptoms and thus made them unlikely, or unable, to enter the larger population i.e. working, socialising, travelling, and most importantly, flying internationally (Hunter 2007).

Another example of pandemic fears being raised was avian flu ("Bird Flu"), which had an extremely crude mortality rate of 60%. Despite initial fears of the possibility of this passing from human to human, it was shown that the disease remains endemic only in the bird population (particularly in south-east Asia), and though the virus can infect humans, in its present form, it has only done so in cases in which a human has been in direct and regular contact with birds. The inability of this virus to spread via airborne droplets led to it not becoming a global pandemic (Hunter 2007).

In 2009, fears of yet another pandemic occurred during the outbreak of "Swine flu". At the time of the initial outbreak, fears were raised as Mexico had reported 152 deaths resulting from swine flu within the first month (though these were unconfirmed cases). Coupled with this, the virus appeared not to affect the very old or very young, which is typical of seasonal flu. This indicated an altogether new strain of the virus. Fears were further raised after the confirmation that the swine flu virus was H1N1 influenza virus, which was the cause of the 1918 Spanish flu. The virus spread rapidly from human to human via air droplets, could not be contained, and was declared a global pandemic. It was later argued that swine flu was little more serious than seasonal flu and the mortality of the flu was little or no higher than seasonal flu. Though, with so many unconfirmed cases, it is difficult to state this categorically (Cohen & Enserink 2009).

In this last decade, 3 viruses have raised serious concerns in the medical community about the possibility of a new global pandemic comparable to the 1918 Spanish flu. These viruses all showed different characteristics that would be required of a virus to cause a pandemic i.e. virulence, high mortality, and ability to spread rapidly from human to human (Hunter 2007). Bird flu failed on the account of its inability to spread from human to human, swine flu failed on the account of having a low mortality; SARS, however, was devastating as an epidemic in its area. Only its rapid onset of symptoms caused it to fail as a global pandemic.

It can be seen from the nature of these viruses and the different characteristics they hold that the concept of a devastating global pandemic occurring in future is not as farfetched as many might believe.

With all 3 of these viruses presenting with 2 or 3, of the 3 necessary requirements to be considered a serious global pandemic, it is clear that the emergence of new viruses

poses a serious threat to the human population globally. Coupled with this, the increasing threat of bioterrorism, as well as the increasing use of explosives for terrorist activities have led to greater enthusiasm for the development of a broad-spectrum medical response that can be rapidly implemented in event of such an incident, such as a portable cytokine reduction technology.

1.4 Pathophysiology:

During the progression of SIRS/sepsis, many physiological factors contribute to the pathology of the disease. Of particular interest to the development of this technology are the inflammatory and coagulation cascades associated with the disease. The physiologies of either septic or aseptic SIRS differ slightly, though many similarities exist. These will be discussed here with some focus towards the prospect of technological intervention.

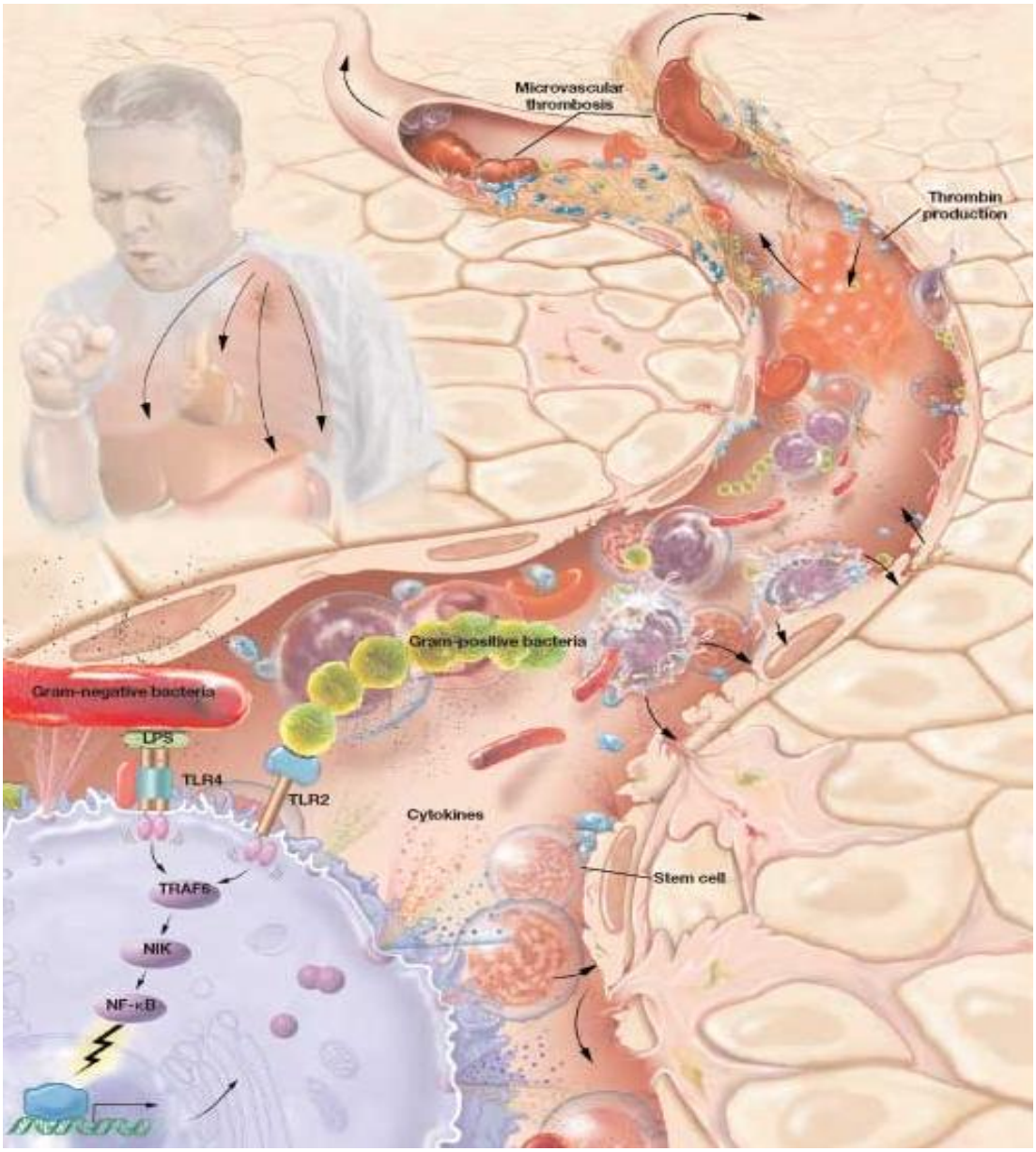

Figure 3 – Molecular mechanisms relating to sepsis.
Taken from http://infections.consultantlive.com/

1.4.1 Damage associated molecular patterns (DAMPs):

Initial response to traumatic injury or infection takes place on the cellular level in the form of damage associated molecular patterns (DAMPs).

When associated with traumatic injury, a DAMP is subcategorised as an "alarmin"; these are endogenous intracellular proteins, which are released by dead cells and recognised by pattern recognition receptors (PRRs); most notably the toll-like receptors (TLRs) found on the surfaces of surveillance cells, such as dentritic cells or macrophages. This molecular signalling pathway provides the body's initial "alarm signals" in response to traumatic injury.

Pathogen associated molecular patterns (PAMPs) are another form of DAMP, not associated with trauma, but infection. Conversely, PAMPs are typically exogenous microbial molecules. A classic example of such a PAMP, discussed regularly in reference to sepsis, is LPS/endotoxin. This molecule is a component of the gram-negative bacterial cell wall released upon the breakdown of such a bacterium, and is considered extremely toxic. Exogenous PAMPs have been studied longer than trauma-associated alarmins (this may be another factor in the imbalance in the literature toward sepsis over aseptic SIRS).

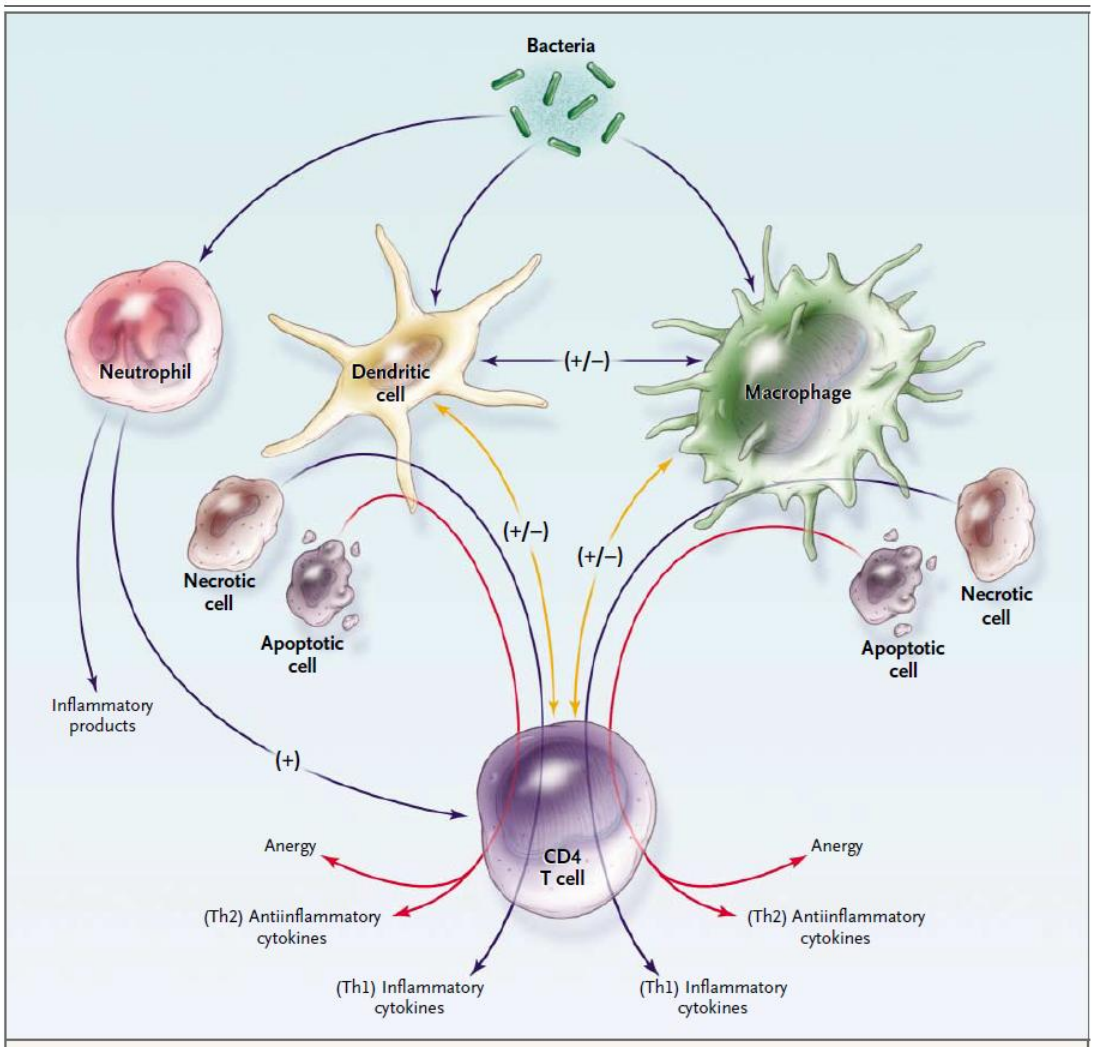

Figure 4 – Cellular mechanisms relating to sepsis.
Taken from the New England Journal of Medicine.

It is being increasingly accepted, however, that pathogens are not the only cause of tissue or cell damage that leads to systemic inflammation. Injury can take many forms, including mechanical, thermal, chemical, radiological or ischemic injuries (Bianchi 2007).

For a considerable period of time, it was felt that since trauma is regularly followed by pathogens invading wounds and leading to subsequent secondary infections, an antimicrobial strategy was appropriate for traumatic injury. However, it has increasingly been shown that though many traumatic injuries exhibit similar physiological and clinical factors to infectious injuries, many show no sign of infectious pathogens present(Jaffer et al. 2010; Pugin 2008). As such, it is now felt

that alarmins and PAMPs together constitute the larger family of DAMPs, and that it is these DAMPs (either endogenous or exogenous) that initiate the inflammatory process. Furthermore, it has been hypothesised that in order for a pathogen to induce systemic inflammation, an exogenous PAMP must be followed by an endogenous alarmin to achieve a systemic response. An endogenous alarmin, however, is sufficient alone to achieve such a response without any pathogen present (Pugin 2008). Alarmins, therefore, are now considered to be the crucial molecular pattern associated with systemic inflammation.

During incidences of cell injury or necrosis, alarmins are released both actively and passively into the extracellular fluid and activate immune cells by way of biochemical interaction with surveillance cells and their TLRs. Many of the alarmin molecular patterns share innate immune cell receptors with PAMPs. Thus, the molecular patterns associated with traumatic and infectious injuries converge at the point of immune cell receptors, and most notably TLRs.

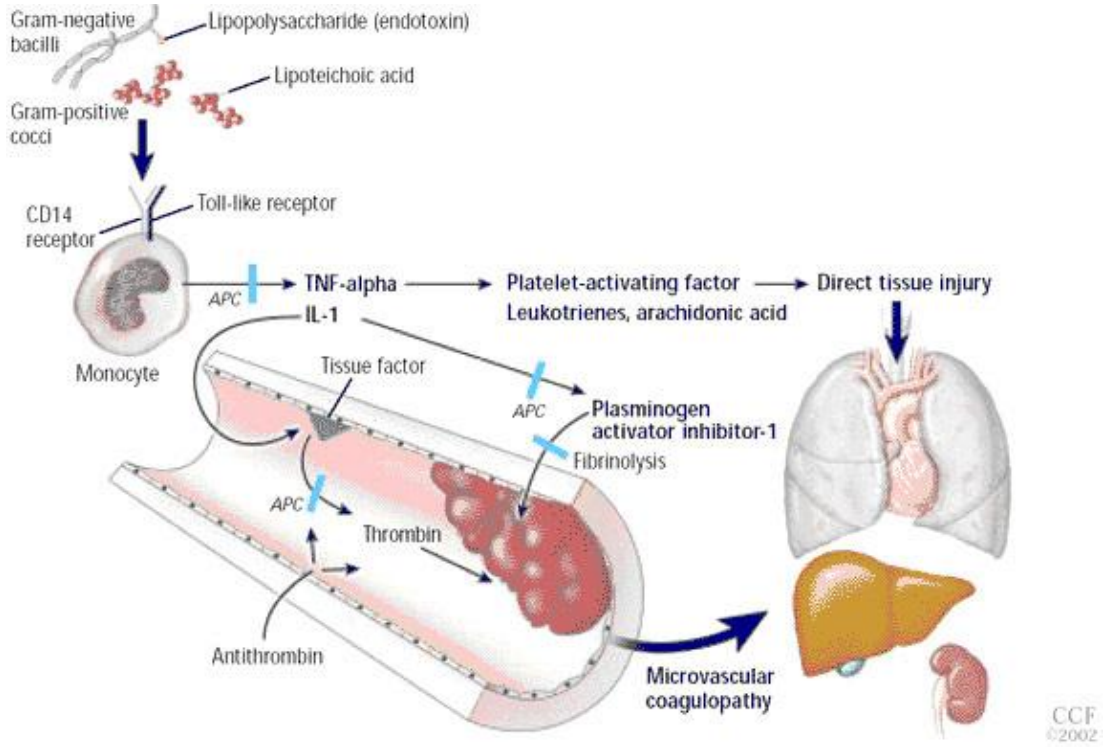

Figure 5 – Triggering of toll-like receptor, leading to cytokine induced pathology. Taken from http://www.clevelandclinicmeded.com/

Comparatively, gram-negative sepsis has been studied in great depth, due in part to the fact that LPS induced sepsis is one of the most regularly used models of sepsis in the laboratory. Thus, the mechanism of gram-negative sepsis is relatively well

understood. TLR-4 has widely been accepted as the "endotoxin receptor" and it appears that TLR-4 is the principal signalling pathway associated with gram-negative infection. It remains highly contentious however, as to whether or not TLR-4 plays a protective or detrimental role in the development of sepsis. It has been suggested that the TLR-4 receptors initially play a protective role in infection but when overwhelmed, localisation of infectious agents fails and systemic manifestation of sepsis occurs (Brunn & Platt 2006).

It has also been shown that lipoteichoic acid (LTA), released upon breakdown of the gram-positive bacterial cell wall, triggers TLR-2, as do many fungi. Though these component molecules of gram-positive or gram-negative bacteria appear to trigger specific receptors, such specificity does not appear to be the case for whole bacteria and it is believed that certain species have a dominant signal pathway e.g. TLR-2 or TLR-4 for gram-positive and gram-negative respectively.

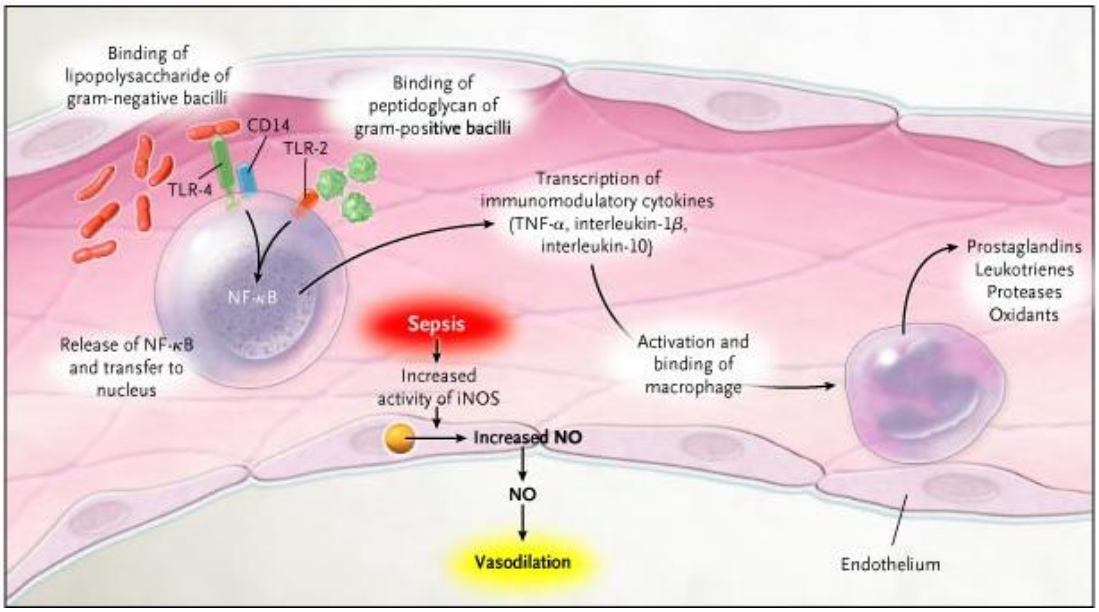

Figure 6 – Triggering of toll-like receptor, leading to endothelial activation.
Taken from http://crashingpatient.com/

Despite the fact that different species trigger different TLRs, after initial molecular and cellular signalling, all pathways lead to the same result; that is, the up-regulation of pro-inflammatory cytokine production and consequently the initiation of the inflammatory cascade (Gao et al. 2008).

1.4.2 Cytokine-induced Pathology in SIRS/sepsis:

Following infection or injury, the triggering of TLRs, and the up-regulation of cytokine production, a patient's progression to systemic inflammation involves a complex immunological reaction, comprising of inflammatory mediators, complement factors and the coagulation cascade; all of which are intimately intertwined.

Cytokines are considered to be fundamental in the mediation of the inflammatory and coagulation processes. Though their purpose is to facilitate the immunological response to DAMPs, and the eradication of insults, these same cytokines are implicated in the degradation of organ function and are believed to contribute significantly to death in both septic and sterile shock. This is what is known as a "cytokine storm". Consistently raised concentrations of cytokines in a patient's circulation are indicative of mortality. Listed below are the cytokines that are considered to be pivotal in the pathophysiology and progression of SIRS/sepsis.

Tumour necrosis factor – α (TNF-α):

Tumour necrosis factor – α (TNF-α) is a pro-inflammatory cytokine, heavily implicated in the progression of systemic inflammation, and in particular, haemodynamic abnormality and coagulopathy. It is considered to be one of the earliest presenting cytokines in plasma prior to SIRS and is therefore believed to be a major facilitating factor in the mediation of further inflammatory mediators (Jaffer et al. 2010). Though essential in combating infection, persistent high concentrations of plasma TNF-α have been correlated with development of MODS, and subsequent mortality (Hotchkiss & Karl 2003).

Interleukin-1 (IL-1):

Interleukin 1 (IL-1) comprises two similar proteins, i.e. IL-1α and IL-1β. Both proteins act on the same receptor; however IL-1β is far more readily implicated in SIRS/sepsis with high concentrations being detected in the plasma during inflammatory processes. Attempts to discover the full function of IL-1β have largely been inconclusive. It has been observed by some that this is due to the fact that other similar proteins may be able to compensate for its function in its absence, e.g. IL-1α, IL-18, and IL-33.

Interleukin-6 (IL-6):

The role of interleukin-6 (IL-6) in SIRS/sepsis is a complicated one but is pivotal in the development of the disease and is widely accepted to be one of the best cytokines to be used as a marker for the severity and mortality of SIRS/sepsis. Some aspects of the function of IL-6 during SIRS/sepsis remain contentious. However, widely accepted is the fact that IL-6 plays a central role in the orchestration of the disease and is most effective when working in synergy with other cytokines. IL-6 induces fever, leukocytosis and thrombocytosis, as well as the release of interleukin-10 (IL-10) and interleukin-1 receptor antagonist (IL-1Ra) (both anti-inflammatory cytokines). Thus, it can be considered to have both pro and anti-inflammatory properties. It is also the most heavily implicated cytokine in coagulopathy. Many research groups specifically target IL-6 as many believe it to be the single most important cytokine in the disease.

Interleukin-8 (IL-8):

The molecule interleukin-8 (IL-8) is a chemotactic cytokine, or chemokine. Raised levels of this molecule in the blood are observed in patients with sepsis and multiple trauma (Lenz et al. 2007). Highly indicative of the development of acute respiratory distress syndrome (ARDS), IL-8 is considered an important cytokine marker for prognosis of those with MODS. Difficulties exist, in the measuring of circulating levels of IL-8. It has been shown in multiple studies that plasma concentrations of IL-8 in patients with SIRS are often undetectably low. This result is deceiving however; as it has been shown that these same patients will have elevated concentrations of IL-8 in their whole blood (measured after the lysing of whole red blood). It has been shown that this is the result of IL-8 molecules binding to both erythrocytes and leukocytes (Reinsberg et al. 2000; Hirani et al. 2001; Steinbach et al. 2007)

Interleukin-10 (IL-10):

Interleukin-10 (IL-10) is a molecule considered to have anti-inflammatory properties. With an ability to inhibit TNF-α, and protect against LPS induced injury, IL-10 has been proposed by many as a potential therapy for inflammatory conditions. However, IL-10 has been also been shown to be highly elevated in patients with major trauma, and especially burns; and appears to correlate with both the progression of systemic

inflammatory response and the likelihood of subsequent secondary infection leading to secondary sepsis. It can therefore be considered a marker in the severity of the development of SIRS/sepsis (Yeh et al. 2000; Jaffer et al. 2010).

1.4.3 Complement Cascade:

Activation of the complement cascade also plays a significant role in SIRS/sepsis and can be triggered by either the classical pathway of antigen-antibody complexes; or the alternative pathway of bacterial cell wall components e.g. LPS. Though the purpose of the complement system is to assist in the removal of pathogens, many molecules of the complement system contribute considerably to the inflammatory process. Of particular note, are the anaphylatoxins C3a and C5a. These molecules are involved in infection and inflammation control and both play their part in the classical and alternative pathways of the complement system. Among their responsibilities, exist the recruitment of neutrophils, the facilitation of phagocytosis, and the opsonisation of bacteria (Lenz et al. 2007).

Like the cytokine response, the complement system is essential in defence, but control can be lost and activation can become non-local and unregulated; this generally contributes to systemic inflammation causing organ failure.

High plasma levels of both C3a and C5a have been shown to correlate with mortality in SIRS/sepsis.

1.4.4 Coagulation Cascade:

Almost all patients with SIRS/sepsis will present with some form of coagulation abnormality, occurring in approximately 50-70% of cases. The severity of this can vary hugely from barely detectable variation in coagulation factors to severe disseminated intravascular coagulation (DIC), in which microvascular thrombosis and profuse bleeding occur simultaneously. It has been shown that DIC is another predictor of mortality and organ failure in SIRS/sepsis cases. DIC is so indicative of mortality and lacking in available treatment, that many clinicians will exchange the meaning of the acronym DIC from disseminated intravascular coagulation to "death is coming". Thrombocytopenia also, is a coagulation abnormality associated with SIRS/sepsis that can be indicative of mortality. Either DIC or thrombocytopenia occurs in approximately 35-50% of cases.

Research in recent years has indicated that it is in fact cytokines that are pivotal in the regulation of the coagulation cascade during systemic inflammation (Levi et al. 1997). Much research has shown that the inflammatory cascade and the coagulation cascade are complexly intertwined and affect one and other significantly. Thus, SIRS/sepsis should not only be considered a systemic inflammatory condition, but a systemic coagulation disorder also.

The initiation of the coagulation system takes the form of either the contact activation (intrinsic) pathway or the tissue factor (extrinsic) pathway. These pathways both converge at the point of factor X; this is the beginning of the common pathway that leads to thrombin converting the soluble plasma protein fibrinogen into its insoluble form fibrin. Deposition of this fibrin leads to a fully formed blood clot.

The tissue factor pathway was formerly believed to have been initiated exclusively outside the bloodstream (hence its traditional name of "extrinsic pathway") and begins with the release of tissue factor. Tissue factor is a 45-kD transmembrane protein that is expressed by many cells around the body. These cells generally do not come into contact with blood and so tissue factor should not either, in healthy circumstances. During insult, however, tissue factor can come into contact with blood by several well known molecular mechanisms. The most well understood molecular mechanism is the direct exposure of tissue factor to blood, subsequent to damage to the integrity of the vascular tissue, therefore allowing the molecule to

enter the bloodstream; the more severe the damage, the more tissue factor is released and therefore clotting occurs more swiftly. More recently, however, it has been seen that tissue factor can be expressed by mononuclear cells circulating in the blood, subsequent to these cells being stimulated by pro-inflammatory cytokines. Thus, it can be seen that the change in name from extrinsic pathway to tissue factor pathway is justified as tissue factor can be expressed from cells intrinsic to the vascular system.

Though damage by either trauma or infection will initially result in the activation of the tissue factor pathway, the contact activation (intrinsic) pathway will later be initiated also as part of the positive feedback loop characteristic of the coagulation cascade i.e. the activity of the common pathway leads to the activation of both the tissue factor and contact activation pathways.

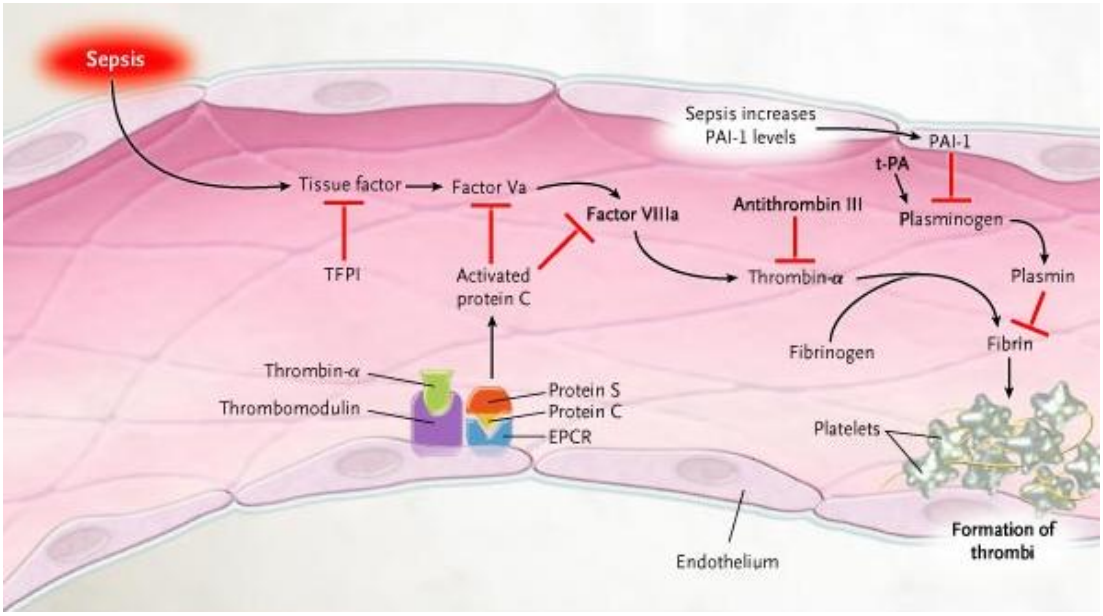

Figure 7 – Coagulation cascade associated with sepsis.
Taken from http://crashingpatient.com/

Typically, in a healthy individual, the coagulation system is regulated by three anticoagulant pathways. These are; antithrombin, the protein C system and the tissue factor pathway inhibitor (TFPI). These pathways of coagulation, as well as the fibrinolytic system, have been seen to be significantly affected by inflammation. During systemic inflammation, all of these anticoagulant pathways can be adversely affected. Significantly reduced levels of these endogenous anticoagulants can be

observed in patients presenting with SIRS/sepsis. These pathways have a "bidirectional" relationship with inflammation that is mediated by cytokines (Levi & Poll 2005). Like the early inflammatory process, many of these coagulation activities are essential in the containment of injury or infection to a localised area. But like the systemic inflammatory response, cytokine-induced coagulation can lead to systemic activity and contribute to the severity of disease. TNF-α, and IL-1 have been shown to have some effect on the coagulation system. But it is IL-6, in particular, that is believed to be deeply involved in inflammatory associated coagulation. Multiple animal studies and human trials have shown that anti-TNF or anti-IL-1 treatments have had limited success in reducing SIRS-associated coagulopathy, whereas anti-IL-6 therapies were able to completely eliminate endotoxin-induced coagulopathy. IL-10 also has been shown to be pivotal in coagulation, presenting an anticoagulation effect, but such data remain in a research phase at this time, and as such, contentious to some extent.

Not only do cytokines affect coagulation. Coagulation factors can contribute to the activity of cytokines. It has been seen by many studies that the presence of thrombin, factor Xa, fibrin, and process of the clotting process itself can activate endothelial cells, and increase production and synthesis of several cytokines, namely the interleukins 1, 6 and 8.

The protein C anticoagulant pathway has been observed in considerable depth and appears to be one of the most significant molecular mechanism by which inflammation and coagulation are interlinked. The introduction of activated protein C into cases of gram-negative sepsis has been seen to attenuate the production of cytokines, and ultimately reduce organ dysfunction and mortality. Disruption of the protein C pathway in animal models has shown evidence of increased levels of circulating cytokines, as well as significantly higher inflammation and coagulopathy. It is these findings that act as the foundation upon which the activated protein C became a significant drug in the treatment of SIRS/sepsis, as will be discussed later.

1.5 Pharmaceutical Treatment:

Attempts to find an effective treatment for SIRS/sepsis have persisted for decades, if not centuries. Little progress has been made, however, developing such a treatment. Though many treatments exist in a supportive context, there has been very limited success in dealing with the cytokine storm that is so central to the pathophysiology of the condition. Supportive treatments have, without doubt, assisted in reducing the mortality of SIRS/sepsis, such treatments include; artificial organ support (renal or pulmonary); antibiotic, antifungal, or anti-viral agents (attempting to eliminate any infectious aetiology); or immunomodulatory drugs. Despite such treatment, the mortality remains high and a great deal of research has been focussed towards finding a novel means of treating the disease. Multiple attempts have been made in the past to achieve a treatment for SIRS/sepsis. Many of these attempts have led to clinical trials with varying degrees of success. With the exception of the case of activated protein C, success in animal trials has not led to success in human trials. Both anti-TNF and anti-LPS therapies have shown promise in animal models but have led to no success in a human trials (Riedemann et al. 2003). Discussed below are the few successes, and some of the many failures, that have been attempted.

1.5.1 Immunomodulatory agents:

In what has been described as a "graveyard for pharmaceutical companies", anti-inflammatory strategies in SIRS/sepsis have often appeared to show success in small uncontrolled phase 1 and phase 2 clinical trials but later fallen at the final hurdle of a large randomised clinical trial. Many believe this may be due to the use of unsuitable models in the earlier stages of the research, such as LPS infusion into animals, which is now generally accepted as inappropriate as a model for human sepsis, though conversely considered a very good model for endotoxic shock (a specific form of sepsis). A major problem in the development of a clinical trial for SIRS/sepsis is the effective inability to classify the immune status of a patient population. Some trials have found that success of an anti-inflammatory agent is dependent on the severity of the condition of the animal or patient. Hence, it is believed that an anti-inflammatory strategy may result in the immunodepression of a patient and exacerbation of the compensatory anti-inflammatory response, leading to death when an organism

overwhelms the host (Minneci et al. 2004). Long term use (i.e. approximately 7days) of moderate doses of corticosteroids were shown to be somewhat beneficial, whereas short term (1 day) use of high doses were shown to be detrimental (Annane et al. 2002).

1.5.2 Activated Protein C:

The use of recombinant activated protein C was conceptualised over a decade ago in an attempt to trigger the anticoagulant pathway in the body that is highly connected to inflammation. The conceptual foundation of this drug is well-founded as the protein C anticoagulant pathway is pivotal in SIRS/sepsis as both an inflammatory and coagulatory pathway. Activation of this pathway has been shown to reduce mortality in some animal studies.

Many antithrombotic strategies were explored for use in SIRS/sepsis. A series of 13 clinical trials, exploring the 3 major anticoagulant pathways relevant in SIRS/sepsis, were conducted. The trials concluded that activation of any these pathways significantly increased the risk of bleeding, and that only activated protein C presented any significant benefit. As such, the protein C pathway was explored more thoroughly, and the use of activated protein C was approved for limited use.

There has been some success over the past 10 years in the development and utilisation of this drug in randomised human trials. However, use of the drug remains highly controversial, with some studies suggesting a reasonable overall mortality reduction of 6.1% (Camporota & Wyncoll 2007); others suggesting an associated 55% increased risk of adverse cardiac event and 181% risk increase of stroke or encephalopathy (Jaffer et al. 2010); as well as one article, albeit a personal opinion, suggesting that the Federal Drug Administration (FDA) and European Medicines Agency (EMEA) approvals of activated protein C should be considered as an example of why the "current drug regulation system needs reforming", suggesting that activated protein C results in higher mortality in many cases of SIRS/sepsis and has been inappropriately approved as "both agencies passively accepted the reassuring interpretations of sponsored investigators and of the manufacturing company itself".

1.6 Emerging Therapeutic Techniques:

As our understanding of SIRS/sepsis has improved over the last two decades, particularly on the molecular scale, novel therapeutic techniques have been proposed. Having progressed from research into the clinical environment, activated protein C has been the most notable example of this.

1.6.1 Cytokines as a Therapeutic Target:

Since the discovery of many of the cytokines pivotal to SIRS/sepsis, such as TNF-α and the family of interleukins, many have believed the specific targeting of these molecules to be a strong conceptual foundation upon which to develop therapeutic techniques.

Initially, the therapeutic targeting of these molecules took the form of pharmaceutical drugs. Typically, the approach of these therapies was to selectively target specific cytokines, for example, anti-TNF-α therapy.

Repeated failure of such therapies under clinical trial conditions resulted in this particular approach to be benched in favour of selective adsorption of cytokines from the blood via an extracorporeal circuit.

This of method of adsorption typically involved immobilising selective antibodies onto the surface of a biomaterial for use in an extracorporeal circuit. This therapy also failed, when tested under clinical trial conditions. It was found that the removal of one specific molecule did not reduce mortality as many other cytokines were able to replace the function of one, either individually or cumulatively. Therefore, this therapy was also benched, this time, in favour of broad-spectrum, unselective adsorption utilising various forms of adsorbents; typically ceramic or polymer, for their desirably porous structures.

Since the discovery of cytokines, their targeting as a therapeutic method has not lost popularity, though no universal method of neutralisation or removal of them has been established.

1.6.2 Treatment by Technological Intervention:

The concept of using haemoadsorption as an extracorporeal technique in blood purification is one that has existed since the 1960s. Limited success and recurring clinical issues, however, resulted in the idea being shelved as a treatment in all but acute poisoning. In the last 20 years or so however, an improvement in the biocompatibility of uncoated adsorbent beads or granules has reignited interest in the use of adsorbents in extracorporeal medical therapies (Mikhalovsky 2003).

The initial extracorporeal use of adsorbents in medical therapy occurred in the 1960s. Though some successful clinical results arose, multiple clinical issues were associated with the delivery of the treatment. Haemoperfusion, at this time, was a novel extracorporeal treatment and the complications that occurred consisted of; hypotension, hypocalcaemia, hypokalaemia, hypoglycaemia, and thrombocytopenia. Not to mention the microemboli threat, that is a consequence of the release of fine particles (dust) from the adsorbent materials. Attempts were made to combat these clinical issues by coating the adsorbent materials in biocompatible membranes. This approach was successful in increasing the biocompatibility, but did so at the cost of the adsorbent efficacy (Mikhalovsky 2003). Significant reduction in the ability of coated adsorbents to adsorb high or middle weight molecules meant that haemoadsorption as an extracorporeal therapy was limited to the treatment of poisoning in only low molecular weight toxins. Paraquat poisoning, in particular, is an example of this and has been documented multiple times with reasonable success (Feinfeld et al. 2006; Lee et al. 2008; Suh et al. 2008).

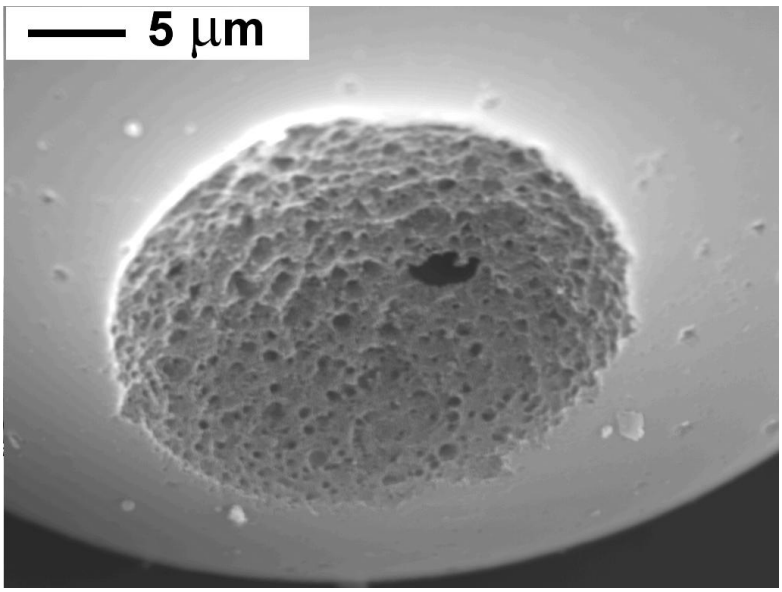

Figure 8 – Puncture in the coating of an adsorbent micro-bead, exposing the underlying porous structure.

1.6.3 Past and Emerging Technologies:

For many years, the adsorbent capability of charcoal in a medical context has been well known. There was much discussion of the use of activated charcoal adsorbents for haemoperfusion/haemofiltration in the 1970s. Some success was made in the ability of such adsorbents to remove exogenous toxins from the body. Increased survival rates in acute poisoning led to the use of charcoal haemoperfusion being encouraged in the clinical setting (Vale et al. 1975).

Figure 9 – Activated carbon adsorbents currently in clinical use.
Taken from http://www.gambro.com/

By the late 1970s the use of these charcoal haemoadsorbents was common place in exogenous intoxications. Their use was limited to this clinical setting however, as many side effects were noticed, such as; thrombocytopenia, leucopoenia, and fibrinogen loss. On top of this, there is a charcoal emboli threat. This emboli threat was overcome by coating the adsorbents in a synthetic hydrogel. Though this encapsulation of beads made them highly biocompatible, the beads had a reduced ability to act as adsorbents. In 1983, a novel technique was developed for selective removal of endotoxin/LPS by utilising polymyxin immobilised fibre (PMF). This

was later followed by the development of continuous haemofiltration (CHF) in the 1990s. Some, albeit limited, success was seen with these technologies in treating multi-organ failure in sepsis (Kodama et al. 1997). Though mortality was not reduced dramatically, the success that did occur, reignited interest in the field and a great deal of work has been carried out in the concept of blood purification for SIRS/sepsis since the turn of the century (Hanasawa 2002; Cole et al. 2002; Covic et al. 2003; Shalkham et al. 2006; Rozga 2006; Sandeman et al. 2008). Early in the 21st century, the concept of plasma separation followed by non-selective adsorption was proposed and initially became popular as a conceptual treatment for sepsis. This technique gained little long-term popularity however, as a result of the complications in the circuitry (the need for plasma separation) as well the complications associated with its implementation, such as, the activation of the complement system from blood-surface interaction and the need to use extensive anticoagulants (Stegmayr 2000). The use of selective adsorbents was proposed later as a possible method of removing specific antigens, known to be associated with SIRS/sepsis, such as endotoxin. Polymyxin B is known to inactivate endotoxin. It is, however, highly toxic to the central nervous system and the kidneys. A biomaterial was developed in which polymyxin B was immobilised onto polystyrene fibre and was safely used in a phase 2 and 3a clinical trial. Such immobilising techniques are expensive and unpopular and little success has been made when neutralising only one factor in SIRS/sepsis (Hanasawa 2002). This last decade has seen the birth of the concept of blood purification for the treatment of SIRS/sepsis by broad-spectrum removal of molecules from the blood. Coupled with this, is the increase in understanding of the molecular mechanisms behind SIRS/sepsis and therefore interventional technological treatment have been conceptualised and, to some extent, developed. Both arterio-venous and veno-venous circuits have been used previously in extracorporeal circuits. Arterio-venous circuits have many risks associated with them and are generally avoided if possible. Consequently, popularity of the veno-venous circuit has resulted in better technologies for this application and the double-lumen cannula has become widely accepted as the safer, more efficient method to achieve extracorporeal circulation (Venkataraman et al. 2003).

Attempts to attenuate the inflammatory response by different extracorporeal methods have been explored by many (De Vriese et al. 1999; Venkataraman et al. 2003; Mikhalovsky 2003; Tsuchida et al. 2006); these efforts have mostly been focused on continuous renal replacement therapies (CRRTs) such as; continuous haemodialysis (CHD), continuous high-flux dialysis (CHFD), continuous haemofiltration (CHF), high-volume haemofiltration (HVH), and plasma exchange (PE). Extracorporeal membrane oxygenation (ECMO), an artificial cardiac-respiratory support system, has also has been proposed a method by which to treat SIRS/sepsis (Fortenberry & Paden 2006). "Haemoperfusion" was proposed as a relatively novel technique in which adsorbents would be placed in an extracorporeal circuit for direct contact with whole blood. By a combination of physico-chemical attractions, such as diffusion, and porous structure, adsorbents are capable of "trapping" molecules and therefore removing them from the blood. With a good engineering process it is possible to manipulate the characteristics of these adsorbents and increase their specificity, narrowing the weight range of molecules capable of being trapped.

In the early stages of haemoperfusion research, the concept of removing the initiating insult was popular. In many studies, this was endotoxin/LPS, which is readily implicated in gram-negative sepsis. Success was made in the removal of this molecule, but the technique did not gain popularity in the clinical setting. Since this treatment would only be suitable for gram-negative sepsis (only a small proportion of all systemic inflammation cases), a more broad spectrum approach was suggested as superior.

Both polymeric and activated carbon adsorbents have been developed and tested for use in SIRS/sepsis (Song et al. 2004; Covic et al. 2003; Yushin et al. 2006; Howell et al. 2006; Sandeman et al. 2008). Both types of adsorbent appear to be appropriate for use and cytotoxicity testing has been performed and has shown that these have no significant toxic effect and are appropriate for extracorporeal use (Sandeman et al. 2005; Barnes et al. 2009; Parker & Clermont 2010; Song et al. 2004).

Researchers in both Europe and the USA, in the last ten years, have been attempting to develop medical devices capable of extracorporeal removal of cytokines. Most notable are; the CytoSorb™ system developed by a team of researchers at the

University of Pittsburgh, in Pennsylvania, USA and the MDS system developed by a team of researchers at Danube University Krems, in Krems, Austria.

Figure 10 – Image of the CytoSorb extracorporeal device.
Taken from http://medgadget.com/

The CytoSorb™ device developed in the USA has recently been approved by the EMEA for use in a large efficacy study and has been shown to reduce circulating IL-6 levels, while contributing no significant harm to the patients. The full results of the efficacy study are yet to be published.

The Microspheres-based Detoxification System (MDS) was developed by a team of researcher in Krems, Austria. (Brandl et al. 2005). Though a technically impressive system, the system is complex and requires many steps for blood to be treated before being re-introduced to a patient's circulation.

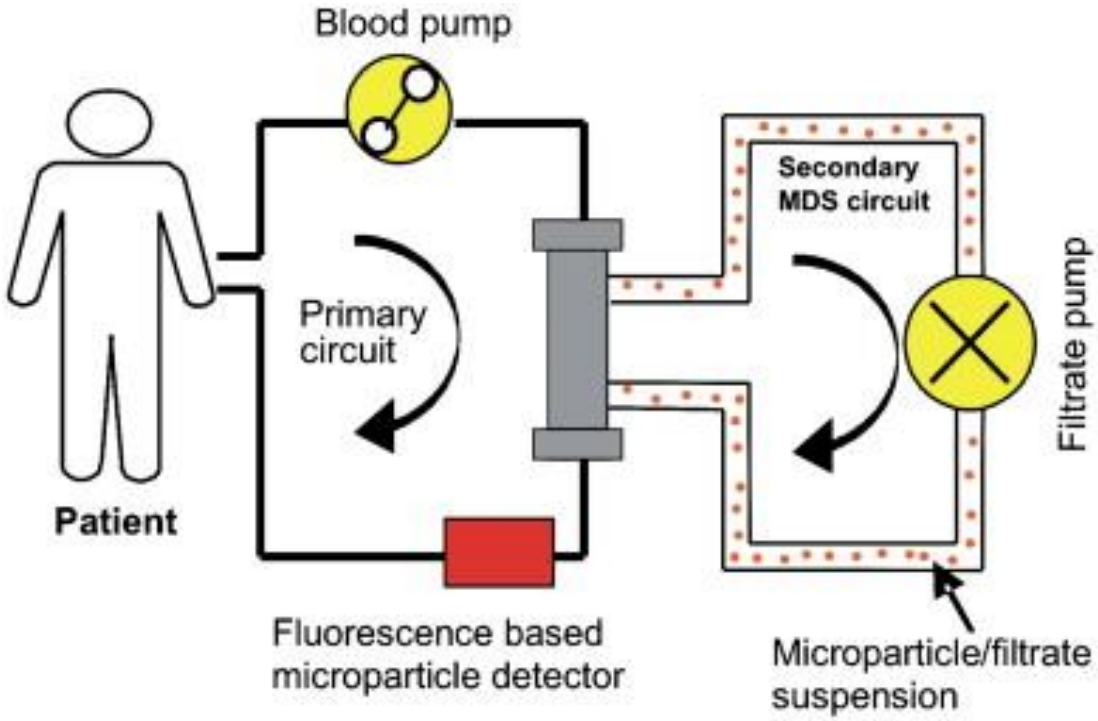

Figure 11 – Diagram of the MDS circuit.
Taken from http://www.sciencedirect.com/

The system developed utilises extremely small microspheres approximately 1-7µm. These microspheres have high adsorption capacity as a result of their high surface area and diffusion distance. However, given their size, they present a considerable embolism threat. Methods by which to minimise this issue complicated the nature of the device circuit. Direct contact with whole blood is not an option as they are approximate in size to red blood cells and therefore can easily enter the bloodstream.

Within the circuit, blood is first pumped from the patient into a primary circuit, and then separated into blood cells and plasma. This plasma is then pumped through a secondary circuit where passive diffusion of cytokines from plasma through a hollow fibre occurs (dependent on concentration gradient). Within this hollow fibre, adsorbent microspheres are suspended and achieve adsorption of cytokines. Once this treated plasma is recombined with the blood cells, this reconstituted whole blood is then passed through a magnetic trap, where any freed microspheres will be trapped before having the chance to enter the bloodstream, therefore eliminating the embolism threat (Brandl et al. 2005; Hartmann et al. 2005; Weber et al. 2007)

1.7 Thesis Objectives:

1.7.1 Objectives:

- To develop a miniaturised and portable blood pumping system for integration in a cytokine adsorption device.

- To immobilise adsorbent microbeads in a manner that allows the continuous flow of blood across these beads without risking embolism.

- To establish experimentally the efficacy of our adsorbent beads, with a view to informing the design of an integrated device.

- To integrate effectively the miniaturised portable blood pumping system with the immobilised adsorbent micro-beads.

- To show the capability of this integrated system to remove the target cytokines TNF-α, IL-6, and IL-10 to a clinically relevant degree.

1.7.2 Proposed Methodologies:

Developing a prototype of a miniaturised integrated blood pumping/adsorbent technology requires a series of engineering design phases.

Initially, an understanding of the engineering and clinical constraints relevant to blood has to be acquired. This can be achieved by referencing the extensive literature base available on the subject of blood/device interaction in the cardiovascular field. Subsequent to gaining an understanding of the biological and mechanical complexities associated with blood as a fluid, a review of available technologies, relating to the development of a portable blood pump, should be performed.

Using the knowledge gained from a literature and technology review of blood and cardiovascular devices, ideas and concepts can be developed for a blood pumping technology which fit our intended application, leading to the conceptual design of a series of potential solutions. Before developing these solutions into models or prototypes however, limitations should be considered, particularly with regard to material choice and method of manufacturing, as these affect the potential for clinical delivery of the device during this project. Based on the limitations of each concept, the chosen concepts can be developed into physical prototypes to demonstrate their performance; or computational models to predict performance.

Concurrent with the development of a portable blood pump, a method by which to effectively immobilise adsorbent microbeads in a manner which allows continuous blood flow across these beads, as well as eliminating any embolism threat should be explored, and developed.

Methods for integrating a portable blood pumping technology and an immobilised adsorbent technology should be explored in conjunction with each concept. The efficacy of the adsorbent technology utilised in this research should be explored under laboratory conditions in order to further inform the design of an integrated device. This can be achieved by initially reviewing laboratory methods carried out by other research groups working in this field.

Finally, a clinical study utilising the fully developed integrated device should be undertaken, if at all possible, in which human blood should be employed to illustrate cytokine reduction rates associated with the deployment of the technology. This is a key step in ensuring that the work is clinically grounded.

Chapter 2: Concept Development

2. Concept Development:

2.1 Blood: An engineering perspective

Blood is an essential material within the body. Vital for multiple functions, blood is responsible for heat regulation, and transport around the body of a huge range of substances, including; gases, hormones, electrolytes, or immunological molecules (including cytokines).

Extracorporeal treatment is a method by which a patient's blood is removed, treated, and replaced in a continuous flow process. Many clinical and engineering factors need to be taken into account when developing or performing such a treatment.

Composition:

Blood is a fluid connective tissue that consists of a suspension of formed elements in a liquid medium, known as plasma. Plasma is 92% water, with the remainder of the medium being made up of soluble plasma proteins, among other solutes. Overall, plasma is considered to be a Newtonian fluid (discussed later) and comprises 46-63% of whole blood. Blood cells and cell fragments constitute the formed elements of whole blood. 99.9% of these formed elements are erythrocytes/red blood cells (RBCs), which consist of a cell membrane containing a protein, haemoglobin, with no nucleus. It is these that are responsible for the transport of oxygen through the body. The remaining formed elements are; white blood cells (leukocytes), responsible for the body's immunological response to foreign bodies, and platelets, which play an important role in the formation of clots. The combination of the plasma and formed elements constitutes whole blood.

Haemodynamics: Haemolysis, Thrombosis and Platelet Activation

An understanding of the nature of blood flow (haemodynamics) is essential in the development stage of a blood pump. The presence of formed elements in blood results in it being considered a non-Newtonian fluid, though Newtonian assumptions are made in many cases, as the interaction between particles, from a mechanical perspective, is considered negligible.

Appropriate flow conditions are necessary to avoid blood damage. Flow regions of turbulence, stagnation, recirculation, high shear stress, and negative pressure can all contribute to blood damage (Mulholland et al. 2000). The most threatening results of

these flow regions are mechanical haemolysis, platelet activation and thromboembolism, though non-haemolytic mechanical damage of RBCs is increasingly considered to be of concern also (Lee et al. 2009). Shear is one the most significant mechanical factors to be considered in haemodynamics and is often discussed as either; shear stress, the measure of the pressure exerted on a fluid at a given point, or shear rate, a measure of the velocity gradient across layers of blood flow. Shear stress is often considered when discussing haemolysis, whereas shear rate is often, more appropriately, considered when discussing thrombosis as it is considered to be more indicative of the kinetics of a fluid. Shear rates and shear stress, in a Newtonian fluid, are directly proportional to one and other, and to viscosity. The presence of the formed elements in plasma results in whole blood behaving in a non-Newtonian fashion. Though for shear rates greater than the relatively low $100s^{-1}$, Newtonian assumptions are considered to be acceptable for most flow predictions to do with blood pumps.

Red Blood Cells:

When RBCs first form in the bone marrow, they have nuclei and irregular shape. As they mature, they discard their nuclei and enter the blood stream in a biconcave shape. These cells then circulate the bloodstream for 120days before changing into a spherical shape when they are haemolysed by macrophages. While travelling in the bloodstream red blood cells dramatically deform into shapes often described as parachute or bullet shapes. Fluid dynamic shear acting on RBCs causes these deformations. However, in static conditions, RBCs form into regular and uniform shapes.

Mechanical haemolysis is defined as the catastrophic release of haemoglobin into the plasma as a result of mechanical damage. In the case of blood pumps, this is usually the result of exposure to high shear stresses, causing rupture of the cell membrane. Other factors, such as thermal, chemical and osmotic, also contribute to haemolysis in a non-mechanical manner but are considered to be negligible in cases involving extracorporeal blood pumps (Apel et al. 2001). The release of haemoglobin into the plasma can initiate the activation of platelets, and subsequently, the coagulation cascade. Severe haemolysis can also lead to kidney dysfunction and ultimately multiple organ failure. The stresses required to induce haemolysis exist over a broad

spectrum, and are time-dependent. 150Pa is widely considered to be the lower end of this spectrum. Rather than the catastrophic rupture of RBCs due to high shear stress, the main mechanism of haemolysis in blood pumps is non-lethal damage to these cells, allowing haemoglobin to leak out of the membrane. This non-lethal damage does not occur as an immediate result of high shear stress but requires time for the RBCs to undergo deformation. As shear stresses increase, RBCs will progressively deform. If a RBCs undergoes shear stress less than a critical value and exposure time, it will recover to its original shape (Evans et al. 1976). Shear stresses as high as 1,000Pa can occur without causing haemolysis, provided the exposure time is only a few milliseconds (P. L. Blackshear & G. L. Blackshear 1987). Over a larger timescale, however, non-haemolytic damage has been documented at stresses as low as 30Pa. Constant shear stress upon a RBC, above this threshold value, results in an unsustainable elongation of the cell, causing it to lose its elasticity and become rigid. This rigidity increases the likelihood of full mechanical haemolysis.

Platelets:

Though flow induced blood trauma has traditionally been focused toward RBCs and haemolysis, recent work has shown that platelet activation and thrombogenicity are far more pertinent factors in blood trauma. It has been shown that RBCs are far more resistant to shear stress than platelets, and that platelet activation is considerably more clinically relevant in cardiovascular devices than haemolysis. Thus, thrombosis is increasingly recognised as the most serious clinical problem associated with such devices (Bluestein et al. 2010).

The mechanics of thrombus formation is dependent on the fluid dynamics of the blood in the surrounding area. Particularly of interest with regards to thrombus formation are shear rates. During low shear rates of $<50s^{-1}$, fibrinogen causes RBC aggregation. As shear rates increase, this fibrinogen and rouleaux formation breaks down. Flow visualisation techniques in animal models have shown that thrombus formation correlates to continuous shear rates of $<300s^{-1}$ and $>1300\text{-}1700s^{-1}$. These different regions of shear rates are believed to correspond to different biochemical mechanisms. The former, resulting from the aggregation of RBCs undergoing extremely low/insufficient shear rates, and the latter is believed to be the result of high shear rates activating platelets which go on to be deposited elsewhere, where the

shear rates are much lower, and flow more stagnant (Yamane et al. 2004). Shear rates' affect on the blood is believed to be time-dependent, as with haemolytic and non-haemolytic damage. Under physiological conditions, shear rates exist as high as $5000s^{-1}$ in arterioles, though this is only for very brief time periods.

The potential development of a thrombus within an extracorporeal circuit is an important factor that requires much attention. Development of such thrombi can result in a potentially life threatening embolism risk to the patient.

Conclusions can be clearly be drawn from past work that optimising the haemodynamics within a cardiovascular device is crucially important in its development in order to reduce the deleterious effects of blood trauma in a clinical situation. There are multiple flow characteristics that must be avoided in the development of a blood pump. Optimising haemodynamics involves avoiding these, while also achieving the fluid dynamics necessary to adequately impart kinetic energy upon a fluid. Achieving this balance is, in many cases, a problematic task.

The goal in the development of a cardiovascular device is to avoid all haemolysis or non-haemolytic damage, as well as avoiding thrombogenicity. Optimising fluid mechanics can contribute significantly to such a goal, especially in the case of haemolysis. Thrombosis, however, often requires further intervention. If haemodynamics cannot be optimised to a level at which thrombogenicity is completely eliminated, other methods must be introduced to the clinical situation. Typically, this would take the form of systemic anticoagulation of a patient, though anticoagulation would ideally be minimised or eliminated entirely, as in the case of bioprosthetic heart valves. One proposed method of achieving this is the coating of medical devices with anticoagulants. This concept has been pursued and tested during the later stages of this project and is discussed later in the thesis.

2.2 Technology Review:

2.2.1 Introduction:

The first goal of this research was to identify whether or not the overall concept is practicable i.e. can a blood pump achieve the hydraulic performance necessary, while remaining miniaturised, portable and clinically safe?; is there an adsorbent available that is capable of adsorbing cytokines?

Firstly, it must be established that these two criteria can be met. Initially, this can be completed under laboratory conditions. But later, it must perform under clinically relevant conditions in order for it to be fit for purpose.

The first method by which to define whether or not the proposal is practicable was to review similar technologies currently available for a range of blood handling scenarios. Although these may not be applied to molecular adsorption, it may be possible to base ideal designs on these successful technologies.

At the outset of this project we were confident that suitable adsorbents existed (Howell et al. 2006; Yachamaneni et al. 2010; Yushin et al. 2006) so therefore, first and foremost, our objective was to design a suitable miniaturised portable blood pump. Discussed throughout this section is a review of the various blood pumping technologies available on the medical market that we envisage as being appropriate for this application.

Further to the review of blood pumping technology, a review of the conditions under which existing adsorbents have been tested was also carried out.

There are many medical adsorbents existent on the market, though the use of such adsorbents in a flowing system has not been explored to the extent to which adsorbents have been tested in static conditions, where only quasi-static diffusive forces take significant effect. Thus, the working conditions typically necessary for clinical relevance are explored in detail in this project.

2.2.2 Blood Pumps: a review

The miniaturised, portable blood pumping component of this technology had to meet a dramatically different set of criteria to most other fluid pumps. Further considerations need to be taken when dealing with the clinical environment, and in particular, when dealing with blood. As a material, blood has complex characteristics, both mechanically and biologically. Thus, during the development of a blood pump, careful consideration must be taken when selecting certain features of the working parts. Despite these complications, there are several types of pumps that are considered to be conventional for use in the clinical setting. Of the most common are; the roller pump, the pulsatile-diaphragm pump, and the rotary pump (either centrifugal or axial flow).

Achieving adequate hydraulic performance from a pump is rarely complicated; control of such a pump, however, often proves more challenging. The interactions of cardiovascular devices and the biological systems with which they interact are strongly influenced by the fluid mechanics within the device. This can be, to some extent, modelled by predictive mathematical models i.e. computational fluid dynamics (CFD). Design and material choice is also crucial. A blood pump must avoid causing haemolysis, immune response, infection, or thrombosis. To achieve this, blood pump design must avoid features such as, sharp edges and corners in flow paths, or blood contacting seals. An appropriately biocompatible material must also be chosen for any blood contacting material. Such choices may complicate the manufacture process however, and potentially raise costs significantly.

Before development of detailed designs for blood pumps, it is important first to review the available blood pumps on the market and draw conclusions as to whether or not these are at all feasible in this particular application. Many blood pumps are available, but many of these are not suitable for use as a part of a portable technology.

Discussed below are the types of blood pumps that are conventional for clinical use. These have been considered to be a conceptual foundation upon which the development of a portable blood pump can be developed. The 2 broad categories of fluid pumps that have been used for blood use have typically been; positive-

displacement pumps i.e. roller or diaphragm pumps; and rotary pumps, i.e. axial-flow or centrifugal pumps.

Roller Pump:

The peristaltic pump (roller pump) is one of the most popular and conventional methods of pumping blood and is typically used in extracorporeal clinical procedures such as haemodialysis, cardiopulmonary bypass and extracorporeal membrane oxygenation.

The roller pump consists of relatively simple, but reliable technologies. Typically, one would consist of a motor (and gearbox) with a roller or double roller mechanism, spring loaded and attached to the shaft of the motor. The roller's radial path would be in contact with a length of tubing within a circular raceway.

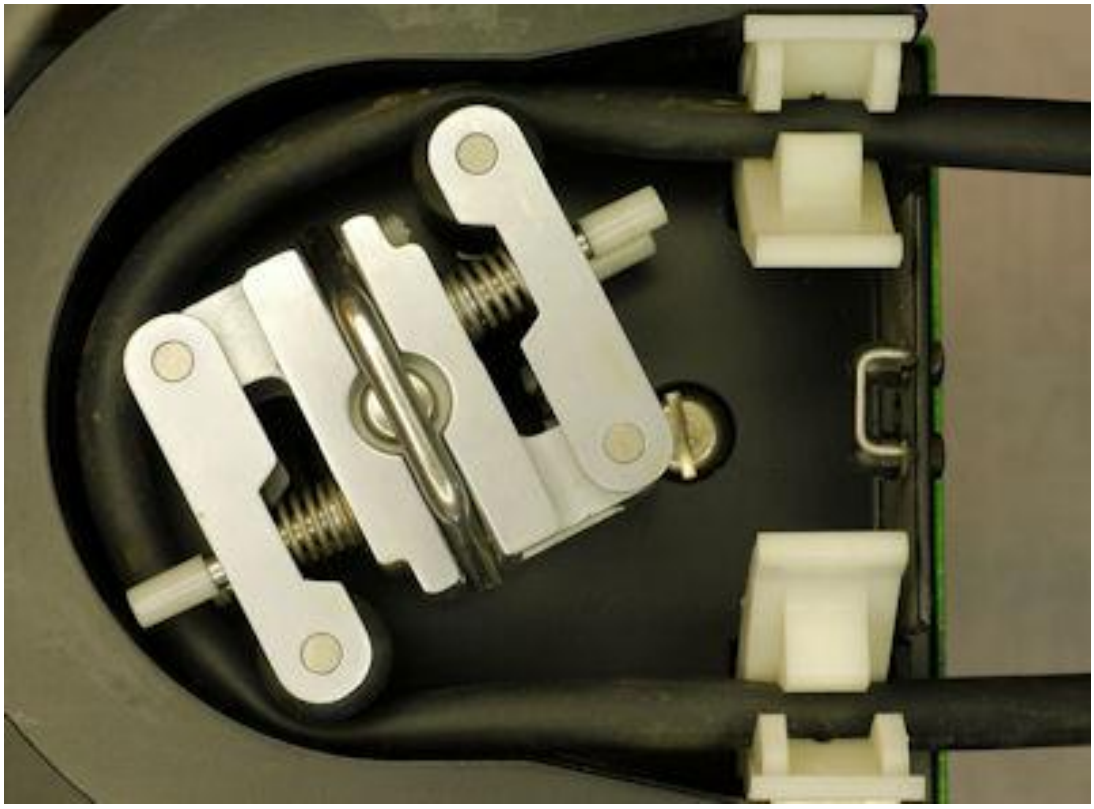

Figure 12 – Image of roller pump, utilising a Watson-Marlow roller head.
Taken from http://www.watson-marlow.com/

As the motor is powered, the roller or rollers travel around the raceway compressing the tubing. As the roller travels along the length of the tube, the blood ahead of, and behind the roller is forced along the tube by a combination of positive and negative pressure. Roller pumps are classified as single, double or multiple roller pumps.

Single roller pumps are used in some cases as they offer pulsatile flow. This technique was common in the 1950s and 60s during cardiopulmonary bypass. However, since the double roller allows for one roller to be causing full compression of the tubing at any given point in time, the double roller has become more popular as a continuous flow pump and is the most common type used for extracorporeal blood pumping. Multiple roller pumps have also been considered for use in blood pumping. However, the increase in the number of rollers has been shown to cause an increase in the level of haemolysis. The flow output of the roller pump is a product of the revolutions per minute and the volume of the tubing that is compressed during one revolution. The volume is subject to the inner diameter of the tube and the length of the radial pathway. Such blood pumps in modern use typically generate flows of up to 600ml/min (Hoenich 2007). Roller pumps have rarely been modelled computationally; however, some recent work has shown CFD of a roller pump (Mulholland et al. 2000). This work showed detailed analysis of the fluid dynamics within a roller pump. These results showed acceptable blood damage predictions, and allowed for further optimisation of the roller pump in future, in order to minimise the blood damage.

Pulsatile-Diaphragm Pump:

Until continuous flow was felt to be superior to pulsatile flow in cardiac assist, many first generation ventricular assist devices (VADs) utilised pulsatile pump technology for clinical use. The diaphragm pump is a form of positive-displacement pump in which a combination of positive and negative pressures cause suction and expulsion of a fluid through a chamber isolated by one-way valves. Initially, negative pressure would be produced by pulling a flexible wall, thus increasing the volume of the chamber and decreasing the pressure within it. This negative pressure draws fluid through the inlet valve and fills the chamber. This step is followed by the compression of the chamber by the flexible wall, reducing the volume and thus increasing the pressure, causing the inlet valve to close and the outlet valve open. Repetition of these steps results in the pulsatile impartment of kinetic energy upon the fluid.

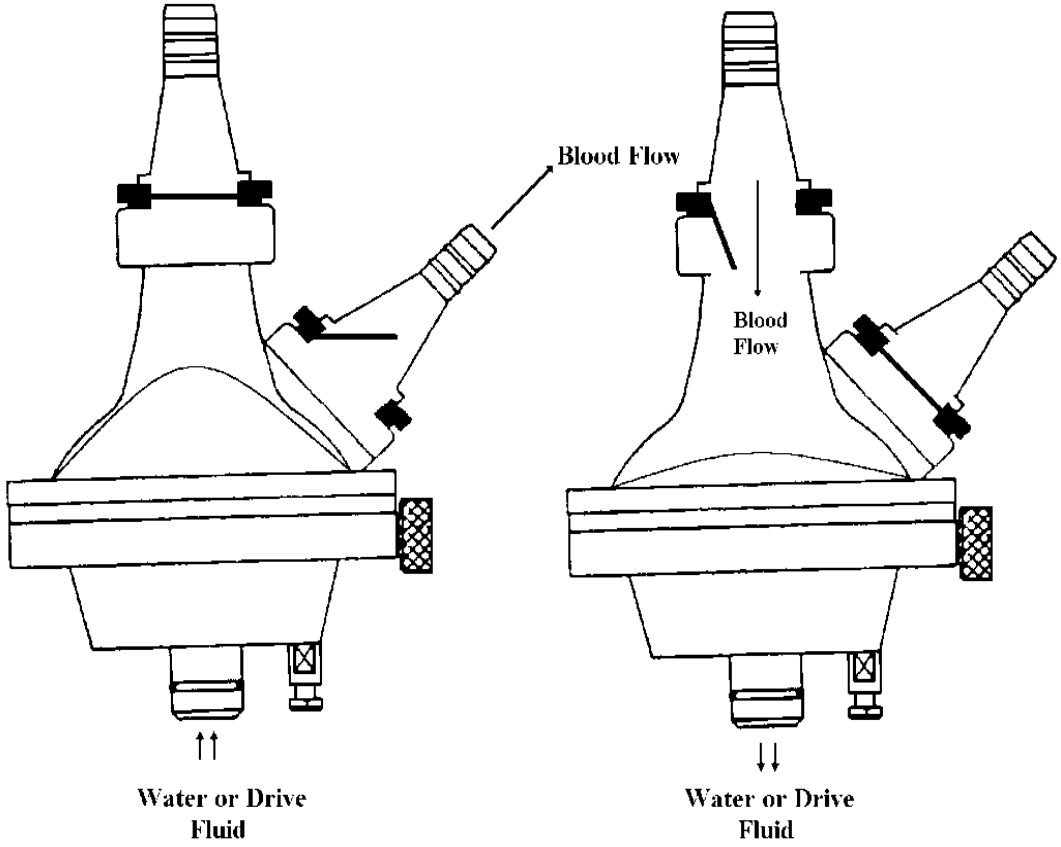

Figure 13 – Diagram of a pulsatile-diaphragm pump.

Figure 14 – Photo of a pulsatile-diaphragm pump.

Left- Fully constructed device

Right- Components of Device

Rotary Pumps:

Rotary pumps are pumps used regularly in industrial engineering. Their use in medicine is more recent and has typically been used for methods requiring higher flow rates, such as the extracorporeal method of cardiopulmonary bypass, or in ventricular assistance, *in situ*, as in the case of long-term implantable ventricular assist devices (VADs).

A typical rotary pump consists of an impeller (the working part that imparts kinetic energy on a fluid by direct rotational contact) encased within an appropriate housing. This impeller is coupled with a powering component in order to create its rotational kinetic energy. Such a powering device would likely be a motor and the method of coupling could either be; direct, with the motor's shaft protruding a bearing and/or seal and achieving direct contact with the impeller, as is the case in what are classified as "2nd generation" VADs. Alternatively, coupling could take the form of magnetic fields, as is the case in "3rd generation" VADs. The magnetic coupling method avoids the complications associated with having a bearing and/or seal in direct contact with blood. It is achieved by implanting a permanent magnet or magnets within the impeller and having it levitated in position by magnetic fields with complex feedback control mechanisms. These magnetic fields are also responsible for rotating the impeller.

In both 2nd and 3rd generation rotary blood pumps, as the impeller rotates, a pressure differential is created across it and it is this that causes blood flow.

The 2 main geometry types for rotary blood pumps, are axial flow pumps and centrifugal pumps. Each of which is discussed further below.

Axial-Flow Pump:

The axial flow impeller is the most common geometry adopted in the development of modern VADs. Their geometry is long and thin in contrast with centrifugal pumps which are relatively flat and wide. The axial flow pump is favoured in certain applications as it is smaller and consumes less power. Higher rotational speeds and lower priming volumes are expected in axial flow pumps compared with centrifugal pumps. This can lead to higher shear stress and therefore potentially more haemolysis. However, the shorter exposure time to the shear rates and foreign

materials is considered to be a positive feature, possibly compensating for these issues.

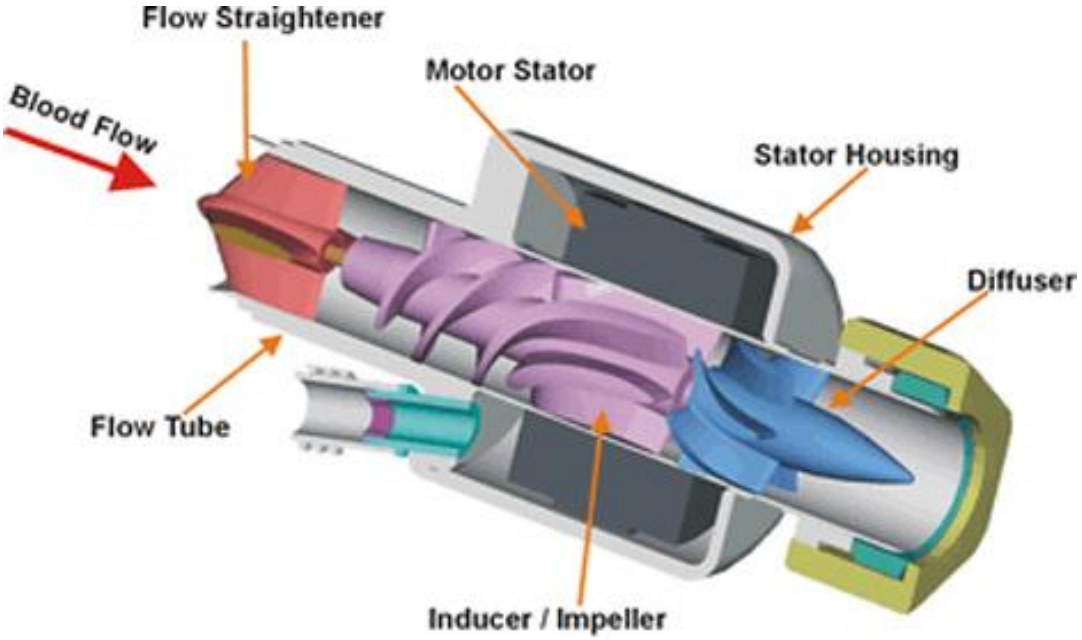

Figure 15 – Diagram of a DeBakey VAD.
Taken from http://www.sciencedirect.com

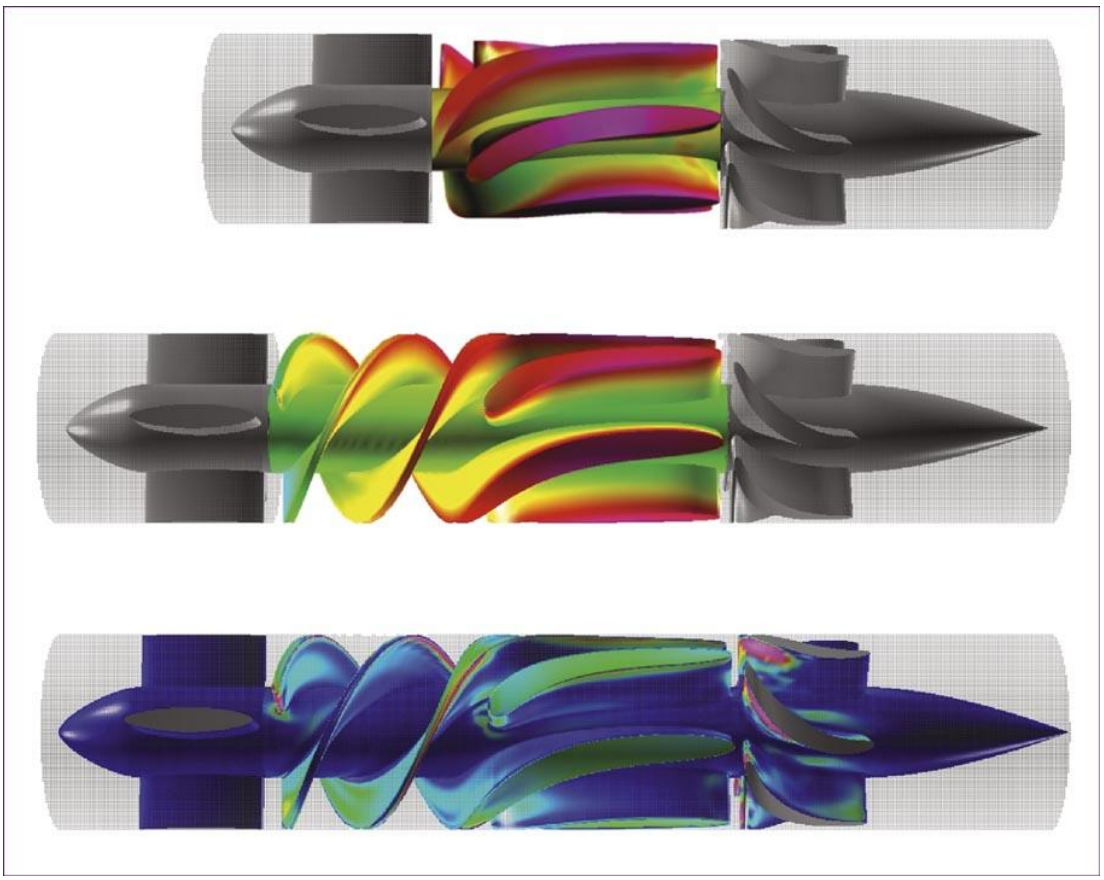

Figure 16 – Various CFD analyses of DeBakey VAD during design reiteration. Taken from http://spinoff.nasa.gov/spinoff2002/hm_3.html

As can be seen here, the axial impeller comprises a varying angle along the vanes of the impeller. This allows for there to exist an inducer region at the lower end that draws blood into the impeller, thus "inducing" flow. The pitch of the vanes on the impeller varies from this inducer pitch, through a mid-region responsible for imparting the pressure differential, to a diffuser which is responsible for converting the pressure differential into kinetic energy.

This pitch variation is done gradually so as not to introduce any dramatic changes in blood flow which may lead to trauma. Prior to the impeller, a flow straightener is placed here in order to avoid swirling flow entering the impeller region, thus perturbing the efficiency of the pump.

Radial-Flow (Centrifugal) Pump:

Use of the centrifugal blood pump is an increasingly common technique of pumping blood during cardiopulmonary bypass, yet differs little from the axial flow pump from an engineering or clinical perspective. The geometry of the impeller is the only major difference. In this type of rotary blood pump, blood enters the impeller chamber through an inlet at the top of the chamber onto the impeller itself. As this impeller is rotated, kinetic energy is imparted upon the fluid, similar to the method by which the axial-flow pump imparts energy, though in this case it is the centrifugal force that results in the fluid forming a radial fluid path, exiting the chamber tangentially.

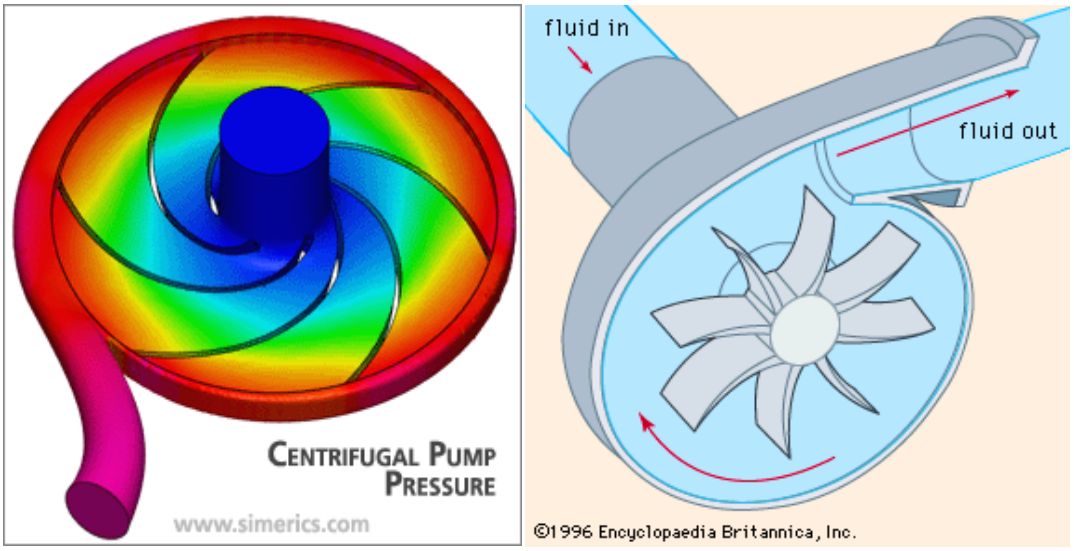

Figure 17 – Diagrams of a centrifugal pump.

Left – CFD of pressure contours in a centrifugal pump. Taken from http://www.simerics.com/gallery_centrifugal_pump

Right – Fluid path through a centrifugal pump.
Taken from http://m.eb.com/assembly/7035

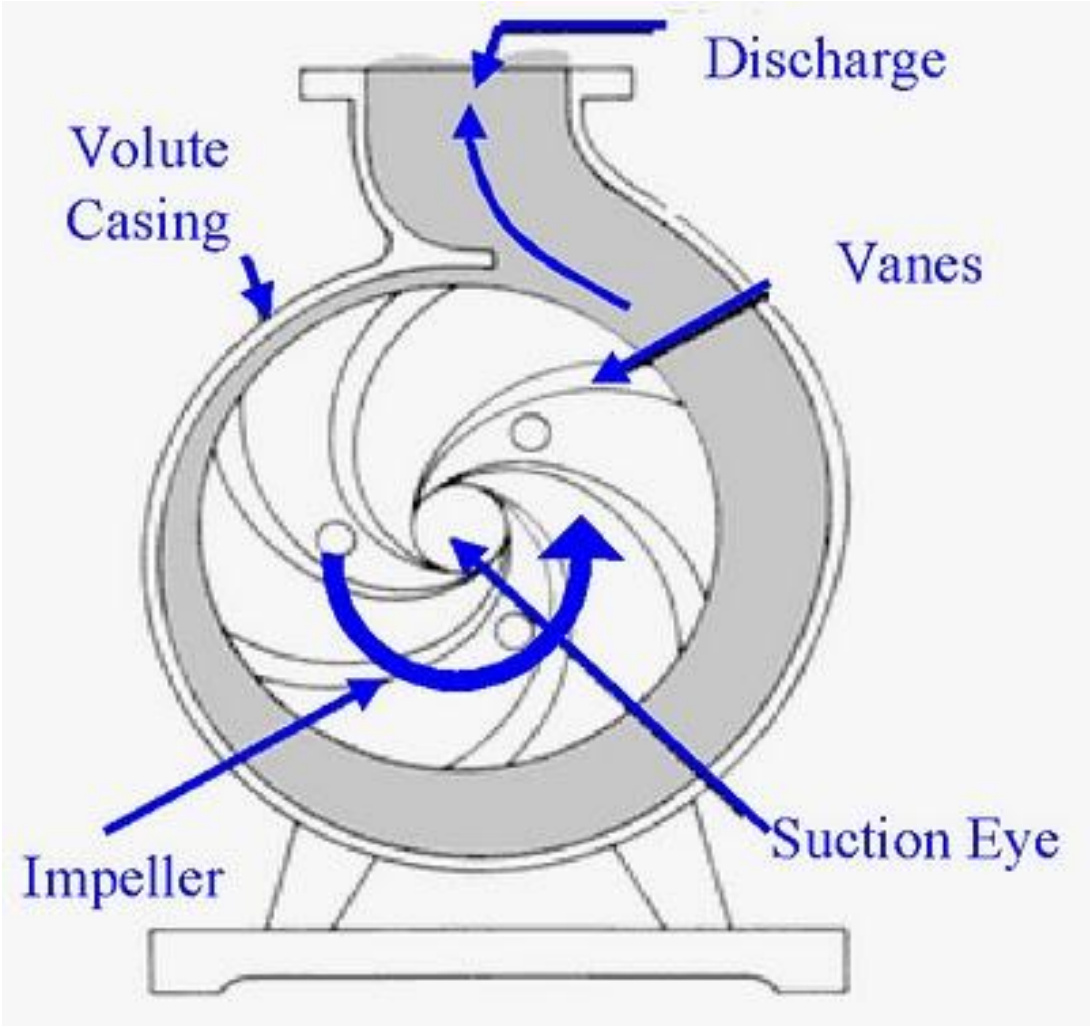

Figure 18 – Diagram labelling various components of a centrifugal pump. Taken from http://engineeringoperations.blogspot.co.uk/2011/05/draw-centrifugal-pump-diagram-and-show.html

Though rotational speeds, shear rates, and power consumption differ between axial-flow and centrifugal impellers, they differ relatively minimally. The methods of powering, controlling, coupling and developing centrifugal and axial flow pumps are essentially the same. The lower rotational speeds of the centrifugal pump and the lack of constraints with regard to the overall size of the pump usually make the centrifugal pump more popular in extracorporeal circuits, particularly cardiopulmonary bypass.

2.3 Conceptual Design:

After reviewing technologies available from within the broad medical devices field, we decided to pursue conceptual designs based on blood pumping technologies used in routine clinical practice. Uppermost in driving this selection process was that knowledge of these technologies, being on the medical market, already meet the taxing restrictions of the medical device regulatory legislation.

2.3.1 Manufacture as a Limiting Factor:

In the context of this project, manufacture is a significant limiting factor that needs to be considered. Manufacture of complex geometries, for example, that of an axial flow impeller cannot be performed by simple machining equipment available in the Bioengineering Unit, such as a lathe or milling machine. Machining such geometry typically requires a computer controlled 5-axis machining tool in which a cutting piece or laser is used to remove materials from a work piece in order to achieve the desired geometry.

An alternative to machining materials is to use one of the many methods of rapid prototyping. Rapid prototyping is a modern alternative form of manufacture, known as additive manufacture, in which a 3D object is built up from a series of 2D "slices". The term 3D printing is increasingly used to describe this process. The process is reliant almost entirely on utilising computer aided design (CAD) models of the desired geometry. Processes and materials used in 3D printing vary hugely. Highly specialised processes are able to utilise metal materials; layering molten metal upon molten metal within a vacuum (to avoid air gaps), eventually building up a solid metal 3D object. This process, impressive as it is, is extremely expensive and the technology and running costs are all too often economically unrealistic for projects that remain in a conceptual phase.

Cheaper methods of 3D printing do exist also, but as the cost reduces so does the quality of the material used and the precision of the geometry. A very common material available in 3D printing is a very brittle form of a polymer. However, though this is accessible, and comparatively cheaper than metal printing, it remains very expensive in comparison to utilising traditional subtractive manufacture (if less than 5-axes).

2.3.2 Blood Pumps: Prototype Development

During this initial stage of the project, conceptual designs were developed for 4 pump types i.e. the 2 rotary pumps (axial-flow and centrifugal), the pulsatile-diaphragm pump, and the roller pump. 3 of these pump designs, excluding the centrifugal pump, were taken to the prototype development stage. This was done in order to gain better insight and understanding of the manufacture constraints and costs associated with each design.

Rotary Pumps:

In the very early stages of the project, the conceptual design of 2 rotary pump impellers was undertaken. Computer aided design (CAD) was employed to model suitable impellers for blood pump use. The designs were based on pumps that are already in clinical use, i.e. a VAD based design was used for the axial flow pump and a cardiopulmonary bypass pump based design was used for the centrifugal pump.

The need to power the rotation of a rotary impeller in order to impart kinetic energy onto blood requires the use of a motor. The speed and torque required in order to achieve acceptable blood flow are commercially available in current motor technology. Conventional motors are capable of delivering such speed and torque, while remaining discrete enough to meet the miniaturised/portable design criteria set out at the start of this process. However, coupling this motor to the impeller proved to be challenging.

Coupling:

It is the coupling of a motor and impeller in a rotary blood pump that generally proves to be the most challenging engineering factor in pump design. Typically, the motor and impeller of a rotary blood pump would be coupled by one of the following methods; direct drive, magnetic coupling, magnetic suspension, or magnetic fluid seals.

Direct drive powering of an impeller in a rotary blood pump comprises a simple mechanism in which the shaft of a motor is connected to the impeller directly, protruding a bearing and shaft seal. This bearing and shaft seal acts as a barrier between the blood and any electro-mechanical working parts. Direct drive is a coupling technique that can be expected in the majority of industrial uses of rotary

pumps, due to its simplicity and the fact that high efficiency is characteristic of such a coupling method (Mitamura et al. 2011). Sadly, in a clinical situation, issues are raised that are not experienced in many industrial processes i.e. the shaft seal is the most common site of blood trauma (both thrombosis and haemolysis). Conventional bearings are used in some blood pumps. However, they have issues with the corrosion of metals within the mechanical bearing, leading to highly toxic microparticles entering the blood stream and posing the risk of both thromboembolism and toxic injury to patients (Hoshi et al. 2005).

The magnetic coupling technique of powering an impeller involves implanting a permanent magnet within the impeller. A further magnet is then placed underneath the impeller, outside the housing. As the outer magnet is spun by a motor, the inner magnet follows, thus rotating the impeller. This technique has been proposed as a method by which to avoid blood trauma and has been shown to improve clinical outcomes. Sadly, engineering this coupling method has resulted in it not being so popular. The distance between the two magnets must be minimised, as the strength of the magnetic field is inversely proportional to the square of the distance between the magnets. So minimising this gap to the lowest possible distance is essential in order to reduce the size of magnet necessary. Finding a material that is both biocompatible enough to be used for direct contact with blood, while also remaining strong enough to be manufactured to extremely thin sections can prove expensive. Finding a lubricant to be placed between the inner magnet and the chamber wall is also necessary and since this will not only be in contact with blood (therefore needing to be biocompatible) it is also in a highly dynamic fluid region, therefore at risk of being dragged into the fluid stream. Additional to this, is the threat of magnetic decoupling, which remains a common concern in magnetically coupled systems. This is when the outer magnet rotates and inner magnet fails to follow. This lag results in loss of efficiency and therefore, in the clinical setting, potentially life-threatening scenarios.

Magnetic levitation (maglev) is an engineering technique, developed in the early to mid 20^{th} century, initially proposed as a concept by which to speed up transport. The method was popularised in the 1980s during the development of maglev trains. The technique was redeveloped for use in 3^{rd} generation rotary blood pumps. It utilises

modern control electronics in order to levitate an impeller in position, while also imparting the necessary rotational energy upon it. This is achieved by integrating the existing magnetic levitation technology with brushless DC motor technology. Such blood pumps have a permanent magnet, or series of permanent magnets, implanted within an impeller, and a series of well controlled electromagnets outside the housing of the impeller, stepped in a manner in which to levitate and spin the impeller. This maglev technology has been shown to reduce the haemolysis rates within blood pumps. And, despite the expensive and complicated control mechanisms, this levitation method eliminates many of the engineering constraints, such as abrasive wear, short life span, and manufacturing difficulties seen in the development of 2nd generation blood pumps. This is largely due to the removal of the need for bearings and fluid seals. Maglev impellers are thus considered to be ideal systems and are common in long-term implantable ventricular assists devices. Unfortunately, magnetically levitating impellers are extremely expensive in comparison to 2nd generation rotary pumps that contain more conventional bearings. This is in part due to the complex electronic control required, but also an increase in manufacturing costs, as magnetic materials must be implanted in specific locations with the impeller itself.

The use of magnetic fluids as bearings/seals in rotary blood pumps is a more novel approach to combating the complications associated with coupling motors and impellers in such pumps. Though chronologically this concept was proposed subsequent to the existence of 3rd generation rotary blood pumps, the use of a magnetic fluid seal as a bearing at the motor-impeller interface is seen to be a promising approach to coupling a 2nd generation style rotary blood pump (Mitamura et al. 2008). This method involves replacing a conventional bearing with a magnetic alternative i.e. a solid magnetic ring, with the inner radius coated with a magnetic fluid. This method provides many advantages over other methods. The fluid inner radius allows for the motor shaft to protrude the bearing easily. The magnetic fluid also provides many desirable properties as a bearing, particularly for this medical application. These properties were; biocompatibility, the ability to remain in a fixed position, and ideal tribology. Early use of magnetic fluids as bearings appeared to suggest that such bearings presented decreased performance or failure when

submerged in liquid. However, a recent study has shown that when a small seal is introduced to protect the magnetic fluid, long-term performance while submerged is dramatically increased and may be appropriate for clinical use (Mitamura et al. 2011).

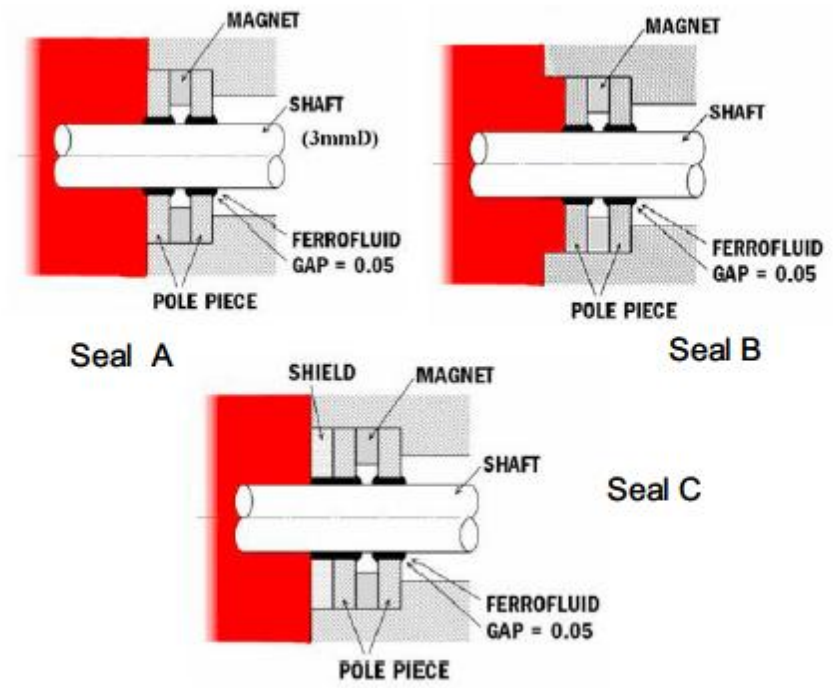

Figure 19 – Diagram showing the placement of a magnetic fluid seal as a bearing. Taken from the Journal of Artificial Organs.

Radial-Flow (Centrifugal) Pump:

An initial design of a rotary blood pump was developed in the software package ProEngineer. This design was of a radial-flow (centrifugal) impeller based design. CAD images of this design are shown below.

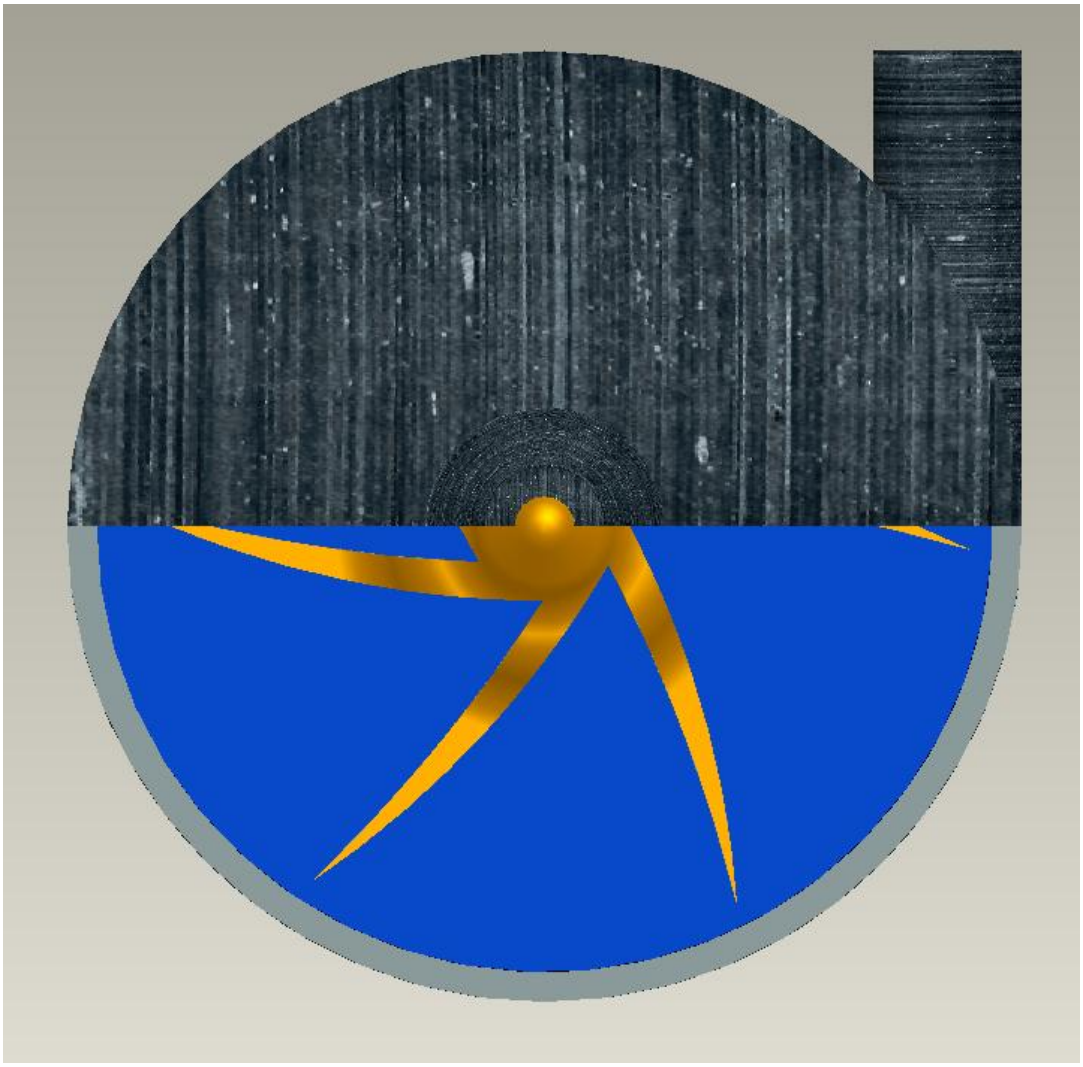

Figure 20 - Top view of a centrifugal impeller, designed in ProEngineer.

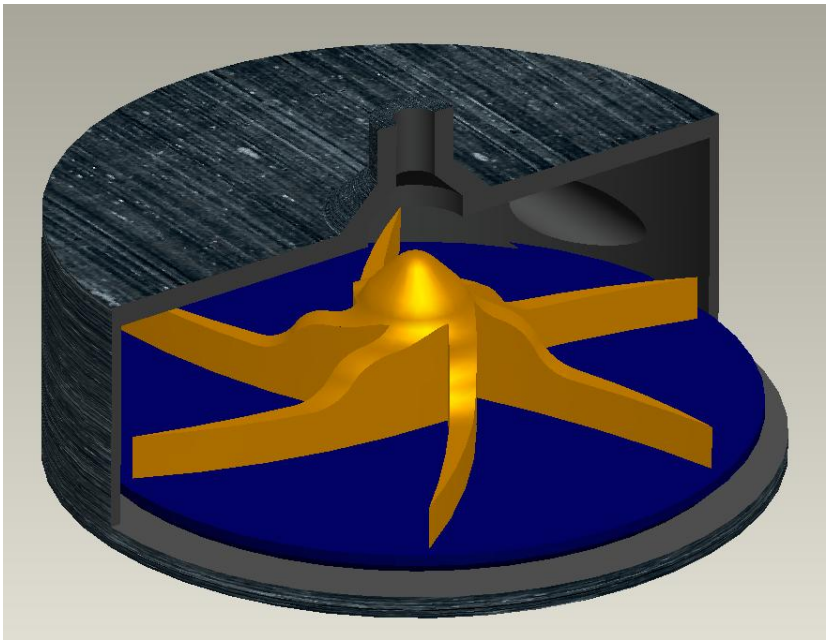

Figure 21 - Angled view of a centrifugal pump, displaying half-cut housing

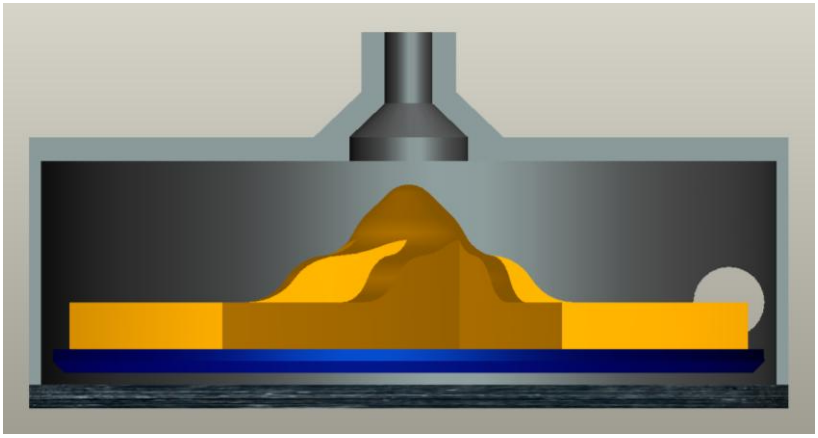

Figure 22 - Side view of a centrifugal pump, displaying half-cut housing.

Though this design had promise and some desirable features, it was not initially pursued, and in the early stages was not taken into a prototyping phase in favour of an axial-flow pump, as it was felt that there was little sense in developing 2 rotary pumps as both would need to combat the same engineering constraints at a later time and the axial-flow pump, appeared otherwise more suitable for use in that it is more discrete and adaptable to an overall integrated device housing, as will be seen later.

Axial Flow Pump:

Our industrial collaborators assisted considerably in rapid prototype development of an axial flow impeller, given the complex geometry of the impeller it was concluded that the manufacturing technique would need to take the form of machining with a 5-axis machining tool. This method is highly technical, expensive and time-consuming. It was thus decided that a different method of manufacture would be necessary for the early stage prototyping. Rapid prototyping by the laser polymerisation technique was opted for as Brightwake Ltd. was able to facilitate this. Fast and relatively low cost rapid manufacture of an axial impeller was achieved. The materials used in this process are appropriate for conceptual design and for laboratory and workshop experimentation but are not appropriate for end use.

The powering/coupling of the axial flow pump became the largest issue needing pursued. No driving/coupling method is both ideal and affordable. Two of the potential methods were pursued into a design phase. These were maglev and direct drive.

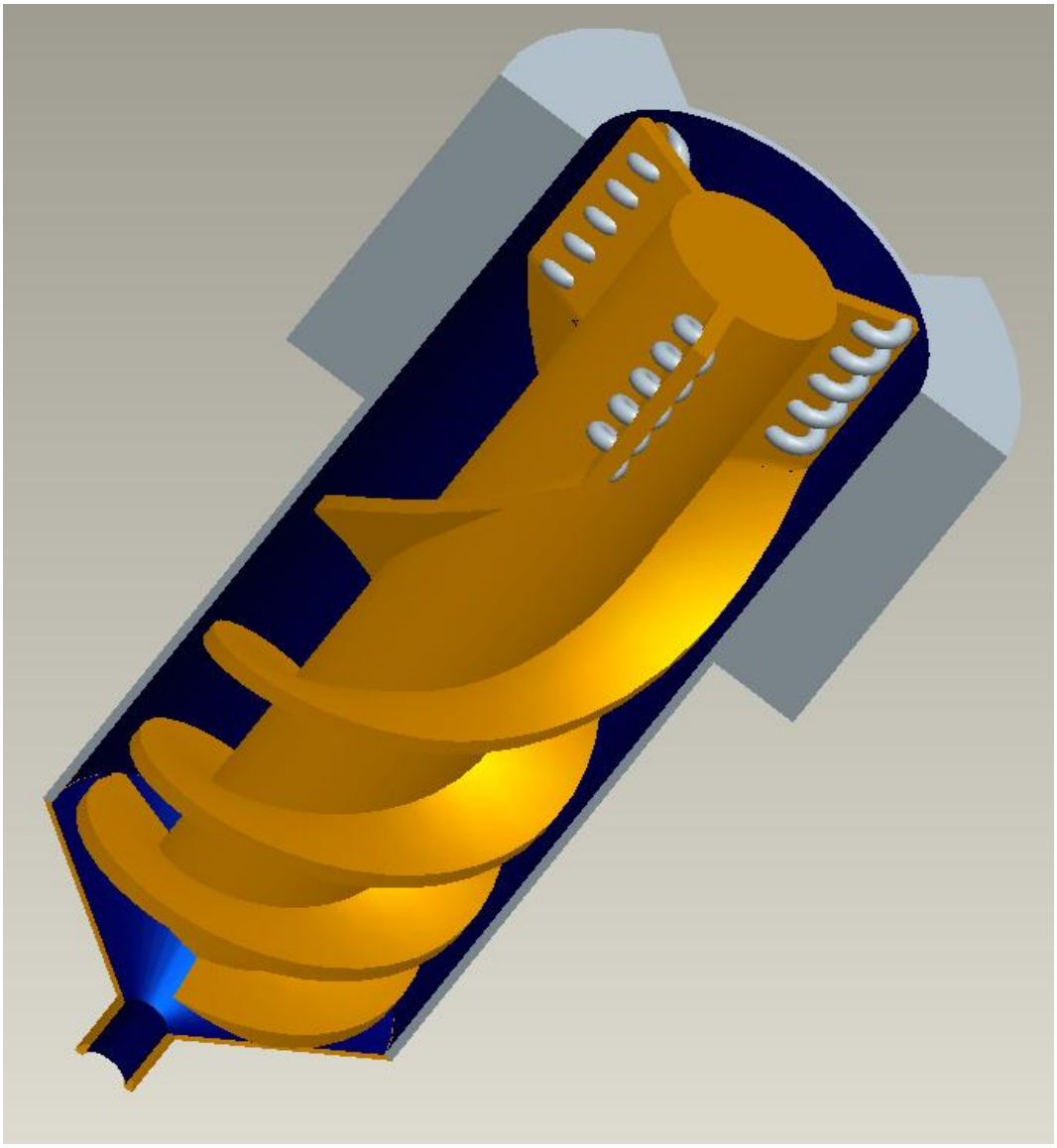

Figure 23 - Angled view of an axial flow impeller, utilising MagLev, displayed in half-cut housing.

Shown above is the design of an axial impeller coupled by magnetic levitation technique. One method of magnetically levitating and driving an impeller is by implanting conducting coils into the impeller blades and placing electromagnets outside the housing the axial pump. These electromagnets can then be powered and controlled by electronic circuits to produce motion by the same method in which an induction motor is powered and driven i.e. the impeller effectively becomes the rotor of an induction motor. Shown above is a CAD image of such coupling. In a prototype, the conducting coils would not protrude the blade as above. The coils have only been exposed in this CAD in order to demonstrate their placement.

Figure 24 - Side view of an axial flow pump, utilising direct drive, displaying half-cut housing.

Shown above here is the much simpler method of coupling that is direct drive. In this concept, a flat motor is used to stabilise, power and spin the impeller. Given that the motor is directly below the impeller, another form of inlet is necessary. Thus, multiple inlets have been drilled around the housing. A channel can easily be machined to allow blood to enter through these inlets. The major complicating factor associated with this design is that in order to isolate the motor from the blood a bearing must be used. This bearing is then in contact with blood and even when using biocompatible materials to produce a bearing, corrosive wear will lead to the release of microparticles, which in turn lead to toxic injury to a patient.

Pulsatile-Diaphragm Pump:

The development of a pulsatile-diaphragm pump was also considered and pursued during this project. This pursuit was, however, short-lived. A combination of a machined metal chamber, a polymer wall, two one-way valves, tubing and a powerful solenoid were used and a portable pulsatile pump was eventually achieved.

Initially, a metal chamber was machined in the Bioengineering Unit with an O-ring to provide a water tight seal. Shown below is a series of CAD drawing of this concept.

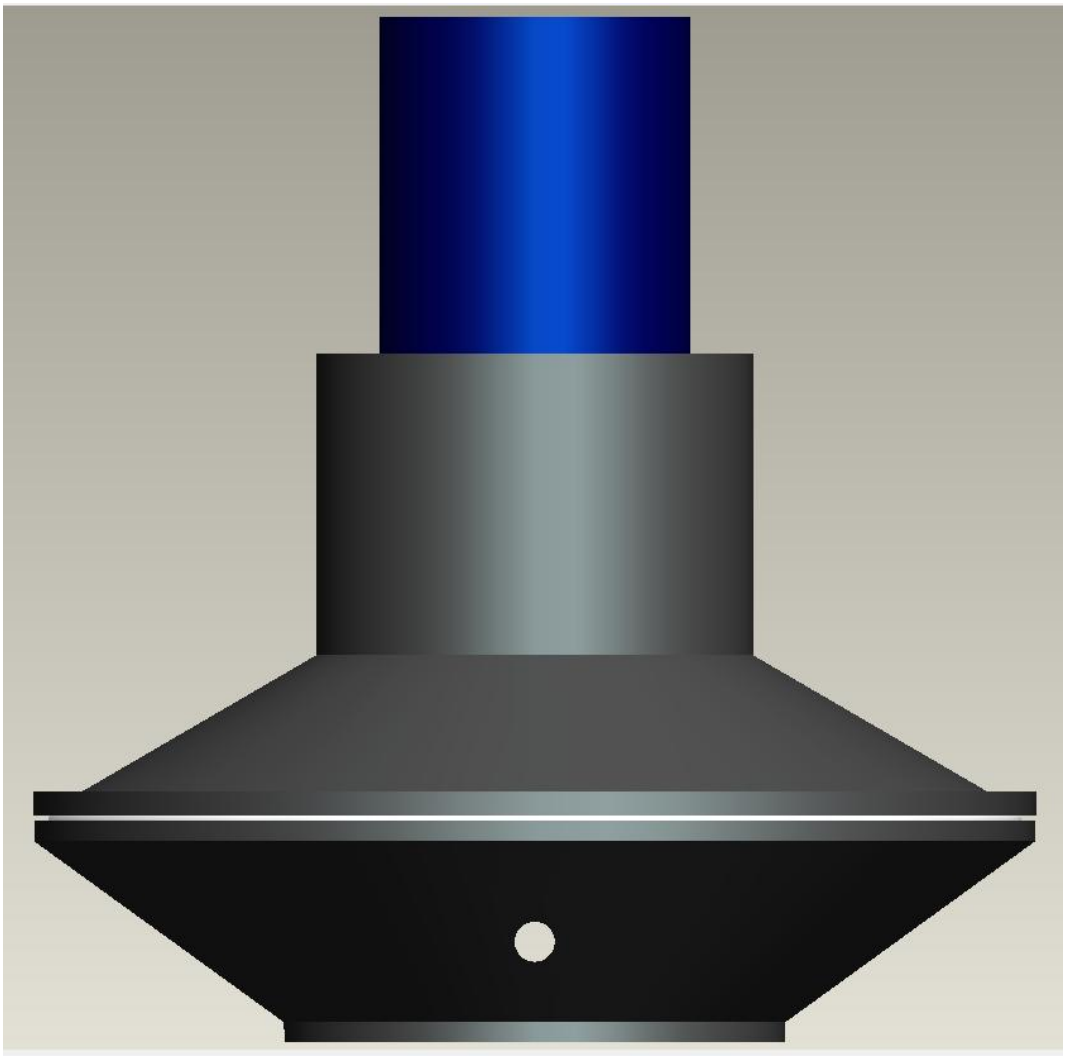

Figure 25 - Side view of a pulsatile-diaphragm pump, displaying full housing

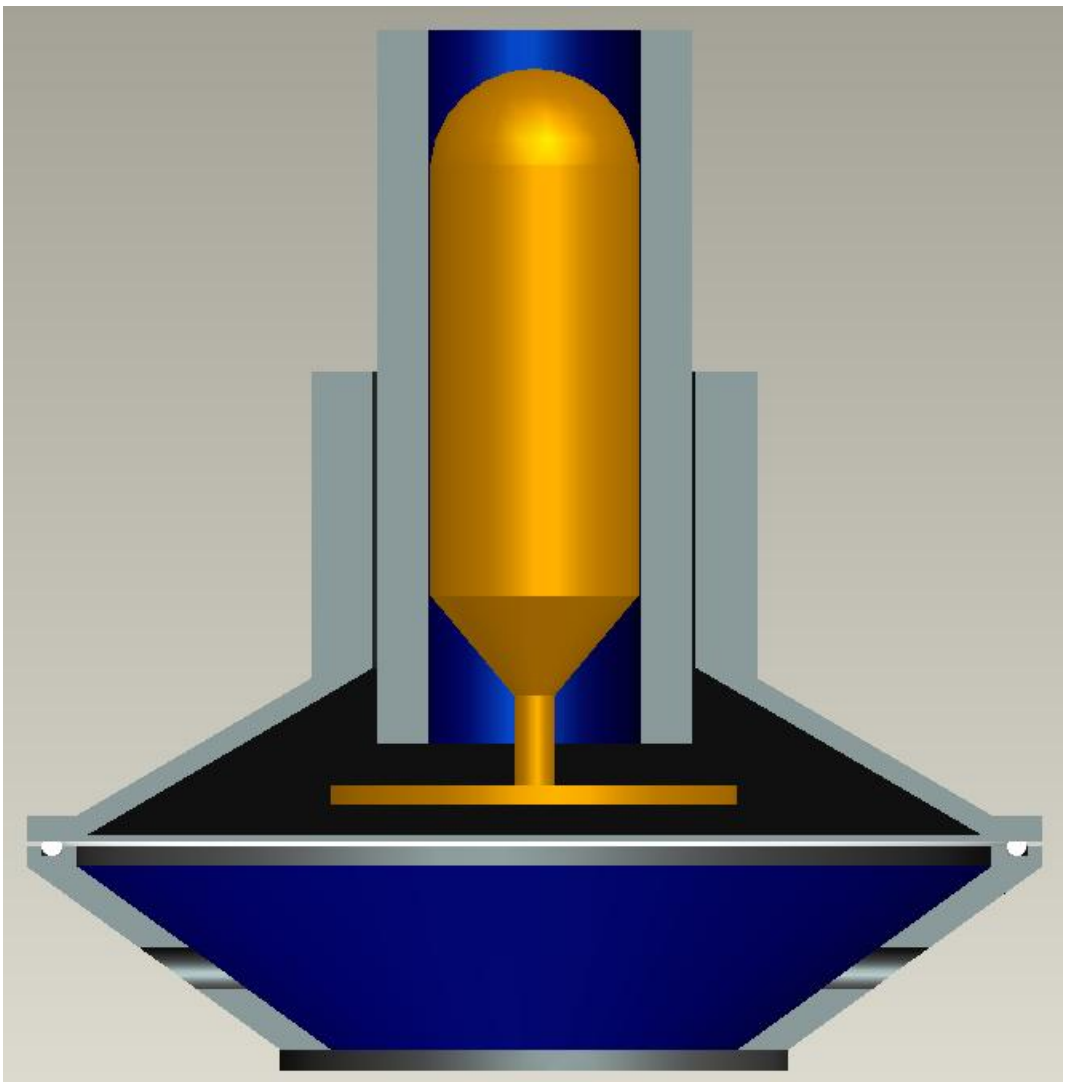

Figure 26 - Side view of pulsatile-diaphragm pump, displaying half-cut housing

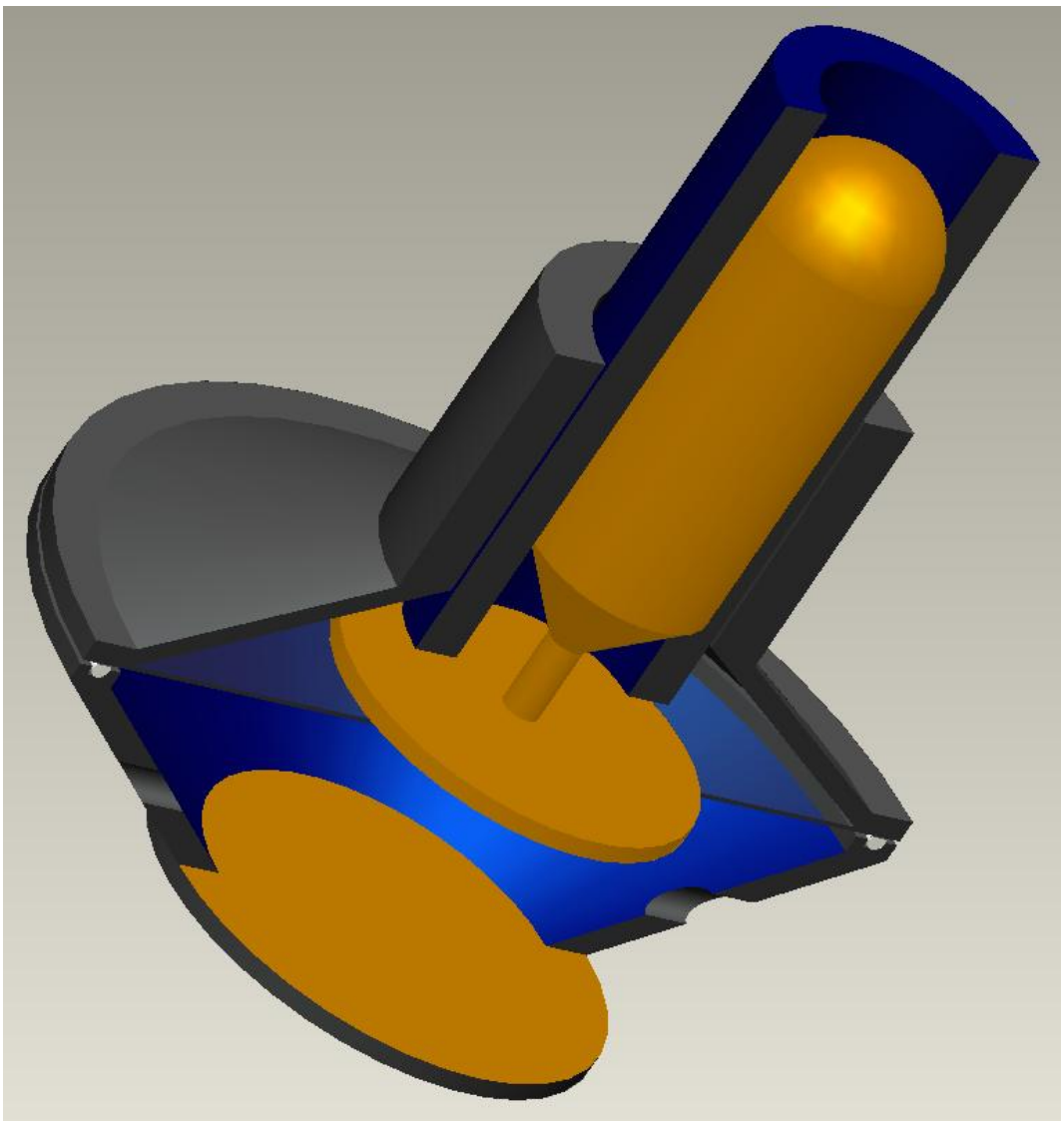

Figure 27 - Angled view of pulsatile-diaphragm pump, displaying half-cut housing, displaying polymer membrane and solenoid working part

A chamber was produced in two parts, a lower part and an upper part. Both of these parts include a flange that facilitates the placement of an o-ring. A rubber o-ring and the flexible polymer are placed between the two parts and the two flange pieces can be compressed by a number of methods, for example, clamping or with nuts and bolts. Inlet and outlet holes were drilled into the chamber and one-way valves were fitted to each of these holes. With the flexible polymer fixed to the centre of the chamber and one-valves fitted to the inlet and outlet holes, the fluid tight chamber was capable of passing fluid in one direction, through the inlet, the chamber and outlet without complication.

Adding working parts to this component in order for it to achieve pulsatile fluid flow caused considerable difficulty. A solenoid (with a square wave electronic control output) was the only appropriate method by which to achieve regular, powerful compression of the chamber, and therefore reliable flow rates.

This concept was quickly abandoned as very large, powerful solenoids were required to compress the chamber fast enough to achieve flow rates of 200ml/min. As well as this, the industrial collaborators of this project were uncomfortable pursuing a technology reliant on electronically controlled solenoids, as they felt that these are unreliable components that are difficult to couple, manufacture and control.

Roller Pump:

In seeking a pumping solution for our design, we considered the roller mechanism as a last resort. The reasoning behind this was that roller pumps remain the most widely used blood pumping solution in medical practice and that their deployment would require the least development. In this regard, the roller pump might be considered to be a fallback device should more novel, high tech solutions prove problematic.

Development of a prototype proved to be quick, reliable, easy to control, discrete, reasonable in cost, and easy to maintain (potentially with newer, more efficient parts available on the market with time).

The working parts of the roller pump consist of a motor, gearbox and electronic controller. In order to pump the blood effectively, the working parts had to meet certain criteria. These criteria were relatively high torque, low speed, and lowest possible profile.

Motors:

There are many types of motors commercially available, although for this application, considerations were limited to only DC motors, as the device must be powered by a battery source in order to be portable. DC motors can be categorised into brushed DC motors and brushless DC motors. Both convert electrical energy into mechanical energy via the electromagnetic force. However, they do so by different mechanisms. Brushed DC motors have fixed permanent magnets implanted in or placed outside the housing with an electromagnetic rotor. They are typically low cost and simple to control. They do, however, have a low lifespan and require high maintenance as a result of their brushes wearing.

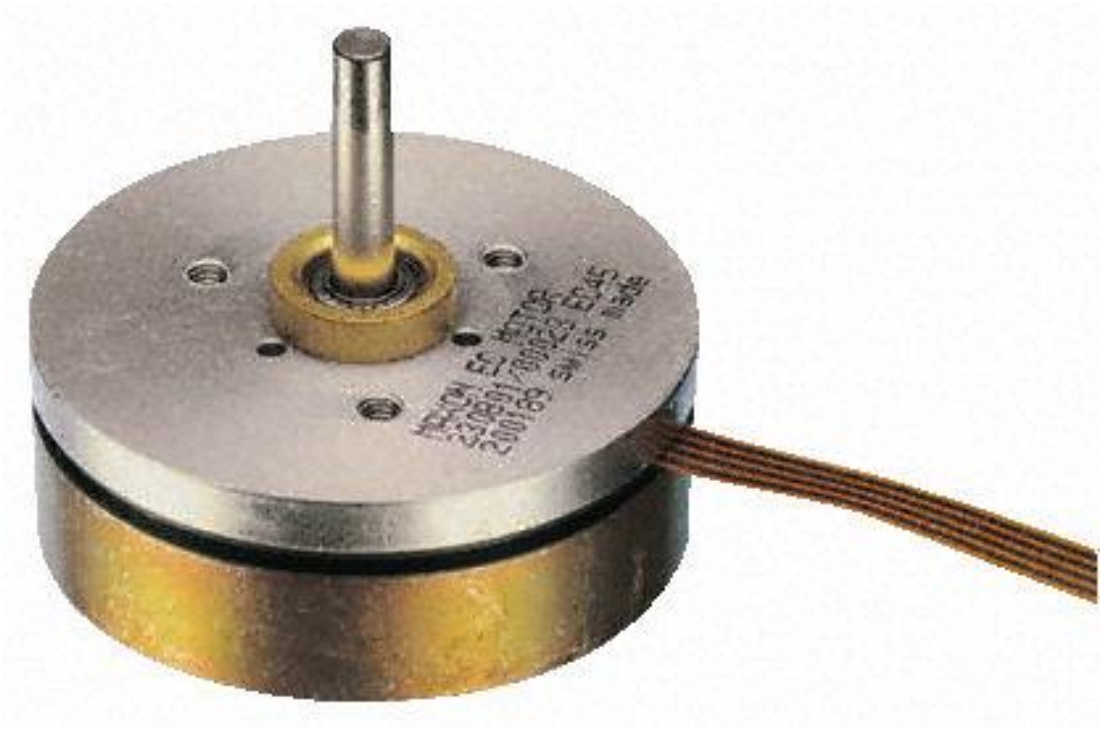

Figure 28 - Image of flat brushless DC motor
Taken from http://www.maxonmotor.co.uk

Brushless DC motors, on the other hand, have permanent magnet rotors with fixed electromagnets in the housing. These electromagnets are stepped in sequence to force the permanent magnet rotor to complete revolutions. To achieve the stepping of the electromagnets with appropriate timing, an electronic controller is required. This also allows for easy torque and speed control. These factors result in brushless DC motors being higher cost and more complicated to control. They do, however, have a longer lifespan, higher efficiency, require no maintenance and often allow for smaller dimensions.

To achieve the required speed and torque as stated above, a gearbox is often needed to lower the speed and increase the torque of the motor. The reason that this is necessary is that the method of building motors typically results in them being high speed and low torque. A gearbox is able to adjust the output of a motor by a set ratio, for example, a ratio of 100:1 would both reduce the speed, and increase the torque of the motor, by a factor of 100. It is very common to assemble a motor and gearbox together in many areas of engineering and is considered the standard approach by which to achieve desired speed and torque.

Tubing:

The material properties required of tubing are that it must be elastomeric and biocompatible. The most commonly used tubing materials are silicone rubber, latex rubber, and polyvinylchloride (PVC). All of these materials are considered to be safe for use in clinical applications and are in common usage, though PVC is increasingly controversial as a result of recent research suggesting that the plasticiser in PVC leaches out of the PVC and is highly toxic; some efforts have made to moderate this migration of plasticiser but its use remains controversial despite some success in these efforts (Hoenich 2007; Gourlay et al. 2010).

Achieving the correct level of compression of the tubing is an issue with roller pumps that needs to be dealt with carefully. Increasing the degree of compression of the tubing dramatically reduces the lifetime of the tubing and also increases the level of haemolysis. This increase in haemolysis is most prominent with latex rubber, and least with silicone rubber (Bernstein et al. 1967). Decreasing the compression of the tubing, on the other hand, reduces the efficiency of the pump and can lead to insufficient and unpredictable flow rates.

Occlusion is a measure of how much the tubing is compressed. This is expressed as a percentage of twice the tubing wall thickness. For example, when 2mm tubing is compressed shut, the collective wall thickness is 4mm; 20% occlusion refers to compression of such tubing to a thickness of 3.2mm i.e. 80% of its diameter when uncompressed. Between 10 and 20% occlusion is considered typical; however this may be increased if softer materials are in use. Occlusion of the tubing can also cause the release of microparticles. This behaviour can be seen considerably in both PVC and silicone rubber (Uretzky et al. 1987). Two methods are in common usage to achieve controlled levels of occlusion during pumping. These are the fixed occlusion roller and the spring loaded roller. The names of these mechanisms are descriptive and are, respectively; a mechanism that has the roller fixed at a set distance from the shaft with a constant gap between the roller and the raceway, resulting in constant compression, and a roller mechanism that is set at a given distance from the shaft with a spring. The spring loaded rollers are considered to be a more elaborate design and widely considered to be the 2nd generation of rollers for peristaltic pumps. The reason for this is that the spring loaded rollers allow for much more variation in

many of the factors, such as variation in the wall thickness or pressure with the system.

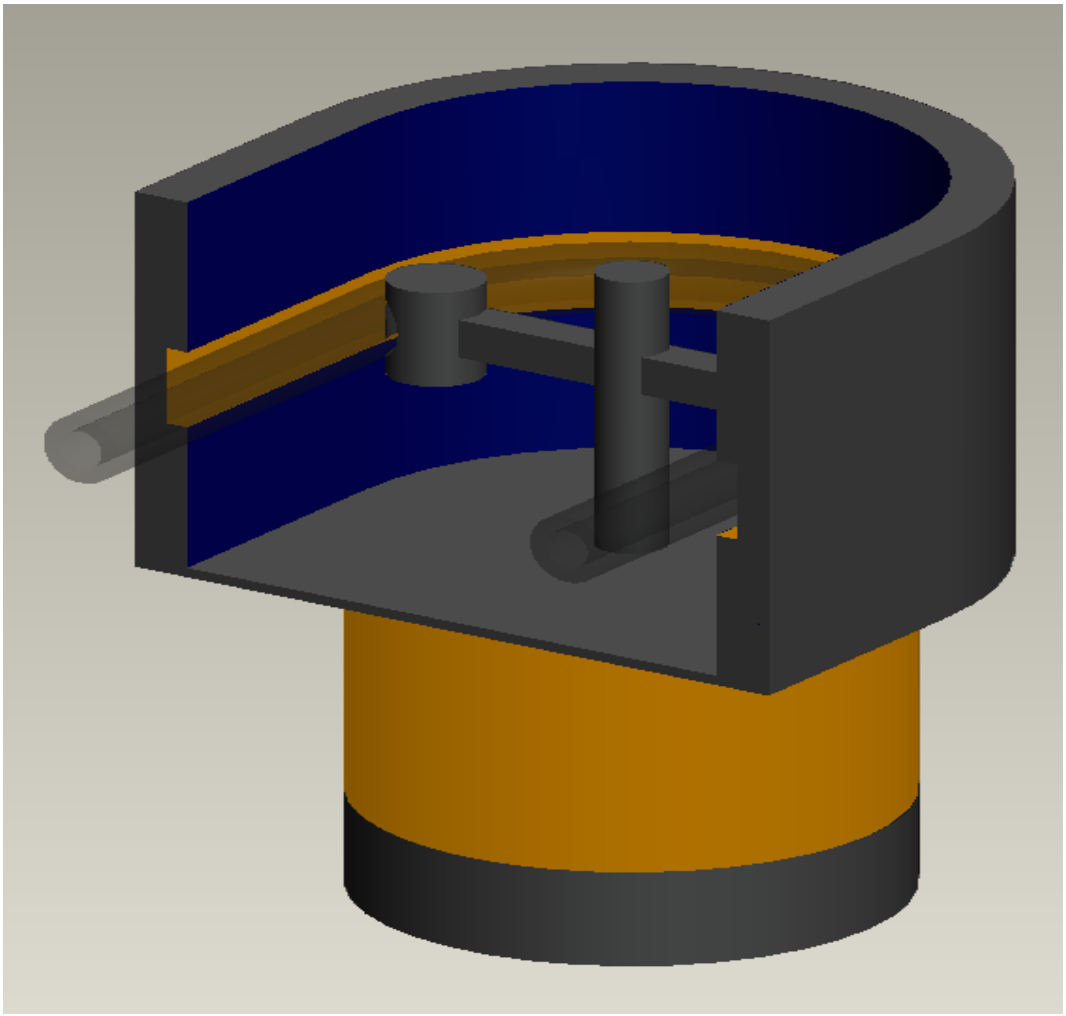

Figure 29 - Angled view of a roller pump.
Displaying motor, gearbox, raceway, tubing and spring loaded double roller

2.3.3 Modelling Blood Pumps: design and performance

Computational fluid dynamics (CFD) is a useful tool in predictive modelling that has been employed in many engineering disciplines since computers have had the processing power to facilitate it. The design methodology involved in rotary blood pumps tends to employ CFD as an optimisation tool. A great deal of work has been carried out in this area. CFD modelling of rotary blood pumps is useful in the design optimisation stage. The main purpose of this is to aid in the prediction of blood damage associated with different geometries, for example, by predicting shear stress under flow conditions. Despite the considerable progress that has been made since the adoption of CFD as a modelling tool, the process remains time consuming and still requires experimental validation of the results. However, given the cost of pursuing a prototype for a rotary pump, it was decided that CFD analysis would be necessary in order to validate the suitability of a rotary pump configuration.

In order to perform CFD analysis on a prospective design, a computer model must first be built using a computer aided design (CAD) package, such as ProEngineer. This model must then be saved in a particular format and exported to another computational package to be "meshed". Mesh generation is a critical step during CFD in which a geometric object is split into numerous discrete "cells". The reasoning behind this is that the governing equations of fluid flow are best solved in simple cases. Thus, a complex geometry must be split into multiple simple geometries and the partial differential equations are solved for each of these discrete cells. The number of cells in relatively complex geometries can easily reach over 1million, therefore considerable computing power is required to resolve complex geometries.

Axial-Flow Pump analysis:

Methods:

A model of an axial flow pump impeller was built in ProEngineer, using a left-handed variable angle helical sweep protrusion along the axis of the impeller. The relatively high pitch angle at the bottom of the impeller induces flow into the impeller, and as the pitch angle decreases along the axis, the rotational velocity of the fluid is gradually converted into axial velocity.

Upon leaving the impeller region, the fluid passes through a diffuser which has a right-handed variable angle. This diffuser converts any remaining rotational kinetic energy into axial kinetic energy.

The blood pump technology of this axial flow impeller was made with dimensions of 50mm in length and 19mm in outer diameter, with a 20mm inner diameter of the impeller housing, which provided 0.5mm clearance between the blades and the housing.

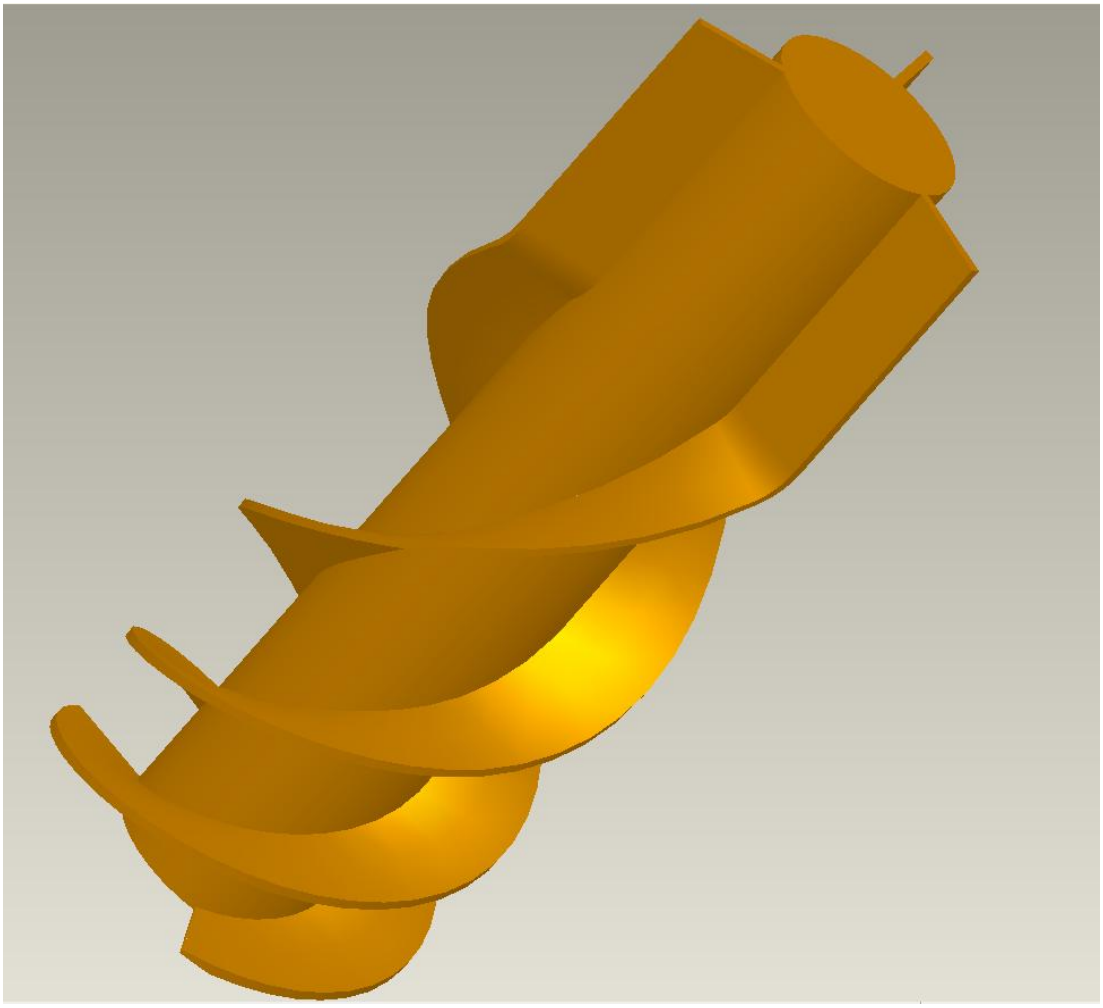

Figure 30 - Angled view of axial flow impeller created in ProEngineer.

After building the impeller and diffuser geometries in ProEngineer, the models were saved as step files and exported to the meshing package Gambit. Further, relatively uncomplicated, geometries were created in Gambit, such as a flow straightener prior to the impeller and an impeller casing. These components comprise the full model for an axial-flow pump.

In Gambit, the various volumes were united, or subtracted, as necessary in order to create a single continuous fluid domain. This fluid domain was then meshed using a tetrahedral mesh with a spacing of 0.5. Such a fine mesh was necessary given the complex geometry of the axial-flow impeller. After meshing, the various faces and the fluid domain volume of the pump were defined before the mesh was exported to the solving package Fluent.

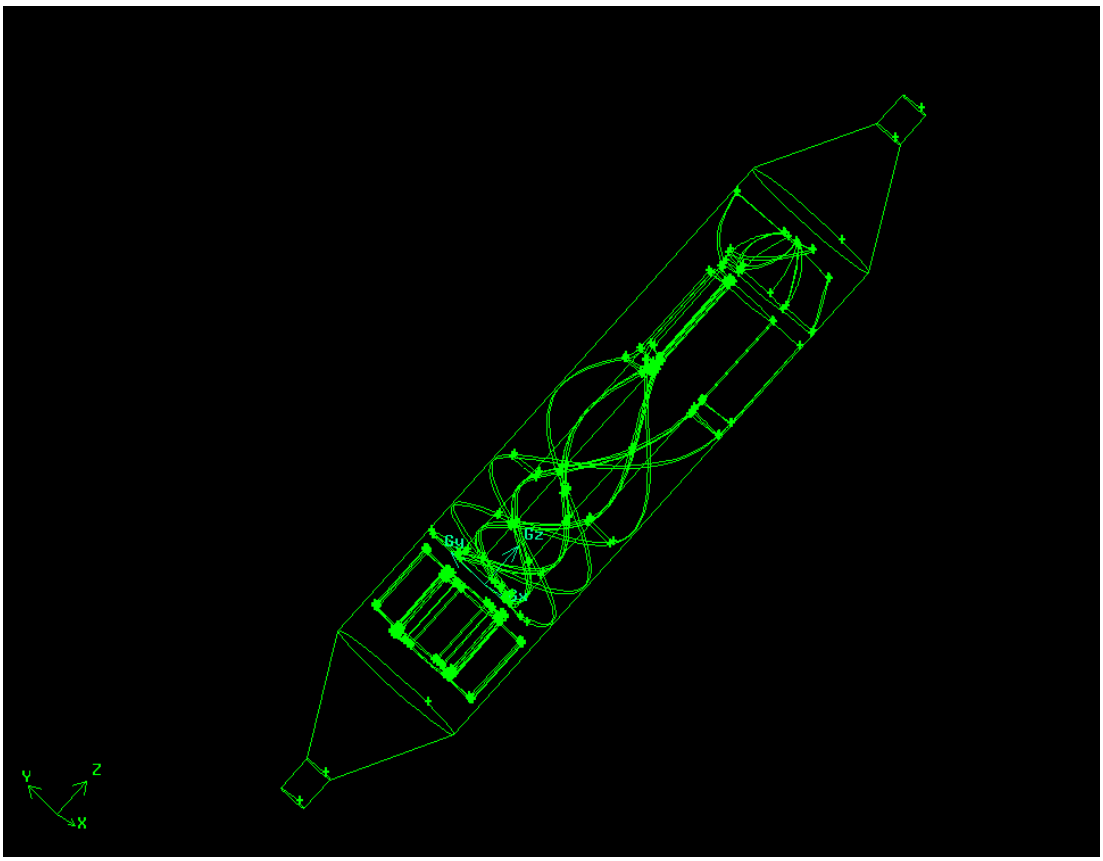

Figure 31 - Gambit view of axial impeller within housing.

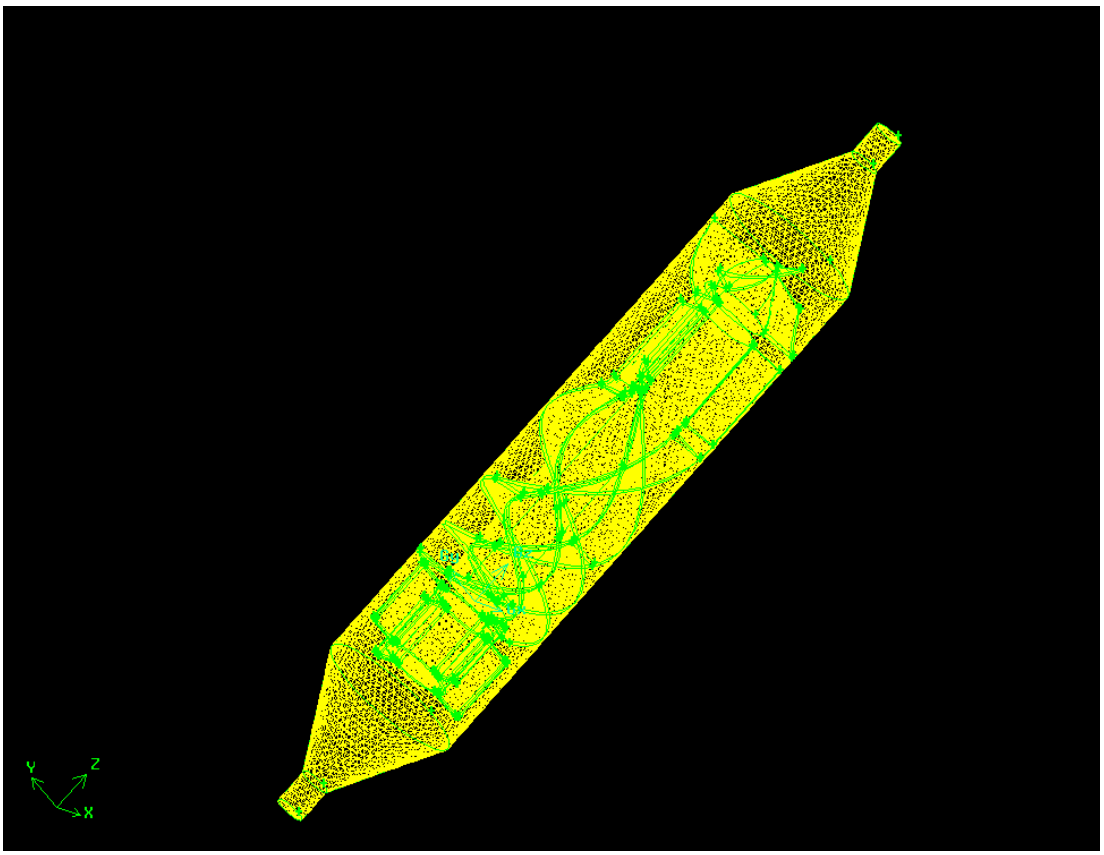

Figure 32 – Gambit mesh of axial flow impeller within housing.

Upon being imported to Fluent, the inlet of the pump was defined as a pressure inlet, set to 0Pa, and the outlet defined as a pressure outlet to 0Pa. These pressures are indicative of the pressure that can be expected in the device when connected via a veno-venous cannula.

Every other face within the pump, with the exception of the impeller, was defined as a stationary wall.

The impeller was defined as a moving wall, with the rotational speed defined at the beginning of each analysis.

Blood was defined at the beginning of the analysis as the continuous fluid region throughout the model and was assumed to be a Newtonian fluid. The mechanical properties of blood were defined as a density of 1050kg/m^3 and constant viscosity of 0.0035Pa.s.

The pump was then analysed for impeller rotational speeds of 1000, 2000, 3000, and 5000rpm. Flow rate and shear stresses on the impeller walls are shown below.

Results:

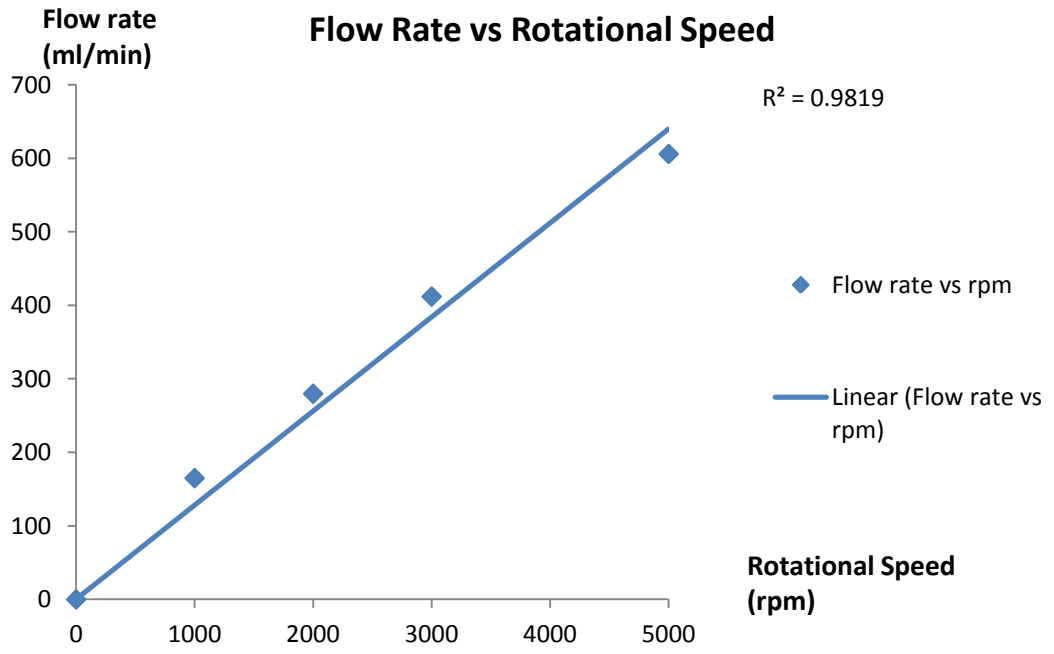

Figure 33 – Flow rate vs. Rotational speed of axial impeller.

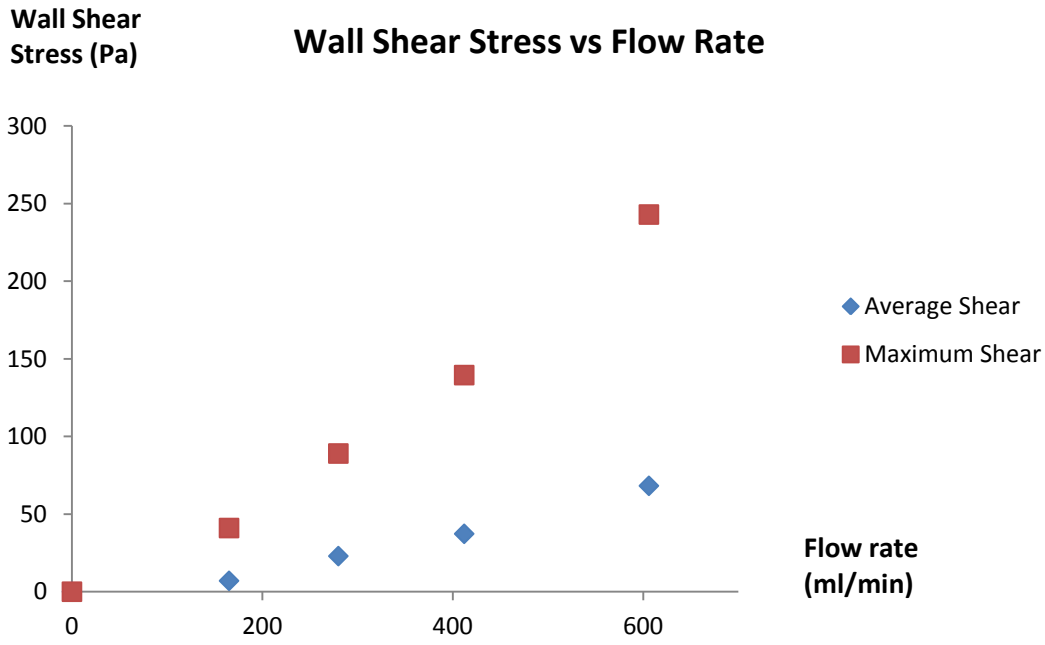

Figure 34 – Wall shear stress vs. Flow rate in axial impeller.

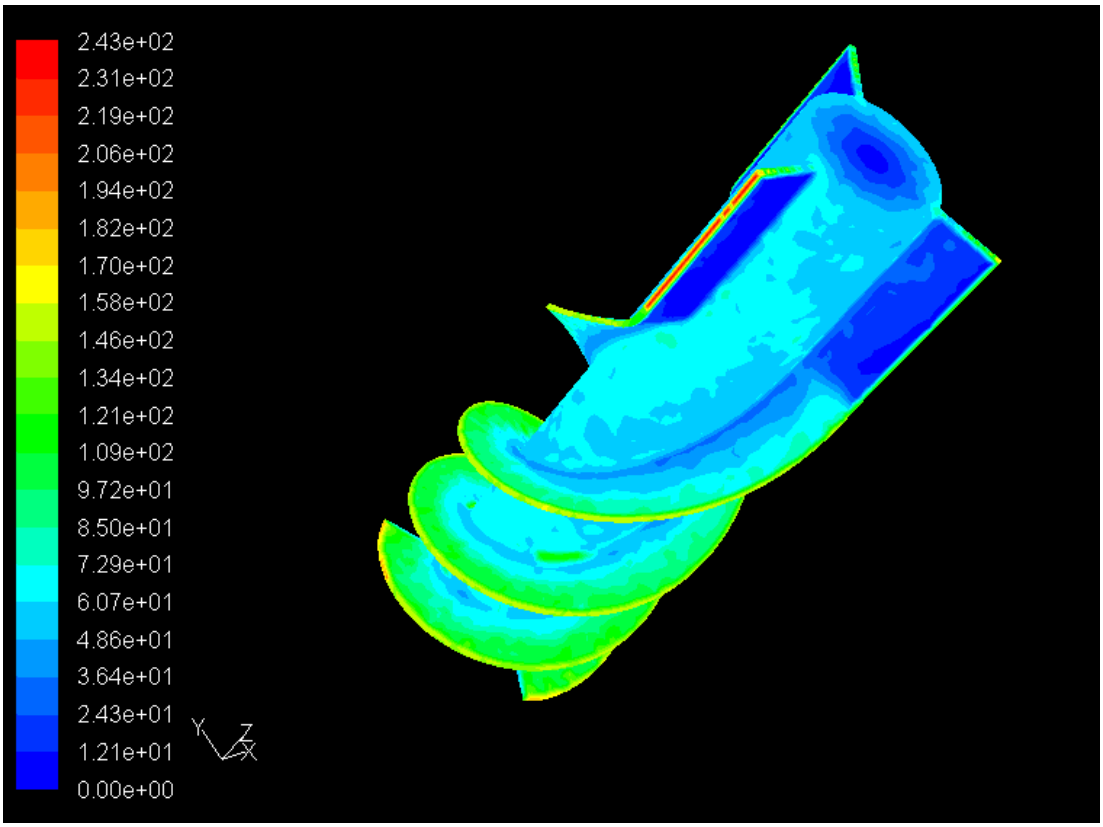

Figure 35 – Contour of wall shear stress (Pa) in axial impeller, modelled in Fluent.

Centrifugal Pump analysis:

Methods:

A model of a centrifugal pump was built in ProEngineer. Firstly, a disc and centre piece was created, followed by a streamlined impeller blade. This blade was then repeated every 60° to give a 6 bladed centrifugal impeller.

The dimensions of this centrifugal pump are 50mm in diameter and 20mm in height. Housing was created to provide a clearance of 1mm in any direction.

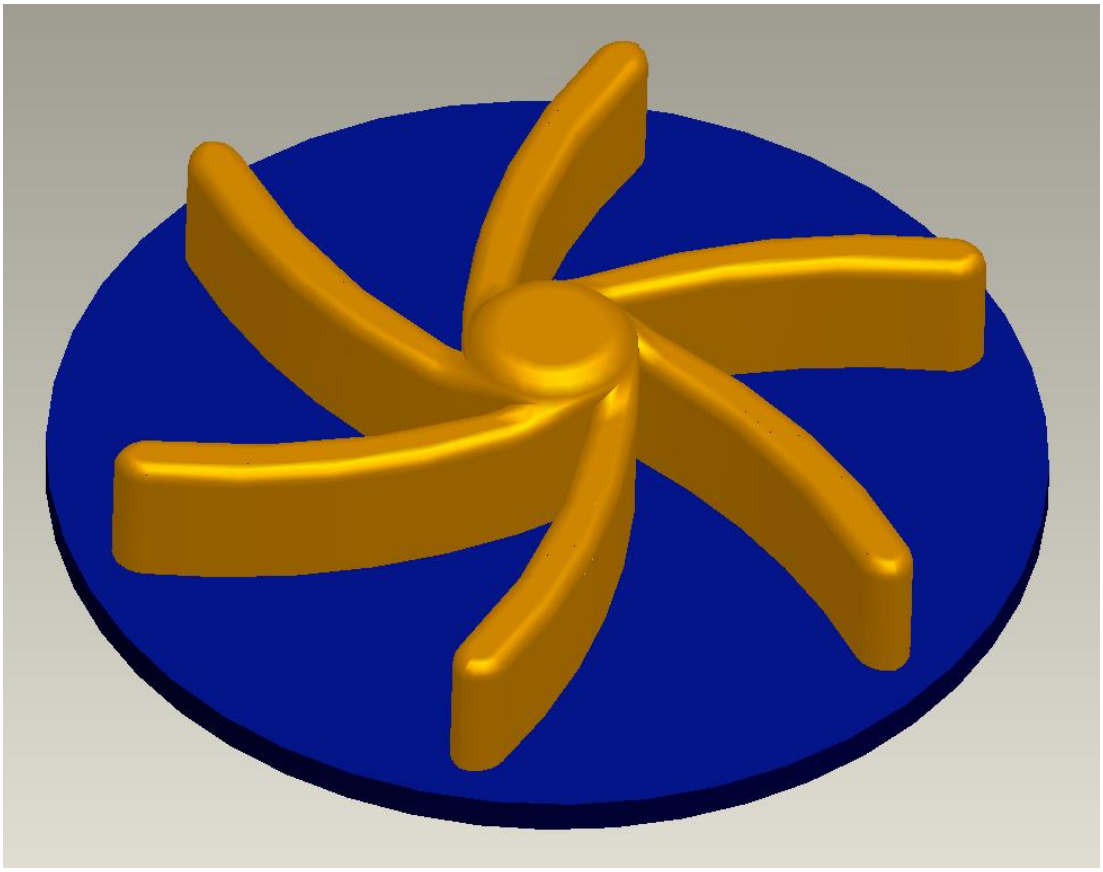

Figure 36 – Angled view of centrifugal impeller created in ProEngineer.

This centrifugal impeller geometry was then saved as a step file and exported to Gambit for meshing. Further, relatively uncomplicated, features were then created in Gambit, such as a casing and an inlet and outlet. The various components created comprised a full model for a centrifugal pump.

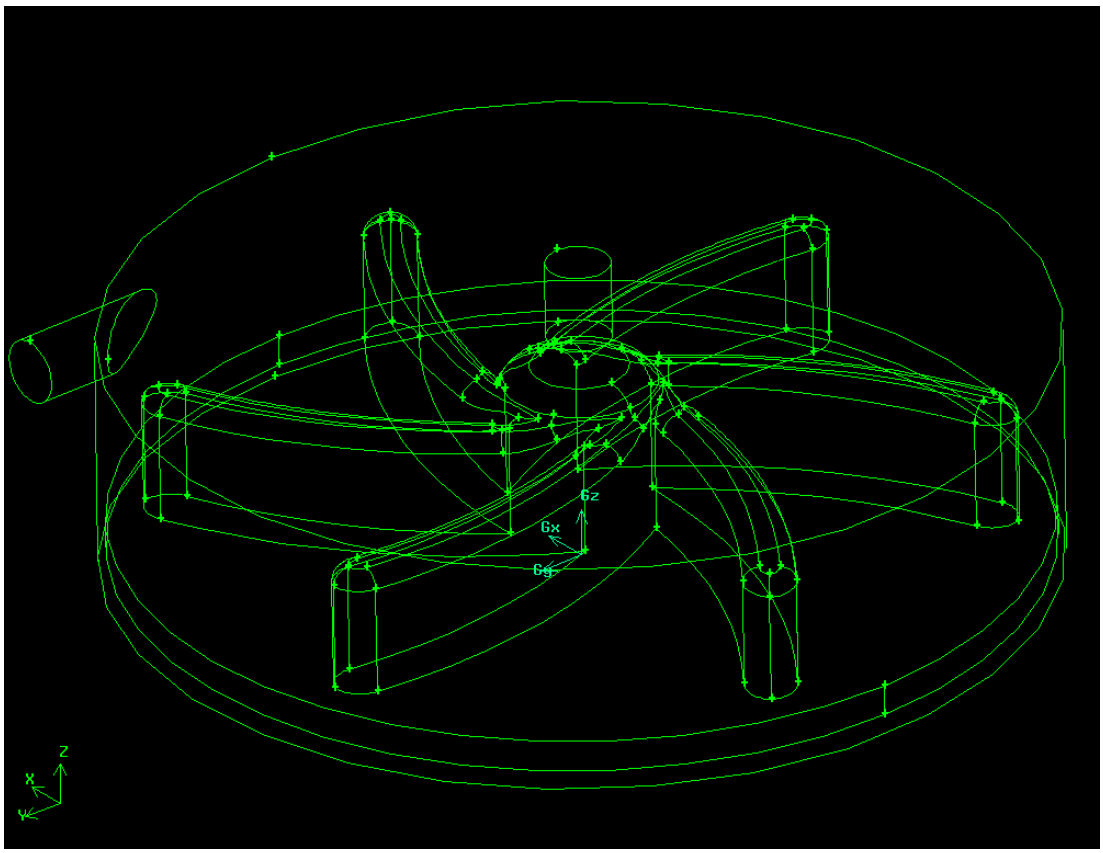

Figure 37 – Gambit view of centrifugal impeller with housing.

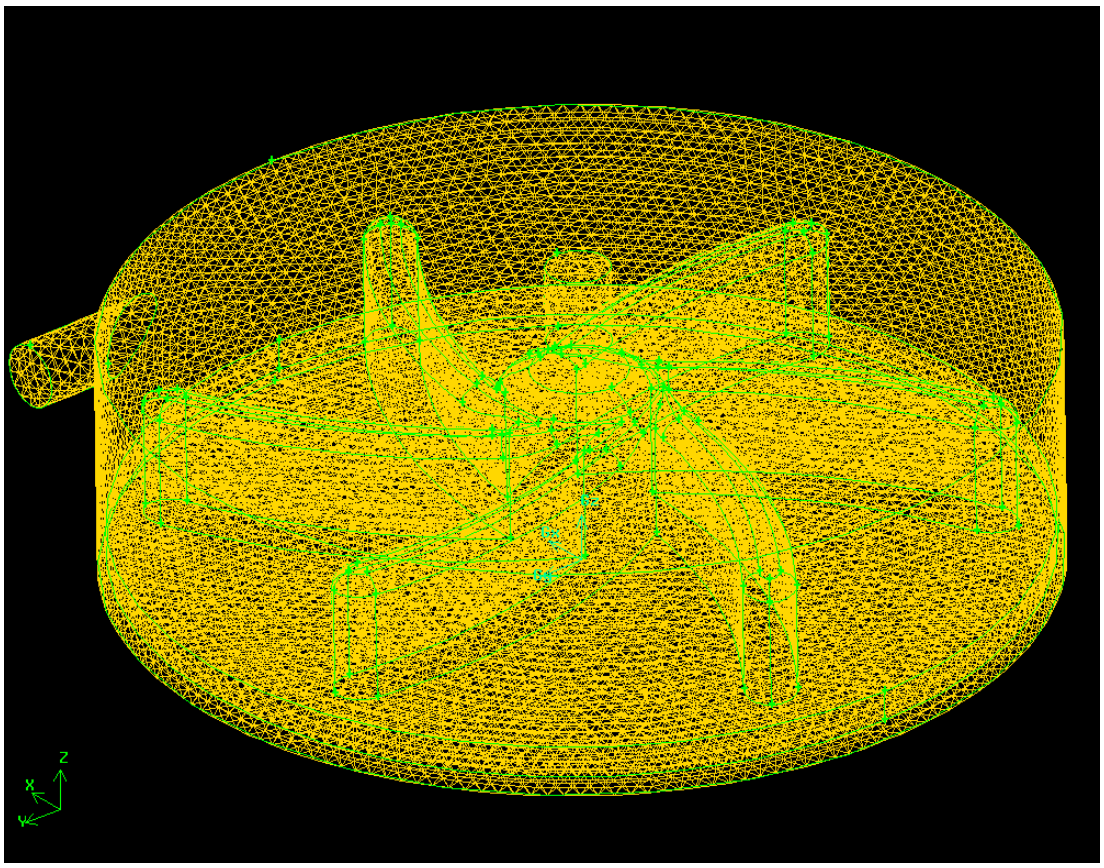

Figure 38 – Gambit mesh of centrifugal impeller with housing.

As with the axial-flow pump, the various volumes were united, and subtracted, as necessary, in order to create a single continuous fluid domain. This fluid domain was then meshed using the default spacing of 1. After meshing, the various faces and the fluid domain were defined before the mesh was exported to Fluent.

As with the axial-flow pump, upon being imported to Fluent, the inlet of the pump was defined as a pressure inlet, set to 0Pa, and the outlet defined as a pressure outlet to 0Pa. Every other face within the pump (with the exception of the impeller) i.e. the housing was defined as a stationary wall.

The impeller was defined as moving wall, with the rotational speed defined at the beginning of each analysis.

The continuous fluid region throughout the device was defined as blood, which was assumed to be a Newtonian fluid with a density of 1050kg/m^3 and a viscosity of 0.0035Pa.s

The pump was then analysed for impeller rotational speeds of 1000, 2000, 3000, 4000 and 5000rpm. Flow rate and shear stresses on the impeller walls are shown below.

Results:

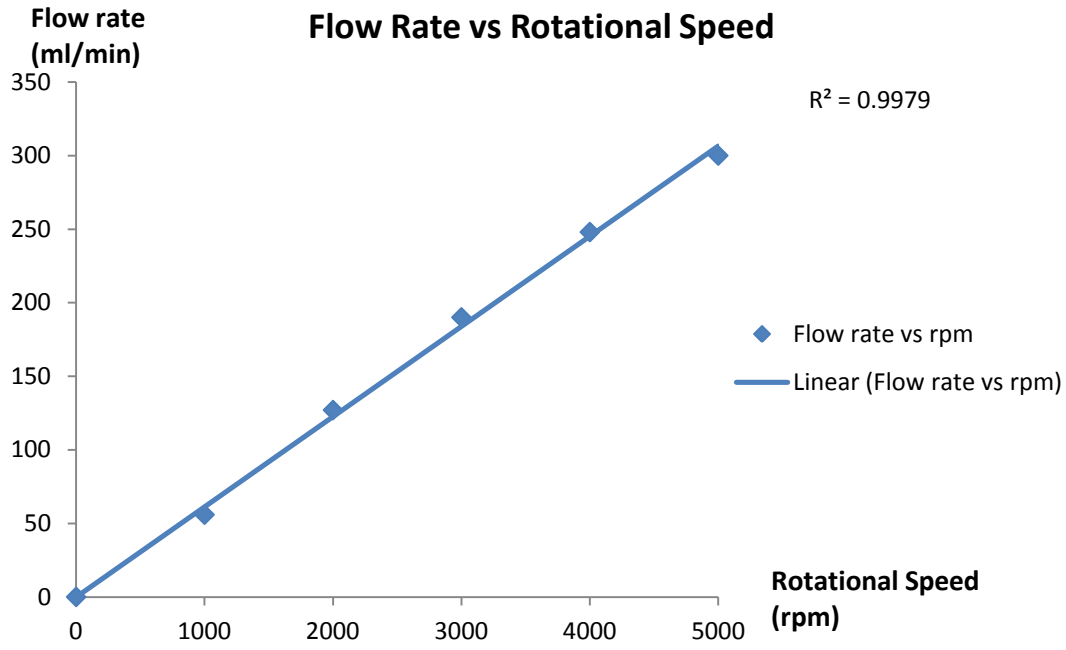

Figure 39 – Flow rate vs. Rotational speed for centrifugal impeller.

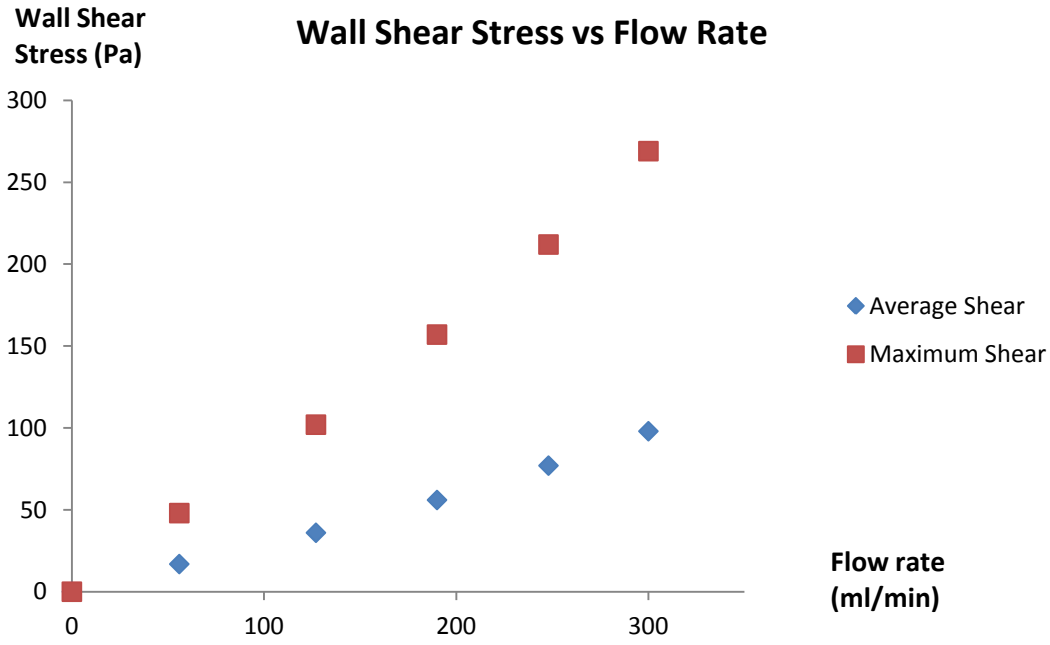

Figure 40 – Wall shear stress vs. Flow rate for centrifugal impeller.

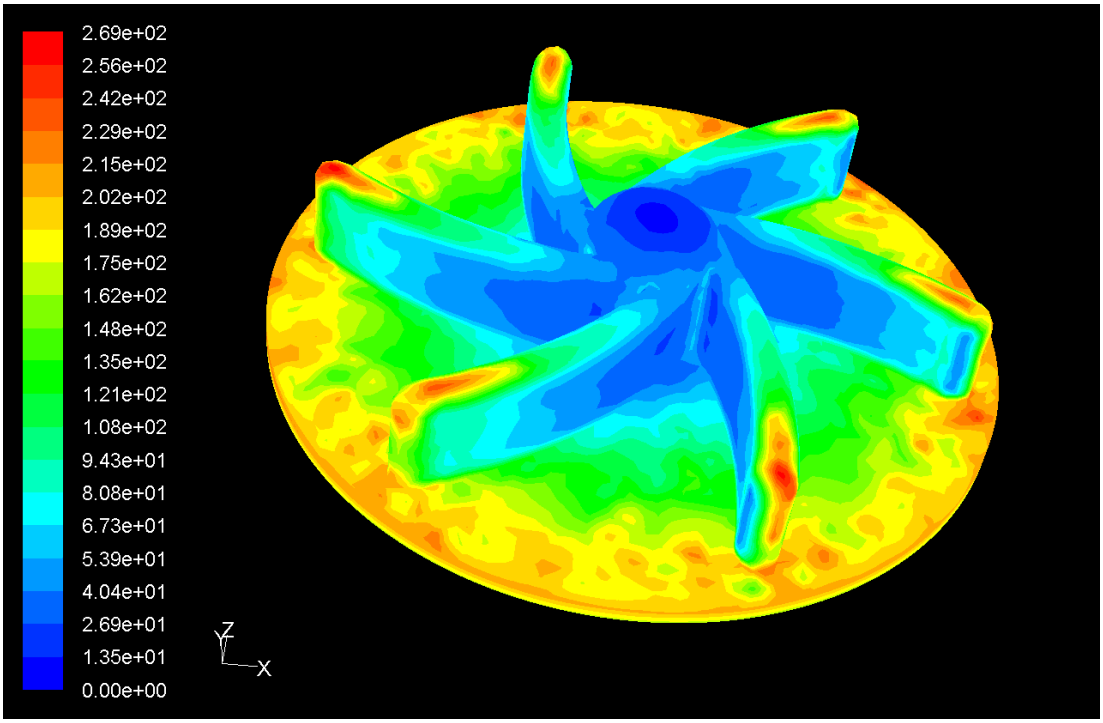

Figure 41 – Contours of wall shear stress (Pa) in centrifugal impeller, modelled in Fluent.

2.4 Integration:

Development of a portable device did not only require the miniaturisation of a blood pump but also a system in which the exposure of an adsorbent to whole blood was feasible. Achieving portability required that we minimise the size of the working parts within the device, thus avoiding using any unnecessary technologies within the device, such as a plasma separation column. This reduces the size of technology bringing us closer to our goal. However, the resultant exposure of whole blood to an adsorbent further complicated the development of the device and thus an innovative solution was necessary in order to avoid thromboembolitic complications i.e. microparticle adsorbents entering the cardiovascular system with returning blood. Ultimately, the only way to truly avoid this complication was to completely immobilise the adsorbent beads by one method or another.

2.4.1 Adsorbent Immobilisation:

Detailed in this section are the various adsorbent immobilisation concepts pursued and how their development (both conceptual and physical) evolved.

Concurrent with the research of blood pumping technology was the development of an adsorbent material appropriate for integration with the blood pump concepts explored.

The initial adsorbent concepts that were explored consisted of; the packed bed formation that is popular within the chemical processing industry, the monolithic adsorbent structure suggested and patented by Mast Carbon Ltd., the loose bed formation isolated by inlet and outlet filters, a “teabag” formation explored and developed between the Bioengineering unit and Brightwake Ltd. And finally a fibrous material immobilising concepts also developed between the Bioengineering Unit and Brightwake Ltd. These concepts were all explored to differing extents and the work pursued is detailed below.

Packed Bed:

An early stage concept for the adsorbent was the packed bed. This concept initially seemed reasonable as it is possible to manufacture the packed bed in any formation, therefore allowing for effectively any geometry of an overall device.

It was quickly decided not to further this concept as we encountered both engineering and clinical complications.

Firstly, the fluid mechanics within the device promoted minimal fluid contact with the adsorbents, due to shunting. The packed bed formation has previously been a popular and effective method of adsorption in the chemical process industry; this however, has largely been successful only compressible fluids i.e. gases.

Secondly, a tiny manufacturing fault in the formation of a geometry could result in "shunting", in which a small channel exists through the geometry, and a huge amount of fluid follows that path of least resistance; again therefore, not achieving reasonable blood contact with the adsorbent.

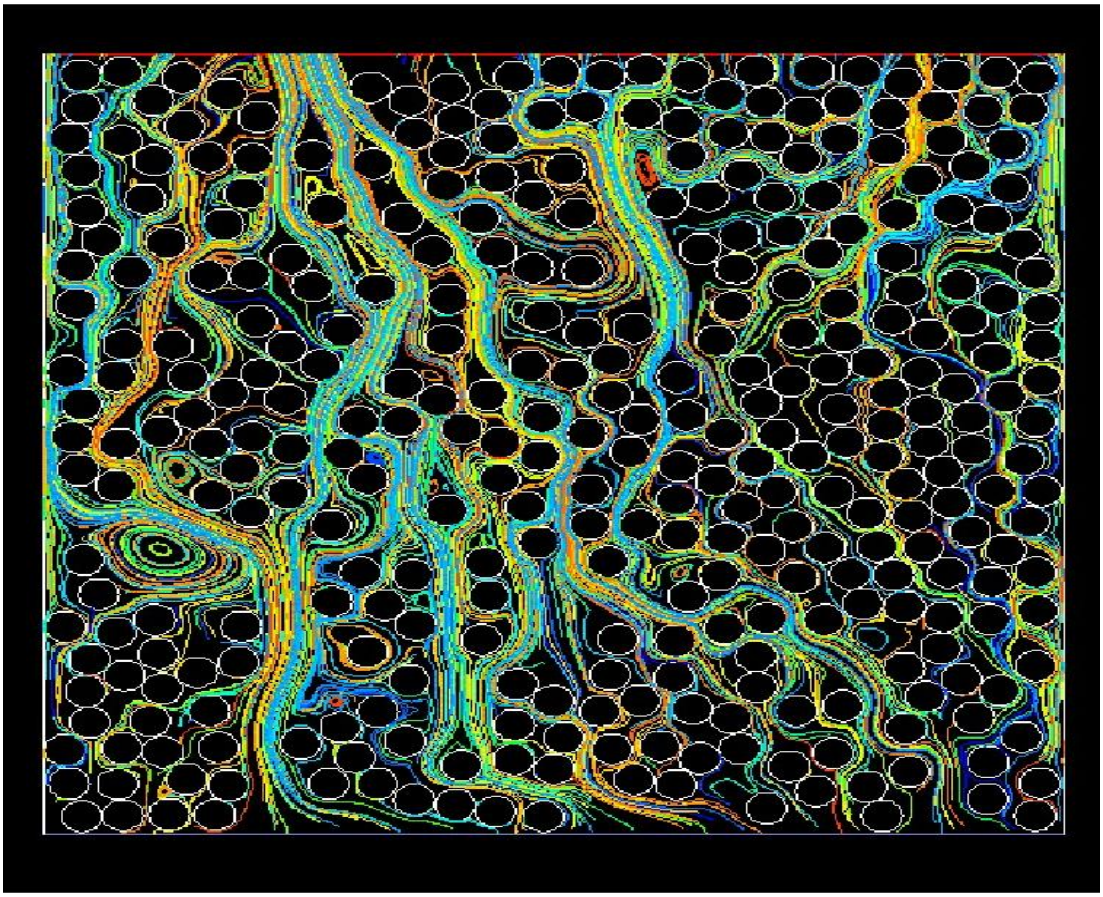

Figure 42 – CFD model of flow through a packed bed, displaying shunting.
Taken from http://sharif.ir/~moosavi/research.html

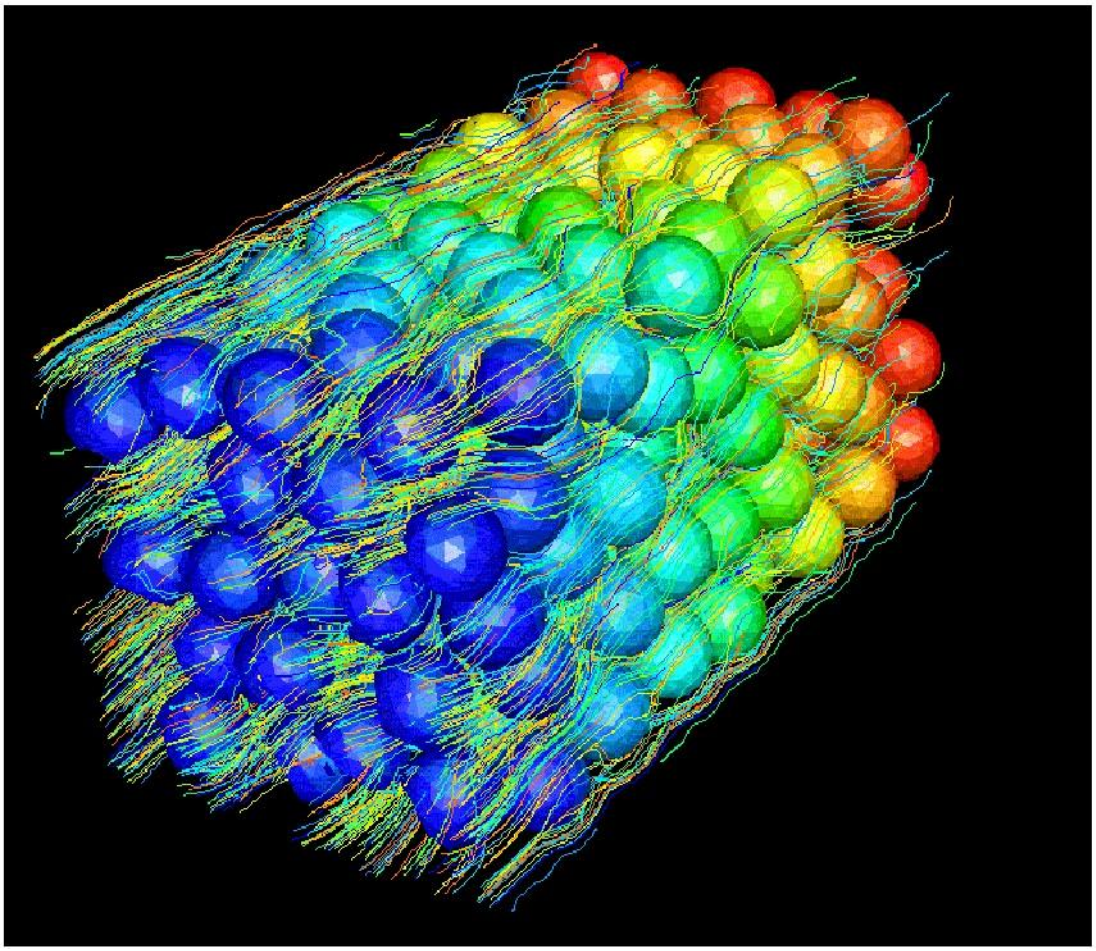

Figure 43 – Pressure drop across packed bed, modelled using CFD.
Taken from http://sharif.ir/~moosavi/research.html

Thirdly, the pressure drop resultant across a packed bed when using blood as fluid is far higher than that which is considered clinical safe. The resultant haemolytic blood damage would be enormous and therefore unacceptable clinically.

Monoliths:

In very early stages of the development phase, the idea of monolithic adsorbent from Mast Carbon Ltd. was suggested. This is another adsorption process that is popular within industry. Below is an image of the Mast Carbon monolithic structure.

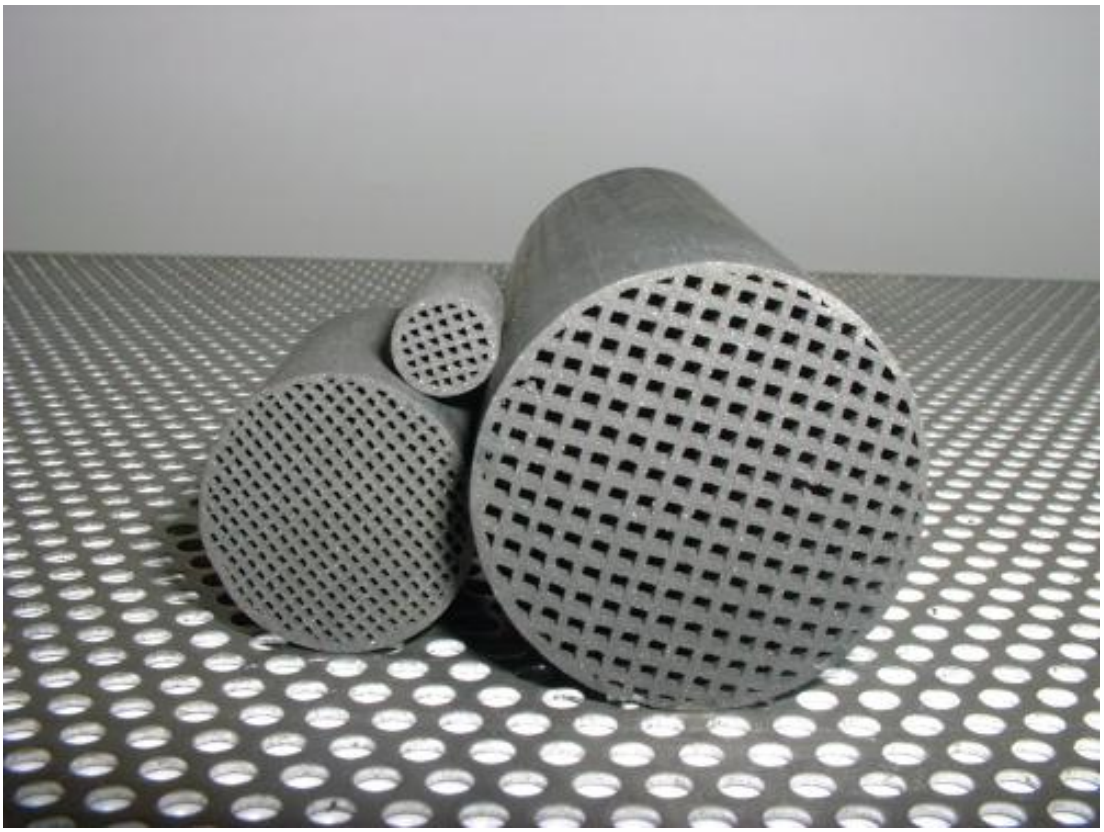

Figure 44 - Monolithic Adsorbent.
Taken from the website of Mast Carbon International Ltd.

It can clearly be seen from the geometry of this design that the flow of blood would enter the monolith and then, as expected, follow the path of least resistance. Thus, the blood would flow freely through the channels within the monolith achieving contact only with the beads on the surface of these channels. Though diffusion will occur to some extent, it is clear that this diffusion aids flow only a small distance into the carbon structure and therefore the majority of the carbon used in this structure is wasted and thus the structure is highly inefficient. Most importantly for a portable device, it is space inefficient.

Teabags:

This similar concept, known as the “teabag” configuration in which beads would be immobilised by being enclosed within a fibrous matrix made of the same 40µm pore filter considered for the inlet/outlet filters. It was the appearance of these matrices that made them become known colloquially as the “adsorbent teabags”. Like the packed bed, one of the benefits of the teabags was that they could be manufactured in almost any configuration, thus easing the restrictions of the overall device configuration. Shown below is the teabag configuration of beads.

Figure 45 - Teabag configuration of immobilised adsorbent.

Top – Adsorbent Teabag configuration

Bottom – Size comparison with a drinking tea teabag.

Adsorbent Mat:

Following on from successful work carried out in the development of adsorbent structures for deployment during high flow CPB, we selected a similar approach in the current technology. One major advantage in adopting this approach was that the biocompatibility and toxicology required for commercial use of the material had already been carried out.

This adsorbent mat was developed in which adsorbent beads were immobilised within a fibrous polymeric matrix. 4 components make up this material, these are;

- A non-woven polymeric fabric.
- Adsorbent microbeads (100-250µm diameter, supplied by Mast Carbon Ltd.)
- An aerosol acrylic adhesive.
- A woven polyester material with a 40µm pore size.

The manufacturing process includes ultrasonic welding of these materials into a sheet. The non-woven polymeric fabric acts as support medium for the beads and has a permeable and open structure that allows the passage of blood without causing undue damage to the RBCs. This porous open structure also provides a large surface area for the attachment of beads, and thus increases the contact between the beads and the blood.

Design Methodology:

- Initially a section of non-woven polymeric fabric is placed in a well ventilated area.
- This fabric is then sprayed with an aerosol acrylic adhesive.
- This adhesive is allowed to partially cure for between 20 and 30 seconds.
- Carbon beads are then sprayed onto this fabric at a low controlled pressure to achieve a weight/area ratio of approximately $4g/m^2$.
- A woven polyester material with 40µm pore size is then placed on both sides. Pressure and ultrasonic vibrations are then applied at multiple points. This results in the layers of material being welded together at these points.

Outcomes:

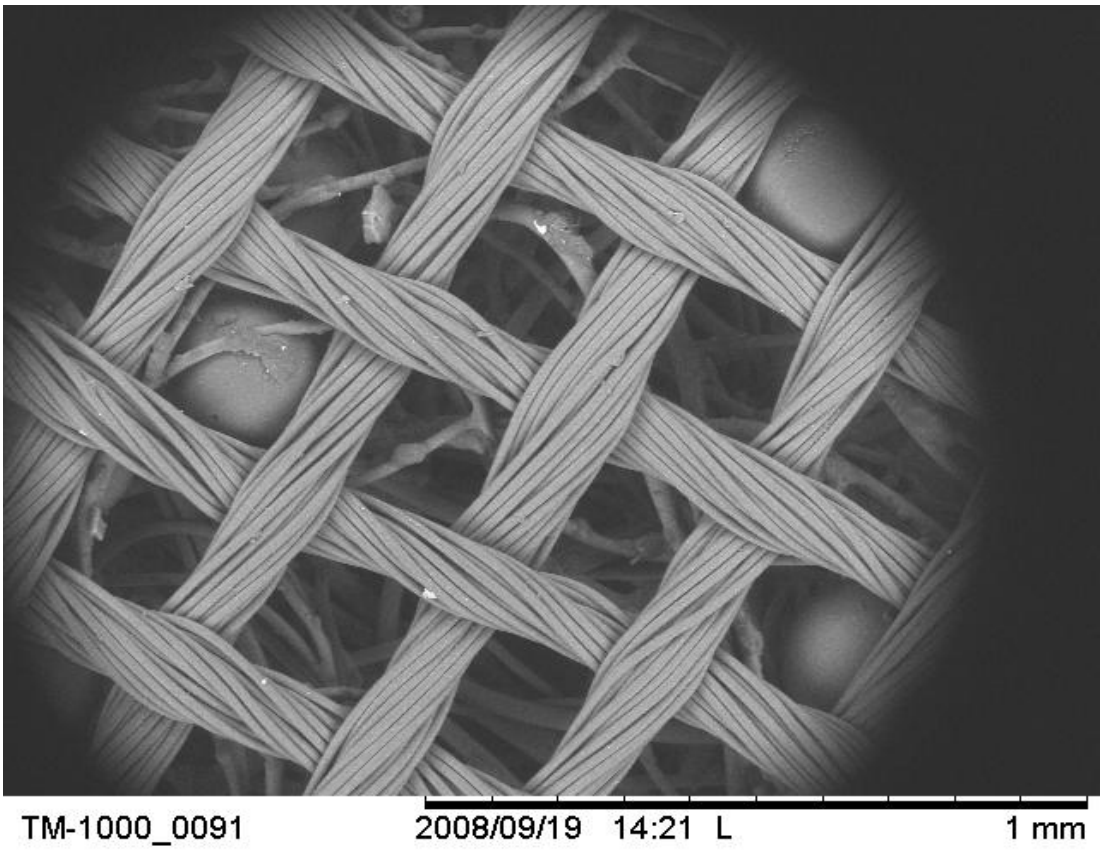

Figure 46 - Scanning Electron Microscope (SEM) image of adsorbent beads trapped within polymeric fibrous matrix.

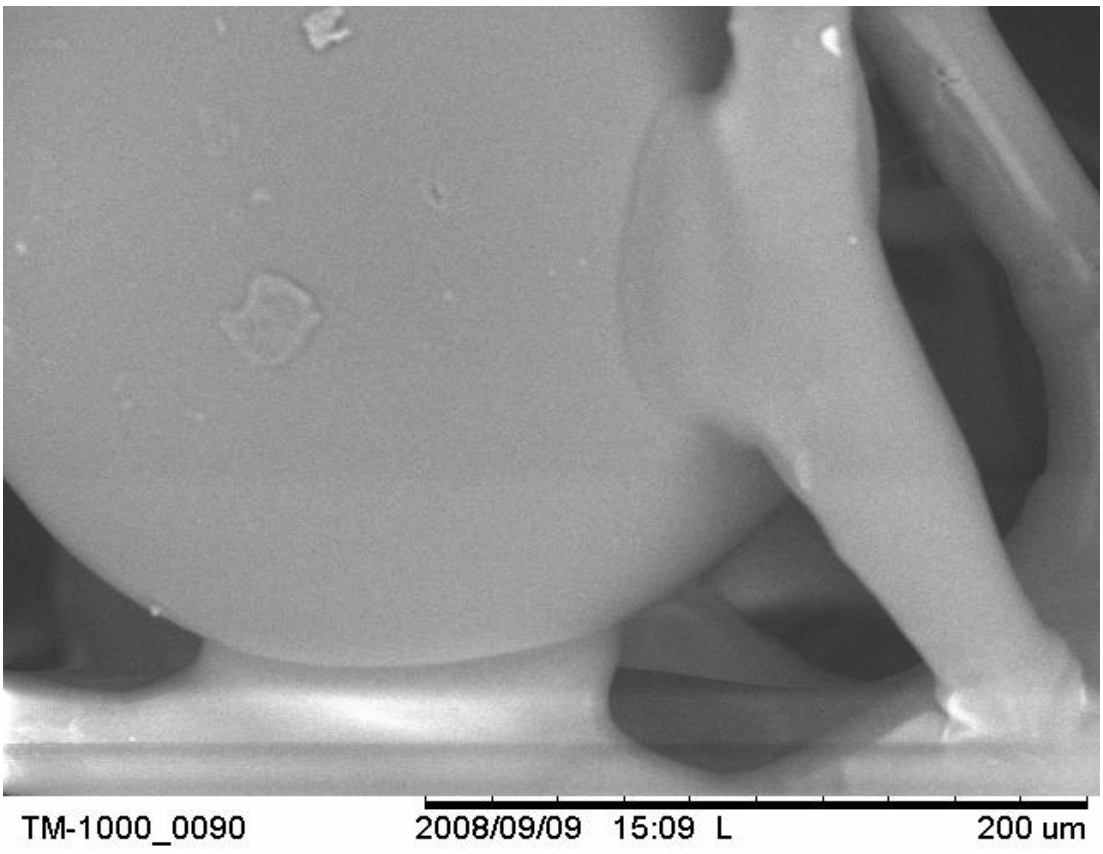

Figure 47 - Enhanced SEM image of adsorbent bead immobilised within non-woven fabric, utilising acrylic adhesive.

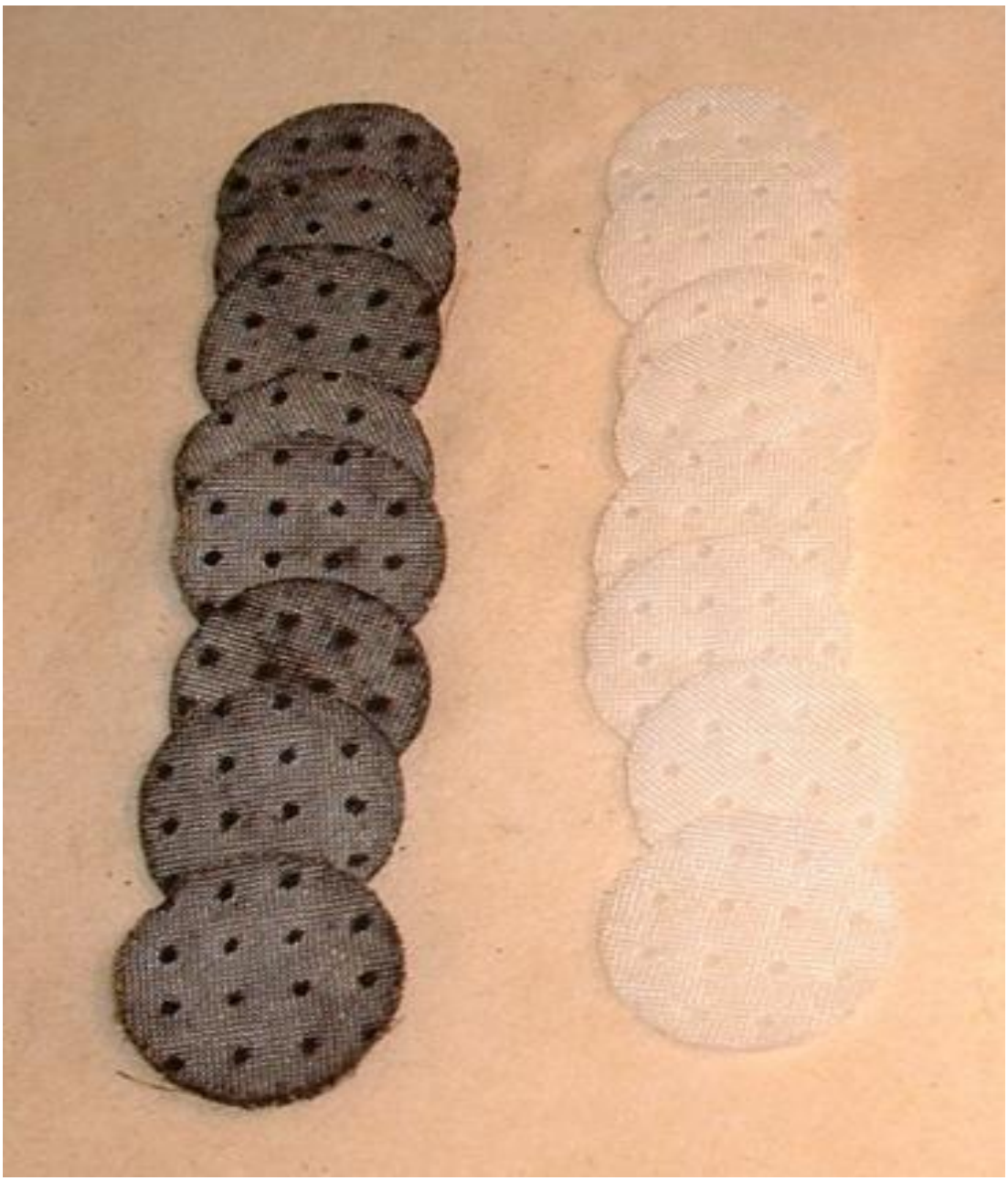

Figure 48 – Photo of non-woven fabric with ultrasonic welding points.

Both materials here comprise of the non-woven fabric with a woven polyester material ultrasonically welded together.

Left - Carbon beads impregnated into the non-woven fabric immobilised using aerosol acrylic adhesive.

Right – Non-woven "sham" fabric, without carbon beads.

2.4.2 Integration of Components:

During the initial stages of the project, concurrent activity was undertaken in the development of many concepts. This involved exploring and developing concepts for portable blood pumping technology, adsorbents, and an overall integrated housing in which these two technologies can be combined. Some, but not all, of these concepts were pursued into a prototype development phase.

At this time the adsorbent component available (supplied by Mast Carbon Ltd.) existed only as a porous carbon-based ceramic bead (approximately 100-250μm in diameter). This technology was the basis of devices deployed in CPB by this group in previous research.

Below are discussed the pros and cons of a series of integration concepts, utilising the four blood pump concepts proposed, and a more detailed discussion of the challenges of this integration.

Military Requirements:

In the initial stages of the project, the United Kingdom's Ministry of Defence (MOD) showed significant interest in the progression of the project. They insisted on the device being portable as part of a soldier's personal load carrying equipment (PLCE), otherwise known as webbing.

Figure 49 - Standard British Army PCLE webbing set.
Taken from http://kmmilitaryarmysurplusstore.wordpress.com/2010/06/06/hello-world/

This is the combat belt carried by a soldier containing mostly emergency equipment, such as ammunition, food, medical supplies, and protection against chemical, biological, radiological, or nuclear (CBRN) weapons. The MOD felt that if a small, portable device capable of rapid, point-of-care treatment was achievable, then such a device should become an integral part of a soldier's personal kit and also suggested that such a device could become a major part of disaster and emergency planning with regard to emergency security situations, such as bio-terrorism.

Though the project began with the proposed concept of removing both inflammatory cytokines as well potential chemical threats, during a visit to the Defence Science and Technology Laboratory (DSTL) at Porton Down, Wiltshire, representatives of the MOD requested that the focus of the treatment be directed toward blast injuries in particular, as a result of the prevalence of improvised explosive devices (IEDs) in the wars in Afghanistan and Iraq, as well as their extensive use in terrorism.

With these design requirements in mind, geometries proposed typically consisted of rounded housings with no sharp edges or corners. Multiple reasons existed for this. First and foremost, rounded geometries do not have any sharp edges or corners on the outside of the device. Thus when carrying such a device on the body, an individual would not have the threat of injury if they were to fall on this equipment; a serious potential hazard for a soldier carrying the equipment around their abdomen in a combat situation. Based on the assumption that the defence/security application was the most significant at this time, this geometry was pursued throughout this project. This geometry is also attractive for a working cardiovascular treatment device.

Concept 1:

Shown here is the 1st concept that was proposed. It is a device which utilises the axial flow pump concept integrated within the hip-flask shaped housing.

Positives:

- The working parts and housing of this concept can all be machined exclusively from biocompatible materials, such as medical grade stainless steel.
- The profile of this concept is highly compact and with the option of multiple forms of pump drive-systems, including maglev, direct drive, magnetic coupling and magnetic fluid sealing.

Negatives:

- This form of blood pump system is costly and complex to manufacture, requiring a 5-axis machining tool.
- The rotary pump used is pre-load and after-load dependent, therefore in the event of unforeseen high or low pressure, the impeller could appear to be working without achieving any hydraulic performance.
- Complexities exist in the positioning of a drive system for the impeller, as in some scenarios this positioning is where the inlet would ideally be positioned also.
- Complex control mechanisms would be required to observe any flow or pressure abnormalities, but may only provide a warning rather than a solution; therefore the device would require constant attention.
- The rotary pump is prone to failure in the event of a significant impact, considered likely when in field use.
- The pressure differential in the axial direction causes great difficulties in coupling a drive system to the impeller, as air can be pulled through a bearing.

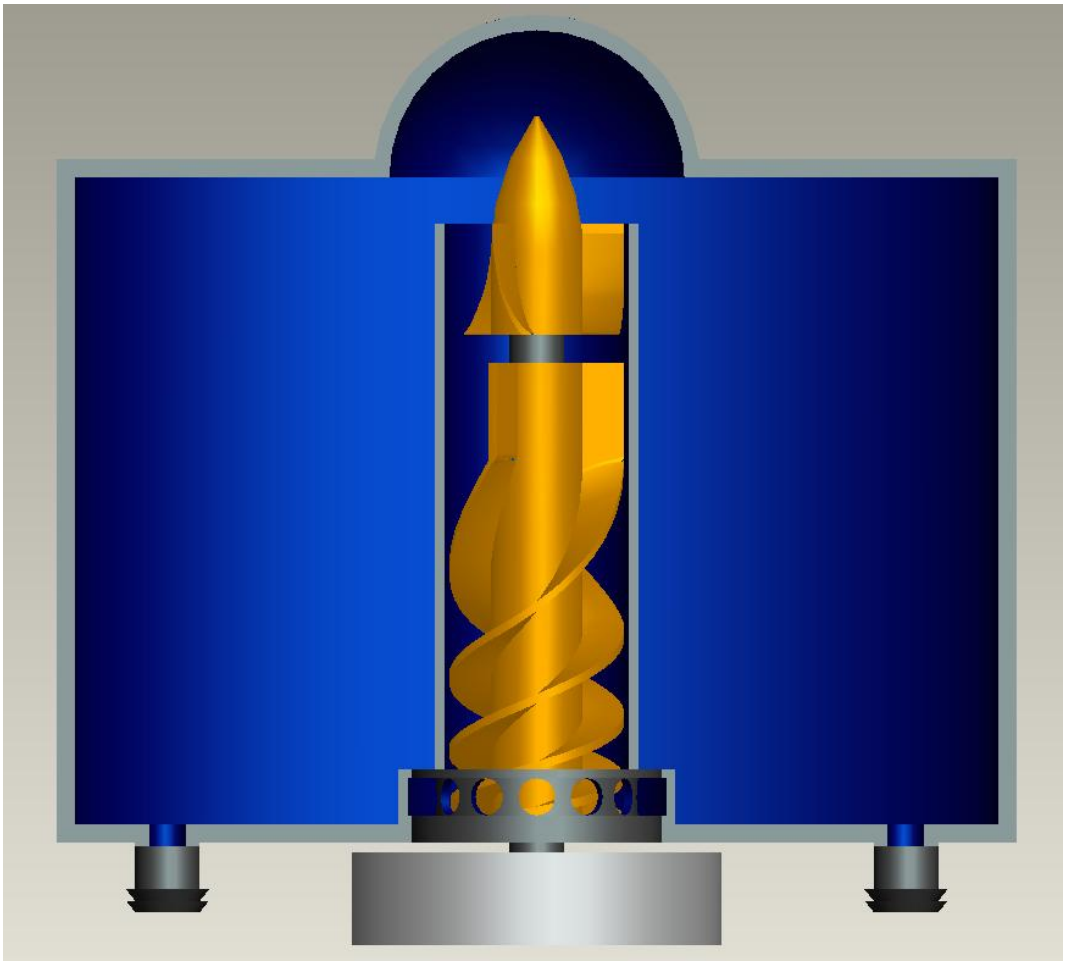

Figure 50 - Front Half-cut view of first concept.

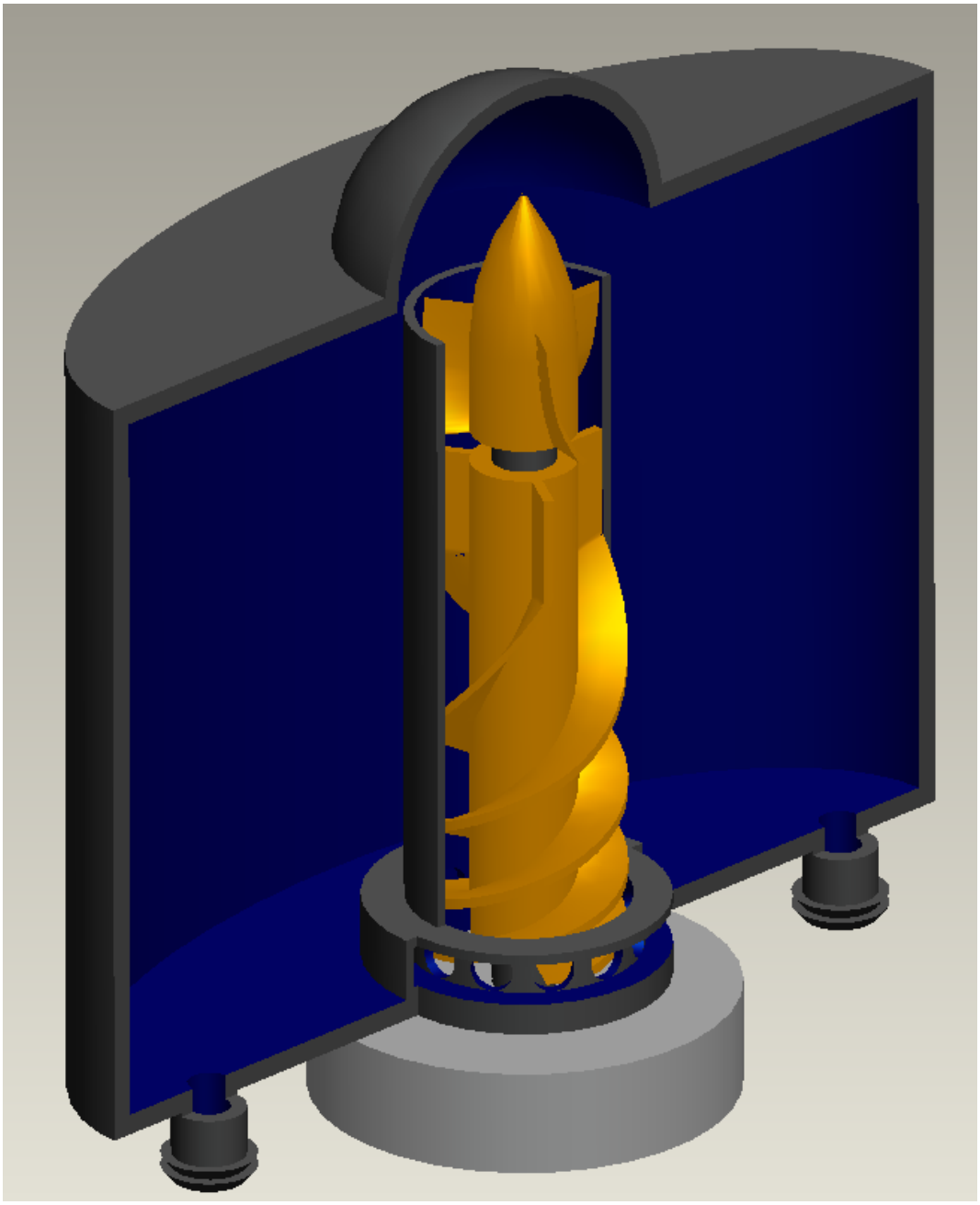

Figure 51 - Angled Half-cut view of first concept.

Concept 2:

The second concept proposed was very similar to that of the first but utilising a centrifugal impeller rather than an axial impeller. The positive and negative of this concept are very similar to the previous concept and are described below.

Positives:

- The working parts and housing of this concept can all be machined exclusively from biocompatible materials, such as medical grade stainless steel.
- The option of multiple forms of pump drive-systems exists, including maglev, direct drive, magnetic coupling and magnetic fluid sealing.
- No complexities or conflict associated with the positioning of the drive system and inlet for the impeller.

Negatives:

- The rotary pump used is pre-load and after-load dependent, therefore in the event of unforeseen high or low pressure the impeller could appear to be working but not actually achieving any hydraulic performance.
- Complex control mechanisms would be required to observe any flow or pressure abnormalities, but may only provide a warning rather than a solution; therefore the device would require constant attention.
- The rotary pump is prone to failure in the event of a significant impact, considered likely when in field use.
- This concept is not as compact as the 1st concept.

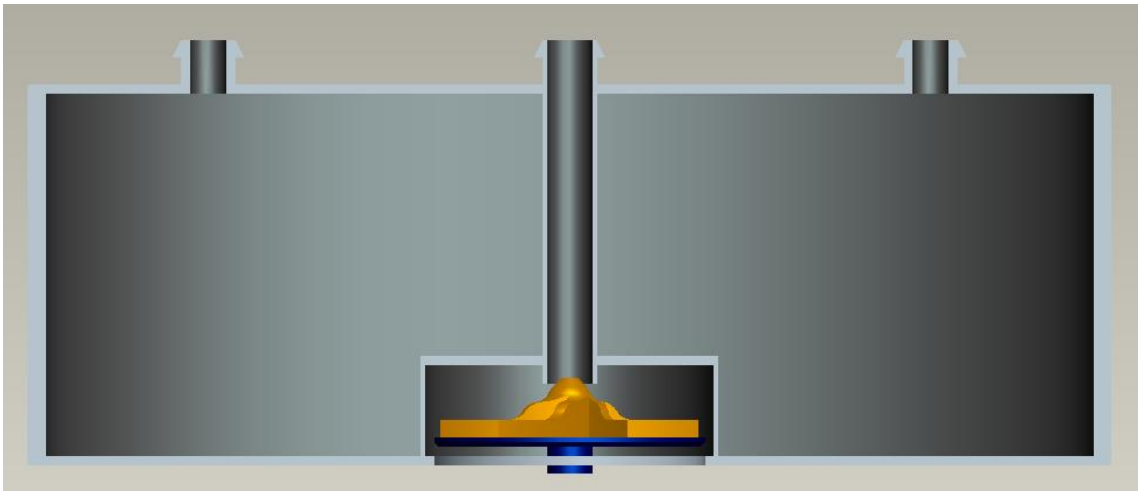

Figure 52 - Front Half-cut view of second concept.

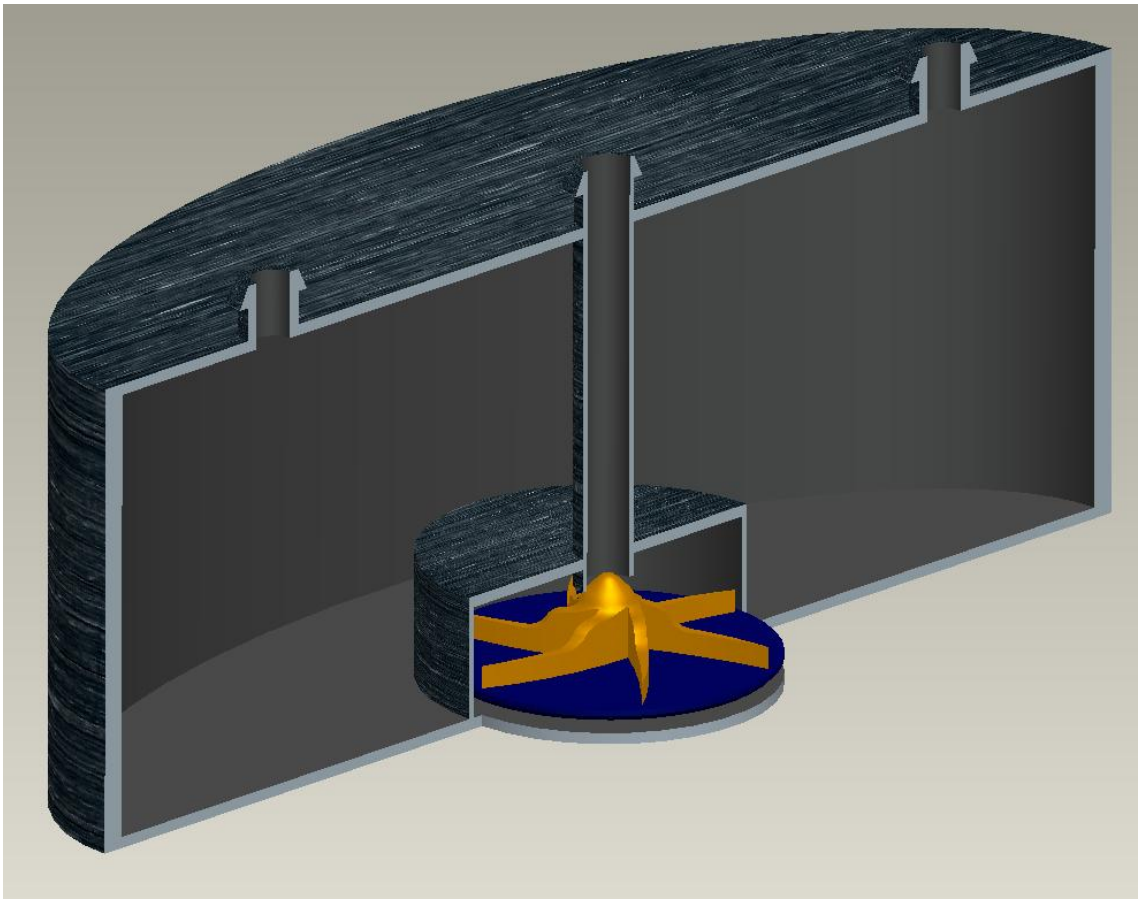

Figure 53 - Angled Half-cut view of second concept.

Concept 3:

Positives:

- As with the 1st and 2nd concepts, the working parts and housing of this concept can all be machined exclusively from biocompatible materials, such as medical grade stainless steel, as well as a biocompatible polymer.

Negatives:

- Use of solenoid technology requires complex electronic control, yet still results in deeply unreliable technology, even in ideal conditions, not to mention potential impact.
- Use of high and low pressures associated with the pulsatile pumping method can result in high haemolysis or rupture of the polymer, causing catastrophic failure.
- The profile of this concept is not compact and the inlet and outlet must be on the outer walls rather than the top of bottom walls, therefore this inlet and outlet are prone to damage.

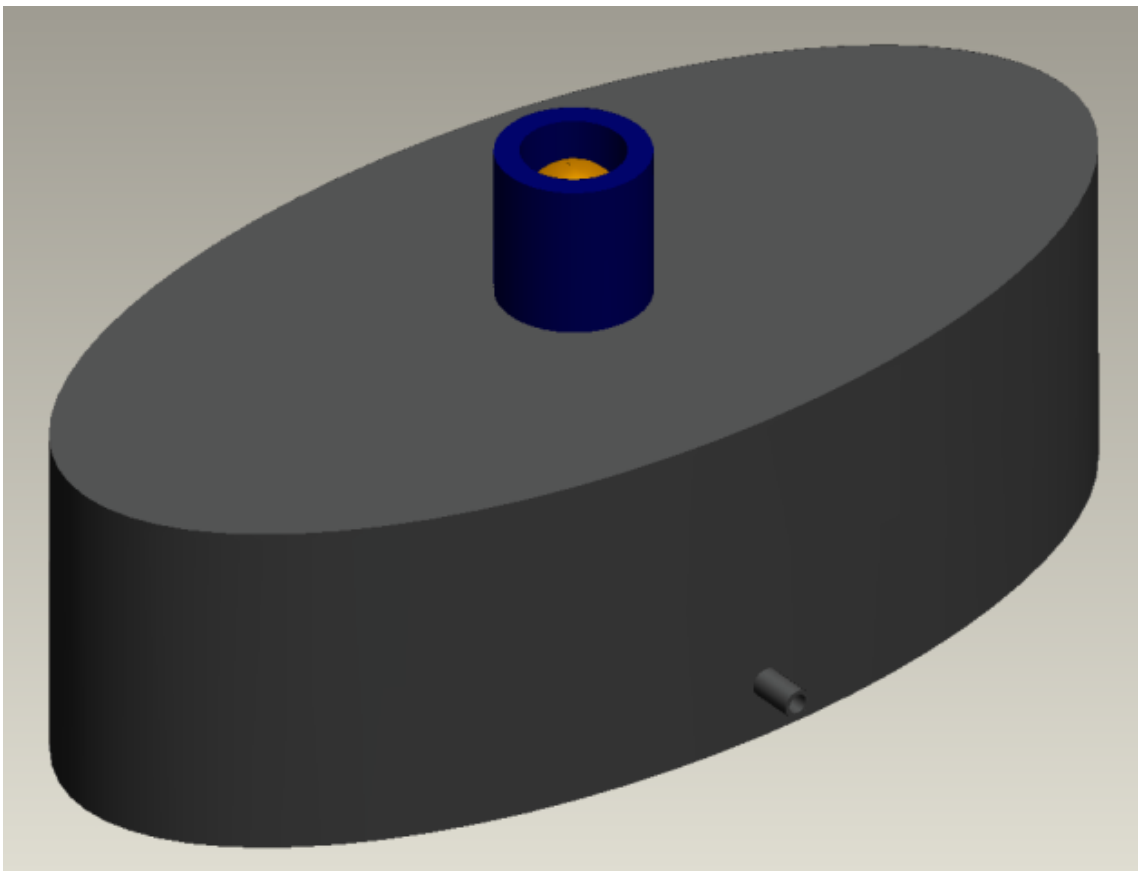

Figure 54 - Angled Full-cut view of third concept

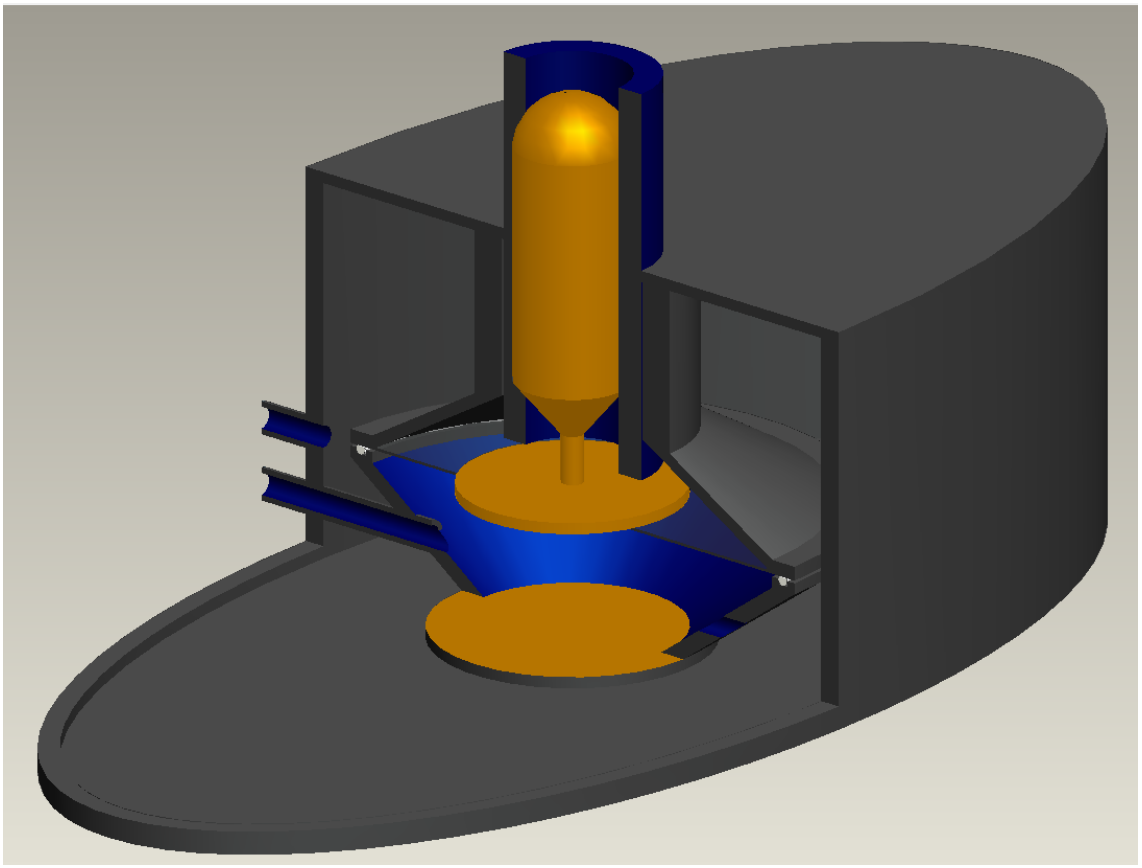

Figure 55 - Angled Half-cut view of third concept.

Concept 4:

The 4th concept pursued was not a fully integrated concept. In this concept a roller pump was chosen as the blood pumping technology. This blood pump and the adsorbent technology were separate entities that could be utilised independently of each other or in combination as per the wish of the user.

Positives:

- Unidirectional flow
- Positive displacement pump, eliminating the need for valves.
- The ability for the components to be deployed independently of each other and replaced easily.
- Ability to retain the blood pumping component after use with no need to clean.
- Very low priming volume
- Highly reliable, and easy to use, working parts.

Negatives:

- Higher profile than some other concepts, as a result of two independent components.
- Limited potential future development.

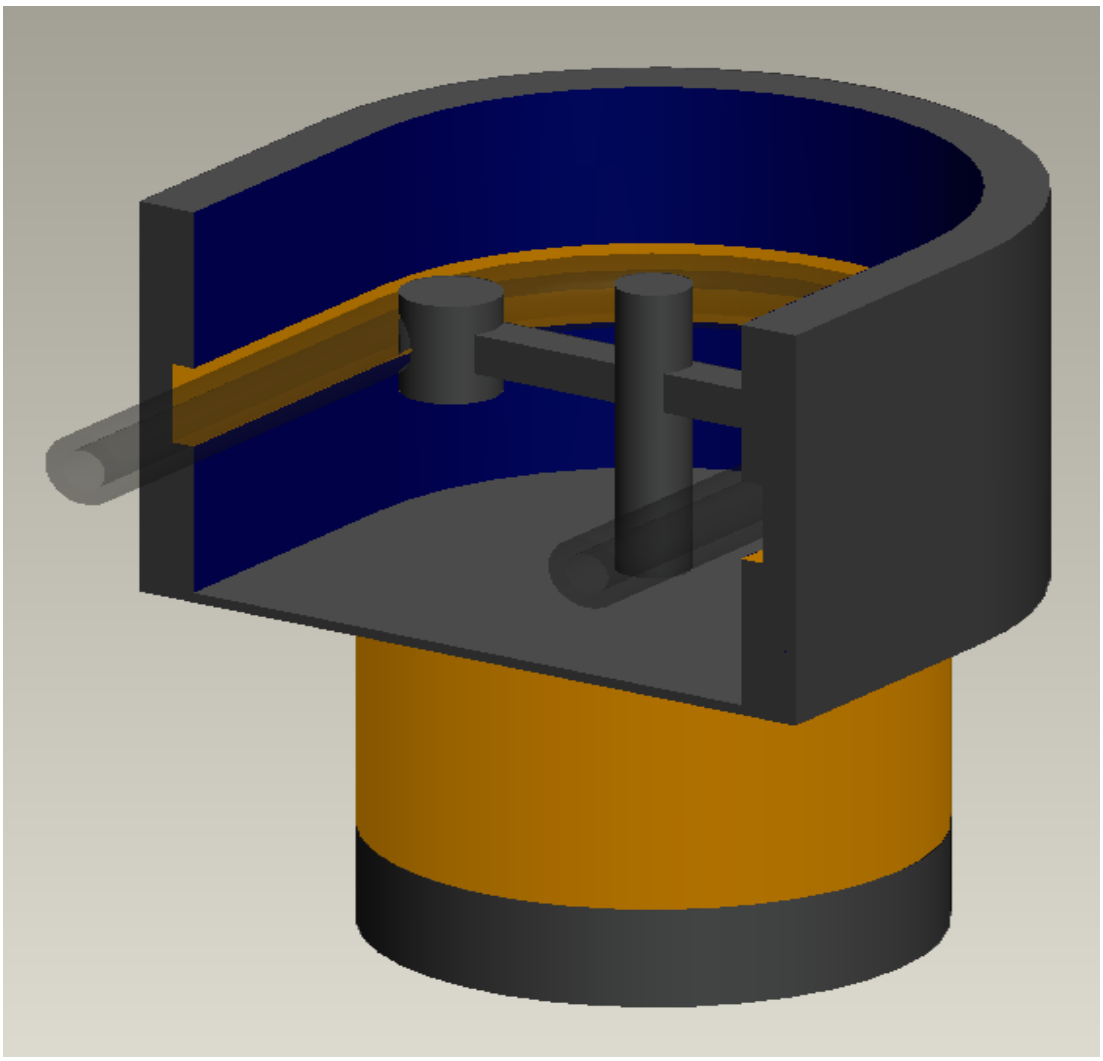

Figure 56 – Angled view of roller pump component of concept 4.

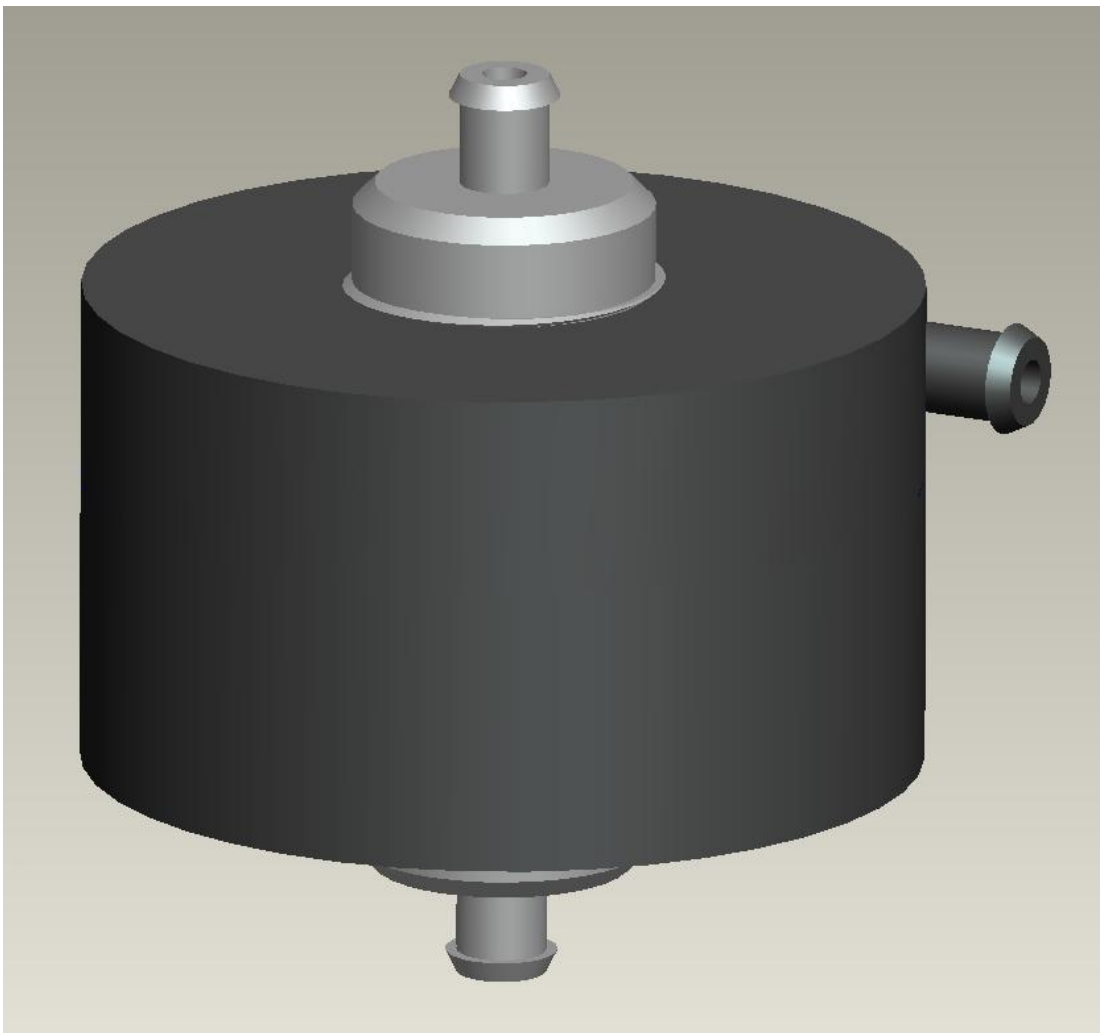

Figure 57 - Adsorbent component of concept 4.
Adsorbent material would be pleated inside.

2.5 Outcomes:

2.5.1 Blood Pumps: Final Design

Despite having developed an early stage rapid prototype of an axial flow impeller, after the assessment of integrated concepts, only the roller pump was continued into the phase of research in which a fully functioning prototype was developed.

The design criteria required for the development of a roller pump prototype were established by looking at existing extracorporeal technologies available on the market. In particular, roller pumps available from Watson-Marlow Pumps Limited (Watson-Marlow Pumps Ltd. Bickland Water Road, Falmouth Cornwall, United Kingdom) were noted as these are used in both clinical and laboratory settings; often offering low flow rate pumps typically used for perfusion in small animal studies. Sadly, the Watson-Marlow pumps are designed for non-portable use and as such, tend to be mains power supplied and are high profile, even when offering low flow rates.

Thus, it was decided to buy working parts available on the market and compose them into a portable roller pump. Based on a review of available technologies, as well as local experience, it was decided that a brushed DC motor would not be appropriate for use due its lifespan, and maintenance required.

A brushless motor was considered to be far more suitable for use. Once control of the motor's speed and torque is established and set to a specified value, the technology requires no attention.

A flat brushless DC motor was chosen for this prototype, in conjunction with its electronic controller counterpart and a compatible 26:1 ratio gearbox. These parts were assembled and provided by Maxon Motors UK Ltd.

A double spring loaded roller head was provided by Watson-Marlow Ltd and silicone tubing was provided by Altec Tubing. The raceway housing for the tubing was machined from aluminium within the Bioengineering Unit. The dimensions of the radial pathway were simply determined by the diameter of the roller and the tubing so as to achieve a suitable occlusion. This raceway was then attached to the working parts with the shaft and double roller protruding into it. Silicone tubing was fed into the raceway and the occlusion was set by adjusting the screws responsible

for the springs. Testing of this prototype in the laboratory has shown it is capable of pumping fluid at a rate of 200ml/min, with a power consumption of 6W.

Figure 58 – Photo of roller pump prototype.

2.5.2 Adsorbent: Final Design

Development of the adsorbent chamber began after the successful development of the adsorbent mat material.

A prototype suitable for multiple flow rates as well as ease of manufacture was considered. For ease of manufacture of the chamber itself an axi-symmetric (circular) adsorbent chamber was finally developed. Rapid prototypes of this concept were printed by laser rapid polymerisation at Strathclyde University's Department of Design, Manufacture and Engineering Management (DMEM). Later superior prototypes were machined from acrylic at the Bioengineering Unit.

Machining circular parts is generally quicker, cheaper, and easier than most other geometries. Choosing a circular geometry allowed for the relatively inexpensive method of using a lathe tool to be used. Though it is less apparent in lower flow rates, a circular chamber is more appropriate for high flow rates. A radial-flow inlet encourages vortex flow within the chamber and thus increases the volume of adsorbent material a fluid must pass through before exiting through the centre of the chamber.

Given that this chamber will be dealing with varying blood flow through it, the threat of air embolism begins to present itself, especially at higher flows. It was therefore decided that 2nd outlet must exist in the device. With the major outlet for blood flow being placed at the bottom of the device, this is the fluid route back to the patient. A 2nd outlet was placed at the top of the device to trap air bubbles and "bleed" the device. Attached to this outlet could also be pressure sensors suitable for detecting air emboli threats.

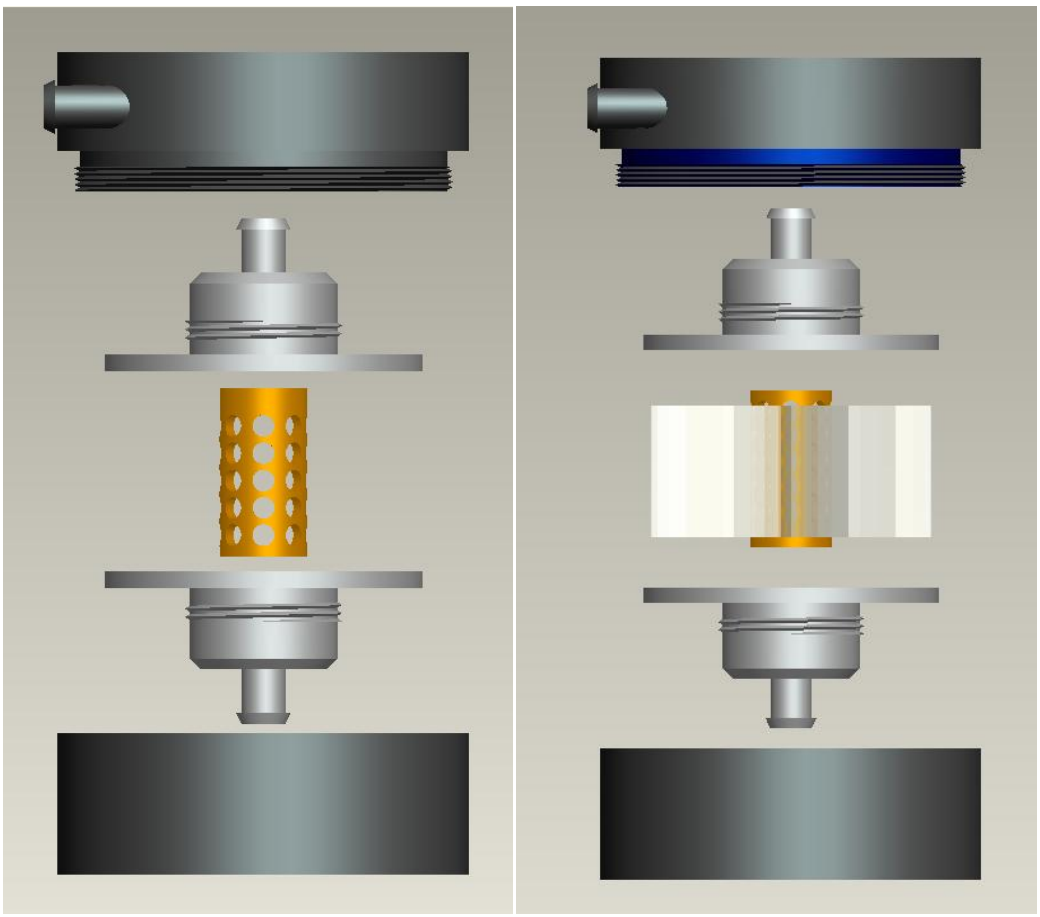

Figure 59 – Exploded side view of adsorbent chamber components.

Left – Exploded view of chamber, without pleated material within, displaying the macroporous diffuser.
Right – Exploded view of chamber, with adsorbent fabric pleated around the macroporous diffuser.

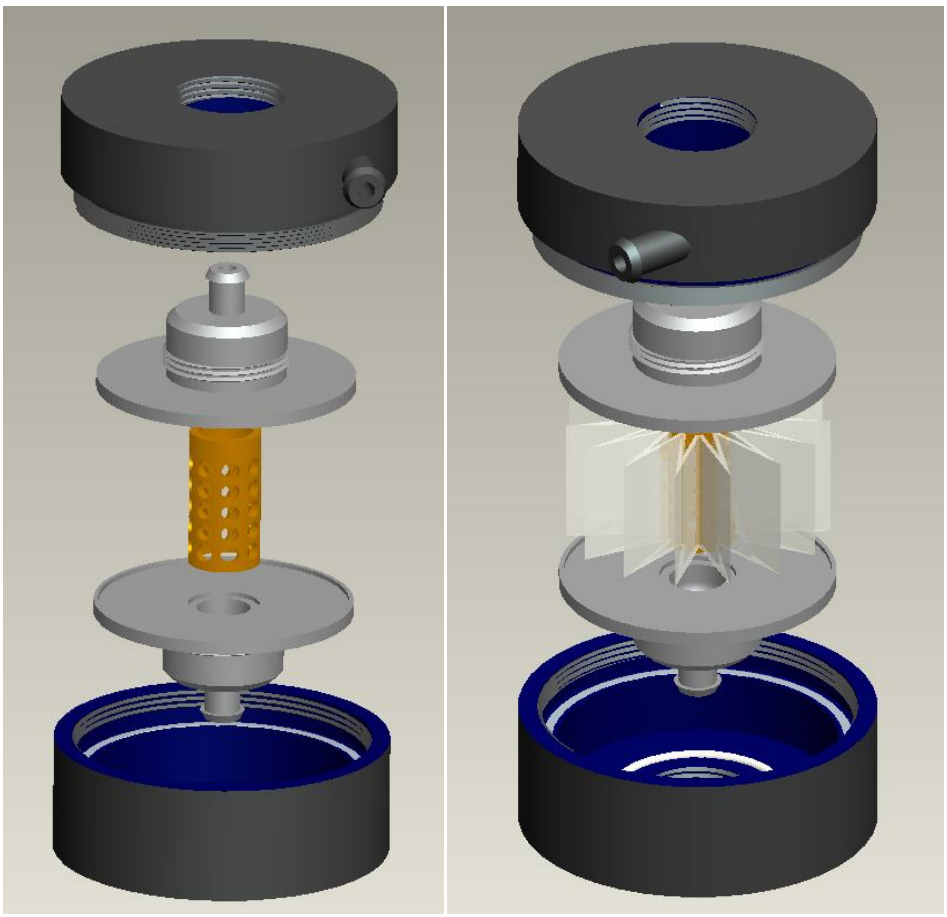

Figure 60 – Exploded angled view of adsorbent chamber components.

Left – Exploded view of chamber, without pleated fabric.

Right – Exploded view of chamber with pleated fabric.

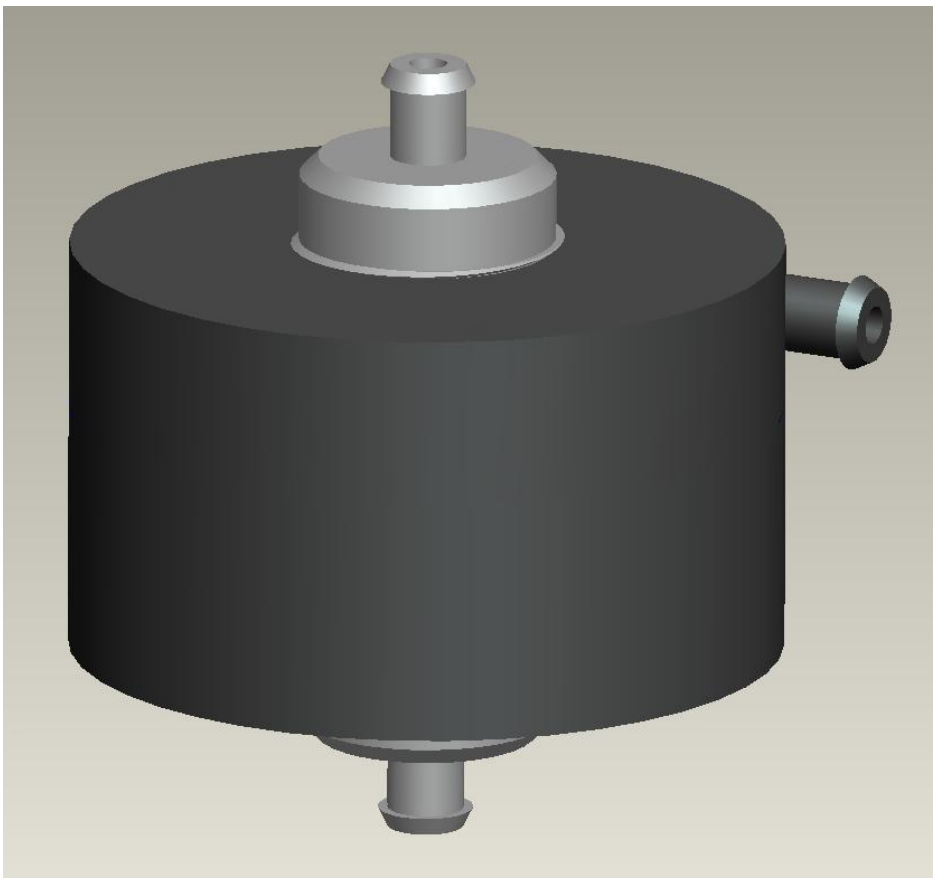

Figure 61 – Full cut view of complete housing

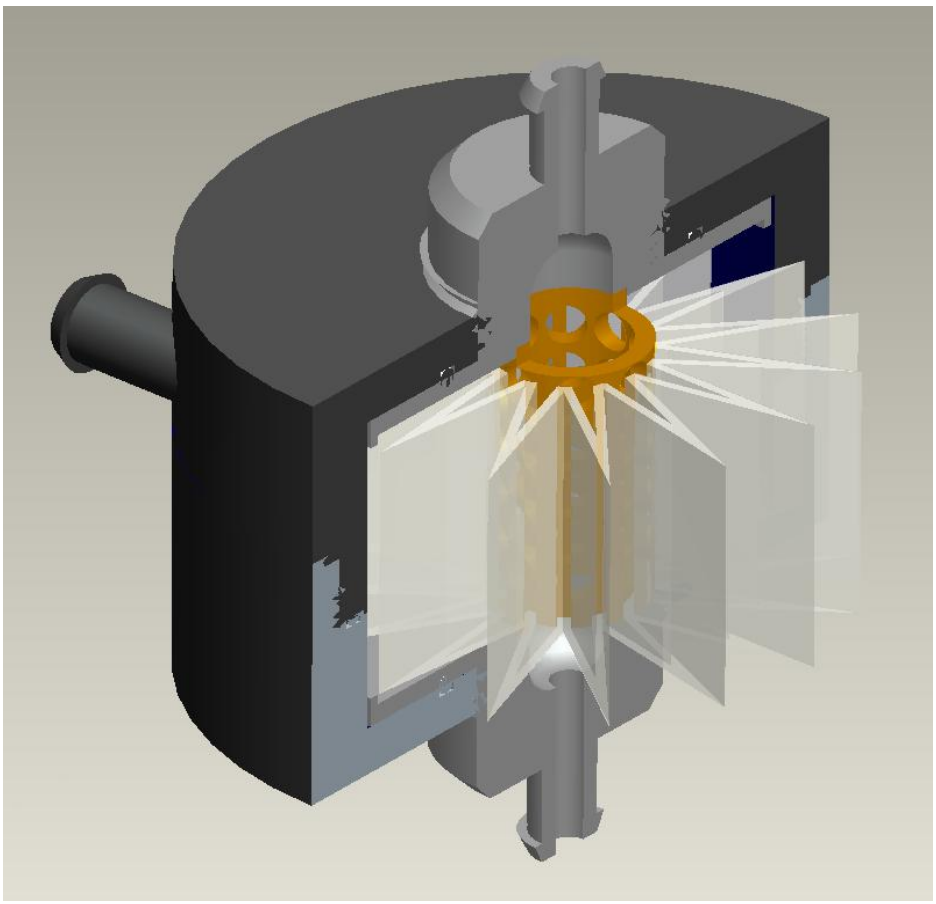

Figure 62 – Half cut view of chamber, showing pleated material.

Between the macroporous diffuser, depicted in gold, and the inner walls of this chamber, depicted in blue, the adsorbent material is inserted in a pleated formation; this material is depicted in semi-transparent white in selected images above. Thus, as the blood enters the chamber through the radial inlet, the blood is forced to pass through the adsorbent material before exiting the chamber through the macroporous diffuser and subsequently the outlet.

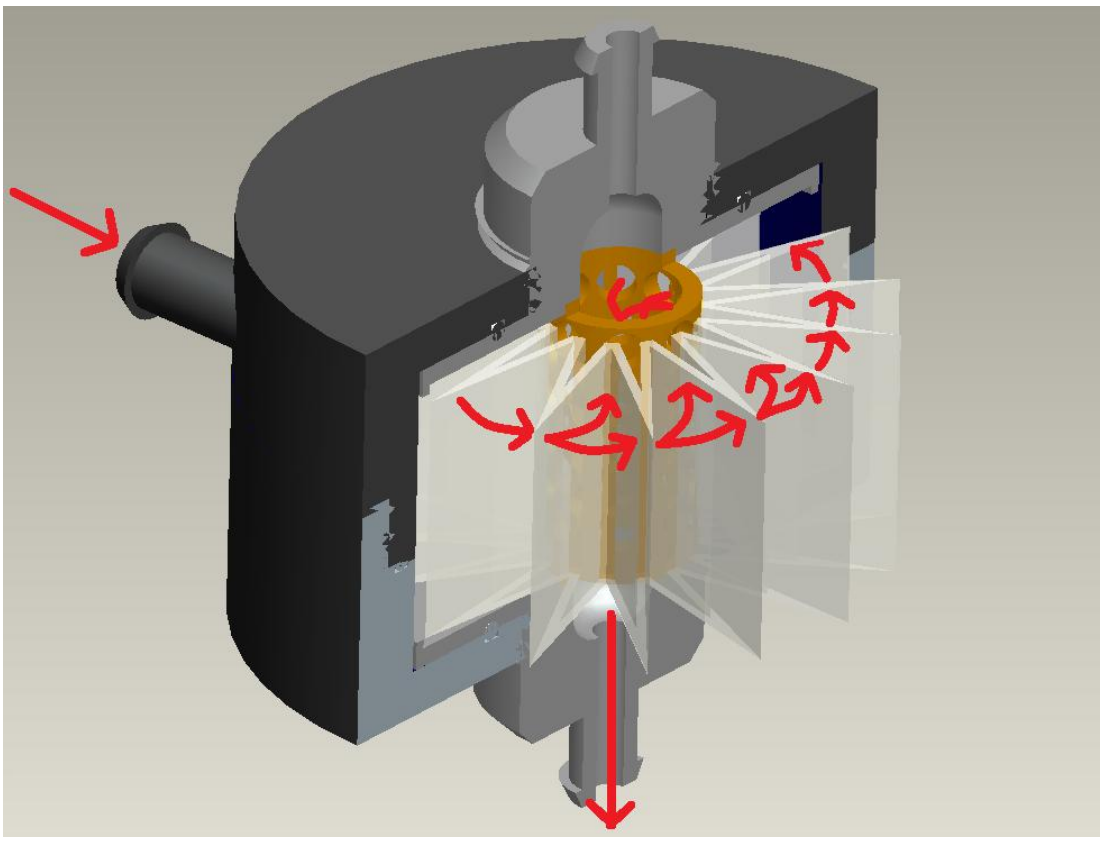

Figure 63 – Blood flow path through adsorbent chamber.

This chamber is made up of 5 metal parts; 2 identical inner parts, a macroporous diffuser, a top part and a bottom part.

It was decided to machine the inlet, outlet and walls of this chamber from 4 parts rather than the possible 2. This was done because the outer top part has an asymmetric feature i.e. the radial inlet. Thus, this piece is difficult to machine from materials such as metals or polymers. Therefore, it was more elegant to produce the outer parts once, and produce disposable inner parts (which are symmetrical around their axis) as many times as is necessary, as these parts are far easier to machine.

Since this chamber is the working part of the device in which blood will pass through, it must be watertight. For this reason, threads and rubber o-rings were introduced into the design. Any two parts that need to be brought together are screwed together by male and female threads. As these threads tighten, an o-ring will be compressed and thus provided a watertight seal.

A “bubble trap” has also been introduced into this design. The result of this is that the 2 inner parts can be machined identically (reducing machining time), as well as

trapping any bubbles brought into the system. This can be seen in the images in the form of a secondary outlet at the top of the device. The rounded feature will act as a trap for any air bubbles and the outlet from this will have a tap that can be used to "bleed" the chamber.

The prototype of this chamber was machined from transparent acrylic and then polished so as produce a highly transparent chamber. This was necessary during the research phase so that it was possible to visualise multiple aspects of its use, such as; flow development, inspection of any mechanical failure, and inspection of coagulation.

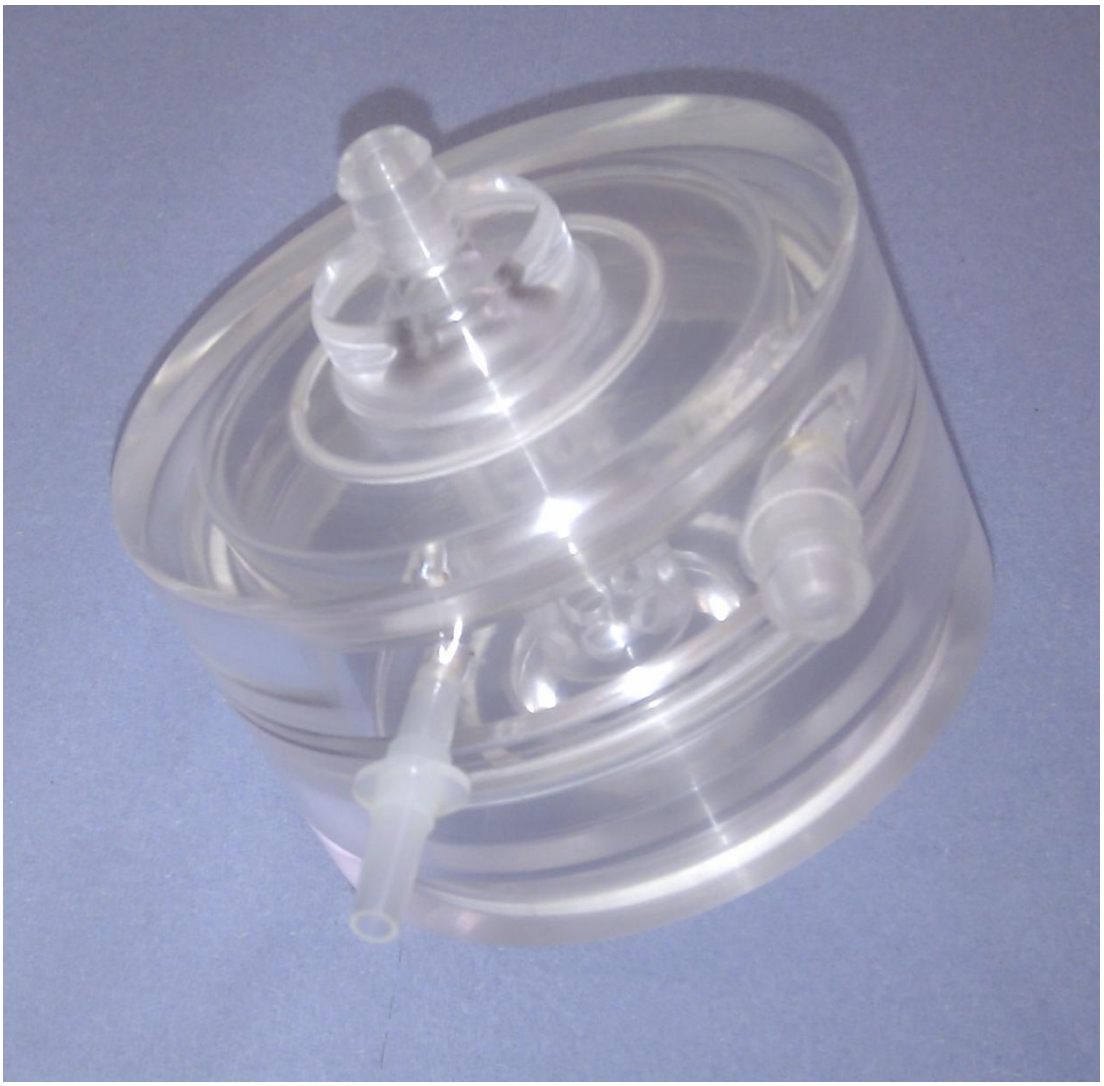

Figure 64 – Photo of adsorbent chamber, manufactured from acrylic.

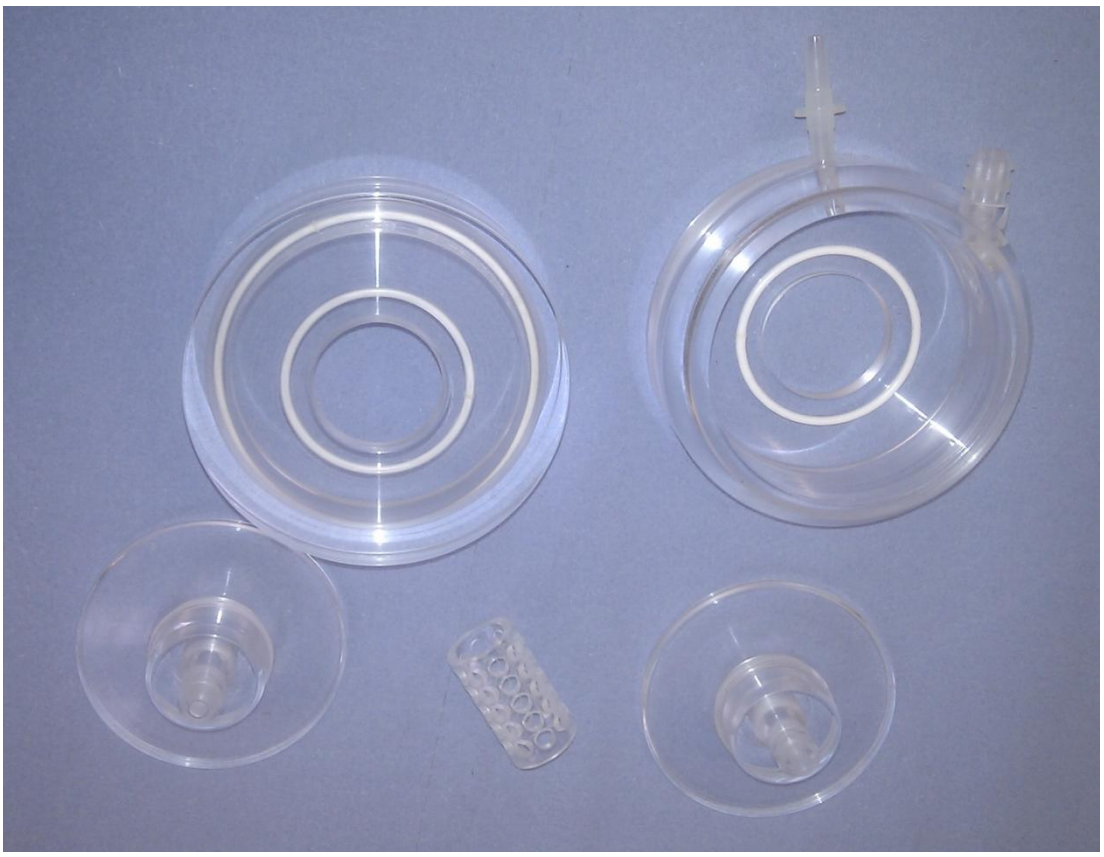

Figure 65 - Component parts of adsorbent chamber.

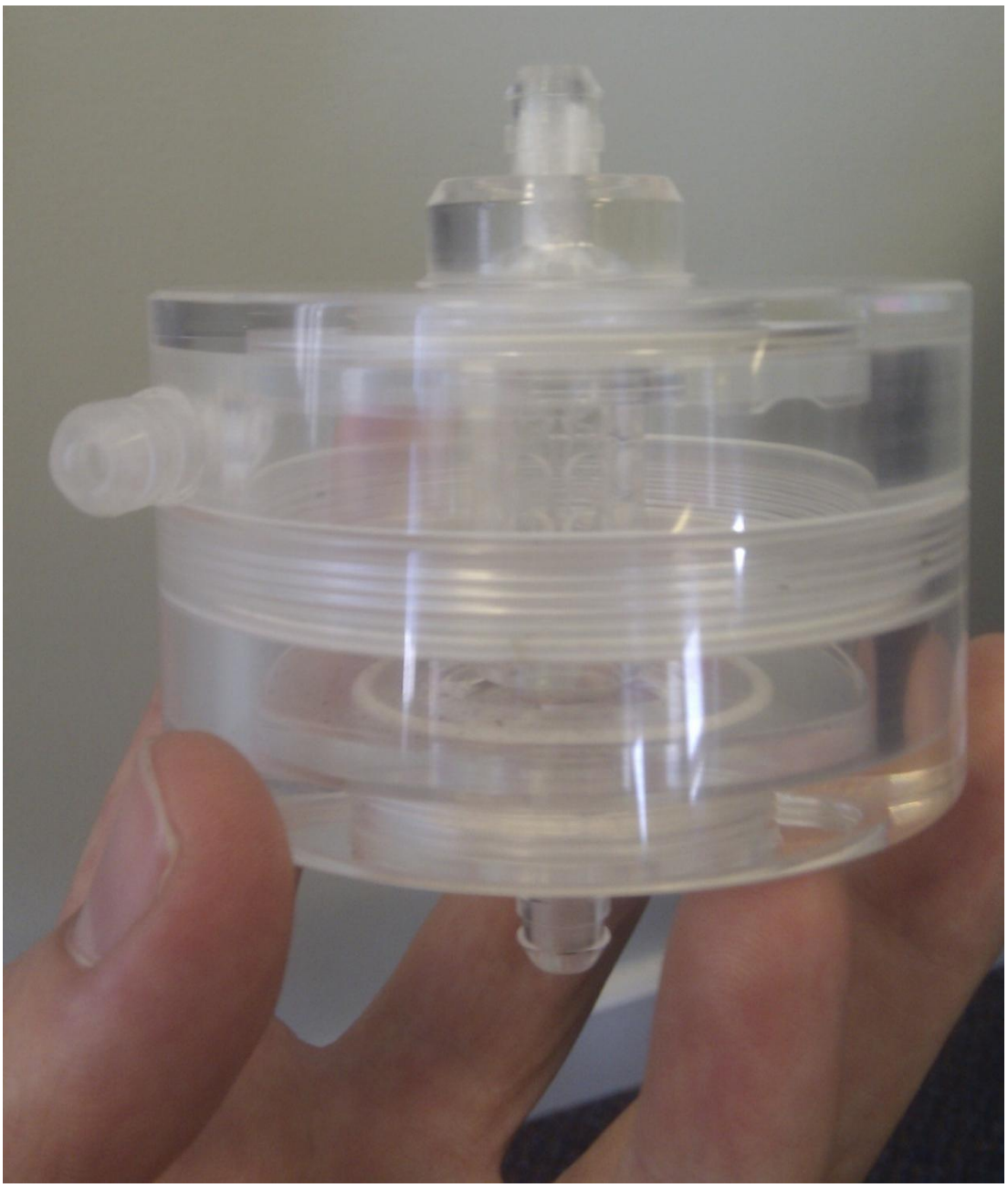

Figure 66 – Full acrylic chamber.

Chapter 3: Adsorbent Testing

3. Adsorbent Testing:

3.1 Introduction:

In order to establish the capability of the adsorbent material in reducing cytokine populations in spiked solutions, a series of laboratory experiments was carried out. The objective of these experiments was to identify the "total" cytokine adsorption capability of the beads and any differential in cytokine species adsorption. Data derived from these experiments would, it was anticipated, inform the design of the finished clinical device, ensuring sufficient adsorption capability across the range of cytokines of interest. In carrying out these studies, a number of commonly used laboratory techniques had to be learned and deployed. These are outlined in the following sections of this thesis.

3.2 Laboratory Techniques:

Media Spiking:

In all experimental procedures regarding the testing of cytokine reduction, a fluid medium was spiked. Different experiments utilised different fluid media, and later different cytokines, but the spiking procedure was identical throughout and is detailed below.

1. Cytokine samples were supplied in solution (frozen on dry ice) by eBioscience Ltd. These solutions were defrosted, then aliquoted into multiple vials upon arrival, in order to minimise waste; only one vial was defrosted for a single experiment, thus avoiding repeated freeze/thaw processes and associated waste. These vials were then frozen at -80°C for later use.
2. Upon the initiation of an experiment utilising recombinant cytokines, samples of cytokines were defrosted and pipetted into a fluid medium to a desired concentration e.g. 1000pg/ml. Henceforth, this shall be referred to as "spiking" a fluid. Throughout the project, many different fluids were spiked; and to different concentrations.

ELISA Technique:

In accordance with the instructions supplied by eBioscience Ltd. the following procedure was followed for each ELISA technique.

1. A 96-well assay plate was coated with 100µl/well of capture antibody suspended in the coating buffer supplied; this was then left to incubate overnight at 4°C.
2. The following morning, the wells of the assay plate were aspirated and soaked with wash buffer (PBS, 0.05%Tween) 5 times, before being blotted on absorbent paper to remove any residual solution.
3. Each well was blocked with 200µl/well of assay diluent and left to incubate for 1hour.
4. The washing step was repeated as before.
5. 100µl/well of appropriate standards and samples were added to the plate, with the standards diluted in assay diluent so as to achieve a standard curve. The plate was then sealed and incubated overnight at 4°C.
6. The washing step was repeated as before.
7. 100µl/well of detection antibody, suspended in assay diluent, were added to the plate and left to incubate at room temperature for 1hour.
8. The washing step was repeated as before.
9. 100µl/well of Avidin-HRP (diluted in assay diluent) were added to the plate. The plate was then sealed and incubated at room temperature for 30mins.
10. The washing step was repeated as before, but repeated 7 times rather than 5 in this instance.
11. 100µl/well of substrate solution TMB (tetramethyl benzidine) were added to the plate. The plate was then incubated at room temperature for 15mins.
12. 50µl/well of stop solution (2M H_2SO_4) were added to the plate.
13. The plate was then read at a light absorbance of 450nm.

3.3 Adsorption Studies: Techniques

Adsorption of cytokines from fluid media is a technique that has been explored by many researchers in recent years. Attempting cytokine reduction has been met with many methods. As discussed earlier, the MDS device utilised an impressive, but complex circuit, requiring multiple working parts, including; primary and secondary circuits (both requiring pumps), plasma separation, transmembrane diffusion, adsorption, and a magnetic trap. The technique utilised in the MDS carried little significance to this project as the device being developed in this project aims to achieve miniaturisation as well as adsorption by direct contact of adsorbents and cytokines i.e. not reliant on diffusion through a membrane, or plasma separation.

In another technology (the CytoSorb device), cytokine adsorption was achieved to significant levels when using horse serum as the medium in which to suspend human cytokines. The use of horse serum intentionally avoided mimicking the clinical conditions likely to be seen *in vivo*, in order to verify a mathematical model designed to predict cytokine adsorption in ideal conditions (DiLeo, Fisher, et al. 2009). And though the experimental verification of the mathematical model was successful, and the reduction of the cytokines was significant, the study lacks clinical relevance as it neglected the complex biological interactions between molecules that occur within whole human blood, such as the interactions between cytokines and their soluble receptors. Another factor distancing this study from clinical relevance was the flow rates used i.e. generally less than 1ml/min. Though a scaled down device was used for these flow rates, the scaling down of the device is only predictive of the mechanical aspects of the cytokine adsorption, again neither presenting the complex biological situation, nor the fluid mechanics that would occur in a "real" sized device. This study showed appropriate methods in which to test the mechanical scenario of cytokine adsorption with the adsorbent beads used, in ideal conditions (as was its remit), but did not present appropriate methods by which to display clinical relevance of a device attempting direct whole blood contact. Although the difference plasma and haemo-adsorption cannot be underestimated, the mathematical model, however, may be useful in discussing some results (DiLeo, Kellum, et al. 2009).

Another study group which attempted cytokine reduction to a much higher clinical relevance, as well as much closer to methods utilised by this project, was a research

group based at the University of Brighton. This research group regularly used human cytokines TNF-α, IL-6, and IL-10 and suspended them in defrosted fresh frozen human plasma, and used this as a medium in which to test the cytokine reduction. Flow rates were also much closer to clinical scenarios and considerable success was had. The industrial collaborators of this research group were Mast Carbon Ltd. (the same provider of carbon ceramic adsorbents to this project). Results from this research group were highly significant to this project as the same adsorbents were utilised and in similar methods as used here. However, a very critical difference exists in terms of the presentation of the adsorbent beads. In the present study, the adsorbent beads were immobilised in a superior matrix material with the intention of achieving whole blood contact.

The Brighton study lacks appropriate controls, which introduces serious limitations in terms of framing the results in both the laboratory and clinical contexts.

3.4 Laboratory Results: Rat Cytokines

TNF-α is a starting point for study of cytokines adsorption, as it has known clinical relevance. Adsorption of TNF-α is well described in the literature and represents a good test bed for the new technology.

Though biologically no (or negligible) cross-reactivity exists between the human and rat forms of the molecule TNF-α and its receptors, structurally the molecules are very similar, both having a molecular weight of 17kDa, being produced in the same manner, and both having had dimers and trimers observed. The rat molecule is an appropriate model by which to test the adsorptive mechanical properties of the adsorbent beads utilised, and should be highly predictive of the efficacy of beads with human molecules.

3.4.1. Aims:

Initially, a simple model of cytokines suspended in fluid was utilised to examine the capacity of our adsorbent beads under near-physiological conditions. PBS was used as the suspension fluid as it mimics the concentration gradients of salts that might be observed in blood, and therefore the diffusion characteristics that can be expected clinically, but lacks other elements of biological complexity that might confound the adsorption processes. In this regard, PBS is an ideal solution to investigate adsorption kinematics.

3.4.2 Initial Results:

Methods:

6ml of freshly prepared PBS, spiked to 1000pg/ml of recombinant rat TNF-α (supplied by eBioscience Ltd.), were added to 4 test tubes. Added to these test tubes were 4 different weights of carbon, these were; 0.05g, 0.025g, 0.0125g, and 0g. The test tubes were placed on a roller mixer apparatus and samples were taken from these test tubes every 15mins for 2hours and were analysed by ELISA for TNF-α concentration in the fluid.

Results:

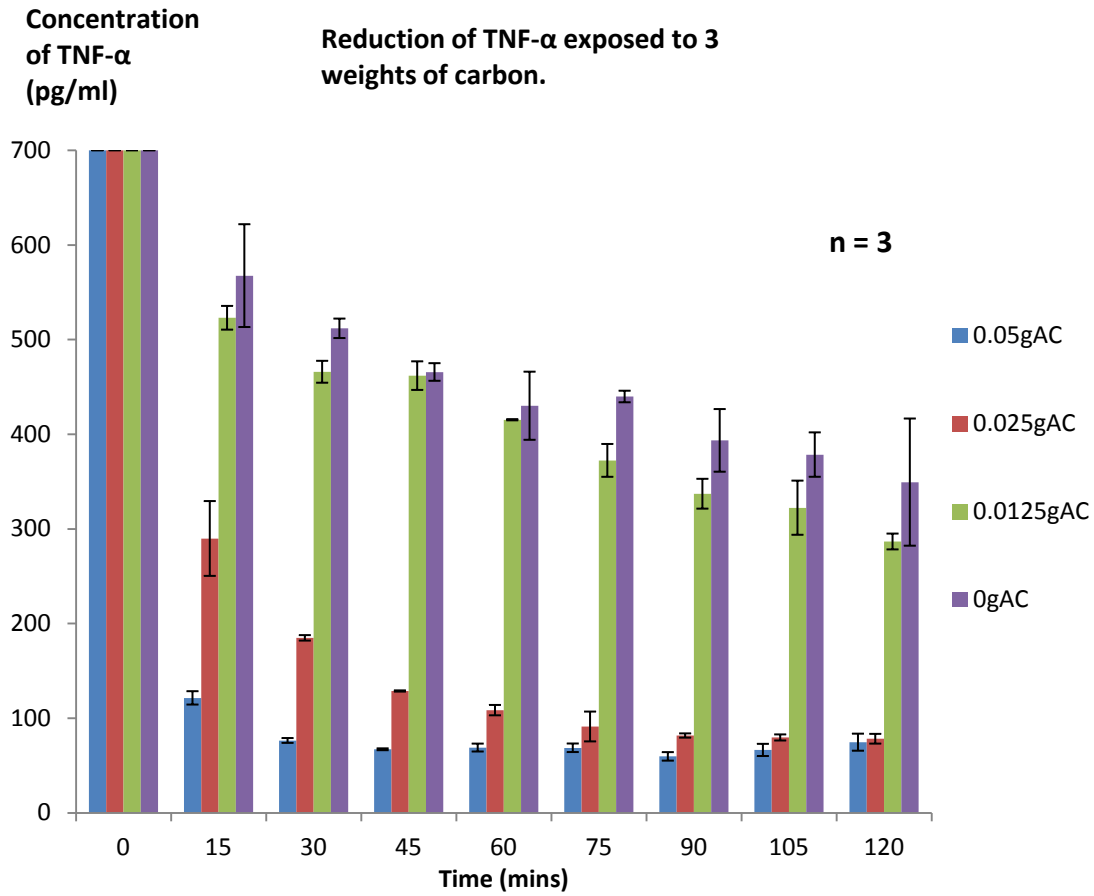

Figure 67 – Cytokine reduction in varying weights of carbon.

It can clearly be seen here that the higher the weight of carbon, the more significant the reduction in TNF-α.

It appears that the highest weight of carbon reduces the concentration of cytokine rapidly in the solution, reducing the TNF-α concentration to minimum values after as little as 30mins.

All groups showed a significant decay in TNF-α over time. The differences between the 0.05gAC and 0.0025gAC were statistically significant after 15mins ($p<0.005$) but were statistically insignificant from 75mins to the end of the process ($p>0.05$). The 0.05gAC and 0.0025gAC were associated with significantly greater adsorption of cytokines than the 0.00125gAC group ($p<0.005$) at all time points with the exception of start (zero time) level. All carbon groups showed a statistically significant difference from the control group. This was consistently the case across the timeline of the study when comparing the 0.05 and 0.0025 groups with control, but only reached significance when comparing the 0.00125gAC group with controls at the final time point.

Interestingly, the TNF-α control appears to be reducing somewhat, over the 2 hours.

3.4.3 Re-Challenging Adsorbents:

Based on previous research undertaken in the department on this adsorbent material, in the initial stages of this project, we were confident that the adsorbent was suitable for this application. So initially, it was decided to repeatedly challenge the adsorbent in order to replicate, to some extent, what might be seen in a clinical environment.

Methods:

3 Eppendorf sample vials were taken and labelled. Each vial had 0.25ml (0.05g) of carbons beads added to them. This carbon was then primed with 0.3ml of PBS. A freshly prepared solution of PBS was prepared and spiked with recombinant rat TNF-α to a concentration of 1000pg/ml, this will henceforth be referred to as stock solution. 0.5ml of this stock solution was taken and placed into each vial.

A sample of the stock solution was taken before the experiment began, and was frozen for later analysis by ELISA.

After 1 hour, 0.5ml of solution was removed from each vial and frozen for later analysis by ELISA.

The 0.5ml removed was then replenished with a further 0.5ml of stock solution in an attempt to re-challenge the adsorbents. The removal and replenishment of samples was repeated after 1 hour a further twice, in order to give 3 samples for each of the 4 time points.

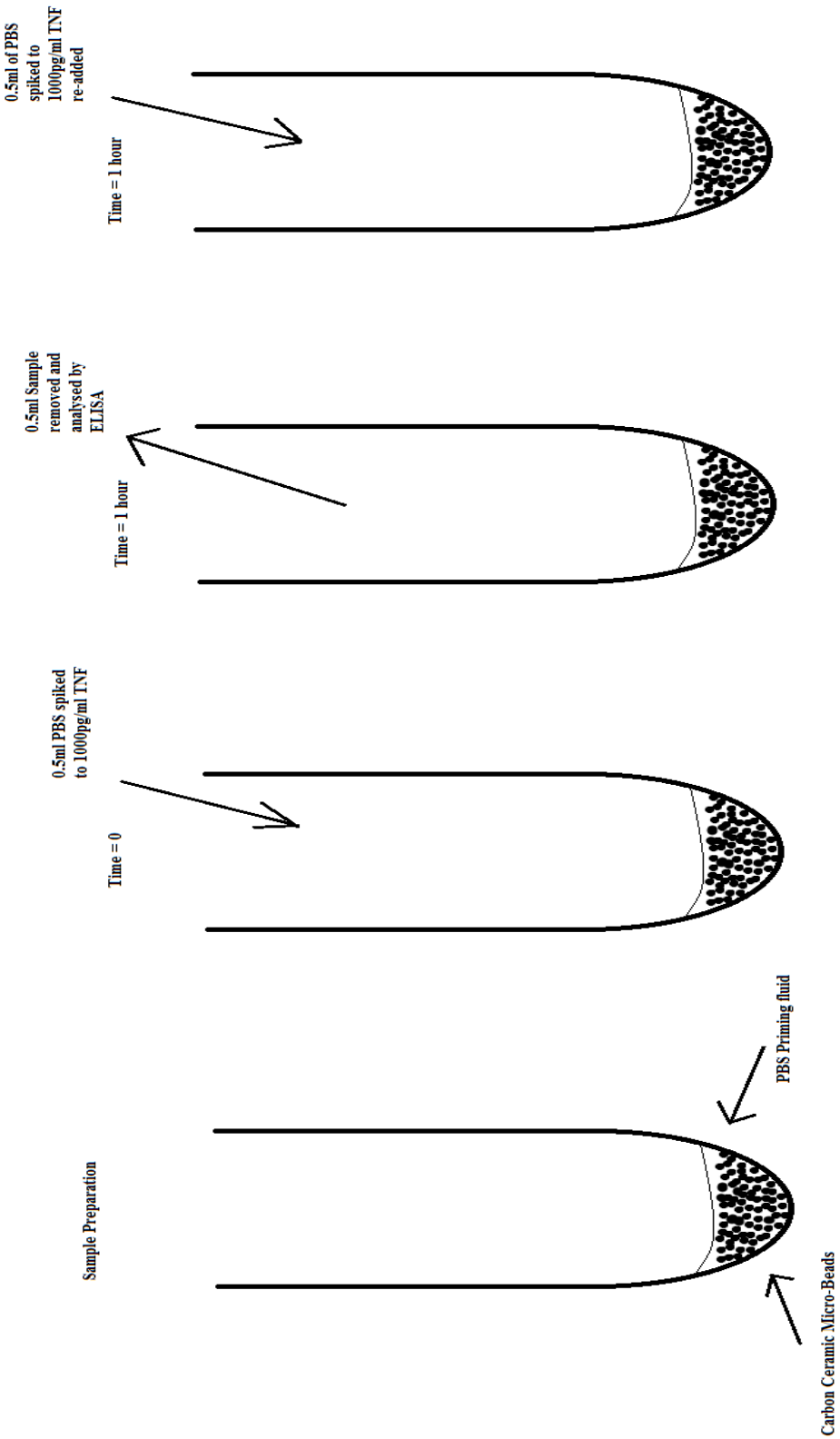

Figure 68 - Experimental flow chart

Based on the concentrations read by the ELISA for samples that had been exposed to an adsorbent for an hour, the concentration of the solution after re-spiking was calculated, based on the known concentration of the stock solution.

This protocol gave a pre-exposure cytokine concentration, followed by a post-exposure cytokine concentration for 4 time points.

Results:

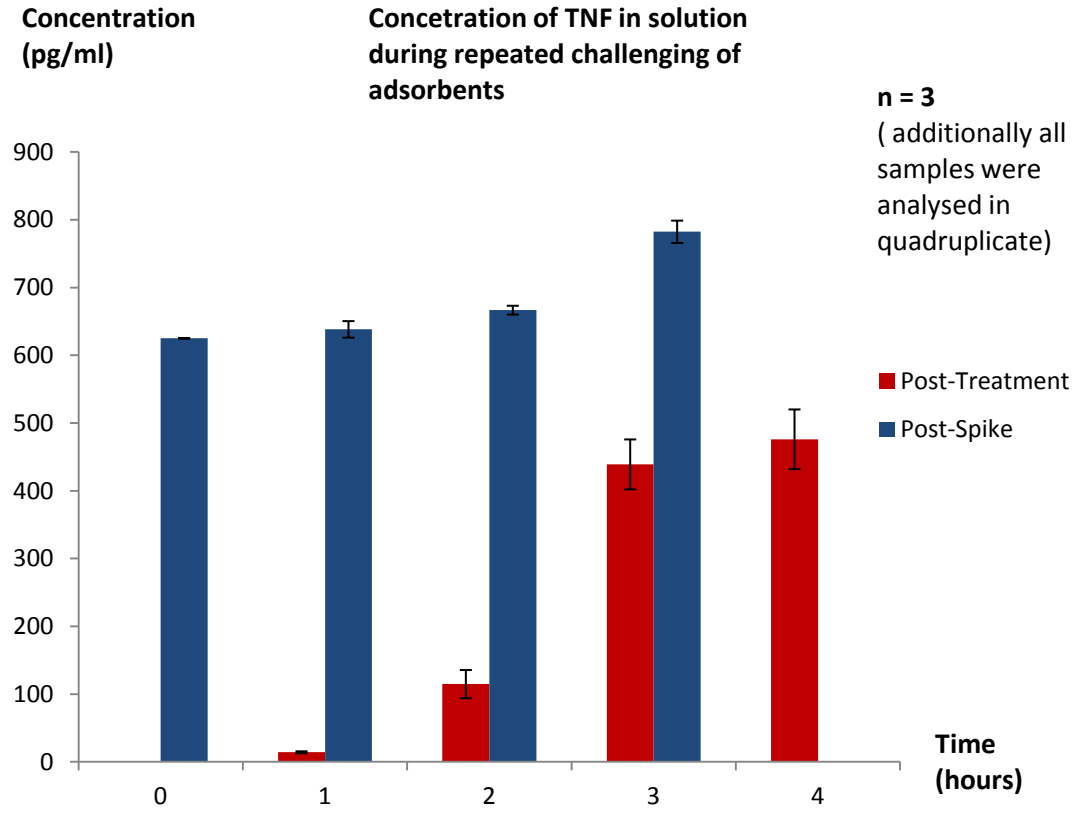

Figure 69 – Cytokine concentration during re-challenging of adsorbents, over 4 hours.

As can be seen here, the first exposure (from time=0 to time=1hour) to adsorbent saw the most dramatic reduction in cytokines in all experiments. After re-spiking the test solution, the rate at which the adsorbent performed reduced over the 4 hours, but does not reach saturation.

An interesting point to note here is that, despite the fact the initial stock solution of PBS with TNF-α was supposedly spiked to a "known" value of 1000pg/ml, it appears only to be reading at a concentration of approximately 600pg/ml. It is clear from the results that the adsorbent is performing, but the concentration of the initial solution is an issue that needs to be addressed. The removal that can be witnessed from this

experiment suggests that over 1300pg of cytokine have been removed, though it is difficult to make such a statement categorically given the concentration of the initial solution.

It was decided to repeat this experiment and perform the experimental protocol for a further 2 hours in an attempt to saturate the adsorbent.

Methods:

Initially, a solution of freshly prepared PBS was spiked with the cytokine recombinant rat TNF-α to the concentration 1000pg/ml. A sample of this stock solution was taken and refrigerated for the duration of the experiment until analysis by ELISA.

0.05g of adsorbent beads was placed in 3 Eppendorf sample vials and was primed with 0.5ml of PBS, to wet the beads.

1ml of the stock solution was added to the vials and they were left to incubate on a plate shaker for 2 hours. During this 2 hour incubation/shaking period, every 15mins, a 1ml sample of solution was extracted from the vials and was replenished with 1ml of the initial stock solution. The samples that were removed were refrigerated until analysis by ELISA was performed immediately after the experiment. The re-spiked concentration in the solution was calculated, based on the concentration of residual 0.5ml in the vials and the concentration of the stock solution analysed at the beginning of the experiment.

Results:

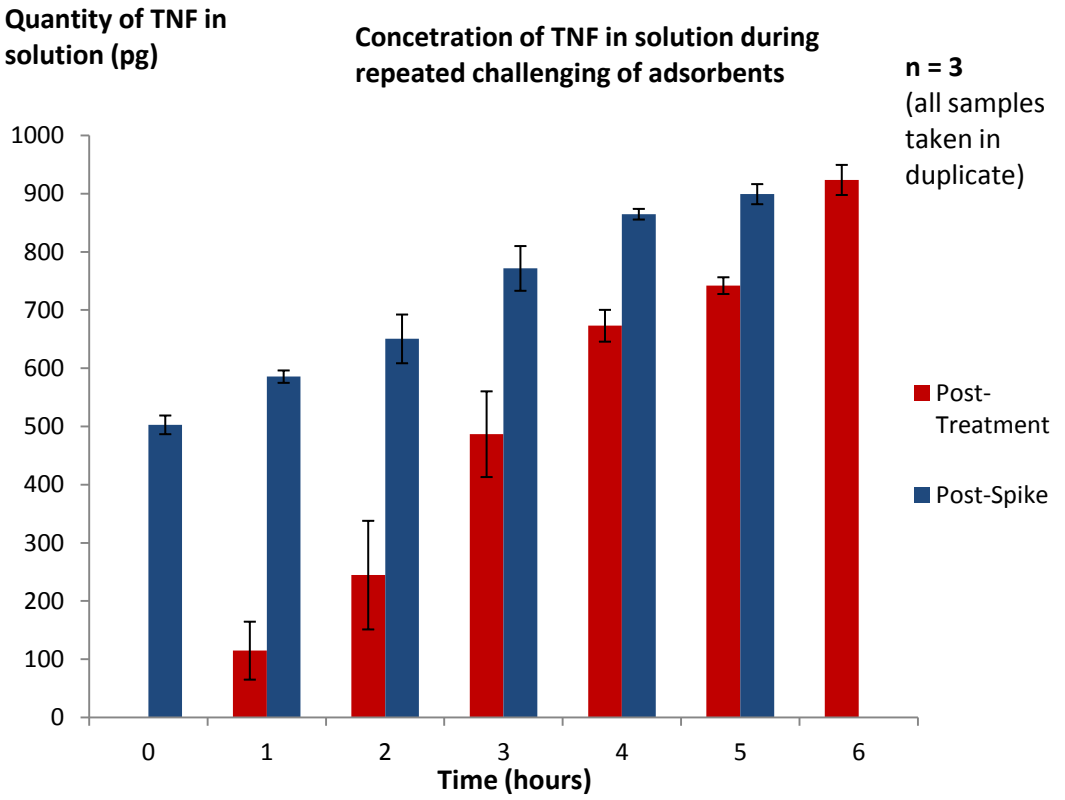

Figure 70 – Cytokine concentration during re-challenging of adsorbents, over 6 hours.

A similar trend can be seen here as before. Again, the control solution that was supposedly spiked to a concentration of 1000pg/ml appears to only be reading at a concentration of approximately 500pg/ml.

Again, it can be seen that the first exposure of adsorbent to cytokine results in the most dramatic reduction of cytokine throughout the protocol. As the adsorbent is exposed repeatedly to further stock solutions, the rate at which the adsorbent performs appears to decrease, until the 5th spike point, at which point there is no significant adsorption.

Also worth of note is that although the post-spike/pre-adsorption concentrations (effectively acting as a control) appear to be increasing, this is due to the remaining 0.3ml of solution in each vial after a 0.5ml sample is taken. Thus, when there is 0.3ml PBS with 100pg/ml TNF solution remaining in a vial (as in the case after 1hour), and 0.5ml of stock solution is added, the resultant solution concentration will increase slightly.

3.4.4 Controls Analysis:

Introduction:

In carrying out these experiments, a number of issues became apparent with regard to quality control of the assay procedure. In particular, a series of control plates was run, in which there were large discrepancies on the control sequences. This resulted in some dissatisfaction with the overall TNF-α assay process, relating to some poor calibration curve construction.

Upon discussing this issue with eBioscience Ltd, it was suggested this may be due to the lack of carrier protein in the solution.

We thought this phenomenon worthy of further investigation, with specific regard to investigation of the molecules used in these experiments and to ascertain that these were indeed appropriate for use, by making a comparison between the recombinant rat TNF-α molecule provided by eBioscience Ltd and used to spike the test solution, and the recombinant rat TNF-α molecule provided as a standard with ELISA kits from eBioscience Ltd and used to calibrate the assay process. This was done as in order to establish whether or not the cytokines that had been thawed, aliquoted, and re-frozen upon arrival, had degraded to a point at which their use is no longer appropriate. This comparison was possible as the molecules provided as standards with an ELISA kit arrived in single use vials and thus did not require a thawing/freezing step. During this analysis, it was also decided to check which solution is most appropriate for use in this application, with particular attention paid toward the presence of carrier proteins, as another variable from the previous experimental protocols is that the standard provided with the ELISA is diluted in assay diluent before being put in an ELISA plate, whereas the molecule provided independently has until now been diluted in PBS rather than assay diluent.

Methods:

Lyophilised recombinant rat TNF-α was delivered from eBioscience Ltd at -20°C. ELISA assay diluent was added in accordance with the manufacturer's instructions in order to reconstitute it to liquid form. This liquid was then diluted and aliquoted into multiple vials and re-frozen at -80°C.

Rat TNF-α ELISA kits were delivered by eBioscience Ltd. Within these kits, were frozen samples of recombinant rat TNF-α, in a non-lyophilised form. These samples were already aliquoted into single use vials upon delivery.
Multiple solutions were prepared for this experimental protocol. These were; PBS, PBS (0.05%Tween20), PBS (1% w/v Bovine Serum Albumin (BSA)), PBS (1%BSA, 0.05%Tween20), PBS (10% v/v Foetal Calf Serum (FCS)), PBS (10%FCS, 0.05%Tween20). These solutions were chosen as they mimic the recipes of various manufacturers' assay diluents. In this case, the PBS with FCS and Tween20 mimics the assay diluent provided by eBioscience Ltd.

Frozen samples of both of the rat TNF-α molecules were taken and defrosted. Both of these samples were then prepared to a concentration of 1000pg/ml for each of the 6 solutions. The top standards were then used to perform series dilutions to provide solutions of the concentrations of; 500, 250, 125, 62.5, 31.25, 15.625, and 0pg/ml for each solution.

Each of these series dilutions, for each molecule, and each solution were all added to an ELISA plate in accordance with the ELISA instruction as though each was a line of standards. After performing the necessary steps in the ELISA protocol, the plate was read in a plate reader and the light absorbance readings are shown below.

Results:

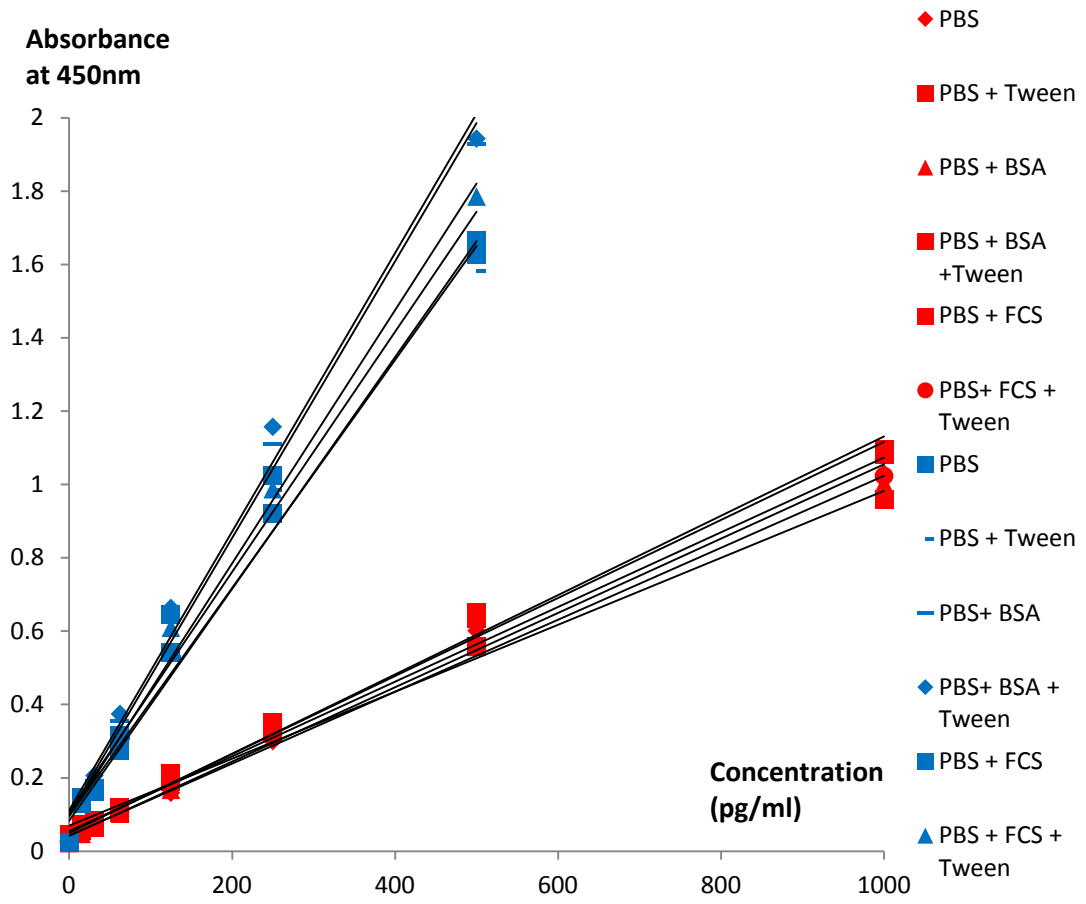

Figure 71 – Control analysis of ELISA protocol.

Shown in blue are rat TNF- α standards provided by eBioscience Ltd. as part of the ELISA kit. These are used in order to calibrate the light absorbance reading with concentration of TNF- α in the liquid.

Shown in red is are Rat TNF-α molecules purchased from eBioscience Ltd. independently of the ELISA kits, prepared according to the instructions, to the same "known" concentrations of the standard curve i.e. 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0pg/ml, in order to observe the difference between the standard curve and control curve.

It can clearly be seen that there is significant disparity between these calibration lines.

Conclusion:

The disparity between the calibration curves a result of which molecule is used clearly suggests that either the ELISA kit is highly unreliable, or the TNF-α molecules provided independently are unreliable. Regardless of which is true, it is not possible to draw reasonable conclusions from experimental procedures using either of these products.

Upon discussing these data with eBioscience Ltd, they responded by saying that the molecules should not be considered to be compatible and that eBioscience would address this issue.

Most previous experiments have been based on the assumption that these two products are compatible. This assumption, however, has been shown not to be true. The impact of this finding is significant, and suggests that although trends in adsorption might be investigated using this combination of molecules, the absolute values have to be called into question, whichever diluent is employed. The implication of this finding may be considerable; as other investigators may have assumed that the molecules are compatible, resulting in a very favourable result when investigating adsorption.

3.4.5 Protocol Refinement:

Subsequent Aims:

Given the issues surrounding the use of rat cytokines *in vitro*, experiments based on the use of human inflammatory molecules were pursued. With this approach, it is possible to continue onto preclinical trials rather than animal models. Also, on discussing the issue with eBioscience, we were assured that in the case of human molecules, the independent recombinant proteins and the corresponding ELISA kits are compatible and fit for use in our proposed laboratory work.

Bearing in mind our experience with rat molecules, the first issue to be considered in planning the subsequent human molecular laboratory work was selection of the most suitable fluid medium for the experiments.

Introduction:

To determine the most suitable fluid medium to use as a diluent for the human cytokines, we constructed a calibration curve for a range of standard laboratory solutions. The level of conformity of these calibration curves with the standard calibration curves derived from the ELISA kits was assessed to ascertain the most appropriate fluid medium.

Initially, 3 solutions were used to in order to gather information on the calibration curves and therefore the suitability of the fluid media. These were bovine plasma, PBS, and assay diluent (multiple recipes exist for this and usually change from provider to provider, but typically consist of PBS, a carrier protein, and occasionally Tween20). Bovine plasma was attempted, with a view to proceeding onto bovine blood, as conducting experiments in bovine blood could mimic the mechanical properties of human blood and could provide a valuable alternative to human blood, which is expensive and challenging to source.

Methods:

Bovine blood was sourced from the local abattoir at no cost and centrifuged down to plasma in batches of 10ml at a time until sufficient plasma was available for the experimental procedure.

A solution of fresh PBS was prepared prior to this protocol by dissolving PBS tablets in distilled water in accordance with the instructions provided with the tablets.

Recombinant human TNF-α, recombinant human IL-6, and recombinant human IL-10 were supplied by eBioscience Ltd. Upon arrival, these frozen solutions were defrosted, aliquoted, and re-frozen at -80°C.
ELISA kits for each of these molecules were also provided by eBioscience Ltd. The standards provided in these kits were also stored at -80°C upon arrival.
A fresh solution of assay diluent was also prepared from the concentrated solution provided with the ELISA kits.

Standard concentrations representing the upper range of each ELISA concentration were prepared for each of the three molecules for each of the three fluids. These top standards were; 500pg/ml for TNF-α, 200pg/ml for IL-6, and 300pg/ml for IL-10.
Series dilutions were then performed for each of these 9 solutions to provide the concentrations necessary to provide calibration curves.
The concentration series for each molecule were then put in their corresponding ELISA plates in accordance with the manufacturer's instructions (see generic methods). After completing the necessary steps in the ELISA protocol, the plates were read in a plate reader.

Results:

TNF-α:

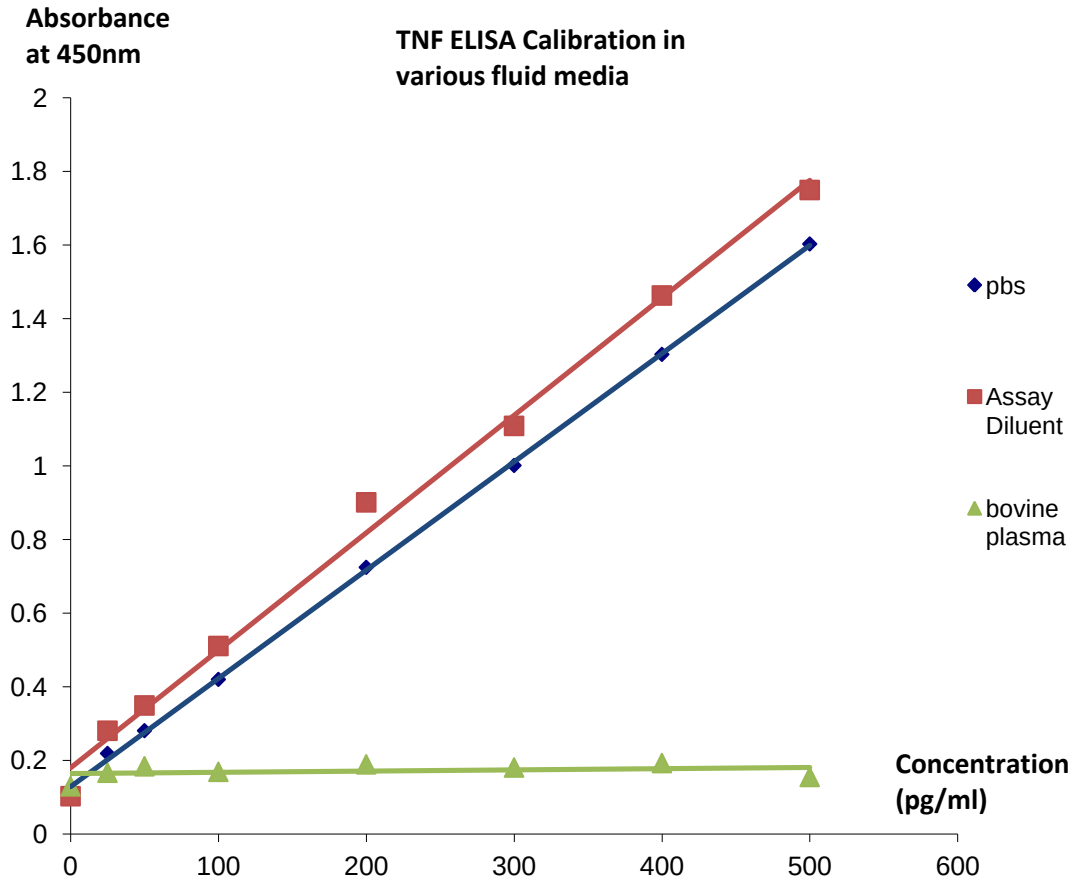

Figure 72 – Control analysis of TNF-α ELISA, utilising various fluid media.

It can be seen that as the concentration incrementally increases, so does the absorbance reading for both PBS and assay diluent in the fashion of a straight line. Such a straight line calibration cannot be seen for bovine plasma.

IL-6:

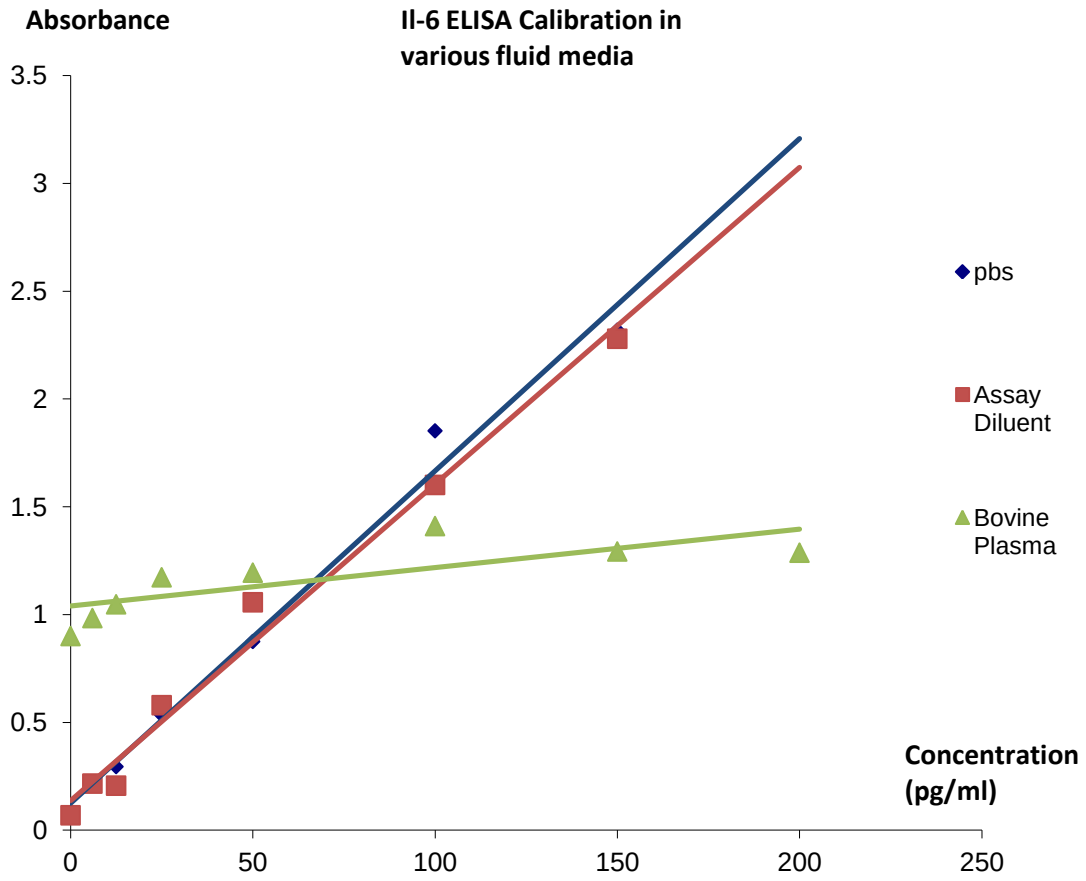

Figure 73 - Control analysis of IL-6 ELISA, utilising various fluid media.

It can be seen that as the concentration incrementally increases, so does the absorbance reading for both PBS and assay diluent in a straight line fashion. A straight line calibration cannot be seen for bovine plasma.

IL-10:

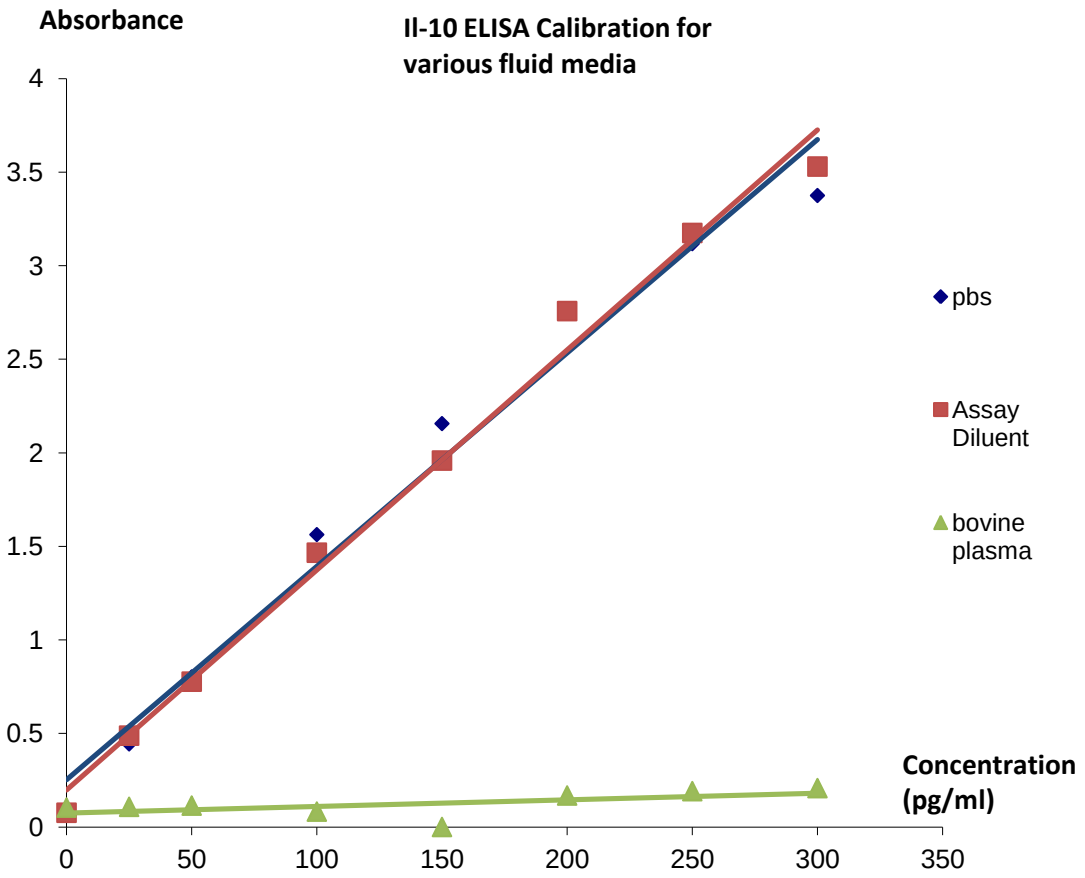

Figure 74 - Control analysis of IL-10 ELISA, utilising various fluid media.

It can be seen that as the concentration incrementally increases, so does the absorbance reading for both PBS and assay diluent in a straight line fashion. A straight line calibration cannot be seen for bovine plasma.

Conclusion:

It can clearly be seen from figures 72 - 74 that both PBS and assay diluent are perfectly appropriate for use, as they provide matching calibration lines, but that bovine plasma does not provide a reasonable calibration curve and should not be considered for use with any of these molecules in our adsorption studies. The straight line calibration curves indicate that a reliable correlation can be drawn between the concentration of a cytokine in a fluid and the light absorbance detected by an ELISA plate reader. For all 3 molecules, the calibration made with PBS provides a straight line in agreement with the recommended standards provided by the manufacturer of the ELISA kits.

This indicates that PBS may be used as an appropriate fluid medium for future experimental protocols. The use of PBS is preferable to the use of assay diluent from the perspective of financial constraints. Readymade assay diluent can be provided by manufacturers but only at considerable cost. That there was no measurable difference between PBS and assay diluent was considered to be a bonus in terms of the future conduct of the laboratory experiments. Its low cost, ready availability and apparent similarity to the assay standards suggest that it is an ideal diluent for the cytokines of interest.

3.5 Laboratory Testing: Human Cytokines

3.5.1 Aims:

The purpose of the following series of experiments is to provide an insight into the performance of the adsorbent technology that is being implemented in this project with a view to informing the design of a portable adsorbent device.

3.5.2 Human Cytokine Adsorption:

Methods:

A 1litre solution of PBS was freshly prepared prior to this experiment. This litre of PBS was spiked with the human cytokines TNF-α, IL-6, and IL-10 to their top standard concentrations i.e. 500, 200, and 300pg/ml respectively.

This spiked solution was split evenly into two separate beakers (500ml in each).

0.5g of active carbon adsorbent beads was added to one of these beakers. The other solution remained without adsorbent as a control.

A magnetic flea was placed in both beakers and spun throughout the duration of the experiment. This provided a dynamic setting for the adsorption process as well as reliable mixing of the fluid throughout the protocol, allowing for both a consistent concentration gradient throughout the experiment as well as reliable readings.

1ml samples were taken every 10mins, until 16 samples were taken from each solution.

The samples were centrifuged to remove any excess carbon beads from the solutions when necessary. Samples were immediately refrigerated until all samples were taken. The samples were then added to a coated ELISA plate and left to incubate at 4°C overnight in accordance with the manufacturer's instructions.

Results:

The following 3 figures show the concentration of various cytokines in a solution of 500ml PBS, initially spiked to concentrations of 500pg/ml TNF-α, 200pg/ml IL-6, and 300pg/ml IL-10. These were the top standards provided in the ELISA kits.

TNF-α:

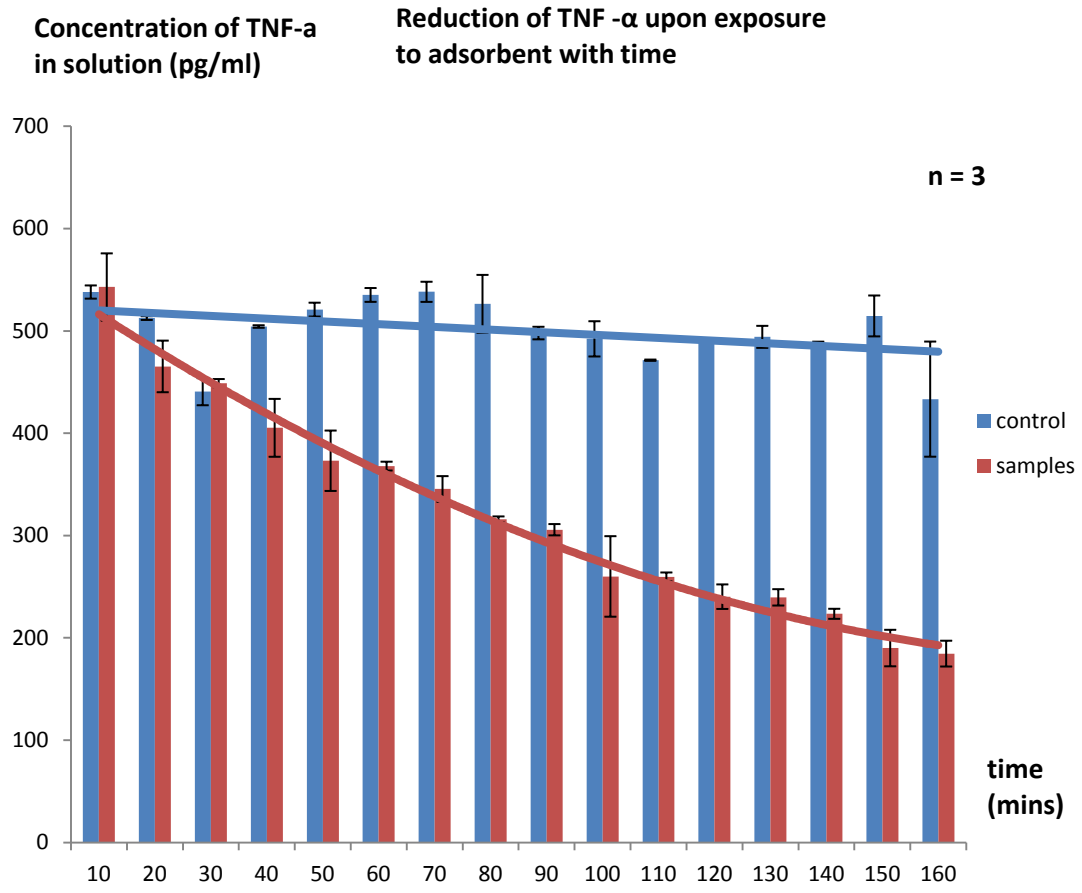

Figure 75 – TNF-α reduction over time upon exposure to adsorbent beads.

As shown in figure 75 it can be seen that the TNF-α control is stable, if a little noisy, and the samples taken clearly show a reduction in the concentration of TNF- α in the solution over the 160mins time period, compared with the control.

IL-6:

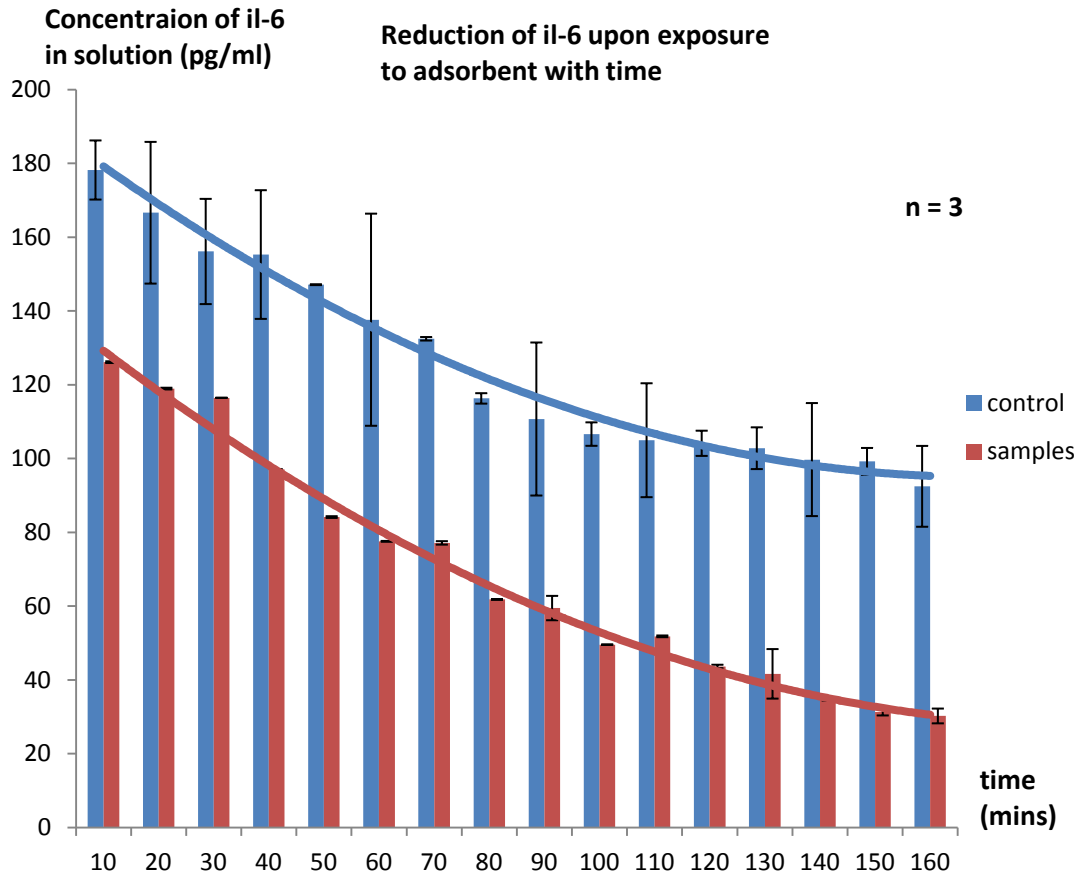

Figure 76 – IL-6 reduction over time upon exposure to adsorbent beads.

IL-10:

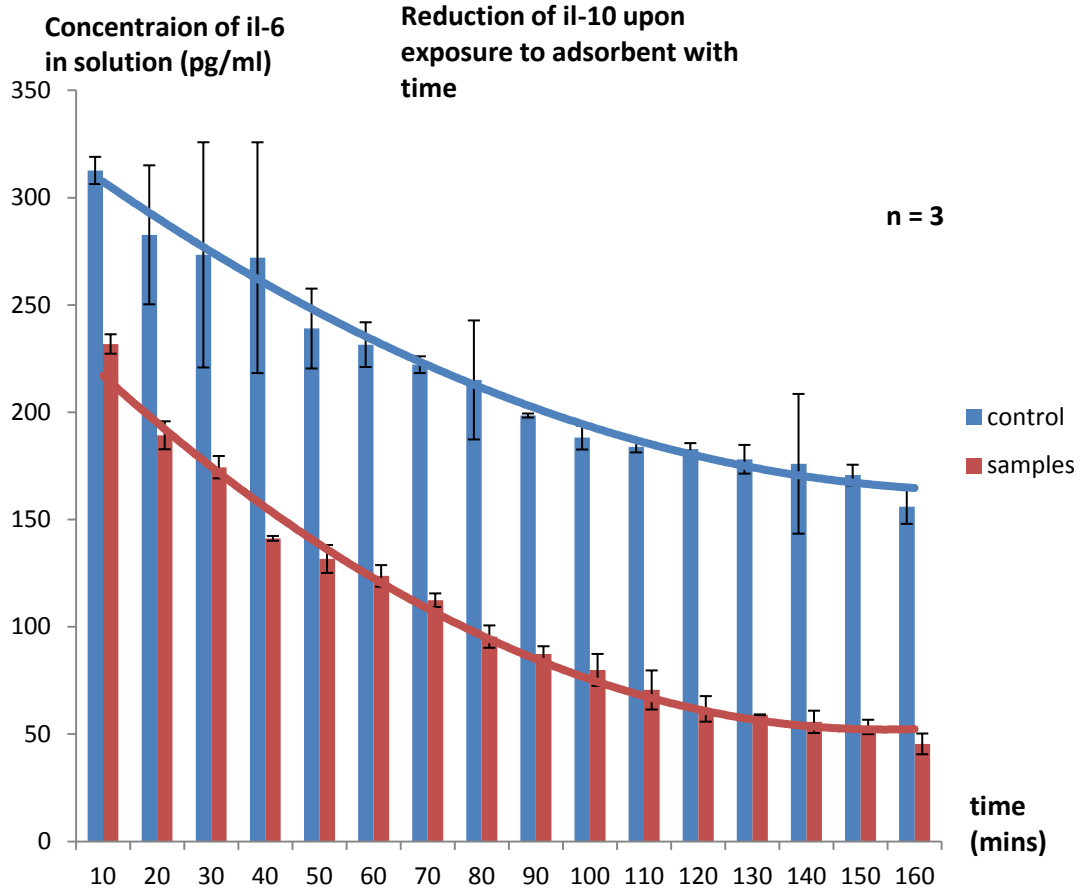

Figure 77 – IL-10 reduction over time upon exposure to adsorbent beads.

As shown in figures 76 and 77, in the case of both the interleukins, despite the fact that the control solutions appear to be decaying, there is significant disparity between the control solution and the sample solution exposed to the adsorbent. This indicates that the adsorbent is performing as expected, although meaningful data about the efficacy of the adsorbent may be, to some extent, unreliable in this case, due to the apparent decay of cytokines under these conditions.

TNF- α, however, does not display this control degradation.

3.5.3 Human Cytokine Control Analysis:

Introduction:

Despite the ability of PBS to be accurately calibrated for cytokine concentration, clearly there appears to be some degradation of the interleukin proteins when in this solution.

In the following series of experiments, various fluid media have been tested in an attempt to find a fluid medium that can be used to avoid this degradation. This has been done for TNF-α, despite it not having this issue, as well as for both the interleukins that appear to have this degradation issue. This was necessary as one single fluid medium must host all 3 molecules in combination for an experiment to have any clinical relevance.

Methods:

At the beginning of this experiment, 4 solutions were freshly prepared. These were, PBS, PBS (10% v/v Foetal Bovine Serum (FBS)), PBS (20% v/v FBS), and assay diluent provided by eBioscience. All solutions were spiked with the molecules TNF-α, IL-6 and IL-10 to their top standards 500, 200 and 300 pg/ml respectively.

These solutions were not exposed to any adsorbent and were left to sit at room temperature for one hour.

Samples were taken at time points 0, 15, 30, 45, and 60mins and later analysed by ELISA for cytokine concentration.

Results:

TNF:

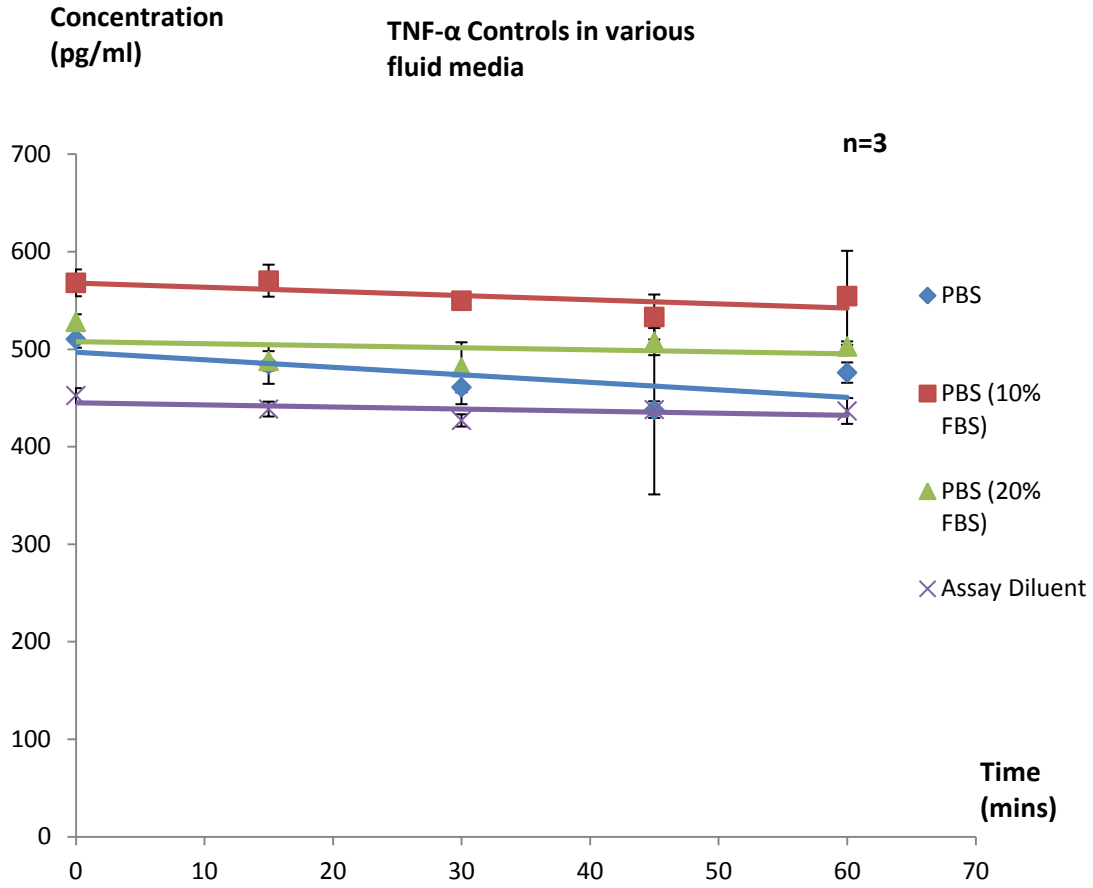

Figure 78 – Control samples for TNF-α in various fluid media.

Shown in figure 78, it can be seen that over a one hour time period the TNF-α molecule does not appear to degrade to any great extent in any of the fluid media. There does appear to be some disparity between the media. This, however, may be due to the fact that the samples are of a concentration close to the top standard. On approaching this concentration, the accuracy of the assay declines.

IL-6:

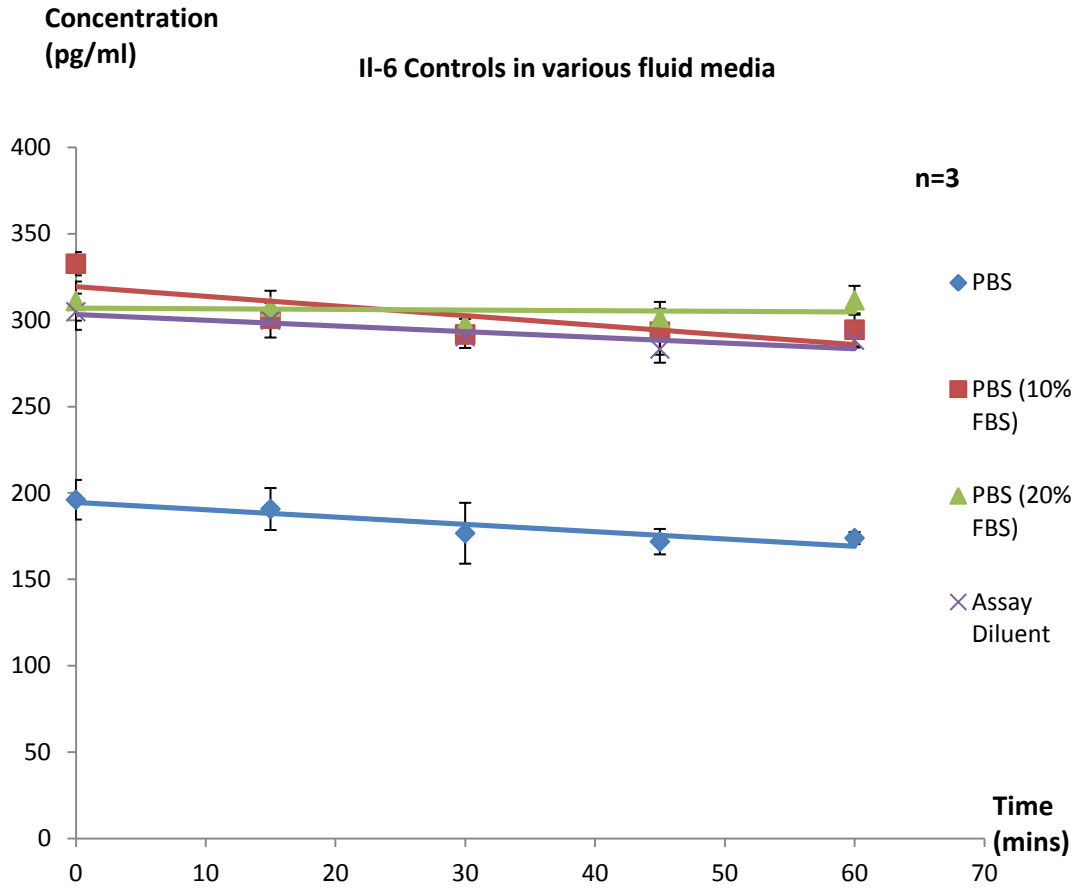

Figure 79 - Control samples for IL-6 in various fluid media.

It is clear from figure 79 here that the fluids containing carrier proteins are all well above the top standard light absorbance reading of 2 for the entire duration of the experiment, and do not appear to be degrading.

The PBS sample of IL-6, however, appears to have a slight decline over the one hour period.

IL-10:

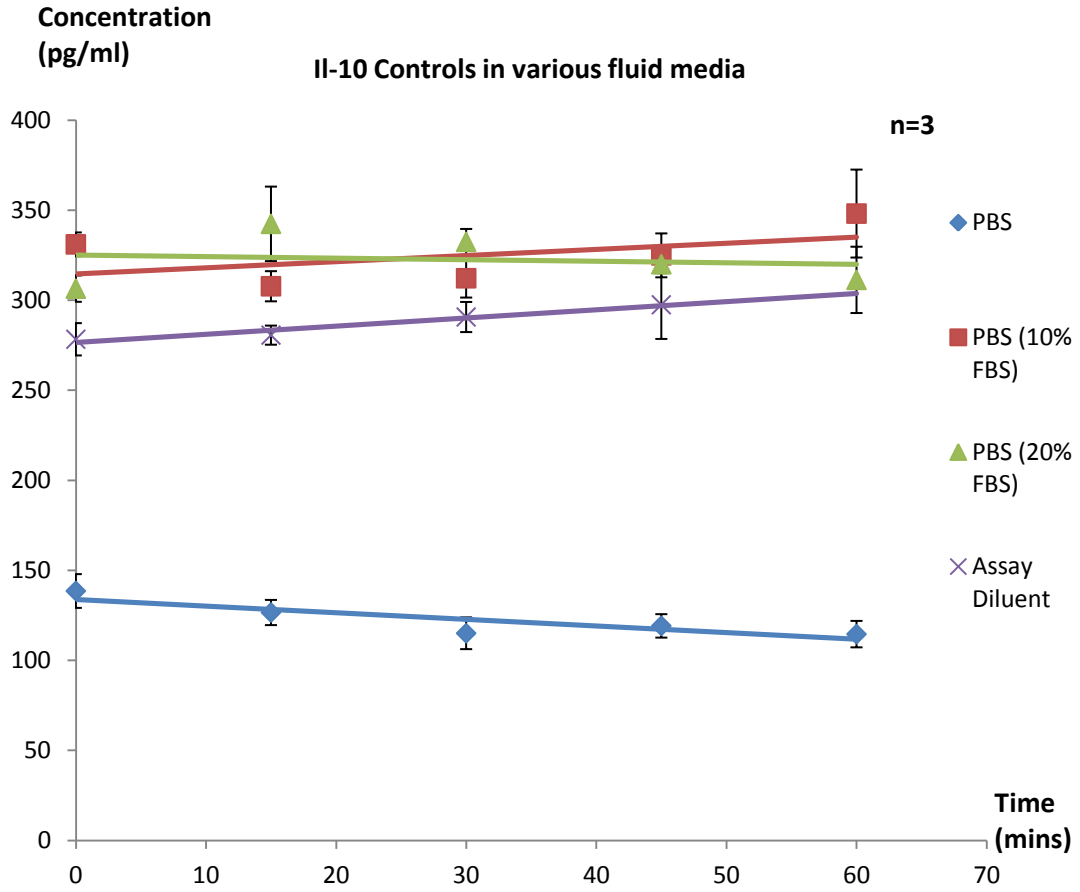

Figure 80 - Control samples for IL-10 in various fluid media.

Seen in figure 80, it can again be seen that all the solutions that contain carrier protein appear to maintain their IL-10 concentration at approximately the top standard light absorbance of 1.52, with the exception of the solution of PBS, which appears to read at a much lower concentration than expected, as well as having a slight downward trend.

Conclusion:

These studies demonstrated it can be seen that in the case of both the interleukin molecules, the introduction of a carrier protein to the solution appears to prevent the interleukin molecules from the degradation previously seen.

In the case of IL-6, the solutions containing carrier proteins appear to have a concentration above the top standard and significantly above the PBS samples. With the interleukins, there appears to be some disparity between the concentrations of cytokines in the solutions with or without carrier proteins, and the solutions without carrier proteins appear to have some degree of degradation over a one hour period.

3.5.4 Effect of Carrier Protein on Adsorption:

Introduction:

In the case of TNF-α, it appears that using PBS as a fluid medium for experimental adsorption protocols is perfectly acceptable. Use of PBS as a fluid medium in experimental protocols is not appropriate, however, in the case of interleukins. Introduction of carrier protein is clearly necessary in such cases. Based on the previous experiments, it was seen that PBS with 10% v/vol. FBS prevented the apparent degradation of the interleukins. It was therefore decided to compare the performance of an adsorbent protocol in both PBS alone, and PBS (10% FBS).

Methods:

1 litre of fresh PBS was initially prepared for this protocol. This PBS was separated into 4 x 250ml beakers. Two of these beakers had 25ml of PBS removed and 25ml of fresh frozen FBS was added instead, giving 2 x 250ml solutions of PBS (10% FBS) and 2 x 250ml solutions of PBS.

One of these PBS solutions was marked as a control and left alone, the other was marked as a sample solution and was spiked to the top standard for each of the human cytokines TNF-α, IL-6, and IL-10.

This was repeated for the PBS (10% FBS) sample, again giving a control solution and a cytokine spiked sample solution.

0.25g of adsorbent beads was added to each of the sample solutions.

1ml samples were taken every 10mins until 8 samples were taken from each solution and refrigerated until later analysis by ELISA.

Results:

TNF-α:

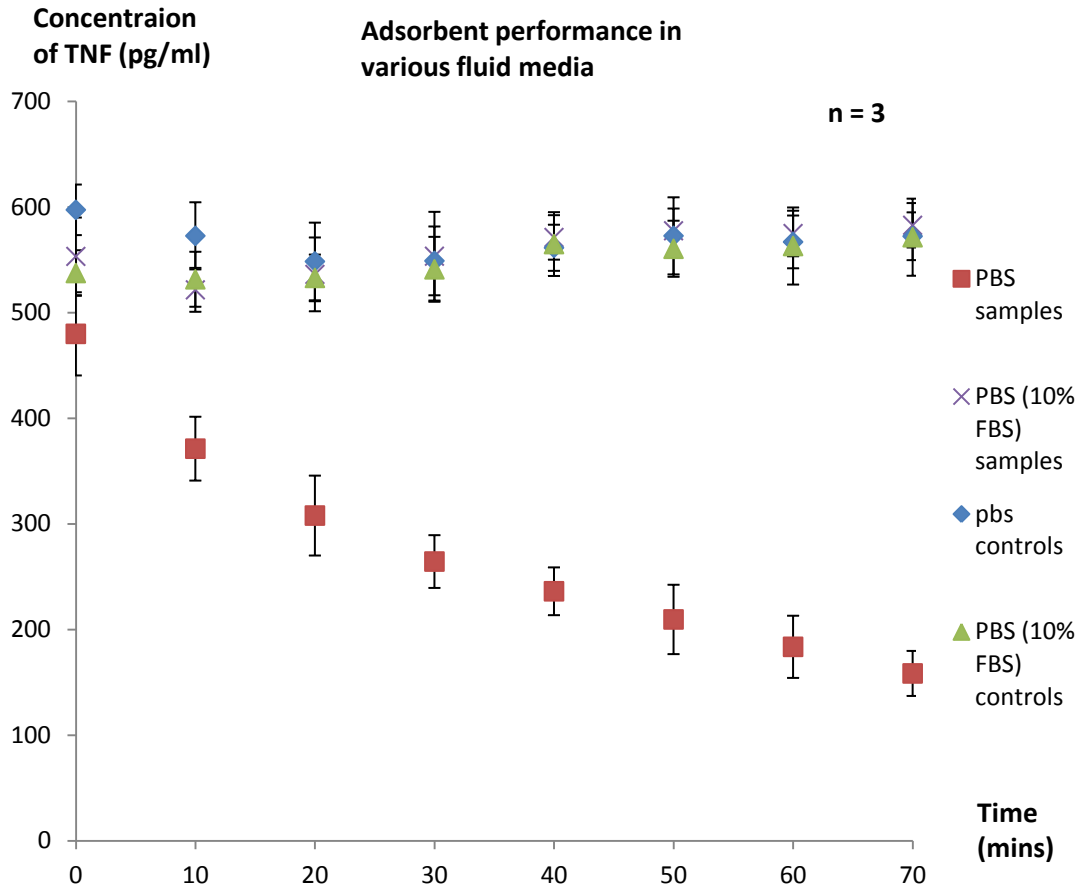

Figure 81 – Adsorbent samples, and controls, for fluid media with and without carrier protein for TNF-α.

These studies demonstrate that for the TNF-α molecule i.e. a PBS control and a PBS (10% FBS) control remained stable over the 70min time period.

The PBS samples that were exposed to the adsorbent can clearly be seen to be reducing over time and therefore show that the adsorbent is performing.

The spiked sample exposed to the adsorbent in a solution of PBS (10%FBS), however, appears not to be reducing. Despite being exposed to the adsorbent in the same conditions to which the PBS samples were exposed, the adsorbent does not seem to be performing in this fluid medium. At this stage, we suspect that the cytokine/carrier protein complex may be blocking the porous structure of the adsorbents, leading to a sub-optimal, or zero adsorption profile.

IL-6:

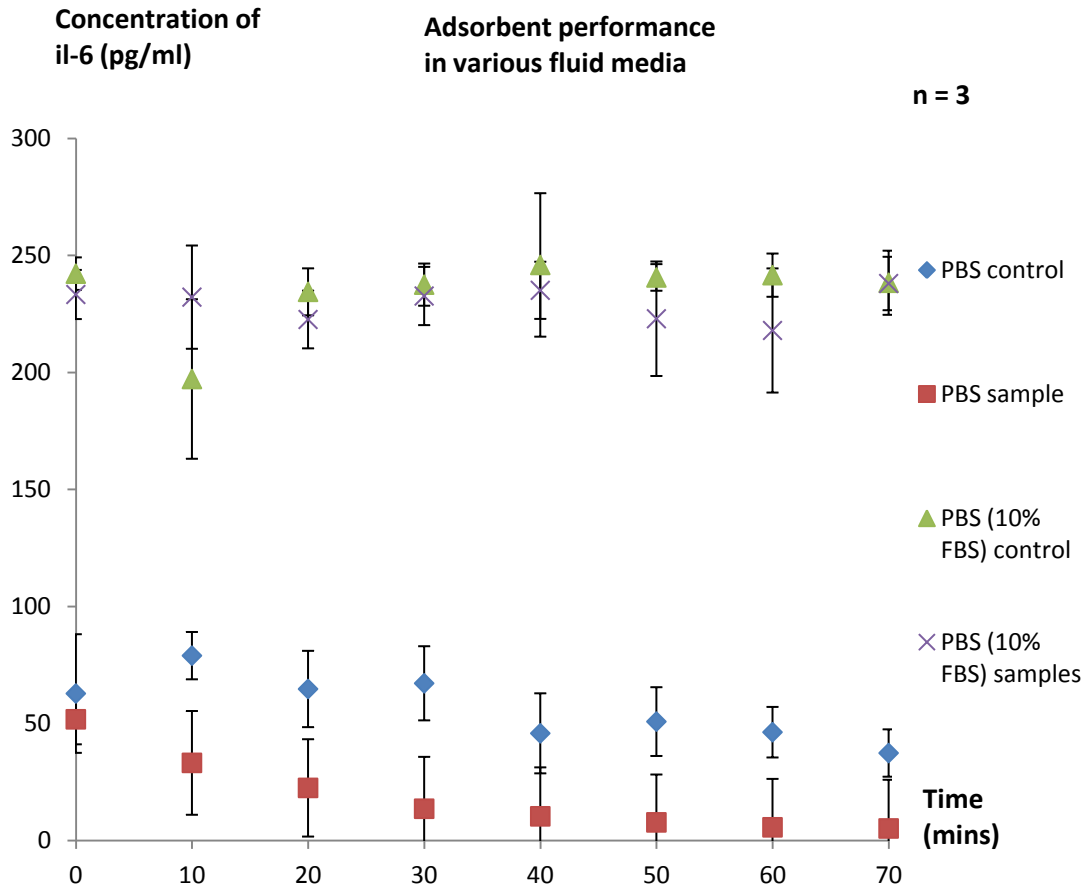

Figure 82 - Adsorbent samples, and controls, for fluid media with and without carrier protein for IL-6.

Here in figure 82, similar trends to before can be seen. As with the case of TNF-α in this experiment, the presence of a carrier protein appears to prevent the adsorbent from performing. Yet in the case of PBS without a carrier protein, the samples that were exposed to an adsorbent appear to be reducing much more quickly than the PBS controls. This suggests that adsorbent is performing in this case but that PBS cannot be used as control to calculate the efficacy of the adsorbent in removing IL-6 from solution.

The challenge presented by the apparent decay of IL-6 profiles in PBS may be a compounding factor in the assessment of adsorption.

IL-10:

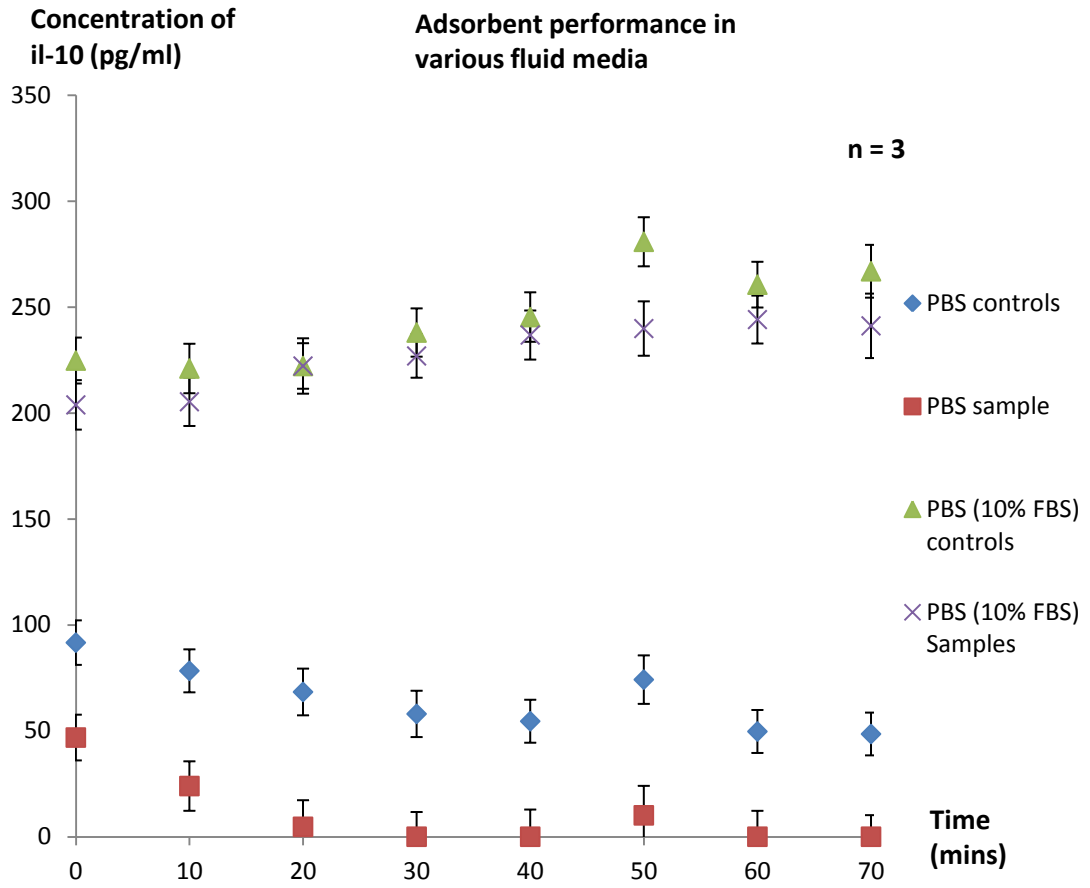

Figure 83 - Adsorbent samples, and controls, for fluid media with and without carrier protein for IL-10.

Similar trends can be seen with IL-10 as with IL-6. It appears that the presence of carrier proteins has prevented the adsorbent from performing. Yet the adsorbent appears to be performing when in the case of spiked PBS. But, yet again, the PBS control appears to be degraded therefore making it impossible to rely upon it to calculate the efficacy of the adsorbent material.

Conclusion:

Similar trends were seen in these data as have been seen before. In the case of interleukins, the introduction of a carrier protein appears to stabilise the control over the time period of the experiment, but seems to prevent the adsorbent from performing properly. Without a carrier protein, the PBS control appears to be degrading, as has been seen before. But again, there appears to be enough disparity between the control and samples exposed to adsorbent to conclude that the adsorbent is performing. Quantification of the efficacy of the adsorbent cannot, however, be drawn from this experiment due to the degradation of interleukins in PBS.

3.5.5 Investigation of Factors Contributing to Cytokine Decay under Laboratory Conditions: Container Material

Introduction:

Based on the reduction of interleukin concentrations in control media, it was decided to check another factor that may be involved. In this case, it was decided to check various container materials used in experimental protocols so as to be sure that the interleukin proteins were not aggregating to the surfaces of specific materials. Or that the materials used did not have their own slight adsorption process occurring as a result of a highly porous polymeric structure.

Methods:

Various containers were found around the lab. Checking for specific materials and manufacturers as much variety as possible was found.

The materials (including manufacturer where appropriate) found were; Eppendorf™ vials, PYREX™, plastic (unlabelled), Azlon™ plastic, and Kartell™ plastic.

Containers of similar volume were taken and ml of freshly prepared PBS, spiked to 200pg/ml of IL-6, was added to each container.

100µl samples were taken every 10mins until 8 samples were taken in total. These samples were put into the appropriate wells of a coated ELISA plate immediately. Upon completing the protocol, the ELISA plates were incubated at 4°C overnight to provide sufficient time for the capture antibody to attach to all available interleukin protein in the fluid samples added. This was done so as to account for the 1 hour of difference between the addition of the first sample and the final sample.

Results:

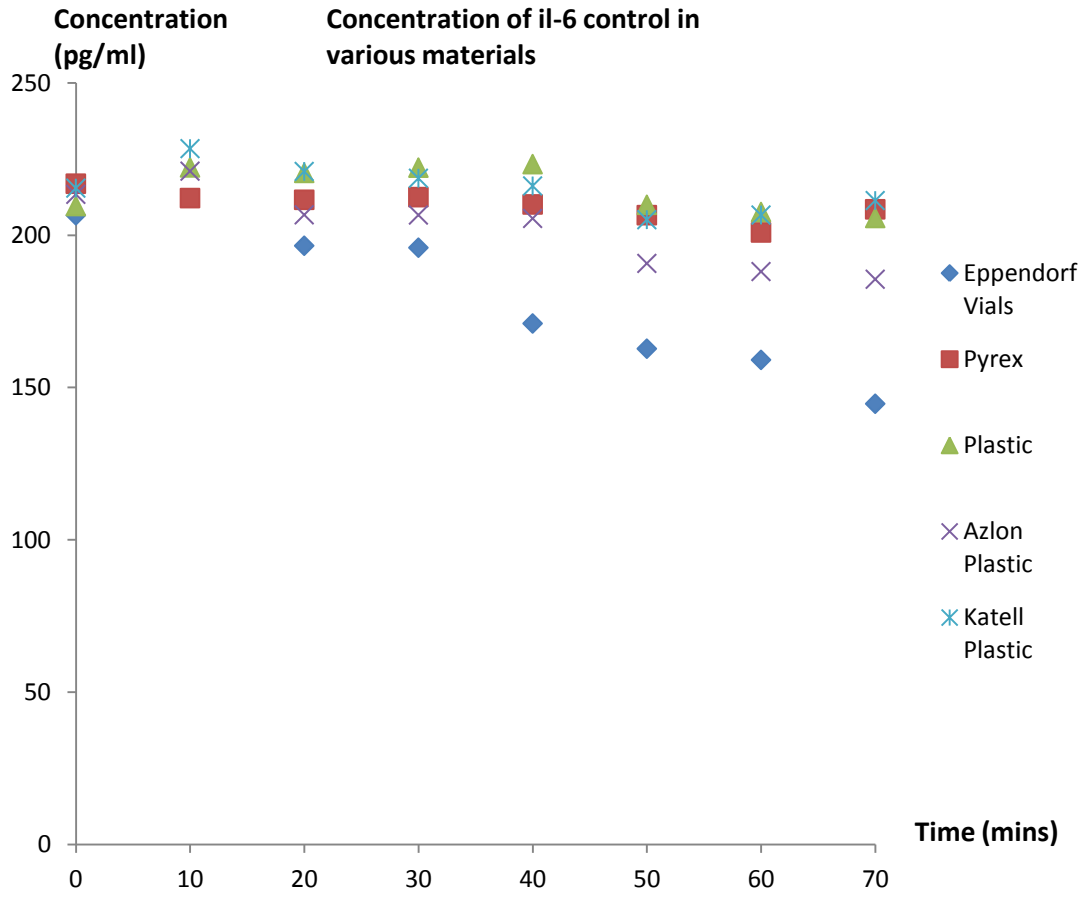

Figure 84 – Decay of IL-6 control in various container materials.

The only material that seems to have some effect on the interleukin concentration are the Eppendorf vials. A fairly clear downward trend can be seen in comparison to all the other controls provided.

The majority of the materials do not appear to affect the interleukin concentration.

Conclusion:

Eppendorf vials are made of a highly porous polymeric material and that this material may cause some interleukin adsorption on its own. Eppendorf vials should be avoided where possible in future cytokine adsorption studies. This is of little retrospective concern, as until this series of experiments, Eppendorf vials had only been used in early rat TNF-α experimental protocols.

3.5.6 Degradation of Cytokines in combination:

Introduction:

To further explore the mechanism behind the interleukin degradation in control media, an experiment was undertaken to investigate the behaviour of each of the three cytokines TNF-α, IL-6, IL-10 when presented in combination in suspended media. And if so, which combination of protein affects the rate of degradation.

Methods:

3 litres of PBS were freshly prepared for this protocol.

1 litre of PBS was spiked with TNF-α to a concentration of 500pg/ml.

1 litre of PBS was spiked with IL-6 to a concentration of 500pg/ml.

1 litre of PBS was spiked with IL-10 to a concentration of 500pg/ml.

Various mixtures were made utilising 250ml of each solution when necessary, ultimately giving the following 7 solutions; 250ml of; TNF-α alone (500pg/ml), IL-6 alone (500pg/ml), and IL-10 alone (500pg/ml); 500ml of; TNF-α + IL-6 (250pg/ml), TNF-α + IL-10 (250pg/ml), and IL-6 + IL-10 (250pg/ml); and 750ml of TNF- α + IL-6 + IL-10 (167pg/ml). Note: the reduction in concentration is due to a dilution factor i.e. 1:1 dilution when 250ml of TNF- α (500pg/ml) solution is added to 250ml of IL-6 (500pg/ml) solution, giving 500ml of 250pg/ml of each cytokine.

This gave each of the three cytokines alone in solution, as well as all the possible combinations of these three cytokines in solution together.

Magnetic fleas were then added to each of these solutions and spun for the duration of the experimental protocol. The solutions were kept at room temperature for 3.5hours, with samples taken every 30mins. The samples were immediately frozen at -80°C for later analysis by ELISA. All dilutions were corrected for after ELISA calibration performed. This protocol aims to give an insight into the degradation of the cytokines dependent on their biological environment.

Results:

TNF-α:

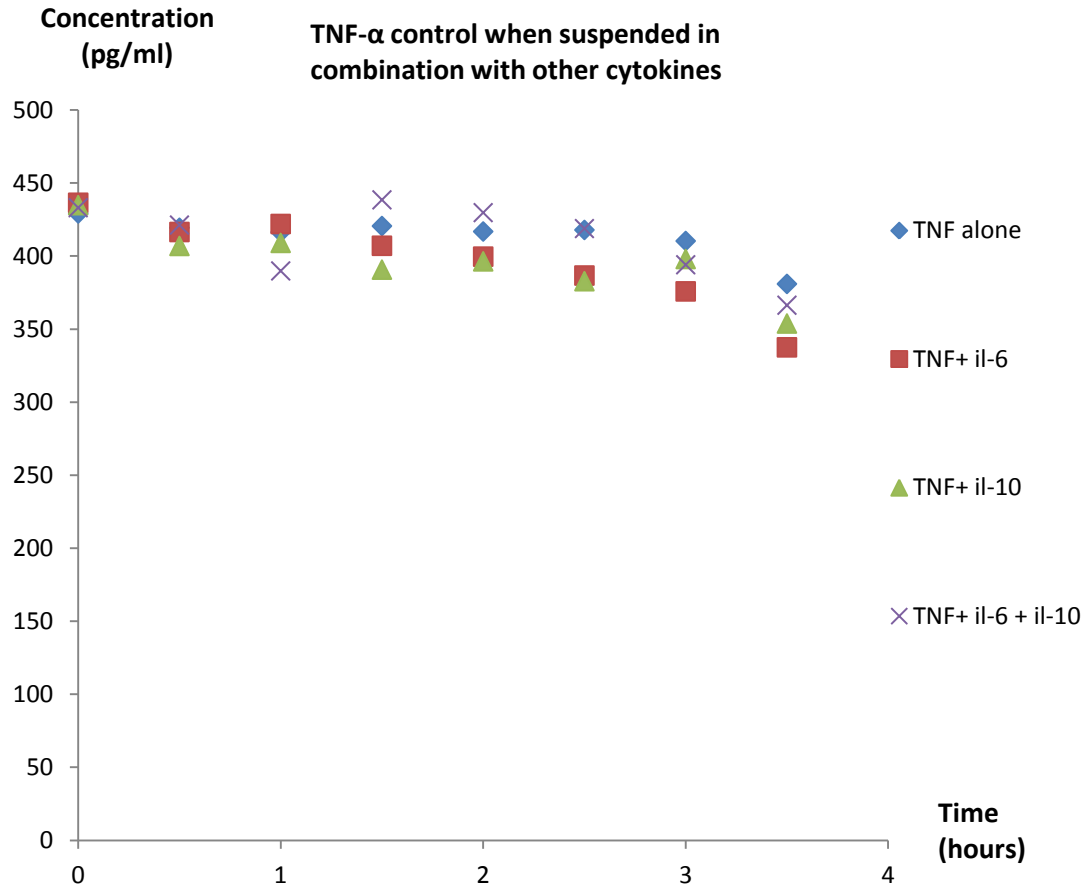

Figure 85 – Comparison between the decay of TNF-α when alone in solution and when in combination.

The TNF- α molecule does not appear to degrade much over the 3.5 hour period (maximum reduction 15.4%) and the behaviour of the molecule does not appear to differ significantly between the various combinations.

IL-6:

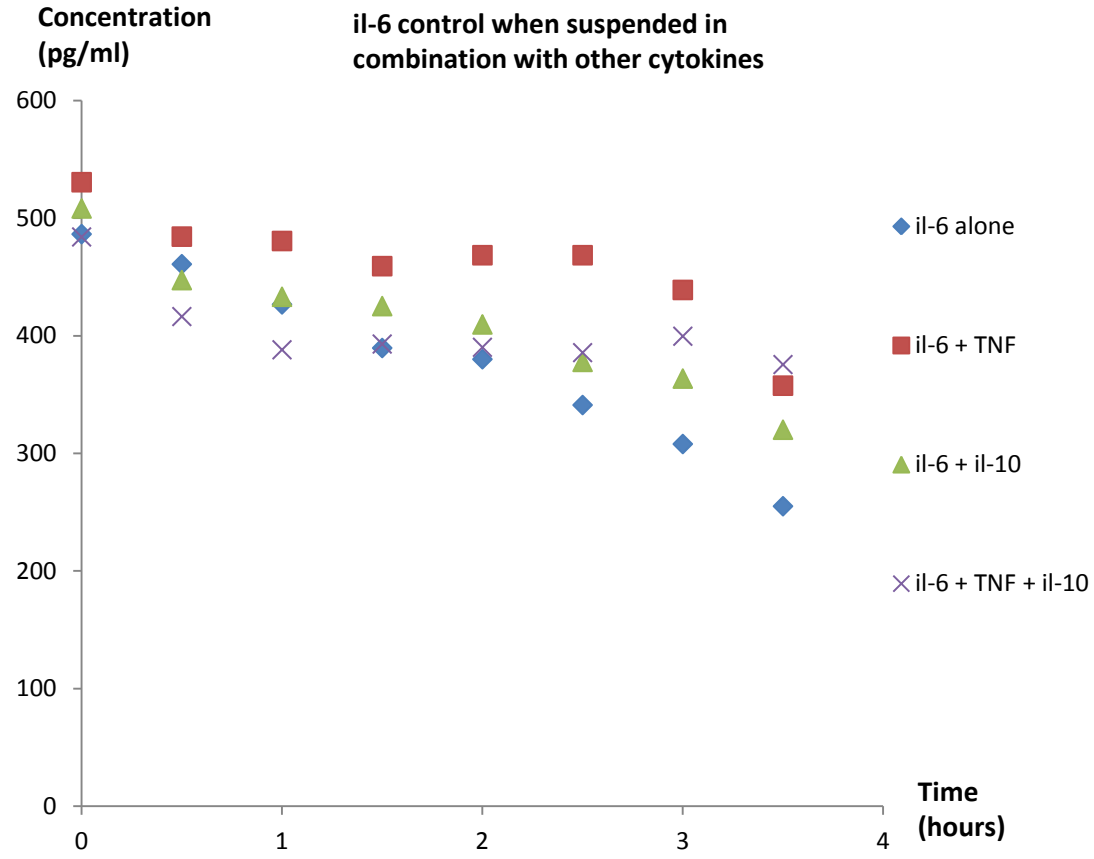

Figure 86 - Comparison between the decay of IL-6 when alone in solution and when in combination.

Reduction of IL-6 alone; 47% over 3.5 hours.

Reduction of IL-6, with TNF-α present; 22% over 3.5 hours.

The results demonstrate that IL-6 alone in a fluid degrades rapidly, whereas the presence of another protein molecule appears to slow this degradation. This is most apparent in the case of TNF-α, which is both the largest molecule and the only cytokine that does not appear to degrade significantly over time. In this case, it appears TNF-α may be acting, to some extent, in the same way a carrier protein does. It appears the presence of other proteins in the fluid is sufficient to slow or reduce the degradation of this interleukin molecule.

IL-10:

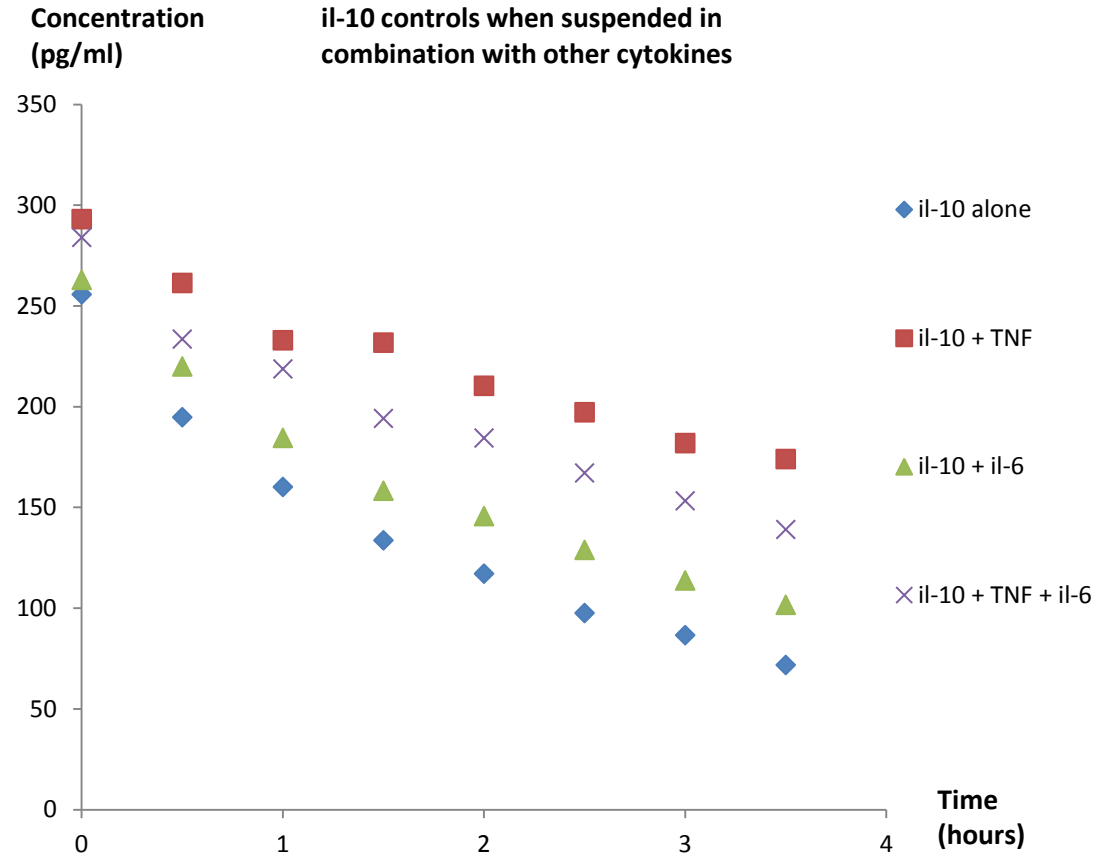

Figure 87 - Comparison between the decay of IL-10 when alone in solution and when in combination.

Reduction of IL-10 alone; 71% over 3.5 hours.

Reduction of IL-10, with TNF-α; 51% over 3.5 hours.

The same trend can be seen here as with IL-6. IL-10 appears to be degrading most rapidly when it is alone in the fluid medium. The presence of another protein appears to slow this degradation. This trend is once again most apparent in the case of TNF-α.

Conclusion:

These studies have demonstrated that when using various fluid media, cytokines, and carrier proteins, that there is significant variation in the behaviour of these molecules, dependent on the environment in which they are used.

These findings suggest considerable limitations to the deployment of cytokines and that considerable caution must be taken when interpreting results to ensure that "environmental" decay is recognised.

It is clear from these data that it is critical to characterise the behaviour of the cytokines and the adsorbents in a near-clinical environment in order to conclude with confidence that this adsorbent is effective at adsorbing cytokines, and that other factors of influence are accounted for.

The *in vitro* data suggest that the complex behaviour of cytokines is highly dependent on the environment in which they are deployed. Their chaotic behaviour (interleukins in particular) makes it extremely challenging to extrapolate from *in vitro* data the performance of an adsorbent in the clinical environment.

Summary of Preclinical Findings:

In the absence of human plasma or blood, it is impossible to continue to study molecular adsorption with this technology in the laboratory. The situation may be different if we had access to human blood. However, upon exploring this with the Scottish National Blood Transfusion Service (SNBTS) it was cost-prohibitive (to the order of £15,000) for a sufficient supply of blood.

Laboratory results were however useful insofar as they suggest that the device as developed is capable of adsorption albeit that the process involved was complicated by external factors, such as the carrier protein, container material, and general decay of interleukins under laboratory conditions.

Critically, the rate of decay is similar to the adsorption profile of other laboratories (DiLeo, Fisher, et al. 2009), which calls into question whether adsorption is taking place or to what extent decay is responsible for these findings by other laboratories. We therefore took the decision to move this technology to the clinical setting where, albeit under different flow conditions, we have seen some success in the past. The chapter details our studies under clinical conditions using whole blood to test cytokine adsorption.

Chapter 4:
Clinical Studies

4: Clinical Studies:

Introduction:

Based on the results of our *in vitro* studies, it is clear that the adsorbent technology used in this research is capable of a high level of performance.

Although the data were promising, the apparent complications associated with cytokine decay resulted in an impasse with regards to investigation of adsorption studies. We therefore decided to undertake studies in the clinical setting to further investigate the technology. We had investigated carrying out studies on fresh human blood, but our contacts with the SNBTS were not supportive. The charge for access to fresh human blood (£15,000) was outwith the budget of this project. Another strategy was therefore required to further these investigations.

Close links with the university hospital Kirikkale in Ankara, Turkey presented an opportunity in this regard. Discussions with the cardiovascular surgery group resulted in the development of a protocol for deploying the technology on patient blood residual in the heart-lung bypass machine upon termination of cardiopulmonary bypass (CPB). The use of these blood products represents two key steps in the investigation of this technology.

1. It is known that cytokine levels in post-CPB blood are elevated and investigation of the ability on our technology to reduce these populations may be of clinical significance.
2. It is possible to spike the blood products to elevate cytokine levels to mimic more challenging clinical conditions and ascertain whether the saturation potential of the device can be reached.

We therefore gained ethics approval to investigate this device under post-CPB conditions.

Methods:

Ethical approval was sought, and subsequently granted, to retain residual human blood from a cardiopulmonary bypass (CPB) circuit post cardiac surgery. From 5 patients, 300ml of blood were retained in the CPB circuit. This blood was then filtered in the portable adsorbent device as detailed below.

Filter Preparation:

5 filter cartridges were manufactured from a single piece of acrylic in the Bioengineering Unit at Strathclyde. This was done almost entirely using a lathe tool, though a bench top drill was necessary to provide a radial inlet to the device. Ultrasonically welded adsorbent filter material was provided by Brightwake Ltd. The concentration of adsorbent in this material was approximately $4g/m^2$. This material was cut to an appropriate width and pleated into 3 of the acrylic chambers, fixed to the top and bottom using medical grade silicone adhesive.

"Blank" non-woven fabric material was also provided by Brightwake Ltd. This material is identical to the material that encapsulates the adsorbent beads, but in this case is lacking the carbon beads. This material was pleated and fixed into a further 2 acrylic chambers, used as control devices.

The following experimental protocol was used in order to test the efficacy of the adsorbent material in these near-clinical conditions.

Experimental Protocol:

1. A blank or carbon filter device was connected in a closed loop circuit to the residual blood (300ml) from the cardiopulmonary bypass circuit.

2. The device was primed, pumping blood into the radial inlet, blocking the axial outlet and releasing the air through the small hole next to the inlet. Once primed the "purge port" was closed using a 3-way tap.

3. The axial outlet was released and the blood was pumped through the circuit at a flow rate of approximately 100ml/min.

4. A 5ml sample was taken at 0mins and 20mins.

5. The blood was then spiked with the recombinant cytokines human TNF-α, IL-6, and IL-10 (purchased from R&D systems) to a concentration of 500pg/ml (150ng of each molecule added). The blood continued to be pumped through the circuit as before.

6. Another 5ml sample was taken at time = 0mins (post-spike) from the circuit, followed by further samples every 10mins for an hour.

7. After one hour, the blood was spiked again with a further 150ng of each molecule, as before.

8. All the blood samples were spun down to plasma in a centrifuge and frozen at -80°C.

9. All samples were labelled for the sample number and the time point e.g. Patient 1, time 1...Patient 6, time 8.

10. Once all samples had been collected, the samples were defrosted, and analysed in duplicate in 3 ELISA plates (purchased from R&D systems), measuring for TNF-α, IL-6, IL-10.

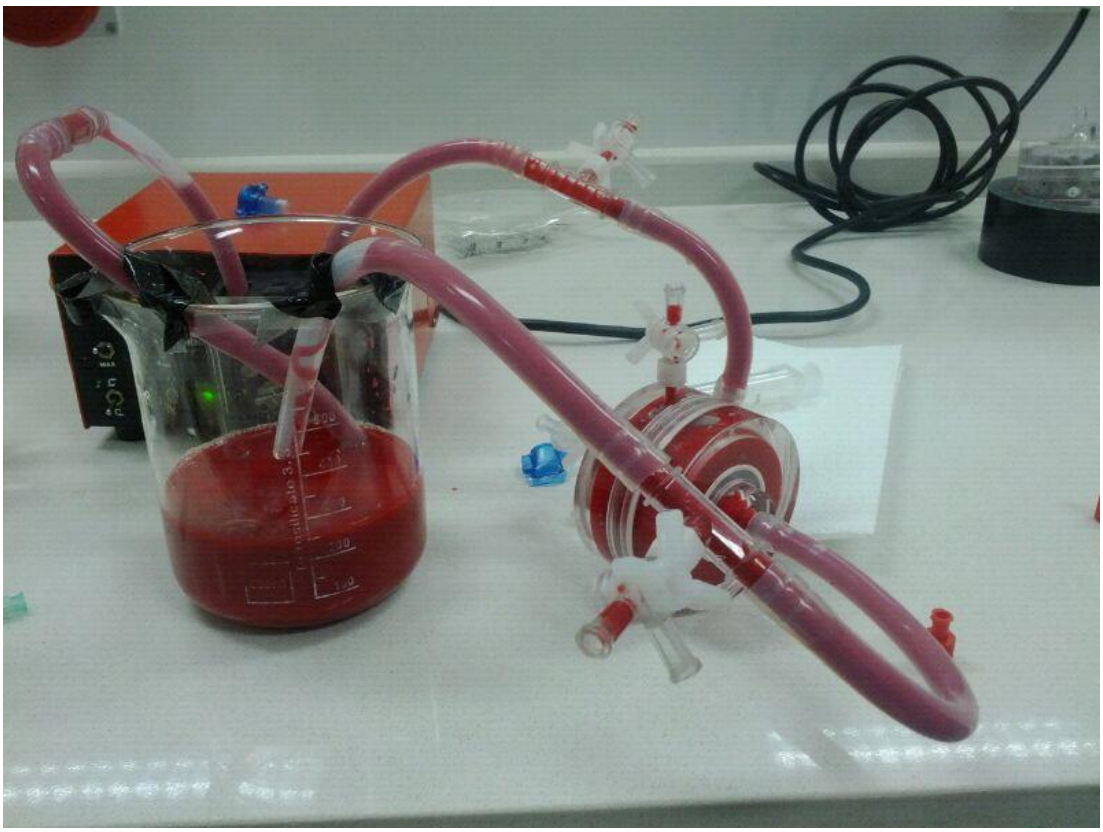

Figure 88 – Photo of experimental protocol.

Results:

Shown below are column charts of the average cytokine concentration profiles associated with; control circuits, and the carbon containing circuits. Due to the limited number of control experiments (n = 2), statistical comparisons were not possible between the carbon and control groups.

TNF-α:

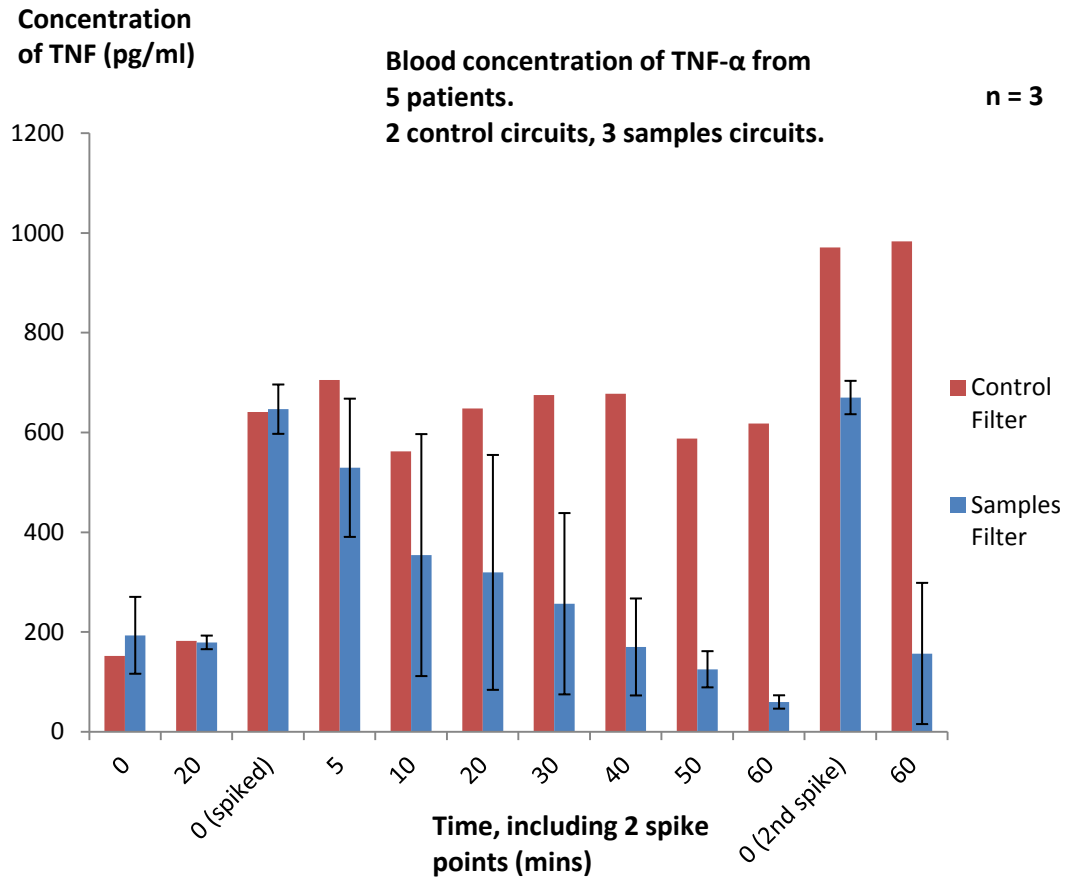

Figure 89 – TNF-α concentrations in blank and carbon filters.

The post-surgery TNF- α levels for all 5 patients are in the region of 200pg/ml. Immediately after taking a reading at 20mins, the spiked concentration of TNF-α in the patients increased to approximately 700pg/ml. For the control filters, this concentration remains relatively constant (with a little noise) for the following hour until the next spike. The carbon filters, however, dramatically reduce the concentration of TNF-α in the blood exposed to them, to below 100pg/ml within an hour. At the end of this hour, the blood was spiked a 2nd time and a sample was taken

at this time and again after 1 hour. The same trend can once again be seen. The blood concentration of TNF-α remains relatively constant in the control filters, whereas it is dramatically reduced in the carbon filters to a concentration of less than 200pg/ml.

In comparing cytokine levels throughout the experiment with the initial spike point, the difference is statistically significant at 20mins ($p<0.05$) and by 60mins the difference has reached its highest statistical significance ($p<0.005$). This statistically significant adsorption continues after a 2^{nd} spike, where the difference between the 2^{nd} spike levels and the levels after 1 hour is $p<0.005$.

IL-6:

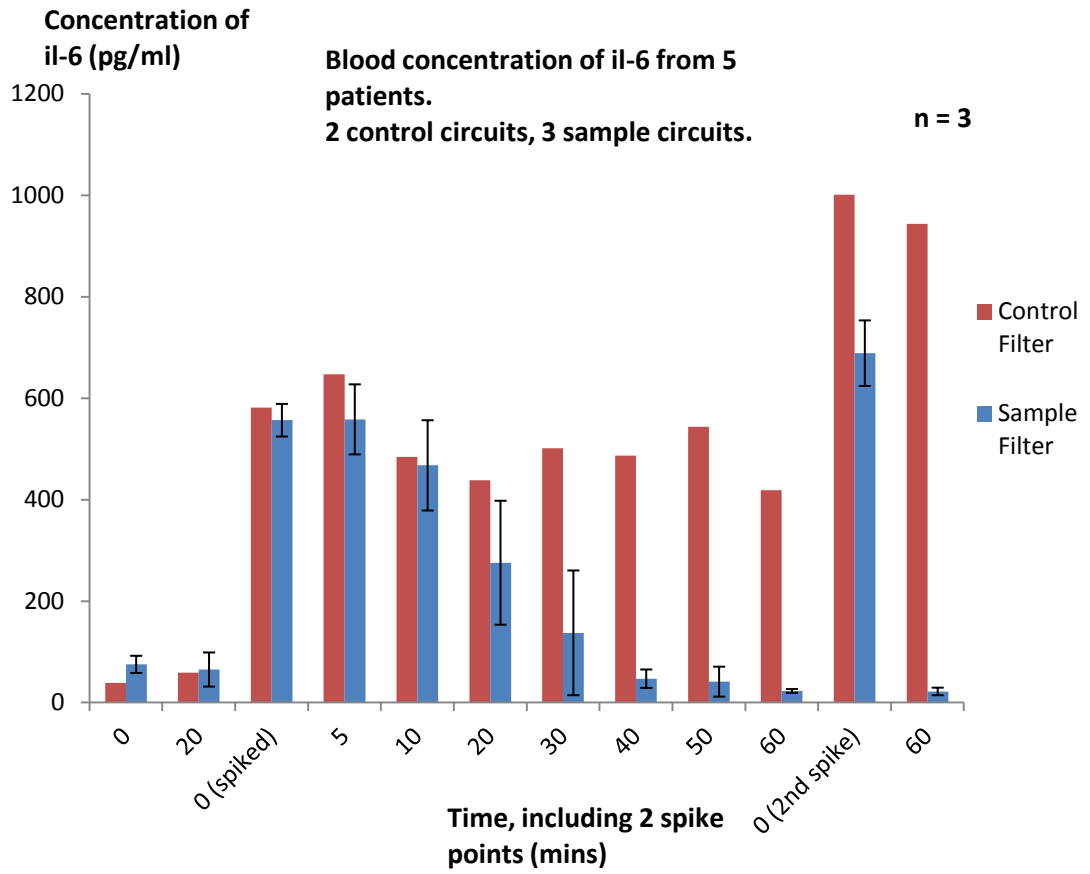

Figure 90 – IL-6 concentrations in blank and carbon filters.

The post-surgery IL-6 blood concentrations appear to be relatively low for all 5 patients, averaging lower than 100pg/ml. Upon spiking at 20mins, this concentration increases to approximately 600pg/ml. For the hour following this spike, the control blood concentration of IL-6 appears to slightly reduce, similar to the degradation that we have seen in the past *in vitro*. It is possible that the reduction seen here could be due to the material used to suspend the carbon adsorbent. These studies suggest that this has some adsorbent properties of its own and is producing some mild adsorption. Despite this slight reduction in the control, it can be seen that the blood concentration of IL-6 has been reduced to near base-line levels, considerably below 50pg/ml. After the 2nd spike, the control again appears to moderately reduce over the 1 hour period. The carbon filter samples, however, have clearly been reduced again to negligible value, far below 50pg/ml, confirming that the carbon beads are effective as adsorbents.

Again, the difference in cytokine levels, when comparing with the initial spike point, gains statistical significance at 20mins ($p<0.05$) and reaches high statistical significance ($p<0.005$) at 60mins. Again, this highly statistically significant reduction can be seen after an hour beyond the 2nd spike point ($p<0.005$).

IL-10:

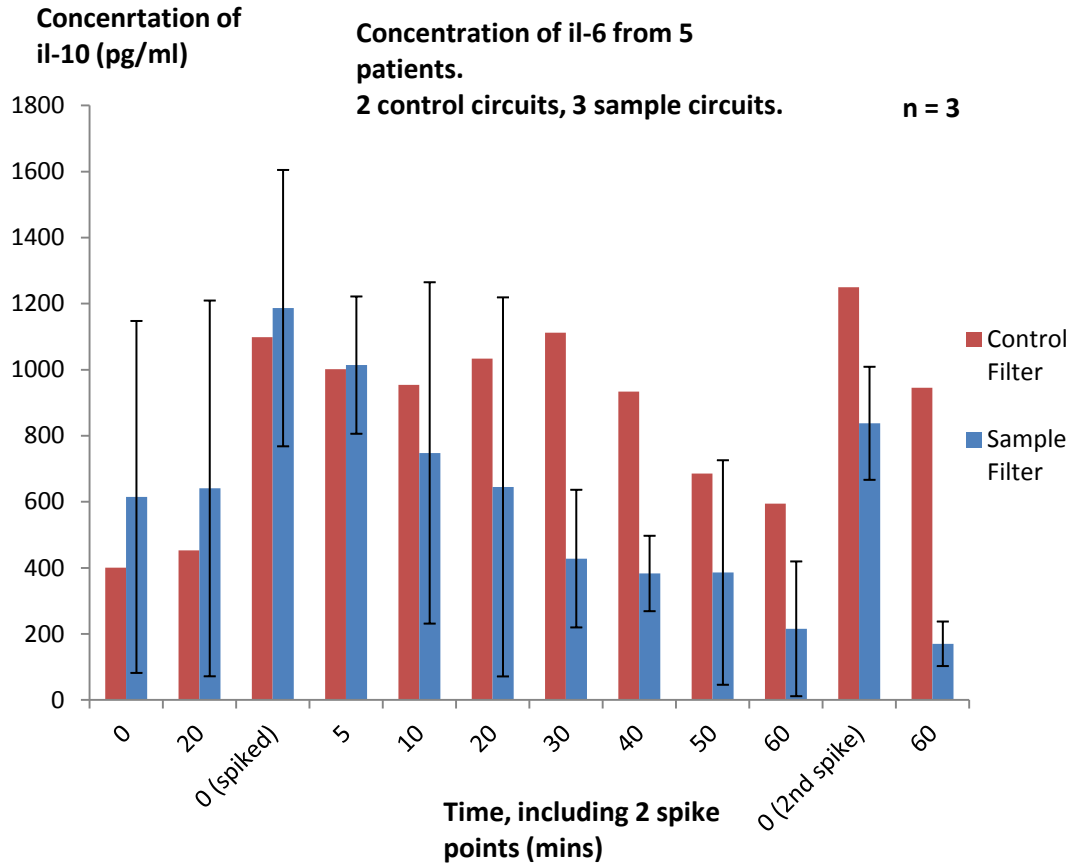

Figure 91 – IL-10 concentrations in blank and carbon filters.

The post-surgery levels of IL-10 differ dramatically between the patients, with one patient displaying levels above 1000pg/ml and another displaying levels below 100pg/ml. The variation in these data resulted in extremely high standard deviation and thus limited our ability to analyse statistically.

Despite this, similar trends to the IL-6 blood concentrations can be seen in the mean values, with the carbon filter dramatically reducing the blood concentration levels of the molecule, but also showing that the control has some reduction. This profile is to some degree similar with that if IL-6 and suggests some level of material-related adsorption.

In the case of IL-10, it is not until 30mins that the difference between the cytokine levels and the initial spike levels gains statistical significance. However, a highly significant reduction can be seen after 60mins, for both the first and second spikes.

Time point	0	20mins	1st Spike	5min	10mins	20mins	30mins	40mins	50mins	60mis	2nd Spike	60mins
Cytokine Concentration (pg/ml)												
TNF-α Sample(mean +/- SD)	193.25 +/- 77	179 +/- 13.6	646.58 +/- 49.4	529 +/- 138.6	354 +/- 242.5	319.4 +/- 235.4	256.6 +/- 181.7	169.9 +/- 97.2	125.19 +/- 36.3	59.6 +/- 13.4	669.9 +/- 33.5	156.9 +/- 141.5
TNF-α Sample (Range)	*139 - 281*	*164 - 190*	*590 - 684*	*409 - 680*	*182 - 631*	*169 - 590*	*56 - 411*	*79 - 272*	*88 - 160*	*49 - 75*	*634 - 701*	*24 - 350*
TNF-α Control Mean*	152	182	640.75	705.3333333	562.4166667	648.25	674.9166667	677.4166667	587.8333333	617.8333333	970.75	982.8333333
TNF-α Control Range	*148 - 155*	*180 - 183*	*588 - 693*	*702 - 708*	*499 - 624*	*635 - 660*	*626 - 723*	*555 - 799*	*570 - 604*	*554 - 680*	*860 - 1080*	*911 - 1054*
Il-6 Samle (Mean +/- SD)	75.2 +/- 16.9	65.1 +/- 33.62	556.7 +/- 32.15	558.3 +/- 69	467.6 +/- 88.96	275.7 +/- 112.1	137.5 +/- 122.9	47.1 +/- 18.21	41.25 +/- 29.6	22.9 +/- 3.82	688.8 +/- 64.7	21.9 +/- 7.46
Il-6 Sample (Range)	*56.25 - 88.75*	*40 - 103*	*533.3 - 593.3*	*493.3 - 630.8*	*378.3 - 556.25*	*155.8 - 400*	*25 - 268.75*	*26.25 - 60*	*13.75 - 72.5*	*18.75 - 26.25*	*631.25 - 758.75*	*13.3 - 26.25*
Il-6 Control Mean*	38.75	58.75	581.25	647.0833333	484.5833333	438.5416667	501.25	486.875	543.75	418.75	1001.041667	943.5416667
Il-6 Control Range	*21.25 - 56.25*	*51.25 - 66.25*	*494 - 669*	*538 - 755*	*404 - 564*	*376 - 500*	*400 - 602*	*526 - 447*	*468 - 618*	*381 - 456*	*983 - 1018*	*768 - 1119*
Il-10 Sample (Mean +/- SD)	614.7 +/- 532.8	640.5 +/- 568.8	1186.4 +/- 418.3	1013.9 +/- 207.9	748 +/- 516.8	645.1 +/- 573.8	428 +/- 208.4	383 +/- 114.1	385.9 +/- 339.9	215.5 +/- 204	837.6 +/- 171.1	170.1 +/- 67.4
Il-10 Sample (Range)	*12 - 1025*	*13 - 1121*	*715 - 1513*	*776 - 1162*	*151 - 1051*	*14 - 1135*	*238 - 651*	*263 - 491*	*128 - 771*	*1.4 - 407*	*646 - 976*	*125 - 247*
Il-10 Control Mean*	400.75	452.625	1098.25	1001.375	954.5	1033.25	1112	933.875	685.125	594.5	1249.5	945.125
Il-10 Control Range	*357 - 443*	*390 - 515*	*977 - 1218*	*788 - 1213*	*891 - 1017*	*977 - 1088*	*893 - 1330*	*742 - 1125*	*361 - 1008*	*343 - 845*	*1106 - 1392*	*857 - 1032*

Table 1 –Mean Cytokine Concentrations +/- Standard Deviation for the three target cytokines, plus range, for controls and filters.

Chapter 5
Discussion

5. Discussion:

The results of this thesis have been presented in relation to the 5 major objectives outlined at the start of the work:

- Achieving portability in the development of an extracorporeal blood pump.

- Effectively immobilising adsorbent microbeads in a material that allows clinically effective and clinically safe use.

- Integration of the blood pumping and adsorbent components into a system that meets the engineering design requirements.

- Establishing *in vitro* methodologies with a view to developing a technique which can effectively ascertain the efficacy of the adsorbent beads, and therefore assist in informing the design of our device.

- A clinically grounded study presenting the capability of the integrated device in a near clinical environment.

Blood Pump:

Why portable technology?

The overriding objective of this project was to develop a miniaturised and portable extracorporeal adsorbent technology, capable of treating SIRS/sepsis. Device miniaturisation was important in meeting this objective, as portability permits ready deployment under a wide range of conditions. For example, a major benefit we envisage of this technology is that is capable of responding to military or emergency situations, such as the deliberate exposure of military personnel or civilians to biological agents leading to inflammatory insult, or the sudden outbreak of a major pandemic. Such a scenario would overwhelm healthcare providers as there is little by way of existing treatment modalities available for such threats. A miniaturised, portable technology could be deployed out-with the hospital environment and could be utilised at the site of insult.

In the case of military blast injuries, so often these injuries occur far from any field hospital, and point of care treatment could potentially respond to the early onset of inflammatory shock.

What were the major challenges for developing a portable technology?

In developing a portable technology there are a number of challenges to be addressed such as;

- Power
- Ease of use
- Weight
- Dimensions.

These factors do not restrict the development of non-portable devices. A portable device should ideally be powered by a battery; a compromise must be met with regards to the size, weight, and the capacity of the battery.

The blood pump component of the device is the only mechanically active component and is thus the only component that requires a power source. Power consumption is therefore a major factor in deciding upon a blood pumping technology. Ultimately, all of the major challenges in achieving portability in the present work can be restricted to the development of a low power-low profile blood pump.

During the development of concepts in the early stages of this project, two rotary pumps were modelled using the software ProEngineer. These were then exported to the software packages Gambit and Fluent, for meshing, and solving, respectively.

In comparing the two rotary pump concepts with the help of this CFD software, it was seen that the axial flow pump design performed far better than the centrifugal pump in all respects. The axial flow pump was able to achieve the target flow rate of 200ml/min at a rotational speed of less than 2000rpm. This flow rate was associated with negligible average shear stress across the impeller, and a maximum shear stress of less than 50Pa. It can also be seen from the displayed contours of shear stress that this maximum value exists for only very small discrete regions along the outer radius of the impeller blades. These values, and their limited presence across the impeller blades, are well within reasonable shear stress associated with blood damage.

In contrast with the axial flow pump, the centrifugal pump required a rotational speed of approximately 3000rpm in order to achieve the target flow rate of 200ml/min. This flow rate was associated with average shear stress across the impeller of over 50Pa and a maximum shear stress across the impeller of over 150Pa. It was also observed that this maximum shear stress exists in many regions across the impeller. These values are well within the region of serious haemolytic damage and it appears that this damage may occur across much of the impeller.

Since the introduction of rotary blood pumps into clinical use, users have generally expected the roller pump to be replaced by the centrifugal pump for the purposes of extracorporeal treatment, in particular cardiopulmonary bypass. Yet, the roller pump remains the most common type of pump to be used for any form of perfusion less than 8h (Mulholland et al. 2005). This is due to the fact that they are simple, predictable, safe and cost effective.

The roller pump has many major benefits over other types of blood pumps. The majority of these benefits stem from the fact the blood only comes in contact with tubing internal to the device. This minimises many of the compatibility issues that occur within blood pumps and allow for the rest of the device to be manufactured from a much broader choice of materials, thus reducing the cost and complications of the manufacture process. The tubing is also the only part that needs to be sterilised

and since it is removable and can be exchanged easily, this is not a cause for any concern. These factors make the manufacture of the roller pump much easier in comparison with other forms of blood pumps. The lack of valves, bearings, and seals also make the roller pump easy to maintain. The shear stress within a roller pump has also been shown to be relatively low and well within acceptable limits (Mulholland et al. 2005). This is often a complicated issue in the design of centrifugal pumps.

Rotary pumps suffer from more than just concern over their potential haemolysis rates. Despite extensive efforts to effectively couple the impeller to a power source, the rotary blood pumps remain pre-load and after-load dependent (fluid pressure load). Excessive rotational speed of the motor can cause the decoupling, and ultimately failure, of the system. Though the axial flow pump remains the most practical option for long term use as an internal artificial organ, rotary pumps in general offer little major benefit over the roller pump with regards to short term extracorporeal use (Hansbro et al. 1999). Rotary pumps, however, have existed in the medical market for a relatively short period of time and as such, design reiteration is highly likely and improved performance can be expected in the relatively near future. The roller pump has been in use for many decades and little more design reiteration can be made. It seems unlikely that the roller pump will ever improve significantly beyond its current design.

In consideration of all these factors, the roller pump was chosen as the blood pumping technology to employ in this technology. Though other pumps were perfectly appropriate conceptually, especially the axial-flow pump, the cost associated with the development of this pump is far beyond the scope of the project funding. However, ultimately the decision to continue with the roller pump was taken mostly for clinical reasons; the roller pump is a technology well understood in the clinical domain, and offers the benefit of continuous unidirectional flow. These are key characteristics for a device designed for deployment in this sector.

Adsorbents:

Based on the engineering design objectives of this project, it was also important to conceptually develop a technology in which it was possible to immobilise adsorbent beads. This was important in order to achieve direct whole blood contact with the adsorbent beads without presenting an embolism threat. Initial concepts were explored both computationally and in some cases manufactured into early stage prototypes. A later, more detailed concept was then explored, tested for biocompatibility and manufactured on a large scale.

From the initial concepts developed for adsorbents, many were discarded for clinical reasons and others for engineering reasons. However, some were considered appropriate for use; it was these immobilisation concepts that were pursued further, to be utilised in fully developed integrated prototypes.

The packed bed and monolithic adsorbent concepts were discarded in the early stages of their development as it was clear that they were incapable of the clinical demand. Concepts for both the teabag and fibrous matrix were pursued further and were both developed into physical prototypes.

The concept known as the “teabag” has promise for some applications. However, the adsorbent mat was considered more appropriate for the current concept.

In the case of the adsorbent mat the attachment of beads to the fibres of the non woven fabric is desirable as it prevents the beads being dislodged from the fabric as blood passes through. Migration of beads from the fabric could lead to accumulation of beads on the porous screen downstream of the non woven fabric, potentially blocking the pores in that screen and affecting blood flow. Good attachment of the beads to the non-woven fabric can be seen in figure 47, in which beads are well attached to fibres of the fabric with pedicles of acrylic adhesive.

Integration:

Having resolved the best-fit solution to each individual component of the system, consideration had to be given to the integration of these into a single device.

As many initial concepts for blood pumps, adsorbents and integrated technologies were developed concurrently, many concepts were achieved in the early stages of research that were either not taken into prototype development or not pursued for later development. Generally, as each blood pump concept was proposed, an adsorbent concept and an integrated technology were developed in conjunction, so that incorporation of these concepts into one integrated technology was relatively uncomplicated.

Since the overall device's geometry was an important factor for some of the proposed applications i.e. the military application, the concurrent development of concepts for the 3 major components often resulted in "trade-offs" and development of the blood pumps, adsorbents, and integrated technologies were all significantly interlinked.

Blood pump concepts were initially regarded in the early stages of the project as the most important component, since producing a blood pump in a portable manner was crucial to developing an overall portable technology as this is the only mechanically active component. After initial blood pump concepts were assessed and deemed feasible for this application, no individual component was regarded as more important than any other component and integrated concepts were evaluated on their performance overall.

Once multiple concepts had been proposed as potential solutions, compromises were reached based upon the positive and negative attributes of each concept to provide a best-fit technology.

Further to the development of integrated concepts, a semi-integrated concept was also pursued, in which both a portable blood pump and a detachable adsorbent filter were developed in a manner enabling uncomplicated integration. However, each element remained individually deployable. This provided a significant advantage over the fully integrated concepts, as the adsorbent chamber can be added to an existing extracorporeal circuit in the clinic, as well as being deployed independently outwith the hospital setting.

Laboratory Studies:

Initially, the laboratory studies were performed utilising the rat recombinant cytokine, TNF-α. The reasoning behind this was with a view to progressing onto a combination of the three target molecules followed by animal models at a later stage.

An initial objective was to establish the adsorbent capacity of the adsorbent beads, with a view to informing the design of an integrated device. These laboratory studies presented some interesting, but unexpected data. This resulted in a series of experimental methodologies that were reactive to the data from previous experiments, an iterative investigation.

Preliminary experiments in which rat TNF-α was suspended in PBS, showed poor controls. Firstly, in an experiment in which a solution of 1000pg/ml of this protein, suspended in PBS, was exposed to 3 increasing weights of adsorbents plus a control, it could clearly be seen that the rate of reduction of TNF-α, between the weights of carbon, was dramatic. Without attempting to draw any conclusion as to the capacity of the adsorbent, this trend clearly indicated that the adsorbent is capable of depleting TNF-α. However, in this experiment, the initial concentration of TNF-α in the solution appeared to be somewhat less than 1000pg/ml upon assay. This immediately raised concern as to the accuracy of the assay or the assay procedure.

Despite underestimations of TNF-α in spiked solutions, we did a series of experiments to determine the saturation point of the beads with respect to TNF-α. Therefore, we carried out a series of experiments in which there were repeated spikes every hour, over 6 hours. At the end of this, the adsorbents ability to deplete TNF-α from test solution appeared to be significantly impaired, suggesting saturation had been reached. However, throughout this test procedure it was apparent that there was a disparity between “known” spiked concentrations of TNF-α (1000pg/ml) and that measured by the ELISA. This was a substantial difference in the region of 40%. We felt that the disparity in measured levels may be masking some of the true adsorption profile, and at the very least, rendered us unable to accurately assess, in molecular volume terms, the adsorbent capacity of the beads.

Clearly, these findings could have significant implication for the ongoing assessment of adsorption capacity and profile. We therefore undertook to liaise with the cytokine and ELISA kits manufacturers with a view to ascertaining whether this was a

recognised issue that might have a simple solution. The response from the manufacturer was that it might be helpful if we were to use a carrier protein when assessing TNF-α in PBS.

Feeling that this issue merited further investigation, we therefore prepared a series of solutions, with and without carrier proteins, and with and without Tween20. This replicated recipes for assay diluent from various manufacturers. With all of these solutions, we prepared standard curves using both the TNF-α provided with ELISA kits and the TNF-α purchased independently of ELISA supplies. From the data, it was clear there was serious disparity between the calibration curves. Though straight lines could be seen for both TNF-α molecules, it was clear at this point that these independently sourced molecules could not be used in conjunction with the ELISA kits as calibration proved unreliable. Upon further discussion with eBioscience Ltd, we were advised that these molecules and ELISAs to be compatible, and that this issue would be addressed by the company in future. In short, these commercially available ELISA kits, sourced for TNF-α measurement could not be relied upon for the measurement of TNF-α with any degree of accuracy.

From these findings, it was clear that we must pursue a different path. Upon discussion with eBioscience, we were assured that the human form of the molecule and its corresponding ELISA were compatible and thus we pursued further adsorption studies utilising these human molecules. Studies involving human molecules correspond more closely to adsorption study methods by other research groups in this field (DiLeo, Fisher, et al. 2009; Sandeman et al. 2008; Weber et al. 2007)

Given our previous experience with controls in PBS, we decided first to establish the most appropriate fluid medium in which to suspend the human cytokines. Although other research groups had, at times, used human plasma, we were not able to pursue laboratory methods utilising this solution, due to difficulty in obtaining a reliable source. We chose to assess the viability of three fluids; PBS, assay diluent, and bovine plasma, as potential fluid media for our laboratory studies. It was immediately clear from the results that bovine plasma could not be used as a fluid medium in the ELISA protocol. The unsuitability of the bovine plasma for an ELISA protocol could be seen in all 3 plates. TNF-α, IL-6, and IL-10. Assay diluent and

PBS both showed matching calibration curves, and it was thus decided to pursue further protocols utilising PBS because of its ready availability, low-cost and apparent reliability.

Following these initial results, adsorption studies were carried out using PBS as the fluid medium suspension. The data from these adsorption studies showed clearly that the adsorbent is effective, but also showed that the controls of the interleukins were decaying over time. Initially, the reasoning behind this was believed to be the lack of carrier protein in the fluid medium, and therefore, the introduction of a carrier protein was investigated. This, however, led to further unexpected data. The introduction of carrier protein into the fluid medium appeared to steady the controls of the interleukins, but also, prevented the adsorption process. This was true of TNF-α, as well as the interleukins. Again, during these experiments the decay in interleukins could be seen in the controls that did not have carrier protein. The reasoning behind this is unclear, as the beads have been shown to perform well in fluids with many proteins present, such as blood or plasma. We believe it may be due to the cytokine-carrier proteins complexes blocking the porous structure of the adsorbent under the laboratory conditions used; or the unselective binding of carrier proteins to the capture antibody of the ELISA, therefore preventing accurate readings

From the experimental protocols performed, in which cytokines were suspended in PBS both with and without a carrier protein, it could clearly be seen that the adsorbent is capable of a high level of performance. Its efficacy, however, cannot be calibrated effectively without a greater understanding of the mechanisms responsible for apparent decay of the interleukins when present alone in PBS. We therefore decided to investigate the mechanisms behind this decay.

Initially, the materials used as containers throughout previous experimental protocols were investigated as the source of target molecule loss. The only material found to have any particular effect on the concentration of the cytokine in solution was the Eppendorf plastic vials. We believe that this is due to the Eppendorf plastic's highly porous polymeric structure. This is one of the many factors that may contribute to the apparent decay of controls, but does not continue solely to this effect.

The decay of controls in *in vitro* studies appears to be an issue that has been largely ignored, or gone unnoticed, in the literature, with many researchers choosing not to compare adsorption profiles with controls (Howell et al. 2006)

Another factor thought to affect the rate at which cytokines degrade was whether or not they were present in PBS in combination or alone. We found that when the interleukins were suspended in PBS alone they would decay rapidly, but when in the presence of other cytokines, this rate would reduce somewhat. This was most noticeable in the decay of the interleukins when in the presence of TNF-α. The presence of TNF-α appears to slow the decay of the interleukins, in the same way a carrier protein also appeared to do so in our earlier work. This may be due to the fact that TNF-α is the largest of the three molecules and appears to have a limited degree of decay associated with it. The decay of IL-10, when alone, in PBS appeared to be associated with the most prominent rate of decay, and the presence of TNF-α noticeably reduced this.

Laboratory findings confirmed that it is critical to compare any adsorption profile with an effective control; otherwise the true adsorption capacity of an adsorbent cannot be verified. We found this to be most acutely noticeable with IL-10, and least so for TNF-α.

The use of human plasma, by other researchers, appears to eliminate this issue, to some extent, in the case of TNF-α and IL-6 (Tripisciano et al. 2011), though the use of IL-10 in these studies appears to have been neglected.

Clinical Studies:

It appears from table 1 that the post-CPB levels of the cytokines TNF-α and IL-6 and was relatively similar for all 5 patients.

In the case of IL-10, however, there was massive variation between the patients' post-CPB cytokine levels, limiting the statistical significance that can be drawn between the control sample and the patient samples at the earlier stages of the protocol.

The first 20mins period of adsorption did not appear to produce any statistically significant decrease in the cytokine concentration, despite what might be expected. This apparent steady level may be due to a combination of factors;

- Adsorption reducing the concentration of cytokine
- Blood contact with the surface of a foreign object increasing the level of cytokines in the blood, as part of an inflammatory response. This is a phenomenon that has been observed previously (Lappegard et al. 2008).

Upon spiking the blood, the cytokine concentrations increased in a highly predictable fashion. Beyond this spike, the control for the TNF-α protein, remained relatively steady throughout the procedure, with a little noise.

Initially after spiking, the blood concentration levels for both the TNF-α and IL-6 molecules appeared to increase slightly from the spike point for 5mins. It is highly likely this is due to these cytokines diffusing through the blood, and the blood mixing to a consistent concentration throughout the fluid sample. Within 10mins, the controls for all 3 molecules appear to trend downwards, followed by upwards. This occurs to a significant level. Though this may simply be noise in the assay procedure, other factors that may be affecting this are;

- The blank material having some moderate adsorptive properties of its own.
- The blood-surface interaction inducing a further inflammatory response.
- The cytokines forming dimers or trimers when in high concentration.

It is likely that a combination of these factors, both reducing and increasing the cytokine concentration, results in the apparent noise seen in the assay procedure, in these near clinical studies.

One hour after spiking, the performance of the adsorbent can clearly be seen to have achieved a statistically relevant reduction of cytokine for all three cytokines targeted.

Overall, in the control samples, the initial 1 hour of the protocol resulted in no significant reduction of the TNF-α. This is also true of the 2nd hour (repeat) of the protocol.

This is not the case, however, in the case of the interleukins. Similar to the trends we have seen in our laboratory studies utilising PBS, the controls for both IL-6 and IL-10 appear to reduce for both the first hour, after the first spike, and the 2nd hour, after the 2nd spike. This reduction in the control was, once again, most apparent in the case IL-10. Again, despite this reduction in the control, there remains a statistically significant reduction of the sample concentrations throughout, for both the first hour after the spike, and the 2nd hour after the re-spike.

Though the statistical significance is limited for the first few time points, particularly in the case of IL-10, the trend in the mean values can clearly be seen and statistical significance can be observed by the end of each hour.

This decay of interleukin controls is an issue we initially believed would be resolved by the use of human blood. However, as we have seen here in our clinical studies, it is clear that this is not the case. Often, other research groups working in this field do not display controls with their data. Many groups find creative ways by which to avoid displaying the raw control data, such as only displaying the percentage reduction at each time point compared with the control. This can be deceptive in that, for example, a 90% reduction would imply a highly efficient adsorption profile; however, at the end of an experimental protocol, the control sample may be considerably decayed, therefore, a 90% reduction may no longer be associated with a high removal of cytokine from a sample.

This decay which we have seen repeatedly in multiple fluid media, including blood, is a critical issue that should be addressed whenever attempting to suspend interleukin proteins in fluid media, and especially when trying to draw conclusions about the efficacy of an adsorbent material.

Given the success in our near clinical data to effectively reduce cytokine concentrations for our 3 target molecules, we believe that this device may represent a genuine therapeutic option for patients with SIRS/sepsis

Another research group working on a cytokine reduction technology, the CytoSorb device, chose to target the IL-6 molecule, and have shown in laboratory studies that they are capable removing this molecule to approximately 50% of its initial concentration, after 1 hour of circulation through their device.

They have later gone on to show in animal models that this adsorption profile is associated with a significantly improved survival. The CytoSorb device in now undergoing a large scale clinical trial and has received European approval under the CE mark and is now sold in the EU.

Many of the research groups working in this field, including those who developed the CytoSorb device, have stated their concern in adsorbing TNF-α, claiming it is the most challenging molecule to adsorb, as it is a much larger protein than the interleukins, particularly in its trimeric form; its most common form (Yushin et al. 2006). The CytoSorb research group went on to investigate the effect of deoligomerisation of the TNF-α protein into its monomeric form. This led to an accelerated adsorption profile of the monomeric form. This group progressed to investigate strategies by which to deoligomerise the TNF-α protein within the device before adsorption occurs (Kimmel et al. 2011).

In their *in vitro* studies they also found that the use of purified BSA as a carrier protein did not adversely affect the adsorption profiles of their target cytokines; but the use of unpurified BSA did. They believe this is due to small molecular weight impurities of the BSA competing for sites in the porous adsorbent. These BSA impurities are present in the PBS at a much higher concentration than the cytokines. BSA is a 66kDa protein, and the trimeric form of TNF-α is 51kDa. Both of these molecular weights appear to be above the distribution of weight which the CytoSorb adsorbents are capable of binding.

In comparing our data with that of this research group we can see that our adsorbent material is capable of binding a higher distribution of molecular weights. This has pros and cons. Having this greater adsorption distribution allows for us to achieve the direct removal of the trimeric form of TNF-α, without requiring any pre-processing

deoligomerisation step. However, this is almost certainly the reason why the use of carrier proteins in our laboratory work adversely affected the adsorption profiles of the target molecules.

The carrier proteins we used included BSA (66kDA), and FBS, which is rich in BSA, as well as a-1-glycoprotein (48.4kDA). This glycoprotein falls well within the adsorption weight distribution of the beads, and these molecules would certainly compete for binding sites on the porous structure at the concentrations that can be anticipated in this solution.

Reported findings (Kimmel et al. 2011) corroborate our suspicion that the use of carrier proteins in cytokine adsorption work *in vitro* adversely affects the adsorption profile of the adsorbent material.

Our adsorbent carbon's ability to bind trimeric TNF-α readily is a significant clinical. It does, however, affect our ability to establish the adsorbent capacity in laboratory studies.

6. Conclusions and Future Work:

This work set out to investigate the development, efficiency and function of a portable cytokine adsorption technology. The outcomes from the work, however, had broader impact. We uncovered complexities in the laboratory investigation of cytokine adsorption, which to our knowledge, had not been previously reported. This somewhat complicated our work, but will act as a guide to future investigators in this area. A number of conclusions have been drawn from this work, including;

- The clinical studies have shown that the adsorbent technology utilised in this research has a considerable ability to remove cytokines from circulating blood and we suggest that this has the potential to treat SIRS/sepsis.

- The work confirms that there are significant limiting factors that need to be addressed when undertaking research involving interleukins. In particular, these laboratory studies show an apparent decay of interleukin controls when suspended in PBS. The introduction of a carrier protein, as recommended by the interleukin providers, did prevent this decay. However, it appeared to prevent the adsorbent beads from performing effectively. It is likely that this is due to the carrier proteins being within the weight range of the adsorption profile.

- The trend for interleukin controls to decay over time was shown again in our clinical data. In this case, the interleukins were in their natural environment, and this apparent decay may therefore be concluded as being associated with the interleukin natural half-life. Though half-life has been discussed in the literature, there does not appear to be any consensus, but it is a factor that must be vectored into any investigation of cytokine adsorption. The magnitude of the decay was such that it could mask or exaggerate the adsorption effects.

- Through this work, we were able to effectively integrate a portable blood pump and adsorbent technology into a miniaturised and integrated system.

This, coupled with the adsorbent's considerable ability to remove cytokines from circulating blood, met our engineering design requirements. This was surpassed by the success in our clinical studies.

Future Work:

Since cytokines are heavily implicated in both inflammation and coagulation, we suggest that a treatment focused on reduction of cytokines will not only moderate the inflammatory response, but also moderate, to some degree, the coagulatory abnormalities also associated with SIRS/sepsis. Previous work, involving suspending human cytokines in animal blood or its derivatives, or even human plasma, cannot draw a full picture regarding the nature of the inflammatory/coagulatory response in the human environment. However, the use of whole human blood may provide an insight as to the effect that cytokines have on coagulation factors, and the result, if any, that reduction of cytokines has on these coagulation abnormalities.

Experimental protocols involving the spiking of whole human blood with cytokines could be expanded to involve the measurement of prothrombin time (PT), activated partial thromboplastin time (aPTT) and activated clotting time (ACT). The PT and aPTT coagulation factors measure the intrinsic pathway and the extrinsic pathways of the coagulation cascade; and ACT is the typical clinical measurement of coagulation during extracorporeal techniques, and as such is highly relevant to clinical procedures.

If ready access to a large supply of whole human blood is available, further research investigating coagulation abnormalities associated with elevated blood concentration levels of cytokines can be performed with our technology.

The filtering of spiked whole human blood through an adsorbent filter and the measurement of cytokine levels in conjunction with coagulation measurement could provide valuable insight into the efficacy of this adsorbent technology to reduce to coagulatory response as well as the inflammatory response.

Through the previous work, we have demonstrated the effectiveness of our adsorbent technology under near-clinical conditions. Given that the technology underpinning this device is now patented and approved for clinical use, there is no restriction on deploying it under clinical conditions. Future clinical studies on SIRS/sepsis patients are now in the planning stage and it is hoped will take place over the next 12 months.

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Appendices:

Attached is the ethical approval of the ethics committee of the University of Kirikkale.

TURKISH REPUBLIC
UNIVERSITY OF KIRIKKALE
LOCAL ETHICS COMMITTEE

APPROVAL FOR RESEARCH APPLICATION

APPLICATION INFORMATION	PROTOCOL NUMBER	12/45		
	PROTOCOL NAME	Use of Carbon Adsorbents to Filter Cytokines in Blood from Post-CPB Patients		
	PRINCIPLE INVESTIGATOR AFFILIATION/NAME	Professor, Serdar Gunaydin, M.D., PhD Professor Terence Gourlay, PhD		
	RESEARCH CENTER	University of Kirikkale, Medical Faculty, Department of Cardiovascular Surgery Strathclyde University, Bioengineering Department, UK		
	ETHICS COMMITTEE APPLIED	University of Kirikkale, Local Ethics Committee		
	SUPPORTING INDUSTRY			
	PHASE	Laboratory Examination		
	PARTICIPATING CENTERS	Multi-center International		
DOCUMENTS EVALUATED	**Document Name**	**Date**	**Version No**	**Language**
	RESEARCH PROTOCOL	20.01. 2012	Version 1.0.	English, Turkish
	INVESTIGATOR'S BROCHURE			
	VOLUNTEER INFORMED CONSENT FORM FOR TURKEY	20.01.2012	Version 1.0.	Turkish
	SECURITY CARD FOR PARTICIPANT			
DECISION INFORMATION	**Decision No: 12/45**	**Date: 23.02.2012**		
	The multi-center research application file and related documents of the abovementioned study that is planned to be carried out in the Department of Cardiovascular Surgery under the supervision of Prof. Serdar Gunaydin, M.D., PhD have been reviewed by our Board according to the aspects, aim, approach and methodology and approved ethically by unanimous vote of participating staff members the main study.			

ETHICS COMMITTEE INFORMATION

PRINCIPLE OF ACTION — **GOOD CLINICAL PRACTICES GUIDELINE**

MEMBERS

Affiliation/Name/Surname Ethics Committee Membership	Specialty	Association	Gender	Conflict of Interest (*)	Participation (**)	Signature
Prof. Serdar Gunaydin, MD Chair	Cardiovascular Surgery	University of Kirikkale	Male	*Yes*	*No*	
Associate Prof. Siyami Karahan, DVM, PhD Vice- Chair	Veterinary Medicine	University of Kirikkale	Male	*No*	*Yes*	
Prof. Aytul Cakmak, MD, PhD Member	Public Health	University of Kirikkale	Female	*No*	*Yes*	
Associate Prof. Hakan Kalender, DVM, PhD Member	Veterinary Medicine	University of Kirikkale	Male	*No*	*Yes*	
Prof. Saadet Atsu, PhD Member	Dentistry	University of Kirikkale	Female	No	*Yes*	
Assistant Prof. Nahit Pamukoglu, PhD Member	Biology	University of Kirikkale	Male	*No*	*Yes*	
Prof. Aydin Yagmurlu, MD	Pediatric Surgery	Ankara University	Male	*No*	*No*	
Mustafa Akin	NGO		Male	*No*	*Yes*	

*Conflict of interest with this study **Participation in the session