Immobilisation of Lactate Oxidase and Deoxyribonuclease I for use within a Bio-Artificial Liver Assist Device for the treatment of Acute Liver Failure

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Declaration

I, Katherine Bethany Lintern, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I can confirm that this has been indicated in this thesis.

Acknowledgements

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This thesis is dedicated to my uncle, Kenneth Dennis Edward King.

Abstract

Constraints of cell supply indicate that proliferating cell lines are likely to be essential components of Bio-Artificial Liver support devices (BAL) for the treatment of acute liver failure. The Liver Group BAL employs clones of cells derived from the HepG2 cell line, which in common with many tumour derived cells, are predominantly dependent on anaerobic glycolysis for energy supply, leading to production of lactate within the bioreactor. The BAL system requires prolonged culture of alginate encapsulated HepG2 cells, and lactate accumulation presents a potential hazard in this system: at ~15 mM, accumulated lactate becomes toxic to the cells in the bioreactor, and also compromises alginate bead integrity by chelating the calcium ions necessary for alginate polymerisation. Furthermore, the tumour lineage of the cells could prove a potential threat to patient safety should any HepG2 DNA enter the patient's system.

It was hypothesised that inclusion of immobilised Lactate oxidase (LOx) to catalyse degradation of lactate into pyruvate could offset these limitations whilst simultaneously providing a potential energy source utilisable by HepG2 cells. In a similar fashion, immobilised Deoxyribonuclease I (DNase I) could be utilised to remove non-patient DNA during the treatment phase of the BAL system.

Here it is demonstrated that functionalised glass beads are a feasible method of immobilising LOx and DNase I. Enzymatic activity was retained even after prolonged incubation at 37°C in the presence of human plasma, offering a means of reducing lactate levels during HepG2 culture, and potentially removing circulating DNA below practically detectable levels, thus facilitating cellular performance and BAL efficiency as a safe and effective potential therapy for acute liver failure.

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List of Abbreviations

4-AAP	4-aminoantipyrine
α-MEM	Alpha minimal essential medium
ANOVA	Analysis of variance
AFP	Alpha-fetoprotein
ALF	Acute liver failure
ATP	Adenine triphosphate
BAL	Bio-Artificial liver assist device
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
cfcDNA	Cell free circulating DNA
DMSO	Dimethyl sulfoxide
DNase I	Deoxyribonuclease I
dNTP	Deoxyribonucleotide triphosphate
ECCC	European collection of cell cultures
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELS	Encapsulated liver cell spheroids
ELISA	Enzyme-linked-immuno-sorbent-assay
FAD	Flavin adenine dinucleotide
FBB	Fluidised bed bioreactor
FCS	Foetal calf serum
FDA	Fluorescein diacetate

FFP	Fresh frozen plasma
FMN	Flavin mononucleotide
GMP	Good manufacturing practice
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HBSS	Hank's balanced salt solution
LoD	Limit of detection
LOx	Lactate oxidase
mtDNA	Mitochondrial DNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHS PEG	O,O'-Bis[2-(N-succinimidyl-succinylamino)ethyl] polyethylene glycol
OPD	o-phenylenediamine
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PERV	Pig endogenous retro-virus
PI	Propidium iodide
QC	Quality control
qPCR	Quantitative real-time PCR
SAP	Serum Amyloid P Component
SDS-PAGE	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
TAE	Tris-acetate ETDA
TRH	Thyroid releasing hormone

Chapter 1. General Introduction

1. General Introduction

The scope of this study falls into a rather niche and specialised field and so it is necessary to thoroughly establish the context, background and ultimately the relevance of this work to the scientific community. The physiology of the human liver will be discussed, followed by the aetiology and treatment of liver failure conditions, particular emphasis being placed upon current liver-assist device development; it is at this point where the process of enzyme immobilisation and the potential use of said enzyme systems in a clinical application will be explored.

1.1. The Liver

The human adult liver is located in the upper right quadrant of the abdominal cavity and is, by mass, the second largest organ of the body, weighing on average around 1.5 kg and thus contributing approximately 2% of the total body mass. The liver is a highly metabolically active organ and thus receives a seemingly disproportionately large percentage of oxygen rich blood from the hepatic artery – up to 25% of the total cardiac output - and is also supplied via the portal vein. Both then subdivide into a network of capillaries to supply the four lobes of the liver, which can be further subdivided into lobules, the functional units of the liver (1). The lobule has a hexagonal structure, comprised of between 1×10^5 and 2×10^5 hepatocytes, arranged in arrays one or two cells thick around the central vein. Between two arrays are specialised blood vessels, termed sinusoids, which are lined with sinusoidal cells and connect the portal venous system to the systemic venous system. Between four and six portal triads surround the lobule, themselves comprising the terminus of portal vein and hepatic artery branches, and a bile duct. Small bile ducts are also found between two hepatocytes, whilst the space between hepatocytes and sinusoidal cells is termed the Space of Disse.

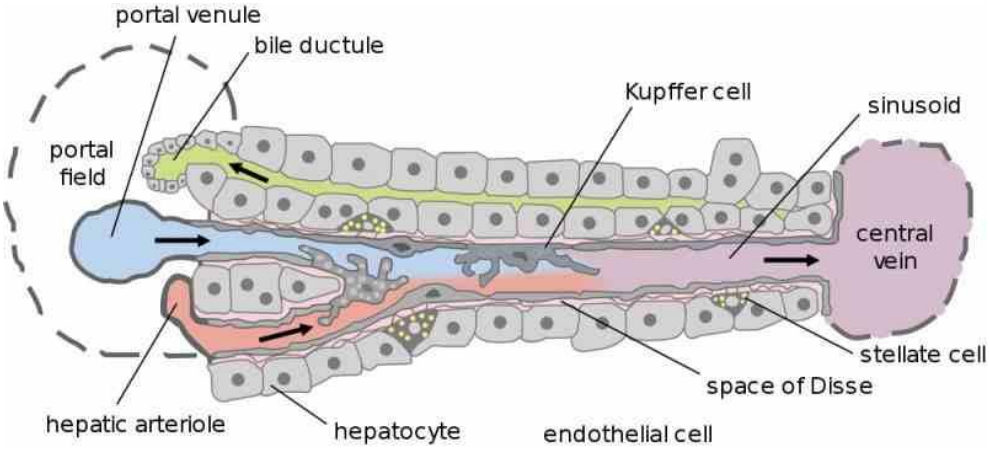


Figure 1-1– Representation of the liver lobule.

Arrays of hepatocytes surround the central vein, from which sinusoids extend radially. Portal triads are found at the lobule periphery, comprising the terminus of portal vein and hepatic artery branches and a bile duct. Hepatocytes, Kupffer cells, sinusoidal and stellate cells are found in the lobule unit.

http://php.med.unsw.edu.au/embryology/images/0/02/Liver_structure_cartoon.jpg

1.1.1. Structure and function of the liver

Benefiting from such an extensive blood supply assists the liver in performing its "cleansing" roles; the liver is responsible for detoxifying molecules which would otherwise prove hazardous to health, in addition to its role as a centre of metabolism and molecular synthesis. In terms of synthesis, the liver is the site of production of bile acids, which are essential for digestion (1). Other molecules to be produced by the liver include proteins such as blood clotting factors, heparin, cholesterol, albumin, oestrogen and somatomedins (2;3). In addition to synthesising bile acids and cholesterol, the liver is also responsible for their filtration and detoxification (4), along with bilirubin, hormones and ingested drugs and poisons. The liver also serves as a filter for the removal of amino acids, dead blood cells and Immunoglobulin A (5).

Multiple cell types comprise the liver (6), including sinusoidal cells, Kupffer cells, hepatic stellate cells and hepatocytes Mitochondria-rich hepatocytes account for 60% of the liver cells and are responsible for many metabolic functions including bile production (up to 15 ml/kg/day in healthy humans) (6) and glycogen synthesis. The liver has the ability to convert lactate back into glucose by means of gluconeogenesis, which if not immediately required as an energy source, can be converted and stored in the liver cells as glycogen. As would be expected of such a metabolically active and productive organ, the number of genes expressed in the liver is second only to the brain (7). The majority of these genes are transcribed in the hepatocyte, but in disease, the increase in gene expression is mostly undertaken by other cell populations such as stellate cells. It is worth noting that the liver is unique among mammalian organs in that, extraordinarily, it is able to regenerate back to its original size from as little as 25% of its initial mass (8).

Taken together, it is evident from the physical and functional complexity of the liver why maladies of this organ can have such devastating consequences. These can range from the effects of inborn metabolic disorders such as the lysosomal storage disorders, Gaucher Disease and Fabry Disease, to infectious diseases such as viral Hepatitis C, to autoimmune conditions (e.g. Autoimmune Hepatitis, Systemic Lupus Erythematosus (SLE)), drug (commonly by N-acetyl-p-aminophenol (Paracetamol)) related damage and lifestyle induced conditions as seen in fatty liver disease and alcoholic liver disease. The implications of liver failure will be discussed in the following section.

1.2. Liver Failure

An estimated 1-2 million people worldwide die each year from hepatic failure (9). There are two basic types of liver failure, chronic and acute. Chronic liver failure is defined as the deterioration of liver function over a prolonged period of time, whereby functional hepatocytes are replaced by

the processes of cirrhosis and/or fibrosis. Acute liver failure (ALF) results following the death of liver cells within a short period of time and is characterised by the rapid loss of liver function, including a decrease in Factor V levels and the onset of jaundice and hepatic encephalopathy (9). ALF represents one of the liver maladies with the poorest prognoses, with orthotopic liver transplantation being the most widely and successfully used method of treatment. Whilst viral infection, particularly with Hepatitis E, is the principal cause of ALF in the developing world (10), drug-induced liver failure is more commonly described as the causative agent of ALF in Western countries. However, the cause of this malady is not always evident and furthermore it can arise as a complication of an underlying liver condition such as cirrhosis, referred to as acute-on-chronic liver failure. Following the onset of symptoms, the condition of ALF patients rapidly declines; mortality can occur after only a matter of days with a particularly rapidly progressive form of liver failure, fulminant liver failure, if not treated appropriately (11), with brain edema and intracranial hypertension being the leading causes of death (9). It is thus necessary that in addition to a rapid diagnosis, there is a mode of treatment which is readily available. Therefore, in recent years great emphasis and effort has been invested within the medical and scientific community to develop an alternative to organ transplantation.

1.3. Liver Failure Treatment Strategies

Currently, if the patient's liver is unable to regenerate following the insult, transplantation is the treatment of choice for liver failure. There are two types of transplant methods, which use either material from deceased or living donors. Unfortunately, the situation is far from ideal as the demand for transplantations far outnumber the availability of donor organs. Data from 2007 shows that in the United States there were some 17,500 patients on a waiting list for liver transplantation; yet annually typically just 6,000 transplantations take place with more than 1,700 patients dying whilst on the waiting list (12). The most recent UK data published by the NHS for the four quarter period 2011/2012 shows that there were 764 liver/lobe

transplant operations (726 deceased donor, 38 living donor) yet a further 507 patients still awaiting a transplantation (excluding those temporarily suspended from the waiting list) (13). Furthermore, a recent study revealed that the proportion of unused donor livers has risen drastically since 2004 (14), with organs being deemed unsuitable due to poor liver condition. This phenomenon has been hypothesised to be linked to an increasing incidence of obesity and an ageing population. In addition to the challenges presented by resource scarcity, patients in need of liver transplantation must also contend with the fact that after transplantation they will be subjected to an immunosupression programme for the duration of their life in order to avoid the new liver being rejected by the immune system.

Therefore it is apparent that an alternative treatment to transplantation be developed, which can be readily made available when and as required, whilst keeping side effects to a minimum.

1.3.1. Biological approaches

The use of non-human organs for transplantation has been practised globally when no suitable human equivalent can be obtained. The caveat here is the risk of immune rejection and also the potential for the transmission of zoonoses and cross-species pathogens, in particular of endogenous retroviruses (15). However, a small study on patients linked extracorporeally to porcine kidneys detected no measurable level of pig endogenous retro-virus (PERV) in the patients following treatment (15).

The transplant of isolated liver cells to support liver function in acute and/or fulminant liver failure patients is an active area of research since the idea was first proposed in the 1970s (16). Although primary human hepatocytes would be preferable they are infamously difficult to proliferate in culture and are heavily reliant on cell-cell contacts and the extracellular matrix (17). In an attempt to overcome the challenges presented by the use of primary hepatocytes other human cell lines have been explored, including

pluripotent stem cells and embryonically derived stem cells, both of which proliferate well (18). However, apart from the obvious ethical issues this raises, there is also the consideration that these highly proliferative cells may pose a tumorigenic risk to the patient. Moreover, to date, there is no suitable and readily available stem cell derived hepatocyte line.

Encapsulating non-human xenografted or human allografted liver cells within a synthetic membrane prior to transplantation reduces the likelihood of rejection by the patient's immune system as the contained cells do not come in to contact with cellular components of the immune system although there remains a body of evidence to suggest that an inflammatory response may still be triggered (19;20). However, this approach must ideally be used only following the clearance of plasma toxins induced by liver failure (21;22), as such substances have a profoundly detrimental effect on the survival and function of the transplanted cells (23). Moreover, the synthetic membrane prevents normally secreted proteins from entering the circulation; with a molecular cut-off of 70,000 kDa the membrane would prevent diffusion of large proteins, such as fibrinogen (340,000 kDa).

1.3.2. Artificial liver support strategies

Artificial liver support devices serve to remove toxins from the patient's blood, a role which the failing liver is no longer able to adequately perform. This is critical, as otherwise an accumulation of products such as ammonia will have a detrimental effect upon the body, often leading to sepsis and / or multiple organ failure, which are common complications of untreated liver failure. Several systems have been explored to fulfil this role, the most prominent of which will be briefly discussed. Generally speaking, these methods fall into one of three categories: those which remove toxins based upon their charge; those which remove toxins by binding to albumin; and, those which involve plasma exchange to remove harmful substances from the patient's system.

Bio-Logic DT utilises the key principal of sorbent technology to remove toxins from the patient's circulation, which were commonly believed to be the principal cause of liver failure associated complications (24). However, this has been shown to not be the case, with a multitude of other entities, including endotoxin, mediators of the inflammatory immune response and other signalling molecules, also playing fundamental roles in the progression of liver failure symptoms. Many of these molecules cannot be removed by simple haemodialysis or adsorbed via charcoal, thus explaining why such approaches have shown little or no benefit in clinical applications (9).

Prometheus® passes blood through an albumin permeable hollow-fibre filter before it enters a series of two resin cartridges, following which the blood is haemodialysed before being returned in a purified state to the patient (25). The rationale is that molecules bound to the patient's albumin will be sequestered by albumin in the filter, thus effectively cleansing the patient's albumin. Although this combination of strategies permits the removal of both water soluble toxins and also albumin bound substances (25;26) there is to date limited clinical evidence that Prometheus® improves the prognosis of liver failure patients (27); a recent study showed no significant improvement of survival rates between acute-on-chronic liver failure patients treated with the Prometheus® device compared to those receiving the standard medical therapy (28).

Molecular Adsorbent Recirculating System (MARS)® is an albumin dialysis based method of performing the blood purification functions of a failing liver, comprising of a dialyzer, a charcoal column and an ion exchange column. It utilises an albumin containing solution to dialyse and remove both albumin bound (bilirubin and bile acids) and soluble molecules (such as urea and ammonia) from the patient's blood across a high flux membrane (29;30). However, ultimately MARS® is only capable of

removing toxins from the liver failure patient's blood and is unable to support the failing metabolic or synthetic functions of the liver.

A further use of activated charcoal to remove blood toxins has been employed whereby the charcoal particles are encapsulated within an inert polymer casing (31), forming an artificial cell which can then be administered to the patient. This method has advantages in that it can be used when standard dialysis methods are not suitable, and also avoids the damaging effect of embolizing particles on cells which occurs when bioadsorbants are used in isolation. Furthermore, in clinical trials this approach successfully saw Grade 4 Hepatic Coma patients recover consciousness (32;33).

1.3.3. Bio-Artificial liver devices

The development of a Bio-Artificial liver device (BAL) would represent a major advance in improving the prognosis of patients suffering from acute liver failure (34;35). Such a device would serve to perform short term liver-specific functions such as protein synthesis, detoxification and synthetic and metabolic homeostasis, allowing sufficient time for the liver to regenerate and recover, or in extreme circumstances, serve as a bridge to liver transplantation. This would be of great significance, as currently there is an insufficiency in the number of donor organs available and a dismally high number of liver failure patients expire whilst on the transplantation waiting list. Furthermore, a biological approach to cleansing the blood has an advantage over carbon based adsorption therapies, as the latter indiscriminately bind molecules, including those which are beneficial to patient health. There are currently several BAL devices being developed, which will be briefly described in this section.

Technically a hybrid of artificial and Bio-Artificial approaches, treatment with HepatAssist^{TM} involves the separation of cellular and plasma

components of blood by plasmapheresis, whereupon the plasma phase passes through a charcoal filter and then warmed and oxygenated before entering a hollow fibre chamber housing some 6 x 10^9 porcine cells (36-38). The charcoal filtration pre-treatment of plasma promotes the efficacy of the treatment as toxic substances which would have detrimental effects on cellular growth and performance, are purged before reaching the cells (37;38). A logical explanation for the lack of success of this method can be attributed to the simple insufficiency of cells; only 6 x 10^9 cells were utilised, representing just 6% of the hepatocyte population in a healthy 75 kg human adult; it has been demonstrated that liver resection procedures are only successful when the postoperative liver volume is at least 35% of the original mass (39).

The Extracorporeal Liver Assist Device (ELAD) also utilises a hollow fibre chamber, although here the cell line of choice is C3A, derived from a human hepatoblastoma (HepG2) line. Although early trials showed no improvement in the outcome for patients compared to the standard mode of treatment for liver failure (40) safety was demonstrated (particularly important in this application as the use of human carcinoma cells had not previously been used in a clinical setting) and the ELAD system continues to be developed and trialled. However, this approach suffers from the poor mass transfer of nutrients and metabolites, owing to the structure of the hollow fibre chamber. Inevitably, this compromises the efficacy of this system, and prevents the possibility of cryopreserving the chamber.

1.3.3.1. The Liver Group BAL

The BAL currently under development by the Liver Group (LG) at the Royal Free Hospital Campus, UCL Medical School, is an extracorporeal device, where the plasma phase of the liver failure patient's blood would enter a double circuit system and pass through a chamber containing three-dimensional spheroids of HepG2 cells which would perform the normal liver functions of detoxification and substance metabolism and synthesis.

The cells are encapsulated within alginate, a non-toxic material, and contained within a fluidised bed bioreactor (FBB). The advantages of using this type of chamber to culture cells are well known (41) but, succinctly, have the benefit of utilising microgravity principles to promote the growth of multicellular spheroids. As spheroids, HepG2 cells have improved cell-cell contacts (34) which are critical for their growth and performance, thus enhancing efficiency of the BAL device. It is accepted that a greater degree of complexity is given to the structure and function of the liver by the effect of the location and microenvironment on the cells of the liver. It is well documented that the metabolic, synthetic and genetic transcript profile of hepatocytes, stellate cells, sinusoidal endothelial and Kupffer cells all vary depending on their specific location within the liver (42) and thus the local concentration gradients of oxygen saturation. By culturing the HepG2 cells as spheroids in a microgravity environment, the mass transfer of metabolic products and metabolites, including oxygen, is optimised, permitting the maximum productivity of the cells and hence efficacy of the treatment. Furthermore, another advantage of using a micro-gravity method of cell culture rather than, for example, growth in a hollow-fibre support matrix, is that the potential failure of the device due to pore blockage is eliminated.

The BAL system utilises alginate bead encapsulated HepG2 cells (34), a human hepatoblastoma line derived from a 15 year old Caucasian male. Primary human hepatocytes would seem to be the ideal candidates for use in the BAL, as it is this cell type which accounts for the majority of the liver mass (6). However, a number of limitations including a difficulty with expanding cell number in culture (43), restricted availability of cells and a loss of transcription of liver specific genes throughout the isolation and culture process precludes their use. HepG2 cells are currently the best available human cell line for use in the LG BAL (34), as HepG2 cells maintain several properties typical of hepatocytes, including the synthesis of albumin and clotting factors (44-46). Furthermore, being of human origin, HepG2 cells are not subject to the safety and biocompatibility concerns associated with the use of xenogenic cell lines. Although this BAL has not

yet reached clinical trials, *in vitro* studies have shown that encapsulated HepG2 cells show good protein synthesis (demonstrated by an increase in albumin, clotting factors and alpha-1 antitrypsin production) and detoxification of bilirubin (34) when perfused with human liver failure plasma. Additionally, the use of HepG2 spheroids encapsulated in uncoated alginate beads allows greater mass transfer of metabolites and nutrients, and it is possible to make a sufficiently accurate calculation of cell number and hence viability and efficiency of the BAL system, a feat which is not possible with hollow fibre based methods, for example.

However, the utilisation of HepG2 cells is not without some drawbacks; this cell line lacks some detoxification functions, including high Cytochrome P450 activity (47;48) and there is a lack of ammonia metabolism. Whereas these issues can be ameliorated, if not completely overcome, by adjusting the growth media of the cells or by genetic manipulation, there are other problems associated with the use of HepG2 cells which must be addressed if they are to be successfully incorporated into a clinically approved BAL device. Among these is the potential threat of circulating HepG2 DNA to the patient; it has been proposed that any DNA released from dead HepG2 cells may trigger an immunogenic response from the patient's immune system. Another key concern is with regards to the high levels of lactate produced by HepG2 cells as a result of aerobic glycolysis, a common phenomenon in many cancer cell lines (49). In addition to having the potential to compromise cellular performance (low pH can be detrimental to enzyme integrity and trigger caspase-induced apoptosis), the Liver Group laboratory investigations have shown that concentrations of lactate in excess of approximately 15 mM cause a loss of alginate bead integrity by chelating the calcium ions required to stabilise the alginate hydrogel. If this occurs, the cell spheroids could break free and become free-flowing within the BAL, jeopardising the efficacy and safety of the treatment. In order to best understand the potential threat lactate and DNA pose to the BAL, the following sections will discuss the nature of these molecules in more detail.

1.4. Lactate

The Liver Group BAL incorporates HepG2 cells encapsulated within sodium alginate ($NaC_6H_7O_6$) beads. Alginate is a non toxic, biocompatible anionic polymer (50) which is polymerised to form a stable hydrogel by the interaction between carboxyl groups and divalent ions such as calcium (50-52). However, this process is reversible and the integrity of the beads is compromised when divalent ions are removed (53) as occurs when lactate levels accumulate within the FBB. Furthermore, lactate has been shown to increase the proportion of free calcium in solution (53) and is believed to achieve this by chelating the divalent ions from insoluble calcium (54).

Lactic acid ($C_3H_6O_3$) is a carboxylic acid with a dissociation constant (pK_a) of 3.86 which when in solution dissociates to form its conjugate base, lactate. It is a chiral molecule, having both an R- and L- form, the latter being the predominant stereoisomer in the human body. It is found in human blood at a base level of 1-2 mmol/L, although this can rise 10-fold during periods of strenuous exercise. A product of glycolytic respiration in oxygen limited conditions or when energy demand is elevated, lactate is produced from the reduction of pyruvate, itself the product of glucose oxidation. Although only two molecules of ATP are generated per glucose molecule instead of the 36 during full aerobic respiration, only the glycolytic pathway is utilised which has far fewer reaction steps and so a more rapid glucose to ATP turnover is possible. The cell line utilised in the BAL, HepG2, favours the glycolytic pathway for the generation of ATP, consequently generating lactate, and therefore highlighting the requirement to establish a means of reducing lactate concentration in the FBB to acceptable levels.

1.5. Deoxyribonucleic Acid

Deoxyribonucleic Acid (DNA) contains the hereditary genetic information of an organism, found mostly in the nucleus of eukaryotic cells and in the cytoplasm of prokaryotes.

1.5.1. DNA structure

The structure of DNA was famously discovered by James Watson and Francis Crick in 1953 although the molecule had been identified in the late 19th century. One of four bases (adenine (A), cytosine (C), thymine (T) and guanine (G)) is joined to a pentose sugar, 2-deoxyribose, and a phosphate molecule to form a nucleotide, the monomer of DNA (55). Polymerisation of these monomers links adjacent deoxyriboses via phosphodiester bond formation, generating an external "back bone" to the DNA strand. The bases are classified by their molecular structure as being a purine (A and G) or a pyrimidine (C and T) molecule; a purine binds with a pyrimidine (A-T and G-C) via hydrogen bonds thus forming "rungs" between two strands of DNA. These two strands are arranged around each other to form a hexagonal coiled-coil, with the complimentary strands running anti-parallel to each other, the phospho-sugar backbone being external, the base pairs internal to the structure. The spaces between strands are referred to as the major and minor grooves, the width of which is 22 Å and 12 Å respectively (56). Proteins such as transcription factors are able to bind to exposed nucleotides in the major groove (57).

There are approximately three billion bases in the human genome, a copy of which is present in the nucleus of every cell. In order for such an expanse of material to be contained within such a small space, DNA undergoes several organisational modifications to become more compact. First, the double strands can be more closely twisted around each other, or supercoiled. The strands are wrapped one - to - three times around an octomer of histone proteins to form 10 nm nucleosomes (representing a seven-fold condensation factor) and are then linked by histone H1 to from a 30 nm coiled rope-like structure (a further six-fold condensation factor), termed chromatin. Chromatin fibres are then further condensed by forming looped structures along a non-histone protein scaffold, which can in turn be further condensed to form chromosomes during cell division (58).

1.5.2. Forms of eukaryotic DNA

As mentioned earlier, DNA is most often located in the nucleus of the cell, but can also be found in the mitochondria and the cytoplasm. Like chromosomal DNA, mitochondrial DNA (mtDNA) is double stranded and comprised of the bases A, C, G and T, but differs from chromosomal DNA in that it is circular rather than forming a double helix. Another difference is that whereas each cell contains just two molecules of chromosomal DNA, there can be thousands of copies of mtDNA within a single cell. Once external to the cell DNA is termed cell-free circulating DNA (cfcDNA). DNA found in plasma is predominantly double stranded (59) and cfcDNA is usually complexed to lipids or protein.

1.5.3. DNA as the carrier of genetic information

The identity of the mechanism by which genetic material is inherited was under debate for some time, with chromosomal protein also being thought to play a role; it wasn't until the 1940s that DNA was finally shown to carry the genetic code (60). Genes are the units of hereditary information, regions of the DNA sequence which encode proteins by way of their amino acid constituent parts. Each of the twenty amino acids are represented by a three base codon (e.g. TGG encodes tryptophan) with the majority of amino acids being represented by more than one codon. Messenger RNA (mRNA) is synthesised during transcription of a gene on the sense strand of DNA, forming a strand of complimentary sequence. This is later translated into amino acids by the action of transfer RNA (tRNA), forming a polypeptide polymer which constitutes the target protein. The target protein may then undergo further post-translational modifications such as the addition of functional groups (e.g. phosphate, lipids or carbohydrates) the formation of disulfide bridges or phosphorylation. Thus, a functional protein is synthesised and can affect its environment depending upon its nature and specific function.

1.5.4. Extraneous DNA as a potential risk to the patient

The use of cells from a non-patient source will never be without risk; induction of an immunological response could occur by either the cells themselves or by their secreted proteins. It has also been postulated that DNA and / or other constituents from the cells may invoke a secondary disease situation. It is therefore necessary to protect the patient from any circulating DNA of HepG2 origin by removing extraneous DNA from the BAL.

Assessing the risk that extraneous cell lines pose to the patient is however not straightforward. First of all, there is no absolute maximum value of non-patient DNA which is deemed tolerable by the regulatory bodies, including the US Food and Drug Administration (FDA). Secondly, it has been reported that only trace levels of DNA can be found in the circulation (61), with levels in healthy individuals reported at 34 +/- 34 ng/ml (62), and so identifying an analysis method with sufficient sensitivity to detect these levels also poses a challenge. To demonstrate this point, a recent study on the use of encapsulated porcine cells in a BAL observed that upon applying the supernatant from cell culture to a population of human hepatocytes, Pig Endogenous RetroVirus (PERV) was found to have infected the hepatocytes (63). Conversely, however, a study undertaken by the AMC-BAL (Academic Medical Centre – Bio-Artificial Liver) group which utilises porcine cells in their BAL system, detected no measurable levels of PERV in chronically immunosuppressed patients having undertaken treatment with the device (64).

Usually restricted to the cell nucleus, DNA is released into the extracellular environment following apoptosis or necrosis, or “metabolic” DNA can be actively secreted by cells (65). In healthy individuals levels of between 59 ± 15 ng/ml of DNA can be detected in the plasma (66), the vast majority of which is cleared by a process of inter-nucleosomal fragmentation (67). As the BAL utilises a large cell population there is the potential for non-

patient DNA, in the form of cfcDNA, to be transferred from the BAL chamber circuit into the patient. The relative risk this would then pose is a cause for contention, with relatively little information being available on the potential for extraneous DNA to cause harm to the host. That withstanding, there is evidence to support the theory that cfcDNA can be incorporated into eukaryotic host cells (68;69). The process by which circulating DNA could enter a host cell could be via the toll receptor pathway (70). By initiating a DNA damage repair response, these DNA fragments can become incorporated into the host genome by a process of homologous recombination. A recent example of this was demonstrated in a study where the plasma from colon cancer patients with a *k-ras* mutation was applied to mouse fibroblasts, after which the *k-ras* oncogene sequence was detected within the mouse cells (71). This finding is of particular importance as the BAL utilises HepG2 cells which are of a tumour lineage; it is quite obvious that a risk of geno-metastasis to liver failure patients can not be permitted.

It is also plausible that circulating DNA could initiate an immune response in the patient, where it is the double stranded nature of DNA which is recognised (72). This stimulates transcription of genes for the major histocompatibility complex (MHC) and interferon regulating factors, which then eventually lead to the transcription of pro-inflammatory factors such NFκβ and chemokines (73) which can be linked to causing tissue damage. Mitochondrial DNA and degraded formidyl peptides have also been suggested to initiate a pro-inflammatory response (74). Another mechanism by which circulating DNA can trigger an immune response is via proteins such as IF116 (75) and DAI (76) which bind cfcDNA and recruit transcription factors, resulting in the expression of IFNβ. Moreover, it has been demonstrated that cfcDNA can evoke an inflammatory response by stimulating IL-6 production in monocytes (77;78). Together, these data demonstrate that measures must be taken to minimise the potential for HepG2 DNA to enter the patient's circulation during treatment with the BAL device.

Although physical filtration is an option for DNA removal, as is supplementation / exchange of the culture media to limit the effects of lactate, there exists a more elegant solution, utilising the natural catalytic activity of specific enzymes to convert these detrimental entities into harmless, or even potentially useful, molecules. This would provide a solution which can be "in line" with the BAL, enabling a continually working closed circuit system and therefore forming the basis of a safer and more time efficient device.

1.6. Enzyme Immobilisation

The catalytic activities of enzymes have long been exploited, dating back to antiquity when enzyme rich media were used, for example, in the preparation of leather, or the use of rennin in cheese production. Today, the applications of enzyme technology are plentiful, covering a multitude of disciplines ranging from the food industry, to the manufacture of cleaning products and pollutant neutralisation strategies. There is also a growing area of research into the field of enzymes as therapeutics, whereby enzymes are administered to patients in order to replace the function of their nonexistent or non-functional native enzyme. This is referred to as enzyme replacement therapy (ERT). The majority of investigations have used enzymes in solution to be administered either orally or as an injectable, with relatively few studies into the use of immobilised enzyme complexes as a potential therapeutic agent. However, the process of immobilisation may facilitate the use of enzymes in a therapeutic context, as it has been reported that this strategy can bestow many beneficial properties onto both the activity and stability of the enzyme. This will be discussed further in this section.

The first recorded account of enzyme immobilisation was in 1916 by Nelson and Griffin who noted that invertase adsorbed onto a charcoal and $Al(OH)_3$ artificial support retained its catalytic activity (79). However, it was not until the 1950s that the potential for immobilised enzyme technology was recognised, highlighted by a steady increase in enzyme immobilisation research (80). The immobilisation of enzymes has received a great deal of

interest in recent years, for use in the pharmaceutical, diagnostic and food production industries amongst others.

1.6.1. Benefits of immobilisation

Regardless of specific application, usually the primary aim of immobilisation is to stabilise the enzyme, facilitating reuse in multiple procedures (thus lowering running costs). The ways in which an immobilised enzyme is stabilised varies depending on the method used, but among the mechanisms of stabilisation are molecular confinement (81) whereby the enzyme is less readily unravelled as a consequence of thermal denaturation, or by adding stability through the formation of new bonds during covalent binding (82). Some methods also confer an increased level of enzymatic activity; the action of multi-point attachment between enzyme and support can result in the enzyme being held in a permanently active conformation (83).

Additionally, for enzymes intended for a therapeutic application, there is the advantage that an immobilised enzyme is far less likely than the free enzyme to initiate an immune response (84). In support of this, studies have demonstrated that compared to the free enzyme, immobilised enzymes provoked the formation of either fewer antibodies or deleterious downstream effects, such as invoking an immunological response or having a toxic effect upon the organism (85;86).

1.6.2. Immobilisation considerations

There currently exists a wide variety of immobilisation techniques, with the requirements of the target enzyme dictating which method should be applied for optimal effect (87). A particular consideration is that the coupling reagent and the support matrix together can potentially contribute to the enzyme's microenvironment, (for example, affecting pH, ionic strength and the hydrophilic-lipophilic balance (88),) which can influence substrate

interaction and product formation. During the immobilisation process the enzyme must be in its active state. The requirements of each application to utilise immobilised enzymes will obviously vary, as will the particular conditions in which an enzyme will function; a system can only be described as "robust" when both the enzyme function and method of immobilisation work effectively for that particular application (89). For use within a biological system, as will be the case with the Liver Group BAL, there are additional criteria to consider when selecting an immobilisation method; for example, as well as binding the enzyme in its active state, the support matrix must be bio-compatible, neither being itself degraded nor causing damage, for example, to blood constituents (e.g. by inducing haemolysis or thrombosis) and must also be composed of non-toxic or carcinogenic materials (80).

1.6.3. Solid support based immobilisation strategies

The majority of immobilisation procedures involve physically or chemically anchoring the enzyme to a support matrix. The benefits associated with this include the ability to control more precisely the duration and location of an enzymatically catalysed reaction. Also, stabilisation of the immobilised enzyme is frequently observed. The most common and significant modes of immobilisation are summarised in the following sections; however, it is to be noted that several methods which have been allocated a separate heading may under certain conditions and applications overlap with other methods. This highlights the growing complexity and ingenuity behind the designs and protocols for enzyme immobilisation.

1.6.3.1. Adsorption & immobilisation via ionic interactions

Often regarded as the simplest means of immobilising an enzyme, adsorption requires very little in the way of chemical modification or pre-treatment of the enzyme/support surface. However, the bonds formed are relatively weak in inorganic solvents and thus leaching of the enzyme is not an uncommon phenomenon (90). Although stronger than adsorption, ionic fixation of an enzyme to a carrier is by nature a reversible process and so

leaching of the enzyme is still a potential hazard. Nevertheless, various groups have utilised ionic interactions to assist binding of the enzyme to the support, namely orientating the enzyme active site into a position whereby it can achieve optimal functionality i.e. ensuring that the enzyme is not immobilised via a residue located in/near to the active site.

Due to the reversible nature of these interactions no further consideration will be given to either of these methods for use in the immobilisation of enzymes in the LG BAL.

1.6.3.2. Covalent attachment

For many applications where leaching of the enzyme cannot be tolerated, the use of irreversible covalent methods of immobilisation are preferred. In addition, conformational flexibility and as a consequence thermal vibrations are reduced, serving to stabilise the protein structure. Enzymes are typically bound at multiple points, strengthening the interaction between protein and support. Frequently the ε-amino group of lysine is used to form the covalent bond between enzyme and epoxide groups on the support (91;92), owing to the tendency of lysine to be found on many enzymes and located on the protein surface. There are a plethora of cross-linking reagents available for mediating the covalent attachment of enzymes to a support, in addition to a wide variety of methods of activating and functionalising the support surface (see sections 3.1.3 and 4.1.3 for further information). Furthermore, there exist multiple commercial supports which can be acquired ready-functionalised, common examples being Eupergit (Evonik) macroporous spheres and Sepabeads (Resindion), which contain surface fissures and thus provide a degree of protection to the immobilised enzyme from shear stress. However, the decrease in flexibility often associated with covalent binding may also hinder enzyme-substrate interactions and hence significantly lower the rate of enzyme activity. When seeking to immobilise an enzyme by means of covalent attachment to a solid support, the choice of support and cross linking reagent is often the result of a compromise between stability

and enzyme activity. For example, although shorter linking groups confer greater stability by limiting vibrations and increasing rigidity, longer linking groups may be necessary to overcome the problems with steric hindrance associated with shorter linking groups.

1.6.3.3. Nanoparticle attachment

The use of nanoscale structures such as spheres, fibres and tubes is one of growing popularity and interest owing to recent advances in nanotechnology. In theory, use of nanosized supports should make optimal use of the available surface area, thus maximising enzyme loading efficiency. Silica based matrices have been used to encapsulate enzymes within nanoparticles (93), the benefits associated with silica including formation under mild environmental conditions, good stabilisation of the immobilised enzyme, variable morphologies and variable pore sizes. An elegant method of this approach utilised the R5 peptide, a synthetic derivative of a silica precipitating protein found in *Cylindrotheca fusiformis*, which was inserted into the protein sequence without obvious detriment to the enzyme to yield an enzyme encapsulated within a silica nanoparticle (mean diameter of ~500 nm) synthesised *in situ*. Other molecules are also capable of catalysing silica formation, such as polyethylenimine (PEI), although there have been reports that the potentially hydrophilic environment created by this particular molecule may limit availability of hydrophobic substrates, a particular problem for lipases. Electrospun polymer nanofibres (reviewed by Wang et al. 2009 (94)) are another option utilising nanotechnology for the immobilisation of enzymes. Advances in reducing the difficulties associated with the recovery and handling of nanoparticles have been addressed for example by means of magnetic microspheres (93). However, it is crucial for the LG BAL application of immobilised enzymes that no particles such as cell debris are able to enter the patient. For this reason, a 0.5 µm filter is an essential component of the LG BAL system, but this would become subject to an unacceptably high back pressure were any nanoparticles to escape and become trapped behind

the filter. For this reason, the use of nanoparticles for enzyme immobilisation will not be given further consideration within this study.

1.6.3.4. Entrapment

Sol-gel technologies are amongst the most frequently used methods of immobilising enzymes using an entrapment strategy, wherein a chemical solution ("sol") is gradually evolved to from a solid network or polymer structure ("gel"). The composition of a sol-gel can vary greatly in material, pore size and mesh network structure, all of which significantly affect their enzyme immobilisation properties. Mesoporous silica remains a popular medium for entrapment owing to the controllability of pore size (mesoporous denotes a pore size of 2-50 nm; microporous <2 nm, and macroporous >50 nm) especially given that the diameter of the majority of enzymes lies between 3 to 6 nm. This quality of mesoporous silica has been successfully used for a range of biomolecule retention and release applications (95) What is more, sol-gels are chemically inert, physically stable and optically transparent, facilitating their use in a wide range of applications. The amine group of the sol-gel theoretically plays a role in maintaining pH and preventing protonisation of enzyme functional groups, thus demonstrating the importance of selecting an appropriate sol-gel for entrapping the chosen enzyme in the required working conditions. Furthermore, sol-gel entrapment has frequently been observed to increase the stability and reusability of the enzymes (96-98).

However, problems with low loading efficiencies are common and there is some concern that the relationship between properties of a sol-gel and the subsequent enzyme activity rates is poorly understood. It stands that there are very few generic sol-gels, each one necessitating refinement and development of both structure and composition for each enzyme studied.

1.6.3.5. Encapsulation

The act of sealing enzymes within a polymer encasing has the significant advantage of conferring protection to the enzymes from their external environment (99;100), permitting their use in a biological context, as they will evade detection and rejection from immune system components (99;101). Furthermore, entrapment methods protect the enzyme from the external environment thus lowering the impact of many physical phenomena such as the effects of gas bubbles and sheer stress. An early experiment to address the need for a liver failure treatment involved the encapsulation of a myriad of liver enzymes within a nylon-polyethylenimine shell (102) to form an artificial cell. Although not tested at a biological level, the artificial cells were demonstrated to reduce ammonia and urea concentrations in solution by converting them into the essential amino acids of leucine, isoleucine and valine, the metabolism of which is often aberrant in fulminant liver failure (103).

Zhang *et al*. reported a procedure whereby a silica shell was used to encase a liquid alginate core containing β-glucuronidase, thus encapsulating the enzyme (104). The resulting sphere was resistant to swelling and enhanced enzyme stability and activity. Unfortunately, there exists the disadvantage of limited diffusion potential, leading to issues with mass transfer, e.g. substrate availability through the encapsulation shell (90).

1.6.4. Support-free methods of immobilisation

Recently, there has been growing research into support-free modes of enzyme immobilisation. These systems alleviate the situation whereby the support matrix can typically account for 90-99% of the volume of the immobilised catalyst (84;105) thus potentially allowing for a more efficient system.

1.6.4.1. Spherezymes

The term "spherezyme" denotes a multi-enzyme structure whereby enzymes are anchored to each other without the assistance of a carrier (90). It has been demonstrated that in four lipases and laccase the formation of a spherezyme leads to a higher activity rate than that observed in the native enzyme (87;90). Protein cross-linkers were utilised in a water-in-oil emulsion of aqueous enzyme solution to form individual spheres of 0.5-10 µm but which tended to form aggregates of approximately 100 µm. These structures were robust enough to be collected by centrifugation and filtration, permitting their reuse. Initial problems were encountered with a loss of activity following cross-linking with glutaraldehyde, although these were resolved by adding the substrate to the aqueous enzyme solution, resulting in an activity rate higher than that of the native enzyme. Addition of ethylenediamine (EDA) increased activity further, likely due to increased linker chain length. From this technology co-immobilisation becomes possible, where one enzyme could theoretically protect a second, more sensitive enzyme beneath it. This configuration would also allow for multi-step reactions, whereby multiple enzymes are immobilised in close proximity and thus facilitating a more rapid interaction between catalysts and substrate(s).

1.6.4.2. Cross-linking of enzymes

Typically, a di-functional agent (e.g. glutaraldehyde) is used to covalently bind enzymes together, acting as their own carrier. Cross-linked enzyme aggregates (CLEAs) are formed by aggregating and then cross-linking the enzymes. The procedure benefits from relatively facile preparation steps and negates the use of expensive supports (93), a disadvantage of carrier based methods of immobilisation. Self-immobilised enzymes (CLEA, cross-linked enzyme crystals (CLEC) etc) typically show 10-1000 times higher volumetric activity (catalytic activity observed within a defined reaction volume (84)) than carrier bound immobilised enzymes. However, problems have been reported with poor reusability for CLEAs. Furthermore, cross-linking is highly likely to be affected by the ability of the enzyme to resist

chemical cross-linking (93), and mass-transfer limitations may become problematic if large aggregates of CLEAs are formed.

1.7. Hypothesis & Aims

HepG2 cells offer high proliferative capacity and are capable of performing many of the liver functions when in 3D culture (44) such as synthesis of albumin and clotting factors whilst retaining viable metabolic and synthetic functions (106-108). Furthermore, it has been demonstrated that the presence of plasma does not have a negative impact upon HepG2 cells; indeed, an increase in proliferation and amino acid uptake rates was observed (109).

However, HepG2 cells lack some key liver functions (48) including ammonia metabolism (110) and furthermore there is a very small risk of tumour metastasis in a patient receiving BAL treatment should the tumour cells enter the patient's circulation, due to the lineage of the cells. Additionally, there is a body of evidence to imply that cfcDNA has the potential to trigger an immunological response.

Another obstacle posed by the hepatoblastoma origins of HepG2 cells is their tendency towards glycolytic respiration (111), leading to elevated levels of lactate production. In addition to the effects on pH, increased lactate levels within the BAL are problematic as lactate is able to chelate calcium and so compromises the integrity of the alginate beads used to encapsulate HepG2 cells. In order to optimise encapsulated HepG2 cell proliferation and function it would therefore be logical to modulate the lactate concentration within the FBB. Although this could simply be removed by repeated media changes, a more elegant approach would be to convert this problematic molecule into an entity which could be utilised by the cells.

The hypothesis of this thesis is that lactate oxidase and Deoxyribonuclease I can be immobilised in order to address the issues of lactate clearance in the bioreactor and the effective elimination of the potential threats from non-self DNA attributed to HepG2 utilisation in the LG BAL.

Consequently, the following aims are proposed:

- Reduce lactate concentration in FBB media to levels which do not compromise either cell proliferation or alginate bead integrity.

- Identify a method of DNA removal to be incorporated into the BAL device, which can function under physiological conditions reminiscent of the patient's body, for the duration of the proposed treatment period.

- Develop methods of immobilising suitable enzymes to assist in the reduction of lactate and DNA concentrations. The enzyme used must not have any detrimental effects on either the cell population or on the patient, nor should the mode of immobilisation. If this is not the case, acceptable measures must be taken to negate said risks.

The following chapters will discuss the strategies and findings generated as a consequence of addressing these aims.

Chapter 2. General Materials & Methods

2. General Materials & Methods

This section outlines the methodology and materials required to conduct the majority of experimental investigations described in the following chapters. Experiment specific procedures will be described within the relevant chapter. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich UK.

2.1. HepG2 Cell Culture

HepG2 cells were originally acquired from the European Collection of Cell Cultures (ECCC) and a cell line has since been maintained by the Liver Group for use within the BAL project. All cell culture work was performed in Class II Microbiological Safety Cabinet using sterile, disposable plastic ware in order to maintain sterile working conditions.

2.1.1. Complete medium preparation

Materials:
α-MEM with ribonucleosides and deoxyribonucleosides (Invitrogen 32571)
10% Foetal Calf Serum (Hyclone CH30160-03)
100 U/ml Penicillin with 0.1 mg/ml Streptomycin (Lonza DE17-603E)
50 µg/ml Linoleic Acid Bovine Serum Albumin
9.5 µg/ml Insulin (Actrapid 8-0201-01-203-3)
1.25 µg/ml Fungizone (Gibco 15290-026)
0.04 µg/ml Hydrocortisone
0.04 µg/ml Thyroid Releasing Hormone
0.002 µg/ml Sodium Selenite

All additives were pooled in a 50 ml centrifuge tube and filtered through a 0.2 µm mesh filter prior to adding to the α-MEM.

2.1.1. Fresh frozen plasma media preparation

Materials as listed above, but with the foetal calf serum replaced by human FFP.

2.1.2. Preparation of human plasma aliquots

Materials:
Human plasma
40 IU/ml heparin (Multiparin, CP Pharmaceuticals)

When the experimental conditions called for the enzymatic reactions to be performed in the presence of human plasma, bags of human plasma of the same blood type (AB positive) were pooled and centrifuged at 1000 g for 20 minutes to remove cellular debris. The plasma was then aliquoted and stored at -20°C until required. When acquired the plasma had already been treated with sodium citrate to prevent the clotting process. However, prior to use, plasma aliquots were treated with 40 IU/ml heparin to replace any loss-of-function caused by the freeze-thaw process of storage, to discourage the process of clot formation.

2.1.3. Monolayer cell culture

This section describes the process by which the HepG2 cell line was maintained and grown prior to experimental application.

Materials:
Complete culture medium
T175 Nunclon surface tissue culture flasks (Nunc)

HepG2 cell suspension was seeded in 30 ml complete culture medium at an approximate density of $2x10^6$ cells/ml in a T175 flask (Nunclon surface, Nunc). Cells were incubated in a humidified incubator at 37°C under 95% air and 5% CO_2 until the monolayer reached approximately 80% confluency. The medium was replaced 24 hours after seeding and every 48 hours thereafter until the monolayer was deemed to be sufficiently confluent for use or reseeding.

2.1.4. Passage of HepG2 cells

Materials:
Liver Group Trypsin:
0.25 g/l Trypsin 0.1 g/l EDTA (Invitrogen, 15400-054)
1 g/l glucose in citrate saline (4.4 g/l trisodium citrate.$2H_2O$, 10 g/L KCl)

Complete culture medium
HBSS (without calcium and magnesium)
T175 Nuncon surface tissue culture flasks (Nunc)
Sterile 21G syringe and needle

Confluent T175 flasks of HepG2 monolayer cells were rinsed three times in 10 ml calcium- and magnesium-free HBSS solution before adding 10 ml of Liver Group Trypsin. Cells were incubated for six minutes in a humidified incubator at 37°C under 95% air and 5% CO_2 to promote trypsin activity, after which 20 ml warmed complete medium was added to inactivate the trypsin. Cell detachment was further promoted by tapping the sides of the flasks. The suspension of detached cells were transferred to 50 ml centrifuge vials and centrifuged at room temperature at 300 g for four minutes. The supernatant was discarded and the resulting cell pellet gently re-suspended in a small volume of complete medium, any clumps being dispersed by gradually passing through a 21G needle no more than three times. This suspension was then used to seed flasks for monolayer growth, the volume of which depending upon the desired seeding cell density.

2.2. Determination of Cell Number

Cell viability and numbers were determined by the Trypan Blue method of excluding non viable cells.

Materials:
Hanks Balanced Salt Solution (HBSS) - (without calcium and magnesium)
2% Trypan Blue in PBS
Haemocytometer

A sample of 20 µl of the previously described HepG2 cell suspension was mixed with an equal volume of Trypan Blue (2% in PBS) and 160 µl of

calcium- and magnesium-free HBSS and incubated at room temperature for two minutes. Of this solution, 9 µl was loaded onto a haemocytometer and viable (unstained) and non-viable (blue stained) cells counted under 10 x magnification. Counts were performed five times in order to obtain mean and standard deviation values. The percentage viability was determined by the ratio between living and dead cells.

2.3. Cell Viability by Fluorescence Microscopy

Propidium iodide (PI) is an intercalating agent and fluoresces in the red spectrum when bound to DNA. As it is unable to pass through the cell membrane, only damaged, i.e. non viable cells will take up the dye. Fluorescein diacetate (FDA) when hydrolysed by esterases within the cytoplasm of metabolically active cells is converted to fluorescin which emits a green fluorescence. The cell membrane is permeable to FDA, and so viable cells will be positive for this stain.

Materials:
1 mg/ml FDA (Sigma F7378-5G) in DMSO
1 mg/ml PI (Sigma 70335-5ML-F) in water
1x PBS containing calcium and magnesium
Microscope slides
Tissue
Coverslips
Nikon Eclipse microscope
DX1200 camera
FDA filter block (excitation filter 465-495 nm, emission filter 515-555 nm)
PI filter block (excitation filter 510-560 nm, emission filter 590 nm)

Approximately 500 µl of ELS beads from Day 11 of a FBB experiment were transferred to a 2 ml microcentrifuge tube. The residual culture medium was aspirated and the beads washed in 1 ml PBS solution (with Ca^{2+} and Mg^{2+}). The beads were allowed to settle, whereupon the wash solution was aspirated and the process repeated. For the final wash the beads were suspended in 500 µl PBS solution, to which 10 µl FDA (Note: FDA is light sensitive and so must be protected from light and stored at 4°C) and

20 µl PI were added. The mixture was incubated at room temperature for 90 seconds, inverting the tube to mix. The supernatant was aspirated and washed in 1 ml PBS as before, with the beads then being suspended in 500 µl PBS to provide an adequate liquid volume to transfer the beads to a microscope slide. A glass coverslip was gently lowered onto the beads taking care not to damage them. Samples were imaged immediately afterwards using Lucia imaging software with a DX1200 camera on a Nikon Eclipse microscope. Images were taken at 4 x on phase contrast, live FDA and dead PI settings, protecting the specimens from light.

The density of fluorescence was determined on the Lucia software using the following macro specifications:

Fluorescein Diacetate Macro:
```
ClearBinary();
WaitText(3,"Click on areas omitted by macro to add them, then right click and click OK");
DefineThreshold(0,106,14,13,255,216,7);
_DefineThreshold();
Threshold();
MeasureField();
_FieldData();
```

Propidium Iodide Macro:
```
ClearBinary();
WaitText(3,"Click on areas omitted by macro to add them, then right click and click OK");
DefineThreshold(106,8,6,255,61,18,7);
_DefineThreshold();
Threshold();
MeasureField();
_FieldData();
```

The viability of cells was calculated according to the equation:

$$Viability\ (\%) = \frac{FDA\ Density}{FDA\ Density + PI\ Density} \times 100$$

2.4. BCA Assay

The bicinchoninic acid (BCA) assay is used to determine total protein present within a sample, and is indicated by a colorimetric change from green to purple, which when measured by absorbance spectrophotometry at the 570 nm wavelength, can be compared to a standard curve to deduce protein concentration.

Materials:
0.5% TritonX 100 in PBS

Reagent A: (in 100 ml MilliQ H_2O, adjusted to pH11.25 with 10 M NaOH):
1 g sodium bicinchoninate (Sigma, D8284)
2 g sodium carbonate (BDH, 102404H)
0.16 g sodium tartrate (Sigma, S4797)
0.4 g sodium hydroxide (BDH, 102524X)
0.95 g sodium bicarbonate (BDH, 102474V)

Reagent B:
0.4 g cupric sulphate 5-hydrate (BDH, 3312-3)

Standard BSA solution (1 mg/ml):
1 ml of 2 mg/ml BSA standard solution (Sigma, P0834-10X-1ML)
1 ml MilliQ H_2O

A series of BSA standards (1000 µg/ml – 31.25 µg/ml) was prepared by a 1:2 serial dilution of the 1 mg/ml BSA solution in a solution of 0.5% TritonX 100 (in PBS).

A working solution was prepared by mixing 50 parts reagent A to 1 part reagent B (e.g. 50 ml reagent A and 1 ml reagent B). To each well of a 96-well plate, 200 µl of this working solution was added.

A sample of 250 µl encapsulated HepG2 cells were combined in a 2 ml tube with 1.6 ml 16 mM EDTA and incubated at 37ºC for 10 minutes to dissolve the alginate. The resulting solution was centrifuged at 13,200 g for five minutes and the cell pellet was first washed in 2 ml PBS and then centrifuged at 13,200 g for 2.5 minutes to separate the pellet from the wash solution. To the dry pellet, 1 ml 0.5% TritonX 100 (in PBS) was added and then vortexed before incubating for 10 minutes at 37°C. To each well, 20 µl

of each standard (in triplicate) was added, including a blank consisting of 0.5% TritonX 100 (in PBS) alone, or 20 µl of each sample in triplicate. The plate was placed on a shaker for 30 seconds, then wrapped in cling-film to prevent evaporation, and incubated at 37°C for 30 minutes. The plate was allowed to cool before the absorbance was measured on a plate reader (Anthos HTCII) at 570 nm.

2.5. MTT Viability Assay

Metabolically active cells reduce methylthiazolyldiphenyl-tetrazolium bromide (MTT) to insoluble formazan by the mitochondrial and microsomal reductases. The formation of dark blue formazan crystals is directly proportional to cell activity and can be quantified by measuring absorbance at the 570 nm wavelength.

Materials:
0.75 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, M5655) in PBS
16 mM EDTA in 0.15 M NaCl
PBS
4 mM HCl in Isopropanol
96 well clear flat bottomed culture plate
Orbital plate shaker

A sample of 250 µl of encapsulated HepG2 cells were combined in a 2 ml tube with 1.6 ml 16 mM EDTA and incubated at 37ºC for 10 minutes to dissolve the alginate. The resulting solution was centrifuged at 13,200 g for five minutes and then washed twice in PBS. The supernatant was aspirated and 500 µl 0.75 mg/ml MTT solution was added to each tube at incubated at 37ºC for three hours, until blue crystals had visibly formed. The crystals were mixed to homogenously resuspend and then 50 µl of this was transferred to 200 µl acidified isopropanol in each well of a 96 well plate. The plate was sealed with a plate sealer and agitated for 30 minutes on an orbital shaker to dissolve the crystals. The absorbance was read using a spectrophotometer at 570 nm.

2.6. Alpha-Fetoprotein ELISA

The production of alpha-fetoprotein (AFP) was used as a marker of liver-specific protein function, making use of an enzyme linked immunosorbent assay (ELISA) to detect the presence of said target protein. The principle is based upon the binding of target protein specific antibody, which is then itself bound by a secondary antibody which catalyses the breakdown of a precursor molecule to generate a coloured compound. The intensity of the coloured compound formed is therefore directly proportional to the quantity of protein in the sample.

Materials:
96 well Immuno coated plates (HiBind, Nunc, DIS-971-030J)
Tween-20 (Sigma, P1379)
2 M sulphuric acid (H2SO4) (Analar, 102760B)
o-phenylenediamine (OPD) tablets (Dako, s204530)
Sodium carbonate (Na2CO3) Analar (102404H)
Sodium hydrogen carbonate (NaHCO3) (Analar, K38347229.805)
Sodium chloride (NaCl) (Analar, 27810.364)
Potassium chloride (KCl) (Analar, 26764.232)
KH2PO4) (Analar, 102034B)
Di-sodium hydrogen orthophosphate 2-hydrate (Na2HPO4.2 H_2O) (Analar, 103834G)
Hydrogen peroxide (H2O2) (VWR, 23615.261)
MiliQ H_2O
Non-fat milk powder (Marvel)
2 mg/ml α Alpha-1-Fetoprotein (Abcam, Ab10071)
Capture antibody (Abcam, Ab10072)
Plate washer
Plate reader

10 x PBS (in 500 ml H_2O):
40 g NaCl
1 g KCl
7.2 g $Na_2HPO4.2\ H_2O$
1 g KH_2PO_4

Coating Buffer (pH 9.6, in 200 ml dH_2O):
0.318 g Na_2CO_3
0.586 g $NaHCO_3$

Wash Buffer:
1 L 1 x PBS
500 µl Tween-20

Blocking buffer:
5% dehydrated non fat milk in Wash Buffer

OPD Solution:
2*OPD tablets
12 ml distilled water
Before use, add 6 µl H_2O_2

The primary antibody was diluted 1 in 1000 in coating buffer to give a final antibody concentration of 2 mg/l. 100 µl of this solution was transferred into each well of a 96 microwell plate using a multipipette and the plate was then wrapped in cling-film and left overnight at 4^{o}C.

A plate washer was used to rinse the plate three times with Wash Buffer and aspirated before adding 100 µl blocking buffer to each well, incubating at room temperature for one hour. Samples, the QC and AFP standards (1:1 serial dilution from 200 mg/ml to 6.25 ng/ml) were diluted in medium.

After first swirling the plate to dislodge any sediment of blocking buffer, 100 µl of each sample, standard and QC was added to each well in triplicate. The plate was then covered and incubated at 37^{o}C for 90 minutes, followed by again using the plate washer to rinse three times with Wash Buffer. The secondary antibody (Ab10072) was diluted 1 in 7000 in blocking buffer and 100 µl of this was added to each well using a multipipette. The plate was again covered and incubated at room temperature for one hour, after which it was washed five times with Wash Buffer and the supernatant aspirated.

To start the colorimetric assay, 100 µl OPD solution was added to each well at timed intervals and the plate covered with foil and left to incubate at room temperature for approximately five minutes or until sufficient colour had developed. To stop the reaction, 50 µl of 2 M sulphuric acid was added at timed intervals. Finally, the plate was transferred to a plate reader and the absorbance intensity read at 492 nm.

2.7. HepG2 DNA Extraction

To obtain high molecular weight human DNA for use in DNase I experiments, the following protocol was utilised, modified from Laird *et al.* (112):

Materials:
Lysis Buffer:
100 mM TrisHCl
5 mM EDTA
0.5% SDS
200 mM NaCl

Proteinase K (lyophilised, from *Tritirachium album*; Sigma-Aldrich, P6556)
100% Isopropanol

T175 flasks of 80% confluent HepG2 cells were washed twice in 50 ml PBS (at room temperature) discarding the supernatant. 10 ml of lysis buffer with 1 mg Proteinase K was then added and the cells incubated for four hours at 37˚C, after which the dislodged cell suspension was removed and mixed with an equal volume of isopropanol and gently inverted for 10 minutes to precipitate genomic DNA. The supernatant was aspirated from the resulting pellet which was allowed to air dry briefly before being transferred to an initial volume of 1.5 ml PCR grade DNase free water (BioLine).

2.8. Proteinase K Treatment of Samples

Prior to applying plasma-based samples to PCR analysis it was necessary that they were first treated with Proteinase K to remove intrinsic plasma proteins which could otherwise interfere with the PCR readings.

Materials:
5 x TTE buffer (50 mM Tris Base, 1 mM EDTA)
2.5% Tween-20
Double distilled H_2O (dH_2O)
Proteinase K (lyophilised, from *Tritirachium album*; Sigma-Aldrich, P6556)

For each sample, 20 µl was applied to 16 µl lyophilised Proteinase K re-suspended in 20 µl 1 x TTE buffer (For 10 ml 1 x TTE: 2 ml 5 x TTE stock, 7.75 ml dH_2O, 250 µl 20% Tween-20) and the resulting solution incubated at 50°C for 45 minutes. Samples were then mixed with 160 µl 1 x TTE buffer and heated at 95°C for 10 minutes to inactivate the Proteinase K. Protein debris was removed by centrifugation at 13,000 g for 10 minutes, the supernatant being carefully aspirated and utilised in further assay protocols.

2.9. Q-PCR

Quantitative Real Time Polymerase Chain Reaction (Q-PCR) analysis was chosen to measure DNA content of samples owing to its well documented sensitivity (67;113), being able to detect and amplify trace levels of the targeted amplicon. The Alu tandem repeat consensus sequence was targeted by using a 115 base pair (bp) primer pair first utilised by Umetani *et al.* (114) to target DNA released from apoptotic cells containing this 115 bp consensus sequence. It is to be noted that apoptotically cleaved DNA is typically 180-200 bp in length. The Alu repeat is found abundantly in the human genome, representing approximately 10.8% of the genome, and is species specific.

Materials:
2x Hot StarTaq® Polymerase (comprising 2x PCR Buffer, 1.5 mM $MgCl_2$, 200 µM dNTP's and 1.25 U Taq Polymerase) (Qiagen, 203645)
28 mM $MgCl_2$
DNase- and RNase-free PCR grade H_2O (BioLine, BIO-37080)
100 µM 115bp Primer (Sigma, bespoke order)
FORWARDS: CCTGAGGTCAGGAGTTCGAG
REVERSE: CCCGAGTAGCTGGGATTACA
10 000 x SybrGreenI (Sigma, S9430)
200 ng/µl certified human genomic DNA (BioLine, BIO-35025)
0.2 ml PCR microcentrifuge tubes (Corbett, Qiagen, 0030 125.215)

1x PCR mix: (Reaction volume = 20 µl (15 µl PCR Mix + 5 µl sample))
10 µl Hot StarTaq® Polymerase
0.5 µl 1/1000 SYBR Green I
1 µl 10 µM Forward Primer (final concentration: 400 nM)
1 µl 10 µM Forward Primer (final concentration: 400 nM)
2.5 µl 28 mM $MgCl_2$ (final concentration: 5 mM)

A standard curve was created and run within each Q-PCR experiment, using standardised human genomic DNA in a five point range of concentrations from 0.01 pg to 10 pg DNA per 20 µl Q-PCR reaction volume.

Samples were run using the RotorGene 3000 (Corbett) with the following cycle programme:

95 °C 5 minutes
95 °C 15 seconds
64 °C 30 seconds
72 °C 30 seconds
72 °C 10 minutes

This was run for 40 cycles. The melt curve data was acquired from 45°C to 95°C at a rate of 1°C per minute.

2.10. PicoGreen® Assay

The Quanti-iT™ PicoGreen® reagent is a fluorescent stain for double stranded DNA (dsDNA), which has been shown to detect and quantify dsDNA in concentrations ranging from 0.25 ng/ml to 150 ng/ml (115).

Materials:
Quanti-iT ™ PicoGreen® dsDNA Assay Kit (Invitrogen, P7589)
PCR Grade H_2O (Invitrogen AM9935)
DNase free 0.2 ml PCR microcentrifuge tubes (Corbett, Qiagen)
RotorGene 3000 (Corbett)

As an alternative method of DNA detection to PCR, the Quanti-iT ™ PicoGreen® assay was adapted for the required application. This method detects dsDNA fragments less than 500 bp in length and may have an

advantage over PCR based methods of detection as it does not require the presence of a specific consensus sequence to detect DNA. To prepare the samples, 100 µl of PicoGreen® reagent (1 x PicoGreen® diluted in 1 x TE buffer (diluted with PCR grade H_2O from a 20 x TE buffer stock solution) from 200 x PicoGreen® stock reagent) was added to an equal volume of sample, giving a total reaction volume of 200 µl per PCR microcentrifuge tube. A total of 15 endpoint fluorescence measurements were taken at 37°C for each sample using the RotorGene software and PCR machine. Standards were produced using bacteriophage λ DNA (diluted to a 2 µg/ml stock with 1 x TE buffer from the 100 µg/ml stock solution supplied with the PicoGreen assay kit) to give a 6-point range of final DNA concentrations from 0 – 1 µg/ml.

2.11. DNA Quantification – NanoDrop

It was necessary to quantify the concentration of HepG2 DNA prior to enzymatic degradation. NanoDrop instrumentation and software was used to gain data values using the nucleic acid absorbance assay (performed as per manufacturers' protocols). A range of serially diluted samples were used in all situations to allow for DNA solubility and hydration limitations which may occur at higher DNA concentrations.

2.12. DNA Gel Electrophoresis

Materials:
2% agarose
0.004% 10 mg/ml ethidium bromide
HyperLadder III (500 bp, Bioline, BIO-33055)
Gel electrophoresis tank (BioRad)
10 x TAE buffer (to 500 ml, in dH_2O):
24.2 g TrisBase
5.71 ml acetic acid
10 ml 0.5 M EDTA

Samples were run at a constant current at 90 volts for 10 minutes (unless otherwise stated) on a 2% agarose gel stained with ethidium bromide in

1xTAE buffer (stock diluted 1:10 in dH_2O). An appropriate molecular weight ladder was used to identify product size. Gels were viewed and images taken using UVP Gel Doc software.

2.13. Preparation of Enzyme Aliquots

In order to gain usable and reproducible concentrations of enzyme solutions, thus minimising intra-experiment variability, each batch of lyophilised enzyme was aliquoted.

Materials:
Deoxyribonuclease I (Type II from bovine pancreas; Sigma D4527)
TrisHCl buffer:
50 mM TrisHCl pH7.4
1 mM $CaCl_2$
1 mM $MgCl_2$

Lactate oxidase (lyophilised, from *Pediococcus* sp.; Sigma LO638)
Phosphate buffer:
10 mM potassium phosphate
0.01 mM FAD

Polypropylene microcentrifuge tubes

A vial of 20 KU Deoxyribonuclease I was re-suspended in 28.5 ml TrisHCl pH7.4 buffer to give a concentration of 0.2 mg/ml enzyme. Aliquots were made on ice of a combination of 10 µl, 100 µl and 1 ml and stored at -20°C until required. Care was taken to minimise shear stress as DNase I is notoriously sensitive to denaturation by physical stress (i.e. vortexing).

Lactate oxidase was suspended in 10 mM potassium phosphate buffer to give a concentration of 2 U/ml. This was divided into aliquots, lyophilised and stored at -20°C until required.

2.14. Solution Lactate Concentration Determination

Analox® GM7 was used to measure the lactate content of supernatant solutions. The detection method utilises an enzymatic biosensor which, upon substrate binding, catalyses the conversion of lactate to generate hydrogen peroxide as a by-product. This is detected by an electrode sensor and a read-out is generated, proportional to the concentration of sample substrate.

Materials:
Analox® GM7 direct glucose and lactate sensor
Lactate Reagent Kit (Analox, GMRD-093)
Positive displacement pipette (10 µl, Rainin, MR-10R)
MilliQ H_2O

The machine was flushed with lactate oxidase reagent for eight cycles prior to calibration by injecting 7 µl 8 mM lactate standard using a positive displacement pipette. The pipette was rinsed between calibration and each sample reading in MilliQ H_2O.

2.15. Statistical Analysis

Where appropriate the unpaired Student's T-test or a one-way ANOVA followed by the Tukey-Kramer test was used to determine significance of the data generated.

Chapter 3. Lactate Clearance

In this chapter modes of Lactate oxidase immobilisation and the efficacy of the systems to reduce lactate concentration in physiologically relevant conditions is experimentally investigated.

3. Lactate Clearance

3.1. Introduction

Lactate is produced as a consequence of oxygen deficient respiration and can be used as an energy source when glucose is unavailable to the cell (116). Yet many cancer cell lines, including HepG2 cells, respire primarily through aerobic glycolysis (49), generating lactate, a shift in the mode of metabolism referred to as the Warburg Effect (117). However, elevated lactate concentrations can impede cell growth and proliferation by lowering the pH (118). Furthermore, as a potent chelator of calcium (53;119), lactate levels in the fluidised bed bioreactor (FBB) should be regulated to below 10 mM in order that the integrity of the HepG2 containing alginate beads is maintained. This is particularly critical during the cell proliferation stage, when encapsulated HepG2 cells are cultured in the FBB prior to their use within the functional BAL, as any decrease in proliferation rate would inevitably lead to fewer viable cells capable of performing the required therapeutic liver cell functions.

The enzyme lactate oxidase (LOx) is an appealing candidate to address the problem of lactate accumulation within the BAL; not only does it catalyse the clearance of lactate but by doing so pyruvate is generated (120;121) which could potentially be used as an energy source for the encapsulated HepG2 cells. In its favour is that unlike lactate dehydrogenase, which also breaks down lactate *in vivo*, lactate oxidase has been demonstrated to restore cytoplasmic pH levels following an increase in lactate concentration (121;122).

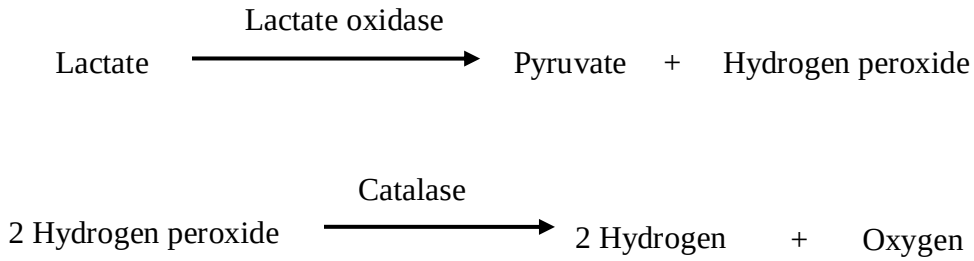


Figure 3-1 - Schematic depiction of the action of Lactate oxidase and Catalase

3.1.1. Lactate oxidase

The (approximately) 98 kDa (123) tetrameric (121) enzyme belongs to the a-hydroxyacid oxidase family, sharing their β8/α8 barrel structure (121) and is functional in the presence of the cofactor, flavin adenine dinucleotide (FAD), a member of the flavin mononucleotide (FMN) group. It is a highly specific enzyme, discriminating between L-lactate and glycolate (121). Studies of the crystal structure of *Aerococcus viridians* LOx have proposed a mechanism of lactate catalysis involving an interaction between lactate, the cofactor and amino acid moieties of the enzyme's active site. Specifically, Arg268, His265, and Tyr40 are absolutely required in the active site domain for successful catalysis where they interact with the cofactor FAD; His265 and Tyr40, along with Tyr146, Tyr215 and Lys211, are also required for the correct orientation and chirality of lactate, facilitating an interaction with, and the consequent reduction of, the FAD cofactor and the degradation of lactate.

LOx has been putatively identified in the inner membrane space of rat liver mitochondria (122) where it is implicated in the conversion of administered L-lactate into pyruvate and hydrogen peroxide (H_2O_2) (120;121), a potent oxidising agent which has been demonstrated to induce apoptosis due to oxidative stress (124;125).

Therefore, application of immobilised LOx for use within the FBB may also require a similar incorporation of catalase in order to convert the produced H_2O_2 to water and oxygen, should the innate catalase activity of HepG2 cell spheroids be insufficient.

3.1.2. Existing methods of LOx immobilisation

Previous strategies for the immobilisation of LOx have generally been for use within or as a biosensor device. Although some methodologies may be transferable between a biosensor design and an application such as the BAL there are a number of different considerations which must be acknowledged. It is an absolute requirement that the materials used for immobilisation of the enzyme must be able to withstand an effective sterilisation method and furthermore that the material can function sufficiently under the perfusion conditions, including compatibility with plasma/growth medium constituents and functionality under flow rates of between 2-600 ml/min. The most relevant methods undertaken for the immobilisation of LOx are summarised in the following sections.

3.1.2.1. Nylon net

In this approach discs of nylon net were surface-modified and lysine used as a spacer by which lactate oxidase from *Mycobacterium smegmatis* was covalently tethered to the discs (126). These were then used to coat the tip of a pO_2 oxygen electrode probe. A specific activity of 50 nmol min^{-1} cm^{-2} was obtained when 1 mg of protein was loaded onto the nylon net and tested at 25°C in 0.1 M citrate buffer at pH6 (126). This technology was shown to function well as a simple electrode sensor for lactic acid in blood serum samples (127) and, furthermore, was also shown to be operational for up to two months when stored in 0.1 M citrate buffer, although it is to be noted that serum samples were diluted 1:20 or 1:50 in citrate buffer prior to taking readings. Despite such encouraging results, the incorporation of a nylon net based method of LOx immobilisation into the BAL is probably unfeasible, if only as a consequence of space limitations within the FBB and the large

surface area of nylon net support that would be required to encompass a sufficient quantity of LOx enzyme. Moreover, this may also act as a binding surface for the proteins secreted by the biomass, meaning the benefit of these to the patient could be drastically reduced.

3.1.2.2. Covalent attachment onto Immobeads

Previous experiments undertaken by the Liver Group sought to immobilise LOx onto Immobeads150 (ChiralVision, The Netherlands). The beads have a diameter of 150-300 μm and comprise a macroporous cross-linked polymer of methacrylate carrying oxirane groups which bind lysine groups. Beads were pre-treated with 40 mM glutaraldehyde for four hours before cross-linking to LOx. However, enzyme activity remained low and active site analysis of *A.viridians* LOx revealed a lysine residue (Lys 241) in close proximity which, although not involved directly with the active site, is required for the correct orientation of FAD, the cofactor. This was unexpected as lysine residues have been frequently targeted to immobilise LOx as they are found on the periphery of the molecule and so can be utilised for binding without altering the active enzyme conformation (128). This could be accounted for by species variation of LOx enzyme; *Pediococcus* LOx was used in this study rather than the characterised *A. viridians* LOx orthologue.

3.1.2.3. Functionalised glass

In their investigation into the effects of the coupling reagent on the immobilisation of enzymes, Tiller *et al.* (128;129) demonstrated a number of methods for immobilising *Pediococcus* LOx onto NH_2 functionalised glass. An ultrathin (in the range of hundreds of nanometres) layer of SiO_2 was applied to the tip of glass rods by the Pyrosil technique (128-130) and following exposure to atmospheric humidity for 1-2 hours polymerised SiO_2 films were formed. These were then treated with 3-aminopropyl triethoxysilane in toluene. A total of thirteen different coupling reagents were investigated, each used in excess, for support activation, to avoid any

cross-linking of the matrix. Variable amounts of cross-linking may result in a variable degree of swelling of the support which can affect the outcome of immobilisation. The activated support was incubated at 4°C for 16 hours in 0.1 ml enzyme solution (1 mg enzyme/litre) to immobilise the enzyme. It was found that the enzyme was stabilised compared to the native enzyme, in particular that the most suitable method for high activity was coupling via 1,3-benzenedisulfonyl chloride and 4,4'-biphenyldisulfonyl chloride onto NH_2-glass (330 and 339 mU/cm^2; Km 0.37 mM). This becomes more relevant when it is considered that glutaraldehyde, a frequently used and popular choice in coupling reagent, yielded enzyme activities of only 175 mU/cm^2; Km 0.4 mM (Km of the free enzyme, 0.57 mM). However, one great disadvantage encountered with this immobilisation method was that LOx could not be stabilised against thermal stress; the enzyme lost 60% of its activity after only 30 minutes at 45°C (128).

Other successful attempts to tether LOx onto a modified glass surface include the use of zirconia coated alkylamine glass beads (131). A *Pediococcus* derived LOx was cross-linked to the glass support using glutaraldehyde to yield 3.2 mg of protein per gram of support, showing maximum activity at pH 6.5. Of note is that complexes prepared in this manner showed excellent retention of activity and were then applied to human serum to monitor lactate concentrations, indicating that this approach would have had the potential to be applied to the BAL; unfortunately, it was not possible to obtain the highly specialised glass beads for this study.

3.1.2.4. Freely circulating enzyme

In complete contrast to the immobilisation strategies discussed above, Bohm *et al*. incorporated a matrix-free method of lactate clearance which did not entail the immobilisation of LOx (132). Instead free enzyme was housed within a section of semi-permeable dialysis tubing with a wall width of 50 µm and a molecular weight cut-off of 20 kDa. This prevented leakage of LOx whilst permitting diffusion of substrate analytes, namely lactate and

oxygen into the enzyme solution and the efflux of hydrogen peroxide and pyruvate. The system enabled a continuous flow method in a biosensor application, with the advantage that LOx could be used at any desired concentration, even in excess, as no enzyme activity/yield reducing immobilisation steps were required. However, enzyme kinetics data was not investigated in the study, thus the possibility remains that the lack of physical support and stabilisation offered by immobilisation may have had a negative impact upon the activity and lifespan of the free enzyme.

The following section will address the hypothesis outlined in the main introduction chapter, namely, whether LOx can be immobilised in order to prevent the accumulation of lactate in the FBB. To do this, a variety of immobilisation strategies will be investigated and their suitability for use within the FBB determined. In order to be deemed a feasible option, an immobilisation method must fulfil the following criteria:

- Lactate concentration must be reduced from 15 mM to under 10 mM following treatment with the enzyme preparation

- The immobilisation strategy must yield enzymes which function in physiologically relevant conditions

3.2. Materials & Methods

3.2.1. Lactate oxidase activity assay

To determine the activity of LOx following immobilisation and also for the free enzyme positive control conditions, a simple and well documented colorimetric assay was used (133), the principle of which is outlined below.

$$L - lactate + O_2 \xrightarrow{LOx} Pyruvate + H_2\,O_2$$

$$2H_2O_2 + 4 - AAP + DMA \xrightarrow{POD} Quinonediimine\ Dye + H_2O$$

(http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/General_Information/2/lactate_oxidase.Par.0001.File.tmp/lactate_oxidase.pdf)

Materials:
Reaction mix
150 µl 200 mM 3,3-Dimethylglutaric acid buffer (adjusted with NaOH to pH 7.4) (Sigma-Aldrich, 98% D4379-25G)
75 µl 15 mM 4-aminoantipyrine (4-AAP) (A4382-10G)
75 µl 24 mM lactate solution (pH7.4)
75 µl 50 U/ml peroxidise, type II from Horseradish (Sigma, P8250)
225 µl dH_2O

0.2% (v/v) N,N-Dimethylaniline solution (DMA) (ReagentPlus®, 99%, 515124) in dH_2O
0.25% (w/v) dodecylbenzenesulfonic acid (DBS) (Aldrich 522953) in dH_2O

Phosphate Buffer (pH 7.4):
10 mM potassium phosphate
0.01 mM Flavin Adenine Dinucleotide, disodium salt (FAD) (Sigma F6625 - ≥95% (HPLC), powder)

0.2 U/ml LOx (Sigma, L0638) (in phosphate buffer)

To each well of a 96-well plate 80 µl of the reaction mix and 20 µl DMA was added, followed by the addition of the sample (20 µl 0.2 U/ml LOx solution for standards and free LOx samples, 20 µl of post-immobilisation

supernatants or 20 µl potassium phosphate buffer (pH 7.4) when assaying immobilised LOx, unconjugated glass beads or for taking blank measurements). The reaction was quenched by the addition of 20 µl DBS after incubation at 37ºC for 10 minutes.

3.2.2. Immobead enzyme immobilisation

Materials:
Immobead 150 (Sigma, ChiralVision, 94129)
4 U lyophilised LOx (Sigma-Aldrich UK Sigma, L0638)
40 mM glutaraldehyde

Immobeads have a diameter of 150-300 µm and comprise a macroporous cross linked polymer of methacrylate carrying oxirane groups which bind lysine groups. 4 U LOx was re-suspended in 10 µl 40 mM glutaraldehyde solution and incubated for two hours at 4°C, before adding 100 mg of dry beads and incubating for 48 hours at 4°C.

3.2.2.1. $NaBH_3CN$ quenching of glutaraldehyde

Materials: As above, but also:
10 mM $NaBH_3CN$ (Aldrich 156159) in 10 mM potassium phosphate buffer

For $NaBH_3CN$ quenching experiments, 100 mg Immobeads and 370 µl 10 mM potassium phosphate buffer was added to the enzyme solution and incubated at 4°C for 24 hours followed by two hours incubation with 1.5 ml 10 mM $NaBH_3CN$ (final concentration of 8 mM).

3.2.3. NH_2 functionalised glass beads immobilisation

Materials:
Glass beads (750-1000 µm diameter, Kisker-Biotech, PGB-075)
Methanol
Isopropyl alcohol
Sonicator
1,3-benzenedisulfonyl chloride (97%, Aldrich, 444642)
Toluene, anhydrous (99.8%, Sigma-Aldrich 244511)
Cyanuric chloride (99%, Sigma-Aldrich, C95501-5G)
N,N-dimethylacetamide (DMAd) (≥99.5%)Sigma-Aldrich, 38840)
10 mM phosphate buffer
Ethanol

3- aminopropyltriethoxysilane (≥98%, Sigma A3648)
Ceramic mortar
Plate shaker
Miniature blow torch
0.1 mg/ml LOx in 10 mM phosphate buffer

A protocol for the functionalisation of glass was adapted from Tiller *et al.* (128) for the treatment of glass beads. 100 mg beads were sonicated in isopropyl alcohol for five minutes and then rinsed in 2 ml isopropyl alcohol followed by a rinse with 2 ml methanol. The beads were left to dry for 30 minutes at 80°C. The beads were then treated with a procedure adapted from the Pyrosil technique used by Tiller *et al*; the beads were placed in an autoclaved ceramic dish on a plate mixer and exposed to the oxidising tip of a flame from a miniature blow torch. The flame treatment consisted of two seconds exposure to the flame followed by a five seconds pause. This was repeated three times to give a total of six seconds flame treatment per batch of beads.

Treated beads were allowed to cool in air overnight before incubating in a 20% solution of 3- aminopropyltriethoxysilane in dry toluene (Figure 3-2**,** 1), incubated with stirring at room temperature for two hours.

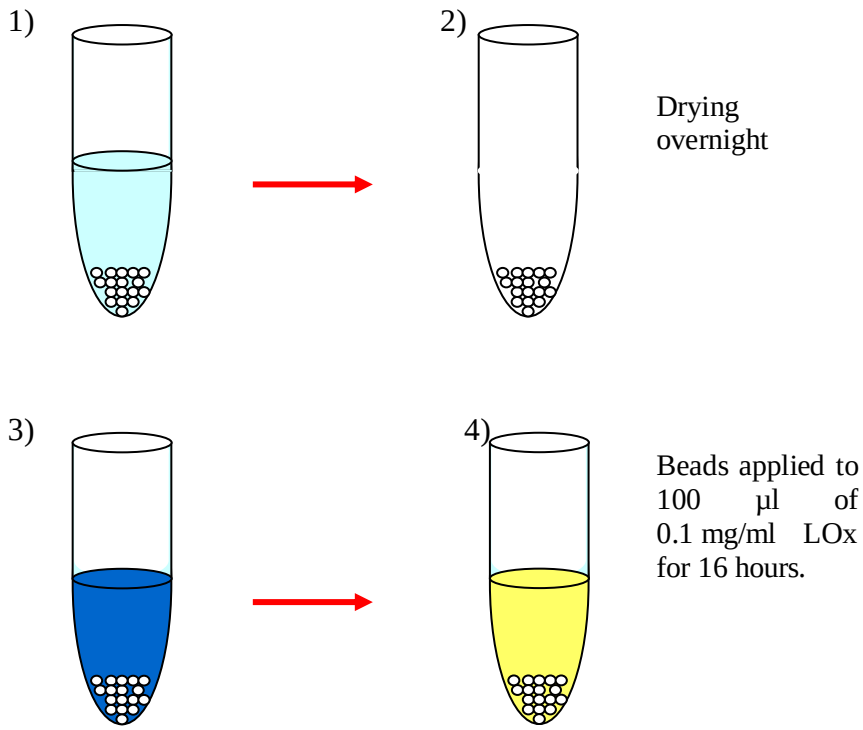


Figure 3-2 - Schematic representation of the NH_2 functionalisation process for glass beads.

1) = beads in 3- aminopropyltriethoxysilane solution; 2) = beads air drying; 3) = beads immersed in cross-linker solution; 4) = functionalised beads applied to LOx solution.

Following this, the beads were rinsed first with 2 ml toluene, followed by 2 ml 1:1 toluene:methanol solution and finally with 2 ml methanol. Beads were dried in air overnight (Figure 3-2**,** 2). For the cross linking stage, the beads were treated with 100 mg of either 1,3-benzenedisulfonyl chloride or cyanuric chloride, dissolved in 10 ml DMAd at room temperature for 15/5 minutes respectively (Figure 3-2, 3). Beads were washed three times with 10 ml ethanol and rinsed in 10 ml chilled 10 mM phosphate buffer (pH7.4) before incubating with 100 µl of 0.1 mg/ml LOx (in buffer, cooled to 4°C) for 16 hours (Figure 3-2, 4)). Finally, the functionalised beads were rinsed three times in 500 µl 10 mM potassium phosphate buffer before being re-suspended in 10 ml of the same buffer solution. It was calculated

that four beads would be required for each individual assay to represent the 0.004 U LOx used in the positive control assay conditions (based on an equal distribution of the total 10 µg LOx used onto 100 mg of glass beads).

3.2.3.1. Immobilised LOx for lactate clearance in FFP media:

Materials:
Immobilised LOx beads, as described previously
FFP culture medium
Heparin (5000 IU/ml, Wockhardt UK Ltd)
1 M HEPES (pH 7-7.6, Sigma, HO887)
200 mM lactate (~98%, Sigma L7022)

Heparin was added to the FFP medium to prevent clotting, at a final concentration of 2 U heparin/ ml of 10% FFP in complete DMEM culture media. A final concentration of 10 µl/ml HEPES was used to buffer the media solution to pH 7.4. 100 mg of beads were used for each experimental condition. 20 µl of the 2 ml solution was replaced with 200 mM lactate solution to spike the media. For the 11 day experiment, the following modification to the lactate spiking protocol was used:

Table 3-1 – Lactate spike administration schedule and protocol.

DAY	200 mM lactate added (µl)	Final concentration (mM)
1	0	0
2	10	0.1
3	5	0.5
4	9	0.9
5	14	1.4
6	19	1.9
7	15	1.5
8	21	2.1
9	31	3.9
10	24	3.1
11	0	2.4
	TOTAL	25.3

3.2.4. PVDF membrane immobilisation of LOx

Materials:
Polyvinylidene fluoride (PVDF) membrane (AnalaR NORMAPUR 081160502)
Methanol
dH_2O
96-well plate (Nunc 735-0291)
0.2 U/ml LOx (in 10 mM phosphate buffer, pH 7.4)

PVDF membranes are typically used in the Western Blotting procedure whereby proteins separated by polyacrylamide gel electrophoresis are transferred to the PVDF membrane. This membrane can then be treated with antibodies to allow the detection and identification of bound proteins. The protein binding property of the membrane could provide a means of physically retaining LOx protein to a designated location within the FBB for lactate clearance. 1.5 mm x 1.5 mm square sections of PVDF were first activated by soaking in methanol for five minutes followed by rinsing in distilled water. Sections were transferred individually to vacant wells in a 96-well plate where they were incubated in 80 µl 0.2 U/ml LOx solution for two hours at 4°C. Sections were then rinsed in 10 mM potassium phosphate buffer before being applied to the LOx activity assay reagent mix for analysis. Modifications to this protocol included varying the incubation time with LOx from the original two hours, to six and 24 hours.

3.2.5. Dialysis tubing for enzyme containment

Materials:
Sodium bicarbonate solution (100 ml):
2 g sodium bicarbonate
200 µl 0.5 M ethylene-dinitrilo-tetraacetic acid (EDTA)

0.2 U//ml LOx in phosphate buffer (pH 7.4)
Polypropylene 1.5 ml and 2 ml microcentrifuge tubes (Appleton Woods, KC024)
Dialysis tubing (Spectra/Por™ 10 KDa, 128106)

Dialysis tubing consists of a synthetic membrane with pores of controlled size. By using a membrane with a suitable molecular weight cut off, it is possible to create a physical barrier between LOx and the circulating

system, thus avoiding release of LOx into the FBB, without resorting to immobilisation and the resulting decreased activity rates often associated with the procedure. Dialysis membrane sections were activated by boiling for 10 minutes in sodium carbonate solution. Sections were rinsed in double-distilled water. A cassette to house the LOx solution was constructed from a 15 mm x 15 mm section of dialysis membrane sandwiched between the lid section of a PCR tube (Figure 3-3).

This cassette was placed base down within the lid section of a 2 ml centrifuge tube containing the LOx activity assay reagent cocktail. This solution was used for the subsequent colorimetric assay.

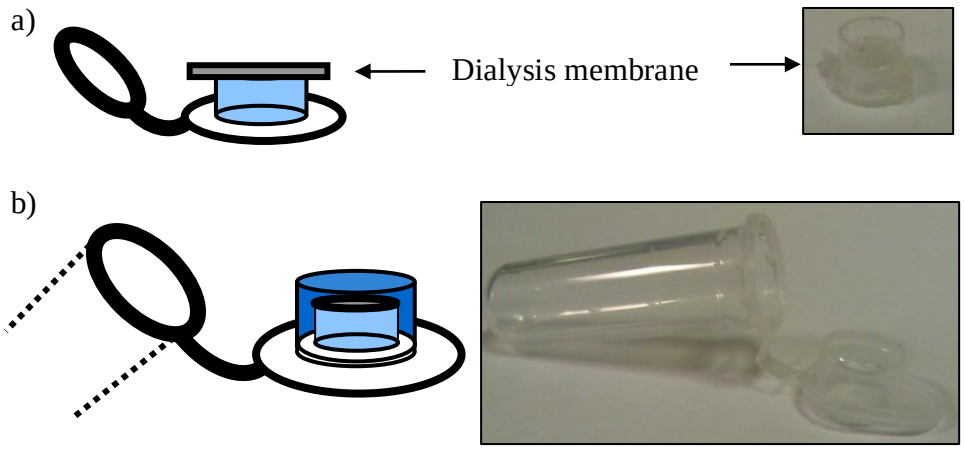


Figure 3-3 – Schematic diagram depicting the assembly of the dialysis membrane cassettes.

(a) The lid section of a PCR tube containing 20 µl 0.2 U/ml LOx solution, covered by a section of activated dialysis membrane, forming the cassette. (b) The cassette was placed membrane face-up within a centrifuge tube "chamber" which contained 120 µl LOx activity assay reagent (section 3.2.1).

3.2.5.1. LOx thermostability

0.2 U/ml LOx solution was incubated at 37°C for 24 hours prior to either application to the cassettes or directly to the reaction mix. All other methodology was as previously described.

3.3. Results & Discussion

3.3.1. Immobead immobilisation

As a preliminary experiment it was necessary to ascertain the storage stability and reusability of free, non-immobilised LOx, to provide a standard against which the activity of immobilised enzyme could be measured. Simultaneously to this, immobilisation with glutaraldehyde was carried out and the beads stored in 10 mM potassium phosphate buffer at 4℃. Samples of beads were taken from this stock every 24 hours and assayed for LOx activity. Activity of immobilised LOx was compared against free LOx solution (0.2 U/ml). The same re-suspended aliquot of LOx was used for the initial immobilisation and also for the analysis of "Free LOx" throughout the time course of the experiment. The data shows that whereas LOx is suitably stable for one week at 4°C, immobilised LOx activity falls rapidly from an initial rate of 0.02 mM lactate cleared in 10 minutes on Day 1 to 0.01 mM by Day 3, representing a fall from around 7% to 2% the rate of lactate clearance of Free LOx (Figure 3-4). Indeed, from Day 3 onwards the amount of lactate cleared was significantly lower ($P<0.05$) than that on Day 1, indicating a fall in enzymatic activity. The amount of lactate cleared by LOx immobilised onto Immobeads was significantly ($p<0.005$) lower than that of free LOx at all times, indicating that enzymatic activity is reduced following immobilisation onto Immobeads.

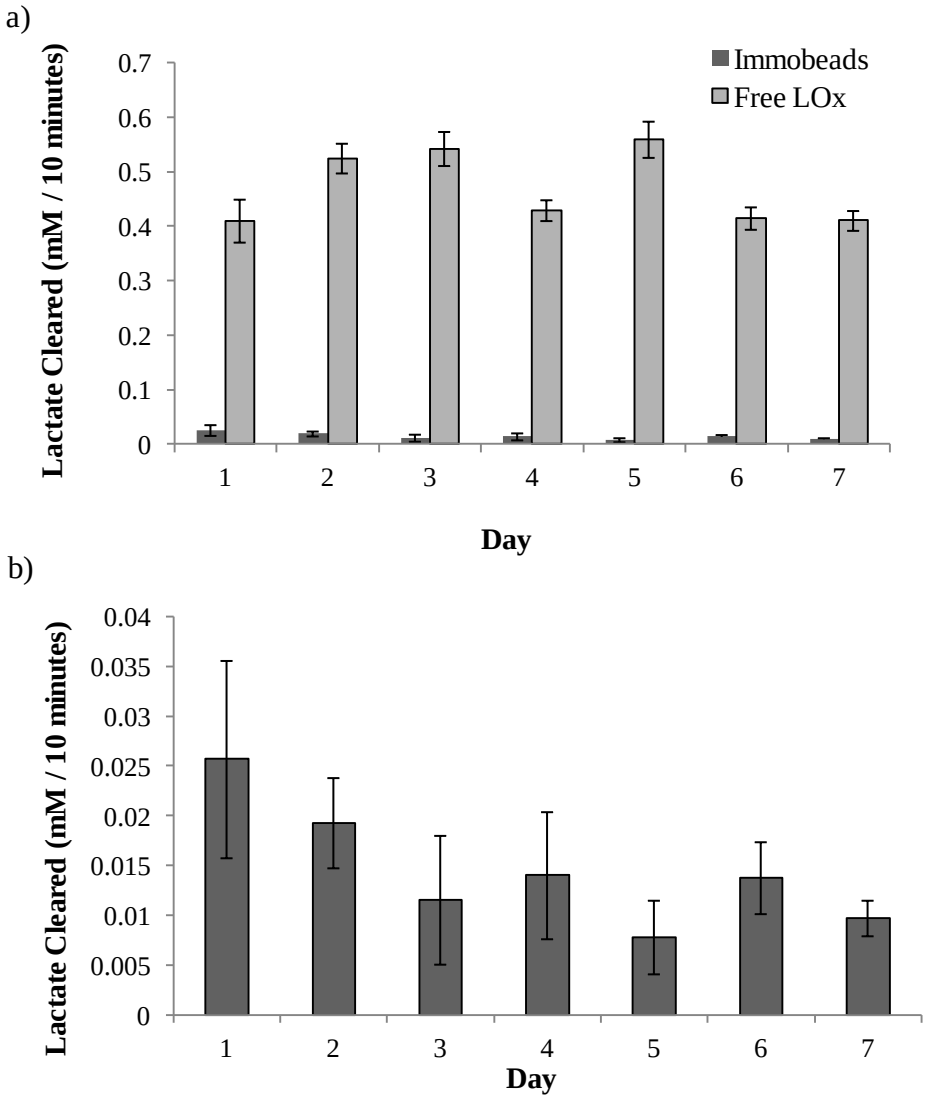


Figure 3-4 - Stability of LOx immobilised onto 100 mg Immobeads.

Free LOx stock was re-suspended on the day of immobilisation and subsequently stored at 4ºC. Data for Immobeads are magnified in 3-4b). Activity determined from colorimetric absorbance at a wavelength of 565 nm. For each day, n=6, error bars depict +/- SD. The extent of lactate clearance between all days from Day 3 onwards is statistically different from that of Day 1. * $p<0.05$

To investigate whether the cross-linking stage could be optimised to gain better levels of lactate clearance using LOx immobilised onto Immobeads, two approaches to the glutaraldehyde pre-treatment of Immobeads were pursued. These were as follows: a) 10 µl 40 mM glutaraldehyde was used to re-suspended 4 U LOx and incubated at 4°C for two hours, before adding 390 µl 10 mM potassium phosphate buffer and 100 mg Immobeads for a

further 48 hours at 4°C; b) 10 µl 40 mM glutaraldehyde was applied to 100 mg Immobeads and 380 µl 10 mM potassium phosphate buffer and incubated at 4°C for two hours, before adding 4 U LOx in 10 µl 10 mM potassium phosphate buffer and incubating for 48 hours at 4°C.

A Students T-test of the data suggests that there is no difference between the two glutaraldehyde treatment conditions, as both yielded activity levels of around 2% that of the free LOx enzyme.

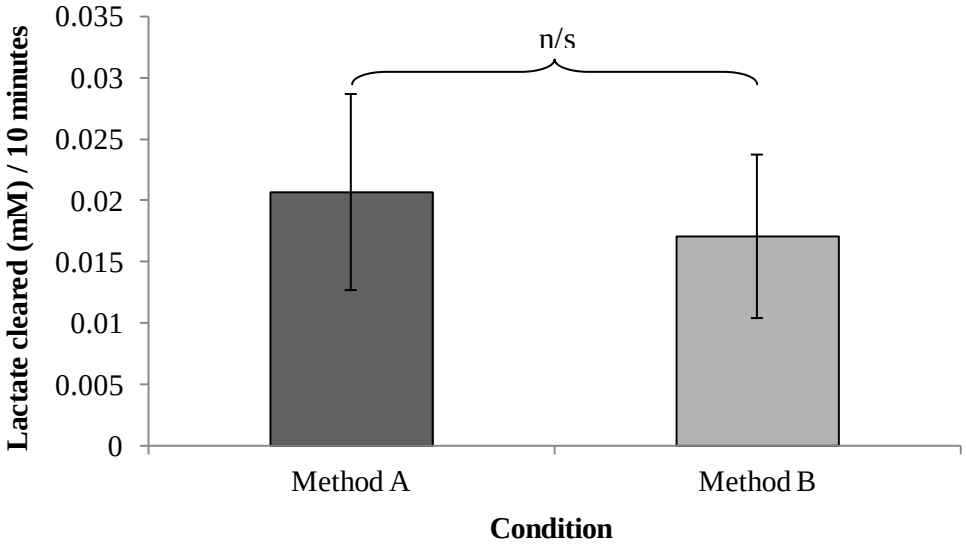


Figure 3-5 - Comparison between two methods of immobilisation of LOx with 100 mg Immobeads.

Method A – 10 µl glutaraldehyde pre-incubated with 4 U LOx prior to addition of 100 mg Immobeads. Method B – 10 µl glutaraldehyde pre-incubated with 100 mg Immobeads prior to addition of 4 U LOx. No significant difference (n/s) was calculated between the methods (Students T-test) n=6 +/- SD

Quenching of the glutaraldehyde cross-linking reaction was investigated in order to determine whether over-binding of the enzyme was limiting the activity of immobilised LOx. A final concentration of 8 mM $NaBH_3CN$ was incubated with 100 mg of glutaraldehyde cross-linked LOx Immobeads for two hours at 4°C, prior to assaying the beads as before with the LOx activity assay.

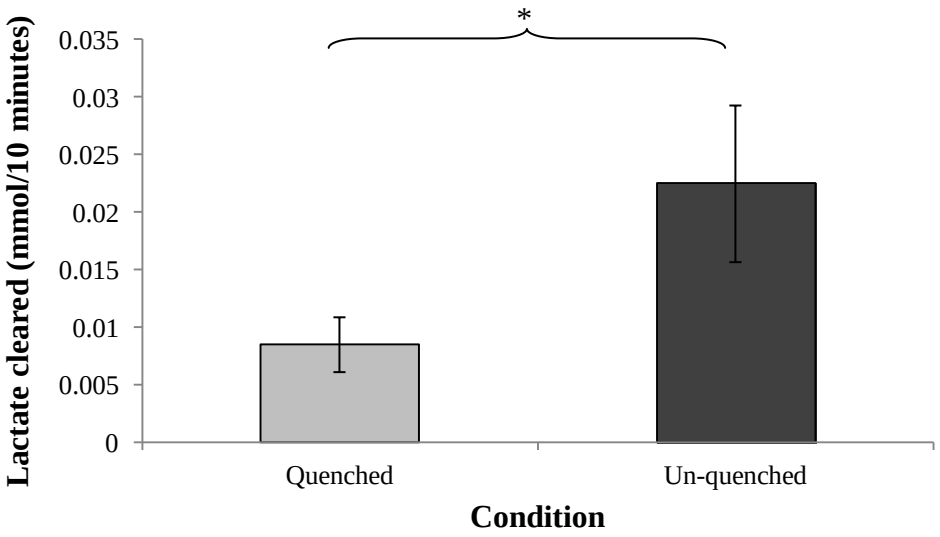


Figure 3-6 – Effect of quenching glutaraldehyde cross-linking on LOx activity.

"Quenched" condition: LOx immobilised onto 100 mg Immobeads with 10 µl glutaraldehyde as previously described, but 8 mM $NaBH_3CN$ added immediately after the immobilisation period in order to quench any further glutaraldehyde cross-linking. "Un-quenched" samples were prepared exactly as previously described, and incubated in phosphate buffer whilst the "Quenched" samples underwent the additional quenching step. Error bars = +/- SD, n=6, *= significant difference (p=0.007).

LOx beads which had been quenched were shown to have cleared less lactate than the unquenched beads (Figure 3-6), implying that excessive glutaraldehyde cross-linking, if it occurs, does not have a detrimental effect on immobilised LOx activity. As the quenched LOx Immobeads had reduced activity, the $NaBH_3CN$ treatment itself may be having a negative impact upon LOx activity.

3.3.2. NH_2 functionalised glass beads immobilisation

The initial immobilisation of LOx onto functionalised glass beads with 1,3-benzenedisulfonyl chloride was encouraging, yielding activity levels of between 10 and 13% that of the free enzyme.

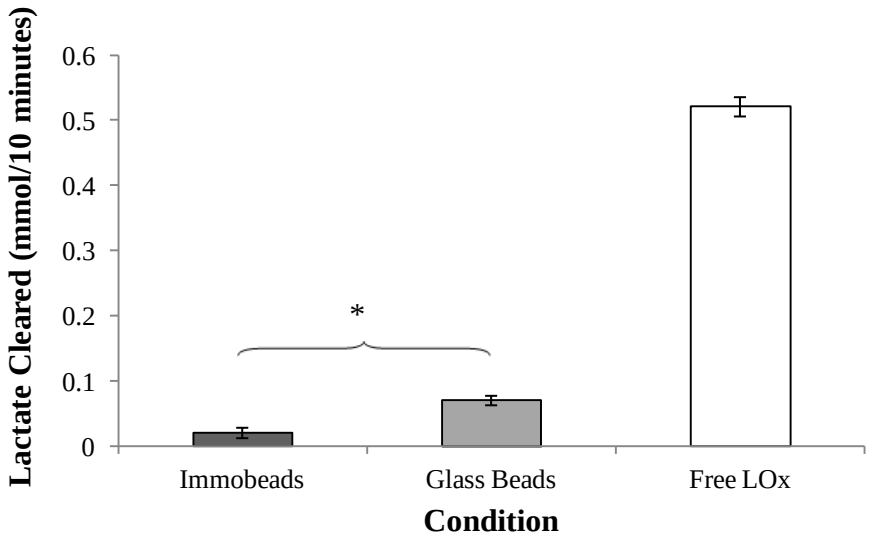


Figure 3-7 – Lactate clearance by LOx-conjugated Immobeads or glass beads.

Activity determined by applying immobilised LOx to the LOx activity assay. Volume of bead suspension / number of beads used per assay was based upon the theoretical quantity of LOx beads required to represent 0.004 U LOx, assuming 100% binding efficiency. Hence, 20 µl Immobead suspension, 4 glass beads and 20 µl Free LOx were used per well. Error bars = +/- SD, n=6. * = significant difference ($P= 2 \times 10^{-6}$ (T-test)).

Moreover, the extent of lactate clearance by LOx immobilised onto glass beads was greater than that achieved when using an equivalent quantity of LOx-bound Immobeads (Figure 3-7). This was also true when using cyanuric chloride as the cross-linking molecule, but a similar level of activity was also observed in the absence of a cross linker, suggesting a degree of non-covalent binding of LOx to the functionalised glass bead (Figure 3-8 a).

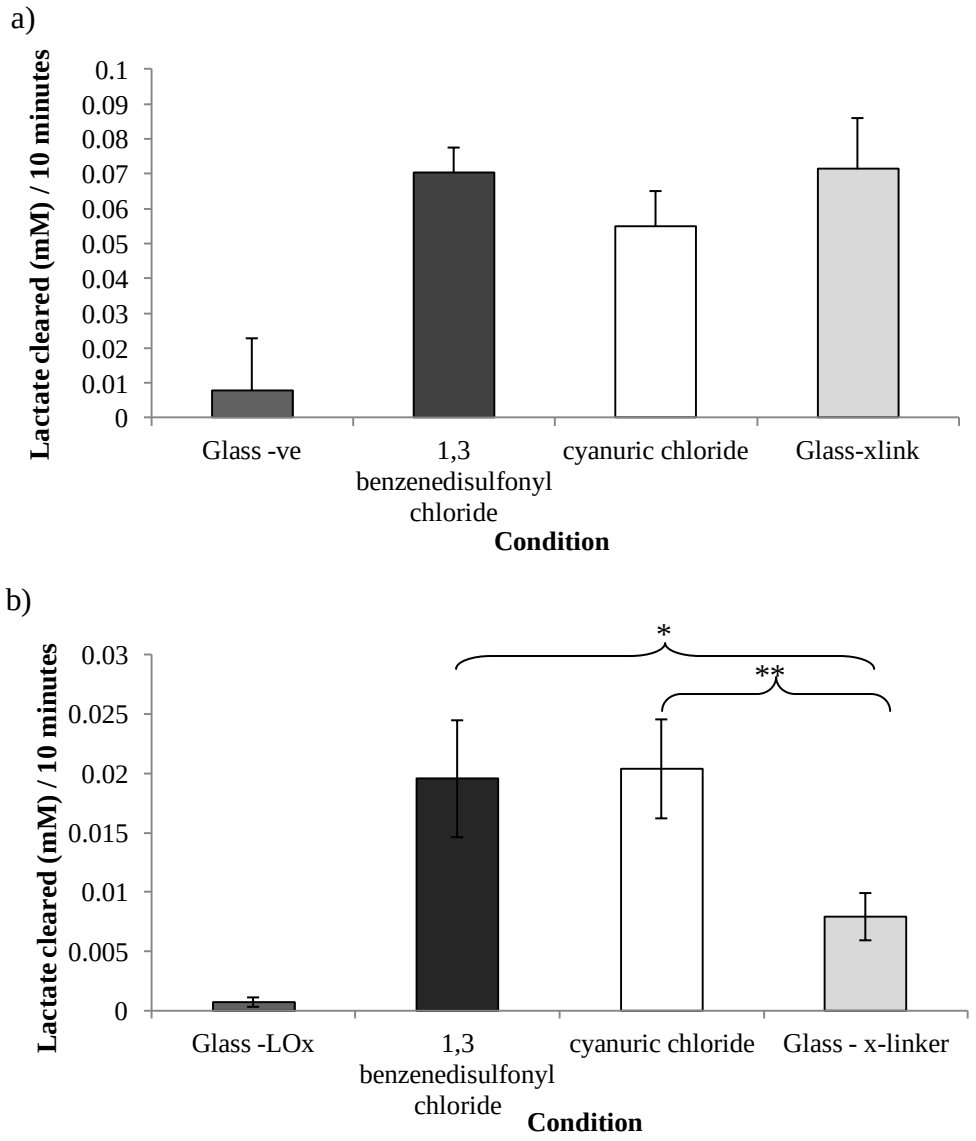


Figure 3-8 – Effect of Cross-linking on Immobilised LOx activity.

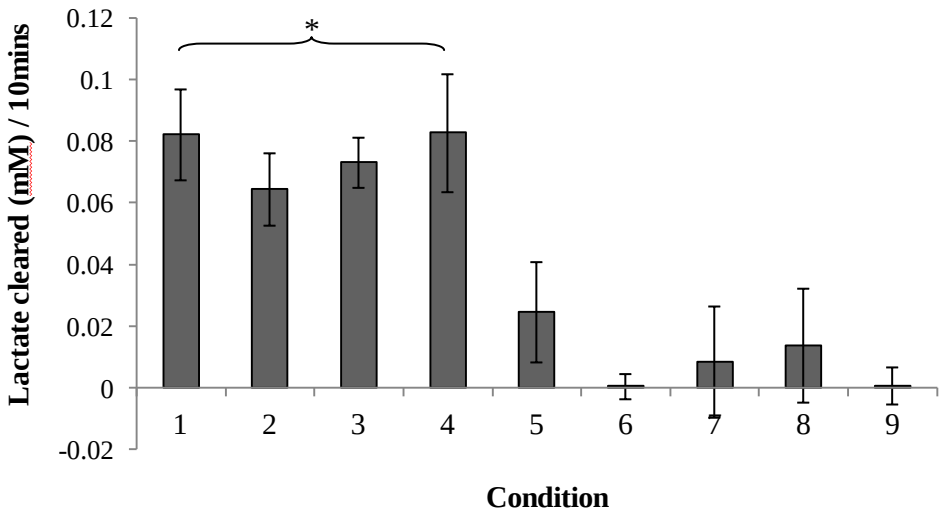
a) Initial LOx activity b) LOx activity following freeze/thaw. Activity determined by applying four glass beads (or 20 µl Free LOx) to the lactate oxidase activity assay. n=6 +/- SD. * = p = 0.001; ** = p = 0.0003.

To test whether cross-linking confers any stability to immobilised LOx, and hence be advantageous over non-covalently bound LOx, the beads were frozen for 48 hours, thawed, refrozen for 24 hours, thawed and assayed for enzyme activity. Figure 3-8b shows that although LOx activity levels had fallen in all conditions, the greatest decrease was observed in the absence of any cross-linking reagent. Whereas no significant difference was observed between cross-linked and non-linked beads initially, activity after freeze/thaw of the non cross-linked LOx beads were significantly lower

($p<0.005$) than both the cross-linked LOx conditions. This implies that cross linking confers some stability to LOx, which has practical implications should the immobilised enzyme be incorporated into a cartridge to be used within the FBB, and necessitate mid- to long-term storage. As the greatest activity levels were observed when 1,3-benzenedisulfonyl chloride was used to immobilise LOx, this molecule was utilised in subsequent LOx immobilisation experiments with the glass bead support.

To identify which point in the bead preparation process was promoting the non-covalent binding of LOx, a series of experiments were carried out omitting various components of the functionalisation process. In brief, the conditions comprised all permutations of the three stages of bead treatment – flame treatment, NH_2 group introduction and cross-linker inclusion, plus or minus LOx as required, generating a total of nine conditions. All reactions were commenced at the same time to allow for a fair comparison between samples, taking into account, for example, any possible degradation of silanol groups on the surface of the flame treated beads over time.

It is shown in Figure 3-9 that there is a significant ($p<0.001$) difference between the amount of lactate cleared by beads treated with NH_2 and LOx compared to those which had not. Although LOx activity was obsereved in the absence of a cross linker, data from Figure 3.8 demonstrates the advantage and requirement for cross linking the enzyme to the functionalised support. There was not a significant difference between conditions which had not involved any NH_2 group surface modification, indicating that NH_2 functionalisation is critical for LOx binding to glass beads.



CONDITION									
Treatment	1	2	3	4	5	6	7	8	9
Pyrosil		●		●	●		●		●
NH_2	●	●	●	●					●
Cross-linker	●	●				●	●		●
LOx	●	●	●	●	●	●	●	●	

Figure 3-9 – Determining the Critical Step for LOx Immobilisation.

Table shows combinations of treatments administered. LOx activity of each treatment is illustrated in the corresponding graph. NH_2 treated beads have significantly greater lactate clearing properties (determined by the LOx activity assay as described in Materials & Methods) than other treatment protocols. n=6, +/- SD. * = Conditions which, as a group, are statistically different to the other treatment conditions. $p<0.05$, ANOVA.

3.3.2.1. Optimisation of LOx immobilisation

In order to increase the activity of immobilised LOx, a comparison was made between static incubation with LOx solution and gentle continuous rotation of the reaction vessel during the immobilisation process. The data indicated no advantage in terms of enzymatic activity to immobilising in a rotating chamber (Figure 3-10). Although non-significant, the P-value was calculated to be 0.055 and so for simplicity it was decided to continue using the static mode of LOx immobilisation.

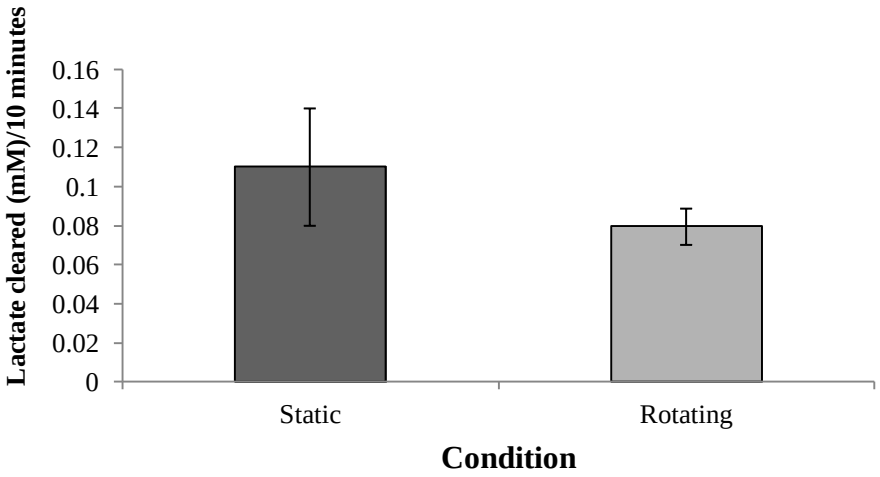


Figure 3-10 – LOx immobilised onto glass beads by static / rotating incubation.
Incubation was carried out at 4°C for 16 hours. T-test value P = 0.055. n=6, +/- SD.

Another measure to increase the activity of the LOx immobilised glass beads was to vary the concentration of LOx enzyme used for each immobilisation. An initial experiment using a ten-fold increase in total LOx protein generated beads which exhibited an approximate 50% decrease in total lactate conversion. The experiment was expanded to include two additional LOx concentrations, with the aim of establishing how close the standard protocol was to the optimal enzyme loading capacity. All steps were performed as originally described except for incubation with LOx solution; the total amount of LOx used was either 0, 0.001, 0.005, 0.01 or 0.1 mg per 100 mg glass beads. All subsequent steps remained as described in the original protocol.

The results obtained (Figure 3-11) show that the current dose of 0.01 mg LOx per 100 mg beads is in fact already close to optimal, conferring almost 30% of the activity of the control LOx solution. Furthermore, as there was no significant advantage to increasing the quantity of LOx used, it is more cost efficient to use the original loading mass of protein.

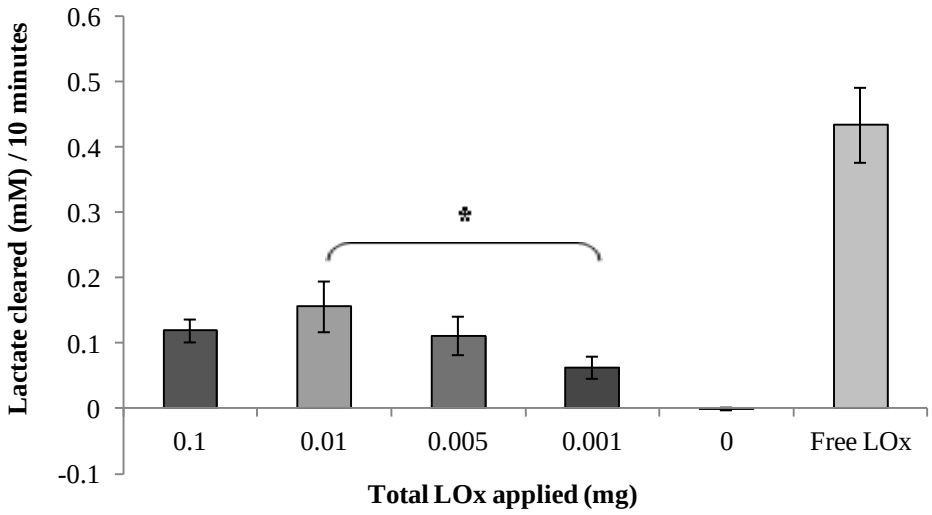


Figure 3-11 – Effect of varying the concentration of LOx used for immobilisation on the activity of functionalised glass beads.

All LOx concentrations yielded levels of activity significantly different (*$p<0.05$) to the un-conjugated (Free) enzyme and the negative control (no LOx added to glass beads). The 0.01 and 0.001 mg conditions were also significantly different from each other at the same level. n=6, +/- SD.

In order to determine the suitability of the beads as a surface for LOx immobilisation, the variability, in terms of lactate clearance, between batches of immobilised enzyme-bead complexes was investigated. Glass beads were prepared as per the original method, in duplicate under identical conditions simultaneously. Again, LOx was applied in a 100 µl volume to all batches of beads, and incubated at 4°C for 16 hours, after which beads were rinsed three times in 500 µl 10 mM phosphate buffer before being assayed by the colorimetric lactate assay described in section 3.2.1. The data indicated that there was no difference between the extent of lactate clearance by batches of immobilised LOx. It can be concluded that the method of immobilisation is highly reproducible in terms of the quantity of LOx immobilised and the resulting activity of the enzyme.

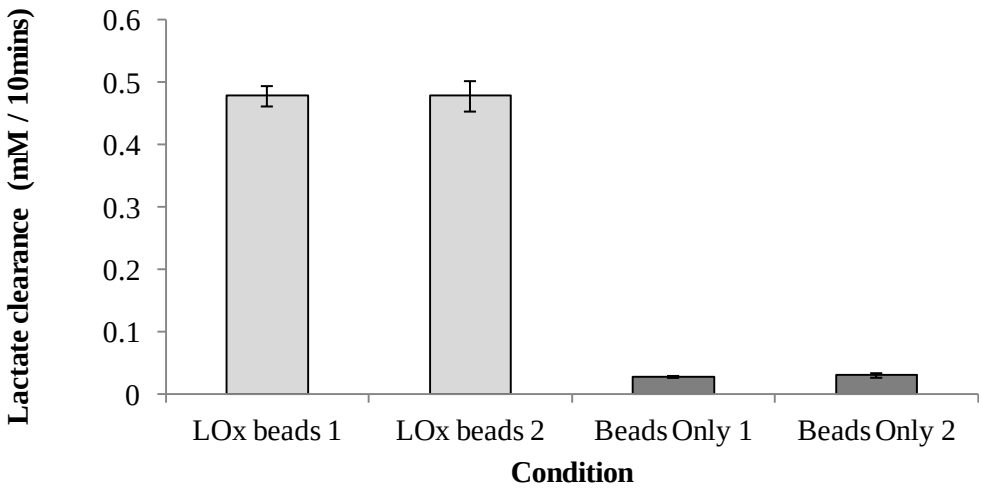


Figure 3-12 – Variability Between Immobilisations.

Four beads were assayed per sample for enzymatic activity using the lactate oxidase activity assay. +/- SD, n=3. No statistical difference was found between LOx bead immobilisations (T-Test, p=0.992)

Furthermore, it was shown in a separate experiment (Figure 3-13) that the relationship between lactate clearance rates (an indicator of LOx activity) and bead number is linear, confirming that there is a fixed amount of LOx is attached to any given bead in an immobilisation process.

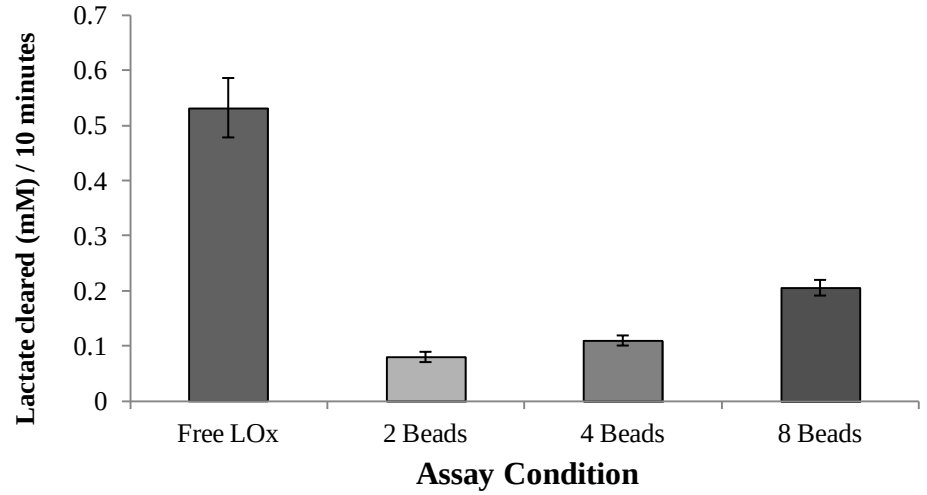


Figure 3-13 – Effect of bead number on the rate of lactate clearance.

Immobilised LOx beads were prepared as previously, and either two, four or eight beads applied to the lactate oxidase activity assay (total reaction volume per well was 120 µl). All conditions were found to be statistically different from one another ($p<0.05$) n=6, +/- SD.

As it seemed that a limit had been reached for the amount of LOx activity that could be yielded from a batch of beads treated with LOx solution, it was investigated whether the remaining supernatant after the immobilisation period could be reused on freshly prepared beads in order to maximise efficiency. For this series of experiments, the LOx solution remaining after the 16 hour incubation period was aspirated and transferred to a batch of freshly prepared beads, which were then incubated with the solution for 24 hours. This was repeated over the course of four days. All other methodology was as described in the original protocol.

While the initial batch of beads on Day 1 exhibited activity levels around 20% that of freshly prepared LOx solution, beads which were treated with the "left over" LOx in the supernatant from Day 1 demonstrated very similar activity levels, around 18% that of freshly prepared LOx (Figure 3-14). However, by Day 3 (the second reuse of the supernatant) LOx activity had fallen dramatically to little over 3.5% that of freshly prepared LOx. By Day 4 LOx activity on treated glass beads was negligible. Free LOx activity had also diminished by Day 4, indicating that a reduction in the activity of the enzyme used for immobilisation was also responsible for the reduced immobilised LOx activity, rather than simply the depletion of available LOx due to previous immobilisations.

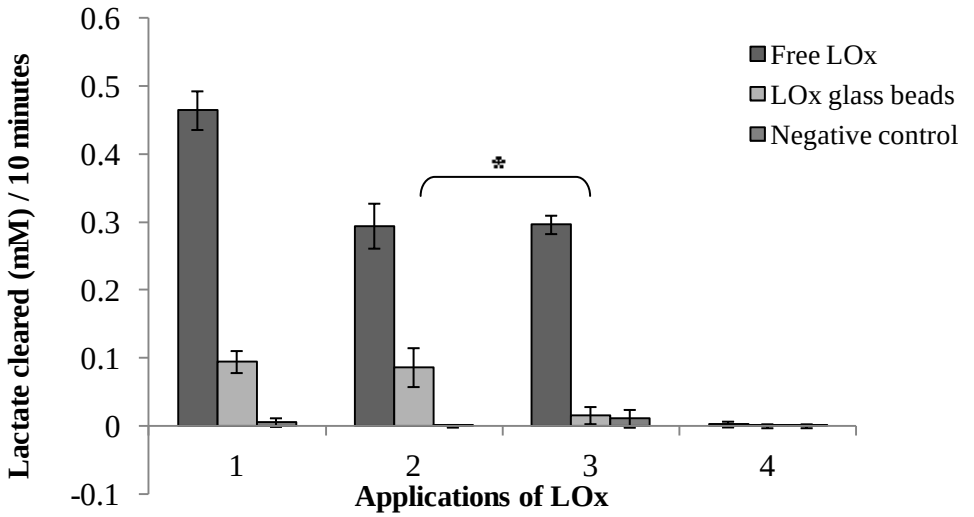


Figure 3-14 – Reuse of the LOx solution supernatant for subsequent immobilisation reactions.

Samples stored at 4°C between readings taken at 24-hour intervals. Lactate clearance by beads cross-linked with Day 2 and Day 3 LOx supernatants are significantly different (*$p<0.05$) n=6, +/- SD.

3.3.2.2. Immobilised LOx activity under physiological conditions

Having determined the parameters for a successful immobilisation of LOx onto the glass bead support, it was necessary to determine how the activity of the enzyme was affected by the presence of plasma, if at all. Glass beads were prepared and incubated with LOx and then rinsed as described previously (section 3.2.3). Beads were then incubated at either 37°C with 2 ml solutions containing a 20 mM lactate spike in either buffer or 100% human fresh frozen plasma (FFP). Samples were taken initially and then hourly for a duration of five hours. Sample analysis was performed using Analox instrumentation. The beads themselves were retained after the time-course experiment, washed and then assayed for residual activity using the colorimetric assay as described in section 3.2.1.

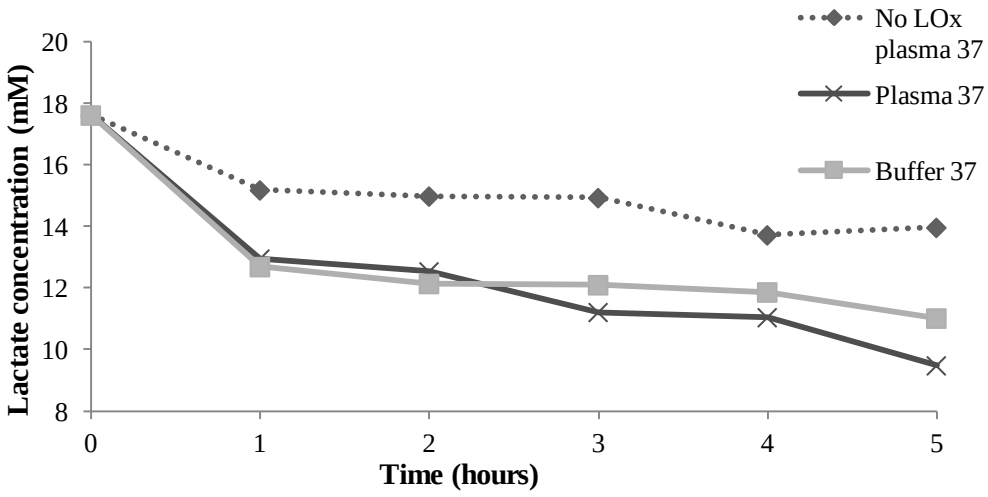


Figure 3-15 – Lactate clearance by immobilised LOx over a five hour period.

Samples of plasma (100% FFP) or buffer (10 mM phosphate buffer) were spiked to a concentration of 20 mM lactate and incubated at 37˚C. Samples were taken hourly for five hours, and analysed using Analox. n=3.

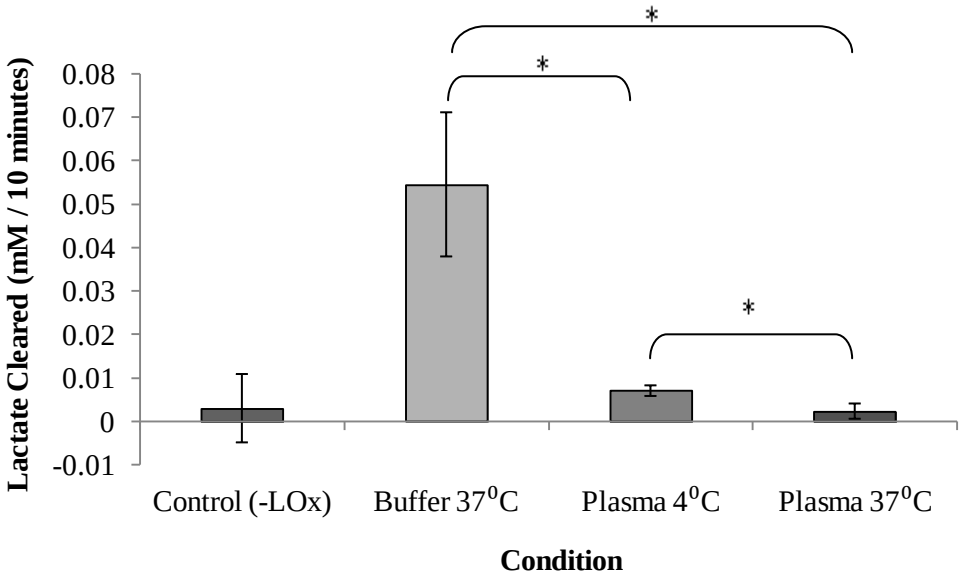


Figure 3-16 – LOx activity after five hours incubation.

Each condition applied to the LOx activity assay after five hours incubation with lactate spiked buffer or FFP at 4°C or 37°C. Extent of lactate clearance was significantly different between the indicated conditions (*$p<0.05$). n=6, +/- SD.

After incubation for five hours, immobilised LOx reduced the lactate concentration in all conditions compared to the negative control (Figure 3-15). Of particular interest is that the rate of lactate degradation by

immobilised LOx in FFP was increasing with time, more so than in buffer. It may be thus concluded that this method of LOx immobilisation is suitable for use in human plasma. However, the residual activity of immobilised LOx exposed to plasma was significantly lower than that of identical beads incubated in buffer (Figure 3-16). Moreover, there was a significant difference between LOx activity following incubation in plasma at 37°C and 4°C, suggesting that a component of plasma is having a detrimental effect upon immobilised LOx after exposure for just five hours. As the immobilised LOx beads will be required to reduce lactate concentrations over a much longer period of time than the five hours tested (up to 11 days), a second experiment was conducted, incubating immobilised LOx beads with lactate spiked FFP / buffer for a duration of 24 hours. The conditions also included samples incubated at 4°C and at 37°C; compromised over extended periods of time at physiological, rather than storage, temperatures.

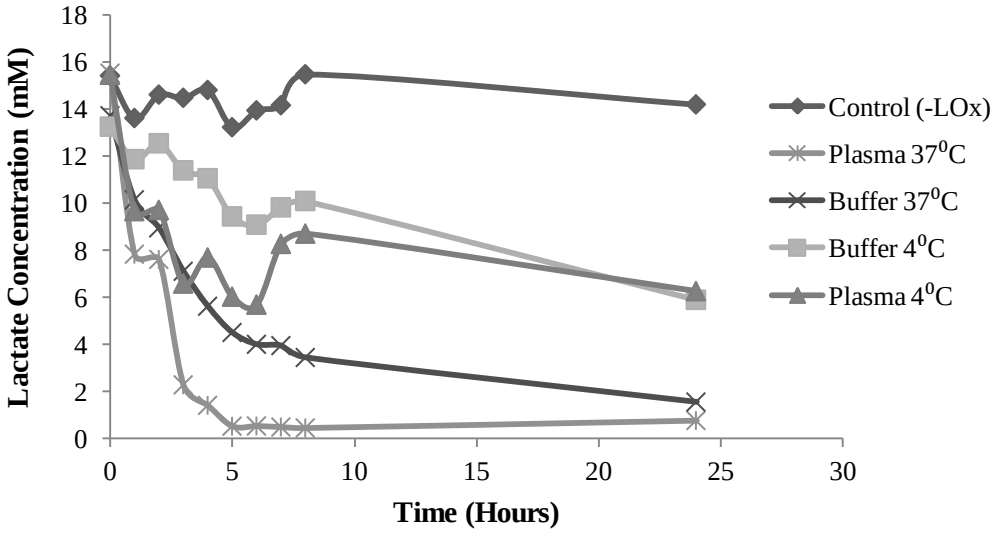


Figure 3-17 – Effect of temperature and plasma on immobilised LOx activity over 24 hours.

Immobilised LOx beads were incubated in either 100% FFP (Plasma) or 10 mM phosphate buffer (Buffer) at 4°C or 37°C for 24 hours. Control consisted of glass beads only in spiked plasma at 37°C. All conditions were spiked with 15 mM lactate and samples taken hourly for seven hours, then finally after 24 hours. Samples assayed using ANALOX.

The results demonstrated that the rate of lactate clearance by immobilised LOx is temperature sensitive, but not compromised by exposure to physiological temperatures (Figure 3-17). Rather, the extent of lactate clearance was greater and more rapid at the higher temperature; as would be expected based on rate of reaction principles.

Interestingly, analysis of the immobilised enzymes after this section of the investigation revealed that although the beads in plasma out-performed those in buffer, after 24 hours the immobilised LOx in buffer was able to reduce lactate levels by a greater extent in the colorimetric assay (Figure 3-18). This could be interpreted to imply that a component of FFP has a detrimental effect upon the functional lifespan of immobilised LOx; although initially functioning at a greater rate, immobilised LOx in plasma lost catalytic activity over time, a phenomena which could have been masked by the initial rapid reduction in lactate concentration

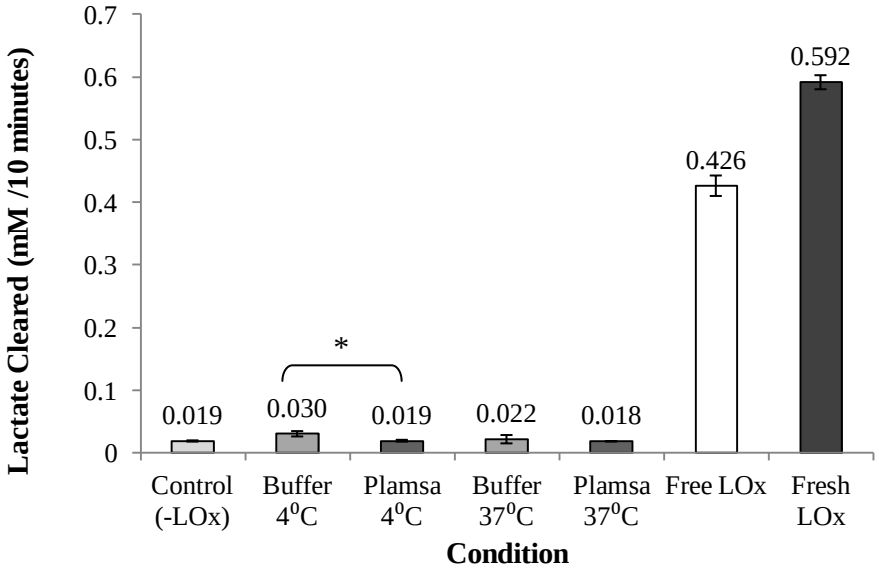


Figure 3-18 – Activity of immobilised LOx beads following incubation in a range of media.

Samples from each condition were applied to the LOx activity assay after 24 hours incubation with lactate spiked buffer / FFP at 4°C / 37°C. Free LOx = enzyme resuspeded from the same aliquot as used for the immobilisation and stored at 4°C until the final asctivity assay. Fresh LOx = enzyme resuspended immediately prior to assay. Extent of lactate clearance was significantly different between the indicated conditions (*$p<0.05$). n=6, +/- SD.

The residual activity of immobilised LOx in all conditions was very much reduced after 24 hours, but less so for LOx which had been in buffer, particularly at 4°C. One interpretation of this could be that the stability of LOx is affected by plasma components; thus, by increasing temperature and consequently the thermal (and hence kinetic) energy of molecules, although an increased rate of lactate clearance was possible, the rate of degradation of LOx by plasma proteases was also increased. Consequently, less LOx would be degraded in plasma at 4^0C than at 37^0C. Although true after five hours incubation (Figure 3-16), there was no significant difference in residual LOx activity between plasma conditions after 24 hours (Figure 3-18), suggesting that by this point, there had been adequate time for substantial proteolysis even at the lower temperature. There was no significant difference at the $p<0.01$ level between residual LOx activity in the buffer conditions. Ultimately, however, it is sufficient to know with some certainty that immobilised LOx can and will reduce lactate levels in physiologically relevant conditions, i.e. at 37°C and in the presence of plasma components, thus permitting this method of immobilisation to be utilised within the BAL.

In the BAL FBB, the LOx beads will be required to function in 10% FFP medium rather than the 100% FFP used in previously described experiments, which have served as an extreme test of whether plasma constituents ameliorate LOx activity; that the immobilised LOx beads have reduced lactate concentrations in the presence of so much plasma provides an indication that they will continue to function in BAL relevant conditions. However, as it was observed that immobilised LOx beads were more active in 100% FFP than in buffer, it could follow that their activity may not be as high in media containing just 10% FFP. Therefore, the activity of LOx beads in either 100% FFP or 10% FFP culture medium was investigated. Furthermore, as lactate production in the FBB will be a continuous, not an initial event, the media received a second lactate spike in a preliminary step to address this issue.

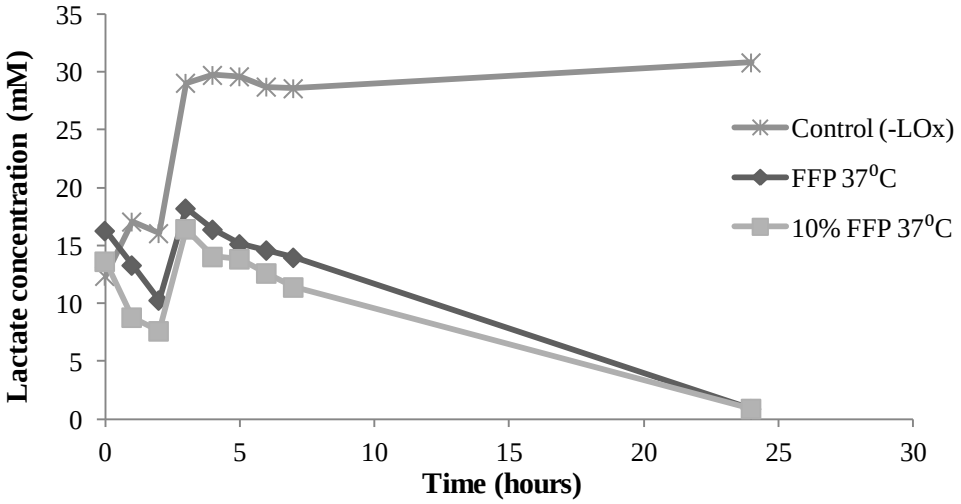


Figure 3-19 – Lactate clearance by immobilised LOx in 10% FFP.

Immobilised LOx beads incubated for 24 hours in either 10% or 100% FFP, spiked at T=0 and T=2 hours with 15 mM lactate. Samples were taken hourly for the first 7 hours, and then a final sample after 24 hours. Control = Glass beads without LOx, incubated at 37°C in 10% FFP culture medium. Lactate concentration determined by ANALOX analysis. n=3.

Following incubation at 37°C for 24 hours in lactate spiked conditions, LOx beads were observed to clear lactate at comparable rates in 100% and 10% FFP (Figure 3-19). It can be concluded therefore, that this immobilisation strategy is, in principle, compatible for use within both the FBB and the BAL device. It is to be remembered, however, that the encapsulated HepG2 cells are cultured for a total of 11 days; if immobilised LOx is to be successfully utilised in this context it must be determined whether it can continue to function for a period of time in excess of the 24 hours here tested. If plasma components, such as proteases, are responsible for the drop in activity of immobilised LOx, a method of protecting the enzyme from degradation should be explored. For this reason, an alternative immobilisation support was investigated.

3.3.2.3. Effect of the glass support on LOx activity

The activity of an immobilised enzyme relies upon a plethora of factors, significantly the physical and chemical profile of the matrix utilised as a support for immobilisation. By carefully selecting the support, the success

of the immobilisation, in terms of enzyme loading efficiency, activity of the immobilised enzyme and enzyme stability, can often be greatly increased. Previous experiments addressed immobilisation of LOx onto glass beads with a mean diameter of 750 µm. It was hypothesised that by maintaining the same quantity of enzyme and mass of beads as previously, but using a larger, porous bead, a greater total surface area would be available for enzyme attachment. Furthermore, immobilising LOx within pores could potentially provide some protection from proteolysis, by physically shielding the enzyme or even excluding proteases from the pore. Moreover, another advantage of using a porous support could be seen if previous rates of lactate clearance were limited by the availability of binding sites (an explanation for why so much LOx remained in the post immobilisation supernatant). Increasing the surface area of the support by the inclusion of pores could allow a greater proportion of LOx to bind and thus result in a greater binding efficiency between enzyme and the support. Additionally, a larger bead would be easier to both manipulate during preparation and also recover from the FBB environment.

Two bead sizes were compared; the 750 µm diameter glass beads and a larger, porous glass bead with an average diameter of 4 mm. Both beads were prepared as previously described in section 3.2.3. The LOx incubation volume was increased to 200 µl to ensure that all beads were fully submerged. After incubation for 16 hours, the supernatants were aspirated and retained for analysis of LOx activity. Beads were rinsed as previously described, before being applied to 2 ml 10% FFP culture medium, spiked to give a concentration of 15 mM lactate, and incubated at 37°C for 24 hours. Samples were taken at 0, 2 and 24 hours, snap frozen and stored at -80°C until analysed by ANALOX.

Analysis of the post immobilisation supernatants revealed that LOx enzyme remained to some extent in the supernatants of all immobilisation conditions (Figure 3-20). There was, however, far less lactate cleared by the post-immobilisation supernatant used on the 4 mm glass bead, indicating that little LOx remained in this solution and hence more LOx had been bound to the beads. ANALOX readings of the samples taken throughout the 24 hour

time course (Figure 3-21) revealed that 4 mm glass beads had lactate clearing capabilities only fractionally greater than the negative control condition. Only LOx immobilised onto 750 µm glass beads brought about a reduction in the lactate concentration.

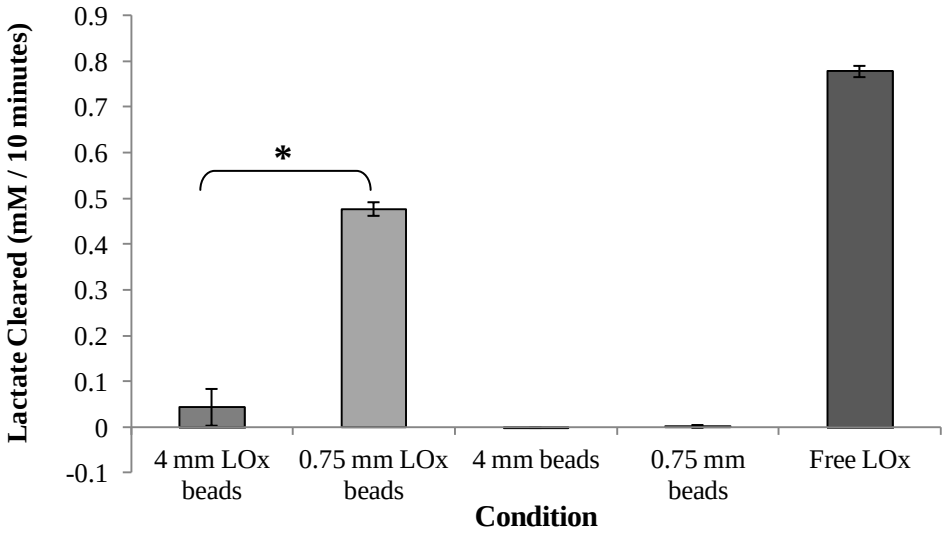


Figure 3-20 – Activity of post-immobilisation supernatant.

Graph depicts activity of the post-immobilisation supernatant (the unbound LOx remaining, in the immobilisation solution) for each LOx immobilisation treatment. "Free LOx" is un-tethered LOx in the immobilisation buffer. Results show mean * = significant difference by T-Test (p=0.0016) +/- SD, n=3.

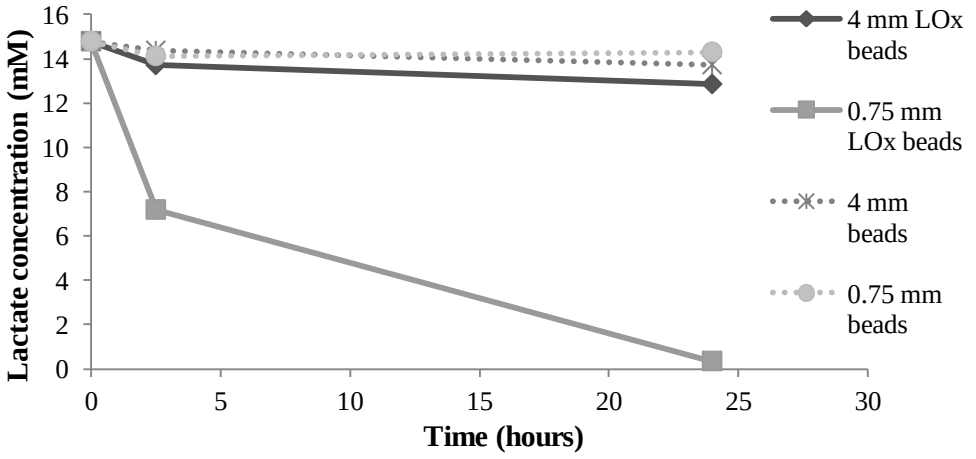


Figure 3-21 – Lactate Clearance by LOx immobilised onto glass beads.

Readings taken at initially, at two and 24 hours after incubation of beads-only or immobilised LOx beads with lactate spiked 10% FFP culture medium at 37℃. Lactate concentrations determined by ANALOX analysis. n=3.

Poor recovery of enzyme in the post immobilisation supernatant would imply that the majority of the enzyme had bound to the support surface. However, this was not quite the phenomenon observed when LOx was conjugated to the larger glass beads; although less enzyme remained in the post-immobilisation supernatant, the LOx beads showed negligible activity. One interpretation of this could be that the enzyme was inaccessible to lactate when tethered within the pores, explaining why little LOx was in the post immobilisation supernatant and yet subsequent activity was so low. It is conceivable also that the enzymatic activity of LOx immobilised onto 4 mm beads was compromised by the presence of cross linking molecules, essentially trapping LOx within the bead pores, or, by binding so much enzyme that each molecule was too densely packed to undergo the conformational changes necessary to participate in the formation of a successful enzyme-substrate complex.

In light of these results, subsequent LOx immobilisations were performed using the original smaller glass bead as a support.

3.3.2.4. LOx activity in FBB relevant models

In order to establish the suitability of immobilised LOx beads for use within the FBB it is critical to answer the two previously posed questions; namely, can immobilised LOx beads continue to function in conditions reminiscent of the FBB (presence of plasma, at 37°C and for a period of up to 11 days), and whether the catalytic activity of the immobilised enzyme decreases over time. Spiking the reaction media at multiple time points with lactate and then assessing the activity of immobilised LOx would serve to investigate the latter point, whilst the first point will also be studied if physiologically relevant conditions are implemented.

Previous experiments demonstrated that immobilised LOx can reduce lactate levels from 10% FFP culture medium spiked initially and after two hours with lactate. As ELS will be producing lactate throughout their culture, rather than just at one time point, it was necessary to establish whether the immobilised LOx beads are capable of removing lactate from

an environment where the levels of lactate will be constantly replenished. For the 24 hour experiment, 20 µl of the 2 ml 10% FFP solution was replaced with 1 M lactate solution and again after two, four and six hours (the negative control received an extra spike after two hours only, in order to remain within the LoD of the lactate detection method (Analox)). Samples (20 µl) were taken hourly for seven hours and again after 24 hours.

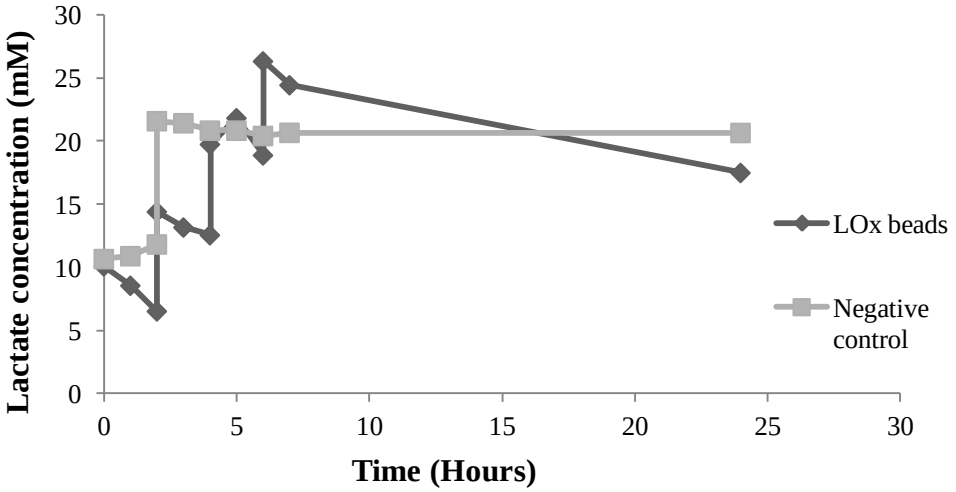


Figure 3-22 – Lactate clearance by immobilised LOx in 10% FFP culture medium at 37ºC.

Media was spiked with 10mM lactate solution; at 0 and 2 hours for the negative control and at 0, 2, 4 and 6 hours for the condition with immobilised LOx.

A reduction in lactate concentration was observed in the presence of immobilised LOx beads, as shown in Figure 3-22. Over a period of 24 hours the sample containing LOx beads had a lactate concentration of just over 17 mM despite having received a total of four 10 mM lactate solution doses. In contrast, the negative control sample, spiked twice with 10 mM lactate but not treated with LOx, showed no reduction in lactate concentration. The rationale behind the difference in lactate spiking regimes for these two conditions was that in the absence of LOx activity the lactate concentration would otherwise rise to levels exceeding the limit of detection for the analysis method (ANALOX) employed.

To further investigate the activity of immobilised LOx in the presence of 10% FFP, the rate of lactate clearance inbetween each hourly sample for the

24 hour experimental period was examined (Figure 3-23). This revealed that lactate clearance (mM/hour) dropped sharply in the first five hours of incubation from 1.8 to 0.4 mM/hour. The average rate after five hours appeared to stabilise at approximately 0.4 mM/hour. The data could support a hypothesis that the reduced rate of lactate clearance could be a consequence of the effects of substrate depletion. A relatively rapid initial rate of lactate clearance would have removed a substantial proportion of lactate from the reaction volume, thus decreasing the lactate concentration and therefore reducing the probability that an immobilised enzyme (which by its very nature will itself have reduced mobility) will encounter a substrate molecule. However, if the process of immobilisation does have a negative effect on the probability of an interaction between substrate and the active site, providing that this does not have an effect upon the total proportion of lactate removed in the conditions found in the FBB, then a compromised rate of reaction can be tolerated. Additionally, the data could suggest that the immobilised LOx had become less effective, for example as a consequence of inactivation accredited to prolonged exposure to a higher temperature.

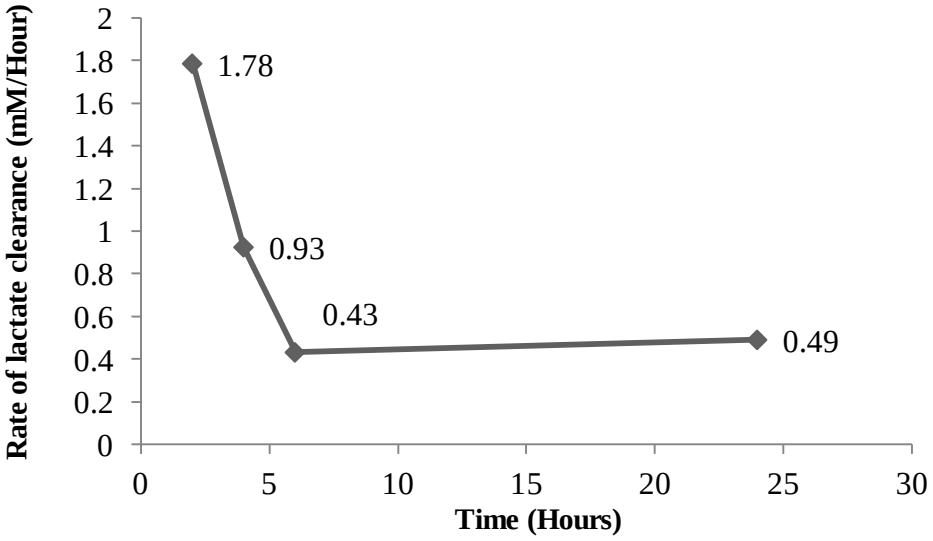


Figure 3-23 – Rate of lactate removal by immobilised LOx.

Study conducted in 10% FFP culture media spiked four times to 10 mM (final reaction volume concentration) lactate, incubated at 37°C with rotational mixing.

3.3.2.5. Activity of immobilised LOx; time-course study

To investigate the functional lifespan of immobilised LOx, a similar experiment was prepared in 10% FFP media buffered with HEPES to pH7.4 and at 37°C, but conducted for a total of 11 days. The lactate spikes administered were calculated to represent the concentrations of lactate produced by encapsulated HepG2 cells within the FBB on a typical day-to-day basis (see section 3.2.31). Samples of 20 µl were taken every 24 hours when possible.

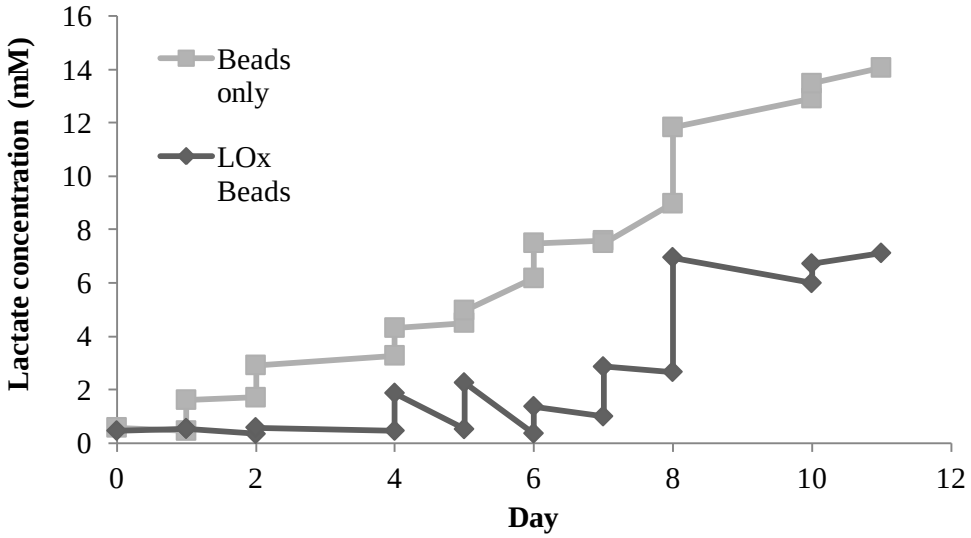


Figure 3-24 – Lactate clearance by immobilised LOx over a period of 11 days.

Lactate spikes were administered to 10% FFP culture medium, maintained at 37ºC for eleven days to mimic daily lactate production in a typical FBB experiment. Mean data values plotted, n=3.

As shown in Figure 3-24, whereas overall lactate concentrations in both the negative control and LOx bead samples rose throughout the experimental time course, samples taken from media with LOx beads were consistently lower in lactate concentration, with a 50% decrease in lactate concentration compared to the negative control by day 11.

3.3.3. PVDF membrane immobilisation

Samples were prepared as described in section 3.2.4 and applied to a lactate containing phosphate buffer solution. Regardless of treatment time, the activity levels of LOx supposedly bound to PVDF membrane were miniscule, to the extent that the values fell below the reliable scope of the instrument used. Therefore, this method was no longer investigated as a potential means of immobilising LOx for use within the FBB.

3.3.4. Dialysis tubing for enzyme containment

Preparation of apparatus and LOx was conducted as described in section 3.2.5. LOx was re-suspended to 0.1 mg/ml in 10 mM phosphate buffer and placed within a cassette-like chamber, such that the enzyme was physically contained by a selectively permeable membrane, through which the lactate solution could pass. The cassettes were directly applied to the LOx activity assay in wells of a 96-well plate. The apparent lower activity rate of LOx contained within the cassette (Figure 3-25) is likely the result of altered rates of reaction owing to substrate/product diffusion rate limitations. Allowing the reaction to continue for a greater time period could potentially see a greater extent of lactate clearance by this immobilisation method; however, the FBB requires a mode of lactate removal that is both effective and sufficiently rapid to remove lactate from the system as it is produced, before the growth and proliferation rates of cultured HepG2 cells can be compromised. Any strategy, including the one described here, which does not meet such criteria would simply be impractical. Additionally, the scaling up of this approach would be particularly challenging, as incorporating a sufficiently large section of dialysis tubing into the FBB to remove lactate would restrict the space available in the FBB for cell culture.

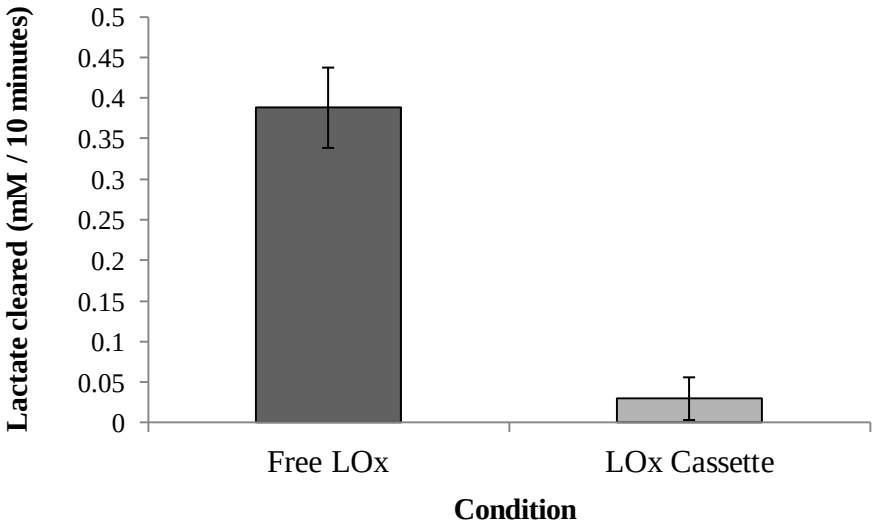


Figure 3-25 – Lactate clearance by LOx contained within dialysis membrane.

"Free" LOx = LOx enzyme re-suspended but not contained within a dialysis membrane cassette. A cassette, or free LOx, was applied to the LOx activity assay. n=6, +/- SD.

3.4. Summary

3.4.1. Immobead experiments

It is generally accepted that immobilisation tends to confer stability to an enzyme, and so it was surprising that over the 24 hour experimental period the immobilised LOx lost activity but the unbound enzyme did not. An explanation for this could be that unquenched glutaraldehyde residues were continuing to react with lysine groups on the enzyme, leading to over-binding and thus compromising the ability of LOx to interact with lactate. However, an assay was conducted with the addition of $NaBH_3CN$ to quench un-reacted glutaraldehyde chains, which lead to a decrease rather than an increase in immobilised LOx activity, indicating that another factor is responsible for this phenomenon. An alternative theory could simply be that physically tethering LOx to the Immobead surface modifies in some way the tertiary structure of the enzyme leading to a decrease in stability. A third possibility is that the drop in activity was an artefact of low level activity, itself the result of an inaccurate method of bead administration. However, there remains the issue that Immobeads retain the dye which is the indicator

of activity in the assay – therefore it is highly likely that the activity levels have been underestimated, although by what extent remains to be deduced. Nevertheless, having regarded the lactate clearing ability of LOx immobilised onto glass beads (Figure 3-7), the Immobead method was no longer perused; not only are the glass beads more effective at lactate removal, but also their larger size makes them easier to manipulate and contain within a designated area of the FBB, and therefore less likely to cause a blockage in the FBB circuit.

3.4.2. NH_2 functionalised glass beads immobilisation

Following two cycles of freeze/thaw, activity of LOx immobilised onto functionalised glass beads had dropped by an average of 65%, and almost 90% for the no-cross linker condition. This suggests that although not absolutely necessary for LOx immobilisation, a cross linker is required to confer greater storage stability to the immobilised enzyme

It was hypothesised that perhaps part of the functionalisation process is making the glass beads susceptible to protein binding, thus accounting for levels of LOx activity in the absence of a cross linker molecule. To address this point, LOx was added to conditions representing a permutation of the functionalisation steps. The data indicated that it is the addition of 3-aminopropyltriethoxysilane, the "NH_2 functionalisation" step, which leads to non-covalent binding. Although this could be further investigated to identify a protocol to minimise these effects, a small proportion of LOx binding directly to the support is not of major concern, providing that LOx and only LOx is binding in a stable fashion, that the majority of LOx is bound correctly and is active, and that the process doesn't lead to a loss of newly synthesised proteins from the BAL when it is used in the patient treatment phase. This is highly unlikely since the concentration of plasma protein is so high, but this will be investigated in a following chapter (Chapter 6).

Following an increase in the amount of LOx used for immobilisation, the activity seen for 0.01 mg LOx was comparable to previous experiments, but

a ten-fold increase in LOx concentration saw roughly a 50% loss in subsequent glass bead activity. The reason for this is unclear; it could be that all potential binding sites on the beads are taken, resulting in overcrowding and thus causing a degree of steric hindrance and so preventing optimal enzyme function.

On the theme of enzyme loading and whether there is a maximum amount of protein which can be cross-linked to the glass bead, the data suggests that the LOx solution can be reused from a previous immobilisation to yield beads with an activity level not dissimilar to those produced by the previous immobilisation. This again suggests that not all the LOx present in the solution is binding to the beads, despite a decrease in total protein used leading to a lower final activity level. This could perhaps imply that it is the bead itself rather than LOx which is the limiting factor in the amount of active LOx which can be immobilised. To test this theory it would be beneficial to further investigate the effects the flame treatment in particular is having on bead structure. Visualisation of the bead surface before and after treatment would be one possible approach, possibly using environmental scanning electron microscopy technology. Importantly, however, the immobilisation method was shown to be reproducible, with separate batches of immobilised LOx having very similar activity levels.

The data from the lactate clearance experiments demonstrate that 100 mg of immobilised LOx beads are sufficient to lower lactate concentration within a 2 ml volume of 10% FFP media by 50% over a period of 11 days when incubated at 37°C under constant rotation of the beads, as would be the case in the FBB. Although encouraging, there is the issue that by day 8 the lactate clearing activity of immobilised LOx was becoming compromised. Identifying the cause for this would be beneficial for optimising the treatment protocol; for example, if the decreased clearance rates are due to enzyme denaturation then immobilisation methods which may confer a greater degree of thermal protection could be investigated. Nevertheless, the data suggests that this is a promising method for addressing the issue of lactate clearance in the FBB. The scaled-down model of lactate production

in 10% FFP culture media indicates that with the addition of LOx immobilised onto glass beads, lactate concentrations can be reduced to around 6-8 mM, levels which are below the 15 mM danger level for encapsulated HepG2 integrity, growth and function. Moreover, eight days of activity may well be sufficient since there is relatively little lactate present during the first few days of HepG2 cell culture; it is as cell number rises with increased culture time that lactate accumulation becomes a greater concern.

3.4.3. PVDF membrane immobilisation

The results obtained using PVDF membrane as a scaffold for LOx immobilisation were disappointing, rendering further investigation into this technique as somewhat superfluous. Further analytical work would be required to establish why this method was so ineffective; initial explanations include (1) the possibility that LOx was binding primarily to the plastic container used as an incubation vessel rather than to the PVDF membrane, and (2) the maximum amount of protein which could be bound to the section of membrane had already done so, but that this was too low an amount to be detectable or indeed of use.

3.4.4. Dialysis tubing for enzyme containment

Despite low conversation of lactate, the use of a cassette incorporating a section of dialysis tubing could still be a potential approach for use within the FBB. Although higher activity levels for immobilised LOx were obtained using other methods, it still remains to be seen whether this strategy could remove a sufficient quantity of lactate given more time for diffusion. If that is the case, there could be a number of potential advantages to using this method over other support-based methods of harnessing LOx activity. For example, as there is no immobilisation required, there is no risk of altering the tertiary structure of the enzyme which may lead to compromised catalytic activity. Also on this theme, without the burden of a physical link between enzyme and the support matrix, there is less scope for

steric hindrance effects in terms of substrate access to the enzyme's active site. However, this method would also forfeit the advantages often associated with immobilisation of an enzyme, the thermostability being of particular concern in the context of the FBB.

3.5. Conclusion

During the culture of encapsulated HepG2 cells for use within a BAL device lactate can accumulate to concentrations which, if left unchecked, are detrimental to the growth, function and proliferation of the ELS. It has been demonstrated that LOx retains catalytic function, converting lactate to pyruvate when immobilised onto the surface of modified glass beads, as observed in the depletion of lactate spiked 10% FFP culture media. Thus the hypothesis has been proven that LOx can be immobilised onto a solid support and used to significantly reduce lactate concentrations to levels which are no longer detrimental to ELS cell growth, function or structural integrity of the alginate bead. The further characterisation of the immobilised LOx will be discussed in Chapter 5, where the kinetics of both the free and immobilised enzyme will be investigated.

Having demonstrated that LOx can be immobilised to reduce lactate concentrations, the next research aim is to apply an appropriate immobilisation technique to harness enzymatic activity to degrade circulating DNA from the Bio-Artificial liver assist device. In the following chapter, the degradation of DNA using enzyme immobilisation technology will be addressed.

Chapter 4. DNA Removal

Methods of immobilising Deoxyribonuclease I are explored, including the effect of cross linker and support on the extent of enzymatic degradation of HepG2 DNA.

4. DNA Removal

4.1. Introduction

The Liver Group BAL is based on the premise that alginate encapsulated HepG2 cells will take on a surrogate role of performing the metabolic, detoxifying and synthetic functions of the liver, until the patient's own liver has recovered or until transplantation becomes possible. However, there is a potential risk of tumorgenesis and initiation of an immune response associated with the use of HepG2 cells, should HepG2 DNA be released from the cells and enter the patient's circulation. This chapter concerns the elimination of this potential risk to patient safety by utilising enzyme technology to degrade circulating DNA fragments.

4.1.1. DNA removal in the Liver Group BAL

There are currently intended to be two approaches utilised for the removal of extraneous DNA; physical removal of DNA by filtration, and removal by enzymatic degradation. Together, these methods will allow compliance with FDA guidelines for DNA levels arising from extracorporeal support devices. These currently stand at 10-100 ng of DNA of host cell origin per parenteraly administered dose for vaccine based therapies. The worst case scenario in the BAL, whereby cell death and DNA release occurs in all of the $7x10^{10}$ cells (which represent 30% of the adult male liver mass and thus the critical cell number required for treatment), would result in 66 µg/ml HepG2 DNA in the 7L BAL volume. (Calculations based on there being 6.64×10^{-12} g DNA in a diploid cell (134)). The amount of DNA released from encapsulated HepG2 cell spheroids in a typical scaled-down model of the BAL has been shown to be 68 ng/ml in 100% FFP over the course of eight hours (group data). Physical filtration has been shown to remove DNA to acceptable levels; therefore, the inclusion of immobilised DNase I will serve as a "fail-safe" mechanism for the BAL device. This thesis is concerned with the enzymatic branch of DNA removal and so no further reference will be made to the filtration approach.

4.1.2. Deoxyribonuclease I

The mammalian phosphodiesterase Deoxyribonuclease I (DNase I), found mostly in the pancreas, is a glycoprotein with a molecular weight ranging from 31-35 kDa, depending on the tissue/species of origin and also the extent of glycosylation (135). It contains two disulfide bridges (136) one of which, between C173-C209 is essential for maintaining the structure and hence activity of DNase I (137). Adjacent to this is one of two calcium binding sites, which, when occupied, have been demonstrated to increase the stability of DNase I (a higher temperature is then required to denature the enzyme) and also protect against denaturation by entities such as urea (137;138). Within the active site of DNase I there are two further divalent ion binding sites to which Mg^{2+} binds in the presence of DNA (139).

The enzyme hydrolyses and degrades DNA by endonucleolytically cleaving double or single stranded DNA depending on the divalent metal ion used (140;141). This occurs via a nucleophilic attack mechanism (142) preferentially at phosphodiester linkages adjacent to pyramidine nucleotides to generate 3'OH and 5'P oligonucleotides (Figure 4-1) comprising at least two nucleotides (142;143).

$$\mathrm{DNA} + \mathrm{H_2O} \xrightarrow{\mathrm{DNaseI}} 5' - \mathrm{Oligodeoxyribonucleotides}$$

Figure 4-1 – Equation depicting the role of DNase I in DNA degradation.

It has been reported that the carboxylate groups H134-E78 and H252-D212 are crucial for binding to the minor groove of DNA molecules (144;145). Interestingly, although the DNA molecule undergoes a conformational change following DNase I binding, the enzyme maintains an almost unchanged configuration (145). The pH optimum for DNase I activity is between 7 and 8.2 (135;146).

DNase I also has a very high affinity for G-actin (147), and when so bound the nuclease activity of DNase I is inhibited (148;149). Glu13 of DNase I is involved in the binding of both DNA and actin which is possible due to the binding site of actin being very close to the active site (150). It is therefore postulated that actin inhibits DNase I activity by a mode of steric hindrance, physically blocking the accessibility of the active site to the DNA substrate. A further role for divalent cations has been proposed, as there is data to suggest that Mg^{2+} concentration can influence the equilibrium point of the ratio of F-actin and the inhibitory G-actin; with decreasing Mg^{2+} more G-actin is present and a greater extent of DNase I inhibition is observed (142). However, divalent cations can also be detrimental to DNase I activity, as the enzyme is inhibited by Zn^{2+} (146).

There have been particular difficulties when engineering a support for the immobilisation of DNase I owing to the large size of DNA molecules (151); the support must permit efficient mass transfer of the substrate whilst also ensuring sufficient contact between the enzyme and the support structure.

4.1.3. Existing methods of DNase I immobilisation

4.1.3.1. Nylon microspheres

Early studies into the use of DNase I for the removal of circulating DNA in systemic lupus erythematosus (SLE) patients (151) utilised activated nylon microspheres with an approximate diameter of 1.1 mm as a support for DNase I immobilisation. The beads were chosen owing to their low patient toxicity, the large surface area available for binding enzyme, structural stability and importantly, because they exhibited minimal thrombogenicity, thus lowering the likelihood of reducing circulating platelet concentrations. The microspheres were gently hydrolysed with 3 M HCl at 30°C for 60 minutes at a flow rate of 30 ml/min before undergoing treatment with 4% glutaraldehyde for 15 minutes to activate the surface for enzyme attachment (enzyme retention on microspheres was calculated to be between

26.8-31.5%). DNase I was immobilised by immersing the beads in a buffer solution containing $MgCl_2$ and 15 mg enzyme for 60 minutes. Following introduction to an extracorporeal circulation system in dogs, immobilised DNase I was shown to accelerate the degradation of circulating DNA *in vitro* and *in vivo* whilst no detectable release of enzyme into the host was observed. The microsphere immobilised DNase I was housed in a chamber where the flow rate of plasma was 200 ml/min. Although this method demonstrates great potential for the use within the BAL, there is the caveat that the nylon microspheres would be very difficult to make or produce in sterile conditions which is a critical practicality for the BAL application.

4.1.3.2. Magnetic hydrophilic polymer particles

Rittich *et al.* (152) successfully immobilised DNase I onto magnetic poly (HEMA-co-EDMA) microspheres, which had a diameter of 1.2 µm and were synthesised in the presence of magnetite powder (ferrous-ferric oxide Fe_3O_4). The advantages of these microspheres included bio-compatibility, low non-specific protein adsorption and furthermore, the magnetic property allowed for easy removal/isolation of the immobilised enzyme from the reaction solution. The immobilisation process was carried out for four hours at room temperature before lyophilising the microspheres, to yield an average of 13.9 mg enzyme per gram of carrier. Interestingly, activity analysis of immobilised enzyme microspheres showed that the degree of activity is dependent upon the identity of the divalent cation used to activate DNase I; these were, as a percentage of original, native enzyme activity 3.4% (Ca^{2+}), 20.5% (Mg^{2+}), 72.2% (Mn^{2+}) and 91.2% (Co^{2+}), when used at concentrations of 10 mM. However, it would not be possible to provide these ions at the required concentration *in vivo* should this immobilisation protocol be utilised within the BAL.

4.1.3.3. Epoxy groups of methacrylate monoliths

Epoxy groups inherently present on macroporous poly (glycidyl methacrylate-co-ethylene dimethacrylate) (GMA-EDMA) monolith columns were used by Bencina *et al*. (140) to immobilise DNase I for use in a bioreactor. These groups form covalent bonds with either amines, sulphydryl groups or hydroxyl groups on the amino acids of DNase I. This immobilisation support system benefits from a large porosity and high mechanical and chemical stability, making it well suited to supporting reactions where high molecular mass analytes (i.e. DNA) are present. The system was observed to function well at flow rates of both 0.6 ml/min and 9 ml/min. However, it is to be noted that flow rates within the BAL system are typically in great excess of these tested values, up to 600 ml/min. DNase I was immobilised by either a static method where the column was immersed in enzyme solution, or by a dynamic method where the enzyme solution was drawn through the column at 15 minute increments for a total duration of two hours. It was concluded that greater enzyme activity could be gained by increasing the duration of the immobilisation procedure and, furthermore, that the speed of immobilisation can be increased by using a dynamic rather than a static method. In terms of physical conditions, the results suggested that pH7 and 22°C were optimal, likely due to a better stability of DNase I.

Despite there having been degrees of success with the above mentioned methods of DNase I immobilisation, the conditions under which the immobilisations and enzyme performance assays were conducted are not suitable for use within the BAL. For this reason, alternative methods had to be developed.

4.2. Materials & Methods

4.2.1. Preparation of Dynabeads®

The high affinity DNA has for binding to glass prompted the use of an alternative immobilisation support matrix. Hydrophilic Dynabeads® M-270 Amine beads (average diameter of 2.8 µm) were chosen for their magnetic property, which would facilitate the removal of the beads from the BAL as well as allowing greater control over the localisation and containment of the beads within the operational BAL. Furthermore, these beads have a low risk of initiating an immunological response, and they could be manufactured in sterile conditions, further minimising the potential risk to the patient. The exposed amine binding site on the beads allowed for simple covalent cross-linking of DNase I onto the beads.

Materials:
Hydrophilic Dynabeads® M-270 Amine beads (Invitrogen, 14307D)
DNase I (0.2 mg/ml aliquot, as described in section 2.13)
0.2 ml polypropylene microcentrifuge tube
Magnetic microcentrifuge rack (Dynabeads®)
Super-fine flat ended gel loading pipette tips (StaLabs, I 1022-2600)

HEPES Buffer (pH7.4):
10 mM HEPES (pH 7-7.6, Sigma, HO887)
1 mM $CaCl_2$
1 mM $MgCl_2$

The basic preparation method was modified slightly from the manufacturers' published protocol in order to minimise the total quantity of enzyme used by scaling down the process, and was as follows: Stock suspension of Dynabeads® was vortexed thoroughly for two minutes to ensure as near as homogenous suspension as possible, before transferring 7.7 µl of the bead suspension to a 200 µl microcentrifuge tube. Each tube was placed within a magnetic rack and left for four minutes to allow the beads to precipitate out of the solution. The liquid phase was carefully aspirated using super-fine flat ended gel loading pipettes and the tubes then removed from the rack and washed by resuspending in 7.7 µl 10 mM HEPES buffer. The tubes were once again applied to the magnet for four

minutes to precipitate the beads; this process was repeated twice. The final wash solution was aspirated and replaced with 10 µl of 0.2 mg/ml DNase I solution (total 2 µg DNase I protein) and the beads incubated at room temperature for 24 hours.

4.2.2. Immobilisation of DNase I onto glass beads

Materials:
100 mg glass beads (750-1000 µm diameter, (Kisker-Biotech, PGB-075)
Methanol
Isopropyl alcohol
Sonicator
1,3-benzenedisulfonyl chloride (97%, Aldrich, 444642)
Toluene, anhydrous (99.8%, Sigma-Aldrich 244511)
N,N-dimethylacetamide (DMAd) (≥99.5%, Sigma-Aldrich, 38840)
10 mM phosphate buffer
Ethanol
3- aminopropyltriethoxysilane (≥98%, Sigma A3648)
Ceramic mortar
Plate shaker
Miniature blow torch

HEPES Buffer (pH7.4):
10 mM HEPES (pH 7-7.6, Sigma, HO887)
1 mM $CaCl_2$
1 mM $MgCl_2$
0.2 mg/ml DNase I

The method for immobilising DNase I onto glass beads was modified from the methodology described in section 3.2.3 to covalently bind LOx onto glass beads; all steps were as described for LOx immobilisation up to the addition of a cross-linker molecule. For the cross linking stages in this method, the beads were treated with 100 mg benzene 1,3 disulfonyl chloride dissolved in 10 ml DMAd at room temperature for 15 minutes. They were washed twice with 10 ml ethanol and then rinsed in 10 ml 10 mM HEPES (pH7.4) or 50 mM TrisHCl (pH7.4) before applying 100 µl of 0.02 mg/ml DNase I (made with 10 mM HEPES or 50 mM TrisHCl and a 10 µl aliquot of 0.2 mg/ml DNase I) and incubated at room temperature for 24 hours. This was based on published literature which demonstrated a longer incubation time at ambient temperature is optimal for DNase immobilisation (140). The

cross-linked enzyme beads were rinsed three times in 500 µl 10 mM HEPES pH7.4 or 50 mM TrisHCl (pH7.4) buffer before use in subsequent applications.

4.2.3. Western blot analysis of samples

Materials:
Blocking buffer:
5% non –fat dehydrated milk powder
1 x PBS (without Ca^{2+} or Mg^{2+})
10% Tween 20

5 x sample buffer (20 ml):
4 ml 1.5 M TrisHCl (pH6.8)
10 ml glycerol
5 ml β-mercaptoethanol
2 g sodium dodecyl sulphate (SDS)
1 ml 1% bromophenol blue
iBlot® Gel Transfer Device (Invitrogen)
Nitrocellulose iBlot® transfer stack (Invitrogen)

1/20 000 Anti-rabbit HRP
1/7000 Anti-DNase I (rabbit monoclonal antibody)
Criterion® pre-cast gradient (4-12%) gel (BioRad)
Full-range Rainbow molecular weight marker (GE Healthcare Life Sciences, RPN800E)
ECL detection kit (Milipore)

For the protein gel, 5 µl 5 x sample buffer was added to 20 µl of the sample and heated at 90°C for 10 minutes to denature protein (i.e. DNase I) which would otherwise prevent the process of protein separation by electrophoresis. Once cooled, 20 µl of this was applied to each well of a gradient gel, loading Full Range Rainbow Marker as a reference for molecular weight. Running buffer was added to the reservoir and the gel run at 250 volts for 45 minutes. The gel was removed and applied to the iBlot® transfer device as per the manufacturer's guidelines and transferred for four minutes. The membrane was blocked overnight at 4°C in blocking buffer to minimise non-specific antibody binding. Anti-DNase I antibody, diluted 1/7000 in blocking buffer, was applied to the membrane and incubated with gentle agitation at room temperature for one hour. The membrane was

washed four times for five minutes in 1 x PBS (with 0.1% Tween 20) before applying anti-rabbit antibody diluted 1/20 000 in blocking buffer. This was incubated with gentle agitation as before at room temperature for one hour. The membrane was rinsed four times in 1 x PBS (with 0.1% Tween 20) before adding equal volumes of the chemiluminescent reagents as per the manufacturer's guidelines for five minutes. Blots were developed using x-ray film for an appropriate exposure time.

4.2.4. Removal of DNA: a time-course study

Materials:
100 mg DNase I beads
HepG2 DNA solution (0.85 mg/ml)
Rotating sample mixer
Liquid nitrogen
HEPES Buffer (pH7.4):
10 mM HEPES (pH7-7.6, Sigma, HO887)
1 mM $CaCl_2$
1 mM $MgCl_2$

An initial spike of 20 µl 40 ng/ml HepG2 DNA in 1 ml 10 mM HEPES buffer solution was added to 100 mg DNase I conjugated glass beads and the tubes incubated at 37°C on a rotational mixer for a total period of 24 hours. Samples of 20 µl were taken initially and hourly for the first four hours and then after 24 hours. Additional samples were taken immediately after a second spike administration (dosage as before) two hours into the experiment. Samples were snap frozen in liquid nitrogen prior to analysis.

4.2.5. NHS PEG cross-linking of DNase I

Materials:
100 mg O,O'-Bis[2-(N-Succinimidyl-succinylamino)ethyl] polyethylene glycol ($M_r \approx 2\,000$, Aldrich, 713783-500MG)
DMSO (anhydrous ≥99.9%, Sigma-Aldrich, 76855)
PBS

100 mg functionalised glass beads (section 3.2.3)

HEPES buffer (pH7.4):
10 mM HEPES (pH7-7.6, Sigma, HO887)
1 mM $CaCl_2$
1 mM $MgCl_2$

100 mg functionalised glass beads were prepared as described in section 3.2.3 up to and including the air drying of the beads following treatment with 3- aminopropyltriethoxysilane in dry toluene. After this, each batch of beads was treated with 1 ml of a solution comprising 100 mg O,O'-Bis[2-(N-Succinimidyl-succinylamino)ethyl] polyethylene glycol (NHS PEG) dissolved in 6 ml DMSO, and incubated for 15 minutes. The supernatant was aspirated and the beads rinsed twice in 1 ml DMSO, twice in 2 ml PBS and finally with 2 ml of 10 mM HEPES buffer (pH 7.4). DNase I was cross-linked and all further steps performed as described in section 4.2.2 (100 µl of 0.02 mg/ml DNase I per 100 mg beads (made with 10 µl of 0.2 mg/ml DNase I in 90 µl 10 mM HEPES buffer).

4.3. Results

4.3.1. Immobilised DNase I activity

In order to establish the extent of DNase I activity the enzyme was incubated with plasmid DNA, which should be a simple substrate, being a smaller, circular molecule rather than the large complex molecule that is eukaryotic genomic DNA. However, the BAL application requires that genomic DNA be removed, which could likely be much longer in length than a simple bacterial plasmid. Calf thymus DNA was used as a preliminary measure to test the ability of DNase I to break down larger and more complex DNA molecules.

Glass beads were prepared and DNase I immobilised as described in section 4.2.2. Free enzyme activity on genomic DNA was compared with that of immobilised DNase I in a 50 µl TrisHCl (with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ pH7.4) solution spiked with 5 µl genomic (10 mg/ml calf thymus, diluted in water to 170 ng/µl) or plasmid DNA (170 ng/µl pEGFP (3.4 kb)). Unconjugated glass beads were included for each DNA condition as a negative control.

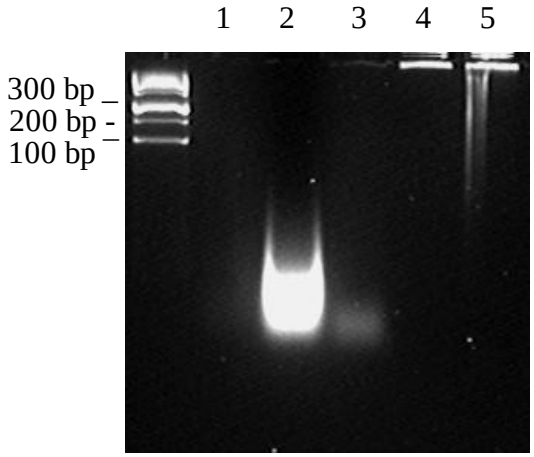


Figure 4-2– Activity of free / immobilised DNase I on plasmid or genomic DNA.

Key: (1) Plasmid DNA + immobilised DNase I; (2) Genomic DNA + immobilised DNase I; (3) Genomic DNA + free DNase I; (4) Plasmid DNA + glass beads only; (5) Genomic DNA + glass beads only. Samples incubated at 37°C for 10 minutes in 50 µl TrisHCl (with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ pH7.4) prior to running on a 2% agarose ethidium bromide gel. Marker = HyperLadder III (Bioline).

Samples were incubated for 10 minutes at 37°C, after which the supernatants were removed and enzymatic activity inactivated by heating for 10 minutes at 75°C. Samples were run on an ethidium bromide stained 2% agarose gel, as described in section 2.12. From this gel (Figure 4-2) it is apparent that free DNase I acts upon genomic DNA and that immobilisation of the enzyme does not completely inhibit this process.

4.3.2. DNA degradation time for immobilised DNase I

Samples of free and immobilised DNase I were prepared and incubated with 10 µl calf thymus DNA (10 mg/ml) in a total reaction volume of 200 µl TrisHCl (with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ pH7.5) at 37°C. Individual experiments were stopped in a time-wise fashion after 0 – 32 minutes, samples taken, heated at 75°C for 10 minutes to inactivate the enzyme in order to ensure a definitive end point to enzymatic activity, and run on a 2% agarose gel as described in section 2.12.

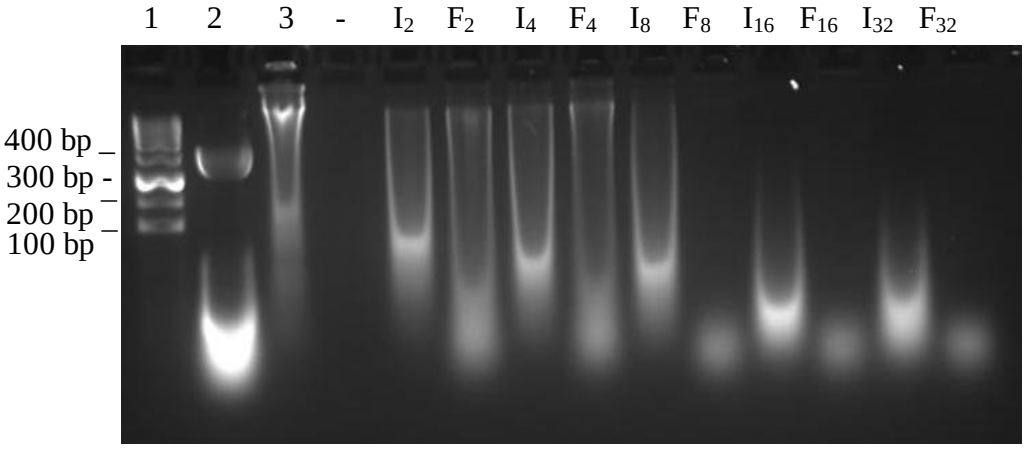


Figure 4-3 – Time required for genomic (calf thymus) DNA degradation by free / immobilised DNase I.

Samples incubated at 37°C for 0-32 minutes in 50 µl TrisHCl (with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ pH7.5) prior to separating on a 2% agarose ethidium bromide gel. Lanes 1 & 2 = molecular weight ladders. Lane 3 = Uncut genomic DNA. - = unused lane. "I" = Immobilised DNase I + genomic DNA; "F" = Free DNase I + genomic DNA. Subscript numbers denote enzyme and DNA incubation time, in minutes.

As predicted, free DNase I brought about a more rapid and more complete degradation of genomic DNA than did immobilised DNase I, with an apparent plateau of activity having been reached after four minutes of incubation with DNA. A similar trend was observed also with immobilised DNase I, although this occurred later, after approximately eight minutes incubation with DNA.

Although useful for ascertaining whether or not DNA had been degraded and allowing a comparison between conditions to be made, the DNA gel method only permits a qualitative assessment of the data; in order to gain a greater insight into the activity of immobilised DNase I compared to the free enzyme a quantitative method of analysis was required. Therefore, analysis of DNA depletion following incubation with DNase I was performed using the PicoGreen assay.

4.3.3. PicoGreen as a DNA detection method

HepG2 DNA was incubated with glass beads in TrisHCl (with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ pH7.4) at 37°C for 15 minutes. Following this the supernatants were removed for further analysis. The PicoGreen assay was utilised to quantify the amount of DNA remaining following treatment with bead-conjugated or free DNase I. This data indicated that virtually no DNA was degraded in the presence of DNase I linked beads and furthermore, that the free enzyme showed little activity (Figure 4-4a). As previous experiments had demonstrated that the immobilisation procedure is effective (Figure 4-2, Figure 4-3), the supernatants following DNA incubation with free and immobilised DNase I were separated on a 2% agarose gel to investigate whether that particular immobilisation had been successful and importantly, whether HepG2 DNA is a suitable substrate for DNase I under experimental conditions. The presence of smeared bands in the DNA gel demonstrated that free DNase I removed more DNA than immobilised DNase I, but that both bands were comparable to those obtained when calf thymus DNA was used as the substrate (Figure 4-4b). An experiment to

determine the suitability of the PicoGreen assay as a means to quantifying enzymatic degradation of HepG2 DNA was thus conducted.

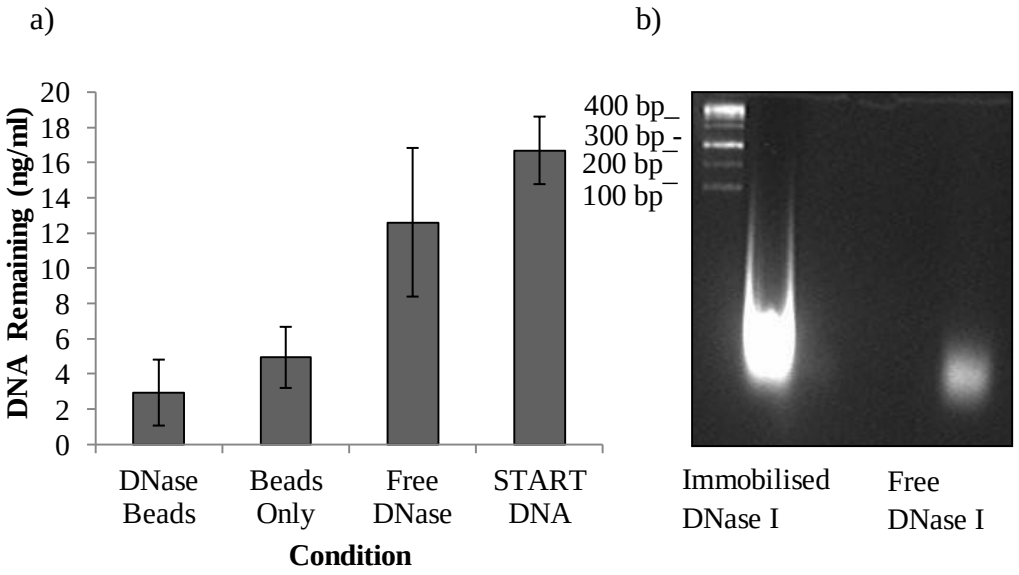


Figure 4-4 – DNA degradation by DNase I.

Remaining HepG2 DNA determined by a) PicoGreen (n=6, +/- SD) and b) Western blot analysis (Samples separated at 90 v on a 2% agarose gel).

4.3.3.1. Linearity of the PicoGreen assay

By plotting the concentration of DNA applied against the concentration of DNA detected, a standard curve demonstrating the linearity of the PicoGreen assay was obtained (Figure 4-5). DNA levels below approximately 25 ng/ml were indistinguishable from each other and were no longer linear. Therefore, this method is not suitable for detecting low levels of DNA. As the detection of low levels of HepG2 DNA is a fundamental requirement of this investigation, the PicoGreen assay method was replaced by Q-PCR for the detection of genomic DNA.

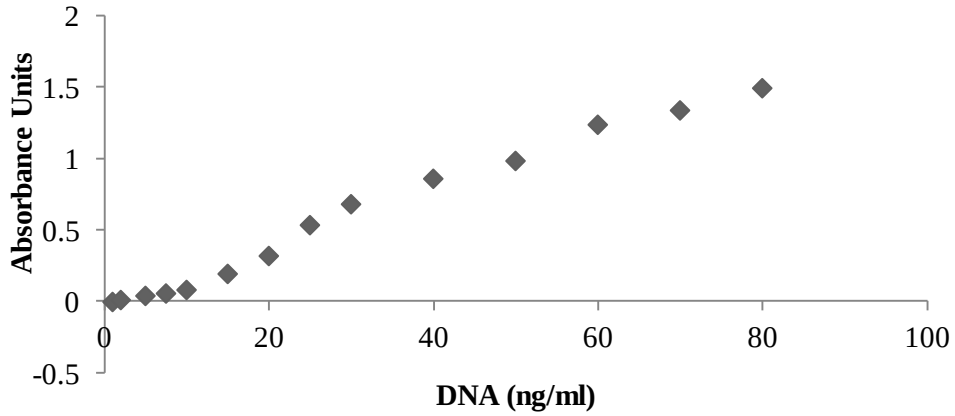


Figure 4-5 DNA detection by PicoGreen assay method.

DNA range from 0-80 ng/ml). Assay performed in TTE buffer. Mean values for each data point plotted. n=3.

The previously described immobilisation of DNase I onto glass beads was repeated, being treated with HepG2 DNA before applying the supernatants to Q-PCR. This time, it was evident that the immobilisation had been successful in so far as HepG2 DNA concentration was reduced following incubation with the enzyme linked beads. Furthermore, the free enzyme was shown to exhibit DNA clearance activity closer to that expected (Figure 4-6).

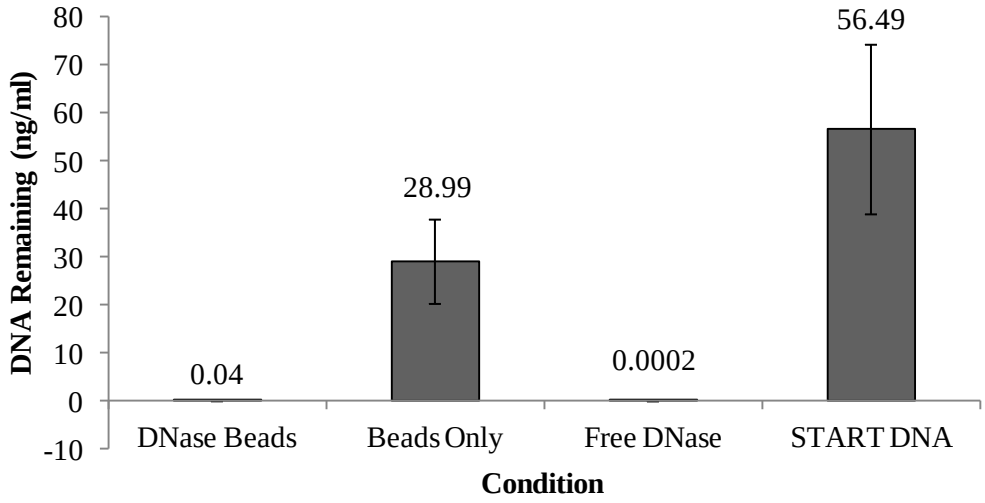


Figure 4-6 - DNA clearance of DNase I immobilised onto glass beads, determined by Q-PCR.

Activity of enzyme determined from the concentration of DNA remaining in solution following incubation for 15 minutes at 37°C in 50 mM TrisHCl buffer (pH7.4) with DNase I, immobilised DNase I or glass beads alone.+/- SD, n=6.

However, it was also apparent that a proportion of DNA was being removed from solution in the absence of DNase I, approximately 50% (Figure 4-6), strongly implying that DNA was binding to the support material and being removed from solution in this non-specific manner. For this reason, an alternative immobilisation support was sought.

4.3.4. Dynabeads® as the immobilisation matrix

Whereas a reduction in DNA concentration was observed in conditions treated with free DNase I, enzyme conjugated to Dynabeads® did not have any effect upon the DNA concentration, compared to the enzyme-free control condition (Figure 4-7). Therefore, although the DNase I enzyme was active, it was hypothesised that this activity was severely compromised during the immobilisation procedure. Dynabeads® utilise amine groups to bind to their target conjugate and it was hypothesised that the buffer medium, TrisHCl, may be having a detrimental effect upon this cross linking chemistry. Consequently, the buffer was exchanged for HEPES, which would also have the advantage in that it is routinely used to buffer carbon dioxide at a physiological pH, which would be of great benefit when undertaking studies in more physiologically relevant conditions (e.g. in the presence of plasma, for extended periods of time).

Exchanging the buffer was observed to improve the activity of Dynabeads® tethered DNase I (Figure 4-8). This was to such an extent that a direct comparison between the DNA removal capacity of enzyme linked to Dynabeads® and the original glass beads was deemed appropriate.

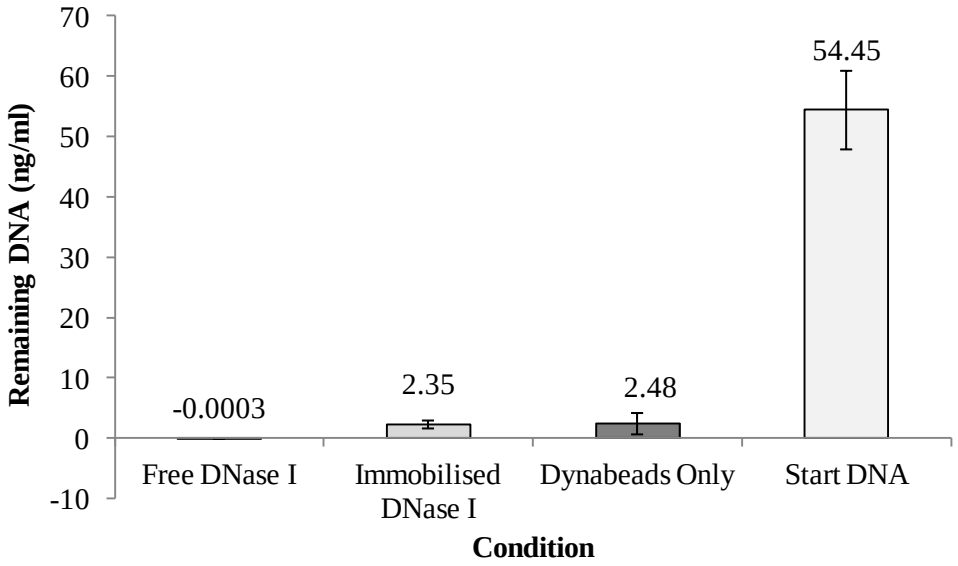


Figure 4-7 – DNA removal by DNase I Dynabeads® in TrisHCl.

DNA digestion conducted in the presence of 50 mM TrisHCl buffer (pH7.4) for 15 minutes at 37°C. Analysis was by q-PCR. +/- SD, n=6

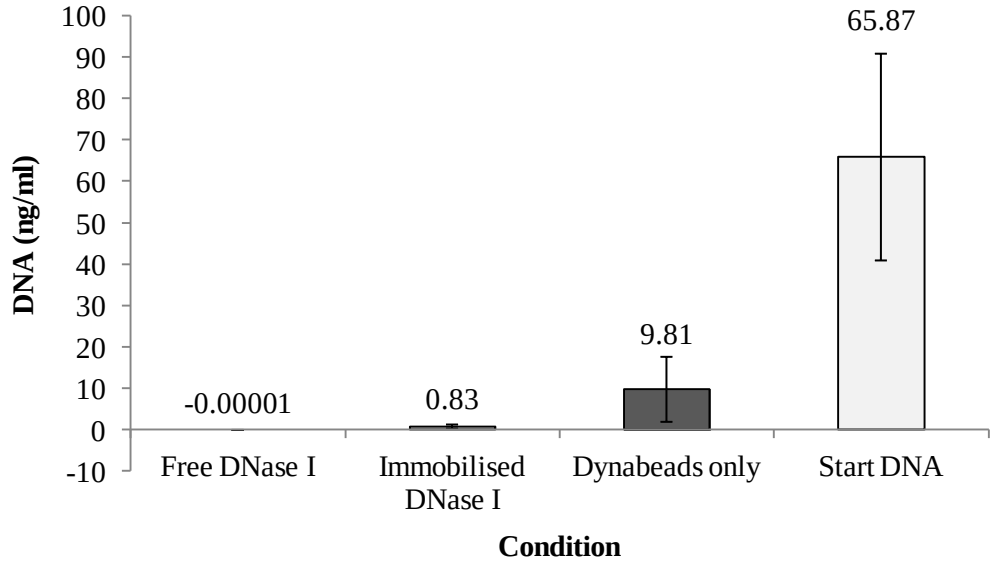


Figure 4-8 – DNA removal by DNase I Dynabeads® in the 10 mM HEPES buffer.

HepG2 DNA digested by immobilised DNase I, free DNase I or the negative control (beads only) in 10 mM HEPES buffer (pH 7.4, with 1 mM $MgCl_2$ and 1 mM $CaCl_2$) for 15 minutes at 37°C. Analysis was by q-PCR. +/- SD, n=6.

4.3.5. DNase I immobilised onto glass or Dynabeads®

A second investigation allowed the direct comparison of DNase I activity when bound to either Dynabeads® or the original glass beads, with the immobilisation protocol for each now being conducted in 10 mM HEPES buffer (pH 7.4). It thus became apparent that DNase I conjugated onto glass beads evoked a more substantial reduction in DNA concentration than did their Dynabeads® counterparts. Furthermore, in experiments when glass beads were used more DNA remained in solution than when Dynabeads® were applied to the DNA solution. Combined data from four experiments demonstrated that DNase I immobilised onto glass beads are more effective at DNA removal than their Dynabead® tethered counterparts (Figure 4-9), with only 0.03 ng/ml DNA remaining after treatment with glass-immobilised DNase I, compared to 0.76 ng/ml with Dynabead®.

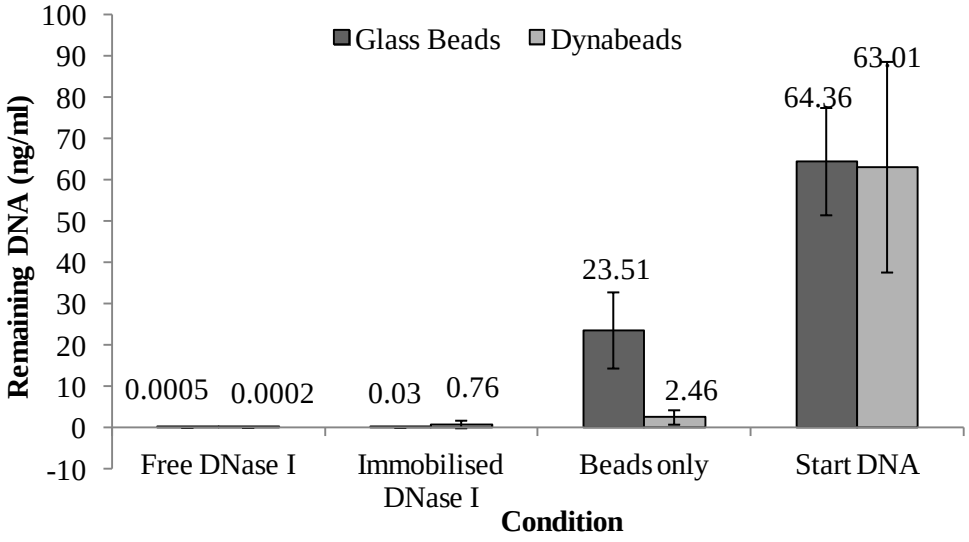


Figure 4-9 – Comparison of DNA degradation by DNase I immobilised onto glass beads or Dynabeads®.

100 ng/ml HepG2 DNA incubated in 10 mM HEPES (with 1 mM $MgCl_2$ and 1 mM $CaCl_2$) for 15 minutes at 37°C with either beads alone, free DNase I or DNase I immobilised onto glass or Dynabeads®. Mean +/- SD, n=12.

Owing to the observation that DNA is removed in the presence of the glass bead support (although to a lesser extent than with Dynabeads®) it was necessary to determine the nature of this interaction. A sample of DNase I

which had been inactivated by heating was cross-linked to glass beads, in addition to a standard experimental condition where active DNase I was cross linked. Following incubation in HepG2 DNA spiked buffer solution, the supernatants were removed before washing the remaining beads in a high pH solution in order to elute any DNA which may have bound to the bead surface. The PCR data in Figure 4-10 shows that although compared to free DNase I there was much DNA remaining in solution following treatment with DNase I conjugated beads, this was itself much less than the quantity remaining after incubation with beads cross linked with inactive enzyme. Analysis of the wash solutions demonstrates that more DNA was eluted from heat inactivated DNase I conjugated bead conditions than from active DNase I beads. Total DNA recovered from the HI DNase I eluent combined with DNA remaining in solution following treatment with HI DNase I beads was not found to be comparable to the initial start concentration of DNA ($p=0.09$, two-tailed T-test).

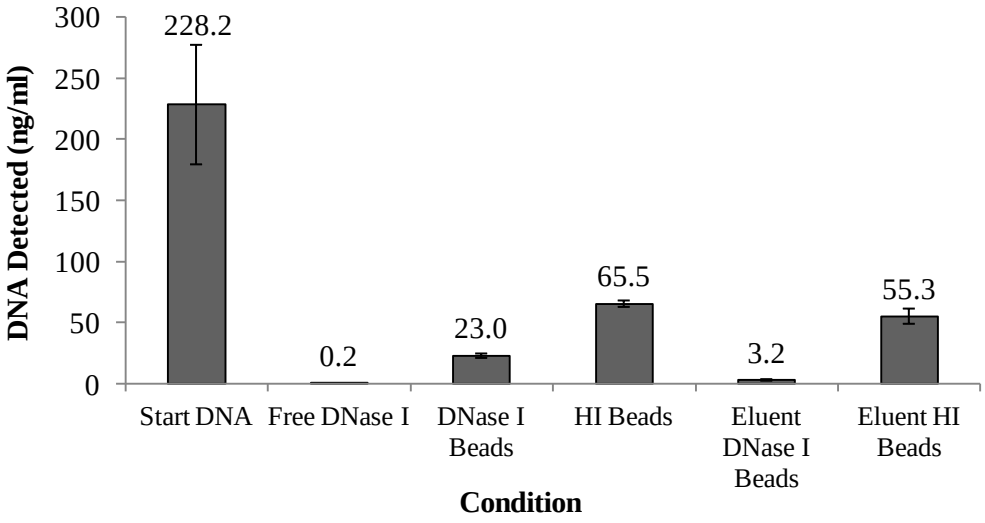


Figure 4-10 – Extent of DNA binding to glass.

Heat inactivated (HI) DNase I was immobilised to glass beads as a control for exposed surface area on glass beads available for DNA binding. DNA was eluted from the beads following DNA digestion (15 minutes at 37°C) with Tris pH 10 and this assayed for DNA content. Prior to applying to PCR, the eluent was pH neutralised to pH7.4 by the drop-wise addition of 0.1 M HCl. Error bars depict +/- SD, n=3.

4.3.6. Cross-linker effect on immobilised DNase I activity

The extent of DNA clearance by DNase I conjugated to glass beads by either benzene 1,3 disulfonyl chloride or by the longer chain molecule, O,O'-Bis[2-(N-Succinimidyl-succinylamino)ethyl] polyethylene glycol (NHS PEG) was determined by Q-PCR analysis of the resulting HepG2 DNA spiked buffer solution after incubation with each set of beads. Beads modified with the cross linking molecules but unconjugated to DNase I demonstrated a greater reduction in solution DNA concentration when exposed to NHS PEG beads than with benzene 1,3 disulfonyl chloride modified beads (Figure 4-11a). Overall, DNase I conjugated to beads with benzene 1,3 disulfonyl chloride brought about a greater extent of DNA depletion in solution than when the NHS PEG ester was used (Figure 4-11b); spiked solutions where DNase I was conjugated to glass beads with NHS PEG had more DNA remaining after treatment than in solution treated by DNase I tethered to beads via benzene 1,3 disulfonyl chloride.

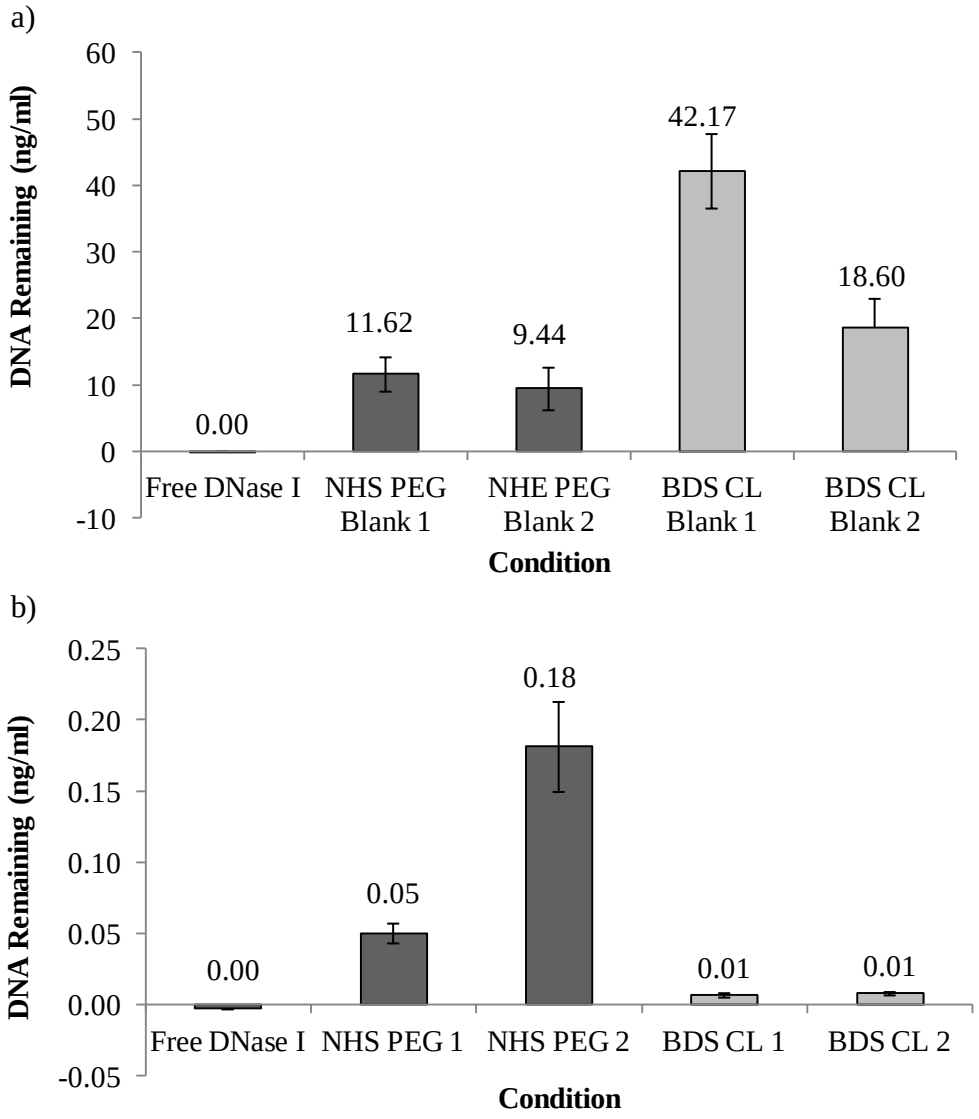


Figure 4-11 – Effect of cross-linker on DNase I activity.

DNA depletion of spiked 10 mM HEPES buffer solution following incubation with glass beads with/ without DNase I immobilised via NHS PEG or benzene 1,3 disulfonyl chloride (BDS CL) cross linkers. (a) Comparison of DNase I activity following immobilisation by the different cross linkers; (b) Effect of cross linker on DNA depletion in the absence of DNase I. Error bars depict +/- SD, n=3 for individual experiments (n=6 for each cross linking condition). Initial DNA spike of 100 ng/ml administered to all conditions and incubated for 15 minutes at 37°C. Supernatants were removed and applied to Q-PCR.

These findings were compared to the corresponding extent of DNase I binding to the glass beads by each method, to give an indication of the specific activity of DNase I conjugated by each method. This was normalised by subtracting the extent of "DNA depletion" in the appropriate

blank condition, to account for DNA being removed from solution by binding to the glass bead rather than by purely enzymatic means. The Western blot data (Figure 4-12) shows that more DNase I tends to be left in solution, i.e. is not bound to the glass beads, when benzene 1,3 disulfonyl chloride is used as the cross linking agent. Also, using NHS PEG seems to yield more variable levels of cross linking. Combining these data enabled a calculation of the specific activity of DNase I immobilised under each condition (Table 4-1).

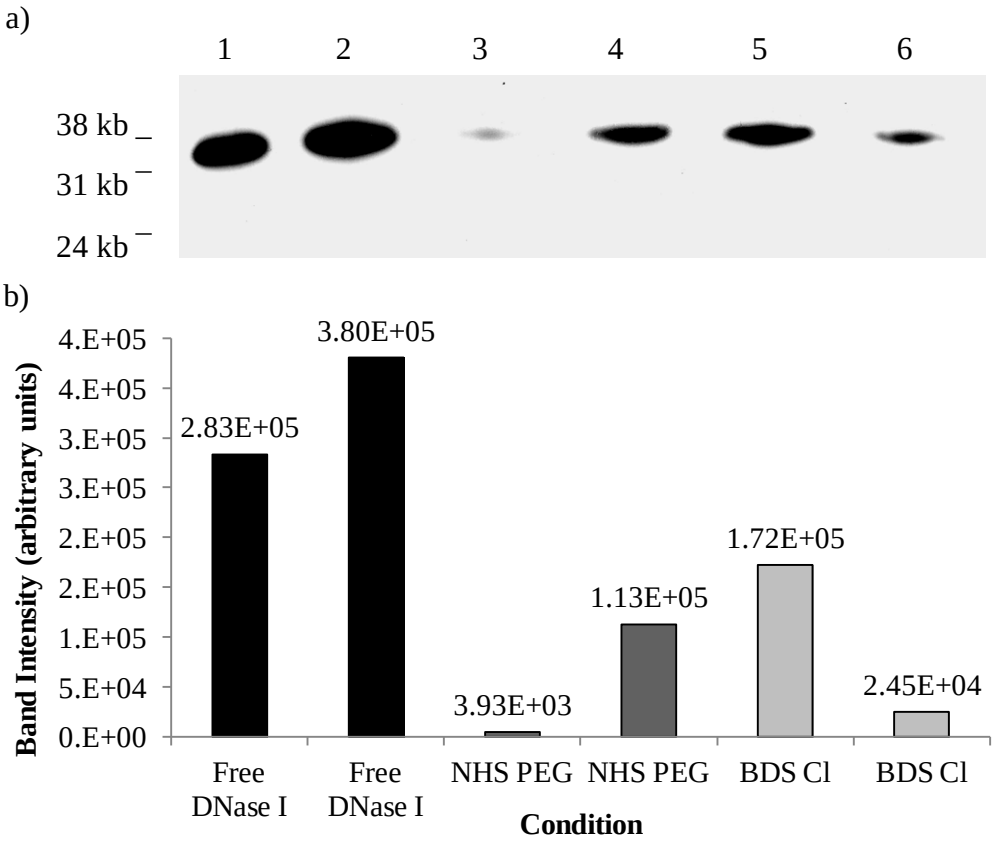


Figure 4-12 – Binding of DNase I to the cross linking molecule.

a) Post-immobilisation supernatants from duplicate conditions loaded onto a gradient pre-cast gel and run for 45 minutes at 250 v. Ladder = Full range Rainbow molecular weight marker. Lanes 1 & 2 = Free DNase I; 3 & 4 = NHS PEG; 5& 6 = Benzene 1,3 disulfonyl chloride. b) Quantification of band intensity (Arbitrary units represent surface area of pixels from scanned blot image). Each condition was performed in duplicate.

Table 4-1 – Calculated activity of DNase I immobilised onto glass beads.

Cross linking molecule used was either NHS PEG or benzene 1,3 disulfonyl chloride (BDS CL). Mean values of duplicate concurrent immobilisations shown. DNA clearance derived from Q-PCR data; Band intensity data obtained from western blot analysis of unbound DNase I following immobilisation.

CONDITION	Mean DNA Cleared (% of Start [DNA])	Mean Band Intensity (Arbitrary Units)	Calculated Specific Activity
NHS PEG	12.17	58312	2.09E-04
BDS CL	35.50	98424	3.61E-04

In light of these findings, the original cross linking protocol was utilised in subsequent investigations.

4.3.7. DNase time-course in buffer

Having established that DNase I can successfully be conjugated to glass beads and that these were then capable of DNA degradation, it was necessary to determine whether these DNase I beads can function in more physiologically relevant conditions. Therefore, DNase I beads were incubated with HepG2 DNA spiked HEPES buffer at 37°C and pH7.4 with continual rotational mixing for a period of 24 hours, and the extent of DNA depletion compared to that in control conditions (Figure 4-13). It was observed that DNA levels were greatly reduced following incubation with both free and immobilised DNase I.

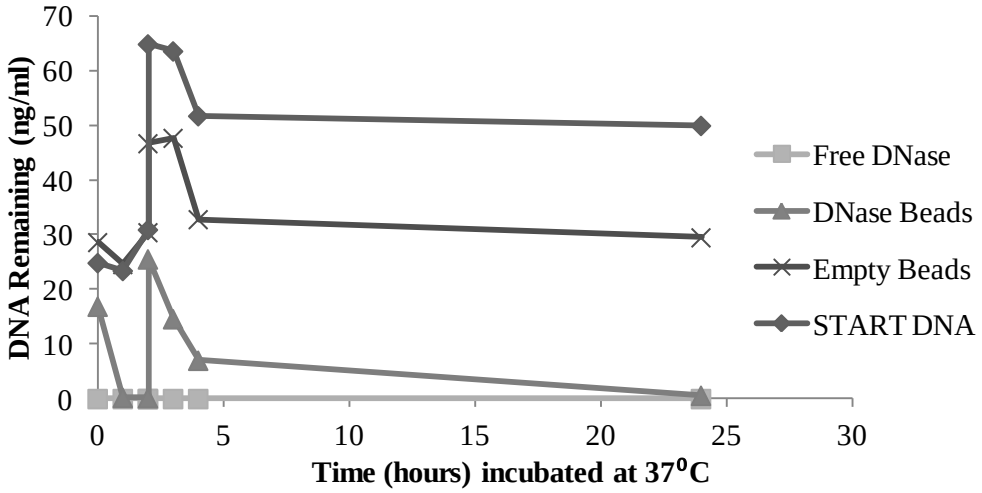


Figure 4-13 – DNA depletion in spiked 10 mM HEPES at 37°C over 24 hours.

Samples spiked initially and after two hours with 20 µl of 0.95 µg/ml HepG2 DNA. Empty beads = glass beads without DNase I (negative control). START DNA = DNA spiked buffer, no beads. DNA concentration determined by q-PCR.

4.3.8. DNase I activity in plasma

The BAL requires that immobilised DNase I be able to effectively degrade DNA in the presence of plasma. In order to quantify the extent of DNA degradation and hence determine the efficacy of the DNase I beads, a suitable Q-PCR protocol had to be established. The difficulties in performing Q-PCR in the presence of plasma are well documented, the various constituents interfering with components of the PCR reaction, such as Taq polymerase. By diluting samples 1/5000 and treating with Proteinase K it was however possible to gain a linear five-point standard curve (Figure 4-14).

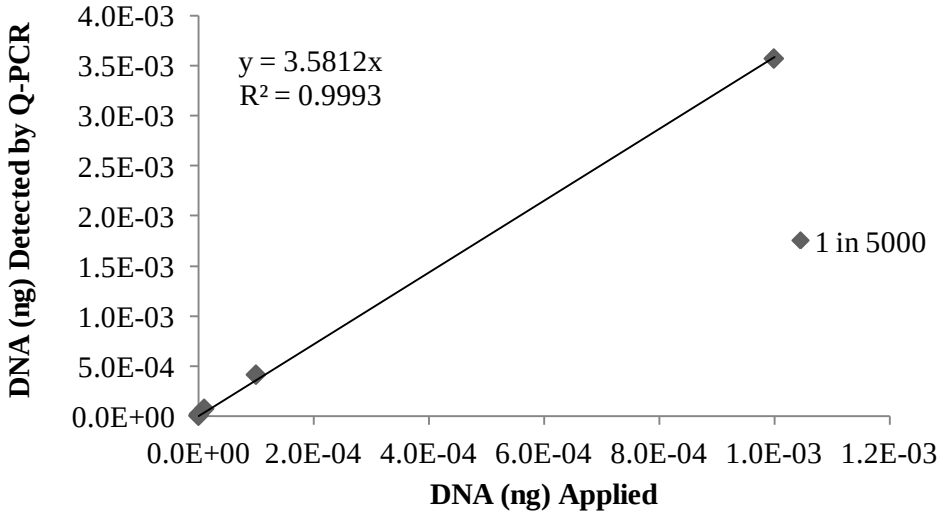


Figure 4-14 – Standard curve of BioLine DNA in FFP.

FFP diluted 1/5000 in dH_2O, spiked with human DNA in a range of concentrations (to give a final range from 1 pg / reaction to 0.0001 pg/reaction) and treated with Proteinase K prior to Q-PCR. n=3.

The enzymatic activity of DNase I was investigated for both free and immobilised DNase I in the presence of plasma, over a period of 48 hours, to determine whether the enzyme can function for extended periods of time in the presence of plasma components which may affect activity. An inactivated form of DNase I, created by heating DNase I for 15 minutes at 75°C and vortexing thoroughly, was included to control for the binding of DNA to available space on the glass bead support. FFP was spiked to a concentration of 100 ng/ml with HepG2 DNA and incubated for 48 hours at 37°C prior to analysing the samples using the plasma Q-PCR protocol. It was surprising to note that the extent of DNA degradation by immobilised DNase I was much closer to that achieved by the free enzyme (Figure 4-15), demonstrating 50% the activity of the free enzyme, compared to the approximate 60-fold reduction in DNA clearance observed when incubations were previously performed in 10 mM HEPES buffer for a shorter period of time.

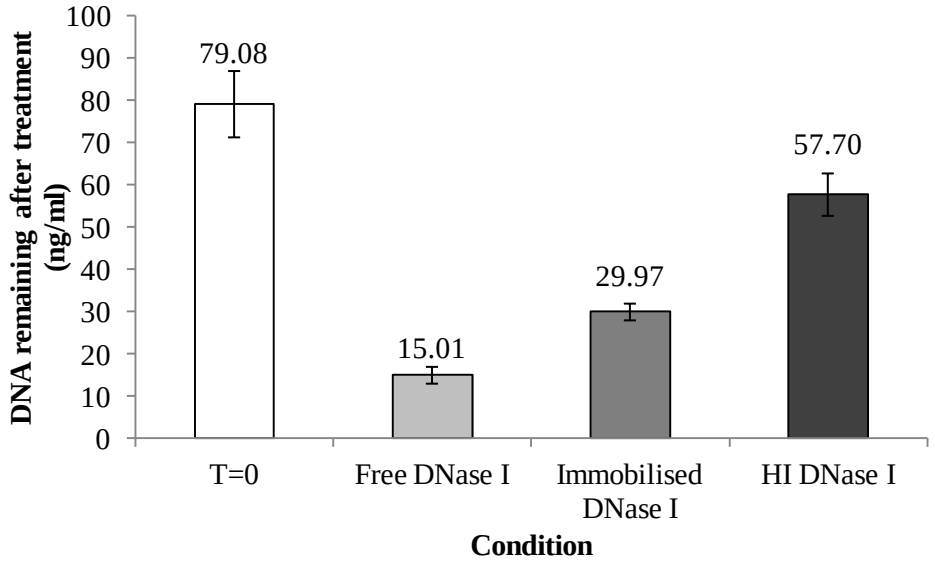


Figure 4-15 – DNA degradation by DNase I in 100% FFP.

FFP was spiked with HepG2 DNA to give a concentration of 100 ng/ml. This was applied to free, immobilised or immobilised heat inactivated (HI) DNase I and incubated for a total of 48 hours at 37°C with continuous rotational mixing. Samples of 20 µl were taken and analysed by Q-PCR. n=6.

4.4. Discussion

4.4.1. DNase I immobilisation onto glass beads

Initial experiments with plasmid and calf thymus DNA revealed that immobilisation of DNase I onto glass beads yielded a satisfactory degree of DNA degradation, as illustrated by DNA gel analysis. Whereas free DNase I unequivocally had brought about a greater extent of degradation on genomic DNA, DNase I immobilised onto glass beads also greatly reduced the average molecular length of both plasmid and genomic DNA compared to the corresponding negative controls (glass beads without DNase I). As the immobilised enzyme had degraded less DNA within the incubation period than had the free enzyme, the question to be next addressed was whether the immobilisation procedure had limited the rate at which DNase I can break down DNA or whether the affinity for the substrate had been reduced. This could be indirectly deduced by allowing immobilised DNase I longer to act upon the same quantity of DNA and observing whether more DNA

degradation is observed with increasing incubation time. A more comprehensive investigation of this is discussed in Chapter 5.

To investigate this, a range of incubation periods for free and immobilised DNase I with DNA were compared. The resulting data can be interpreted to signify that immobilised DNase I may still have the potential to deplete a solution of DNA to the same extent as the free enzyme, but that it requires a greater length of time to do so. This could be due to physical restriction of the enzyme by the cross-linking molecules utilised in the immobilisation procedure, or simply that by no longer being able to freely diffuse through solution, the immobilised enzyme is less likely to encounter and thus interact with the substrate. Although not ideal, an immobilised enzyme with a slow turnover rate may not be of detriment to the BAL system, providing that DNA is cleared efficiently, and importantly, that the enzyme does not lose activity during the extended incubation period required to achieve this. This could be a real issue in the BAL, which is required to function in the presence of plasma, rather than an enzyme-friendly Tris or HEPES based buffer, due to the plethora of plasma components which have been observed to be detrimental to DNase I activity, examples including proteinases and actin molecules.

Investigations into the extent of DNA removal due to binding to the glass bead support, rather than through enzymatic degradation, revealed that this phenomenon does occur. Figure 4-10 demonstrates that after incubation with DNA, washing glass beads in a high pH solution eluted the bound DNA, confirming that a degree of DNA will inevitably bind to the support. Interestingly, heat inactivated DNase I beads released much more DNA than the active DNase I beads. This could give an insight into the method of DNA clearance by immobilised DNase I; the data could suggest that DNA binds to the glass support and it is from there that the tethered enzyme interacts with and consequently degrades DNA. For this reason the quantity of DNA eluted from DNase I cross linked beads is much lower than that for the inactive immobilised enzyme, as the glass-bound DNA had been mostly removed already by the action of DNase I. Alternatively, less HI DNase I

may have bound to the glass bead, thus providing a greater surface area to which DNA could bind.

4.4.2. DNA detection methods

When developing a system for removing DNA contaminants from the BAL it is crucial to have a satisfactory means of determining and quantifying how much DNA was present initially and then what proportion of this remains following treatment with the system. There is hence a great emphasis on selecting a suitable detection method; it must be sensitive, robust, and reliable even in the presence of plasma constituents. Initially, the PicoGreen assay was utilised. Although Q-PCR is a highly sensitive method of DNA detection, PicoGreen was chosen as a) unlike PCR, it does not rely upon enzymatic activity which may be compromised in the presence of plasma, and b) is less likely to bias (the nature of PCR means there is a tendency to disproportionally amplify smaller DNA sequences). However, the PicoGreen assay was discovered to not be sensitive enough in the detection of lower DNA concentrations, and so Q-PCR was henceforth used for the quantitative detection of DNA. The issue of interference from plasma constituents was overcome by modifying the PCR pre-treatment protocol and diluting samples from incubations conducted in plasma 1 / 5000 and treating with Proteinase K to remove protein contaminants which may compromise PCR reliability. This approach has been shown by our group to be a robust and reliable method for detecting and quantifying HepG2 DNA.

4.4.3. DNase I immobilisation onto Dynabeads®

Due to the observed phenomenon of DNA binding to glass, an alternative support matrix was investigated. Dynabeads® are small magnetic spheres, pre-modified with an amine group for binding the target protein. It was their pre-modified, ready to use state along with their magnetic nature which made them an ideal choice for an immobilisation support for DNase I; being magnetic, they would allow greater control of the containment of DNase I should the approach be utilised within the BAL.

Initial difficulties in terms of a lack of enzymatic activity were overcome by performing the immobilisation in 10 mM HEPES instead of the TrisHCl buffer. The rationale behind this decision was based on the chemistry of the reactive group on both the Dynabeads® and DNase I. Tris contains amine groups which can compete for binding sites and so interfere with the conjugation of enzyme and support. Exchanging the buffer solution lead to improved performance of cross linked DNase I, although this was then shown to be inferior to the levels of DNA degradation achieved when glass beads were used as the immobilisation support. Therefore, subsequent investigations into optimising DNase I immobilisation utilised glass beads rather than pursuing Dynabeads® any further.

4.4.4. Cross-linker effect on immobilised DNase I activity

For the immobilisation of DNase I onto glass beads two cross linking molecules were investigated. The first, 1,3-benzenedisulfonyl chloride, is a relatively small, aromatic molecule, comprising two sulfonyl chloride moieties attached to a benzene ring.

a) b)

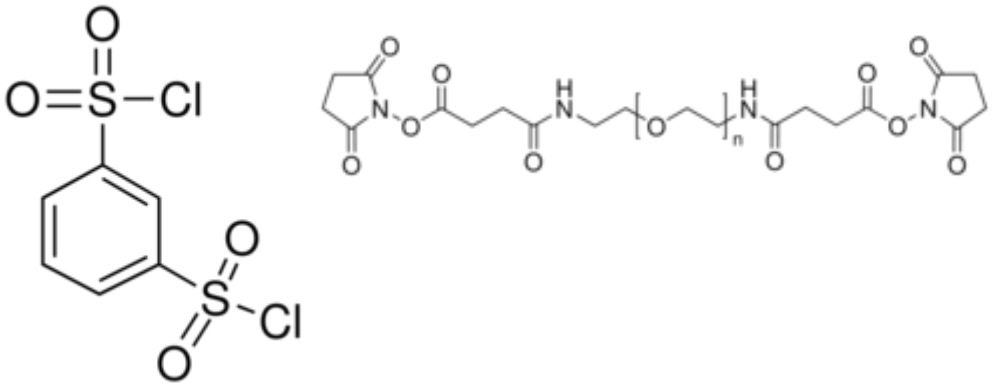


Figure 4-16 – Molecular structure of cross-linking molecules.

a) 1,3-benzenedisulfonyl chloride and b) O,O'-Bis[2-(N-Succinimidyl-succinylamino)ethyl] polyethylene glycol. "n" depicts a repeating polyethylene glycol (PEG) unit, generating a cross linker molecule with a molecular weight of approximately 2000 Daltons. (Images taken from www.sigmaaldrich.com)

The second is a homobifunctional molecule, containing two succinimidyl termini separated by a polyethylene glycol repeating unit, or spacer arm, with a MW of 2 000 daltons. Both target primary amines, which are present on the N-terminus of peptide chains and also on lysine residues, which are usually on the outer surface of proteins owing to their charged and hydrophilic nature (137). This therefore makes them ideal targets for cross linking; it is unlikely that the enzyme would be denatured following structural changes, which may be the case if the target residue was more internally located. DNase I has nine lysine residues (137) and so the potential for a successful cross linking reaction is reasonably high. It would seem that the reaction had been successful when 1,3-benzenedisulfonyl chloride was used to tether DNase I to glass beads, as the resulting enzyme complex was observed to reduce DNA concentration in a spiked solution. However, as this was significantly less than the activity of the free enzyme, it was hypothesised that a large proportion of the enzyme was not being bound and so a more effective method of cross linking was investigated. The NHS PEG ester was chosen because succinimidyl esters are an established means of binding primary amines, and furthermore, the long spacer arm was hypothesised to increase the activity of bound DNase I by permitting greater scope for movement of the enzyme and thus facilitating an interaction with DNA.

However, subsequent investigation revealed that this was not the case; using NHS PEG as the cross linking agent actually saw less DNase I activity than when the original method of cross linking was employed. An explanation for this could be that the NHS PEG bound DNase I at multiple sites, thus holding it in a rigid conformation and preventing substrate interactions. This becomes particularly relevant when taking into account the DNase I active site. This comprises an exposed loop region which interacts with the minor groove of DNA, whilst positively charged residues on either side of the loop interact with phosphate groups of the DNA strands; one of the required loop region residues is Lysine 74. It is therefore apparent that should this residue become involved in tethering the enzyme to a physical support, the properties of the lysine, and hence those of the active site would be altered, hence affecting the affinity, formation and interaction of the

enzyme - substrate complex. However, it must be considered that there are a total of nine lysine residues, all of which are accessible, i.e. found on the outer regions of DNase I (137), and so theoretically there stands an 11% chance that this residue will be targeted – assuming that the enzyme is cross-linked by only one cross-linking molecule. Although this scenario could account for an overall reduction in DNase I activity following immobilisation, it does not address the issue of why the longer cross linking agent was less effective than the shorter molecule.

Alternatively, the long length of the NHS PEG cross linker may likely have become detrimental rather than beneficial to DNase I activity. For example, it may possibly have allowed sufficient flexibility in the orientation of the enzyme that it was able to twist around so that the active site faced the glass support, thus vastly decreasing the possibility of a successful interaction with DNA. It is also plausible that the length of NHS PEG (having a molecular weight of over 2 000 Daltons) made it possible for both ends of this homobifunctional cross linking molecule to bind different NH_2 modified sites on the glass bead support, rather than binding to DNase I. This may have limited the available cross-linking sites for DNase I and thus reduced the overall potential efficacy of any given bead. Moreover, the resulting "mesh" of double-bound cross-linker may have created a matrix within which DNA could become caught, or, acted as a barrier between DNA and DNase I, therefore preventing its interaction with immobilised DNase I.

4.4.5. DNase I activity in plasma

It was unexpected to observe that DNase I immobilised onto glass beads demonstrated levels of DNA clearance only 50% lower than those of free DNase I in the presence of 100% FFP; this had not previously been the case when investigating DNase I activity in buffer solutions, where free DNase I typical degraded from 60 up to 200 times more DNA within the given incubation time than the immobilised enzyme. It must be noted that this change is not due to increased activity of immobilised DNase I, but rather a

marked reduction in DNA degradation by free DNase I, compared to digestions carried out in buffer. An explanation for this could be that the process of immobilisation confers stability to DNase I, by protecting it from detrimental effects of interactions with plasma constituents; without this protection, the free enzyme is compromised, possibly by inactivation following binding with inhibitor molecules such as G-actin. If immobilisation is having a beneficial effect upon DNase I activity in plasma, the potential use of the DNase I glass beads in the BAL to reduce circulating DNA over an extended treatment period is feasible. This will be addressed in Chapter 6, where a scaled-down model of the BAL will be used to assess the efficacy of immobilised enzymes to remove DNA in physiologically relevant conditions.

4.5. Summary

There is a potential risk to the patient from circulating HepG2 DNA when undergoing treatment with the BAL. One approach to eliminate this risk is to utilise DNase I to degrade DNA. It has been demonstrated in this chapter that DNase I can be immobilised onto glass beads which, when incubated with DNA spiked solution, results in a reduction of the DNA concentration. Although the extent of DNA degradation in spiked buffer solution was less when treated with immobilised DNase I than with the free enzyme, it has been shown that the immobilised DNase I beads also reduce DNA concentration in spiked FFP. The next logical question to be addressed is therefore whether the immobilised DNase I can reduce DNA concentration in a more physiologically relevant model, at 37°C, in the presence of plasma, for periods of up to eight hours – essentially reproducing the working conditions of the BAL during patient treatment.

In order to determine the length of time required by immobilised DNase I to clear a given amount of DNA, the kinetic profile of both the free and bead conjugated enzyme must be deduced. This will be addressed, along with that of lactate oxidase in the following chapter.

Chapter 5. Enzyme Kinetics

Kinetic data is calculated for the activity of free and immobilised Deoxyribonuclease I and Lactate oxidase, enabling an estimation of the DNA and lactate clearance capacity of the systems in the BioArtificial Liver Device / Fluidised Bed Bioreactor.

5. Enzyme Kinetics

5.1. Introduction

In order to fully comprehend and assess the usefulness and practicality of using the immobilised enzyme preparations it is necessary to determine their functionality compared to the original, non immobilised enzyme. For this to be possible, the determination of the rate at which the enzyme catalysed reaction occurs is required. This chapter seeks to address this question.

Enzymes can be referred to as highly specific "biological catalysts"; they increase the rate at which a reaction occurs by lowering the activation energy (E_a) threshold which must be exceeded in order for the reaction to occur. For this to be possible, the enzyme must have a greater affinity for the transient state form of the substrate rather than the uncomplexed substrate molecule, so that a progression from enzyme + substrate to an enzyme-substrate complex to the formation of product(s) can occur (153). It is to be noted that the addition of a catalyst does not affect the relative quantity of product formed by the reaction; the ratio of product to substrate remains unchanged from that encountered in the absence of any catalyst, only the number of successful reactions per unit time is increased. In other words, the rate at which the reaction occurs is affected but the position of the equilibrium between substrate and product concentrations is not.

It has been frequently demonstrated that the affinity an enzyme has for its substrate and/or the rate at which the reaction takes place is altered by immobilising the enzyme. Typically, both will be lower in the immobilised version of the enzyme as the quaternary structure of the protein is tethered in such a way that the active site is restricted and thus less likely to adopt the conformational change often required to correctly interact with its substrate. Furthermore, binding the enzyme to a bulky support matrix will frequently have a negative impact on the diffusion and accessibility of substrate to the enzyme. However, there are of course advantages to

immobilisation; other than conferring greater stability to the enzyme (thermal, structural and even chemical), instances have been reported whereby enzymatic activity is in fact improved following immobilisation (87).

5.1.1. Determination of enzyme kinetics

The efficacy of an enzyme to catalyse a particular chemical reaction is determined by two main factors; how readily the enzyme forms a complex with its substrate and how transient this interaction is.

$$ES \xrightarrow{k_{cat}} E + P$$

Figure 5-1 – Schematic representation of an enzyme catalysed reaction.
ES = Enzyme-substrate complex; E = regenerated enzyme; P = product formed.

In the most simple scenario, a graph depicting an enzyme catalysed reaction would demonstrate that with increasing time the substrate concentration is depleted whilst the concentration of the product formed increases, until the curve plateaus when the substrate is depleted and becomes rate limiting. In terms of the velocity of an enzyme catalysed rate of reaction, plotting the rate of reaction as a function of the substrate concentration will often produce a curve. This represents that the rate of reaction rapidly increases with increasing substrate concentration until the curve levels off, indicating that increasing the substrate concentration will no longer increase the rate of reaction as the enzyme is functioning at its maximum capacity. This point is referred to as the V_{max}, or the maximum velocity at which a particular enzyme catalysed reaction can occur. It is related to the "turnover number", (as this value is expressed as V_{max} divided by the product of total enzyme concentration and time) representing the number of substrate molecules converted to product by each enzymatic active site per unit time. The K_m of a reaction is the Michaelis-Menten constant and refers to the concentration of substrate which causes a rate of reaction half that of the V_{max}. It describes the dependence of the initial rate of reaction on the availability of the

substrate, and in simple enzymatic reactions is equal to the dissociation constant of the enzyme-substrate complex, hence describing the affinity an enzyme has for its substrate (153).

5.1.1.1. Criteria for calculating K_m

It is not always possible to deduce the K_m of an enzyme for its substrate in a given context. This most often occurs when one or more of the key assumptions which are necessary in order to calculate the K_m cannot be reasonably made. These criteria are:

- The production of product is linear with time during the time interval used
- The concentration of the substrate is greatly in excess; i.e. is not limiting the maximum rate at which the reaction can occur
- The product is formed only by a single enzyme and formation of the product in the absence of that enzyme is negligible
- Substrate binding to an active site of an enzyme has no effect upon the activity or affinity of an adjacent site
- Enzyme activity is neither affected by the substrate nor by the product.

It is not possible to confidently assume that the immobilisation of an enzyme onto a solid support will have no effect upon the affinity of the enzyme for its substrate, nor that multiple enzymes may be bound onto the support or to each other in such a way as to influence the formation of enzyme-substrate complexes in their immediate environment. Furthermore, it has been demonstrated in previous chapters that DNA binds to the glass immobilisation support; in this manner, both DNase I and its substrate are in effect immobilised. Therefore, the proceeding experiments sought to determine an estimation of the K_m and V_{max} of immobilised DNase I and LOx – referred to as the "apparent K_m"(K_m[app]).

5.2. Materials & Methods

5.2.1. Lactate oxidase

Materials:
Phosphate Buffer (pH7.4):
10 mM potassium phosphate
0.01 mM Flavin Adenine Dinucleotide (FAD) (≥95%, Sigma, F6625)

0.02 U/ml LOx (0.02 U in 1 ml 10 mM phosphate buffer)
Plate reader at 570 nm wavelength
96-well plate (Nunc)
Immobilised LOx glass beads (see section 3.2.3)

For the free LOx enzyme, a modified version of the lactate oxidase activity assay described in section 3.2.1 was performed. 20 µl of 0.02 U/ml LOx solution was added to each well of a 96 well plate, containing 80 µl assay reagent mix (as described in section 3.2.1) and a range of lactate concentrations prepared from a serial dilution of a freshly prepared lactate stock. The plate was incubated at 37°C and a total of 40 single end point measurements for each well were taken at 570 nm wavelength using the Anthos HTIII plate reader coupled with Manta software. Final substrate concentrations used per well were 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0 mM. Each series of substrate concentrations was replicated three times.

Methodology for immobilised LOx was as described previously, using LOx glass beads prepared as in section 3.2.3. Four glass beads were used per well in place of 20 µl of 0.02 U/ml LOx solution, the volume being substituted by 10 mM potassium phosphate buffer.

5.2.2. Deoxyribonuclease I

Materials:
HEPES buffer solution (pH7.4):
10 mM HEPES (pH 7-7.6, Sigma, HO887)
1 mM $MgCl_2$
1 mM $CaCl_2$

4 U/µl Deoxyribonuclease I (Type II from bovine pancreas, Sigma Aldrich D4527) (from 25 µl frozen aliquots of 21.8 µg/µl (40 U/ µl) DNase I (original stock of 1836 U/mg protein)).

Assay Reagent:
500 µl 1M sodium acetate buffer pH5
250 µl 100 mM magnesium sulphate
3.65 ml purified water
600 µl 0.033% (w/v) DNA solution:

2 mg calf thymus DNA (Sigma, D3664)
dH_2O
96-well plate (UV Transparent, ThermoFisher)
FLUOstar OMEGA plate reader (BMG Labtech)

To individual wells of a 96-well plate 125 µl assay reagent was applied. Calf thymus DNA diluted in purified water was used at the following range of concentrations: 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 mg/ml. For each condition a further 3.65 ml purified water was added to complete the reaction mixture. This was incubated for five minutes at 25°C after which 25 µl 10 mM HEPES buffer or 25 µl DNase I enzyme (resuspended in HEPES buffer) was added to each well (total 100 U DNase I per well). Absorbance measurements were taken continually at the 260 nm wavelength over a 10 minute period at 37˚C using the OMEGA plate reader.

5.2.3. Enzyme kinetics calculations

All calculations were performed using non-linear regression and the Michaelis-Menten formula on GraphPad PRISM® 4 software programme. The Michaelis-Menten equations are as follows, where υ = reaction rate, d = the derivative function, P = product, S = substrate, and t = time:

$$v = \frac{d[P]}{dt} = \frac{V_{\max}[S]}{K_m + [S]}.$$

Figure 5-2 – Equation for the Determination of K_m.

5.3. Results

5.3.1. Lactate oxidase

Free lactate oxidase was found to have a K_m value of 0.01911 mM and a V_{max} of 0.07523 mM/min. Immobilised lactate oxidase enzyme exhibited an apparent K_m of 0.01491 mM and a V_{max}[app] of 0.003172 mM/min respectively (Table 5-1).

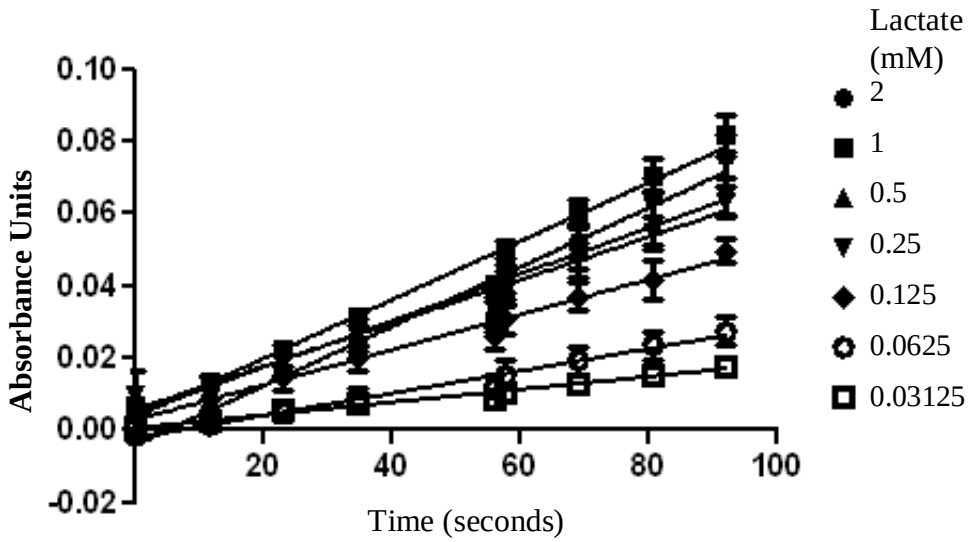


Figure 5-3 – Change in absorbance at different lactate concentrations (Free LOx).

20 µl of LOx (0.02 U/ml in 10 mM phosphate buffer) used per 120 µl reaction volume. Samples incubated and absorbance measurements taken continuously at 570 nm at 37°C. n=3 for each substrate concentration condition.

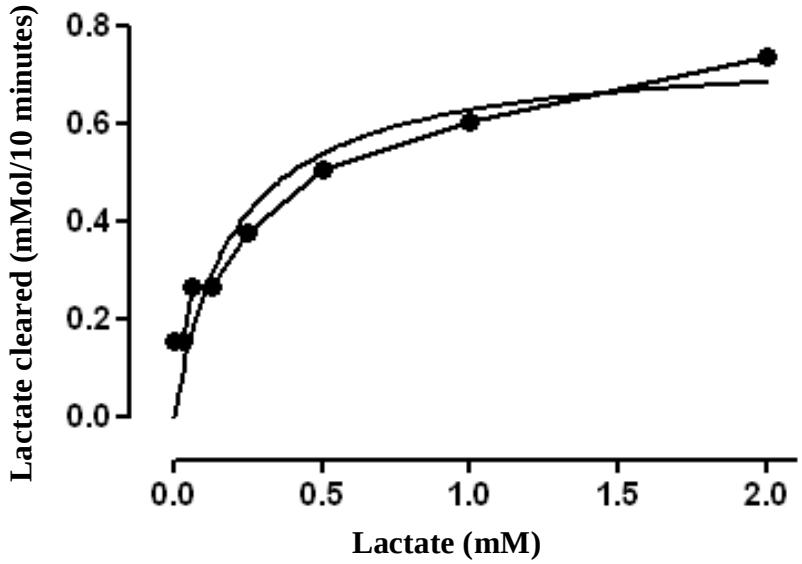


Figure 5-4 – Rate of lactate clearance with Free LOx.

20 µl LOx (0.02 U/ml) used per 120 µl reaction volume. Samples incubated at 37°C and absorbance measurements taken continuously (570 nm). Lactate concentrations from 2-0 mM. Line of best fit modelled by non-linear regression. n=3

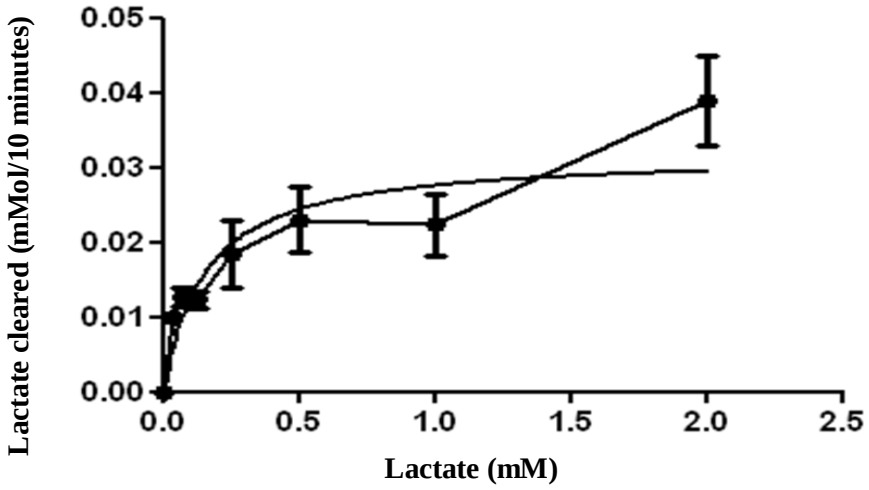


Figure 5-5 – Rate of lactate clearance by immobilised LOx.

4 LOx beads used per 120 µl reaction volume. Samples incubated at 37°C and absorbance measurements taken continuously (570 nm). Lactate concentrations from 2-0 mM. Line of best fit modelled by non-linear regression. n=3

Table 5-1 – Vmax and Km data for free and immobilised LOx.

Calculated using GraphPad Prism4 software.

	V_{max} (mM/min)	K_m (mM)	Std.Error V_{max} (mM/min)	Std.Error K_m (mM)
Free LOx	0.075	0.019	0.005	0.004
Immobilised LOx	0.003	0.015	0.0004	0.006

5.3.2. Deoxyribonuclease I

In contrast to traditional kinetic studies which measure the rate of product formation to determine kinetic data, it was necessary to perform kinetic studies on the rate of substrate depletion for DNase I; since DNase I catalyses a degradation reaction, there is no product formation *per se*. For this reason a time course experiment was prepared, using fluorescence to compare the amount of remaining DNA after increasing time exposed to DNase I. However, the change in absorbance was very slight and tended to fluctuate (Figure 5-6), particularly at lower substrate concentrations, and so establishing a rate of enzymatic activity proved challenging.

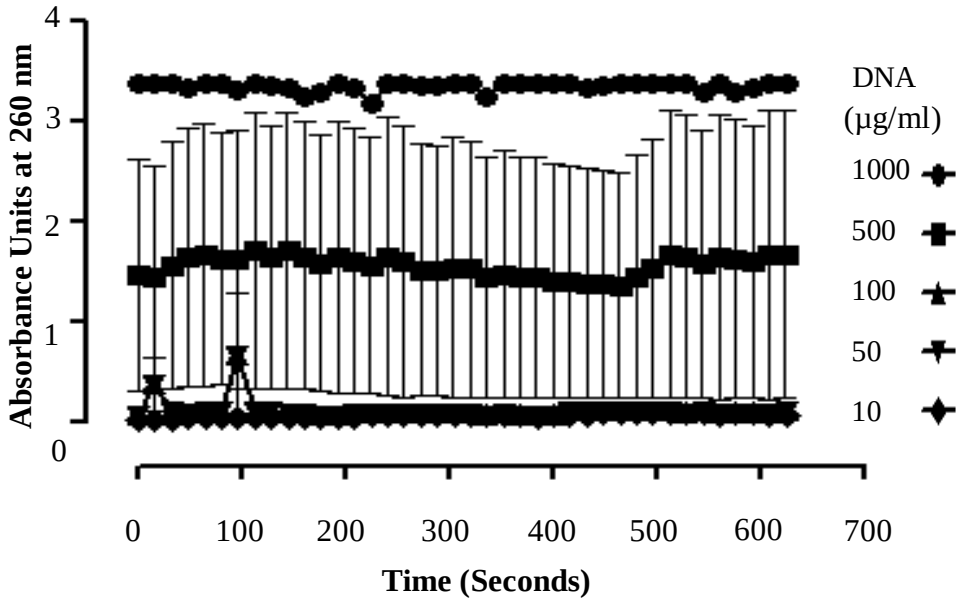


Figure 5-6 – Change in absorbance at 260 nm of DNase I treated solutions.

DNA concentration of solutions were 1000, 500, 100, 50 and 10 µg/ml. 1.77 Units free DNase I incubated with each condition for 10 minutes at 37°C prior to the start of the time course, where absorbance readings were taken continuously for a total of 624 seconds using the OMEGA plate reader at a wavelength of 260 nm at 37°C. n = 3 for each substrate concentration.

However, an approximation was possible for free DNase I by plotting the difference in absorbance at 260 nm between 0 and 624 seconds, using a higher quantity of enzyme; 1000 Units per reaction rather than the original 1.77 Units as used in all previous experiments with free and immobilised DNase I. In this manner a K_m of 54.02 µg/ml and a V_{max} of 228.7 µg/ml/min were obtained.

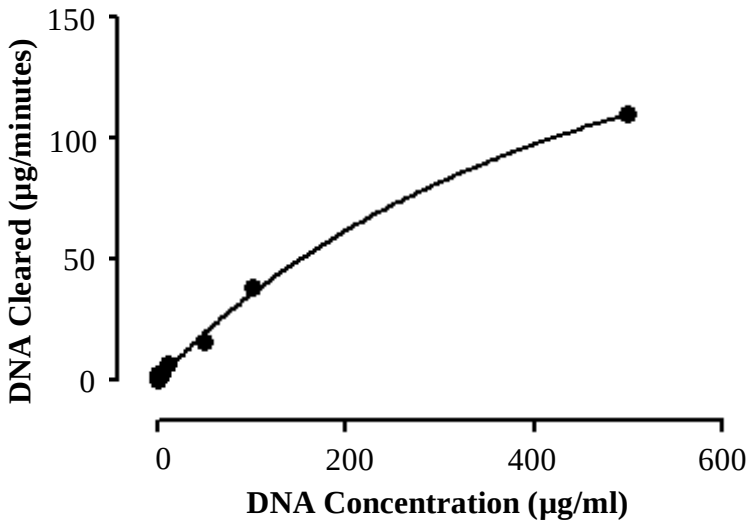


Figure 5-7 – Rate of DNA degradation by 1000 U free DNase I with increasing substrate concentration.

DNA degradation determined by a decrease in absorbance at the 260 nm wavelength. 10 00 Units free DNase I incubated with DNA (500, 100, 50 and 10 µg/ml) for 10 minutes at 37°C prior to the start of the time course. Absorbance readings were taken continuously for a total of 624 seconds using the OMEGA plate reader at 260 nm wavelength at 37°C. n = 6 for each substrate concentration.

Table 5-2 – Rate of DNA depletion by 1000 U DNase I.

Km and Vmax values, as calculated using non-linear regression with GraphPad Prism 4 software, are also included.

DNA Concentration (µg/ml)	DNA Cleared (µg/minute)
500	109.821
100	37.837
50	15.521
10	6.750
5	1.8750
1	1.859
0	0.000

Calculated V_{max} (µg/ml/min)	**228.7** (+/- 23.54)
Calculated K_m (µg/ml)	**54.02** (+/- 9.72)

Determination of true kinetic values for immobilised DNase I was not only theoretically impossible due to the failure to meet the Michaellis-Menten criteria, but also on a practical level, as the observed change in absorbance of a DNA containing solution over time was very slight (Figure 5-6). This is illustrated in Figure 5-8, where the activity rate of immobilised DNase I is compared to that of the free enzyme.

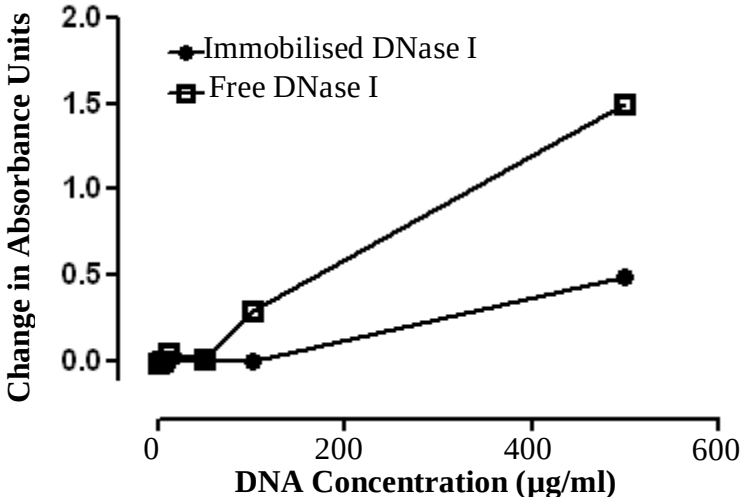


Figure 5-8 – Rate of DNA depletion by DNase I at varying substrate concentrations.

DNase I incubated with DNA (500, 100, 50 and 10 µg/ml) for 10 minutes at 37°C prior to the start of the time course. Absorbance detected using OMEGA plate reader (260 nm) at 37°C. Change in absorbance units calculated from T=0-T = 624 seconds. Data for free and immobilised DNase I are both shown. n=3 for each condition.

5.4. Discussion

For immobilised enzymes the true K_m and V_{max} could not be obtained and so the apparent values for these were derived. This was necessary as the state of being immobilised rendered the traditional model unsuitable for the bound enzymes; as described previously, part of the assumed criteria when calculating enzyme kinetics is that both enzyme and substrate are freely diffusible in solution, which is clearly not going to be the case when dealing with enzymes immobilised to a physical support.

It is evident from the gradient of the curve generated by the activity of lactate oxidase on a range of substrate concentrations that the immobilised enzyme is not as efficient at degrading lactate as is the free enzyme. However, it is to be noted that although there is a great difference between the $V_{max}/V_{max}\{app\}$ of these conditions (0.07523 mM/min and 0.003172 mM/min for free and immobilised LOx respectively), somewhat surprisingly the K_m values are similar (0.01911 mM for free and 0.01491 mM for immobilised lactate oxidase). This can be interpreted to signify that there is a large decrease in activity, but that there is no change in the affinity the enzyme has for its substrate; although the process of immobilisation results in the enzyme being unable to catalyse as many reactions per unit time as its free counterpart, physically tethering lactate oxidase to a support does not seem to have affected the ability of the enzyme to form a complex with its substrate molecule via the active site. Therefore, the immobilised enzyme could catalyse the conversion of lactate once it has bound its substrate, but perhaps mass transfer issues are hindering the frequency of an interaction between substrate and immobilised enzyme. As it stands, using the V_{max} of immobilised LOx (0.003172 mM/min), 100 mg of immobilised enzyme would require 2362 minutes (almost 39.4 hours) to clear 7.5 mM of lactate. However, increasing the quantity of immobilised LOx beads to just 1 gram could theoretically clear 7.5 mM lactate in under four hours.

Although the K_m obtained for an enzyme can vary between batches of isolated protein from the same source, it is of use to know what typical values could be expected to be yielded, in particular for the free, unaltered

enzyme. It has been reported elsewhere that for *Pediococcus* LOx, a K_m value of 2.3 x 10^{-4} M and V_{max} of 0.11 µmole/minute can be obtained (131). In the same study, LOx was immobilised onto alkylamine glass beads with a conjugation yield of 3.2 mg per gram of support and K_m and V_{max} values of 3.6 x 10^{-4} M and 0.22 µmol/minute respectively were calculated. This is in comparison to the data obtained in this study, with K_m of 1.91 x $^{-5}$ M and V_{max} of 75.2 µmol/min for the free enzyme and K_m of 1.49 x 10^{-5} M and V_{max} of 3.7 µmol/min for the immobilised LOx, indicating that the immobilisation conducted in this study resulted in a more active LOx than by the alkylamine glass method.

Issues surrounding mass transfer limitations could be countered when the immobilised LOx complexes are introduced into the FBB circuit – a continuous flow system, rather than the static conditions used in the assay to deduce the kinetic data, will very likely increase the frequency of substrate presentation. The implications for this, in terms of the suitability and practical considerations of using immobilised lactate oxidase within the bioreactor, are unclear based on this data alone. It could be hypothesised that if given adequate time the immobilised enzyme will be sufficient to reduce lactate concentrations within the bioreactor, without having to increase the total quantity of protein (and hence the number of beads) compared to the hypothetical amount of free lactate oxidase that would be required. However, this will have to be deduced experimentally, identifying a compromise point between time of treatment and the number of immobilised LOx beads required within the FBB chamber. Furthermore, the kinetic data was deduced in 10 mM phosphate buffer, and so may not reflect the kinetic profile of LOx in the presence of plasma constituents.

The determination of kinetic data for DNase I proved more problematic than for LOx. The major obstacle was that the criteria could not be met which was required for modelling K_m and V_{max}; not only was the enzyme no longer freely diffusible once immobilised, but there was also evidence to imply that the substrate, DNA, was also not entirely free in solution, as it too tended to bind to the glass beads. Therefore it was not possible to assign a true K_m to

immobilised DNase I. Nevertheless, an approximation of enzyme activity was possible by taking the specific activities of free and immobilised DNase I and comparing the typical extent of DNA clearance from a spiked solution for each enzyme condition. In this manner, a rough approximation of how much DNA could be expected to be cleared per unit time by immobilised DNase I was calculated.

Attention is drawn to Figure 4-12 of section 4.3.6 (page 110) which concerns the determination of the specific activity of immobilised DNase I. From the blot quantification it was possible to approximate the proportion of DNase I bound to the glass beads. By dividing the percentage degradation of a known quantity of DNA in a given time by the proportion of bound enzyme, an activity value could be designated per immobilised DNase I molecule per unit time, as well as per unit of activity (Table 5-3). Although these values are by no means a representation of actual K_m or V_{max} data, they can provide a guideline as to the proportion of DNA that could potentially be cleared by immobilised DNase I within a given time period. Our laboratory data has demonstrated that typically in a 10 litre working volume (containing 7×10^{10} cells) over an eight hour treatment period with ALF plasma, 47 µg/ml DNA can be detected after eight hours. The hypothetical calculations suggest that (assuming a linear rate of increase) a single 100 mg batch of immobilised DNase I would be able to clear 3.5 ng/ml/min DNA; extrapolating this further indicates that 100 times more beads, 10 grams of glass beads would require just 12.3 minutes to clear this concentration of DNA. However promising this seems, it must be remembered that these figures are hypothetical, having extrapolated existing data in the absence of strong kinetics data. Moreover, the kinetic analyses were conducted using standardised calf thymus DNA, whereas a crude extraction from HepG2 cells has been used for the majority of DNase I work in this thesis, as this represents the most probable form of DNA present in the BAL. Owing to the inhomogeneous identity of the HepG2 DNA extract it would not have been practical to utilise this for determining DNase I kinetic data. Therefore, the rate of HepG2 DNA digestion, particularly in plasma, may vary from the K_m and V_{max} values calculated in this chapter.

Table 5-3 – Hypothetical rates of immobilised DNase I activity, compared to free DNase I.

* = Based on the use of 100 µl 0.02 mg/ml DNase I for the immobilisation procedure, as described in section 4.2.2. ** = Data derived from analysis of Q-PCR analysis of samples incubated at 37˚C for 10 minutes with immobilised / free DNase I in a 200 µl reaction volume spiked to give a HepG2 DNA concentration of 100 ng/ml. ***= the DNase I powder (Sigma, D4527) comprised 2.8 mg of 3533 U/mg protein. Working solutions were diluted in 10 mM HEPES buffer to give a stock of 70.6 U/ml (0.02 mg/ml).

	Free DNase I	Immobilised DNase I
Total protein / reaction* (µg)	2	0.28
DNA cleared in 10 minutes** (ng/ml)	99.99	35.50
DNA cleared in 1 minute (ng/ml)	9.99	3.55
DNA cleared / µg protein (ng/ml/min)	4.99	12.62
Units DNase I / reaction***	7.06	2.10
DNA cleared / DNase I Unit (ng/ml/min)	1.42	1.69

5.5. Summary

Irrespective of the method used to approximate the kinetic profile of immobilised DNase I and LOx, it is apparent that the activity of the immobilised enzyme is reduced following immobilisation. However, the data acquired suggests that this residual activity may still be enough to clear circulating DNA and reduce sufficient lactate from the BAL, although the effects of plasma constituents and a dynamic, rather than static, reaction environment must be considered. This will be investigated in the following chapter, where a scaled-down representation of the BAL is used to model the conditions within the BAL device.

Chapter 6. Immobilised Enzymes in the BAL

The efficacy and practicality of using immobilised Deoxyribonuclease I and Lactate oxidase in the BioArtifical Liver Device / Fluidised Bed Bioreactor is investigated, careful consideration being given to downstream effects on encapsulated HepG2 cells and circulating protein concentration.

6. Immobilised Enzymes in the BAL.

6.1. Introduction

It has been demonstrated in previous chapters that enzymatic activity can successfully be harnessed by immobilising these biological catalysts onto a solid support. The motivation for this is the desire to utilise immobilised enzymes within a Bio-Artificial liver assist device (BAL), both in the cell production stage of the fluidised bed bioreactor (FBB) and in the actual patient treatment stage, in order to optimise the efficacy and safety of this device in the alleviation of ALF. However, although both immobilised LOx and DNase I have been investigated under conditions reminiscent of those to be encountered in the intended application (physiological pH and temperature, and in the presence of human plasma) there are other factors and challenges to be considered before it can be more accurately determined whether these complexes will function well in the proposed BAL system. Both enzymes must be able to withstand any potential sheer stress induced by the constant fluidisation of the BAL/FBB circuit; in terms of resistance to denaturation by physical forces, as well as ensuring that the immobilised enzyme beads are in an appropriate setting to actually encounter their target substrate and more importantly, catalyse its degradation.

6.1.1. Protein depletion

There is also the matter of the glass support interacting unfavourably with and sequestering plasma proteins; it is of high importance that any measure introduced to enhance BAL productivity does not compromise either its function or patient safety. ALF patients typically present with depleted levels of liver synthesised proteins circulating in their plasma, such as clotting factors (38). The purpose of treating with a BAL, rather than a purely artificial (filtration) approach, is to replace this loss of function by utilising a liver derived cell line to perform the metabolic and synthetic functions of the patient's damaged liver. Therefore, it is critical to the success of the BAL that the newly synthesised proteins are not being

sequestered or in any other way reduced in functionality by either the BAL itself or by any component of the device.

6.1.2. Hydrogen peroxide production

There is a potential issue with the equimolar generation of H_2O_2 from the degradation of lactate by LOx (122). H_2O_2 is a known inducer of oxidative stress in cells and so it follows that elevated concentrations within the BAL system should be avoided. However, it has been previously reported that ELS show upregulated production of the enzyme catalase, which is naturally present in the liver and catalyses the breakdown of H_2O_2. Therefore, it may be that the BAL ELS can metabolise an elevation in H_2O_2 concentration, although the extent, if any, of this must be thoroughly assessed.

6.1.3. Small-scale BAL experiment

Having demonstrated the efficacy of immobilised LOx and DNase I to reduce levels of lactate and DNA respectively in physiologically relevant conditions, the next logical step would be to apply these technologies simultaneously in a system which mimics the BAL as closely as possible. In addition to incubating for up to eight hours at a physiological pH, temperature and in the presence of plasma, immobilised enzymes were applied to a mini-column system, in order that the effects of flow rate and sheer stress, if any, could be investigated.

6.2. Materials & Methods

6.2.1. Protein depletion

Materials:
Enzyme conjugated glass beads (see sections 3.2.3 and 4.2.2)
FBB conditioned media
15 ml polypropylene tubes (Corning®)
1.5 ml microcentrifuge tubes
Liquid nitrogen
Incubator
Rotational mixer

Enzyme linked beads were prepared, as described in previous sections, in 100 mg batches and each applied to 10 ml of conditioned media, taken from the media change on Day 7 of encapsulated HepG2 spheroids cultured in the FBB. Control samples contained conditioned media only. These were incubated at 37°C for eight hours with constant mixing to mimic the BAL environment. Samples of 100 µl were taken at hourly intervals and frozen prior to assaying for alpha fetoprotein by ELISA (see section 2.6 for detailed methodology).

6.2.2. Effect of H_2O_2 on ELS

Materials:
Day 10 ELS
10% FFP media
Hydrogen peroxide (VWR, 23615.261)
6-well plate (Nunc)
1 M HEPES (pH 7-7.6, Sigma, HO887)

For this experiment, ELS which had been growing in the FBB for ten days were used. 1 ml of cells were transferred to a well of a 6-well plate containing a total volume of 4 ml fresh 10% FFP media spiked with H_2O_2. Two concentrations of H_2O_2 (Mr = 34) were tested; 7 mM and 0.07 mM, obtained by adding 28.6 µl of 979 mM stock or 9.79 mM pre-diluted H_2O_2 respectively to the FFP media. To the control cells, H_2O_2 was substituted with 28.6 µl H_2O. For each condition, a cell-free counterpart was also in place as a negative control for H_2O_2 degradation. All wells received 40 µl

1M HEPES to buffer pH to 7.4. Plates were incubated at 37°C for 10 minutes, after which the supernatants were removed and retained for subsequent analysis. Cell samples were also taken to compare their viability with those prior to experimentation.

6.2.3. Siliconisation of glassware

The process of siliconisation utilises dichlorodimethylsilane to replace the hydrophilic hydroxyl groups on the surface of glass, resulting in a hydrophobic surface which is far less likely to interact and bind via hydrogen bonding with molecules such as proteins and DNA.

Materials:
Dichlorodimethylsilane (Sigma, 33009)
Dichloromethane (Sigma-Aldrich, 270997)
Ethanol
dH_2O

A 5% solution of pre-chilled dichlorodimethylsilane was made in dichloromethane, working in a fume cupboard as HCl vapour is liberated during this procedure. Glassware was immersed in the solution for 30 minutes, whereupon it was rinsed in dichloromethane, ethanol and then dH_2O. Treated glassware was allowed to air dry and stored in clean autoclave bags until required.

6.2.4. Mini-column experiment

A miniaturised working model of the BAL allowed small scale experiments into the effectiveness of various measures to improve functionality of the system.

Materials:
Glass columns of length 185 mm and internal diameter 10 mm (15 ml volume)
Polypropylene screw cap, diameter 15 mm with an 8 mm circular opening in the top
Tapered glass fitting (12 mm base, 1 mm apex)
200 micron mesh filters (comprised of two rubber O-rings, diameter 12 mm)
Silicon tubing of length 75 cm and 50 cm
Pump tubing (2.7 mm bore size, 0.5 mm thickness)
250 ml polypropylene reservoir chamber bottle
Watson-Marlow 520Du peristaltic pump with ten channel pump
Sampling septum
Sterile 5 ml needle
100 mg DNase conjugated glass beads
100 mg LOx conjugated LOx beads
FBB conditioned medium / FFP with 40 IU/ml heparin
Lactate
HepG2 DNA

All glassware was siliconised prior to use, as previously described. Each mini-column circuit was arranged as shown in Figure 5-9. In summary, for each mini-column circuit: filter discs (comprising a 200 micron nylon mesh adhered with silicone glue between two 12 mm rubber O-rings) were positioned at either end of the glass tube, held in place by polypropylene caps which also housed the tapered glass connectors. To the connector on the top section of the column one end of a 75 cm silicone tube was attached, the other being connected to a luer fitting in the lid of a 250 ml bottle (the reservoir). From another luer fitting in the lid a T-piece was joined, to which a septum and a section of 50 cm silicone tubing was connected. The pump tubing was connected to the terminus of this tubing, which was threaded into a multichannel pump. On leaving the pump, the tubing was joined to the 75 cm silicone tubing, thus completing the circuit.

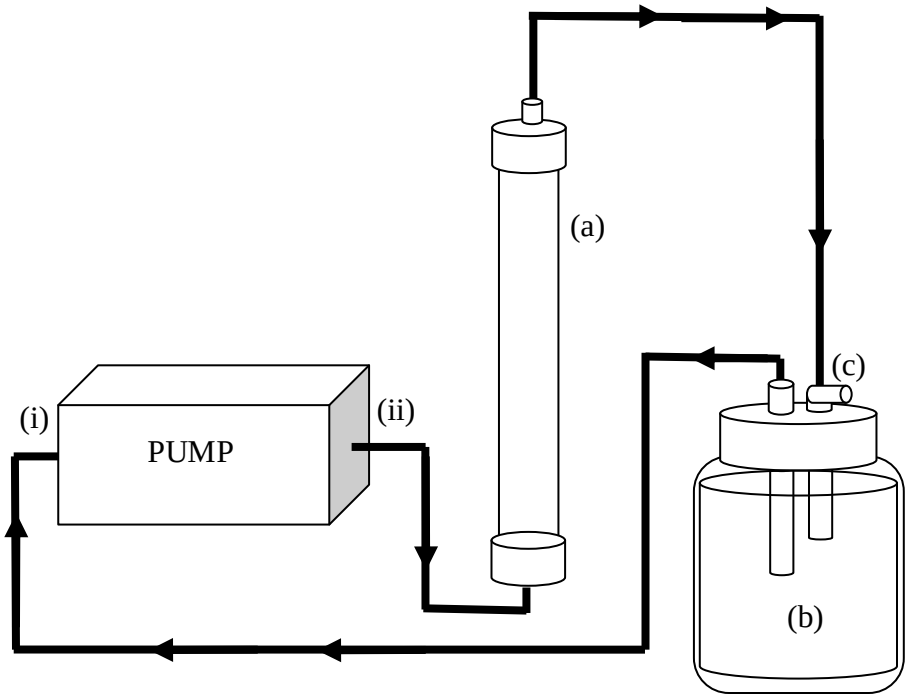


Figure 6-1 – Schematic diagram of the mini column circuit.

For clarity only one column is depicted; multiple column circuits were included by attaching their tubing to the pump at points (i) and (ii). Enzyme cross-linked glass beads were housed in the base of the column (a). A reservoir of 10% FFP media / 100 FFP (b) fed the system which was drawn through the column by a multichannel pump. Samples were taken using a syringe from the septum in the reservoir lid (c).

A solution comprising 0.864 g lactate dissolved in 10 ml dH_2O and pH adjusted to 7.4 was added to 800 ml Day 10 FBB media, giving a final lactate concentration of 12 mM. To this 5.2 µl heparin and 150 µl HepG2 DNA were added and 200 ml of the resulting solution transferred to each mini-column circuit. 100 mg each of DNase / LOx conjugated glass beads were prepared as described previously and the total 200 mg transferred to each glass column. For the control, 200 mg treated but non-enzyme conjugated glass beads were used. The pump was run at a rate of 18 ml/min for six hours at 37°C. Samples were taken from the septum using a needle and 2 ml syringe every hour and snap frozen in liquid nitrogen to await analysis.

6.3. Results

6.3.1. Protein depletion

In order to determine the extent to which plasma proteins are sequestered by the immobilised enzyme beads, 100 mg of these beads were incubated in Day 5 conditioned media and the protein content after a six hour incubation with constant mixing at 37°C compared with that of the initial, pre-treatment value. Media samples without any added beads were also incubated, to control for any loss of protein which may occur as a consequence of external factors such as heat, time and the affects of the plastic-ware utilised. Results are shown in Figure 6-2.

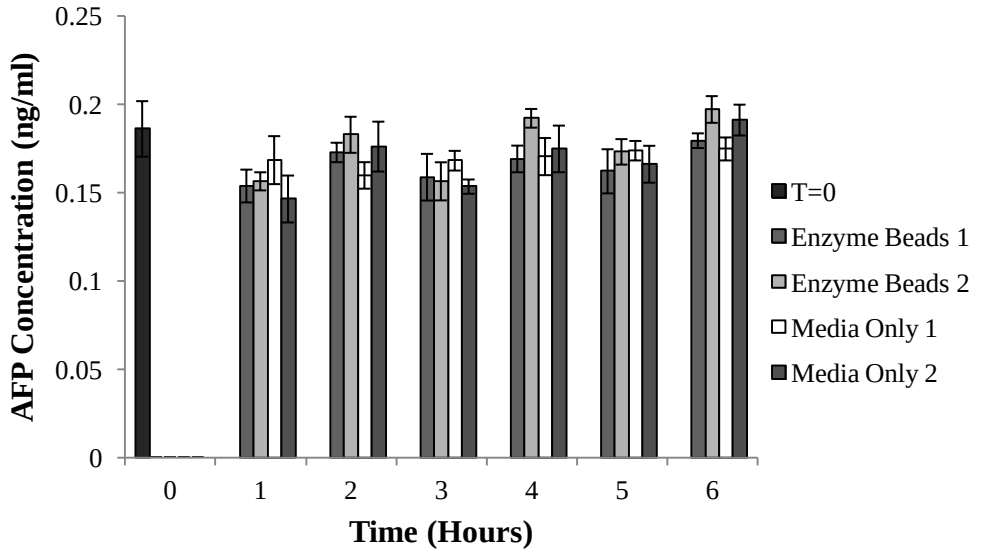


Figure 6-2– Effect of immobilised enzyme beads on plasma protein content.

100 mg enzyme linked glass beads (DNase I and LOx) were incubated for a period of six hours with 10 ml conditioned media which was obtained from the media change on Day 5 of encapsulated HepG2 cell growth in the FBB. Hourly samples of 100 µl were snap frozen prior to screening for AFP in an ELISA. Samples also taken from conditions where conditioned media alone was incubated ("Media Only" – negative control). Conditions run in duplicate, n=3 for each. Error bars denote standard deviation of the mean.

6.3.2. Effect of H_2O_2 on ELS

The data, particularly from the higher H_2O_2 dose condition, demonstrates that H_2O_2 levels were lower when incubated with ELS for 10 minutes than when incubated in the absence of cells (Figure 6-3).

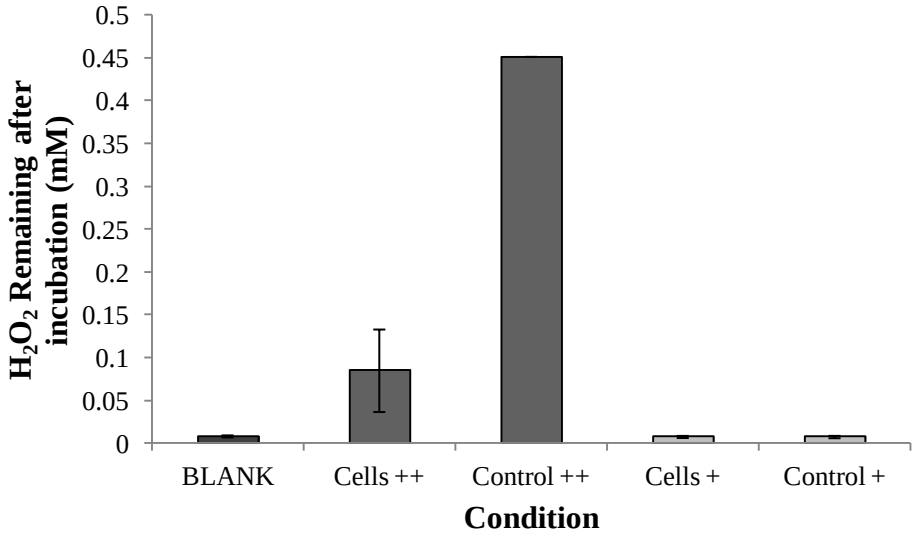


Figure 6-3 - Intrinsic hydrogen peroxide clearance by encapsulated HepG2 cells.

Key to labels: BLANK = assay reagent only (equivalent hydrogen peroxide volume substituted with water); + = 0.07 mM hydrogen peroxide dose; ++ = 7 mM hydrogen peroxide dose. Control condition contained reaction solution only. n=3 for each condition Error bars depict +/- SD.

MTT assay data shows that although ELS subjected to H_2O_2 showed reduced mitochondrial activity, suggestive of metabolic stress, there was little difference between the high and the low H_2O_2 dosed conditions. However, once this data was corrected for by normalising to protein concentration determined by BCA assay, there was no statistical difference between cells from the two H_2O_2 conditions. It is to be noted that the untreated ELS demonstrated slightly better metabolic activities than their H_2O_2 treated counterparts.

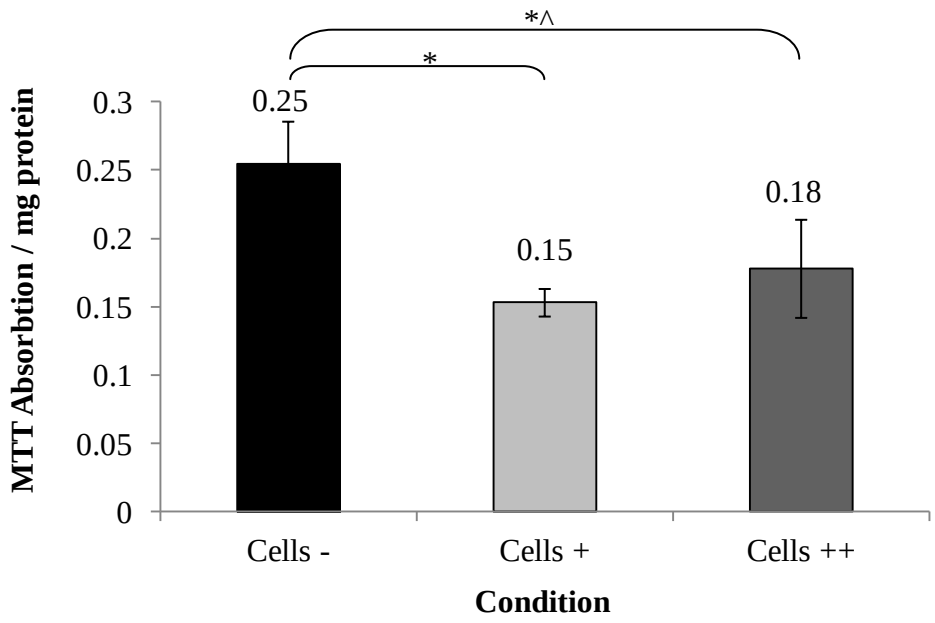


Figure 6-4 – Metabolic function of encapsulated HepG2 cells following hydrogen peroxide treatment.

Normalised to protein concentration, determined by BCA assay. Key to conditions: - = no hydrogen peroxide administered; + = 0.07 mM dose; ++ = 7 mM dose. n=3 for all conditions, +/- SD. T-Test (two-tailed, equal variance) *p<0.05 *^= value obtained of 0.0505

Fluorescent microscopy of ELS before and after the treatment shows that although the proportion of viable cells was lower after H_2O_2 exposure, the viability of untreated ELS was also lower than before the commencement of the experiment, suggesting that at least a proportion of cell death was due to factors other than exposure to H_2O_2 (Figure 6-5).

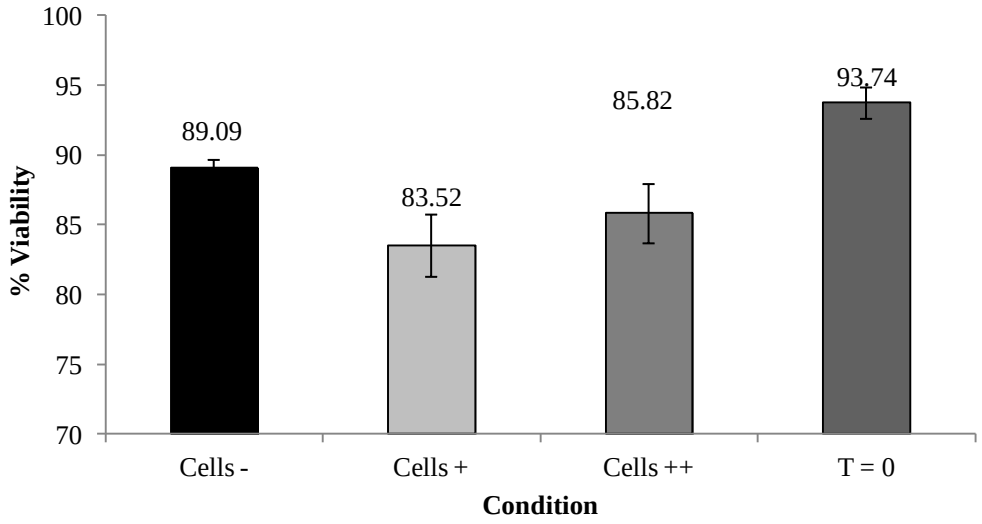


Figure 6-5 – Effect of H_2O_2 on encapsulated HepG2 cell viability.

Day 11 encapsulated HepG2 cells were assayed for viability before and after treatment with hydrogen peroxide spiked 10% FFP culture medium. Viability determined using fluorescent microscopy with propidium iodide (excitation filter of 510-560 nm, emission filter of 590 nm) to mark non-viable and fluorescein diacetate (excitation filter of 465-495 nm, emission filter of 515-555 nm) to mark viable cells. Samples were imaged using Lucia imaging software with a DX1200 camera on a Nikon Eclipse microscope. Images were taken at 4x on phase contrast, live FDA and dead PI settings. Key to conditions: - = no hydrogen peroxide administered; + = 0.07 mM dose; ++ = 7 mM dose; T = 0 = viability of cells before commencing the experiment. n=3 for all conditions, +/- SD.

6.3.3. Small-scale BAL experiment

The first mini-column experiment was conducted as a representation of the conditions likely to be found in the BAL during patient treatment, although for this initial experiment, ELS beads were not included. Conditioned 10% FFP media from Day 10 of an FBB experiment was used, as this had been exposed to growing ELS beads for a period of three days and so would contain protein and molecular components, including some DNA, of HepG2 origin. DNA was not added as human plasma is known to contain some background levels of DNA (typically 10.2 ng/ml (61)). Together, these factors should be representative of the majority of DNA likely to be presented to the DNase I beads during an eight hour BAL treatment period, assuming minimal cell damage and hence negligible DNA release into the circulation. A lactate spike was added to the media in order to fully

investigate the lactate clearance capacity of immobilised LOx in the mini-columns; although such high lactate levels are unlikely to be encountered in the BAL, the LOx beads would have first had to catalyse the conversion of lactate from the FBB, where lactate concentrations would be much higher than in the BAL, and so lactate was added to mimic this earlier role.

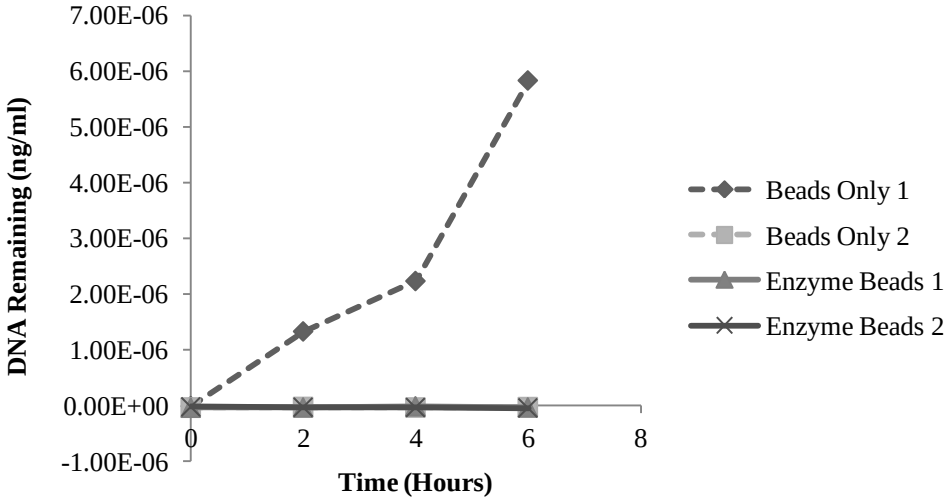


Figure 6-6 – DNA concentration in 10% FFP media mini-column experiment.

100 mg DNase I conjugated glass beads ("Enzyme Beads") or 100 mg glass beads ("Beads Only") were housed in the base of individual mini-column circuits containing Day 10 conditioned FBB media (10% FFP) buffered with HEPES to pH7.4 and treated with heparin to prevent coagulation of plasma proteins. Samples incubated at 37ºC for a total of six hours with a flow rate of 18 ml/min. 500 µl samples were taken initially and then at hourly intervals. DNA concentration determined by Q-PCR analysis. Mean values plotted for each condition, n=3.

The same samples were also analysed for lactate concentration, in order to establish whether the LOx conjugated glass beads were effective under the mini-column conditions.

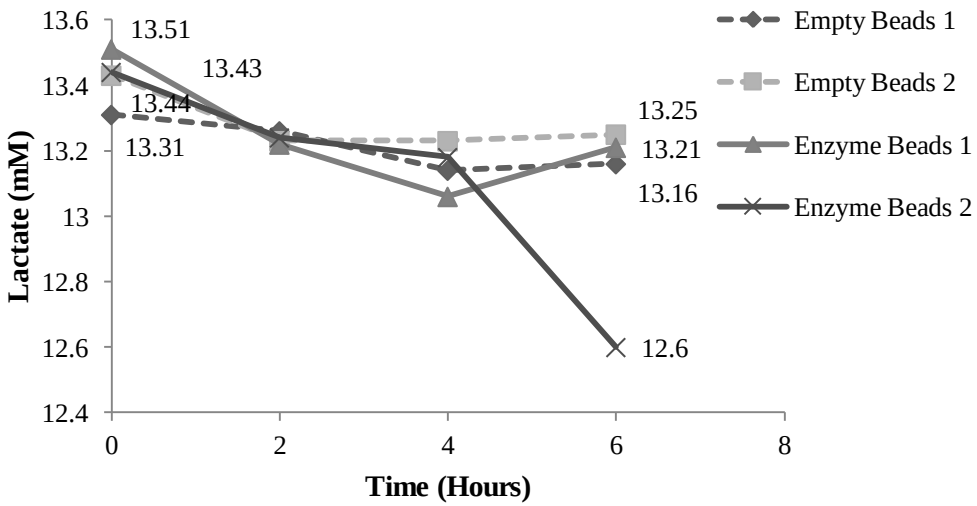


Figure 6-7 Lactate concentration of samples taken from the mini-column experiment.

500 µl samples of lactate spiked Day 10 FBB culture media were taken initially and then every two hours from each mini-column system. "Empty Beads" denotes the negative control, of glass beads without enzyme; "Enzyme Beads" refer to conditions where 100 mg each of DNase I and LOx conjugated beads were housed within the mini-column. Mini-columns were maintained at 37°C at a flow rate of 18 ml/ min. Media was treated with HEPES and heparin to buffer to pH7.4 and prevent clotting, respectively. Mean values shown for each data point, n=3.

In the next experiment, 5 ml of ELS beads were included with the immobilised enzyme beads in the mini-column, with 100% FFP, to represent the closest imitation of the BAL conditions. All other methodology and analysis was as previously described, except that samples were taken at T=0, after one hour, and then at 30 minute intervals up to four hours, after which hourly samples were taken. The results of this investigation are shown in Figure 6-8.

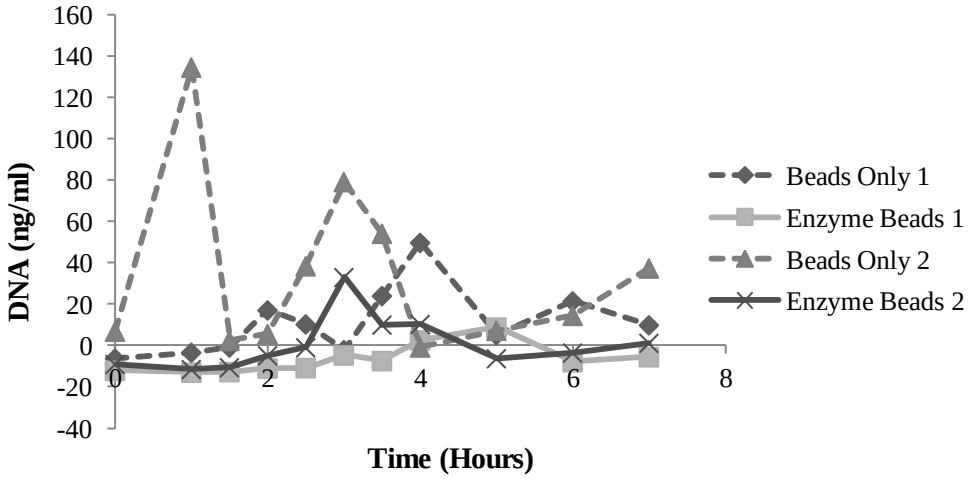


Figure 6-8 - DNA concentration in the mini-columns – with ELS.

500 µl samples of lactate spiked FFP were taken initially, after one hour and then every 30 minutes up to four hours, after which hourly samples were then taken from each mini-column system and assayed for DNA content by q-PCR. "Empty Beads" = negative control (glass beads without enzyme); "Enzyme Beads" = 100 mg each of DNase I and LOx conjugated beads were housed within the mini-column. Mini-columns were maintained at 37ºC at a flow rate of 18 ml/ min. FFP was treated with HEPES and heparin to buffer to pH7.4 and prevent clotting, respectively. Mean values shown for each data point, n=3.

Samples were also assayed for lactate concentration, the data for which is shown below.

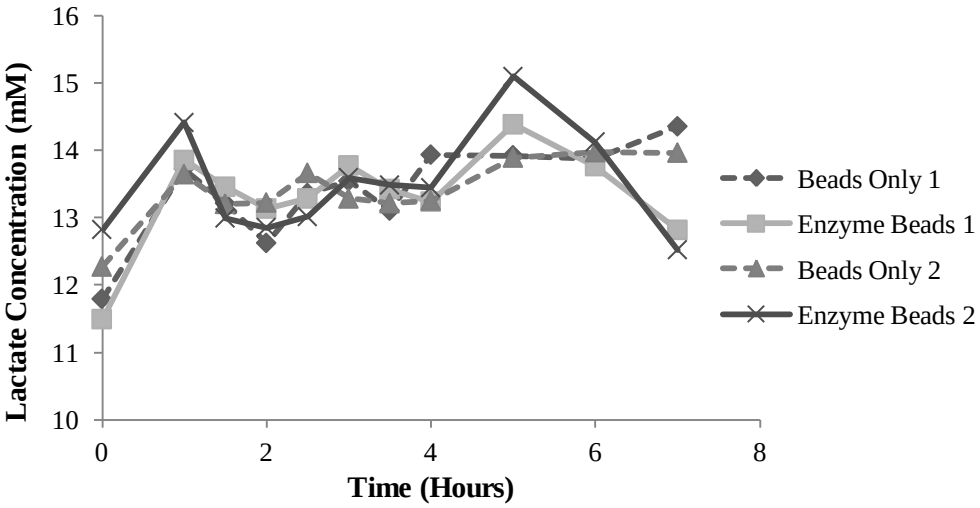


Figure 6-9 - Lactate concentrations within the mini-column with ELS.

From the 500 µl samples taken during the mini-column experiment 3.5 µl were assayed for lactate using the Analox® apparatus. Readings were taken in at least duplicate per sample. Mean values of readings which fell within 0.1 mM of each other are shown.

Once plotted, the lactate concentrations revealed that there was great fluctuation and variation of the data points both within and between samples. To better visualise this change, the cumulative lactate removed from the system between all time points was plotted for each sample (Figure 6-10). Furthermore, as lactate appeared to have been added to the system over time, the difference in lactate concentration between the start and end of the experiment was plotted (Figure 6-11).

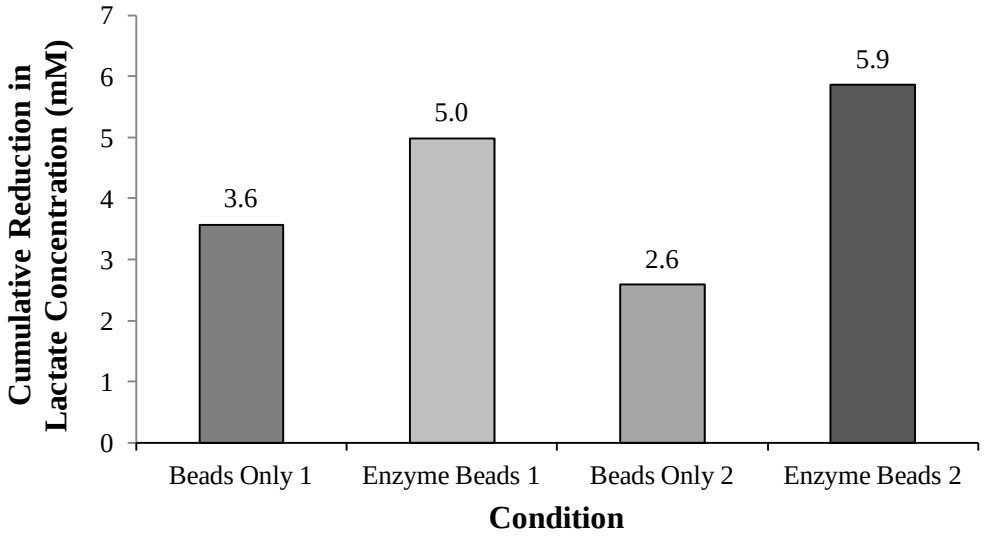


Figure 6-10 – Cumulative reduction in lactate concentration.

In calculating the cumulative lactate cleared, only positive differences between sample times (i.e. representative of a reduction rather than an increase in lactate concentration) were included.

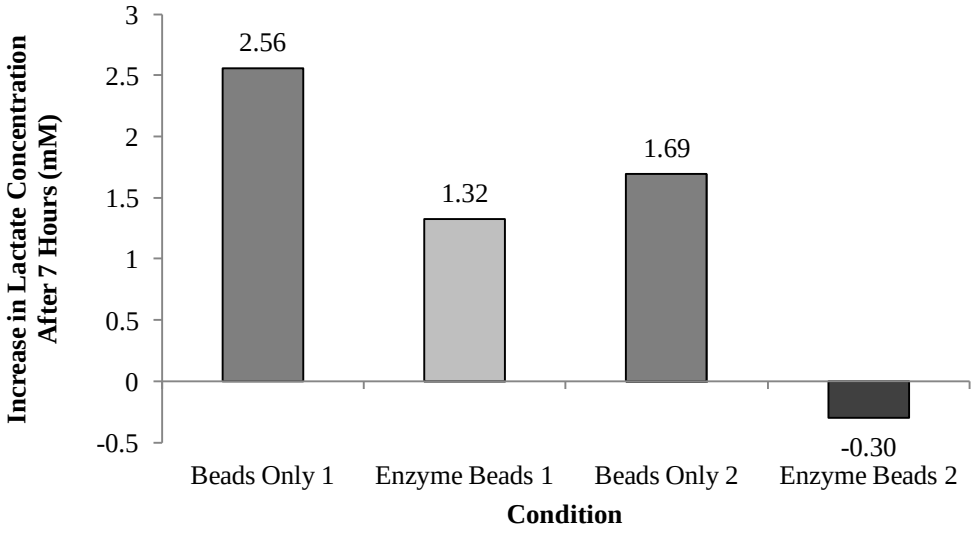


Figure 6-11 – Increase in lactate concentration after the experimental period.

Values obtained by subtracting T=7 hours lactate concentrations from those at T=0 for each condition. Negative values, as in "Enzyme Beads 2", represent an overall reduction in lactate concentration.

6.4. Discussion

6.4.1. Protein depletion

A significant advantage that the BAL would have over a purely artificial method of liver failure support is that in addition to removing hazardous components, the plasma can also be supplemented with metabolites and proteins synthesised by the encapsulated HepG2 cell spheroids. Therefore, it is essential that the inclusion of immobilised enzymes into the BAL device does not have a detrimental impact upon the protein content of the plasma / growth media. Alpha fetoprotein (AFP) was chosen to represent plasma protein content as previous analysis undertaken by the Liver Group has demonstrated that encapsulated HepG2 cells synthesise detectable levels of AFP during FBB culture, which is not usually found in normal human plasma, thus permitting even small background levels to be measured.

The data demonstrated two key phenomena: firstly, that no significant difference in AFP levels can be seen between control and immobilised enzyme conditions, and secondly, that after two hours, there is no longer a significant difference between the T=0 and subsequent hourly samples, indicating that AFP is not being sequestered by the enzyme linked glass beads. This investigation also revealed that there is a degree of variability between replicates of the same experimental condition; this could represent the non-specific manner of protein adhering randomly to the plastic-ware.

6.4.2. Effect of H_2O_2 on ELS

The degradation of lactate is required in order to ensure lactate concentrations in the FBB do not accumulate to such an extent that they have detrimental effects on ELS proliferation or compromise the integrity of the alginate beads themselves. The use of immobilised LOx is an elegant approach to achieving this, as from the conversion of lactate, pyruvate is generated, which could be used as a metabolic substrate by HepG2 cells. However, in the enzymatic conversion of lactate, hydrogen peroxide, a known agent of oxidative stress, is also produced in a stoichiometric fashion. For this reason, it was crucial to determine whether this would prove detrimental to cell performance. When incubated with a relatively high dose of H_2O_2, the addition of ELS saw a reduction in supernatant H_2O_2, indicative of an active catalase function in these cells. The significance of this finding is that the intrinsic catalase activity of HepG2 cells may well be sufficient to clear any additional H_2O_2 produced as a by-product of immobilised LOx driven lactate conversion. Although metabolic activity decreased in cells in the presence of the lower H_2O_2 dose, there was no significant difference between cells given the higher H_2O_2 dose and the negative control, implying that HepG2 cells would unlikely be affected by the presence of 7 mM H_2O_2.

6.4.3. Small-scale BAL & immobilised enzyme efficacy

Despite performing the mini-column experiment on two separate occasions, both investigations suffered from inconsistent results. It was observed that, for the first experiment, one sample replicate for each parameter, lactate or DNA concentration, generated results which would have been expected based on previous work, yet the partner replicate did not. For both parameters, the negative control "Beads Only 1" and the test condition "Enzyme Beads 2" appeared to follow the general trend expected of those conditions; namely, DNA and lactate levels remained mostly unchanged in the negative control and were reduced when immobilised enzyme beads were present. However, the replicate condition for each did not follow this, nor any, pattern. This could, in part, be due to the low levels of DNA present in the test conditions; having normalised the data by subtracting the NTC (i.e. plasma only condition) the values obtained were very low, often running into the negative value range. It is therefore unlikely that there was any real difference in DNA concentrations between the four mini columns; on such a small scale, even the apparent peak of the "Beads Only 1" condition could potentially be an artefact of PCR and low level DNA "noise".

The apparent decrease in lactate concentration in the "Enzyme Beads 2" column is more likely to be a genuine result, as the value falls comfortably within the limit of detection of the analysis method used. Nevertheless due to the frequent leaking of solution from the mini-columns, fair test conditions could not be adhered to as some columns had to be removed from the circuit for repair work, thus altering the duration of treatment and also the volume of solution to be treated by the immobilised enzyme or empty glass beads. The inter-condition, and indeed, inter-sample variation (see fluctuation of data points in Figure 6-6 and Figure 6-7) is likely a result of the assembly of the mini-columns themselves and the technical difficulties they presented. All four mini-column circuits were subject to leakage of varying degrees; notably, for the circuit assigned "Enzyme Beads 1", the pump had to be stopped on multiple occasions, for periods of up to 30 minutes, in order to try to stop leaks from either the base or top of the

glass column chamber. As a consequence, it would be unjustified and impossible to conclude that the immobilised enzymes had any benefit over the negative control in this experimental situation.

The mini-column experimental protocol underwent some modification for the second experiment; notably, the inclusion of 5 ml encapsulated HepG2 cells. However, the experimental set up once again proved problematic and leaking mini columns meant that uniform experimental conditions could not be maintained. Furthermore, it was observed that seven hours into the experiment, almost complete depolymerisation of the alginate beads had occurred. This was likely due to citrate, which was added to the FFP during collection from the human donor; citrate is a potent chelator of divalent ions, and so in this fashion sequestered calcium away from the alginate matrix, leading to bead depolymerisation. Another consequence of this chelation, is that DNase I activity could likely have been negatively affected, by removing the calcium and magnesium ions upon which the enzyme is dependent for catalytic activity. Hence, should this experiment be repeated, calcium should be titrated into the FFP to negate the action of citrate. It should be noted that during the course of treatment with the BAL, the patient would not be treated with citrate as an anticoagulant, but heparin, thus avoiding this situation.

A loss of volume due to a leak would result in a lower pressure within the mini column circuit, hence decreasing the extent of fluidisation of the ELS and so subjecting them to stressful conditions. The delay caused when the pump had to be stopped in order to repair columns is likely to have contributed to the rising lactate levels demonstrated in Figure 6-9; when no longer fluidised the HepG2 spheroids would not have sufficient oxygenation and so would rely more upon glycolytic respiration, generating lactate. The extent of fluidisation, and hence oxygenation would have been affected by the time for which the pump was stopped and also the extent of leaking, which would vary between columns and hence could account for at least some of the inter sample variation in trend for the same condition. Interestingly, by studying the cumulative lactate removed from each column

during the treatment period, it would seem that the LOx beads are functional, as both enzyme bead conditions show greater lactate clearance than the negative controls (Figure 6-10). It is clear that the ELS were producing lactate as a response to poor fluidisation, as lactate levels rose above that of the initial spike. Yet, plotting the overall rise in lactate over the experimental period reveals that this was less so, or indeed, lactate was reduced, in the columns containing immobilised enzyme beads, again indicating that they were functional in this context. However, this was not sufficient to reduce lactate levels below the ~ 10 mM threshold.

It must be considered that this does not necessarily imply that LOx beads would be ineffective in the BAL; rather that more than the seven hour treatment time is required to clear 15 mM lactate. This experiment represented a "worst case scenario", in other words, the treatment of a critically ill patient with exceptionally elevated lactate levels; however, the LOx beads would only be absolutely required to clear lactate during the 10 day growth phase of the encapsulated HepG2 cells, which would see gradual lactate production and thus much more likely to be cleared by the LOx beads (note the results shown in Chapter 3 which demonstrated an appropriate reduction in lactate concentration). During the treatment phase of the BAL, should such high lactate levels be present in the patient's plasma, other, traditional treatments (such as the addition of buffering agents) could be administered to prevent the depolymerisation of the alginate beads.

A rise in DNA levels seen in the negative control columns is also likely due to cellular stress, damage and death caused by the lack of fluidisation, and also physical cell damage when passing through the cap filters once the cells were no longer within the alginate beads. It is interesting to note that DNA concentrations remained much lower in columns which contained immobilised DNase I than in the negative controls (Figure 6-8) strongly suggesting that the DNase I is effective, if not functioning optimally under these unintentionally harsh experimental conditions. However, as in the previous mini column experiment, the very lowest DNA values run into the negative values. This demonstrates a limitation of Q-PCR as a means of

DNA detection in plasma; in order to avoid reaction inhibition, it was necessary to dilute all samples 1/5000 prior to applying to the PCR. Whilst possible to compensate for this when generating the standard curve by adding more DNA prior to dilution, this is not only practically unfeasible to do with samples (the amount of DNA required to spike the total volume of each mini column circuit to the concentration used in the previous DNase I experiments (100 ng /ml) would be in excess of 20 µg) but also defeat the purpose of trying to determine whether the immobilised enzyme can clear levels of DNA typically released from HepG2 cells under normal BAL conditions. Having been so heavily diluted, small values are distorted, particularly after normalisation against the NTC, and so any pattern observed may be an artefact.

In conclusion, the data generated by this series of experiments cannot empirically support nor reject the hypothesis that enzyme immobilised beads can reduce levels of DNA and lactate in a BAL context. Before this can be addressed, it is first critical that the mini-column apparatus and methodology is fine-tuned and improved in order that it can be assured that the experiment will not be compromised by technical errors and inaccuracies. Only then can the experimental protocol be repeated and the efficacy of DNase I and LOx conjugated beads truly tested in conditions as reminiscent as possible of those in the BAL device.

Chapter 7. General Discussion & Future Work

7. General Discussion & Future Work

The aims of this thesis were to develop a means of reducing concentrations of lactate and circulating DNA of non-patient origin. An enzymatic approach was chosen whereby the chemical tethering of the enzyme onto a variety of solid supports was investigated and the extent of substrate clearance used as an indicator of the efficacy of the immobilised enzyme complex. As these complexes would be required to function in the presence of plasma constituents within a physiological temperature and pH range, experimental situations were devised to mimic such conditions as closely as possible.

7.1. Lactate Clearance

Lactate is produced by HepG2 cells as a by-product of glycolytic respiration, which, unless measures of intervention are taken, could accumulate within the BAL chamber to concentrations in excess of 15 mM. At these elevated levels of lactate a detrimental effect is observed upon cell proliferation rates (group data). Additionally, in 10 mM lactate, ELS beads visibly swell, indicative of a loss of integrity due to chelation of calcium ions, thus compromising the integrity of the alginate beads used to house populations of HepG2 spheroids. In order to maintain the proliferative potential and structural integrity of the ELS, and hence maximise efficiency of the BAL unit, it is therefore desirable to employ a means of ensuring lactate concentration does not exceed a critical threshold. One approach would be to perform frequent media changes of the FBB; however, this may be considered wasteful of media, and is both time and labour intensive.

In this study an enzymatic approach was elected to bring about a “real time” reduction in lactate, by utilising the enzyme lactate oxidase to catalyse the conversion of lactate in the presence of oxygen to pyruvate. It has been demonstrated in this thesis that LOx immobilised onto glass beads retains enzymatic activity in the presence of plasma and over a period of at least 11 days to reduce lactate levels below the 10 mM threshold, whereby it no longer poses a threat to the integrity of alginate encapsulated HepG2 cells.

7.1.1. Role of LOx in the BAL

Although lactate oxidase was immobilised for use within the BAL primarily in the cell growth phase of ELS culture in the FBB in order to overcome detrimental effects on the alginate encapsulated cell spheroids, there exists a potential for this technology in other applications. The second phase of BAL treatment, whereby the patient's plasma is passed through the BAL device, could in particular benefit from incorporating immobilised lactate oxidase to reduce lactate concentrations in the plasma before returning it to the patient. Lactate concentration is an established prognostic tool in the determination of patient outcome in Acute Liver Failure (ALF), particularly in acetaminophen-induced cases (154). In healthy individuals lactate is converted by the process of gluconeogenesis in the liver but in ALF a net splanchnic release of lactate is observed (155). Elevated lactate concentration is strongly correlated with increased mortality rates; patients with serum lactate levels above 2 mmol/L which persist for over 24 hours, have been shown to have a 70% increased mortality rate (156). Lactic acidosis occurs following accumulation of lactate, produced via the anaerobic metabolism of glycolysis in hypoperfused tissue; with prolonged oxygen deficit, acidosis ensues and the body's buffering capacity is exceeded (157;158). Although a mode of providing glucose rapidly, anaerobic glycolysis (utilising the glycolytic pathway) is much less efficient than standard aerobic respiration, generating just two molecules of ATP, compared to the 15 generated in aerobic respiration, per glucose molecule (159). It is therefore in the interest of the patient to repay the oxygen debt and restore lactate concentrations to the normal range of 0.5-1 mmol/L. However, the situation is exacerbated in liver failure patients; the liver normally converts blood lactate via gluconeogenesis to glucose, which is then used as an energy source for the hepatic cells. Yet the ability of the liver to take up lactate is concentration dependent, the extent of uptake being negatively proportional to the blood lactate concentration. Therefore, by removing less lactate from the blood, a failing liver not only limits its own energy supply, but also contributes to the progression of hyperlactemia.

Historically, bicarbonate has been used to buffer the excess anions in patients presenting with lactic acidosis, but this has now been phased out since it frequently proved more harmful than beneficial, owing to an increase in carbon dioxide and a further lowering of pH. Carbicarb, an equimolar mix of sodium bicarbonate and sodium carbonate, is used to buffer blood serum ions, and hemodialysis is another option for restoring lactate levels. However, the inclusion of immobilised LOx within the BAL unit would permit the restoration of normal serum lactate levels as part of the complete approach, negating the need for separate treatments and thus potentially having time, economic and patient comfort benefits, contributing to an improved QUALY (quality adjusted life years) score.

Moreover, including LOx in the BAL could potentially provide a means of preventing a loss of function of the immobilised DNase I, by keeping lactate levels as low as possible and thus minimising the possibility of divalent ions being sequestered and made unavailable to DNase I, the activity of which is highly dependent upon calcium and magnesium ions.

The benefits of using immobilised LOx to reduce lactate levels in patients would need to be clinically assessed, to investigate whether any improvement in prognosis could be observed by lowering lactate concentration in isolation of the plethora of other complications that a failing liver would present. One way of determining this could be to investigate the levels of ATP synthesis as a marker for improved cellular respiration, which would at least ameliorate the burden on a failing liver.

7.2. DNA Removal

There is the potential risk to patient safety if HepG2 DNA is released from damaged cells and then enters the circulation via the BAL system. A physical barrier is planned to be used within the BAL circuit to prevent this but as a fail-safe method, this project sought to immobilise the DNA degrading enzyme, DNase I, to minimise this risk to become practically

negligible. It has been demonstrated in this work that DNase I immobilised onto glass beads retains catalytic activity in physiologically relevant conditions (pH7.4 and at 37°C) although not to the same extent as the native, free enzyme.

DNA removal occurs naturally within the body, with several strategies being utilised to clear superfluous DNA, particularly from damaged and apoptotic / necrotic cells. In addition to the action of macrophages, DNase I is intrinsic to plasma where it acts to scavenge and degrade circulating DNA. Another example is that of serum amyloid P component (SAP), a pentraxin protein which has been shown to bind to chromatin (160), extracellular accumulations of which are coated with SAP (161). SAP has been suggested to play a role in facilitating endocytosis in macrophages, thus contributing to the extracellular clearance of DNA (162). Moreover, the protein contains a nuclear localisation sequence, leading to the proposal that SAP can enter the nucleus of a damaged cell (i.e. a compromised plasma membrane) to bind DNA before it is released from the cell (163). Despite this, it would not be acceptable to assume that these intrinsic methods of DNA removal would be sufficient to remove completely and hence minimize risk from any additional DNA of non-patient origin. For this reason, taken with the data that immobilised DNase I functions well in plasma, the inclusion of immobilised DNase I beads will be a suitable measure to ensure that patient safety is maintained during treatment with the BAL device.

7.2.1. DNase I immobilisation

As DNase I is intended to be used within the BAL device itself, it was appealing to elect an immobilisation platform such as magnetic Dynabeads® which could be easily retained and recovered, as this would minimise the possibility of the enzyme linked beads entering other components of the BAL and potentially causing a blockage in the circuit. However, experiments showed poor activity of the subsequent immobilised enzyme, and so the same basic protocol was used as had been previously

demonstrated to be successful with LOx immobilisation. The lysine targeting approach appears to successfully cross-link DNase I to the glass bead support, although a reduction in activity is observed. A reduction in activity is not uncommon after immobilisation, as the process of immobilisation often distorts the physical conformation of the enzyme, thus affecting the ease and mode of interaction with its target substrate (164), although the large size of DNA, the substrate, could also have played a role. In the catalysis of DNA and the formation of the enzyme-substrate complex, it is, unusually, DNA which undergoes a conformational change, not DNase I (165). It is not improbable that the "mesh" of cross-linker, particularly in the case of the long NHS-PEG ester, formed an impermeable barrier between immobilised enzyme and DNA, or created an environment in which DNA could become entangled and thus prevented from having the degree of flexibility required to form a successful interaction with DNase I. An interesting alternative mode of immobilisation would be to engineer a tagged form of DNase I, such as has been done recently for lactate dehydrogenase (166); in this way, the orientation and site of cross-linking would be certain, minimising the need for long cross linking molecules and thus reducing the steric hindrance effects upon DNA.

7.2.2. Additional clinical uses for immobilised DNase I

The use of immobilised DNase I as a therapeutic for the treatment of other diseases is a possibility. There exists already much research into the use of DNase I as a means of reducing circulating DNA levels in SLE patients and thus ameliorating the symptoms of an autoimmune response (141;151), and also for removing DNA deposits in cystic fibrosis patients (141;142;167). Recently, it has been demonstrated that administration of RNase and DNase I can bring about an almost complete elimination of metastases in tumour bearing mice and also leads to an immunomodulatory effect (168). However, before research into these areas could go ahead, it would be necessary to devise first a mode, rather than passing the plasma over the DNase I beads, which is far less invasive and thus more practical and feasible for use within these particular clinical contexts.

7.3. Existing Clinical Applications for Enzyme Immobilisation

The majority of investigations have used enzymes in solution to be administered either orally or as an injectable, with relatively few studies into the use of immobilised enzyme complexes as a potential therapeutic agent. Immobilised enzymes are frequently found in the bio-clinical field as an analytical tool, most often in the form of biosensors, but less often as therapeutics in their own right. However, a selection of prominent examples will now be discussed, demonstrating that there is a need and a role for immobilised enzymes in therapeutic strategies.

7.3.1. Glucocerebrosidase & Gaucher's Disease.

Type I Gaucher's Disease is a lysosomal storage disease (LSD) defined by a deficiency in the enzyme glucocerebrosidase (GCR) (169) which is required for the metabolism of glycosphingolipids (170;171). In this disease, the sphingolipid accumulates in macrophages which infiltrate primarily the viscera and bone marrow where the major symptoms are manifested, namely anaemia, an increased risk of infection due to low leukocyte numbers, and a low platelet count (170;172). It has been demonstrated that GCR can be successfully immobilised into spherical microspheres by pre-adsorbing GCR onto a calcium titanium phosphate powder before mixing this enzyme conjugated ceramic powder with a sodium alginate solution (173). This was then polymerised into spherical microspheres of a homogenous size which exhibited a steady release of the enzyme. Although this has not yet been tested in vivo, it serves as a promising start to a role as a drug delivery mechanism for immobilised GCR as a treatment for a range of glycosphingolipidoses, not just Gaucher's Disease (174).

7.3.2. Deoxyribonuclease I & cystic fibrosis

Cystic fibrosis (CF) is one of the most common autosomal recessive genetic disorders, affecting 1 in 2000 Caucasian live births. It affects endothelial cells' ability to clear pulmonary secretions, which accumulate to create an environment prone to infection. It is the retention of pulmonary secretions which give the disease its characteristic symptom of a thick, viscous sputum, a result of neutrophil derived DNA (167;175). Deoxyribonuclease I, an endonuclease which degrades double stranded DNA, has been utilised for some time in the management of CF. Traditionally, it has been administered as a "raw" enzyme suspension via nebulisation, but a recent study encapsulated the enzyme within poly(lactic-co-glycolic acid) nanoparticles (176). The results demonstrated that the nanoparticles had no significant cytotoxic effects and had a satisfactory level of nebulising efficiency, but immobilised DNase I activity decreased by a third in comparison to the original enzyme. Intended as a carrier for drug delivery, this approach remains to be tested in a clinical trial situation.

7.3.3. L-asparaginase & acute lymphoblastic leukaemia

The use of L-asparaginase (ASP) to treat Acute Lymphoblastic Leukaemia (ALL) has been implemented for over 40 years (177), aiming to reduce abnormal lymphoblast numbers by depriving them of L-asparagine via catalysis of its breakdown into aspartic acid and ammonia (178). However, to overcome issues with immuno-incompatibility when injected, a recent study has developed a method for immobilising ASP within a glycidyl methacrylate-co-ethylene glycol dimethacrylate (GMA-co-EDMA) monolithic column as part of an extracorporeal shunt system (179). The proposed treatment would be to pass patients' plasma through an extracorporeal circuit containing ASP where levels of L-asparagine would be depleted before the plasma is returned to the patient. Thus direct contact between ASP and cells of the patient's immune system would be eliminated, therefore avoiding an immune response. Preliminary data showed that following an incubation period of eight hours, ASP was successfully immobilised onto the monolith, with kinetic data demonstrating that the

conjugated enzyme actually had a lower K_m value than the free enzyme, indicative of an increased affinity for the substrate molecule (179;180). The remarkable finding however, was that when human serum spiked with L-asparagine was applied to this system, a rapid increase in serum aspartic acid and an almost complete absence of L-asparagine was detected after 30 minutes (179), and that no significant decrease in immobilised ASP activity was apparent after a period of 21 days. This indicates that the use of immobilised ASP is a promising alternative to traditional ALL chemotherapy treatments, benefiting patients whilst avoiding many of the common detrimental side effects including hepatic toxicity, errors with blood clotting factors and adverse immunological reaction (181).

7.3.4. Urease in artificial kidney devices

Urease catalyses the conversion of urea to ammonia and carbon dioxide and has been utilised in an immobilised form in several artificial kidney device designs, where it is contained within an extracorporeal circuit to cleanse the plasma of urea. Non toxic and biocompatible polymers (xanthan and alginate) have been used to entrap urease into microspheres, an approach which saw the retention of 75% the activity of the original enzyme (182). The protocol called for polymerisation of the alginate-urease mixture in a Ca^{2+} solution containing gelatine, which was subsequently used to cross-link urease using glutaraldehyde. The immobilised enzyme demonstrated greater stability to pH and temperature *in vitro*, and the physical integrity of the microspheres was not compromised after multiple uses. Furthermore, only 25% activity was lost following the reuse of the microspheres for a total of 20 times over a period of 40 days, indicating that the urease microspheres are suitable for use over a prolonged period, a phenomenon which would hold potential at the theoretical level for use in a therapeutic device.

A more recent study also utilised an alginate-urease suspension to form microspheres which did not exhibit enzyme leaching after 48 hours (183). Although some activity was lost during immobilisation, when applied to a urea-containing solution, designed to mimic blood, the concentration of urea

was reduced to 25% of the initial concentration after five hours of perfusion (183), again demonstrating the potential that alginate microspheres have for clearing urea in renal failure patients.

Chen at al. utilised a poly(vinyl)alcohol (PVA) membrane to immobilise urease (184) which, with an ion-exchange membrane, formed a membrane reactor-separator, for the removal of urea from solution. *In vitro* studies demonstrated that this system effectively reduced pre-treatment levels of urea in citrate buffer whilst preventing the backflow of ammonium ions into the pre-treatment solution.

One approach which has been tested experimentally *in vivo* (in sheep models) saw the co-encapsulation of PEG derived urease and alanine dehydrogenase within ovine erythrocytes (185). Once injected into sheep on a urea supplemented diet, the enzyme-packed erythrocytes continued to retain activity for six days (compared to two days when not encapsulated), effectively lowering urea concentration directly in the blood stream, without evoking an immune response. The longevity of immobilised enzymes in this treatment is probably associated with the production of NADH and pyruvate by erythrocyte metabolism, molecules which are required for the regeneration and hence continued use of the encapsulated enzymes.

7.3.5. Bilirubin oxidase & liver failure

One common symptom of liver failure is jaundice, attributed to the accumulation of bilirubin due to substantial hepatocyte depletion and hence a deficit of bilirubin degrading enzymes. To alleviate this, a potential therapy has been proposed whereby bilirubin oxidase is conjugated to 4% Sepharose beads within a packed bed bioreactor (186) through which the patient's blood would be passed as part of an extracorporeal circuit. This system was shown to convert more than 90% bilirubin to biliverdin *in vivo* in rat models and although a 20% reduction in red blood cell number was

observed, no significant decrease in white blood cell or platelet number was recorded. Also, the products formed were not found to have any tumour inducing or cytotoxic effects. This was also the case for human blood tested *in vitro*, demonstrating a potential role for this strategy in the management of liver failure.

7.4. Future Work

The work presented in this thesis has demonstrated the proof of principle behind utilising immobilised enzymes to convert undesirable molecules into harmless or potentially useful substrates for the ELS of the BAL. However, were more time and resources available, there remain a number of questions which must be addressed before this approach can be fully incorporated into the BAL for patient treatment. These are outlined and discussed in the following sections.

7.4.1. Determination of efficiency of LOx immobilisation

Antibodies against DNase I have been used in this study to determine the extent of enzyme immobilisation to the glass bead support. However, this information has not been possible to directly ascertain for immobilised LOx, owing to the lack of a suitable antibody. Should time and funding have permitted, it would have been desirable to create such an antibody, to allow for an optimisation of the immobilisation procedure.

7.4.2. Pyruvate utilisation by ELS

The enzymatic degradation of lactate leads to the synthesis of hydrogen peroxide and pyruvate. Although it has been herein demonstrated that the hydrogen peroxide produced does not appear to have a significant detrimental effect upon the survival or synthetic activity of ELS, it remains to be seen whether the pyruvate generated bestows any growth advantage to the cells, compared to growth rates in media in the absence of

supplementary energy substrates. In theory, the availability of additional pyruvate, which is a substrate for the citric acid cycle (also Krebs cycle), could mean that there would be a greater availability of energy sources to the ELS. However, it is likely that any growth advantage would only become apparent were the glucose availability limited in some way; as the media in which the ELS are cultured is currently supplemented with a high glucose concentration, it would follow that this would rarely be the case, but the glucose balance could be investigated and optimised further. Indeed, recent data from our group suggests that a lower glucose concentration in fact promotes cell proliferation.

7.4.3. Storage stability of immobilised enzyme complexes

The nature of Acute Liver Failure (ALF) necessitates a mode of treatment which can be made readily available, as and when a case is presented. In order to achieve this, the BAL device is being developed so that the entire chamber, containing sufficient numbers of functional ELS, can be frozen for storage and then thawed and utilised when required, in effect providing an "off the shelf" service. Therefore, it would be desirable that the immobilised enzyme cartridge also be frozen or prepared in another manner for mid- to long-term storage; although the immobilisation process is not as time consuming as is the culture of functional ELS, this nevertheless imposes a significant delay in patient treatment which could prove fatal.

Although it has often been reported that immobilisation confers a degree of structural stability to an enzyme, the extent of damage incurred by the process of freezing and thawing the enzymes must be determined empirically. A proposed method to determine this would be to compare the enzymatic activity of immobilised enzyme complexes after storage in frozen conditions for varying periods of time with freshly prepared immobilised enzyme beads.

Furthermore, deamination of asparagines and glutamine residues on DNase I is known to occur over time when stored in vials and is often

concurrent with a decrease in enzyme activity (143). This is another factor which should be investigated in future work.

7.4.4. Full-scale system of lactate / DNA removal

In an attempt to assess the usefulness and efficacy of using immobilised LOx and DNase I within the BAL, scaled-down models of the BAL were utilised, using a mini-column circuit within which ELS and both immobilised LOx and DNase I were cultured in human plasma for a period of eight hours (representative of the proposed patient treatment time).

Another factor for consideration is the optimal positioning of the immobilised enzyme complexes within the BAL unit. Current mini-column experiments have been simplified models of the full-scale BAL chamber and its circuit; issues of practicality meant that it was most appropriate to house the beads in the glass column, in order to ensure sufficient flow that the immobilised enzymes were exposed to as much of the substrate as possible. This meant sharing space with the ELS in the second mini-column experiment; although no mixing of immobilised enzyme beads and ELS was observed, as the former (being much denser) were not fluidised and remained at the base of the column, this would not be ideal in the more complex, full-scale BAL system. Therefore, a series of further experiments would be required to account for the relative effects upon flow rate and pressure that introducing a cartridge to house the immobilised enzyme complexes would incur. In terms of the positioning of this cartridge, it would be logical to have this after the chamber containing the ELS, although as the BAL system is based upon a double circuit, it may also be possible to have this cartridge elsewhere, if necessitated by issues of pressure, space and other practical considerations.

Before moving the current model to a full-scale BAL chamber experiment it would first be necessary to devise a protocol for the scaled-up production of the immobilised enzymes. Currently, 100 mg of glass beads have been required per “dose” when treating a 200 ml total volume within the mini-column circuit. These beads are produced in individual 100 mg batches (as

described in the General Materials & Methods chapter); however, although it would not be feasible to prepare multiple batches simultaneously using this current methodology to produce the total quantity of immobilised enzymes which would be required in a full-scale BAL experiment, a scale-up process of immobilisation would permit this. Alternatively, recent research has demonstrated that the use of UV light may be a practical and effective way of immobilising enzymes in larger quantities (187), which would also have the advantage of being a "green" method of immobilisation, as the use of harsh chemicals for immobilisation is vastly reduced. This would represent an interesting line of research, in terms of optimisation of the immobilisation process for LOx and DNase I.

7.4.5. Optimisation of immobilised enzyme activity

The covalent immobilisation of DNase I and LOx onto glass beads has been demonstrated in this thesis to be an effective mode of reducing DNA and lactate concentrations in 10% FFP media and human plasma. In both circumstances, the activity of the immobilised enzyme was found to be less than that of the corresponding free enzymes, although sufficient activity was retained in order to bring about a significant reduction in substrate concentrations, compared to the relevant negative control. Nevertheless, in order to optimise the efficacy and efficiency of treatment with immobilised LOx and DNase I, an investigation into the use of modified variants of the enzymes and/or alternative cross-linking strategies would have been conducted had time permitted. For example, it has been frequently stated in the literature that LOx is an unstable enzyme, owing to its lack of thermal stability even when immobilised (188;189), a phenomenon which was also observed in this study, as exemplified in Figure 3-4 (page 60). To overcome this, an approach of using directed evolution has been used by others, for example, comprising a random mutagenesis step followed by error prone PCR and DNA shuffling, to generate a mutated form of LOx which exhibited a half life 36-times greater than that of the wild type enzyme (190). Manipulation of the enzyme prior to immobilisation in order to improve functionality has also been used to generate a galactose oxidase

variant with a ~four-fold improvement in K_m and V_{max}, by a process of error prone PCR and subsequent screening (191).

Moreover, by modifying the enzyme, for example by introducing a cysteine mutation (192) or by incorporating a peptide tag at a region unlikely to affect the enzyme's active site, which is specific for the immobilisation support (166) – termed "orientated immobilisation" – greater retention of enzymatic activity can be observed following immobilisation. Another example demonstrating the merits of orientated immobilisation is a cytochrome c reductase assay which revealed that enzymes tethered in an orientated manner displayed three-fold greater activity levels compared to randomly immobilised enzyme controls (193).

In the light of this data, it could thus be possible to achieve a more efficient immobilised LOx and DNase I enzyme by genetically altering the initial enzyme. In addition to potentially improving specific activity of the enzyme, another advantage of engineering would be that a specific antibody against the enzyme could be generated, facilitating quality control measures; of particular interest for LOx, as to date, no antibody is readily available.

7.5. Conclusion

The aim of this thesis was to devise a means of immobilising enzymes to address the deleterious effects of high lactate levels and DNA of non-patient origin from a Bio-Artificial Liver support device. Through the experiments undertaken, it has been demonstrated that by using glass beads as an immobilisation support, Deoxyribonuclease I and Lactate oxidase can be covalently attached to the matrix. The process of immobilisation in both instances does have a negative impact upon the V_{max}, the rate at which enzymatic catalysis of the substrate occurs, compared to the native free enzyme. However, both enzymes have been shown to retain an acceptable level of activity, leading to significant reductions in both lactate and DNA concentrations in spiked solutions. This is also the case when in the presence of human plasma and in physiological conditions reminiscent of those likely to be encountered in the FBB / BAL. Although investigations

conducted on a small-scale replica of the BAL failed to provide any conclusive evidence that the immobilised enzyme complexes improve lactate and DNA levels, it is probable that flaws in the methodology prevented a true indication of what was actually occurring in these conditions. In spite of this, from the data it can be postulated that, although more verification work needs to be undertaken using improved methodology for confirmation, enzyme immobilisation is a viable approach to reducing lactate and circulating DNA levels within the BAL, in order to improve the safety and efficacy of this device as a therapy for patients with acute liver failure.

8. References

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