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Development of a Rapid Immunoassay for Human Pathogenic Markers

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Abbreviations

AIDS	Acquired Immuno-Deficiency Syndrome
APACHE	Acute Physiology and Chronic Health Evaluation
Ap	Aptamer
C	Controls
CAT	Computerised Axial Tomography
CM5	Carboxymethylated Dextran Sensor Chip
CRP	C-Reactive Protein
DAA	Drotrecogin Alfa Activated
DIC	Disseminated Intravascular Coagulation
DW	Distilled Water
EDC	N-ethyl-N (dimethyl-aminopropyl)-carbidimide
ELIFA	Enzyme Linked Immunosorbent Flow-Through Assay's
ELISA	Enzyme Linked Immunosorbent Assay
ELONA	Enzyme Linked Oligonucleotide Assays
ESR	Erythrocyte Sedimentation Rate
EtOH+NaOH	Ethanol & Sodium Hydroxide
FC	Flow Cell
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HCl	Hydrochloric Acid
HEW	Hen Egg White
IAI	Intra-Abdominal Infections
ICU	Intensive Care Unit
IFC	Integrated μ-Fluidic Cartridge
IL	Interleukin
MODS	Multiple Organ Dysfunction Syndrome
MRI	Magnetic Resonance Imaging
MRSA	Multi-Drug Resistant *Staphylococcus aureus*
N/S	No Staining
NAG	N-acetylglucosamine
NAM	N-acetylmuramate
NHS	N-hydroxysuccinimide
NSB	Non-Specific Binding
OD	Optical Density
PCT	Procalcitonin
RI	Refractive Index
RU	Response Units
S	Sample
SA	Streptavidin Coated Sensor Chip
SELEX	Systematic Evaluation of Ligands by EXponential Enrichment
SOFA	Sepsis-related Organ Failure Assessment
SPR	Surface Plasmon Resonance
TIR	Total Internal Reflection
TNFα	Tumour Necrosis Factor α
URTI	Upper Respiratory Tract Infections
UTI	Urinary Tract Infections
WBC	White Blood Cell
WBTC	Wash Buffer, Tween and Casein

Appendix

Abstract

A demand exists for a fast, sensitive, reliable and economical test for pyogenic sepsis that provides a “real time” bed-side assessment. Detection of significant intra-abdominal sepsis can be particularly problematic in the ICU setting and in patients with multi-system organ failure.

Lysozyme, first reported by Fleming (1922), is a bacteriolytic enzyme released during phagocytosis. Previous studies have shown significant correlation between lysozyme levels and the presence of intra-abdominal abscess in both animals and humans. A method which determines and quantifies lysozyme as part of an assessment of an acutely ill patient in whom major sepsis is suspected; would significantly aid diagnosis and prescription of the most effective form of treatment. To date measurement of lysozyme has been by turbidometry, with consequent poor sensitivity and reliability. Other methods of assay include fluorescence, radial immunodiffussion and enzyme linked immunosorbent assay (ELISA).

This study reports a modified ELISA technique which provides a cheap, sensitive and reliable method of lysozyme determination, producing results in <100 minutes. The ELISA has been tested with ~200 clinical samples provided by the patients at the Great Western Hospital, Swindon. Two ELIFA techniques were also developed for lysozyme and *E.coli* detection. These techniques also provide a cheap and rapid alternative to the more traditional immunoassays. Results from the ELIFA and mini-ELIFA were obtained qualitatively after only 10 minutes. An SPR detection technique was also devised. The BIAcore 3000 was used to create a biosensor for serum lysozyme using an artificial receptor in the form of an aptamer. This system was tested with clinical serum samples, is reusable and took <80 minutes to immobilise a ligand on a blank sensor and analyse a serum sample for lysozyme.

Although further research and development is required on the mini-ELIFA and lysozyme biosensor, the ELISA detection system may prove a useful tool in the diagnosis of sepsis in critically ill patients.

Chapter 1 - General Introduction

1 Sepsis

Sepsis is the leading cause of death in Intensive Care Unit (ICU) patients worldwide; in excess of 18 million cases of sepsis are recorded each year. Mortality rates have decreased only slightly over recent years, but incidence has continued to rise to an unacceptable rate. Even with advances in critical care medicine, numerous treatments developed and profound investment, 56% of all septic ICU patients die each year; suggesting that more immediate exploratory work is needed (Edbrooke *et al*., 1999).

In 2002, the Surviving Sepsis Campaign was spearheaded by the European Society of Intensive Care Medicine, International Sepsis Forum and Society of Critical Care Medicine. Their mission was to create a clearer clinical definition, develop and instigate better standards of care leading to enhanced diagnosis, effective management and ultimately to increase the number of sepsis survivors. Currently sepsis is placing a huge burden on worldwide healthcare resources. It is estimated that ICU expenditure for sepsis alone is costing €7.6 billion annually in UK and Europe and a staggering €16.7 billion in the US each year. Clearly this huge investment is not having the desired effect of reducing death by sepsis and alternative solutions are required without delay.

1.1 Introduction to Sepsis

Sepsis was defined in 1914 by Schottmuler "as a state of microbial invasion from a portal of entry into the blood stream, which causes illness". A more recent definition by Bochud & Calandra (2003) distinguishes between sepsis, septic shock, septicaemia, and severe sepsis. Sepsis is the condition resulting from the presence of bacteria or their toxins or pathogens, in the blood or tissues.

1.2 Classification of Sepsis

Basic signs and symptoms of systemic sepsis show 2 or more of the following; fever, due to the resetting of hypothalamus; chills, resulting from muscular contraction to generate heat; heart rate above 90bpm; respiration rate above 20bpm; pCO_2 less than

32mmHg and leukocyte count greater than 12,000cells/ml^3 (Sharma & Eschun, 2004). Septicaemia is the presence of dividing bacteria caused by a persistence of pathogenic organisms or their toxins in blood. Septic Shock is sepsis as defined above, (resulting from bacteria/toxins present in blood/tissues) along with hypotension and may also include, lactic acidosis, oliguria (reduced urination) or an acute alteration in mental state (Bochud & Calandra, 2003).

Septic shock is most commonly associated with Gram positive bacteria, fungi and endotoxin-containing gram negative bacteria. In the 1970's, Gram positive bacteria accounted for 10% of all causative bacteria, now it is 50% (Bochud & Calandra, 2003). This increase is said to be caused by the increased use of intravascular devices (Daikos, 1994). The commonest Gram negative bacteria which cause sepsis include *E.coli*, Klebsiella species, and *Pseudomonas aeruginosa*. Gram positive bacteria include *Staphylococcus aureus* and coagulase negative staphylococci, as well as *Streptococcus pyogenes*, *pneumoniae* and viridans streptococci. Sepsis caused by fungi (most commonly Candida) only account for 5% of cases, but like Gram positive bacteria this is increasing with time.

Gram negative bacteria usually cause infections in the lungs, abdominal cavity, blood stream, and urinary tract. Whereas Gram positive bacterial infections occur in the skin, soft tissue, primary blood stream, upper respiratory tract and are also associated with infections of intravascular devices (e.g. vascular grafts), (Bochud & Calandra, 2003). Of these bacteria, the products/components that are released include endotoxin, teichoic acid antigen, and exotoxins. All these stimulate the release of endogenous mediators from endothelial cells, plasma cells (which also include dendritic cells, natural killer cells, monocytes, macrophages, and neutrophils) and plasma cell precursors. These mediators have their effects on the heart, vasculature, and body organs and produce the clinical picture of sepsis. The next stage is severe septic shock followed by death caused by singular or multiple organ system failure (Vosylius *et al.,* 2004).

Early administration of antibacterial therapy is essential. A broad spectrum therapy is used because Gram negative sepsis can develop to death within two days, which is not

enough time to identify the causative bacteria, as microbiological confirmation takes 48 hours. However, the administration of antibiotics so soon into an infection may mask bacteriological diagnosis. This is also true for postoperative infections. The symptoms of the primary illness (i.e. the reason for the surgery) can mask the presence of the infection.

1.3 Diagnosis of Sepsis

A patient's response to infection is unique, no two patients present with the same symptoms, which can therefore make diagnosis of sepsis difficult. The symptoms that do present in a patient can be indicative of other diseases. An example of this is put forward by Petersdorf *et al.* (1983). This report found that 18% of appendectomies that were based on acute abdominal pain turned out to be negative.

Diagnosis is made by analysing the patient history, a physical examination, Gram stains from biological fluids and results from blood cultures. Signs and symptoms can include hypotension, inadequate organ perfusion, fever, chills, tachycardia (>100bpm), tachypnoea (excessively rapid breathing), and altered mental state. The circulatory insufficiency is characterised by a lower systemic vascular resistance along with a reduced contractile function and pooling with altered distribution of blood in the microcirculation (Daikos, 1994).

1.4 Signs & Symptoms of Intra-abdominal Infections (IAI)

Intra-abdominal infections are the commonest form of sepsis. Three classifications of Intra-abdominal infections are observed; primary, secondary and tertiary peritonitis. When primary peritonitis occurs the infection is localised, but it can develop into a life threatening disease with high morbidity (percentage of disease leading to disease) and mortality (percentage of death), but this is rare.

Primary peritonitis is especially prevalent in infants, children, cirrhotic patients, the immuno-compromised and patients with ascites and diabetes. Monomicrobial infections are caused by Enterobacteriaceae, Pneumococci, Streptococci, Staphylococci, other anaerobes, or Candida, (Barie, 1999). These organisms are derived from the

intestinal tract, ascension via the female genital tract or by direct transmural migration into the peritoneal cavity. Where peritonitis is associated with chronic ambulatory peritoneal dialysis, causative organisms include vancomycin resistant Staphylococci, Candida and other opportunistic fungi and Pseudomonas.

In secondary peritonitis, peritoneal infections are due to intra-abdominal infections caused by perforation of hollow viscous bowel, *(60-80% of all cases),* other causes include ischemic bowel wall necrosis, infection of intra-abdominal organs, secondary infection (e.g. pancreatitis), tumours, trauma, appendicitis, diverticulitis, perforation of peptic ulcers or post-operative infection associated with suture leakage. Polymicrobial infections in the intestines are usually caused by *Bacteroides fragilis*, enteric Gram negative bacilli and isolates of Clostridia, Enterococci and Pseudomonas. Infections in the liver and biliary tract are caused by *E. coli*, Klebsiella species, Enterococci and anaerobes in patients over the age of 70 or with Diabetes mellitus (Barie, 1999). Polymicrobial infections of the female genital tract are caused by Chlamydiae, *Bacterioides fragilis* and enteric gram negative bacilli. Mortality rate where peritonitis is due to untreated appendicitis is 8%. However, in secondary peritonitis, the progress is also dependent on pre-operative physiological derangement, age and pre-existing diseases. Farthman & Schöffel (1998) in their study showed that 25% of all Intra-abdominal infections cause multiple organ failure.

Tertiary peritonitis is characterised by bacterial contamination of the peritoneal cavity. The progress from contamination to infection of the peritoneum depends on growth characteristics of the bacterium, adherence capabilities, and the invasiveness of the particular bacterial species; which explains why there are over 400 species present in the human bowel yet only 10-20% are commonly found in intra-abdominal infections. Tertiary peritonitis is caused by Enterococci, Pseudomona, *Staphlyococcus epidermidis* and Candida, however as tertiary peritonitis is characterised by the failure of host defences in the peritoneal cavity is debatable whether more could be added to this list, (Barie, 1999). As previously mentioned IAI has a high mortality rate, when IAI occurs in the peritoneal cavity, as a result of colonic perforation of an abscess, the mortality rate is 25% however, where patients with Multiple Organ Dysfunction Syndrome

(MODS) (the physiological failure of several interdependent organs) are associated with IAI the mortality rate exceeds 90%, (Sharma & Eschun, 2004). Even if radiological techniques or surgical exploration techniques are employed, the mortality rate is still 33-71% (Burgess *et al.,* 1994). Successful bacterial characteristics include adaptability to environmental changes, metabolic changeability to anaerobic conditions present in the peritoneal cavity, resistance to antibiotics and adhesion to serosal surfaces. When intestinal perforation occurs most bacterial species are killed due to the lack of suitable environment outside the bowel lumen. Most bacteria that survive are saprophytic organisms, not causing infection but contributing to providing the right conditions for the few organisms which are able to adhere to the peritoneum and initiate the inflammatory response.

Mechanisms of the host defence are subsequently activated. Immediate clearance is a dual effort of peritoneal absorption and phagocytosis by peritoneal macrophages. The result may be complete control of the infection. However, if adjuvants are present bacterial numbers may thrive. The adjuvant's primary function is to obstruct host defence mechanisms and increasing the immunogenicity of the antigen, mainly by preventing phagocytes reaching their targets. Bacterial infection grows progressively over time without host defence intervention. In 24 hours 100million organisms per milligram serosal tissue will develop. By this point the adhesion will be a stable complex pathogenic flora resistant to lavage (Jonjic *et al.,* 1992).

1.5 Incidence of Sepsis

Edbrooke *et al*., (1999) reported the incidence of sepsis in the UK with their study of patient related costs in intensive care units. Their study found that 56% of patients who were admitted to the ICU with sepsis died within 3.3days. The same mortality rate was shown in patients who developed sepsis after 1 day in the ICU, but they lived 13.2days longer before death. Patients who developed sepsis after their 2nd day in the ICU had a mortality rate of 60%. The Edbrooke *et al.*, (1999) study showed patients with sepsis, or who developed sepsis while in hospital, stayed in the ICU for prolonged periods of time, at considerable expense to the hospital and resulted in high numbers of deaths.

Daikos (1994) reported the incidence of sepsis in the US to be 400,000 cases of sepsis per year, 200,000 cases of septic shock per year, and 100,000 cases that resulted in death. Gram negative bacteria are the leading cause of sepsis but sepsis by Gram positive bacteria have increased by 20% over the last 25 years (Daikos, 1994). Werden (1999) also reports sepsis and septic shock as the leading cause of death in intensive care units. Bochud & Calandra (2003) reported severe sepsis to account for 2-11% of all hospital admissions in the US and Europe. Bochud & Calandra's (2003) study also reported that severe sepsis is the cause of 30% of all hospital deaths and more than 60% of hospital deaths being caused by septic shock. Currently diagnosis of IAI relies on a doctor's interpretation, observations of signs and symptoms from the patient, radiological investigation and blood analysis. The result in some instances is a diagnosis with grey-areas of uncertainty. This leads to unnecessary antibiotic use and unnecessary surgery. As sepsis has such a high mortality rate, additional methods of diagnosis are crucial for a more dependable diagnosis.

In addition to the high death rate caused by sepsis a number of key factors mean the situation could pose an even greater threat in future years. These factors include the rise in life expectancy in developed countries. Due to the ever increasing sophisticated medical technologies, society is living longer, and as a result of this their risk of developing sepsis increases, as sepsis-induced mortality increases with age. The rise in bacterial resistance is also an important factor. Multi-drug resistant bacteria are becoming a huge problem in both nosocomial and community-acquired infections. And the numbers of immunocompromised patients are also increasing due to acquired immuno-deficiency syndrome (AIDS). All significant factors have lead to sepsis being the threat it is today, and will continue to increase mortality rates unless action is taken.

1.6 Sepsis Diagnosis & Treatments

A major problem with diagnosing sepsis is due to sepsis being a multifactorial condition and the course of sepsis varies from patient to patient. Currently diagnosis of sepsis is a collaboration of blood analysis and the signs and symptoms of the patient. Appendix A shows the sepsis grading system, as adapted from Elebut & Stoner (1983) which is an example of a scoring system. The signs and symptoms and blood analysis of the patient

are simply recorded over time and correlated with the sepsis grading system to determine the severity of the infection. Appendix A does not take into account dysfunction of the pulmonary and cardiovascular system, which are highly important in early sepsis diagnosis (Bosscha *et al.,* 1997); thus a superior diagnostic tool is required. The Acute Physiology and Chronic Health Evaluation (APACHE) (Zimmerman *et al*., 2005) system takes into account neurological, cardiovascular, respiratory, renal, gastrointestinal, metabolic and haematological variables along with incorporating comorbid conditions such as Diabetes mellitus and cirrhosis. The APACHE system is a comprehensive evaluation of the patient's condition.

Management of sepsis usually begins with admittance to an intensive care unit if the patient is haemodynamically unstable (bp, respiration rate etc), and prompt administration of a broad-spectrum antibiotic. Sepsis can develop rapidly so immediate administration of antibiotics is essential. Bacteriological tests to identify the causative bacteria can take >2 days to be performed. If the antibiotic administration was delayed until after these tests and administered based on antibiotic sensitivities to the specific bacteria, the infection would inevitably be far worse and increase mortality. On the other hand, unnecessary use of broad spectrum antibiotics can cause multi-drug resistance leading to a more severe form of sepsis by opportunistic pathogens.

Other immediate treatments include fluids through an intravenous catheter, inotropic drugs (such as catecholamines) to raise blood pressure and organ support such as artificial ventilators or continuous external filtration of the blood for kidney support. Identifying the source of the infection also helps in the determination of treatments to be prescribed. This process involves white blood cell counts, microbiological cultures (of urine, blood, sputum, aspirates, from intra-abdominal, joint or wound sites), Erythrocyte Sedimentation Rate (ESR) indicative of the acute-phase response to infection, malignancy or trauma, C-Reactive Protein (CRP) an acute phase marker released in the immunological and inflammatory response and Disseminated Intravascular Coagulation (DIC) where clotting factors are exhausted leading to severe haemorrhaging.
Identifying the source of sepsis may be supported by a number of radiological techniques. Chest x-rays may show pneumonia, intra-thoracic collections, effusions

(seepage of bloody fluid into body cavity) or empyema (pus in body cavity). Abdominal x-rays may show free intra-abdominal gas indicating a visceral perforation. Computerised Axial Tomography (CAT) and ultrasound scans are also highly sensitive techniques to identify collections or abscesses within the thorax or abdominal cavity. Magnetic Resonance Imaging (MRI) is useful if infection is suspected in bone (osteomyelitis) or in soft tissue or joints. Labelled white cell scans can also be used when a source of infection is suspected but identifiable by other screening methods. The labelled white cell scan involves radio-labelling the patients white cells, and re-injected into the patient. The white cells congregate at the infection site, and are detected using a gamma camera to indicate sepsis (Vezina, 2004). Again this identification process takes time to execute and analyse, and some patients are simply too ill to under go some of these investigations. Also false positives are common among patients with haematomas (mass of extravasated blood in tissues) and inflammatory bowel disease and false negatives may occur in chronic sepsis (Vezina, 2004). When critically ill patients, in an intensive care unit setting are suspected of having intra-abdominal sepsis which cannot be confirmed by non invasive techniques; a blind laparotomy (incision into abdominal wall) is required but is associated with a very high mortality rate (Polk *et al.,* 1993).

Thus there is a need for a real time bed side test which will aid the clinician managing a critically ill patient in determining whether there is significant sepsis present (www.survivingsepsis.com).

1.6.1 Novel Management Techniques

A study by Kubler *et al.* (2006) demonstrated a significant decrease in mortality with the use of Protein C (Drotrecogin Alfa [Activated] – DAA) in severe septic patients in Poland. The Kubler *et al.* study showed Protein C treated patients had a 31% reduction in their risk of death compared to non treated patients with the same diagnosis. This addition of protein C is supported by a report by Lindstrom *et al.* (2006) which showed that in acute pancreatitis patients a significant decrease in protein C was correlated with multiple organ failure.

A further novel treatment is reported by Orozco *et al.* (2006). This study describes the use of Molgramostim (human granulocyte macrophage colony-stimulating factor - GM-CSF) in addition to antibiotics for the treatment of abdominal sepsis. The report concluded that the addition of GM-CSF to the antibiotic therapy reduces the rate of infectious complications, decreases length of stay in hospital and overall costs to the health service.

1.7 Potential Indicators of Sepsis

A potential early indicator of sepsis includes white blood cell counts. A noticeable increase ($>12x10^9$/L) or a marked decrease ($<4x10^9$/L) of white blood cells is an indication of a septic condition (www.survivingsepsis.com). However, white blood cell counts are inconsistent, for example patients with appendicitis often have normal white blood cell counts.

Other early indicators of sepsis which have been proposed include increased C-reactive protein levels. CRP is released by hepatocytes in response to infection and tissue inflammation in pneumonia patients. Studies have been conducted to show the usefulness of CRP levels to be unreliable in the diagnosis of sepsis (Meer *et al.,* 2005). A comparison by Meisner *et al.* (1999) also showed that CRP was not a useful indicator. This study compared the CRP with procalcitonin (PCT) in two groups of patients with MODS and system inflammation. All 40 patients were assessed with the Sepsis-related Organ Failure Assessment (SOFA) (assessment of the severity of organ function impairment, Vosylius *et al.,* 2004). Those patients with high SOFA scores had significantly raised PCT concentrations, after 4 days of MODS. However, the MODS patients and systemic inflammation patients all had elevated CRP regardless of their SOFA score. Therefore, PCT is a better indicator of sepsis than CRP, but is only an indicator after significant progression of sepsis and cannot distinguish between viral infections and inflammation, (Chan *et al*., 2004). Harbarth *et al.* (2001) also reported a study on the use of PCT as a marker for sepsis. This study showed that PCT can distinguish between sepsis, septic shock, and severe sepsis conditions. PCT increases 2-4 hours after bacterial/endotoxin challenge is initiated. However, the Harbarth *et al.* study also showed that PCT is not a reliable sepsis marker in patients who have

undergone kidney transplants or cardio pulmonary bypass surgery and has only yet been tested with patients undergoing antibiotic therapy; concluding that PCT is not a definitive test for sepsis but may, if combined with already established tests, give a clearer prognosis.

The Harbarth *et al.* (2001) study also investigated the use of the cytokines Interleukin 6 (IL-6) and interleukin 8 (IL-8). Interleukin 6 implements the proliferation of acute phase proteins and interleukin 8 is a pro-inflammatory cytokine. Both IL-6 and IL-8 are inflammatory factors and this study showed they could not be used as indicators of sepsis because they couldn't distinguish between non-infectious and infectious conditions.

A study by Collighan *et al.* (2001) also showed that the cytokines IL13 and Tumour Necrosis Factor α (TNFα) expression only increased for 3 days in septic shock patients but then decreased to non-septic levels; concluding that neither were effective markers for sepsis.

Neutrophil levels have also been studied as an indicator in sepsis patients. An increase by 10% in immature neutrophils (left shift) can be an indication of infection. Hansen & Andersen's study (1973) relates primarily to the release of lysozyme and neutrophil turnover rates. This study distinguished between intra-neutrophil lysozyme and serum lysozyme. Their findings showed intra-neutrophil lysozyme to decrease with increasing severity of infection. This was also heightened by the decrease in the neutrophil granule production in infectious patients (Hansen & Andersen, 1973).

A more recent study by Guo *et al*., (2002) showed myeloperoxidase levels increasing 10 fold in rats using ceacal ligation/puncture models. β1 and β2 integrin content on blood neutrophils also showed a dependent 10 fold increase in the lungs; mechanisms which were only induced in the septic state.

Lysozyme as a possible indicator for sepsis, has also been extensively explored, and due to its promising potential has been chosen in this study for further investigation.

1.8 Discovery of Lysozyme

Lysozyme (1, 4-N-acetylmuramidase, E.C.3.2.1.17) is a small cationic protein first reported by Laschtschenko in 1909, (Burgess, 1973). Lysozyme's discovery however is attributed to Alexander Fleming, a bacteriologist in London, who was the first to report the finding to the Royal Society of London. His report stated that lysozyme was a "powerful bacteriolytic element found in human tissues and secretions" (Fleming, 1922). Fleming was at the time suffering from a cold and he is reported to have allowed drops of his nasal secretions to have fallen onto a blood agar culture plate which was thickly colonised with bacteria. The plates were incubated at 37^{o}C for 24hrs; bacteria grew extensively. There was complete radial inhibition of about 1cm beyond the nasal mucus on the plate. This experiment was later termed the lysoplate. Fleming concluded that the nasal secretions contained an enzyme capable of bacterial lysis; later named Lysozyme.

With the success of his preliminary experiment, Fleming continued to work with Lysozyme testing its antibacterial properties with several different bacteria. Fleming reported the discovery of a small round bacterium, particularly vulnerable to the effects of lysozyme. This bacterium was named *Micrococcus lysodeikticus* (now referred to as *Micrococcus luteus* or *M. luteus*) due to its ability to display lysis (Fleming, 1922). Fleming diluted the nasal secretions in saline and added it to a thick suspension of the *M. luteus*. Within minutes of incubation at 37^{o}C the opaque bacterial solution had cleared. This experiment was later termed the Turbidimetric test, (Fleming & Allison, 1927). Fleming (1922) is quoted as saying his experiments "clearly demonstrated the very powerful inhibitory and lytic action which nasal mucus has upon *Micrococcus lysodeikticus*". Fleming also showed increased levels of lysozyme in patients with pyogenic infections (pus producing) such as meningitis; the first indication that lysozyme could be a marker for sepsis.

1.9 Lysozyme Structure

Since Fleming's report, the potential of lysozyme has attracted considerable interest over many years (Gorin *et al.*, 1971). Hen egg white (HEW) lysozyme is very similar to human lysozyme with 129 amino acids (Jolles, 1969). Human lysozyme has ~148

amino acids and is 3-4 times more reactive than HEW lysozyme as tested with the turbidimetric test. However, due to its structural similarity, availability and inexpensiveness, HEW lysozyme has been used as a model for human lysozyme experiments, making it one of the most studied enzymes (Lollike *et al.,* 1995).

In 1965, the 3 dimensional structure of lysozyme was determined by David Philips and his colleagues (Blake *et al.*, 1965). A molecule of lysozyme can be analysed in 2 parts. The 1st 40 residues form the right-hand wing. This region coils around a core non-polar segment twice to stabilise the conformation. Residues 41-95 form the left-hand wing. These contain many polar residues and form a less rigid conformation. As for the remainder of the chain this slightly narrows the gap between the right and left wing, whilst wrapping around the right (Jolles, 1969).

1.10 Lysozyme Activity & Function

Meyer continued Flemings work by purifying lysozyme from acetone dried egg white describing it as a "basic polypeptide having a nitrogen content of 15.3%" (Meyer *et al.*, 1946). Human Lysozyme is a ~148 amino acid single polypeptide chain with a low molecular weight of around 14.6kDa (Cabellero *et al.,* 1999). Its elevated isoelectric point (pI 11) means lysozyme is a cationic protein, positively charged at biological pH's (Sophianopoulus & Sassa, 1965). Lysozyme is recognised to be non-dialyzable, soluble in water and weak saline, insoluble in alcohol and ether, resistant to heat and desiccation, and stable at room temperature (Burgess, 1973), stable at acid pH and labile at alkaline pH (Jolles, 1969).

Human Lysozyme is present in the lysosomes of phagocytic cells, granulocytes and monocytes (Burgess, 1994). It is released as part of the non-specific immune response, and exists among cells of the blood system, especially leukocytes. Lysozyme is found in all stages of the maturation of the myelocytic series, but not in the myeloblast, eosinophil or basophil, (Davis, 1971). Monocytes contain large amounts of lysozyme, but none is found in the lymphocytes.

In tissues lysozyme is mainly found in bone marrow, lungs, intestines, spleen, and kidneys. Lysozyme exists here due to the breakdown of neutophilic granulocytes in these organs, (Hansen *et al*., 1972). Tissue macrophages discharge lysozyme into serum, nasal and lacrimal secretions along with various other bodily secretions.

Figure 1.1 shows the phagocytosis of bacteria. At stage one the bacterium attaches to the pseudopodia of the phagocyte. By stage two the bacterium has been ingested inside a phagosome, a small pocket inside the phagocyte. Granules in the phagocyte act as storage for lysozyme, acid hydrolases, myeloperoxidases and complement activators, (Farthman *et al.,* 1998). These granules move towards the phagosome and merge. At stage 3 the phagosome membrane fuses with the granules, releasing the lytic enzymes into the phagosome. By stage 4, digestion by the lytic enzymes begins and at stage 5 the digested bacterium is released from the cell.

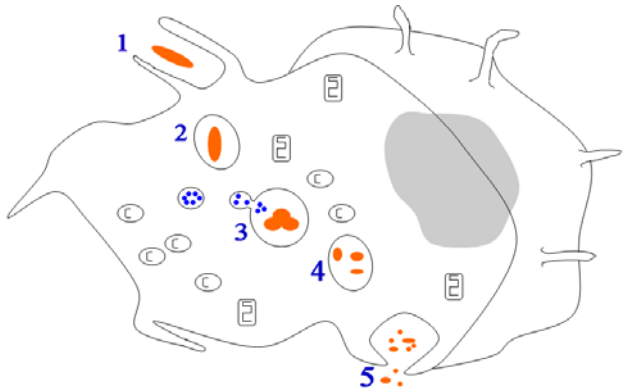


Figure 1.1 Phagocytosis, adapted from Goldsby *et al*., 2000. The attachment of the bacterium to the pseudopodia (1), encapsulation in the phagosome (2), merging of phagosome and granules (3), digestion of bacterium (4) and excretion (5).

Lysozyme exerts its antibacterial activity by promoting the degradation of the prokaryotic cell wall (Francina *et al.,* 1986). When the bacteria's cell wall is broken down the cell bursts due to the high internal osmotic pressure and the cell lyses. Bacterial cell walls are highly complex macromolecular structures, resistant to most solvents. The cell wall is composed of polysaccharide chains N-acetylglucosamine (NAG) and N-acetylmuramate (NAM). Muramic acid residues attached by amide bounds through the carboxyl groups to the peptide moieties act as bridges linking the polysaccharide chains, known as the "murein sacculas" (Weide & Pelzer, 1964).

Lysozyme exerts its effects on the tetrasaccharide structure of the cell wall and hydrolyses the NAG and NAM β 1-4 glycosidic linkage. The result is a disaccharide and consequently the death of the bacterium, (Sharon, 1967).

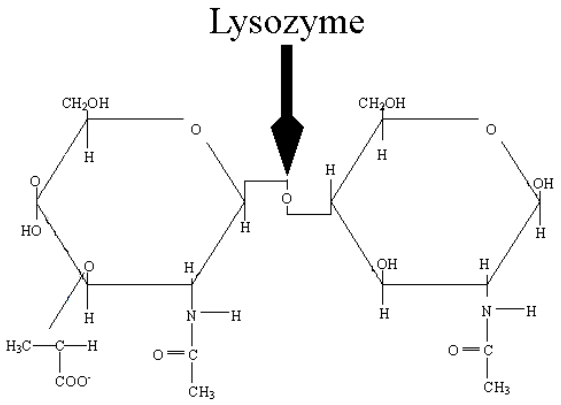


Figure 1.2 Hydrolysis of Bacterial Cell Wall by Lysozyme. Diagram shows the hydrolysis of the tetra-saccharide cell wall construction.

Gram positive cell walls are especially susceptible to the lytic action of lysozyme, but its effectiveness is limited, due to a bacterial capsule. Bacterial resistance is well known and reports of resistant strains of *Streptococcus* were first reported by Fleming & Allison in 1927. Weeks after their initial lysoplate experiments, bacterial colonies began to appear in the zones of inhibition. Over the course of 9 months their colonies were repeatedly sub-cultured at intervals of 1-2 weeks and tested with HEW lysozyme. Fleming & Allison found that these colonies were then totally resistant to further treatment by lysozyme and remained resistant over the entire 9 months. The colonies were then resistant not only to the tissues and secretions which they had grown in contact with but all other lysozyme containing tissues and secretions from plants and animals.

Fleming & Allison's work continued into the nature of the bacterial resistance. Several strains of bacteria were tested with the tears from Fleming's patients. Fleming found lysozyme was not effective against a range of bacteria. These included *Haemophilus influenzae*, *Neisseria meningitidis*, *Escherichia coli*, *Streptococcus pneumoniae, Klebsiella pneumoniae* and group B Streptococci. Lysozyme resistance is caused by an inability to break down bacteria that are encapsulated with a gelatinous polysaccharide layer. This dramatically limits the effectiveness of lysozyme to bacteria that have no

capsule. Lysozyme is therefore of little value as a therapeutic agent against bacterial growth but the release of lysozyme as a product of phagocytosis and white blood cell turnover may be a useful marker for sepsis.

1.11 Determination of Lysozyme

A study by Burgess *et al.*, (1994) showed that Lysozyme levels increased in the presence of Intra-abdominal Abscess. This study showed, where high serum lysozyme levels were recorded, they were correctly correlated with Intra-abdominal Abscess diagnosis. Serum lysozyme levels were high upon onset, predating any signs and symptoms of the infection. This is thought to suggest the sequestration of leucocytes, phagocytes and pus formation at the infection site. Lysozyme was not shown to increase after surgery, trauma, or major lung infections; and is therefore a good indicator of sepsis onset. There are currently several tests on the market which measure Lysozyme content in biological fluids.

1.11.1 Turbidimetric

The first tests to be discussed are based on Fleming's turbidimetric assay. This assay is based on a clearing phenomenon. A bacterial suspension is prepared and the clinical sample is added. The rate of optical density reduction is measured to determine the lysozyme content of the clinical sample (Ronan *et al.,* 1975).

Many of the turbidimetric methods used are relatively similar and differ only with respect to the lysozyme activity and sample preparation. These differences might include buffer composition, pH, ionic strength, concentration of *M. luteus* substrate, temperature, duration of incubation and preparation of enzyme (Houser, 1983).

Klass *et al.* (1977) described the turbidimetric technique as having a high sensitivity and a rapid turnover rate, as each sample only takes one minute to process. The Klass *et al.* (1977) method has a detection limit of 1µg/ml whereas the Ronan *et al.* (1975) method has a detection limit of 1.5mg/ml. The Klass *et al.* method is the preferred method for routine testing of clinical samples, but is not the most reliable as it produces a high inter-batch variability of results, with a precision of 2.5% as obtained by Gorin *et al.*,

(1971). Gorin actually did not look favourably on this precision as they compared their data with that of Bergmeyer (1965) who achieved a higher rate of precision. This study, by Gorin *et al.,* (1971) also showed that not all commercially available lysozyme substrates (e.g. lyophilised *M. luteus* from Sigma, Calbiochem etc.) produce the same effects, even though they may all be labelled *M. luteus*. This study showed that different companies produce different preparations of the bacteria, giving varying degrees of clearing rates, with a reported difference of up to 30%. This is caused by the various physical and chemical treatments they subject their bacteria to, which consequently alters the vulnerability of *M. luteus* to lysozyme and ultimately leads to unreliable results for diagnosis of diseases. Regardless of its unreliability, this assay is easy to perform, requires small sample volumes, can be used with serum, urine and tears and is sensitive.

A method which was built on the principles of the turbidimetric assay was devised by Caballero *et al.* (1999). Cabellero *et al.* described a micro-particle enhanced nephelometric immunoassay using serum and urine patient samples. This assay involved the use of polystyrene particles covalently bound with anti-lysozyme antibodies. The polystyrene particles formed larger particles due to the binding with free lysozyme in the sample which scatters light. The scattered light at the start of the reaction was compared with that at the end and used to calculate the lysozyme concentration in the patient sample. The assay detection limit was 0.58mg/l. Even with this assay Caballero *et al.* recognised there was still room for improvement with the long incubation periods, need for biological fluid pre-treatment and poor detection limit.

Terry *et al.* (1971) published work using an automated turbidimetric assay. This assay involved the use of 2 colorimeters which corrects the urine and serum discolouration problems experienced with controls (causing turbid solutions for blank samples). The bacterial suspension and clinical sample were continuously stirred and standards were run simultaneously. This automated assay was able to process 20 samples per hour and had an increased (undisclosed) sensitivity.

An assay currently on the market that employs the principle of turbidimetric assays is the EnzChek® Lysozyme by Molecular Probes *(Leiden, Netherlands).* This test measures lysozyme in solution at levels, as low as 20U/ml (equivalent to <0.5µg/ml). The assay measures lysozyme activity using *M. luteus* cell walls on substrate. The bacteria's cell walls are specially labelled with a fluorophore in such a way that the fluorescence is quenched. Activated lysozyme reduces the quenching thus increasing the fluorescence. The fluorescence therefore is proportional to the lysozyme activity. The increase in fluorescence is measured using a spectrofluorometer, mini-fluorometer or a fluorescence microplate reader. Each assay takes around 30 minutes with less than an hour preparation time. The assay lays claim to being "simple and sensitive" (www.probes.com).

1.11.2 Lysoplate

The lysoplate method, Fleming's second experiment, has also been used as the basis for assay development. However, Fleming's experiments were more complex than the methods used today. Fleming began by boring a well into the agar in a petri dish. A mixture of molten agar and nasal secretions containing lysozyme were added to the wells and a second layer of agar was applied covering the whole plate. The top layer of agar was then inoculated with a layer of *M. luteus* bacteria and incubated for 24 hours. Fleming's experiment showed that lysozyme was able to diffuse through the agar and prevent growth of the bacteria (Fleming & Allison, 1922).

Osserman *et al.,* (1966) showed that Fleming's lysoplate technique still worked even when simplified. In these experiments the agar was mixed with heat killed inactivated bacteria so that it appeared turbid, allowed to solidify and then wells were bored into the agar. Lysozyme samples from serum and urine were added to these wells and allowed to diffuse over 12-18 hours. The zone of turbidity clearing is proportional to the concentration of lysozyme (Osserman *et al.,* 1966). The Osserman *et al.* experiments involved serum and urine samples taken from patients with monocytic leukaemia. They found large quantities of lysozyme in serum samples, 40-150µg/ml, and 7µg/ml in their control samples taken from healthy patients. A big advantage of using this method is its simplicity. Very little training is required to complete the test in a clinical environment.

However, this advantage is marred by lack of sensitivity, large volumes of samples used and the long incubation time. A test currently on the market which uses this technique is produced by ARUP Laboratories (Mexico, South America). They offer a service using the lysoplate method which takes 2 days before results are received. When diagnosis may depend on these results this service does not seem a viable option. The lysoplate is also said to have a further flaw pointed out by Reitamo *et al.* (1981). This paper states that the lysoplate cannot be automated for high through-put use, and where the agar contains sulphates, the chemical complex forms mucins which affect determination of lysozyme (Reitamo *et al*., 1981).

1.11.3 Immunoassays

Fleming's turbidimetric and lysoplate techniques have been shown to lack sensitivity and have lengthy performance times; immunoassays offer an important alternative to the determination of lysozyme. Immunoassays rely on the reaction between the target analyte and a specific binding molecule of biological decent (the antibody). They can produce both quantitative and qualitative results and have shown considerable improvements in sensitivity (Ekins *et al.,* 1997).

Porstmann *et al.* (1989) developed an enzyme immunoassay for the detection of lysozyme in patients with Crohn's disease and rheumatoid arthritis. This study took urine samples from patients and these were tested using 3 variations of the same method. The method which showed the highest sensitivity involved pre-coating a microtitre plate with anti-lysozyme IgG overnight at 4^{o}C, 2 hours incubation at room temperature with the clinical sample and IgG-HRP conjugate. The assay was ended with 15 minutes incubation with o-phenylenediamine. The detection limit for this assay was 0.2µg/l.

Francina *et al.* (1986) also reports of an immunoassay developed to test lysozyme secretion in serum of acute myeloid leukaemia patients. A microtitre plate was incubated at 4^{o}C overnight with anti-lysozyme IgG. After a wash, the plate was incubated for a further 1 hour at 37^{o}C with the clinical sample. Biotinylated anti-lysozyme was added and the plate was again incubated for a further 1 hour. The plate

was washed and incubated with avidin peroxidase solution for 10 minutes at room temperature. The assay was ended with 5 minutes incubation with an enzyme substrate. The total assay time was ~14.5 hours. The assay time was reduced by decreasing the two 1 hour incubations to 20 minutes and the enzyme substrate incubation to 5 minutes, but the total assay time was still over 12 hours. The detection limit for the standard assay was 0.1ng/ml and for the rapid assay 1ng/ml.

Taylor *et al.,* (1992) also presents an immunoassay for the detection of lysozyme. This test was developed to measure lysozyme in healthy adult's serum and urine samples. The method involved pre-coating a microtitre plate with rabbit anti-human lysozyme, then adding the clinical sample and incubating for 90 minutes at room temperature. A conjugated sheep anti-human lysozyme was added and incubated for a further 90 minutes at room temperature. Finally enzyme substrate p-nitrophenyl phosphate sodium was added and incubated for 30 minutes at room temperature. The total assay time was 15 hours and the detection limit was 1µg/litre.

Immunoassay kits available on the market include the Biomedical Technologies (Stoughton, USA) EIA Kit for Human Lysozyme. This sandwich ELISA detects lysozyme from serum, plasma, urine, tears and saliva. The reference value for human lysozyme from serum ranges from 3-10µg/ml. In this assay, specific lysozyme antibodies bound to polystyrene wells are incubated with a sample. A second human lysozyme-specific antibody is added as well as a horseradish peroxidase conjugated secondary antibody. The total test time is $4^{1}/_{4}$ hours and costs around £275 for 96 tests.

Orgentec (Mainz, Germany) is another company that supplies a kit known as the ANCA Combi kit for lysozyme in serum and plasma. This test only requires 10µl of patients sample and involves a plate pre-coated with antibody. The antigen from the patients sample is added along with a horseradish peroxidase conjugate and then TMB (a colour substrate). The reaction is stopped using Hydrochloric acid and the total time for this assay is 2 hours, due to the plates being purchased pre-coated. During this time 96 patient samples can be processed.

All these kits have the advantage of conducting analysis on several patient samples using minimal biological fluid. This not only alleviates discomfort and distress to patients, but also the costs to lab facilities.

1.11.4 Other Detection Methods

Besides the turbidimetric and lysoplate assay for the determination of lysozyme there are other methods described in the literature. Montagne *et al.* (1998) reports of a microparticle enhanced nephelometric immunoassay for lysozyme primarily in breast milk. The method is based on the measurement of scattered light during the competitive immuno-agglutination of microparticle-lysozyme conjugate with an anti-lysozyme antiserum. Poly-functional hydrophilic microspheres were covalently coated with human lysozyme and mixed with breast milk taken from lactating patients. After 1.5 hours at room temperature the scattered light from the mixture was measured and the lysozyme content calculated. The assay was adapted for serum and urine samples, but all procedures had to be carried out on the day of testing due to the microparticle-lysozyme conjugates agglutinating during storage. The assay had a detection limit of 0.8μg/ml.

Another lysozyme determination method was put forward by Gao *et al.* (1995). The Gao *et al.* research concerned the faecal lysozyme expression in patients with inflammatory bowel disease indicative of Crohn's disease. This assay is a sensitive solid-phase competitive luminescence immunoassay. Polyclonal capture antibody coated to polystyrene beads were used with an acridinium ester-labelled human lysozyme was used as a tracer. Patient's samples were incubated with the beads and tracer over night at 4^{o}C. After a washing phase the emitted light was measured by a luminometer for 2 seconds to determine the lysozyme content. The assay sensitivity is 0.02μg/ml.

A final method for the determination of lysozyme was established by Yuzuriha *et al.* (1979). Yuzuriha *et al.* reported three methods using radioimmunoassay and enzymoimmunoassay techniques. In the first competitive radioimmunoassay I-labelled human milk lysozyme was mixed with antiserum and incubated for 2 hours at 37^{o}C.

Dextran coated charcoal was added and centrifuged for 15 minutes. The radioactivity of the solution was then measured to determine lysozyme content.

The second radioimmunoassay was a sandwich configuration. Standard human milk lysozyme was added to a microtitre plate pre-coated with antibody and incubated for 2 hours at 37^{o}C. I-labelled antibody against human milk lysozyme was added to the plate and incubated for a further 1 hour at 37^{o}C. Sodium hydroxide was added finally and the radioactivity measured to determine lysozyme content. Concentrations from 5-250ng/ml were measured.

Yuzuriha *et al.* (1979) reported a sandwich enzymoimmunassay. Antibody against human milk lysozyme was incubated over night in a microtitre plate well at 4^{o}C. Human milk lysozyme was reacted with pre-coated plates for 1 hour at 37^{o}C. Alkaline phophatase-antibody conjugate was added and allowed to bind for 1 hour at 37^{o}C before washing. The alkaline phosphatase activity was then measured as increased levels indicated increased lysozyme concentration in the human milk sample. This assay was also tried with human urine, placenta, serum and leukocyte lysozyme samples. The lysozyme detection range was 5-250ng/ml and over other methods reported by Yuzuriha *et al.* showed better precision, reproducibility, and simplicity.

1.12 Aims & Objectives of Project

Sepsis has been described as one of the most frustrating diseases of modern medicine. Even with the advances in intensive care, growth in understanding of the human immune system and inflammatory mechanisms and increasing developments in antibiotics; the overall prognosis of septic patients has hardly changed. One of the highest causes of death in the Western World still remains to be by sepsis (Hack *et al.*, 1999). Early identification of sepsis would be clinically useful, particularly in intensive care units. Lysozyme has been shown to be released before the signs and symptoms present in a patient. Therefore a test for lysozyme would speed up the diagnosis process and increase the patient survival rate.

The aim of this project was to produce a rapid, sensitive, inexpensive, and accurate method to aid in the diagnosis of sepsis. At present the methods used have a lengthy turn around time which means the patient is given non-specific antibiotics and in some cases surgery before the infection has been clinically confirmed. A test that is rapid, with the possibility of being carried out at the patient's bedside would reduce antibiotic resistance, speed up diagnosis, and aid management of seriously ill patients.

Surface Plasmon Resonance technology, the more traditional immunoassay based techniques and modified/optimized immunoassay-based techniques; were explored in order to propose a new test for sepsis in this project. Due to the promising potential of lysozyme as an indicator for sepsis, this assay would then be further evaluated by measuring serum lysozyme concentration from septic patients at the Great Western Hospital, Swindon and correlating the responses to the occurrence of sepsis. Ethical approval was gained by the Swindon Research Ethics Committee prior to commencement.

1.13 Specific Aims & Objectives

1.13.1 ELISA (Chapter 2)

The aims of the ELISA work were to produce and optimize an in-house ELISA system. Once a working immunoassay had been developed, several clinical samples were assayed and the lysozyme concentrations recorded. The clinical samples were taken from patients at the Great Western Hospital in Swindon. These patients were suffering from a range of illnesses related to sepsis. Samples were also taken from patients in the hospital who had been deemed non-septic and also control samples were collected from non-hospitalised patients. A comparison will be made between these groups, comparing both controls with the septic lysozyme concentrations. All samples will then be assayed using a commercially available kit. A comparison between the results gained from the kit and the ELISA will then to be made. An assessment will also be made between the commercially available kit and the ELISA. Consideration will be given to the repeated freezing and thawing of the serum samples. As the serum samples have been frozen after collection and then defrosted before transportation to the University, an experiment will be carried out to test the viability of lysozyme after several freeze-thaw cycles.

Along with the serum samples provided by the Great Western Hospital, routine blood analysis results will also be provided including white blood cells, haemoglobin levels, ESR, CRP where available. Patients were classified according to the presence of sepsis dependent on positive microbiological and radiological evidence. An assessment will then be carried out to evaluate the lysozyme level results from the ELISA and the actual diagnosis of the patients. Attention is also to be paid to the blood analysis results and any relationships between those and the lysozyme concentrations.

1.13.2 ELIFA (Chapter 3)

The main aims of the ELIFA section of work were to build on the progress from the ELISA immunoassay. As the ELISA produced good results the aim was to capitalise on the good aspects of the assay and to improve on the aspects which show room for improvement. The aspects of the ELISA which could be improved upon included the total time the assay took, the level of skill required to carry out the assay and the cost of the overall assay.

Initially the aim was to develop the ELIFA system with lysozyme in buffer samples. If time allowed clinical samples would also be assayed with this system. A method for accurately recording the ELIFA results would also be sort after.

1.13.3 Mini-ELIFA (Chapter 4)

The aims of the mini-ELIFA work were mainly to develop the ELIFA system further. The ELIFA system had many good aspects over and above the ELISA system. These aspects included, cost effectiveness, ease of use and its fast turn-around time were again to be capitalised upon when developing the mini-ELIFA system. Also the disadvantages of the ELIFA system, including the non-specific binding, were addressed and solutions were sort to eradicate these problems.

Preliminary experiments would include *E. coli* solutions for immediate binding onto the membrane. This system would build on the idea of using *E. coli* as an alternative indicator for sepsis. Initially a series of *E. coli* dilutions will be used to create a

calibration curve, with the assay being modified for the use of lysozyme concentration measurements and then possibly for lysozyme determination in serum samples.

1.13.4 Lysozyme Biosensor (Chapter 5)

The aims of the biosensor work include the development of a detection method for lysozyme using SPR technology. With the ELISA method already established the next step was to produce a detection system with a greater through put and a greater limit of detection.

The work in this section will be based on the IgE Biosensor work in Appendix B. Procedures for the use of a BIAcore SPR affinity assay instrument would be established using IgE and the aim will be to produce a similar system using lysozyme as the target. Another aim of this section of the work will be to incorporate the use of aptamers into the detection system. An anti-lysozyme aptamer would be used as a receptor for lysozyme in buffered samples. Part of the lysozyme Biosensor method will include the regeneration of the sensor chip for further lysozyme measurements. Once this system had been established and optimized then serum samples provided from the Great Western Hospital in Swindon will again be tested. A comparison of the lysozyme content as measured by the ELISA and the biosensor method with the actual clinical diagnosis of the patient will also be carried out.

Optimization results will include a calibration curve for samples containing known amounts of lysozyme in buffer, as well as known amount of lysozyme in serum samples (where lysozyme was not present) and a calibration curve of results from serum samples which are known to have high levels of lysozyme present (as established by the ELISA method). The results gained from these tests would then be compared with those gained from the ELISA and the clinical diagnosis of the patients.

Chapter 2 -ELISA

2.1 Introduction

The first detection system to be employed for the measurement of lysozyme was the traditional Enzyme Linked Immunosorbent Assay (ELISA). The ELISA experiments in the project involved directly immobilizing lysozyme onto the surface of a microtitre plate. With the addition of primary and secondary antibodies along with a substrate, a colour reaction was produced which was then read with a spectrophotometer wavelength to indicate the presence of lysozyme. This type of assay is known as an indirect immunoassay.

2.1.1 ELISA Use

The ELISA assay was used to quantify lysozyme in both lysozyme buffered samples and serum samples provided by the Great Western Hospital, Swindon, (Appendix C & D). Samples were randomly taken from patients with a variety of illnesses and conditions over a 12 month period. Including patients who had developed abdominal tumours (n=10), pneumonia (n=28), leukaemia (n=9), pregnancy (n=6), chronic renal failure (n=4), abdominal pain (n=15), appendicitis (n=5) and septic patients (n=42 including patients with multi-drug resistant *Staphylococcus aureus* (MRSA) positive infections, urinary tract infections (UTI) and upper respiratory tract infections (URTI)). Two control groups were used; in the first group serum was taken from non-hospitalised volunteers (n=14) who were deemed healthy and the second from hospitalised patients who were negative for sepsis (n=109). All serum samples were used with the ELISA protocol upon arrival. The serum was then divided into aliquots and stored at -20^{o}C until further use.

Patient demographic details were collected but remained anonymous. Patient data including, age, gender, date of sample, haemoglobin count, platelet count, differencial white blood cell count (WBC), neutrophil and lumphycyte count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and their clinical diagnosis were collected and stored on a password protected computer file (Appendix E). Samples were also taken from

patients who subsequently died while in hospital (n=37). Patients who were deceased were recoreded and the causes of death, from the list above were noted.

2.2 ELISA Variety

This section describes all the different configurations of ELISA's which can be produced. Descriptions of non-competitive, competitive, direct, and indirect ELISA's will be included (Figure 2.1).

Figure 2.1 Types of ELISA; (a) Direct ELISA, (b) Indirect ELISA, (c) Non Competitive Direct Sandwich ELISA, (d) Non Competitive Indirect Sandwich ELISA, (e) Sandwich ELISA, (f) Competitive Antigen-Enzyme Conjugated ELISA & (g) Competitive Enzyme-Labelled ELISA.

2.2.1 Non-Competitive Assays

The first type of non-competitive assay described here is known as the single site non-competitive ELISA or Non-Competitive Direct Sandwich ELISA (Figure 2.1). With this assay an antibody is immobilised to a solid phase. Test antigen from a sample is added and binds to the antibody. An enzyme-labelled antigen is then added and this binds to any immobilised antibody that has not reacted with the sample. An enzyme substrate is added and the enzyme-product concentration is inversely proportional to the concentration of the test antigen. There are in fact two forms of this single site non-competitive assay. In the second assay the test antigen is incubated with an enzyme-labelled antibody. This mixture is then transferred and added to an immobilised antigen. The antigen then binds to any un-

reacted enzyme-labelled antibody. The concentration of the enzyme product is inversely proportional to the test antigen (Butler *et al.*, 2000).

A second type of non-competitive immunoassay is the Non-Competitive Indirect Sandwich ELISA (Figure 2.1). Here an antibody is immobilised onto the solid surface of the microtitre plate. The test antigen is added and this binds to the antibody. A secondary antibody that is unlabelled sandwiches the antigen together. An enzyme labelled antiglobulin is added and directed to the secondary antibody. When the substrate is added a colour change occurs and the reaction is quantified in relation to known controls.

The final type of non-competitive assay is called either the two site assay or the Sandwich ELISA in which the antibody, not the antigen is immobilised on a microtitre well. An antigen (from a patient's sample) is added and allowed to bind and then an enzyme-linked antibody is added to react with the antigen. The substrate is then added to produce the colour reaction. The enzyme product is directionally proportional to the antigen content.

2.2.2 Competitive Assays

Besides Non-Competitive assays there is the Competitive ELISA. There are two forms of this assay that can be used, incorporating either the antigen-enzyme conjugate or the enzyme-labelled antibody. In the Competitive Antigen-Enzyme Conjugate version an antibody is attached to the bottom of the well. An antigen labelled with enzyme is incubated with this antibody along with free antigen present in the analyte sample. There is then competition between the labelled and unlabelled antigen to bind with the antibody. Once washed, the enzyme substrate reaction is initiated, run for a fixed time, and stopped. The measured enzyme product concentration is inversely proportional to the concentration of the standard or test antigen. This means that if there is no analyte (or test antigen) the assay output will be high and if there is a high analyte content then the colour output will be low.

The second form of Competitive assay is the Competitive Enzyme-Labelled antibody version. Here an antigen conjugated with BSA is attached to the surface of the well. An enzyme-labelled antibody is then incubated in this well with unlabelled analyte antigen. When the antigen and antibody bind, the enzyme conjugated to the antibody subsequently produces a colour change. Again if there is no analyte (test antigen) then a high colour change will occur, but if there is high analyte content a low colour change will occur.
All these assays can be described as analytical comparative assays as all tests carried out using these methods are measured against standards. The benefits of using these assays have been recognised by companies who have produced kits for the biomedical sciences.

2.3 Commercial Kit

A commercial kit was purchased to compare with the ELISA. The commercial kit was a non-competitive indirect ELISA bought from Biomedical Technologies Inc. (USA). The microtitre plate supplied was pre-coated with monoclonal human lysozyme antibody. The antibodies supplied would then bind to any lysozyme in the serum sample that had bound to the plate surface. A colour reaction was produced which was directly proportional to the amount of lysozyme in the sample.

Before the commercial kits were applied, another ELISA test was carried out on the serum samples, to check the lysozyme was still present and detectable in the samples as they had been frozen for some time. The ELISA test showed that lysozyme was still detectable and in comparable quantities to the results gained when samples were assayed on receipt.

2.4 Materials & Methods -ELISA

All buffer recipes can be found in Appendix G.

2.4.1 Lysozyme Coating

Purified Lysozyme (Sigma-Aldrich Ltd, Dorset, UK) from human milk (10% lyophilized powder in sodium phosphate/sodium chloride) was dissolved in a 10ml coating buffer solution to make a 75µg ml^{-1} stock solution. The lysozyme stock was stored at -20°C until use, (Appendix G). Dilutions ranging from 0.05µg ml^{-1} – 0.25µg ml^{-1}, were diluted in coating buffer. This range was used to create a calibration curve.

An aliquot (150µl) of lysozyme (0.05-0.25µg/ml) was added to each well of a 96 well microtitre plates (Immunolon 4HBX, Thermo Labsystems, Helsinki, Finland). Each dilution was pipetted from a fresh Petri dish to prevent cross contamination of the dilution, with an 8 channel pipette. The microtitre plate was sealed and then incubated for 30 minutes at 37°C. After incubation the plate was washed 4 times using an 8 Channel Immunolon plate washer (A/S Nunc, Roskilde, Denmark) and a wash buffer. Excess wash buffer was removed by inversion and taping.

2.4.2 Blocker

A blocking agent was added to each well (250µl) and the plate was sealed and left at room temperature for 15 minutes. After incubation the plate was washed twice with wash buffer and inverted.

2.4.3 Primary Antibody Coating

Rabbit Antihuman Lysozyme (A0099, Dako, Cambridge, UK) 7.1mg/ml was diluted 1:500 with the wash buffer as described above. Aliquots of 150µl was added to each well and incubated for 30 minutes at 37°C shaking at 100rpm (iEMS incubator/shaker HT, Helsinki, Finland). Following incubation the plate was washed 4 times as described previously.

2.4.4 Secondary Antibody Application

The secondary antibody, Peroxidase Conjugated Goat Anti-Rabbit 0.3mg/ml (P0448, Dako, UK) was diluted in wash buffer 1:1000. Secondary antibody (150µl) was added to each well and the plate was incubated for a further 30 minutes at 37^{o}C, and shaking at 100rpm. Following the incubation the plate was washed using the plate washer 4 times.

2.4.5 TMB Application

A TMB cocktail (150µl) was added to each well whilst in the fume hood. The plate was then incubated for 6 minutes unshaken at room temperature in the dark. The reaction was stopped with 50µl of 1M sulphuric acid. The absorbance was then read at 450nm (Dynex Plate Reader, MRX Revelation, Thermo Labsystems, Helsinki, Finland).

2.4.6 Calibration Curve

A calibration curve was constructed using the results from the range of known Lysozyme samples, (0.05µg ml^{-1} – 0.25µg ml^{-1}). This was then used to determine the Lysozyme content of all unknown samples.

2.4.7 Serum Samples

Whole blood samples were collected from patients in accordance with ethical approval (SW 87/2003) from the Great Western Hospital, Swindon, UK (Appendix C & D). Samples were taken from a range of hospitalised patients (listed in section 2.1.1), from patients who were negative for sepsis and from healthy volunteers. Whole blood samples were collected in Heparin tubes, centrifuged and the serum stored at -20^{o}C in the Hospital for 24 hours after collection from patients. The serum samples were then transported back to Cranfield University where work was undertaken. The serum were divided and stored at -20°C in 100µl aliquots, diluted $^{1}/_{200}$ in 25mM pH8 Tris buffer. Serum Lysozyme was quantified using the above protocol omitting the blocking step (2.5.2) from the procedure. The blocking step was used with buffered lysozyme samples to block unbound lysozyme sites, this step is not required when using serum. Four replicates of each serum sample were

executed using the ELISA protocol. The results were then quantified using the calibration curve produced with known lysozyme samples.

2.5 Commercial Kit Materials

The Human Lysozyme EIA kit (BT-630) was purchased from Biomedical Technologies Inc., USA (Appendix H) and consists of a 96 well microtitre plate coated with a monoclonal human lysozyme antibody. The kit was used according to the manufacturer's instructions. A summary of the protocol is given below.

A 1000ng lyophilised Human lysozyme standard was provided and a dilution range (0.78-50ng/ml) was prepared in Phosphate Saline washing buffer. A human lysozyme control (urine, lyophilised) reconstituted in 1ml washing buffer was also provided. All serum samples were diluted in 1/2000 in washing buffer. An aliquot (100µl) of each of the standards, control and serum samples was pipetted in duplicate, into the wells of the plate and the plate was sealed and incubated at room temperature for 2 hours. After incubation the plate was washed 3 times with the automatic plate washer and washing buffer. Lysozyme antiserum (100µl) was added to each well, covered and then incubated at room temperature for a further 1 hour. The plate was then washed as above and 100µl of Donkey anti-Goat IgG peroxidase was added to each well. The plate was resealed and incubated for a further 1 hour at room temperature. The plate was washed for the final time and 100µl of a 1:1 mixture of TMB and Hydrogen peroxide solution was added to the wells. The plate was then placed in dark storage for 15 minutes at room temperature. A 100µl of the Stop Solution was added and the absorbance measured at 450nm.

2.5.1 Additions to Kit Supplied

Reverse osmosis water was used to dilute the washing buffer supplied. The Dynex reader as mentioned in the above section was used to measure optical density at 450nm. The microtitre plate washer used with the ELISA method was also used.

2.5.2 Calibration Curve

When the assay was completed, a calibration curve was produced. Each kit came with a set of standards (which had known amounts of lysozyme) to be assayed. Therefore a separate calibration curve was produced for each kit to determine the lysozyme content of unknown patient samples.

2.6 ELISA Results

This section details all the results gained from the ELISA and commercial kit experiments. Some graphs contain Standard Deviation error bars.

2.6.1 Reference Range

Table 2.1 shows the lysozyme levels from 14 non hospitalised healthy volunteers. This data was then used in the comparison of hospitalised patient's lysozyme levels with the patient's clinical diagnosis. The control lysozyme level range was 2-5.8µg/ml, with an average of 4.37µg/ml.

Table 2.1 Lysozyme concentrations from healthy patients, used as a reference range.

Patient ID	Lysozyme Concentration (µg/ml)
V1	3.7
V2	2.0
V3	3.8
V4	4.6
V5	5.8
V6	5.7
VN1	3.7
VN2	4.4
VN3	3.8
VN4	5.4
VN5	5.4
VN6	5.4
VN7	3.8
VN8	3.7
Mean	4.37
Standard Deviation	1.07

2.6.2 Calibration Curve

Figure 2.2 was used to determine the lysozyme levels for all patient samples. Several sets of lysozyme standards were carried out using the ELISA's (n=17) during the

assaying of the patient samples. This data was then collated in a table and the averages calculated. Figure 2.2 below was then used to determine the lysozyme concentration for all patient samples.

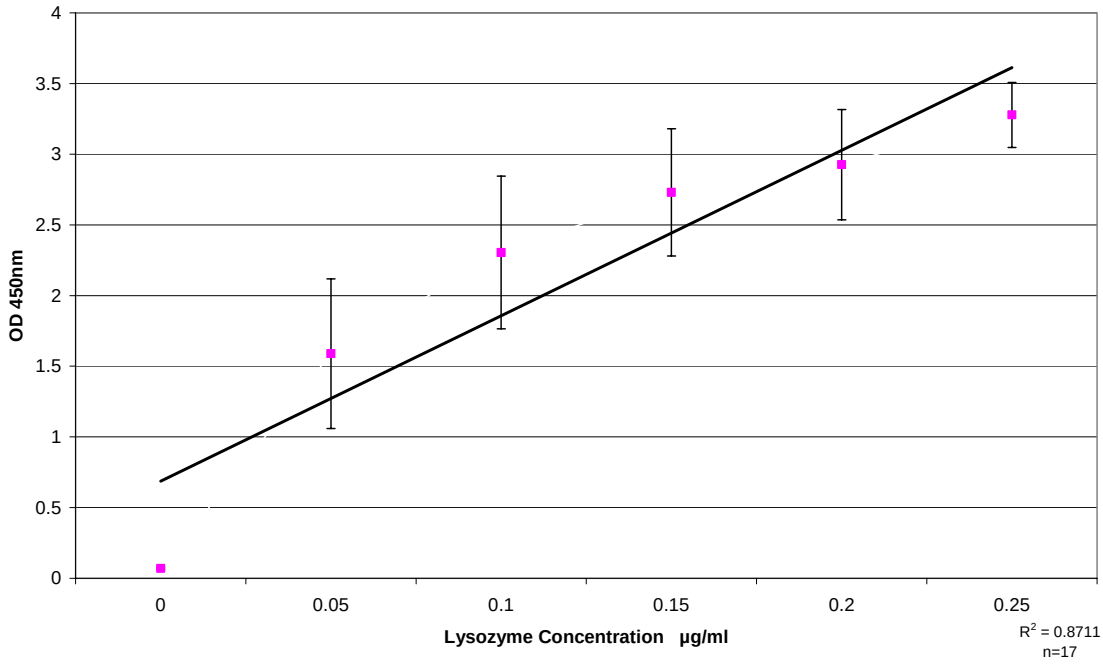


Figure 2.2 Calibration Curve for ELISA, this graph was used for the determination of unknown samples.

2.6.3 Freeze-Thawing Effects

Figure 2.3 shows the results from a series of assays completed over 10 weeks. Ten random samples were chosen from the clinical samples batch and assayed on 6 occasions during a 10 week period. Each sample was allowed to thaw fully before each assay and to freeze fully after each assay process. The results show the freeze thaw process had very little effects on the overall results. Even with repeated freezing and thawing the lysozyme content was virtually the same as when the sample was first assayed after being drawn from the patient. The small differences that were seen can be put down to assay variation.

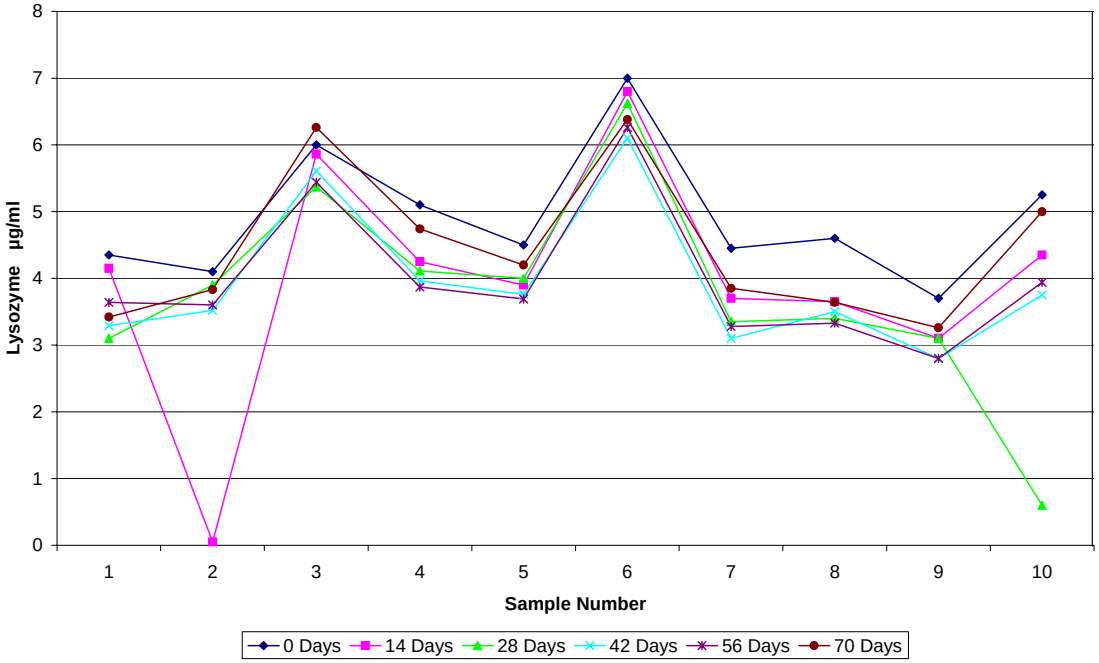


Figure 2.3 Results of Freezing and Thawing serum Samples over 10 Weeks, graph shows very small variations.

2.6.4 Commercial Kit Results

The table below (Table 2.2) shows the results from the clinical samples. Bacteraemia patients included all patients who were positive for sepsis, septicaemia, MRSA, UTI and URTI. All samples were assayed using the two techniques; ELISA and a kit purchased commercially. The results show the mean lysozyme levels in each group of patients.

The commercial kit results show the highest levels of lysozyme to be in the pregnancy patients; where as the ELISA results show the highest levels to be in the pneumonia patients. The commercial kit also shows that the lowest levels of lysozyme in the patients with positive blood cultures; whereas the ELISA revealed the lowest lysozyme levels to be in the chronic renal failure and non septic patients. Appendix I shows the results (in pink) gained from the commercial kits. As these graphs show, the commercial kit results did not compare with the ELISA results, in any of the cases. All commercial kit results were much lower than those from the ELISA kit.

Table 2.2 Comparison of the mean clinical sample results generated by two different assays; ELISA and Commercial Kit.

Diagnosis	Sample No.	ELISA Mean LZ µg/ml	Std. Dev.	Com. Kit Mean LZ µg/ml	Std. Dev.
Tumours	10	6.05	1.77	0.00016600	0.000135
Bacteraemia	42	6.14	2.06	0.00003259	0.000102
Pregnant	6	6.10	0.89	0.00425000	0.000724
Non Septic	109	5.69	1.48	0.00023800	0.000269
Pneumonia	28	7.06	3.26	0.00006092	0.000124
Leukaemia	9	6.59	2.22	0.00009100	0.000183
Deceased	37	6.30	3.00	0.00009073	0.000220
CRF	4	5.28	2.00	0.00014700	0.000194
Appendicitis	5	5.84	1.28	0.00007300	0.000155
Abdo Pain	15	6.13	1.28	0.00012300	0.000256

2.6.5 Confirmation Assay

Figure 2.4 below shows the results from three assays carried out on the same set of clinical samples. When the commercial kits were purchased the samples had been in the freezer for several weeks. This assay was to check immediately before the commercial kits were used, that the samples still had detectable lysozyme. The first set of ELISA Lysozyme results were completed when the clinical samples arrived in the lab. The second ELISA lysozyme results (repeats) were completed directly before the commercial kits were carried out. The results show that the commercial kit distinctly lacks sensitivity. Both ELISA's showed a very similar lysozyme content regardless of the time lapse between uses. No trend can be seen between the results either; where the lysozyme levels are high in the ELISA results the Lysozyme levels are low in the commercial kit results.

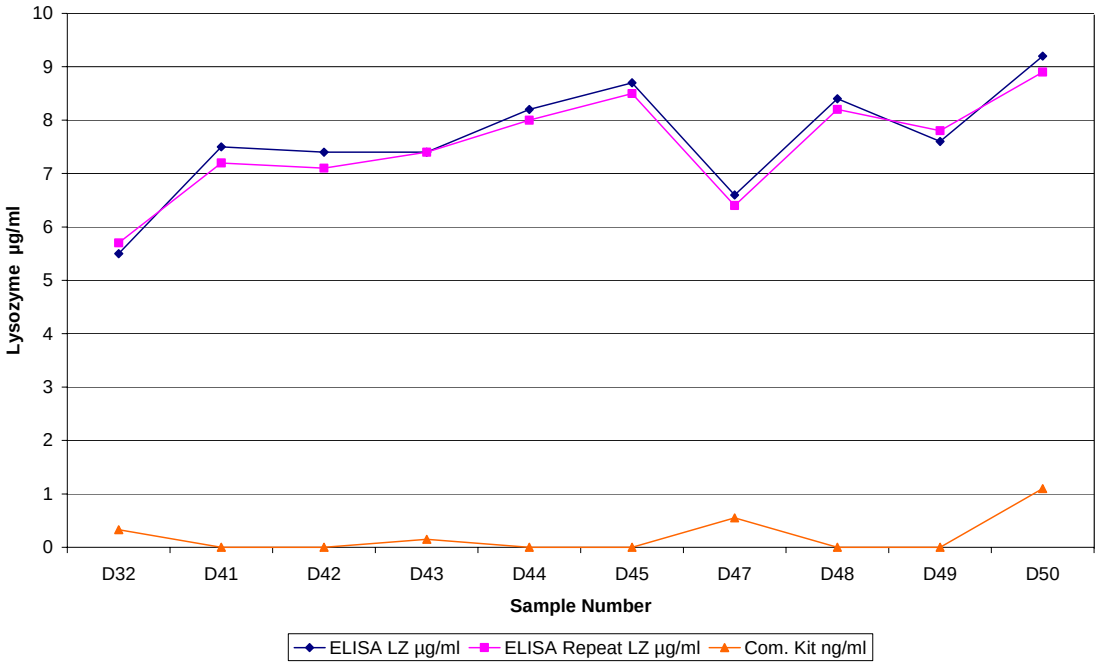


Figure 2.4 Confirmation Assay, to compare the levels of detectable lysozyme in serum samples tested with two ELISA's and the commercial kit; graph shows lysozyme was present in the serum, but not detectable by the Commercial kit.

2.6.6 Commercial Kit Calibration Curve

The graph below (Figure 2.5) shows the standards used in the commercial kit. Each kit came with its own set of 7 known lysozyme dilutions (0.78-50ng/ml), which were assayed (listed as "Average Achieved") and compared with the OD which was expected to be achieved from the standard curve graph which was provided with the kit (listed as "Com. Kit Standard"). The OD of the controls were very similar to the expected results. Figure 2.5 illustrates that the kit was working to an adequate standard. The lysozyme in the standards was detectable and the results comparable to those expected. This graph shows that the kit should have been able to measure the lysozyme in the serum samples, but for unexplainable reasons the kit produced results which indicated that there was far less lysozyme in the samples than the ELISA revealed. This suggests that the commercial kit lacks sensitivity in serum samples. The problem could be the contaminants in the serum. Many of the patients sampled had bacterial infections. The serum was not centrifuged or prepared in any way other than dilution before use in the commercial kit. The bacteria in the samples could be hindering the adherence of the lysozyme to the pre-coated micro-titre plate and also reducing the reactivity to the

primary antibody. Other causes could include drugs the patients were taking or abnormal plasma proteins; these could have caused the lack in sensitivity.

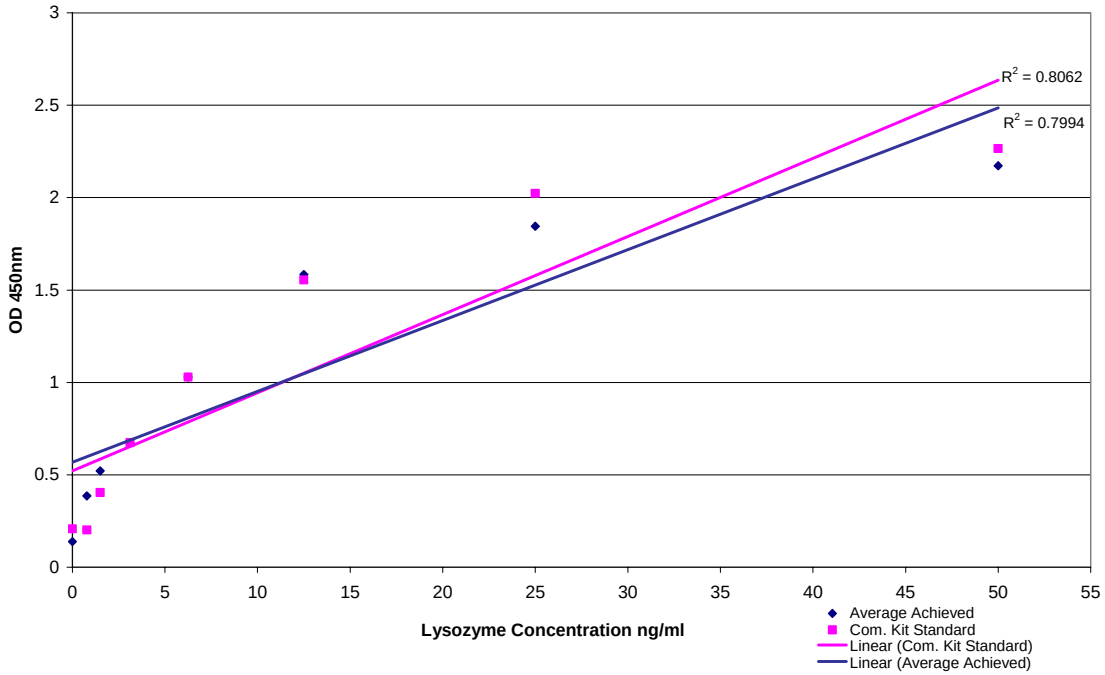


Figure 2.5 Graph showing a calibration curve constructed from commercial kit standards; this graph as used to determine the unknown lysozyme content of samples.

2.6.7 Clinical Sample Results Summary

Table 2.3 Blood Analysis Summary; table showing the mean response to increases in Lysozyme for patients with various conditions

Parameter	**Patient's Condition**						
	Abdo. Pain	*Append.*	*CRF*	*Leukaemia*	*Pneumonia*	*Pregnant*	*Tumour*
Haemoglobin	↓		↓	↓			↓
Neutrophil	↓		↓	↑		↓	
Lymphocyte	↑	↓	↑	↓			
WBC	↓			↑			↓
Platelets			↓	↑	↓	↓	
CRP					↑		

*↑ & ↓ indicate an increase & decrease in parameter value

2.6.8 Clinical Sample Results – Group Analysis

2.6.8.1 Lysozyme Analysis for All Patient Groups

Figure 2.6 below shows the lysozyme levels from all the clinical samples processed with the ELISA. The control group, of non hospitalised, healthy patients clearly have lower lysozyme levels than any of the other groups sampled. The highest lysozyme levels were seen in the leukaemia patients, pneumonia patients, the abdominal pain patients, and the deceased. The non septic group contained all patients who were deemed non septic by the sepsis scale but were hospitalised for other reasons not disclosed and who's true diagnosis was not disclosed. The lysozyme levels for this group were not significantly lower than other groups possibly due to these undisclosed illnesses which were affecting the patient's lysozyme levels.

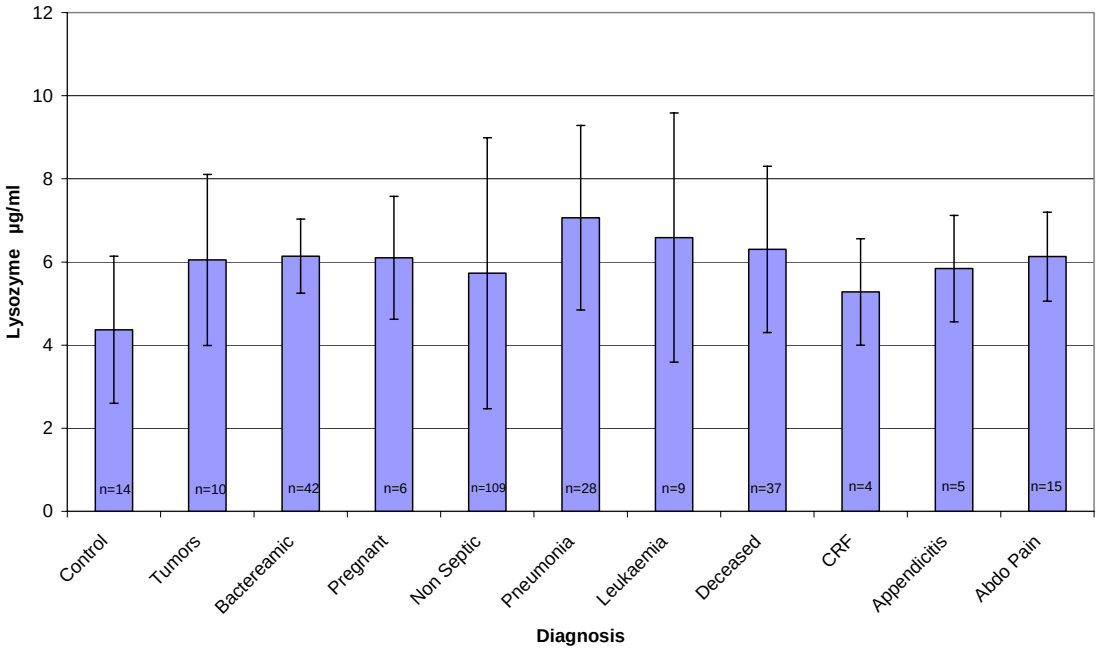


Figure 2.6 Graph showing the lysozyme levels in all patients sampled.

2.6.8.2 White Blood Cell Count Analysis

This graph (Figure 2.7) shows the highest white blood cell count to be in patients with leukaemia. Deceased and appendicitis patients also have elevated white blood cell counts. The main observation of Figure 2.7 is that white blood cell counts do not show a correlation with sepsis. The control group was not included here as these blood samples were taken from non hospitalised patients so blood cell counts were not available, however, this would be a good comparison for future work.

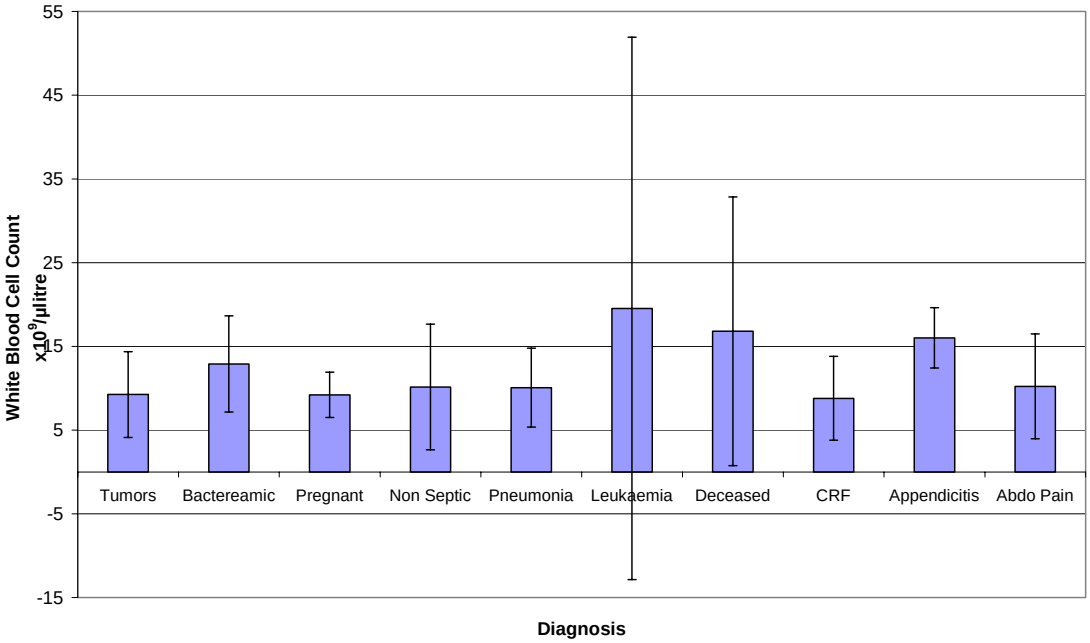


Figure 2.7 Graph showing the white blood cell levels for all patient groups sampled. Stand deviation also shown.

2.6.8.3 Lysozyme & White Blood Cell Count Analysis

Figure 2.8 shows the comparison between lysozyme levels with white blood cell levels from all patients. The graph shows lysozyme levels increasing with increasing white blood cell counts.

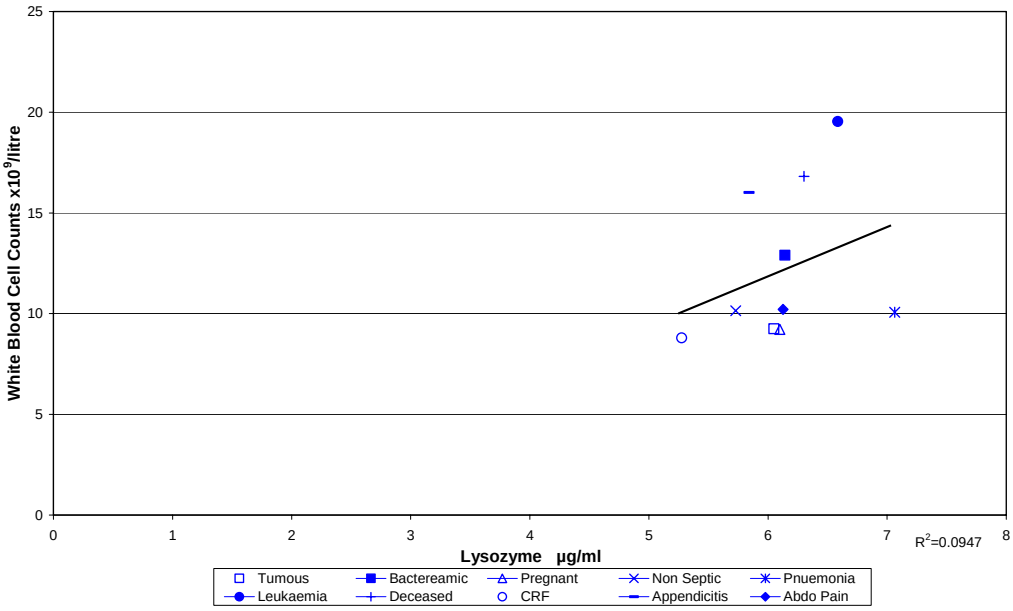


Figure 2.8 Comparison of mean lysozyme with mean white blood cell counts for all patients sampled.

2.6.8.4 Percentage Lysozyme Increase in Sepsis Patients

Figure 2.9 shows the percentage increase in lysozyme in patients with sepsis compared to the non septic and control groups. Even though the non-septic patients were deemed to have no sepsis from their signs and symptoms, from the doctor's observations and from blood analysis, their lysozyme levels clearly show an increase which may have been overlooked by current tests as their mean lysozyme concentrations were 31% higher than controls. Pneumonia patients display the largest increase in serum lysozyme concentration with a 62% increase. However, all groups had elevated lysozyme concentration when compared to controls, (bacteraemia 41% increase and appendicitis 34% increase). This graph shows that a difference is seen between controls and patients with proven sepsis and that lysozyme could be used to aid diagnosis.

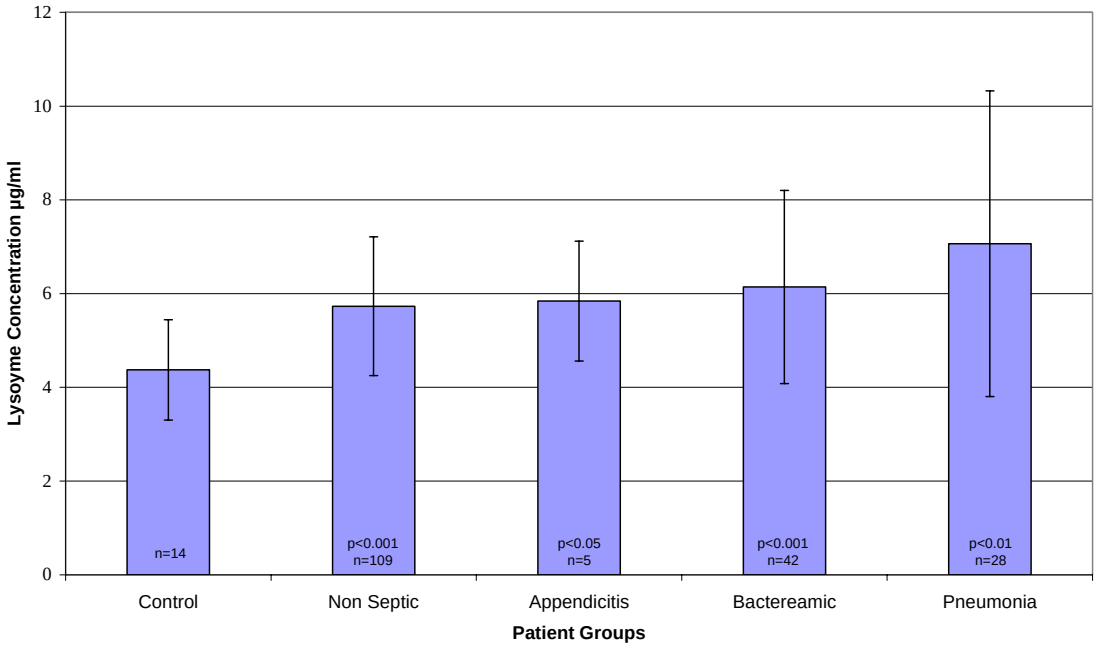


Figure 2.9 Lysozyme Increase, graph shows the lysozyme increase for septic patient groups and compares them with two control groups.

2.6.8.3 Lysozyme & Neutrophil, CRP, Platelets & Lymphocytes Analysis

Figure 2.10 a, b, c & d shows a negative correlation between neutrophil levels and lysozyme; whereby neutrophil levels decrease as lysozyme increases. The figure also shows positive relationships between CRP, platelets and lymphocytes with lysozyme concentration.

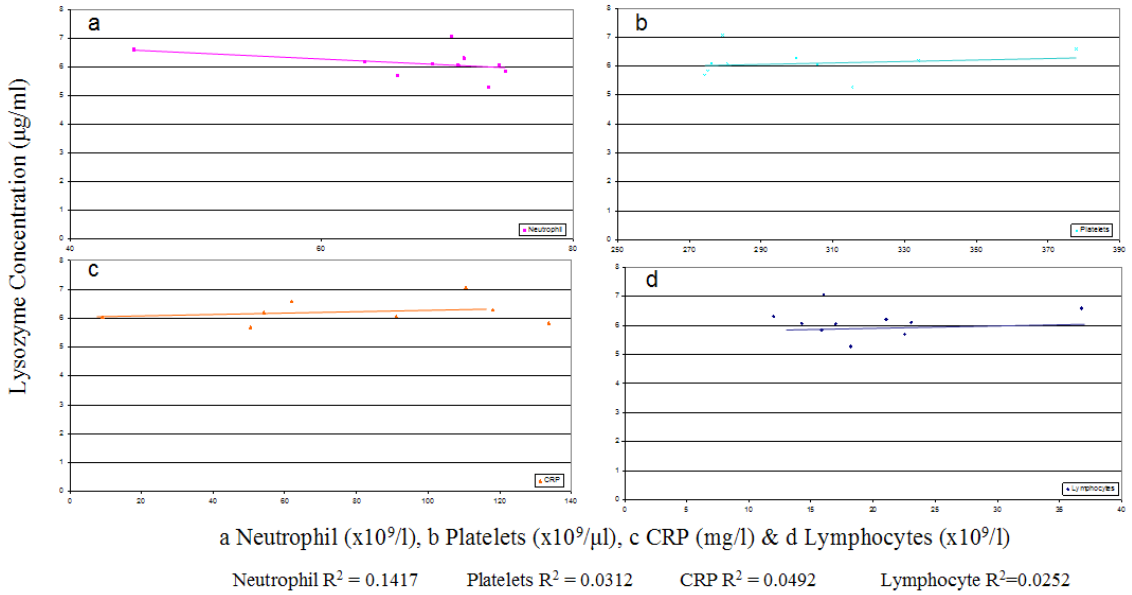


Figure 2.10 a, b, c & d Comparison of mean lysozyme levels with Neutrophils, CRP, Platelets & Lymphocytes for all patient samples.

2.6.9 Additional Sampling

Analysis of specific groups of patients and specific parameters can be found in Appendix I. The suggestive relationship between the clinical condition of the patient and the results from blood assessments is given. No correlations could be seen for the following blood results and diagnoses:

- Lysozyme from abdominal pain patients was compared with platelets and CRP.
- Lysozyme from appendicitis patients and haemoglobin, neutrophils, white blood cells, platelets and CRP.
- Lysozyme from chronic renal failure patients was also compared with white blood cell counts and CRP.
- Lysozyme levels were compared with CRP from leukaemia patients.
- Haemoglobin, neutrophils, lymphocytes, and white blood cell counts were compared with lysozyme from pneumonia patients.
- Lysozyme from pregnant patients was compared with haemoglobin, CRP, neutrophils, lymphocytes and white blood cell counts.
- Patients diagnosed with tumours had their lysozyme compared with neutrophils, lymphocytes, platelets, and CRP.

- Lysozyme from bacteraemia patients, non-septic and deceased patients were compared with all measured parameters (haemoglobin, neutrophil, lymphocytes, white blood cells, platelets, and CRP).
- No correlation between male and female and age lysozyme levels for any of the patients conditions.

2.6.10 Statistical Analysis, T-Test

Table 2.4 shows the results from a statistical analysis test. The Student t-Test was carried out on all the patient sample groups independently, rejecting the null hypothesis if the probability value was $p<0.05$. The null hypothesis stated that there was no difference between each groups mean lysozyme and the control group. Each diagnostic group was compared with the control group and the statistical difference was recorded. The statistical difference was also recorded for the control group v's the mean of all lysozyme levels from all hospitalised groups. The results showed that the majority of the lysozyme levels from each group were statistically different when compared to the lysozyme levels of the controls. The only exception was the chronic renal failure group. This group had lysozyme levels which were very similar to the control lysozyme levels so therefore were not significantly different. Patients with sepsis had mean serum lysozyme levels significantly higher compared to healthy controls.

Table 2.4 Table showing Student t-Test results for all Patient Samples

Diagnosis	No of Samples	Mean	Std. Dev.	t-Test Result	P Value
Controls	14	4.37	1.07		
Tumours	10	6.05	1.77	2.44	$p<0.05$
Bacteraemia	42	6.14	2.06	4.133	$p<0.001$
Pregnant	6	6.1	0.89	3.736	$p<0.01$
Non-Septic	109	5.73	1.48	4.212	$p<0.001$
Pneumonia	28	7.064	3.26	3.963	$p<0.001$
Leukaemia	9	6.587	2.22	2.793	$p<0.05$
Deceased	37	6.303	3	3.388	$p<0.01$

CRF	4	5.28	2	0.875	N/S
Appendicitis	5	5.84	1.28	2.296	p<0.05
Abdominal Pain	15	6.13	1.28	4.021	p<0.001
All Patient Samples	279	5.96	0.468	5.422	p<0.001

*N/S, Not Significant

2.6.11 Statistical Analysis, F-Test

Table 2.5 shows the results from the ANOVA statistical F-test, rejecting the null hypothesis if the probability value was p<0.05. Each test confirmed that the null hypothesis was to be rejected with the exception of ESR v lysozyme. The null hypothesis states that lysozyme is affected by each condition equally. The alternative hypothesis stated that one of the conditions had a greater affect on lysozyme levels more than the others. In all cases of Table 2.5, the null hypothesis was rejected meaning there was a significant difference between factors.

Table 2.5 Table showing F-Test ANOVA results for all Patient Samples

Diagnosis	**ANOVA Result**	**P Value**
Controls v Tumours, Bacteraemia, Pregnant, Non-Septic, Pneumonia, Leukaemia, Deceased, CRF, Appendicitis, Abdominal Pain	2.19	P<0.0190
Control v Non-Septic, Bacteraemia, Pneumonia & Appendicitis	5.59	P<0.0003
CRP v Lysozyme	4.12	P<0.0453
White Blood Cells v Lysozyme	6.59	P<0.0143
Neutrophils v Lysozyme	7.2	P<0.0081
ESR v Lysozyme	2.15	N/S

*N/S, Not Significant

2.8 Discussion

This section discusses the results in 2.6.

2.8.1 Reference Range

Table 2.1 shows the lysozyme results from the non-hospitalised, healthy control group. The lysozyme average was 4.37µg/ml, which was the average taken from 14 volunteer's results. The results ranged from 2-5.8µg/ml. This range was established from a group of non-hospitalised volunteers who deemed themselves to be healthy at the time of the test.

Appendix J - Cited Lysozyme Reference Ranges, lists all the lysozyme reference ranges cited in journal publications. The mean cited reference value for lysozyme was 7.43µg/ml with a range of 0.54-25µg/ml. These lysozyme values are much higher than the level established by the lysozyme ELISA of this project. Appendix J shows that different experimental methods give dissimilar lysozyme ranges. This therefore means that the ELISA reference is not necessarily wrong because it is different from those cited; it is just correct for this particular method and particular biological fluid type and should only be used with the ELISA protocol with serum. This is reinforced by comments made on other kits (IgM ELISA, Research Diagnostics Inc.) that their kits only relate to specific assay ranges provided with the kit.

2.8.2 Freeze-Thawing

The freeze-thawing test was carried out due to the time scale the clinical samples were processed. The clinical samples would arrive unfrozen in the lab and a selection of them would be assayed immediately. The rest would be frozen and then thawed before assaying. They would then be frozen and thawed a second time before being assayed with the commercial kit. Figure 2.3 shows a selection of clinical samples which were frozen and thawed 5 times over 70 days. The results from this experiment showed very little variation in the lysozyme content of the samples. The commercial kit was able to process 40 samples per run, but did not arrive until some time after all the samples had been assayed with the ELISA, this meant the serum samples had to undergo another round of freezing and thawing before assaying with the commercial kit. This lead to the

question of whether the samples were being affected by the freezing and thawing process.

The results from this experiment showed that the serum was not being significantly affected by the freeze-thaw process and the commercial kit assay results should be unaffected. This is conducive with the results from Cavalleri *et al*. (2004). Their study measured free radical concentration in serum samples which had been repeatedly frozen and thawed over a two year period. The free radical concentration did not significantly alter, leading to the conclusion that the freeze-thawing process was not detrimental to serum. A study by Comstock *et al*. (2001) also showed that serum samples which underwent <3 freeze-thaw cycles (-20^{o}C & -70^{o}C) were not affected. Serum which was subjected to more than 3 cycles were more affected showing 2-4% changes in hormone levels with even less changes in cholesterol and most micro nutrients. Serum antibodies were also frozen and thawed several times by Pinsky *et al.* (2003) in the detection of measles, mumps, and rubella. Pinsky *et al.* (2003) found "no clinically or statistically significant effect on measured antibody levels" and concluded by saying their experiments found "no discernible detrimental effect on the ability to measure these antibodies by enzyme-linked immunoassays". Therefore, the effects of freeze thawing on lysozyme in serum samples should be negligible and this is shown in Figure 2.3.

2.8.3 Commercial Kit Results

The Confirmation Assay (Figure 2.4) was carried out due to the poor quality of the results from the commercial kit. A random selection of clinical samples were processed, both with the ELISA and the commercial kit. The ELISA results were in-line with those previously reported by the ELISA kit (Figure 2.4), but the commercial kit results were all far lower than expected.

The calibration curve (Figure 2.5) for the commercial kit showed the kit was working properly. The kit came with its own calibration curve and the results from the standards from the assay were compared with this. The graph shows that the kit was working as it should be. The kit was used as the instructions dictated and the only difference was the amount of clinical samples used. The kit was designed to assay 10 samples, but here, due to budget constraints 40 samples (2 duplicates) were processed with this kit.

However, this should not have affected the overall performance of the kit in any way. The references on the instructions for the commercial kit assay (Appendix H) do not relate to the actual testing or development of the assay. Therefore it is hard to say why these results did not compare with the ELISA results. One explanation of why the commercial kit did not work as effectively as it should is because of its poor instructions. In the "Other supplies required" section of the Appendix H, it clearly states that a 37^{o}C incubator is required. However, no where in any of the "Assay Procedure" section of Appendix H does it say when to use it. Usually the 37^{o}C incubator is used during the initial stages of the assay, during the ligand immobilisation, but this assay comes with a pre-coated plate, so this stage is missed out. Also as the controls on the plate (Figure 2.5) showed the assay was working normally, it suggests that it was the serum which was the problem. But as Figure 2.3 and 2.4 show the serum was fine, regardless of the amount of freeze-thawing it had under gone. In conclusion, the incubator may simply be a typing error and not actually supposed to be used in this assay, but it also may be the reason behind the poor results.

2.8.4 Comparison of Commercial Kit & ELISA

All ELISA protocols have a similar setup, in that they have immobilisations of ligands and analytes, enzyme-substrate reactions, washing phases and incubation periods. Even though all ELISA systems are different they still have the same disadvantages; they all have "position dependent differences in microtitre plates" (Goa, 1995) meaning the position of the well can dictate the quality of the results produced due to poor washing methods used. The assay has very specific timing for the incubations which are critical to the overall result. Most ELISA's consist of more than 2 steps in the protocol giving the assay a degree of complexity warranting training.

2.8.4.1 Analytical Sensitivity

The ELISA has an analytical range of 1-400ng/ml and a limit of detection of 0.36ng/ml. The commercial kit has an analytical range of 0.78-50ng/ml and a sensitivity of 0.78ng/ml. As the limit of detection of the commercial kit is not disclosed it is difficult to say for certain if the commercial kit lacks sensitivity, however, as it could not detect

lysozyme in the serum samples then it's the only conclusion that can be drawn. This was also confirmed by the kits producers.

2.8.4.2 Number of Samples Tested

The number of samples each assay can test is the same due to the number of wells in a 96 well micro titre plate. The number of duplicates for each sample can vary. With the commercial kit due to a limited budget, the number of samples processed by each kit was high (40 samples/plate) as each kit cost ~£300. As the ELISA equipment and reagents were in ample supply each sample was replicated 8 times and an average taken for each.

2.8.4.3 Ease of Performance

The ELISA protocol took 96 minutes in total to complete one assay. The preparation time varied depending on the number of clinical samples being processed, but was generally no more than 60 minutes. The commercial kit protocol took 4.5 hours to complete one assay and preparation time was more than an hour each time. The commercial kit was a very laborious assay to carry out, and very demanding. With the ELISA, 2 assays could be run simultaneously by being staggered by 30 minutes. This would allow several assays to be carried out in one day, and if necessary repeat assays could be carried out and the clinical samples would not have to undergo another freeze-thaw process. The commercial kit, however, took so long to prepare and execute that there simply wasn't enough time in the day to carry out more than 2 assays.

The commercial kit did not arrive with all the reagents needed. Due to the number of clinical samples due to be processed with each kit extra buffer had to be purchased; thus adding further to the expense of the kit. The commercial kit instructions also had in its requirements list, a 37^{o}C incubator. The protocol however, never mentioned the use of this. This was a typing error which should not have been included as confirmed by the kits producer.

The ELISA and commercial kit both had easy reagent preparation methods, an easy to use test procedure, required very little training on the equipment, used safe procedures and the results were easy to interpret.

2.8.5 Clinical Sample Results – Group Analysis

2.8.5.1 Lysozyme & White Blood Cell Count Analysis

Figure 2.8 shows the white cell count versus the lysozyme level in all hospitalised patients. The graph shows the increasing white blood cell count with the increasing lysozyme level. This is due to the number of white blood cells releasing lysozyme to fight the infection. Figures 2.6 and 2.7 also show the distribution of lysozyme and white blood cell expression in the various patients sampled. Leukaemia and pneumonia expressed the most lysozyme with leukaemia and deceased patients expressing the most white blood cells. The pneumonia patient's lysozyme levels do not correlate with the white blood cell counts. This shows that white blood cell counts are not a good indication of sepsis in this group. The bacteraemia and appendicitis patients white cell counts are also not elevated also showing that white cell counts are not indicative of sepsis. During leukaemia, the body produces high levels of white blood cells which are characteristically dysfunctional; these then leak the lysozyme causing high lysozyme levels (Davis, 1971).

2.8.5.2 Percentage Lysozyme Increase in Sepsis Patients

Figure 2.9 shows the percentage increase in lysozyme in those patients with sepsis. The statistical analysis of these results will follow this discussion. Pneumonia patients had the highest increase in lysozyme levels of 62% above controls. When compared to non-septic lysozyme levels, also shown in this figure the increase in pneumonia lysozyme levels is also significant. This figure also shows the lysozyme increases in patients with appendicitis (34%) and bacteraemia (41%). The figure shows these increases also to be significantly higher than the controls. Lysozyme is therefore a good indicator of sepsis in patients with proven sepsis.

2.8.5.3 Lysozyme & Neutrophil, CRP, Platelets & Lymphocytes Analysis

Figure 2.10 a, b, c & d show the relationships between mean lysozyme levels and neutrophils, CRP, platelets and lymphocytes for all ill patients. The figure shows a negative relationship between neutrophils and lysozyme, suggesting a defect in defence cell production caused by surgery stress. There is a positive relationship between platelets, CRP and lymphocytes with lysozyme. CRP is released in response to

inflammation, platelets are involved with blood clotting and lymphocytes release upon infection, therefore in septic conditions a positive correlation between these and lysozyme is to be expected.

2.8.5.4 Statistical Analysis

Table 2.4 shows the results from the student t-test, carried out on the clinical sample results. Each patient group was statistically compared with the control (non-hospitalised, healthy) patient group. Table 2.4 shows that only one group, the chronic renal failure patients had lysozyme levels which were not significantly different from the control. This is because the lysozyme levels were very similar to the control group. Also the amount of patients in this group was very small therefore, more testing of more chronic renal failure patients should have been carried out to make more substantiating conclusions. All other patient groups, (Tumours, Bacteraemia, Pregnant, Non-Septic, Pneumonia, Leukaemia, Deceased, Appendicitis, and Abdominal Pain) had lysozyme levels which were statistically significantly different when compared to the control group. For a more accurate result more patient samples would have to be carried out. But these results do show that the ELISA method is capable of distinguishing non-septic and septic serum samples.

The f-test also showed there was a statically significant difference between healthy control samples and diseased patients. There was also a negative relationship between neutrophil and lysozyme levels and a positive relationship between white blood cells and lysozyme in bacteraemia patients.

2.6 Advantages of ELISA

The total ELISA assay time was 96 minutes. This was a great improvement on current lysozyme immunoassays on the market. Each ELISA assay can process 40 clinical samples per run with two replicates equalling an average of 2.4minutes per sample. Preparation time only usually amounted to an hour before the assay started due to buffers being very stable and lasting for several weeks before needing to be remade. Clean up time once the assay had finished, was also very short, as most of the equipment was single use only and therefore disposed of.

All immunochemicals and reagents are commercially available. The total cost of one assay is around £17.00 for 40 samples. This is a considerable difference in price compared to commercial kits available on the market.

There are several types of immunoassay based on this idea of recognition and these are divided into competitive and non-competitive assays. However, with all immunoassays the results obtained are quantitative. These can then be compared with known standards. With every assay known concentrations of controls are carried out so a direct comparison can be carried out leading to accurate results every time (Torrance, 1998).

2.7 ELISA Synopsis

The ELISA work reported was both a development of an in-house immunoassay and the progression into clinical sample testing for diagnostic use. The work initially began with establishing a reference range for the ELISA assay. The ELISA reference range was 2-5.8µg/ml. The freeze thawing of serum samples showed no effects on the lysozyme at $4^{o}C$ and showed the method used for thawing and refreezing the samples was having no detrimental effects on the lysozyme.

A commercial kit was also used in the ELISA work. This kit was bought to test the validity of the ELISA method. All clinical serum samples were assayed using the ELISA initially and then the commercial kit. The freeze-thaw experiment was therefore used to confirm lysozyme was not being affected during this process. However, the commercial kit was far less sensitive than expected and the results generated did not compare to the ELISA results.

A comparison of the two immunoassays was also carried out. The ELISA proved to be far more sensitive than the commercial kit, the overall cost of the ELISA was much less than the commercial kit and the ELISA was both far easier to execute and takes less than half the amount of time to complete the assay than the commercial kit. This therefore leads to the conclusion that the ELISA kit has many attractive characteristics over other commercially available kits.

The lysozyme levels from all clinical samples were compared to the blood results provided with each clinical sample. The appendicitis patient's results correlated with the expected results for this condition. High lysozyme, lymphocyte and neutrophil levels were shown in these patients due to infection and the surgery-stress response.

The abdominal pain group although had negative bacterial cultures showed an increase in white blood cell and lysozyme which may have indicated the start of an infection, possibly leading to appendicitis.

The results from the leukaemia patients showed the white blood count markedly increased due to the characteristic mass production of dysfunctional white blood cells. The decreased haemoglobin levels results link with many patients suffering from anaemia and the high lysozyme is indicative of infection, to which leukaemia patients are particularly susceptible. The prevalence of infections in the leukaemia group was also evident due to the high neutrophil levels.

The results from the pneumonia group showed increased lysozyme levels representing phagocytosis. Pneumonia occurs due to a suppressed immune system and an opportunistic infection. The CRP levels in pneumonia patients show increasing levels with increasing lysozyme, which is indicative of infection.

The pregnancy patient's lysozyme results also indicated their physiological condition. The patients displayed increased neutrophils and lysozyme, either due to infection or their immune system heightening as a preventative defence against infection.

Results from the patients with tumours showed low haemoglobin levels and a depressed white blood cell count. Lysozyme levels were raised in these patients indicating possible infections or the release of lysozyme during white blood cell lysis.

The collaborative group analysis data showed white blood cell count increasing with lysozyme and the highest distribution of lysozyme and white blood cells was in leukaemic and pneumonia patients.

Statistical analysis was carried out on each diseased patient group and the lysozyme level was compared to the non-hospitalised control group lysozyme level. The results showed all diseased patient groups were statistically different. The only exception was the CRF group due to small numbers. Pneumonia patients had the highest mean lysozyme increase over other groups sampled. Sepsis groups were compared to healthy controls showing there was a statically significant difference. These results showed the ELISA was a reliable diagnostic tool for detecting elevated lysozyme levels in septic patients.

The ELISA therefore has been tested with both lysozyme in buffer and serum lysozyme from both healthy and diseased patients. The ELISA has also been quantitatively and qualitatively compared to a commercially available anti-lysozyme kit and has succeeded to show it is the better test. Further clinical samples should be assayed using the ELISA, but the results from the current clinical samples show a promising use for this diagnostic tool in aiding the diagnosis of future septic patients.

Chapter 3 -ELIFA

3.1 Introduction

The basic principle of Enzyme Linked Immunosorbent Flow-Through Assay (ELIFA) is to filter immuno-concentrates onto the surface of a membrane. With the success of the ELISA assay, the main idea behind the ELIFA system was to build an assay which was faster, easier to use and cheaper to run. The ELIFA has the potential of high levels of ligand binding compared to those assays where ligand are immobilised on a plastic surface, (Hermanson *et al.*, 1992). In theory the ELIFA, limits the diffusion at the surface of the porous materials, but allows the measurement of analytes in low concentrations.

The ELIFA is an indirect immunoassay incorporating diffusion which takes place by the analyte when it reacts with the ligand immobilised on the solid surface. The diffusion can be limited in the liquid-solid interface due to the difference in viscosity. The ELIFA overcomes this because all ligand and analyte are deposited on the membrane and all solutions are sucked through the pores of the membrane. A primary antibody then binds to any analyte present and a secondary conjugated antibody binds to the primary antibody. A substrate then produces a discolouration on the surface of the membrane when all three are present. The discolouration is directly proportional to the amount of lysozyme in the initial sample. Each step is followed by a washing phase which is sucked through the membrane via a peristaltic pump. The end product of the ELIFA is a soluble substrate solution which can be measured using a spectrophotometer.

The ELIFA apparatus consists of a porous membrane in which the reaction occurs. Feadda (2003) trialled 5 different membranes, which were either neutral-hydrophilic or negatively charged. This report found the Polyamide nylon membrane had up to 80% greater binding than other membranes; this membrane was therefore used in the ELIFA protocol. The membrane was held immobilised in position by a flow cell. The flow cells consisted of an inferior and a superior part in which a membrane was clamped. Tubing was connected to the cannula attached to the underside of the interior and to

tubing attached to the peristaltic pump; this allowed all fluid to be removed from the loading chamber of the superior flow cell (Figure 3.1).

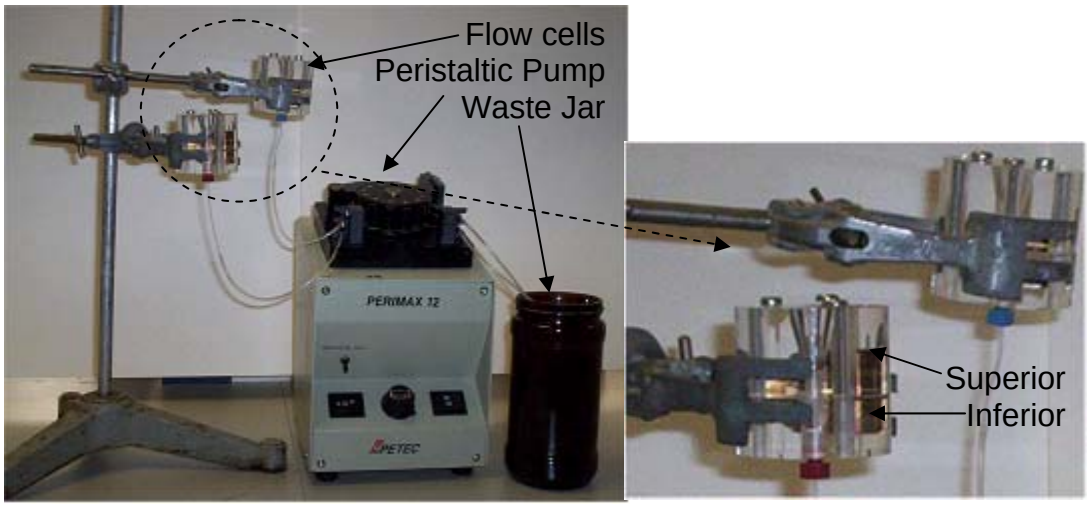


Figure 3.1 The Working ELIFA System; Photograph shows the flow cells assembled and linked via tubing to the peristaltic pump. All fluids are sucked along this tubing from the loading chamber of the flow cell to the waste jar.

3.2 Potential Improvements of the Lysozyme ELIFA compared to the ELISA

Technically the ELIFA design was intended to produce homogeneous and slow flow through rates past the immobilised membrane. This was to allow even distribution of lysozyme to be immobilised and to allow maximum binding with the primary and secondary antibodies; which means the ELIFA would have the potential to be more sensitive than the ELISA.

The distribution of these reagents should also be uniform due to the peristaltic pump pumping rate being constant for all flow cells. This could produce consistency to the delivery of the antibodies, whereas the ELISA is managed by operator control, which could lead to inconsistencies.

3.3 ELIFA Application

A general ELIFA system can be purchased from Pierce Science. The "Easy-titre ELIFA system" can be used with any analyte and for any target for a costly sum of £1,000. The assay takes 25 minutes to complete and a 96 well arrangement is used allowing multiple replicates and controls.

An ELIFA assay proposed by Valkirs & Barton (1985) was for an early pregnancy test measuring the human choriogonadotropic hormone (HCG). This ELIFA was a two-site immunoenzymometric assay, in which an antibody was seeded to a membrane, and the antigen (from either urine or serum) was passed through and a substrate was applied to create a colour reaction if the hormone was present in the sample. The test could detect as low as 4µg/L in urine and 2µg/L in serum. The high ratio of surface area to volume ensured short diffusion distances and a sensitive test.

3.4 Materials & Methods -ELIFA

All buffer recipes can be found in Appendix G.

3.4.1 General Reagents

Hybond N (Amersham, UK) was used as hydrophilic neutral nylon membrane (0.45µm diameter). A Blocker Casein in TBS from Pierce Science UK Ltd. (Tetterhall, UK) was used to minimize non-specific antibodies binding to the membrane. The washing step was used to remove any unbound antibodies/antigen from the membrane.

3.4.2 Peristaltic Pump Calibration

A Spectec Perimax 12, 4 channel peristaltic pump was calibrated with water through both the sample and control flow cells. A calibration curve of ml/minute at particular speeds of the pump was constructed. The pump rate from the calibration curve was then varied during different parts of the immobilisation, injection of antibodies, staining and washes. PVC tubing (0.76mm from Anachem, UK) connected to 0.8x1.5mm Teflon tubing (Ismatec, Zurich) was connected to the cannula of the flow cells and the peristaltic pump. A Gilson Minipuls, 2 channel pump (Gilson, USA) was also used during these experiments.

3.4.3 Flow Cell Construction

Perspex flow cells were purpose designed (John Bolbot, Cranfield University) and made by Model Products Ltd (Bedfordshire, UK). A circle of membrane was cut and placed in between the inferior and superior part of the flow cell. The flow cell components were joined together by three screws to clamp the membrane in place. A tube 0.76mm internal diameter (Anachem, UK) was connected to a cannula on the inferior part of the flow cell and attached to the peristaltic pump (Figure 3.2).

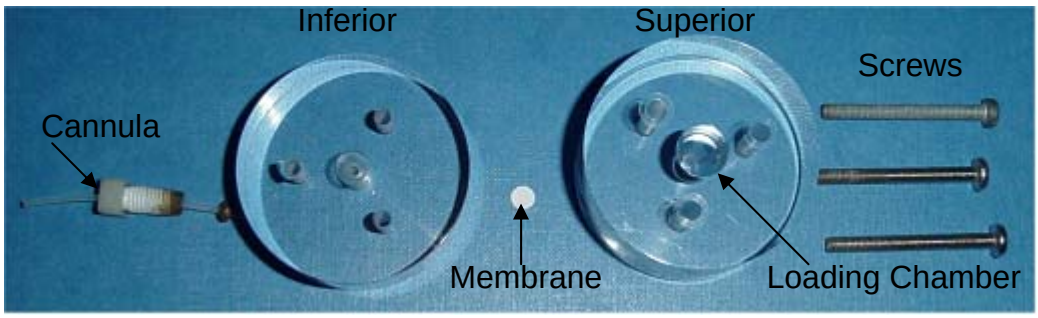


Figure 3.2 Parts of a Flow Cell; Picture shows all flow cell components including the inferior and superior elements, loading chamber and cannula. The membrane is placed between the inferior and superior and held in place with three screws. The cannula is screwed into the underside of the inferior element, and is connected to further tubing and a peristaltic pump.

3.4.4 Lysozyme Coating & Blocking Step

Lyophilised Lysozyme from human milk (L6394 Sigma-Aldrich, UK) was diluted in Coating buffer (Appendix G) and aliquots frozen at -20ºC. An aliquot (500µl) of the lysozyme was loaded into the loading chamber of the superior flow cell. This was pumped through the Hybond-N nylon membrane (Amersham Pharmacia Biotech, UK) at a flow rate of 0.04ml/min. Blocking agent (200µl) was added to the flow cell and pumped through at a flow rate of 0.04ml/min.

3.4.5 Antibody Application

Antibodies used in the ELIFA were from DAKO (Cambridge, UK) (1^{o} A0099 & 2^{o} P0448). The primary antibody (section 2.4.3) concentration was 1:6000 and the secondary antibody (section 2.5.4) 1:4000. Both the primary and secondary antibodies were diluted using the WBTC, (Appendix G). Primary antibody (200µl; 1:6000) was pipetted into the loading chamber and pumped at 0.02ml/min. The secondary antibody was loaded next. A dilution of 1:4000 diluted in WBTC was prepared and 200µl was pumped through at a flow rate of 0.04ml/min. The washing solution (200µl) was pumped through at 0.4ml/min and repeated 3 times.

3.4.6 Colour Reaction

Undiluted TMB cocktail (150µl) (T8665 Sigma-Aldrich Ltd, UK) was loaded into the chamber and pumped through the membrane for 30 seconds at 0.04ml/min. The reaction was then allowed to continue under static conditions for a further 5.5mins. The

reaction was stopped by adding 50µl, 1M sulphuric acid to the remaining fluid in the flow cell.

3.4.7 Collecting the Sample

The sample was collected from the loading chamber by reversing the flow direction of the pump so the fluid in the tubing was forced back into the flow cell. The sample was then collected via a pipette into a microtitre plate and optical density was read at 450nm.

3.5 ELIFA Development Results

This section presents the initial ELIFA results. Several tests were carried out to test and optimise the protocol. The objective was to produce a calibration curve with a range of lysozyme concentrations, through a series of stained membranes and using the optical density of the filtrate. Several abbreviations were used in the following tables; C refers to Controls, S refers to Sample and N/S refers to No Staining. Where results are repeated for each test the characters (i), (ii) and (iii) are used.

3.5.1 Preliminary Experiment

The preliminary experiment was to test the equipment and the reagents. The Gilson two channel peristaltic pump was the first peristaltic pump to be used in these experiments. The assay was very fast only taking 37 minutes. Results showed the reagents and antibodies were viable. Discolouration was only seen on the sample membrane but no difference was seen between the optical density of the control and sample filtrate (Table 3.1).

Table 3.1 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for the Preliminary Experiment.

LZ Conc.	OD	Membrane Staining (C, control; S, sample)
0µg/ml	2.444	C N/S
20µg/ml	2.781	S discolouration

3.5.2 Equipment Changes to Enhance Results

3.5.2.1 Back Flush Test

Due to the success in the preliminary experiment, the lysozyme concentrations were lowered to see if more discolourations could be seen on the membrane with lower levels of lysozyme and to see if this would reveal a difference in the optical density of the filtrates.

The Gilson pump was again used in this second test with a wider range of dilutions of lysozyme. A back flush method was also introduced, which involved pumping the TMB through the membrane for 0.5 minutes and allowing the reaction to take place in stationary conditions for 5.5 minutes. The pump direction was then reversed so the

sample in the tubes flowed back into the loading chamber of the flow cells where it was pipetted out into a micro-titre plate for the optical density to be read.

The back flush was incorporated as non-specific binding was suspected. This is where binding of the HRP-conjugate is taking place not only on the membrane filter but also along the inside of the tubing, causing a purple discolouration. When the TMB was administered it was not only binding to the HRP-conjugate on the membrane but also inside the tubing. Therefore when the whole sample (lysozyme, 1^{o} & 2^{o} antibodies, and TMB) was eluted from the flow cells and tubing at the end of the experiment, an inaccurate sample was collected because so much was lost to the lining of the tubes and flow cells. The results from Test 3.5.2.1 show that the back flush alone was not the solution to the non-specific binding (NSB).

The results for all dilutions of samples tested showed no difference between the filtrates regardless of the lysozyme content (Table 3.2). Membranes from all samples and controls were also unstained.

Table 3.2 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.5.2.1.

LZ Conc.	OD	Membrane Staining (C, control; S, sample)
0µg/ml	1.615	All C N/S
0µg/ml	1.328	All S N/S
0µg/ml	1.167	
10µg/ml	1.213	
0µg/ml	1.158	
15µg/ml	1.103	
0µg/ml	2.766	
20µg/ml	1.536	

3.5.3 Reagent Changes to Enhance Results

3.5.3.1 Buffer Contamination

In the third set of tests, contamination of the buffer solutions was considered. All solutions had been kept out of the fridge on the bench during experiments and over

night. When new buffers (including wash solutions, WBTC and blocking step) were made these were kept at 4^{o}C to limit contamination from bacteria.

The 5µl/ml dilution was tested several times because contamination was suspected. After this dilution, all solutions were changed and stored as mentioned above. The other dilutions (10, 15 & 20µg/ml) performed in this test produced promising results. The results showed the control OD was low and the samples showed some pattern that corresponded to the actual lysozyme content. The control was in line with those expected results from Appendix K. The results for the lysozyme dilutions were also in line with those from Appendix K, with the exception of the 5µg/ml dilution which was higher than expected. No staining was seen on any of the membranes with the exception of the 20µg/ml.

Table 3.3 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.5.3.1.

LZ Conc.	OD	Membrane Staining (C, control; S, sample)
0µg/ml 5µg/ml	1.530 2.054	All C N/S S 20µg/ml discolouration
0µg/ml 5µg/ml	1.740 1.517	
0µg/ml 5µg/ml	2.357 2.146	
0µg/ml 10µg/ml	0.614 2.387	
0µg/ml 15µg/m	0.436 2.145	
0µg/ml 20µg/ml	0.764 2.494	

3.5.3.2 Further Contamination Testing

In a further test for contamination a fourth set of experiments were carried out where the only difference from the method used in Test 3.5.3.1 above was that the solutions were stored at 4^{o}C over night then allowed to reach room temperature two hours prior to the

start of the experiment. For (i) (Table 3.4) no staining was visible on the membranes which may be an indication of a lack of binding taking place on the actual membrane, suggesting a problem with non specific binding either on the flow cells or adherence inside the tubing.

Table 3.4 shows the repeat (ii) of the 5µl/ml dilution from Test 3.5.3.2. The repeat results (ii) show a small difference between the control and sample OD. The sample membrane was fully stained, with the control remaining clear. The optical densities for both (i) and (ii) however, did show a difference between control and sample in all the dilutions tested. But no correlation could be established; the OD did not increase with increasing lysozyme levels.

Table 3.4 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.5.3.2. Initial tests (i) and repeats (ii).

LZ Conc.	OD	Membrane Staining (C, control; S, sample)
(i) 0µg/ml	0.421	All C N/S
(i) 5µg/ml	0.803	All S N/S
(i) 0µg/ml	0.420	
(i) 10µg/ml	1.915	
(i) 0µg/ml	0.185	
(i) 15µg/ml	0.481	
(ii) 0µg/ml	0.344	C N/S
(ii) 5µg/ml	0.791	S 5µg/ml discolouration

The assay seemed to be working in Test 3.5.3.1 with the addition of freshly made buffers in terms of OD. However, in Test 3.5.3.2 the assay also seems to be working to a certain extent with old buffers that were stored at 4^{o}C. The filtrate OD for the sample and control for (ii) were in line with Appendix K. This seemed to suggest the assay was working from these initial results, but the problem of stained flow cells was still an issue. The Perspex flow cells also had a very distinct purple staining. As a result the flow cells were washed in 3M hydrochloric acid (HCl) to remove non-specific binding. The non-specific binding meant the current wash procedure (overnight soak in 1M HCl)

was not effectively cleaning the bound antibodies and may be interfering with the results by increasing the control filtrate OD and causing a decrease in the sample OD.

3.5.4 Wash Procedure Changes to Enhance Results

3.5.4.1 Use of Harsher Acids in Wash

The flow cells were soaked over night in the 3M HCl. Table 3.5 shows a low control OD and a difference between the sample and control OD for both tests using 5µg/ml of lysozyme. The controls were reduced in optical density but were still slightly higher than expected (Appendix K). The flow cells seemed to be adequately washed using the 3M HCl as no flow cell staining took place. But as the sample OD was very low when compared to Appendix K, this still did not provide the answer to the reduced sensitivity of the assay.

Table 3.5 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.5.4.1.

LZ Conc.	OD	Membrane Staining (C, control; S, sample)
0µg/ml	0.576	All C N/S
5µg/ml	0.759	All S N/S
0µg/ml	0.506	
5µg/ml	0.753	

3.5.4.2 Use of Detergent

The flow cells were then washed in Ariel biological hand wash liquid (1 cap/10litres). The Ariel contained proteases which degrade proteins that might have adhered to the flow cells causing the staining of the Perspex and the NSB. The results from this test are promising as the control is very low at 0.275 OD showing no unwanted binding. The sample also yielded a promising result in line with those expected (Appendix K). The limitation of this cleaning method was the time taken. The flow cells were washed in the Ariel for 24 hours before used. This meant that the assay took around 26 hours to complete one sample.

The buffers made in Test 3.5.3 were used for Tests 3.5.3 and 3.5.4. Test 3.5.3.1 (Buffer Contamination) results suggested that making the buffers fresh before running the assay would produce results in line with Appendix K. However, Test 3.5.4.2 (Use of

Detergent) results show that this is not required. Reusing refrigerated buffers also produced good results and shows that it was not an interfering factor.

Table 3.6 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.5.4.2.

LZ Conc.	OD	Membrane Staining (C, control; S, sample)
0µg/ml	0.275	All C N/S
5µg/ml	1.210	All S N/S

3.5.4.3 Reduction of Arial Wash

Section 3.5.4.2 (Use of Detergent) produced promising results, however the flow cell wash time needed to be reduced to speed up the assay. One hours soak in the Ariel was tried in Section 3.5.4.3. The tubing was also changed in this test as it appeared restricted due to the clamps holding it in place in the pump channels. The tubing needed changing quite often in this assay, as restriction meant the assay would run much slower than was intended making the conditions variable between assays. The results in Test 3.5.4.3 show that the Ariel is removing the proteins adhered to the flow cells however not to the extent that a 24 hour soak, as the OD of the controls increased, ranging from 0.921-2.406; far higher than Appendix K. The lysozyme samples showed no pattern either, as they did not increase with increasing lysozyme concentration. The 5µg/ml dilution was repeated twice with the OD from the second 5µg/ml being almost double the OD from the first dilution. This clearly shows that non-specific binding was taking place as the lysozyme from the first dilution was still present when the second dilution was measured. This test shows that the flow cells need washing between lysozyme measurements.

Table 3.7 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.5.4.3.

LZ Conc.	OD	Membrane Staining (C, control; S, sample)
0µg/ml	1.076	All C N/S
5µg/ml	1.754	All S N/S
0µg/ml	2.323	
5µg/ml	3.312	
0µg/ml	0.921	
10µg/ml	3.211	
0µg/ml	2.406	
15µg/ml	2.625	

3.5.4.4 Increased Rinsing of Flow Cells

This next test consisted of two injections of 15µg/ml lysozyme. The control OD for both was very high and the samples OD were also higher than expected. The same conditions as used in Section 3.5.4.3 (Reduction of Arial Wash) were applied. The results show the 1 hour of Ariel (1 cap/10litres) did not clean the flow cells well enough, resulting in poor reproducibility. The flow cells were not visibly dirty so the cleanliness of them when used in this assay was unknown. As a consequence of the results in Section 3.5.4.2 (Use of Detergent), 4 flow cells were left in Ariel overnight, and rinsed for half an hour before use in 3.5.4.4. As the same dilutions were used with these flow cells, the assay results do show a degree of consistency, but the cleaning procedure was either not washing the flow cells adequately or was not being rinsed adequately and was masking the actual binding of the antibodies.

The controls for both 15µg/ml were far higher than expected from Appendix K. As the controls were high the true optical density of both 15µg/ml dilutions was again being masked by the non-specific binding either to the flow cells or the tubing. Again, no staining on the membrane was seen.

Table 3.8 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.5.4.4.

LZ Conc.	OD	Membrane Staining (C, control; S, sample)
0µg/ml	1.711	All C N/S
15µg/ml	2.938	All S N/S
0µg/ml	1.153	
15µg/ml	2.757	

3.5.5 Combination Cleaning of Flow Cell

3.5.5.1 1M HCl & 3M HCl

The experiment in Section 3.5.5.1 was carried out to study the effect of a combination of an overnight soak in 1M HCl and a wash with 3M HCl. It is clear from all other tests that the use of either Ariel on its own or HCl on its own was not sufficient for cleaning the flow cells. Therefore, initially two applications of HCl were used. The flow cells were simply soaked in 3M HCl for 30 minutes prior to the experiment along with the 1M HCl overnight soak. The results show that the HCl alone did not remove the bound proteins adequately as the control OD was extremely high, suggesting that the HCl treatment was not sufficient for removing the bound proteins.

Table 3.9 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.5.5.1.

LZ Conc.	OD	Membrane Staining (C, control; S, sample)
0µg/ml	2.177	All C N/S
5µg/ml	1.989	All S N/S

3.5.5.2 1M & 5M HCl & Ariel

The flow cells were flushed with 5M HCl, soaked overnight in 1M HCl, soaked for 10 minutes in Ariel, and then thoroughly rinsed under running boiling water for 5 minutes. This combined effort was due to the results from Sections 3.5.4.2 (Use of Detergent) and 3.5.5.1 (1M HCl & 3M HCl) showing more vigorous treatments were needed to clean the flow cells of protein binding. The results still show high OD for the control sample at 1.169 and 2.791. Again no staining was seen on any of the membranes.

In this test, consideration was also given to the solutions used. All buffers were remade and stored at 4^{o}C and then incubated at room temperature before the experiment began. The control filtrate OD's were still high, therefore the test was still not delivering the required performance. The experiment shows that even raising the concentration and duration of the soak in hydrochloric acid and washing with Ariel was not effective at removing bound protein. This was indicated again by the high control OD and the true sample OD being masked by the presence of the bound protein.

Table 3.10 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.5.5.2.

LZ Conc.	OD	Membrane Staining (C, control; S, sample)
0µg/ml	1.169	All C N/S
15µg/ml	1.838	All S N/S
0µg/ml	2.791	
15µg/ml	> 3.5	

3.5.5.3 Ariel, 1M HCl & 5M HCl Flush

In this next test an hour long soak in Ariel was followed by a 30 minute rinse. In addition to the overnight soak in 1M HCl a flush with 5M HCl was tried. In this test two sets of flow cells (total=4) were washed using the above method and only used once for each assay. The first test results (i) showed the flow cells were being cleaned by the Ariel as the control OD was low and the 15µg/ml was low but close to the expected value (Appendix K). However, the second set of results (ii) do not follow this pattern as the control OD was far too high at >3.5. The 5µg/ml of (ii) was also far too high when compared to the expected results of Appendix K. As both sets of flow cells were washed using the same protocol, the same concentration of Ariel and hydrochloric acid at the same time, this was not anticipated. No discolouration was seen on any of the membranes.

As half the flow cells showed some elements of a working assay, it was concluded that the flow cells work better with a longer soak in the Ariel, as the control was reduced. This was also seen in Section 3.5.4.2 (Use of Detergent).

Table 3.11 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.5.5.3.

LZ Conc.	OD	Membrane Staining (C, control; S, sample)
(i)0µg/ml	0.746	All C N/S
(i)15µg/ml	1.949	All S N/S
(ii)0µg/ml	> 3.5	
(ii)5µg/ml	3.260	

3.6 Calibration Curve Results

Figures 3.3-6 show the attempts at calibration curves using the current method.

3.6.1 Four-Channel Peristaltic Pump

At this point a new 4 channel peristaltic pump was trialled, the Spectec Perimax 12. This pump was able to process three samples and a control per run. The flow cells (total=12) were soaked for two hours in the Ariel and rinsed under boiling running water for 30 minutes, in addition to a 5M HCl flush and 1M HCl overnight soak.

In the first trial of this pump (i), the control reading reduced to an expected level which looked promising. However, as (ii) and (iii) show the controls were not all as low as the control in (i). Even though all flow cells were washed using the same protocol at the same time it did not produce the same effect across all flow cells. The lysozyme dilutions did not correlate with the actual amount of lysozyme present either. The membrane discoloured was also random, with heavily stained membranes for all control and sample membranes.

The flow cell construction was unique in its delivery of the receptor and analyte in that it does not mix the different dilutions. Therefore the only conclusion as to why the lysozyme concentrations did not result in high lysozyme concentrations producing high membrane staining and high OD, and alternatively low lysozyme concentrations producing low level membrane staining and low OD, is that the lysozyme was not depositing on the membrane effectively, causing less antibodies to bind and subsequent low levels of staining on the membrane in the filtrate.

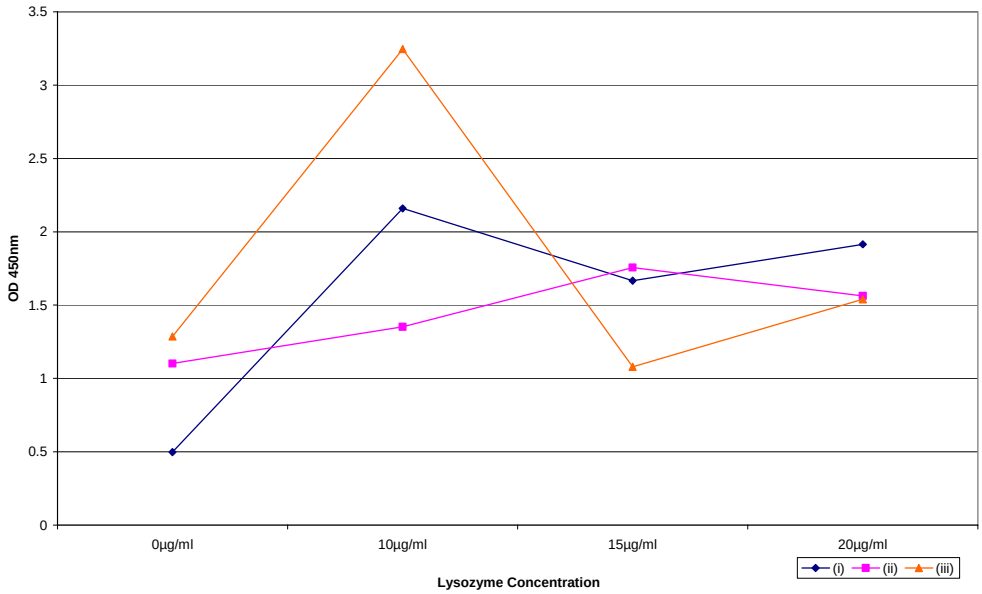


Figure 3.3 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.6.1.

3.6.2 Lysozyme Reactivity

The flow cells were soaked overnight in 5M HCl, soaked in Ariel for 1 hour and rinsed thoroughly. Due to situations beyond control, the laboratory temperature dropped to 16ºC. The lab temperature may have affected the reactivity of the lysozyme with the antibodies, but as the control contained no lysozyme, low control OD's were expected. Again the problem of cleaning the flow cells from protein build up from previous experiments was an obvious problem. The control OD's were still high at ranging from 1.102-1.286.

The laboratory temperature may have affected the lysozyme reactivity as all lysozyme concentrations were less than 2.0OD, (with the exception of (iii) 10µg/ml). However, the issue of cleaning the flow cells was more significant, to the long term prospects of the assay.

Again all flow cells were washed for the same duration, in the same concentration of Ariel and HCl and in the same bucket. All buffers and antibody solutions were made up fresh on the day therefore, as all variables were kept constant, the results should have tallied.

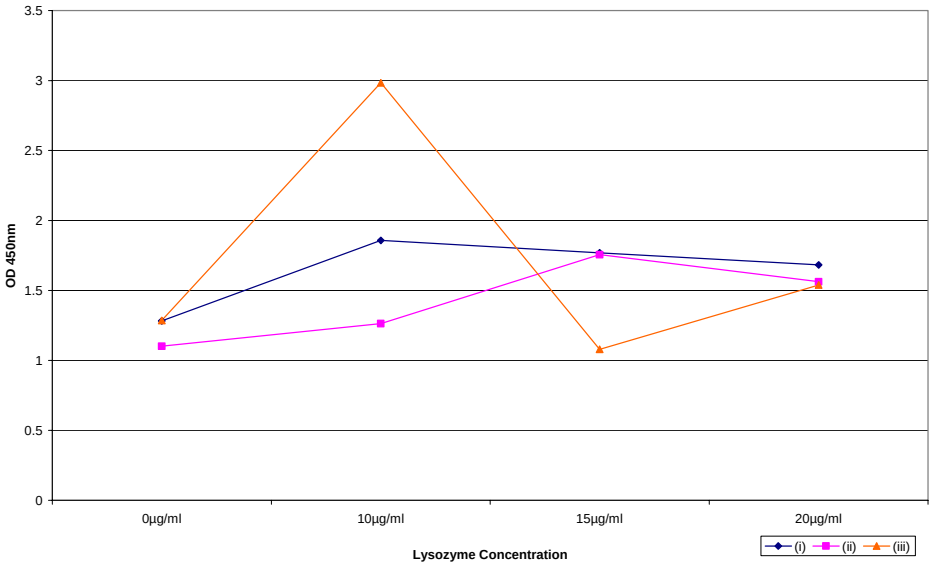


Figure 3.4 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.6.2.

3.6.3 Manual Cleaning of Flow Cells

The laboratory temperature remained 16ºC for this experiment also. The flow cells here were physically scrubbed with Ariel and cotton wool buds and then soaked in Ariel for 1 hour. They were rinsed for 30 minutes and then dried ready for use. The results showed a small difference in the control and sample OD as with the test in Section 3.6.2. However, the control OD was still high compared to Section 3.5.4.2 (Use of Detergent) and Appendix K. The laboratory temperature may have been affecting the reactivity of the lysozyme and antibodies as suggested in Section 3.6.2 (Lysozyme Reactivity). Nonetheless, the cleanliness of the flow cells was clearly a major problem.

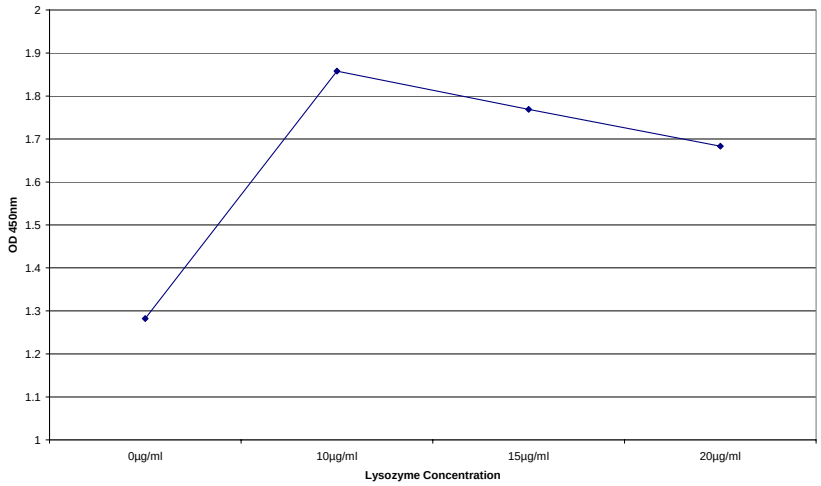


Figure 3.5 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.6.3.

3.6.4 Run Through of Complete Experiment

The equipment was transferred to a 20ºC environment and the experiment was conducted twice, to eliminate the allegation that the lowering of the temperature was affecting the reactivity of the lysozyme. The same wash protocol for the flow cells was used for this test as used in Section 3.6.3. i.e. physically scrubbed with Ariel and cotton wool buds and soaked in Ariel for 1 hour. A difference in OD between the control and the lysozyme dilutions is seen in part (i) of this experiment. The control OD in (i) was much lower than previous Tests and a distinct difference was seen between the OD of the control and samples. However, when repeated with the second set of flow cells, the results in part (ii) did not show a difference in control OD and did not suggest specific antibody binding was taking place.

The flow cells used in part (i) were the same as those used for part (ii). Those used in part (i) were simply washed using the protocol outlined above i.e. physically scrubbed with Ariel and cotton wool buds and soaked in Ariel for 1 hour. When part (i) was finished the flow cells were washed as above and reused for part (ii). As this was the same protocol the results in (ii) should have been similar to those obtained in part (i).

Yet again, the only variable was the new wash protocol. It appears that the bound proteins were not being cleared from the Perspex flow cells and each assay was building

up a coating of protein that was not removed by the cleaning procedures. Therefore the non-specific protein binding in (i) was not removed during the wash procedure and remained for part (ii), which is why the OD for the control and samples in part (ii) are almost double those seen in part (i). A different method for cleaning the flow cells is needed to ensure the flow cells are free from bound proteins for each new assay carried out.

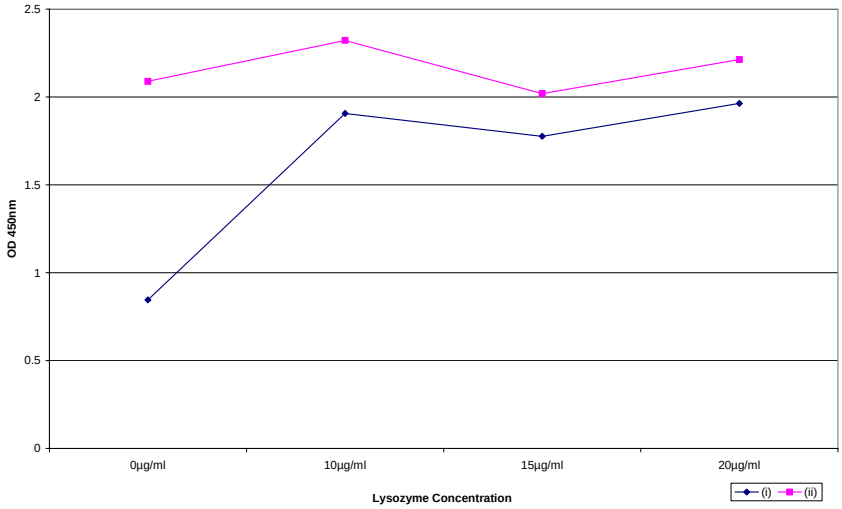


Figure 3.6 ELIFA Results, shows the OD and membrane discolouration of various concentrations of lysozyme in buffer for Test 3.6.4.

3.6.5 Validation Test using ELISA

To test the reactivity of the chemicals, antibodies and the lysozyme being used in these experiments an ELISA was carried out. The results from this (Figure 3.7) show that all chemical reagents were working sufficiently.

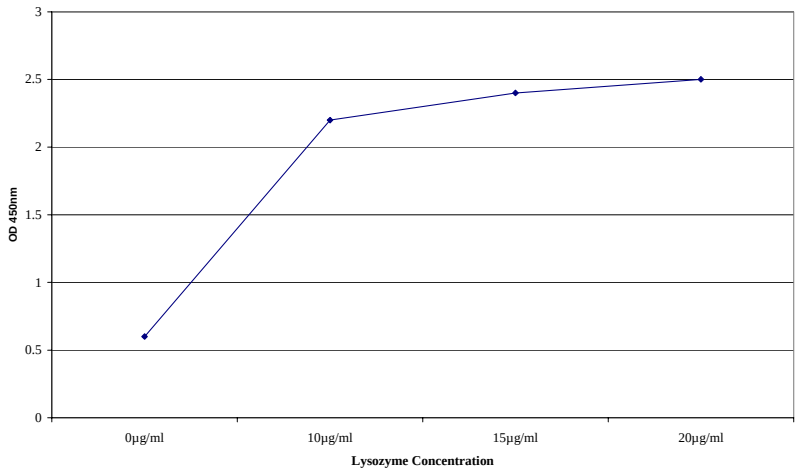


Figure 3.7 Validations ELISA for ELIFA Comparison

3.7 Discussion

This section discusses the results in 3.5.

3.7.1 ELIFA Comparison with ELISA

Section 3.5 details the ELIFA method optimisation that was undertaken. The basic ELIFA protocol was derived from the lysozyme ELISA. Both the ELISA and ELIFA are indirect immunoassays wherein the antigen (lysozyme from a biological sample) is bound to a solid surface, either the bottom of a micro-titre plate or a membrane, and then bound to primary and secondary antibodies before reacting with a substrate to produce a colour reaction. The expected advantage of using the ELIFA was to improve the sensitivity of the assay by using the membrane to trap and immobilise all available lysozyme antigens from the sample. This would potentially produce the maximum response. A further objective with the ELIFA was to reduce the total time of the assay. The ELISA time was 96 minutes, which rivals other commercially available lysozyme ELISA's, but it was felt there was room for improvement. With the elimination of the incubation periods, the ELIFA ran much faster than the ELISA, with an assay time of less than 40 minutes.

3.7.2 ELIFA Results

The ELIFA results are very limited due to problems with the optimisation. Principally because of non-specific binding (NSB), it was not possible to obtain a calibration curve of lysozyme concentrations in buffer or in serum. The possible nature of this problem and solutions will be discussed later in this chapter.

3.7.3 Analysis of Advantages of ELIFA

The ELIFA was designed to eliminate the disadvantages of the ELISA test. The total ELISA assay time was 96 minutes whereas the ELIFA took 40 minutes due to the short incubation times at room temperature. This was a great improvement on the ELISA time. However, it is hard to comment on the actual antigen-antibody binding produced with the ELIFA, LOD and sensitivity. The problem of NSB concealed the binding events to an extent that it is not known whether the lysozyme in the sample would have bound and produced good results for this system.

The ELIFA is also fast because of the limited wash steps in the protocol. Most steps in the ELIFA do not incur a wash phase as any unbound analyte passes through the membrane into the waste chamber. However fast, the ELIFA could not compete with the amount of samples the ELISA could process in one 96 minute run. A total of 40 samples (2 replicates of each), 8 control and 8 standards could be carried out in an ELISA experiment compared to the ELIFA which was only capable of processing 3 samples plus one control per run with no replicates.

Another potential advantage of the ELIFA was that all equipment used in the test was reusable. The initial idea of the ELIFA was to cut costs by using equipment that could be washed and reused for subsequent tests. The ELISA did not offer this as all materials used were single use only. This was also true for all commercially available ELISA kits on the market. The ELIFA did not fulfil this advantage as so many problems were faced with the actual wash procedure. This meant that the ELIFA itself became a single use assay also. However, if the NSB problem were to be tackled successfully, then after the initial cost of the flow cells the overall cost was around £5 per sample.

A further advantage is the total assay time for the ELIFA. The assay time was reduced to 40minutes; from 96 minutes with the ELISA. The preparation of this assay was very laborious and usually amounted to ~2 hours, which included the setting up of apparatus and the cleaning of the flow cells and far exceeded that of the ELISA. Clean up time after the assay would also take as long as 24 hours due to the extent of non-specific binding on the flow cells. The range of cleaning procedures employed is described in section 3.5. This section shows that no matter what how long the flow cells were left in a variety of solutions for up to 24 hours they could not be cleaned effectively, which lead to the poor results. Without a solution to the non-specific binding problem and an adequate reduction in preparation time the ELIFA system offers no improvement over the ELISA.

3.7.4 ELIFA Problem

Section 3.5.4 shows how the flow cell problem was tackled by changing the wash procedure several times. The flow cells were repeatedly washed with hydrochloric acid (1M & 5M) for up to 24 hours and also washed with a biological detergent Ariel for up to 2 hours as well as being physically scrubbed with cotton wool.

Sections 3.5.5.3-3.6.2 describe several flow cells being washed simultaneously and then one set used immediately after washing while still wet and the other set(s) left to air dry until use. According to Hayes *et al.* (1998) a phenomenon occurs during the drying of protein to solid surfaces. This suggests that a flow cell which has not been dried will continue to elute protein from its surface and therefore at the end of the assay have less of a NSB build up. Whereas a flow cell which is left to dry before use in the assay will have a dried layer of residual protein not removed by the wash phase and will therefore continue to bind NSB at a greater rate.

The problem of non-specific binding of protein onto the Perspex flow cells was made worse with the harsh wash solutions. The flow cells were used many times during the optimisation of the ELIFA and undertook several wash phases. Overtime it appears the flow cell surface may have been degraded by the wash solutions and therefore encouraged further binding, which is why the results became progressively worse.

3.7.5 ELIFA Problem Solving

The poor results in sections 3.5 and 3.6 led to the conclusion that the cleaning method for the flow cells was actually degrading the Perspex and encouraging further protein binding. A solution to this problem was to have the flow cell made using a different material, which would limit protein binding and withstand a wash phase that removed bound protein and did not degrade the surface. Ideally the flow cells would be made of glass, but the cost of them to be commissioned was far too high.

Another possible solution was to coat the loading chamber (Figure 3.8) with the membrane to eliminate all possible contact with the antigens/antibodies had with the Perspex. If the membrane was custom made to be cone shaped it would prevent direct

contact with the Perspex and possibly reduce NSB. The problem with this solution was again the cost of having the membrane cut to the exact shape. An alternative was to make them in house, however the solution was not considered until after the solution, discussed in Chapter 4 was found.

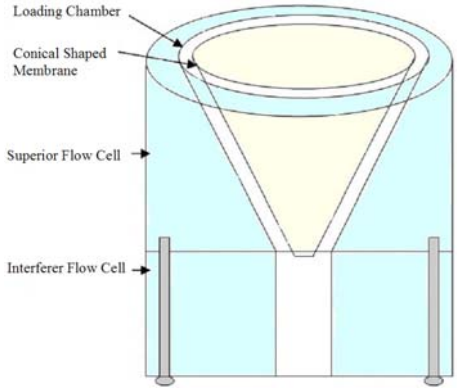


Figure 3.8 Flow cells with conical shaped membrane to prevent NSB to Perspex

A further consideration came in the way of syringe filters (Fisher, UK) using the ELIFA protocol. The syringe filters/ELIFA assay did have the advantage of being fast but the syringe filter membrane was pressure sealed inside the plastic filter and could not be retrieved. This led to problems with recording the results as over time the stop solution (sulphuric acid) degraded the membrane within the filter casing. This meant that the membrane could not be photographed as the purple discolouration from the antigen-antibody binding was lost with the application of the stop solution. Additionally, the syringe filter membranes were not encased in a clear colourless plastic. The colour of the plastic casing of the syringe filters denoted the pore size of the membrane. The membrane was already difficult to photograph due to the degradation from the stop solution but the coloured plastic filter also hampered photographing and interpreting of the membrane, as when wet the membrane took up some of the colour of the plastic.

At this point the Mini-ELIFA (Chapter 4) filters were purchased and tried with the ELIFA protocol. These filters had the potential advantage of being auto-clavable, so no cleaning between assays was required, they were extremely cheap to buy, no peristaltic pump would be required which would reduce running costs and eliminate the interference of restricted tubing and the membrane could be retrieved for documenting.

3.7.6 ELIFA Synopsis

The principle behind the ELIFA suggests that this method has many advantages over the ELISA system. The most significant advantage is the sensitivity. The ELIFA system involves the concentration of ligand and analyte on the non-porous membrane surface. This allows low concentrations of ligand to be measured accurately producing a sensitive assay. However, as the assay was not successful the sensitivity could not be established. If more time and money was expended to explore the potential of the ELIFA system then it may yield better results provided that the problems highlighted were addressed.

The laboriousness of the ELIFA assay due to the problems with NSB did hinder the progress of the assay. However, solutions to over come this problem and the successes that followed are documented in Chapter 4.

Chapter 4 -Mini-ELIFA

4.1 Introduction

The mini-ELIFA is an adaptation of the ELIFA method and is a further technique for the measurement of lysozyme. This method was devised to capitalise on the positive aspects of the ELISA, such as the effective binding of the antigen & antibodies; and to eradicate some of the problems experienced with the ELIFA, such as the NSB and to improve on the assay time, LOD and ease of use. One major problem with the ELIFA was non specific binding of protein to the Perspex flow cells. This problem was tackled by exchanging the Perspex flow cells for Swinnex filters (Figure 4.1) which were auto-clavable and were thought might eliminate the problem.

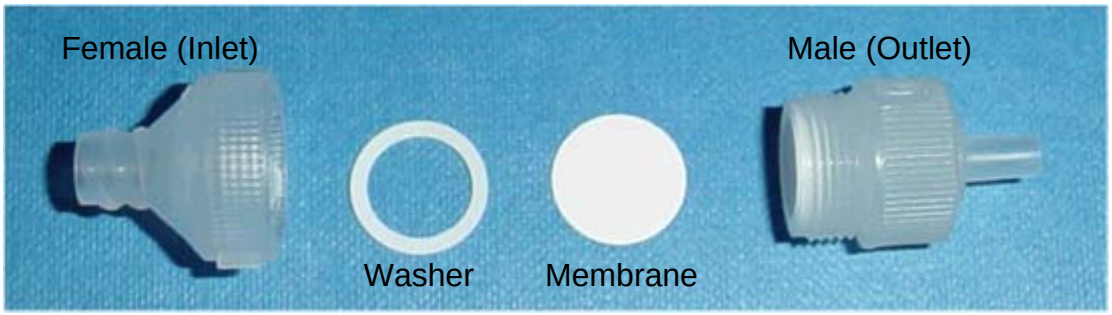


Figure 4.1 Swinnex Syringe Filter; Consisting of an inlet and outlet which are screwed together securing a membrane disc.

4.2 Filter Construction

The Swinnex filter construction is much simpler and quicker than the ELIFA flow cells. A pre-cut disc of membrane is inserted along with the rubber washer into the female inlet (Figure 4.1). The outlet and inlet are then screwed together and sat on to a universal collection tube. Syringes (1ml & 5ml) connected via the inlet, were used to inject the analyte samples, antibodies, wash solution and substrate through the membrane. Discolouration on the membrane was directly proportional to the amount of analyte in the sample.

4.3 Preliminary Experiments -Bacteria

Initially the mini-ELIFA was tried with bacterial samples. This was to broaden the focus of sepsis diagnosis. Instead of measuring a chemical secreted in response to infection, such as lysozyme, this test would measure the actual presence of the bacteria

causing the sepsis. *Escherichia coli (E. coli)* was chosen due to its prevalence in urinary tract infections. Once established with bacteria, the mini-ELIFA would then be tried with lysozyme samples.

4.3.1 Urinary Tract Infections (UTI)

Eighty percent of all UTI are caused by *E. coli* due to its abundance in faecal flora. Other bacteria which cause UTI's include *Proteus mirabilis, Pseudomonas aeruginosa* and *Streptococcus faecalis* (Andrews *et al*., 2002). When a patient complains of urinary tract infection symptoms (frequent micturition, painful voiding and haematuria) a mid stream urine sample is taken and a urine analysis dipstick for nitrites is used. Gram negative bacteria reduce dietary nitrate into nitrite of which the dipstick detects. However, they are known to produce false positives and do not detect Gram positive bacterial strains (Kumar & Clark, 1990).

Urinary microscopy is the usual follow up test along with bacterial cultures of the urine sample and sensitivity testing for the appropriate antibiotics. These follow up tests however, take up to 2 days, by which time the infection is established and the patient is undergoing treatment with non-specific antibiotics. The aim with the mini-ELIFA is to produce a fast, easy to use bedside test which identifies the causative bacteria so immediate use of specific antibiotics can be used for treatment.

4.4 Potential Advantages of Mini-ELIFA

The remit of the mini-ELIFA was to improve sensitivity, reduce assay time so results could be obtained at the patient's bedside and produce a test which was easier to use. The mini-ELIFA is built on the same basic principle as the ELIFA, in that the analyte in the sample and antibodies are forced to bind on to the surface of the membrane because the diffusion rate is limited by the suction through the membrane. This has the potential advantage of utilising all available analyte producing a highly sensitive assay.

A further potential advantage is the speed of the assay. The mini-ELIFA does not use a peristaltic pump to produce suction through the membrane. Instead syringes are used to drive the reagents through the membrane. The basic principle and effects are the same as when the peristaltic pump was used with the ELIFA, but the assay time is shorter.

The main problem with the ELIFA was the NSB on to the Perspex flow cell. The mini-ELIFA do not require any pre-treatment prior to autoclaving, therefore this should produce reusable filters for this assay.

4.5 Applications

A second ELIFA system was proposed by Nogrady *et al.* (1998) for the measurement of *Bacillus macerans*. The concentration was estimated using an "enzyme-linked immunofilter assay". The total assay took <1 hour to complete. Thiele *et al.*, (1992) also used the basic principle of ELIFA for their assay for monoclonal capture and biotinylated monoclonal detection of *Coxiella (C.) burnetii*. Their assay was quoted as being fast and highly sensitive with a minimum dose of 2500 *C. burnetii* particles.

4.6 Materials & Methods -Mini-ELIFA

4.6.1 Bacteria Mini-ELIFA

The wash buffer with Tween and casein (WBTC), washing step solution, antibodies, TMB and sulphuric acid were all made to the same concentration as those used in the ELIFA method outlined Appendix G.

The membranes used in the mini-ELIFA were not the Hybond N membrane used in the ELIFA. This is because a pre-cut membrane specially designed for the Swinnex filters could be purchased. This eliminated the potential problem of compressing the membrane during the cutting procedure, which could result in a membrane with fewer pores.

4.6.1.1 Bacteria Preparation

Liquid broth and agar were made as stated in Appendix G. All agar and liquid broth were autoclaved before use. Concentrations of the *E. coli* JM83 K12 solution were prepared. These included neat, ($8.04x10^8$ cells/ml) $^1/_{100}$, $^1/_{1000}$ and a control (no *E. coli*).

4.6.1.2 Filter Construction & Analyte Immobilisation

Syringe disc filters (0.45µm) from Fisher, (Leicester, UK) were purchased to filter the *E.coli*. Swinnex filter holders by Millipore (UK) along with Supor membrane, 0.45µm, 13mm diameter was purchased from Pall, (Hampshire, UK). Both 2ml and 5ml BD Plastipak syringes were used along with 0.8mm x 16mm lancets from Fisher. The syringe disc filters were placed over a 10ml universal tube to collect the filtrate. Aliquots (0.5ml) of each dilution of *E. coli* was passed through the syringe disc filter using a 50ml syringe. A fresh syringe was used for each suspension.

4.6.1.3 Antibody Application

The step above was followed by 0.2ml primary antibody (1:6000 in WBTC), 0.2ml secondary antibody (1:4000 in WBCT) and then a 0.6ml washing step solution. Anti-*E.coli* antibodies (primary) were purchased from Dako B0357 (Cambridge, UK), along with a secondary antibody *E. coli* HRP P0361 (Dako).

The filter holder with membrane, was then removed from the universal (Solution 1) and a fresh universal was placed underneath the filter (Solution 2).

4.6.1.4 Substrate Application & Quantification of Results

TMB was pumped through the filter (150µl) and allowed to react for 6 minutes. The reaction was stopped by passing 50µl sulphuric acid (1M) through the filter. The Optical Density (OD) of the solution collected in the universal was measured at 450nm and a digital photograph taken of the filters.

4.6.2 Lysozyme Mini-ELIFA

The WBTC, washing step solution, antibodies, TMB concentrate and sulphuric acid were all made to the same concentration as those used in the ELIFA method outlined in Appendix G. The basic method in the lysozyme mini-ELIFA is the same as the ELIFA method.

4.6.2.1 Filter Construction

Swinnex filter holders by Millipore (UK) along with Supor membrane, 0.45µm, 13mm diameter was purchased from Pall, (Hampshire, UK). Both 1ml and 5ml BD Plastipak syringes (Fisher) were used. The Swinnex syringe filters were fitted together by inserting a rubber washer and disc of Supor membrane inside the male outlet. The female inlet was then screwed tightly. The filter was placed over a 15ml universal tube to collect the filtrate.

4.6.2.2 Lysozyme Coating

Purified Lysozyme (Sigma-Aldrich Ltd, UK) from human milk was dissolved in Coating buffer (Appendix G). The lysozyme was stored at -20°C. 0.5ml of each Lysozyme dilution (0.01-75µg/ml) was passed through the syringe disc filter using a 1ml syringe. A fresh syringe was used for each dilution.

4.6.2.3 Antibody Application

The DAKO Antibodies (Ely, Cambridge, UK) used were rabbit anti-human lysozyme immunoglobulin 7.1mg/ml (Primary) and horseradish peroxidase (HRP) conjugated goat anti-rabbit immunoglobulin 0.3mg/ml. Both antibodies were diluted in WBTC, to 1:6000 and 1:4000 for the primary and secondary respectively.

The lysozyme coating was followed immediately by 0.2ml primary antibody (1:6000 in WBTC), 0.2ml secondary antibody (1:4000 in WBTC), and then 0.6ml washing solution. The filter holder with membrane, was then removed from the universal (Solution 1) and a fresh universal was placed underneath the filter (Solution 2).

4.6.2.4 Colour Reaction

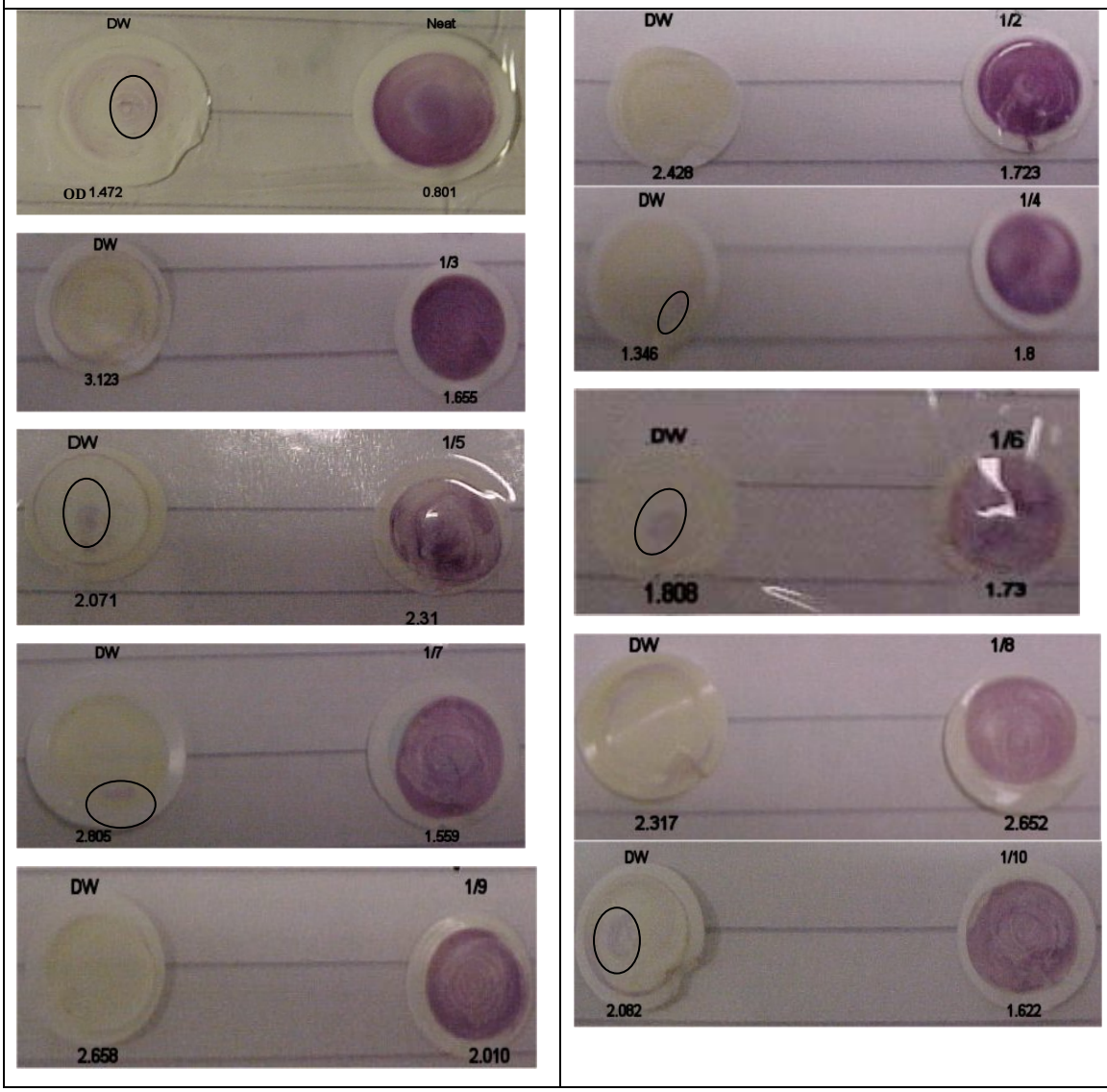
Undiluted TMB was pushed through the filter (150µl) and allowed to react for 6 minutes. The reaction was stopped by passing 50µl 1M sulphuric acid through the filter. The Optical Density of Solution 2, collected in the universal was measured at 450nm and a digital photograph taken of the upper side of the filters.

4.7 Bacteria Mini-ELIFA Results

The results below (Plate 4.1) show photographs of the membranes used in the bacteria Mini-ELIFA. A photograph of the membrane and the optical density (OD) of the filtrate was the method chosen for documenting the results from these experiments.

Plate 4.1 Bacteria Mini ELIFA Raw results, Plate 4.1 show the membrane discolouration and optical density of filtrate for several bacteria samples and control (distilled water, (DW)). The concentration for each *E. coli* sample is shown above each membrane and the OD for each sample is shown underneath each membrane.

Plate 4.1 Photographs of Membrane



4.7.1 Membrane Descriptions from Plate 4.1 & Optical Density of Filtrate

Table 4.1 below shows the optical densities of all filtrates and describes the membrane staining for all *E. coli* dilutions.

Table 4.1 Optical Density of *E. coli* Filtrates & Descriptions of Membranes

Conc. of Bacteria	Control OD	Sample OD	Description
Neat	1.472	0.801	The Neat ($8.04x10^8$cells/ml) bacteria sample showed considerable binding on the membrane. A small amount of discolouration is seen on the control membrane as highlighted. The OD of the filtrates showed a higher reading for the control than the sample.
$^1/_2$	2.428	1.723	The ½ dilution shows considerable binding and discolouration across the entire membrane. No binding can be seen on the control. The control filtrate had a higher OD compared to the sample.
$^1/_3$	3.123	1.655	The $^1/_3$ again showed considerable binding across the entire membrane. When comparing membrane ½ with $^1/_3$, there is a distinct difference in the staining, with the higher dilution producing a darker stain. The OD of the control was greater than the sample.
$^1/_4$	1.346	1.800	The binding of the $^1/_4$ sample membrane is less discoloured than ½ and $^1/_3$ but is distinct from the control. The control membrane had a small amount of binding which is highlighted. The control OD was lower than the sample.
$^1/_5$	2.071	2.310	Discolouration is present on the sample $^1/_5$ membrane but is less distinct when compared to higher dilutions (½ and $^1/_3$). A small amount of binding is present on the control membrane as highlighted above. The sample OD was greater than the control OD.

$^{1}/_{6}$	1.808	1.730	Heavy discolouration is present on $^{1}/_{6}$ membrane. The control membrane has a small amount of binding present as highlighted. The OD of the control was lower for the control compared to the sample.
$^{1}/_{7}$	2.805	1.559	Discolouration across the entire membrane is seen on $^{1}/_{7.}$ A small amount of discolouration is seen on the control membrane as highlighted above. The control OD of the filtrate was far greater than the sample.
$^{1}/_{8}$	2.317	2.652	A distinctly paler discolouration is seen on the $^{1}/_{8}$ membrane, when compared to $^{1}/_{7}$. The control filtrate OD was less than the sample OD.
$^{1}/_{9}$	2.658	2.010	Discolouration was seen on the $^{1}/_{9}$ membrane. When compared to $^{1}/_{8}$, $^{1}/_{9}$ was actually darker in colour. The control filtrate OD was far greater than the sample.
$^{1}/_{10}$	2.082	1.622	Discolouration was seen across the entire membrane of $^{1}/_{10}$, and was to a less extent when compared to $^{1}/_{8}$ and $^{1}/_{9}$. The control membrane did have some binding present as highlighted above. The control filtrate OD was far greater than the sample.

4.8 Bacteria Mini-ELIFA Analysis

4.8.1 Optical Density of *E. coli* Filtrates

Solution 2, collected during the TMB administration, was used to measure the optical density (Table 4.1). The control OD should have been greater than the OD of the sample. A probable reason why this was not so may have been that the bacteria and antibodies were trapped on the surface of the membrane, causing the membrane discolouration. The sample filtrate did not contain these bound bacteria and antibodies and so did not produce a distinguishable colour change compared to the control filtrates. The control filtrate OD was high in most cases because there was residual binding of the antibodies and TMB taking place. With the majority of concentrations the OD was greater for the control compared to the sample. This shows that the membrane was

doing its job and trapping all bacteria present in the sample; allowing ample binding of the 2 antibodies and TMB.

It was also expected that a calibration curve of filtrate optical densities could be produced from the control results, showing an increase in OD with increasing concentration of bacteria. However, the results did not show this pattern. No correlation between filtrate OD and concentration was present.

4.8.2 Membrane Staining

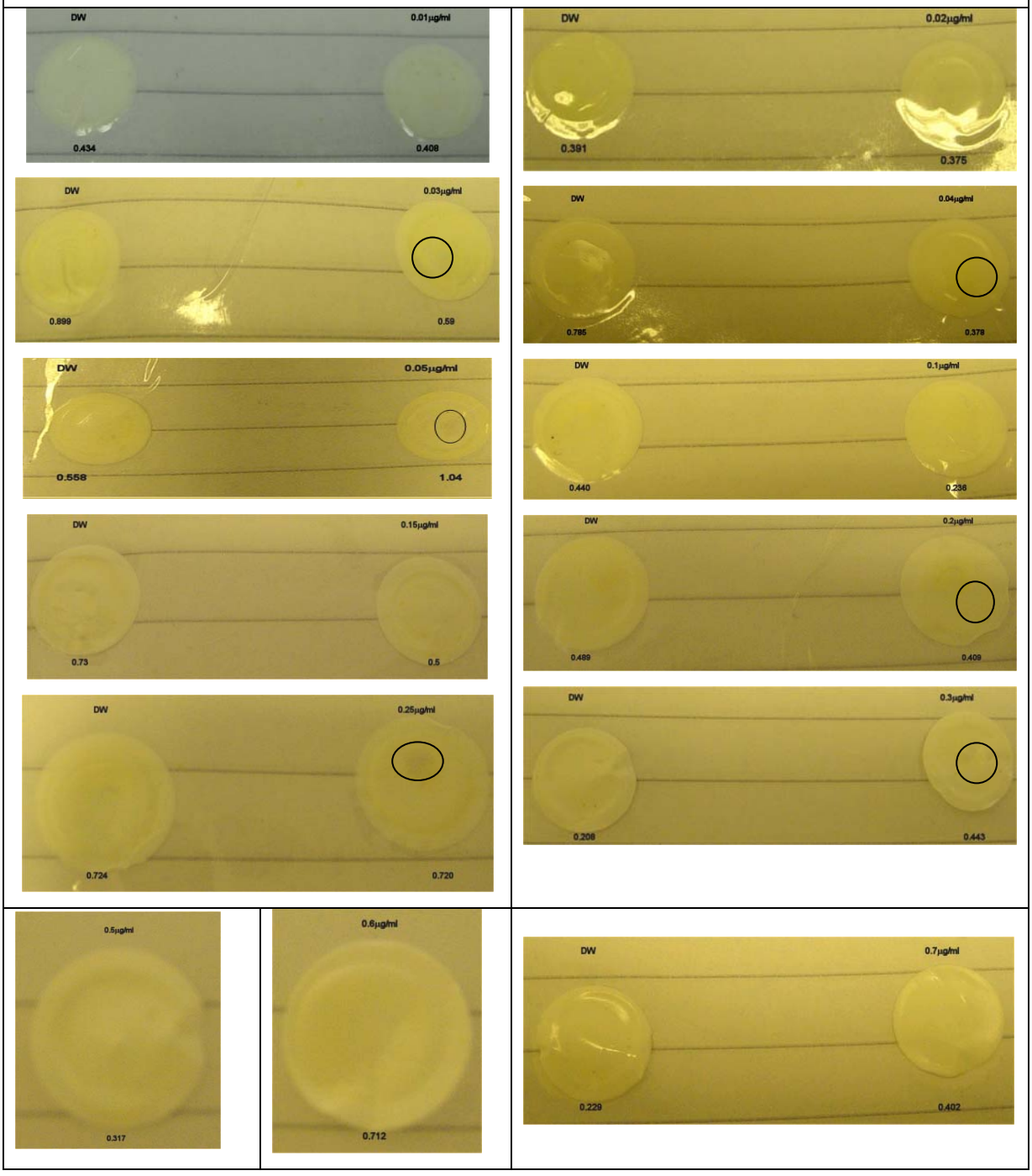
The stained membranes are a more reliable indication of bacterial presence compared to the OD of the filtrate. The discolouration of the membranes (Plate 4.1) was caused by the entrapment of bacteria on the surface of the membrane. This would then react with the primary and secondary antibodies and also the TMB, causing the purple staining. There was very little binding on the control membranes. This meant a clear distinction between the sample and control could be made. The darkness of the staining increased with increasing *E. coli* concentration. All distinctions could be made with the naked eye in a matter of seconds, making assay analysis relatively effortless for the interpreter.

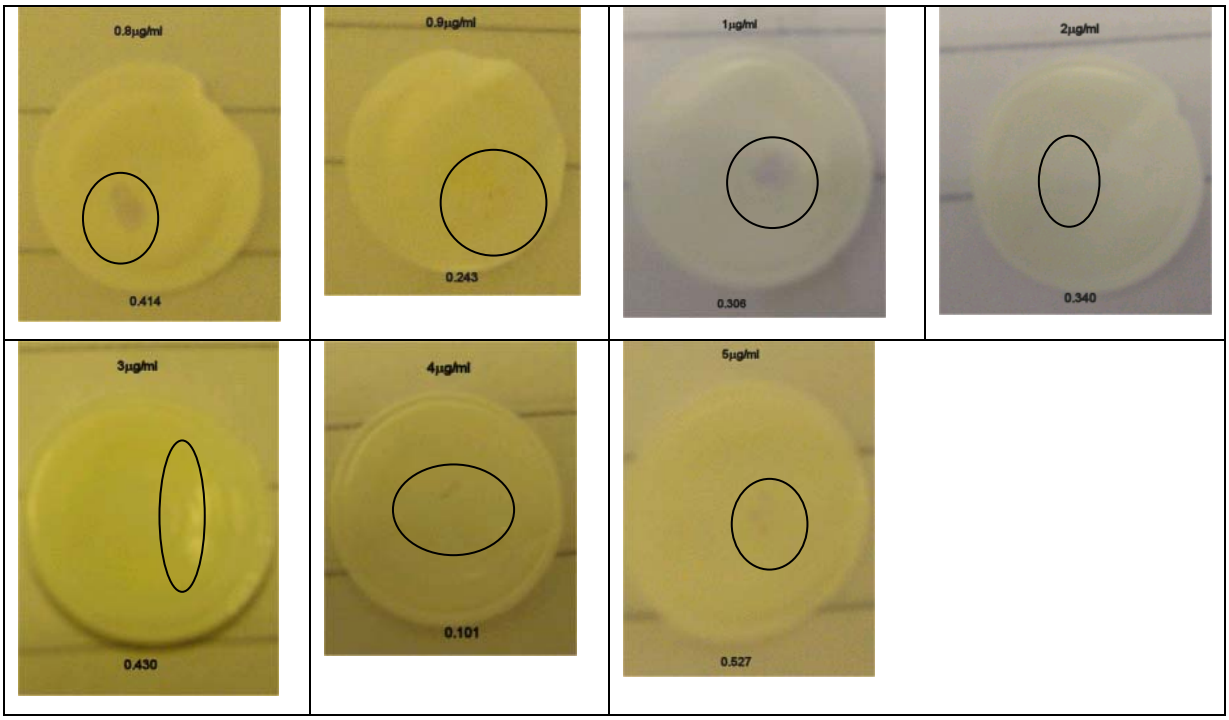
4.9 Lysozyme Mini-ELIFA Results

The results below (Plate 4.2) show photographs of the membranes used in the lysozyme Mini-ELIFA. A photograph of the membrane and the optical density (OD) of the filtrate was the method chosen for documenting the results from these experiments.

Plate 4.2 Lysozyme Mini-ELIFA raw results; OD of filtrates is shown.

Plate 4.2 Photographs of Stained Membrane & Descriptions





4.9.1 Membrane Descriptions from Plate 4.2

The table below describes all the membranes used in the Mini-ELIFA, and lists the OD.

Table 4.2 Lysozyme Membrane Descriptions & OD for the Mini-ELIFA

Lysozyme Conc.	Control OD	Sample OD	Membrane Description
0.01µg/ml	0.434	0.408	The sample and control membrane show no discolouration, and very little difference between the OD of the filtrate for 0.1µg/ml.
0.02µg/ml	0391	0.375	The sample and control membrane show no discolouration, and very little difference between the OD of the filtrate for 0.2µg/ml.
0.03µg/ml	0.899	0.590	The sample membrane shows a small area of discolouration. The discolouration is highlighted on the membrane but is very faint in appearance. No discolouration was seen on the control and the control filtrate OD was greater than the sample.
0.04µg/ml	0.785	0.378	A small amount of discolouration is seen on the

			0.04µg/ml sample membrane, which is highlighted above. The control filtrate OD was greater than the sample.
0.05µg/ml	0.558	1.040	A small amount of binding is seen on the sample membrane and is darker in colour to the staining on 0.04µg/ml. The sample filtrate OD was far greater than the control.
0.1µg/ml	0.440	0.236	No discolouration was seen on either membrane, and both filtrate OD's were particularly low.
0.15µg/ml	0.730	0.500	No discolouration was seen on either of the membranes. The control filtrate OD was much greater than the sample.
0.2µg/ml	0.489	0.409	A small amount of discolouration was seen on the sample membrane as highlighted above. No discolouration was seen on the control membrane. The control filtrate OD was greater than the sample filtrate.
0.25µg/ml	0.724	0.720	A small amount of binding was seen on the sample membrane, as highlighted above, to a greater extent compared to any other LZ dilution. The control filtrate OD was slightly higher than the sample.
0.3µg/ml	0.208[(i)]	0.443	A small amount of binding was seen on the sample membrane. The sample filtrate OD was greater than the control.
0.5µg/ml	0.208[(i)]	0.317	No discolouration of the sample membrane above was seen. The control membrane (Plate 4.22) also showed no discolouration but had a lower filtrate OD than the sample.
0.6µg/ml	0.208[(i)]	0.712	No discoloration was seen on the sample membrane above. The control (Plate 4.22) had a much lower filtrate OD compared to the sample.
0.7µg/ml	0.229[(ii)]	0.402	No discoloration was present on the sample and

			control membranes. The control filtrate OD was much lower than the sample.
0.8µg/ml	0.229[ii]	0.414	Distinctive discolouration is present on the sample membrane, as highlighted above. The control filtrate OD was much lower than the sample.
0.9µg/ml	0.229[ii]	0.243	Discolouration is present on the sample membrane as highlighted above. The discolouration is not as distinct as the 0.8µg/ml LZ dilution. The control filtrate from Plate 4.25 had a very similar filtrate OD.
1.0µg/ml	0.229[ii]	0.306	Distinct membrane discolouration is present on the sample membrane, to the same extent as Plate 4.26. The control OD from Plate 4.25 was less than the sample filtrate OD.
2.0µg/ml	0.229[ii]	0.340	Discolouration was present on the sample membrane, but not to the same extent as Plate 4.26 and 4.28. The control filtrate OD was much less than the sample filtrate OD.
3.0µg/ml	0.229[ii]	0.430	Discolouration was present on the sample membrane, but was very faint. The filtrate OD from the sample was much greater than the control.
4.0µg/ml	0.229[ii]	0.101	Discolouration was present on the sample membrane, but was very faint. The filtrate from the sample had an OD less than any others seen, and was much lower than the control.
5.0µg/ml	0.229[ii]	0.527	Binding was clearly present on the final sample membrane. But again was very faint compared to Plates 4.26 and 4.28.The control filtrate OD was much less than the sample filtrate OD.

*Samples 0.3-0.6µg/ml used the same control indicated (i).
Samples 0.7-5.0µg/ml used the same control indicated (ii).

4.10 Lysozyme Mini-ELIFA Analysis

4.10.1 Optical Density of Filtrate

The membranes should have trapped the lysozyme-1^{o}antibody-2^{o}antibody-TMB complex causing a stain on the membrane. The sample filtrates should not have contained this complex and should have had a lower optical density then the control. The control was expected to have the 1^{o}antibody-2^{o}antibody-TMB complex and with some residual binding taking place this would result in a higher OD compared to the sample.

Samples from 0.01-0.25µg/ml, in the majority of cases, did have a control OD which was greater than the sample. However, where the higher concentrations were tried the control was always (apart from 4.0µg/ml) lower than the sample OD. The only reasonable conclusion from this half of the results (0.3-5.0µg/ml) was that the filtrate of the sample contained the lysozyme-1^{o}antibody-2^{o}antibody-TMB complex.

The previous problem of non-specific binding to the Swinnex filters did not seem tore-occur. The filters were autoclaved as per the instructions from the manufacturers, no harsh acids, nor biological washing liquids were used, so the integrity of the plastic filters was never in question. This washing procedure was also used with the bacterial mini-ELIFA, with success. The dilutions of the antibodies and the lysozyme were the same as those in the ELIFA. Therefore the antibodies should have reacted with the lysozyme. The sensitivity issue therefore leads to the conclusion that the lysozyme was not successfully trapped by the membrane, or was trapped and rendered un-reactive in some way, leading to a lack of binding.

4.10.2 Membrane Staining

The membranes above (Plate 4.2) show very little actual bound lysozyme. In comparison to the bacterial mini-ELIFA membranes this assay lacks sensitivity. The membranes did show a small amount of binding indicated by purple discolouration, but only from the higher lysozyme dilutions, >0.8µg/ml. The membrane had a standard 0.45µm pore size which is recommended for proteins greater than 10kDa. Therefore this membrane should have effectively trapped the lysozyme without any problems.

This discolouration was seen more regularly on the higher lysozyme dilutions but the extent of the discolouration was not in correlation with the dilution itself. This meant that when comparing a high dilution e.g. 3µg/ml with 0.3µg/ml there was binding in both cases, but no more binding was seen on one membrane compared to the other. This assay is therefore not capable of quantifying the amount of lysozyme in the sample. However, it may indicate that the washing procedure was too vigorous for the assay, as it was washing too much of the lysozyme away. This situation could be improved if the membrane was seeded with a monoclonal antibody which would adhere to the lysozyme and hold it firmly to the membrane during the washing phase, as with a Non-Competitive Indirect ELISA (Figure 2.1). This could produce an assay with a much clearer result and still have the advantage of a fast assay giving a yes/no answer.

As it stands this assay is clearly not sensitive enough to give a definite positive or negative answer as to whether lysozyme is present in the sample, due to the faintness of the discolouration on the membranes. All control membranes did not show any binding, but the reliability is always in question due to the absence of discolouration where lysozyme was present. Even with some high lysozyme dilutions, (0.5-0.7µg/ml) discolouration was not seen.

4.11 Discussion

This section discusses the results in Section 4.7 and 4.9.

4.11.1 Bacteria Mini-ELIFA

The bacteria mini-ELIFA was a very successful assay. The remit for the mini-ELIFA was to produce an assay which could qualitatively reveal whether bacteria were present in a buffered sample. The membrane discolouration was a clear and accurate representation of bacterial presence and in the majority of cases showed the amount of bacteria present; i.e. dark staining where there was a high concentration of bacteria and no staining for controls. The membrane staining could also be read with the naked eye without training, making analysis of the results very fast.

An assay that is very similar to the bacteria mini-ELIFA is reported by Paffard *et al.* (1997). Their assay for whole bacterial cells uses an antibody bound bacteria, trapped by an immunofilter, and detected by a secondary antibody and third enzyme-conjugated antibody in a chemiluminescent assay. This assay took under 55 minutes and values were determined using the reflectance of developed x-ray films placed over chemiluminescent membranes. This method of quantifying results may be a technique that could be incorporated in future work on the mini-ELIFA to produce results showing actual amounts of bacteria in patient samples.

A good method for measuring the reaction which was occurring on the surface of the membrane, (between the bacteria and antibodies) would have been to take an OD measurement of this fluid. A method to test the residual fluid on the surface of the membrane was not discovered, simply because it was in such small volumes. The "back flush" used in the ELIFA would have been the way to harness this solution and measure its OD, but again it was in such small volumes that not enough could be harnessed for even a diluted sample. Whether the bacteria-1^{o}antibody-2^{o}antibody-TMB complex would have released from the membrane during this back-flushing would also have come into question; but could have been further investigated if more time was invested.

Even though the bacteria mini-ELIFA was a success it could not be used on its own for diagnosing sepsis. Only 30-35% of septic patients present with positive bacterial cultures (Wheeler & Bernard, 1999). Therefore, the place for this assay would be in conjunction with existing diagnostic procedures, i.e. signs and symptoms. The assay maybe used in bacterial identification and quantification for UTI, for example in GP surgeries. The benefit of using this assay would be its speed of execution. In less than 10 minutes the assay can be preformed from scratch giving a result at the patient's bed side.

4.11.2 Lysozyme Mini-ELIFA

The lysozyme mini-ELIFA was not as successful as the bacteria mini-ELIFA. Again the remit of these experiments was to devise an assay which was fast, cost effective and produced an assay which could be qualitatively assessed to reveal lysozyme content in a sample. The assay was successful to a certain extent, but it lacked sensitivity. Some membranes from Plate 4.2 do show some discolouration indicating lysozyme presence, but this was only clear for high lysozyme concentrations (0.2-5µgml) and even then they were not distinct discolorations. The assay was also a success in that where discolorations did occur; no staining was seen on any of the control membranes. An assay developed by Shields *et al.* (1991) also found that binding proteins to a nitro-cellulous membrane in an ELIFA format resulted in a reduced sensitivity. Their study concluded that conformational changes took place during adsorption to the membrane causing limited binding and consequently an assay lacking in sensitivity. A possible method which could have been employed if more time was allowed would have been to use a capture antibody, seeded onto the surface of the membrane which actively captured the lysozyme from the sample. This may have increased the sensitivity of the assay ensuring that all lysozyme from the sample was harvested and immobilised for 2^{o} antibody binding.

4.11.3 Advantages of Mini-ELIFA

The mini-ELIFA was developed to eliminate the problems faced with the ELIFA work. The total time for the mini-ELIFA was ~9 minutes. Preparation time only amounted to 30 minutes as most of the equipment used was disposed of after use. This is a far faster

assay compared to the ELIFA or the ELISA. This assay fulfils the initial remit of the work; to produce a yes/no answer of whether bacteria or lysozyme is present in a biological sample in real time.

The ELIFA was also limited to the amount of samples and controls that could be carried out due to the peristaltic pump only having 4 channels. This meant that a maximum of 3 samples and 1 control could be processed at any one time. As the mini-ELIFA did not have the limitations of a pump, disposable syringes were used to drive the immunochemicals through the membrane; upwards of 11 samples and 1 control (due to the number of Swinnex filters purchased) could be processed per run.

The ELIFA had the disadvantage of being single use only. This was due to the problems encountered with the flow cells irremovable protein build up. The solution to this problem was the Swinnex filters. These have the advantage of being auto-clavable so protein could never build up and would never interfere with subsequent uses.

As the filters were cleanable and the only disposable material was the membrane this assay was very economical to run. After the initial costs of the filters the overall cost per sample was around £1.30. This is a considerable difference when compared to the ELIFA's cost.

4.11.4 Mini-ELIFA Synopsis

The bacteria and lysozyme mini-ELIFA has many advantages over the ELISA and ELIFA systems. The mini-ELIFA is cheap to run, rapid, high throughput due to the speed of the assay and the results can be read with the naked eye and by an untrained operator. These attributes are highly sort after in a kit which is to be used in a hospital situation, or in the case of the bacteria mini-ELIFA in the home or GP surgery situation to test for urinary tract infections.

For future work, the assays both need to be tested with clinical samples and the issues with the lysozyme mini-ELIFA need to be addressed to produce a more sensitive test. Additional membrane comparisons are detailed in Appendix F.

Chapter 5 –Lysozyme Biosensor

5.1 Introduction

Immunosensors offer an alternative to currently existing immunoassays, as a method for measuring lysozyme. Immunosensors are analytical devices incorporating a biological element that is either intimately associated with, or integrated within a physicochemical transducer (Homola *et al*., 1999). They work on the same principles as an immunoassay, where the measurement is not necessarily of the analyte itself but of the analyte occupying a receptor. There are 2 forms of immunosensors, the non-competitive, and the competitive (covered in more detail in Section 2.2). In the non-competitive sensor an analyte signal is produced when binding sites are occupied. However, in the competitive immunosensor an analyte signal is produced when binding sites are unoccupied due to competition between signal producing molecules and the test analyte. Immunosensors are compact versions of immunoassays which can offer greater ease of use.

5.1.1 History of Surface Plasmon Resonance (SPR)

The phenomenon of surface plasmon resonance was first described at the beginning of the 20th century. But it was not until the late 1960's that optical excitation of surface plasmons was demonstrated by Kretschmann (1968) using a method of attenuated total reflection. In the late 1970's, SPR was shown to be effective for the monitoring of processes on metal surfaces and thin films. Nylander & Liedburg demonstrated its use for gas detection (Liedberg *et al*., 1983). Otto, Kretschmann and Raether, Agerwal and Swalen brought understanding and showed the versatility of the techniques, (www.BIAcore.com). The first SPR biosensor on the market was developed by the Swedish company, BIAcore AB, in 1990. In later years this has been further defined in terms of speed, throughput and accuracy and followed by the introduction of a number of instruments by other companies.

5.1.2 Surface Plasmon Resonance

SPR has become a recognised optical method for the measurement of molecular interactions (Rich & Myska, 2000). This technique gives researchers the chance to

explore protein function, identify binding partners as a target and also provides real time quantitative data of binding kinetics, affinity, specificity and the concentration of active molecules in a sample. SPR monitors the interactions occurring on a bio specific surface, by measuring the changes in refractive index (RI) (Rich & Myska, 2000). It quantifies changes in the solute concentration as a result of any interactions that take place. SPR methods can be used to measure the interactions of proteins, oligonucleotides, oligosaccharides, lipids as well as phage, viral particles and cells (Rich & Myska, 2000).

The main detection approaches commonly used with SPR include:

- The measurement of the intensity of the optical wave near the resonance, (Liedburg *et al*., 1995).
- The measurement of the resonant momentum of the optical wave inducing angle and wavelength interrogation of SPR, (Jorgensen & Yee, 1993).

5.1.3 Principle

SPR measures changes in the refractive index at the external surface of a metal film. An increased concentration of sample being passed across the surface causes an increase in refractive index and also a change in the refractive angle (Liedburg *et al.*, 1995). Figure 5.1 shows this change in refractive index due to the change in surface chemistry.

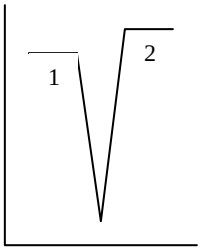


Figure 5.1 Changes in the Incident Light Angle. Diagram shows the change in refractive index; when an analyte is passed across the sensor surface the RI changes from (1) to (2).

An SPR instrument consists of an optical system, usually a prism; a transducing medium, which incorporated the optical system and the biochemical domains; an electronic system which supports the optoelectronic components of the sensor and processes the data, (Homola *et al.*, 1999).

Along with the refractive index, SPR also provides concentration, purity information and can identify the product. Many of these techniques rely on the prior knowledge of the analytes critical angle for comparison; e.g. as antigens bind to antibodies on the metal surface the refractive index at the sensor surface will change and affect the SPR coupling conditions of the incident light entering the SP mode. Increases in the protein concentration will create a refractive index change that is proportional to the mass loading; e.g. increases in protein by $1ng/mm^2$ causes a change in the refractive index of $1x10^{-3}$RIU (Refractive Index Units) and an SPR coupling change of 0.10^{o}. With SPR the minimum detectable surface protein concentration is $50pg/mm^2$, (Earp & Dessy, 1996).

5.1.4 Optical Sensors

5.1.4.1. Optical Phenomenon & Wave-Guides

A wave-guide is a physical medium which light can be guided through. When a beam of light of higher refractive index comes into contact with an interface of a lower refractive index, at an angle of incidence that is above a critical angle, the light is totally reflected and moves out into a high refractive index medium. At total internal reflection the total amount of light that enters the wave-guide leaves the wave-guide because it has been totally confined. In Figure 5.2, for the light to pass through the wave-guide, the wave-guide (N1) must have a higher refractive index than the surrounding area (N2). When this takes place Total Internal Reflection (TIR) occurs, (Figure 5.2). Snell's law says that a TIR can occur at the interface between any two surfaces as long as the critical angle of reflection is met. The critical angle is the minimum angle of incidence of a particular interface. Figure 5.4 shows how total internal reflection can occur when the incident light is equal to or greater than the critical angle, and will be discussed further later.

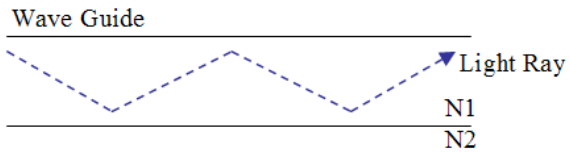


Figure 5.2 Simplified Interpretation of a Wave Guide. Diagram shows total internal reflection, because the light passing through N1 has a higher refractive index than N2.

Also during TIR an evanescent field is created. The reflected beam loses energy as it leaves the interface. This energy loss is in the form of an electrical field intensity called the Evanescent Field Wave. When the TIR interface (surface) is coated with a metallic layer the evanescent wave penetrates the metal layer and excites the electromagnetic surface plasmon waves. If the interface is coated with a non-metallic layer such as gold the plasmon waves will become polarized which creates an enhanced evanescent wave, (Figure 5.3).

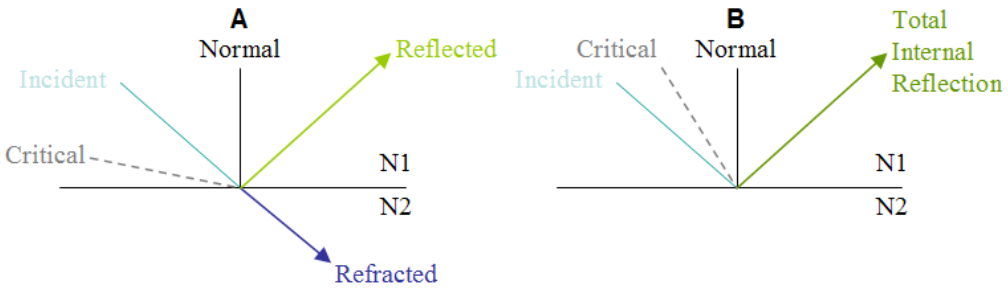


Figure 5.3 Simplified Interpretation of Kretschmanns Prism Arrangement; A- shows the incident light to be less than the critical angle, therefore TIR is not created, B- shows total internal reflection because the incident light is greater than or equal to the critical angle.

5.1.5 Kretschmann's Prism Arrangement

Kretschmann's Prism Arrangement (Figure 5.4) is defined as a structure where light is coupled into a surface plasmon mode, which exists on a solid metal film (Kretschmann *et al.*, 1968). This is the sensor surface onto which a biological sample is placed. The film can either be coated directly onto the surface of the prism or coated onto a glass slide and brought into contact with the prism using refractive index matching fluid. A prism is used to increase the wave vector, which is a mathematical expression describing the propagation of light. A surface plasmon mode is an oscillation of electrons on the surface of a solid conductor.

This solid conductor can be in the form of the gold or silver thin metal films. The support film is then coated on to a glass slide and optically coupled to a wave-guide.

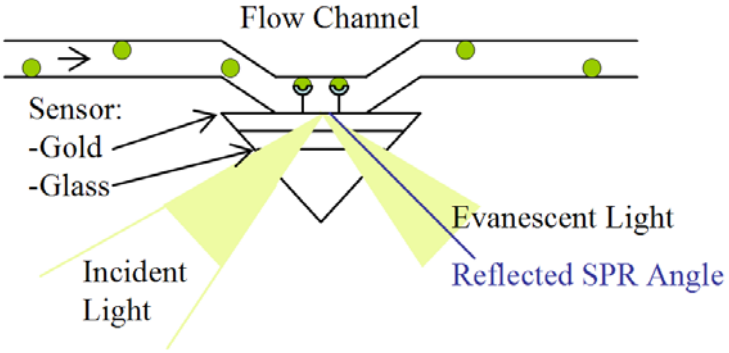


Figure 5.4 Kretschmann Prism Arrangement, showing the polarised light entering the prism and due to the binding event taking place on the surface at the bio-system level (sensor surface) the refractive index of the reflected light is altered.

Coating gold onto glass can prove difficult as it has poor adhesion properties. Chromium can be used to overcome this problem (BIAcore; Sensor Surface Handbook, 2003). Many other materials can be used to support the surface plasmon. However, the main criterion for a good support material is the dielectric permittivity has to be negative. The dielectric permittivity is a measurable physical parameter which detects the optical properties of materials.

The dielectric material surrounds both sides of the metal film containing the surface plasmon. One of these dielectric materials will be the wave-guide media and the other the analyte sample. The interface between the metal and dielectric material is where the surface plasmon exists. Through this interface will be an electric field current created by the light as well as the surface plasmon mode. The surface plasmon mode will be attached to electrons oscillating within this field of electricity that is within the metal layer. Light that is polarized with respect to the metal surface is launched into the prism and coupled to the surface plasmon mode within the metal film. Changes in the amount of light reaching the photo-detector, indicates a sensor output. Only p-polarised light can be coupled into the plasmon mode due to its electric field vector orientation. Some light is also partially reflected off the metal to an optical photo-detector. The dielectric permittivity in contact with the metal surface can alter the surface plasmon the dielectric permittivity is equal to the square of the refractive index of the material (Liedburg *et al*,. 1998). When a change occurs at the bio-system layer a change also occurs in the refractive index of the metal layer. If these values change, the coupling efficiency of the light in the plasmon mode also changes. The response time for the results is close to

instantaneous. The SPR device measures the angle of incidence light (Earp & Dessy, 1996).

5.2 BIAcore

Pharmacia Biosensing started out life in 1982 as a new company within Pharmacia. Researchers from the original company worked along side Linkòping University physicists and biochemists to develop a unique bioanalytical instrument which analysed interactions between biomolecules. The aim was to replace the current ELISA technique with an optical sensor to achieve high levels of sensitivity and specificity. Pharmacia Biosensor launched its first biosensing system the BIAcore in 1990. The BIAcore is a fully automated biosensor; it performs sample handling, immobilisation, SPR analysis, and regeneration of the sensor surface, (Liedburg *et al*., 1995). BIAcore components will be discussed in depth in Section 5.5.

5.2.1 Other Biosensing Instruments

In the late 1990's Texas Instruments (Dallas, Texas) launched its own SPR device TI-SPR-1 commonly known as the Spreeta (Appendix L). This SPR device offers a miniature optical bench and uses semiconductor manufacturing techniques. SPR excitation is initiated by a light-emitting diode mounted inside the light-absorbing plastic casing along with the light polarizer. The sensing surface is a gold layer on the external of the plastic casing. The light is then directed onto the sensing surface and reflected via an internal mirror onto a photodiode array within the sensor chip. This system has the advantage of being portable for field analysis and low purchase and running costs, making it an attractive device for researchers (Earp & Dessy, 1996).

Several other SPR instruments are available on the market, these include Autolab Esprit and Springle low cost SPR detection units (www.ecochemie.nl). Also Reichertai (www.reichertai.com) and Yamagata DKK (www.dkktoa.net) also produce SPR instrumentation.

5.3 Immobilisation Techniques

Firstly in any immobilisation technique, the sensor surface is activated by a sequence of reagents, to prepare the surface for the ligand solution injection. Free ligands that do

not bind are deactivated by injection of a suitable agent and to avoid saturation pre-concentration of the bound ligand is implemented. Electrostatic attraction between negative charges on the surface of the chip and the positive charges on the ligand allow the measurement of pre-concentration and also allows successful immobilisation of solutions with very dilute ligand concentrations.

5.3.1 Ligand Immobilisation

Using BIAcore CM sensor chips the following immobilisation chemistries are useable:

- Amine coupling using reactive esters.
- Thiol-disulphide exchange.
- Biotinylated ligand binding to immobilised streptavidin.
- Hydrazine activated surface used to couple aldehyde.

5.3.1.1 Amine Coupling

In amine coupling the carboxymethyl groups are modified due to an introduction of N-hydroxysuccinimide (NHS) and N-ethyl-N (dimethyl-aminopropyl)-carbidimide (EDC), resulting in N-hydroxysuccinimide esters (BIAcore; Sensor Surface Handbook, 2003). These esters can then form covalent bonds between the ligand and the surface. The method favours ligands with uncharged amino groups and by high pH. This is generally the first coupling method to be tried. Where other methods are preferred, situations can include acidic ligands with a low pH during their pre-concentration phase, which cause the amino groups to protonate and reduces the coupling efficiency. The other situation when non-amine coupling is preferred is where ligands possess very reactive amino or nucleophilic groups which may lose their biological activity during immobilisation.

5.3.1.2 Streptavidin-biotin Coupling

Avidin from bird egg albumin and streptavidin from Streptomyces cultures, are proteins which have binding sites with high affinities for biotin. The biotin itself can be physically attached to biological molecules for this purpose. Where biotinylated ligands are to be immobilised on the sensor chip an avidin or streptavidin is firstly immobilised on the sensor chip. The avidin-biotin affinity is extremely high therefore binds the

ligand to the chip well (Tombelli, 2001). Changes in pH, chaotrophs or manipulations via multiple washing will not disturb this bond.

Avidin has 4 binding sites per molecule. This can be a major problem because it attracts non specific binding of other molecules due to the presence of the sugars in its structure (N-actetylglucosamine) and its isoelectric point being so high (pI~10). Streptavidin on the other hand has 4 binding sites but no carbohydrates in its structure. The pI is 5-6 and has low non specific binding. When streptavidin binds with biotin it goes through several processes including hydrophobic and van der Waals interactions (weak non-chemical bonds of attraction) as well as extended hydrogen bonding network, (Freitag *et al*., 1997). These plus a deep cleft in which the biotin binds inside the protein ensures a high affinity between protein and biotin. Biotin is a small molecule so when it reacts with streptavidin it only uses the biocyclin ring. The carboxylic group can therefore be modified and conjugated to many macromolecules without altering the overall size, characteristics, and biological activity (Gitlin *et al*., 1990).

The streptavidin-binding coupling method is an alternative technique for binding ligands to amine and thiol methods, and should be used when the other methods are not suitable. This method has the advantage of being able to be performed above the isoelectric point of the ligand and does not rely on electrostatic pre-concentration at the sensor chip. Another advantage is that biotinylated probes can be stored at -20^{o}C without loss of activity for longer periods than compared to radioactive probes, (Tombelli, 2001). Ligands where streptavidin coupling is useful include: nucleic acids; polysaccharides and glycoconjugates; acid sensitive proteins; peptides and other small ligands where amine and thiol groups are absent; and where the ligand is to be bound to a streptavidin-biotin receptor the ligand always has to be modified to be biotinylated. This essentially means that the method is longer than the amine or thiol methods and not only this but also optimisation of these steps has to be included too.

5.4 Aptamers & Aptasensors

Aptamers are a new development in the field of artificial receptor technology. Before the development of aptamers, the most popular group of molecules for molecular

recognition were antibodies. Antibodies have been around for more than half a century and have subsequently had a huge involvement in diagnostic assays and become essential in routine clinical tests. Conventionally, ligands for non-nucleic acid targets have incorporated antibodies for analysis of proteins. But recently work has involved aptamers due to their high affinity to bind to target proteins (Liss *et al.,* 2002). Aptamers differ from antibodies but they mimic antibody properties. The demand for diagnostic assays to assist in management of current and up-and-coming diseases is growing. Aptamers have the potential to fulfill the recognition requirements in future assays (Jayasena, 1999).

5.4.1 History of Aptamers

In 1990, Joyce (La Jolla, USA), Szostak (Boston, USA) and Gold (Bolder, USA), simultaneously and independently reported the development of an *in vitro* selection and amplification technique, which brought about the discovery of specific nucleic acid sequences which are capable of binding to non-nucleic acid targets with high affinity and specificity that is often superior to those of antibodies (Joyce, 1989; Ellington & Szostak, 1990; Tuerk & Gold, 1990). The technique was termed SELEX; the Systematic Evaluation of Ligands by EXponential enrichment. SELEX is the technique used to isolate functional nucleic acids by screening vast libraries of oligonucleotides by *in vitro* selection and amplification to produce artificial receptors, i.e. aptamers as well as catalytic nucleic acids such as ribozymes and aptazymes (Luzi *et al.,* 2003). The process results in oligonucleotides referred to as “aptamers”; “aptus” from the Latin word meaning “to fit” (Jayasena, 1999). The progress of research on aptamers has grown over the last 10 years into an extensive range with claims for increased abilities over antibodies in terms of selectivity, specificity and affinity. Exploitation of aptamers in the coming years is to be expected.

5.4.2 Definition of an Aptamer

Aptamers are defined as, “artificial nucleic acid ligands generated against amino acids, drugs, proteins and other biological molecules” (O’Sullivan, 2002). Aptamers are an emerging class of molecules that pose novel approaches for new analytes. Aptamers have the capacity to recognise and bind to virtually any class of target molecule with

high affinity and specificity (Jayasena, 1999). These include small organic molecules to supramolecular structures and organisms, because aptamers are capable of folding into 3D structures to become receptors for a huge array of targets (Gold, 1995). Aptamers can also react with protein targets, which do not react with nucleotides as part of their original function. They also have the advantage of being generated against a wide range of targets, namely, amino acids, drugs, and proteins (Luzi *et al*., 2003). The target diversity for aptamers is quoted as being somewhere in the range of 10^1-10^5 times greater than the largest phage display populations that have been generated. As well as being 10^4-10^8 times greater than the number of antibodies in the human immune system, (Chambers, 2002).

5.4.3 Systematic Evaluation of Ligands by EXponential Enrichment (SELEX)

The SELEX (Figure 5.5) process begins with the synthesis of a single stranded library of oligonucleotides. Each oligonucleotide consists of a 5' and 3' region of defined sequence and in-between a central region of random sequence. The library therefore comprises of many oligonucleotides with unique sequences. The starting library (Figure 5.5 (1)) contains 10^{14}-10^{15} individual oligonucleotides and is usually screened within a couple of days, giving a high probability for selecting an aptamer for the analyte of interest.

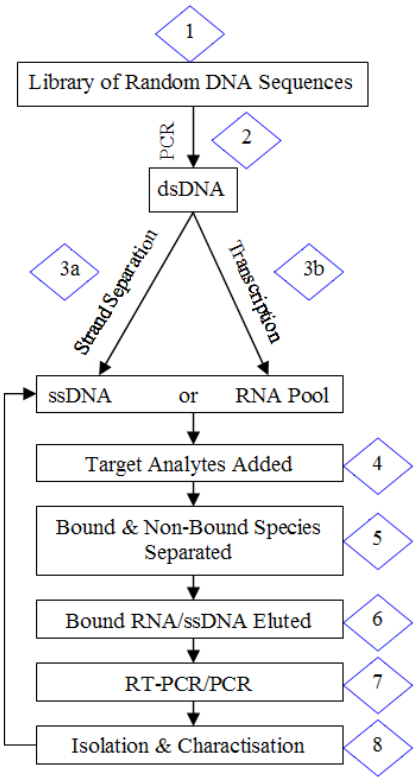


Figure 5.5 SELEX Process. Adapted from Luzi *et al.*, 2003. Diagram showing SELEX from the starting DNA library, through PCR, strand separation, transcription, biding, elution of bound nucleotides, amplification and ending, after several cycles with the isolation of an aptamer.

The process then either creates a single stranded DNA pool which is amplified through PCR to produce a double stranded DNA pool (2). This is either transcribed for strand separated for DNA selection (3a) or RNA selection (3b). This starting library is then incubated with the analyte of interest (4). All non-specific or nucleotides which have a low binding affinity are removed by a washing phase (5). All nucleotides which do bind to the RNA/ssDNA are eluted (6) and amplified by RT-PCR (for RNA libraries) and PCR (for DNA libraries) (7), (Luzi *et al.*, 2003).

The enriched pool of either RNA or ssDNA is then transcribed *in vitro* and used for the next amplification cycle. The total number of cycles required is dependent on the activity of the interactions between the target and aptamer. This usually takes between 8-15 cycles before an oligonucleotide population that binds to the target the best is selected as the successful aptamer (8). In the initial cycles a tiny percentage of 0.1-0.5% of individual sequences bind. These sequences are then separated from the rest of the unbound nucleotides, by affinity chromatography or some other filter binding

technique. The Beckman Biomek 2000 performs an automated SELEX process, producing an aptamer for a given target in just 2 days (Luzi *et al.*, 2003).

5.4.4 Applications

Aptamers can mimic antibodies in many applications; they can substitute for antibodies in ELISA, immunobead assay, cell sorting, fluorescence microscopy, western blotting, and affinity layers for biosensors (Liss *et al.*, 2002). Where aptamers can be used in place of antibodies in ELISA assays, they are referred to as Enzyme Linked Oligonucleotide Assays (ELONA). The ELONA technique was patented in 1997 by Nexstar (now Gilead Sciences, California). Various formats can be employed:

- The aptamer can be used as the capture molecule and the antibody as the receptor.
- The aptamer can be used as the capture molecule and the labelled antibody binds to the aptamer-analyte immunocomplex as a reporter molecule.
- The aptamer is the capture molecule and the labelled aptamer against the aptamer-analyte complex is the reporter molecule.

Another application is the use of aptamers as molecular beacons in aptasensing. Molecular beacons contain two structural components, a loop, and a stem (Yamamoto & Kumar, 2000). The loop is the probe and the stem has two arms of complementary sequences, one florophore and the other a quencher. When the florophore and the quencher are in close proximity no signal is created. But when a target molecule is encountered the probe binds. The probe-target hybrid is stronger than the binding of the probe and the stem, therefore it releases the probe and increases the proximity of the quencher and the florophore; resulting in fluorescence. An example of this is Yamamoto & Kumar (2000) who immobilised a molecular beacon aptamer that fluoresces in the presence of TAT-1 protein derived from HIV-1 and HIV-2, (O'Sullivan, 2002).

5.4.5 Regeneration, Reusing the Aptamer

An antibody coated chip when regenerated (with 0.2M glycine-HCl pH 2.2) can result in the complete release of the antibody and the analyte (Liss *et al.*, 2002). This provides a chip that is free from ligands, but if repeated experiments are to be carried out then immobilisation has to be applied after every use. However, an aptamer-coated sensor

chip can in principle be regenerated for further testing. For example experiments by Liss *et al.,* (2002) involved a chip coated with an aptamer that was rinsed with 50mM EDTA. The rinsing dissolved the analyte, leaving the aptamer intact on the chip. Aptamers unfold in the presence of EDTA and then reassemble into their 3D formation in the presence of metal ions, producing a chip that is ready for use with further antigen.

5.4.6 Comparison of Antibodies & Aptamers

5.4.6.1 Limitations of Antibodies

The generation of antibodies is normally begun in an animal. Certain molecules such as toxins are not well tolerated in animals therefore it is difficult to generate antibodies against them. Additionally, antibodies against molecules which are naturally less immunogenic are difficult to produce. The isolation and manufacture of monoclonal antibodies is both an arduous and often a costly process. This is especially true for rare antibodies, requiring screening of large numbers of colonies. Antibody production also poses the possibility of accidental loss or death of a cell line. Usually multiple stocks of frozen antibody-producing cells are stored at multiple sites to avoid this. Where high yields of monoclonal antibodies are required, they are typically grown in the peritoneal cavity of animals. It poses its own inconveniences as it can be difficult to grow some hybridomas *in vivo*.

Generally, the production of hybridomas is limited to rat and mouse; this limits the use of antibodies in therapeutic applications. Heterophilic antibodies (human antibodies that recognise antibodies from non-human origin) have the potential to bind a capture antibody with a non-human detector antibody in the absence of an analyte; leading to false positive results. Auto-antibodies and rheumatoid factors also have the potential to behave this way and interfere with immunoassays. Batch to batch variation can hinder the performance of antibodies. This can lead to an immunoassay being re-optimised with each new batch of antibodies purchased. Finally antibodies are subject to irreversible denaturation, are sensitive to temperature changes have a limited shelf life and kinetic factors of target-antibody interactions cannot be altered easily (Jayasena, 1999).

5.4.6.2 Advantages of Aptamers

Aptamers have a high specificity. They can detect subtle differences between targets, between methyl or hydroxy groups or D- vs L-enantiomeric configuration of the target. This is due to adaptive recognition. In solution aptamers unfold and refold upon association with the target forming a new 3D complex. An example of this is an anti-caffeine aptamer which showed 10,000 fold lower affinity for theophylline, a molecule which differs from caffeine by just 1 methyl group (Luzi *et al.* 2003). The specificity results from a phase in the SELEX process called "counter-SELEX". During this phase sequences that bind two closely related analogues of the target, are eliminated thus producing a highly specific oligoma (Jayasena, 1999).

Advantages in aptamer technology can be seen in the field of affinity sensing. K_{on} and K_{off} rates can be adapted and controlled according to the nature of the transducer and the desired assay time. Sandwich assays can be employed instead of competitive formats, as they are far more successful at detecting small molecules. Aptamers also have the advantage of allowing modification during the immobilization process or when labelling with a reporter molecule, without affecting its affinity. They can additionally be subjected to repeated cycles of denaturation and regeneration without affecting their reactivity (O'Sullivan, 2002).

An advantage of aptamers over antibodies is their small size. This means that a dense layer of aptamer can be formed increasing the sensitivity. The receptor layer can be highly ordered as the aptamer can be modified with functional groups allowing convalent, direct or indirect immobilization. The arrangement of aptamers onto a chip is highly linear and ordered. Aptamers can produce a dense layer that saturate the chip. The usable aptamer concentration quoted by Liss *et al.* (2002) was 10mg/l and the usable antibody concentration on the chip was >1mg/l. This shows that aptamers form a dense receptor layer that is far more sensitive than antibody arrangements.

Aptamers are produced *in vitro* or in machines, therefore no animals are required. This allows the production of aptamers to affine ligands, non-immunogenic or toxic proteins, with more flexibility. This also means no or very little batch-to-batch variability is seen.

An additional advantage is that the SELEX process is much faster than the typical time spent generating a cell-line to produce monoclonal antibodies and purified antibodies. Multiple SELEX experiments can also be carried out simultaneously presenting fast, high throughput discovery of aptamers (Jayasena, 1999).

Aptamers are more resistant to denaturation and degeneration and have a longer shelf life compared to antibodies (Liss *et al.*, 2002). Experiments by Liss *et al.* (2002) showed this. They immobilized antibodies onto quartz crystal biosensors and regenerated with 0.2M glycine-HCl pH 2.2 solution. This regeneration did release the analyte from the bound antibody but also irreversibly damaged the antibody. In contrast their aptamer quartz crystal biosensor was washed with 50mM EDTA, releasing the analyte leaving the aptamer to be completely reusable.

Aptamers can be stored for several weeks without loss of sensitivity when attached to the surface of a biosensor. The surface stability of an antibody coated sensor chip is vulnerable compared to an aptamer coated sensor chip. The antibody's vulnerability was shown in an experiment by Liss *et al,* (2002). The aptamer coated quartz was subjected to 30 minutes 100^{o}C and retained 25% of its sensitivity yet the antibody coated quartz crystal lost all activity.

5.4.6.3 Disadvantages of Aptamers; Nuclease Resistance

For diagnostic and analytical purposes aptamers need to be stable in biological fluids. However, nucleic acids are very prone to degradation when associated with enzymes such as nucleases. Unmodified RNA has a half life of 1 minute in serum. Its half life has been increased to 15 hours by modification (O'Sullivan, 2002).

Approaches which have been adopted to prevent cleavage include:

- Alteration of nucleotide bases; modifying pyrimidines at the 2'positions amino/fluoro groups.
- Alteration of the phosphodiester backbone; through use of α-thio substituted deoxynucleoside triphosphates.

- Use of spiegelmers (enantiomeric aptamers); with a chemical mirror image of the target, selecting the complementary aptamers and creating a chemical mirror image of the SELEX selected aptamer (Sampson, 2003).

5.4.7 Lysozyme Aptamer

The Lysozyme enzyme is found in a range of body fluids and is released in response to bacterial presence to degrade the cell wall and irradicate its presence. The current work has included an ELISA which detects lysozyme in serum and takes ~100minutes to obtain results, rivalling commercial and clinical tests. However, as Section 5.4.6.2 describes, aptamers have several advantages over antibodies. Therefore a test which incorporates the benefits of aptamers with the knowledge of lysozyme and its usefulness in the diagnosis of sepsis, should produce a much improved test challenging current diagnostic tests.

The Lysozyme aptamer sequence used was derived from a paper by Cox & Ellington (2001). The template was used to create the RNA anti-lyosyzme aptamer, which was then biotinylated at the 5' and amine coupled to the surface of a sensor chip.

5.4.8 Advantages of the Lysozyme Biosensor

The main advantage of a lysozyme biosensor would be the result being produced in real time. Immediately after the injection the concentration of lysozyme in the sample can be calculated. The ELISA, commercial immunoassay kit, ELIFA, and the mini-ELIFA cannot produce results in such a short time.

The lysozyme biosensor described here using the BIAcore 3000 can process 1 sample and 1 control per run with 2 replicates of the sample. This system has huge time saving potential above and beyond any immunoassay. Potential advantages of this system also include;

- Reduced non-specific binding; The CM5 series chips have a negative charge caused by the inactivated carboxyl group. When the ligand is immobilised this negative charge can be counter balanced as the ligands are positively charged. Ionic

solutions in the sample can minimise the effects of the positive ligands on the surface.

- Regeneration; The process of removal of non-covalently bound analytes. The ligand cannot normally be removed without destroying the surface of the chip nor can it be removed during the regeneration process.
- Biological Activity of the bound ligand; The activity of the ligand after the immobilisation process, must be retained for the chip to be useable. When the ligand is coupled to the matrix and not to the surface, it is basically suspended in a gel solution. The advantages of this is the conditions are said to be more favourable as they are freely accessible in a three dimensional matrix. However, the biological activity of the ligand in the matrix may be affected due to the coupling reaction. Steric restrictions may be a consequence of large ligands.
- Reproducibility; when a ligand has been bound to the sensor chip it can be repeatedly used for testing. The sensor chips are usually highly consistent. The experiment of adhering the ligand has also a high consistency between batches with a coefficient variation of <10% for CM5 batches of chips.
- Ligand Modification; A further advantage is that ligands can undergo modifications. The ligand structure has disulphide groups introduced by reaction with its amino groups and NHS-activated heterobifunctional reagents for example SPDP or SMPT, or by reaction with its carboxyl groups with PDEA. Where acidic proteins are to be immobilised, modification of the carboxyl groups are useful as the reaction increases the isoelectric point of the protein and consequently supports electrostatic pre-concentration on the sensor chip surface.

5.5 Materials & Methods –Lysozyme Biosensor

5.5.1 BIAcore Sensor Surface Chip

The BIAcore sensor chip is made of a glass slide housed within a plastic support casing to provide integrity and alignment within the BIAcore instrument and with the optics and flow system. The visible glass on the chip is $1cm^2$ with a 50nm layer of gold coating. On top of the gold layer is a linking layer to aid the binding of the biomolecules. The immobilized molecules need to be linked to the compatible linking layer of carboxymethylated dextran, by covalent linking. Use of an unmodified metal film would result in a low number of immobilized sites.

A typical sensor chip can be used for >50 measurements without sensitivity loss. Two types of chip are commercially available from BIAcore. The first is a certificated grade chip that has statistical variability in immobilized capability of less than 5% and the second type is a research grade chip with a variability of less than 15%; e.g. CM5 and SA (Earp & Dessy, 1996).

5.5.2 BIAcore Flow Injection System

The BIAcore flow injection system is a micro-fluidic system consisting of miniaturised sample loops (Earp & Dessy (1996), Liedberg & Johansen (1998)) valves and conduits to reduce sample and reagent volumes. The unit is comprised of 3 plastic plates welded together with the channels running between the plates for the samples etc. The chip is then fixed into the flow cell for accurate delivery of the sample. Each flow cell has a dead volume of 60nL.

5.5.3 The Sensorgram

SPR is a combined oscillation of electrons with respect to the nuclei in the near surface region of the metal film. The oscillation at the surface plasmon is an optical wave driven by an external light source. The wave is both strong and localized along the interface between the metal and the medium e.g., the buffer or the liquid sample. The signal that is sensed in an SPR experiment is change in the angle of incidence. This signal is equal to the changes in the refractive index and the mass concentration of the biomolecules at the metal film. The sensorgram is a term created by BIAcore, for the

recording of these events in the form of a graph. When producing a sensorgram for any experiment, the first step is to record the background baseline, during continuous flow of the running buffer. The ligand is then injected, resulting in a refractive index shift. After rinsing with buffer the analyte is injected and a new baseline is formed and recorded. The SPR signal is determined by the changes in the second and first baseline.

5.5.4 BIAcore Components

The pump consists of a syringe which measures down to 1µl-ml and a stroke capacity of 500µl. Liquid is pumped from a reservoir to a connector block inlet which is directly connected to the Integrated µ-Fluidic Cartridge (IFC) (Figure 5.6). BIA certificated buffer (HBS) or a custom made buffer, is used as the running buffer. Refilling takes place at the end of each stroke.

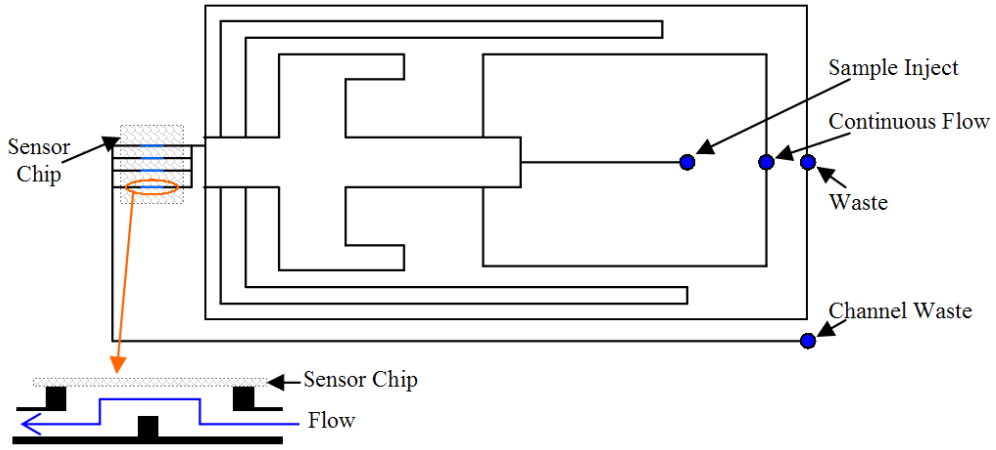


Figure 5.6 Integrated µ-fluidic Cartridge, (adapted from BIAcore Methods Manual, www.BIAcore.com). Diagram shows the delivery of samples to the flow cell of the sensor chip, via the channels in the IFC.

The Connector Block is the sample inlet and outlet chamber. This has two inlets; the pump inlet and the injection port for loading the sample. The sample is injected using a standard Gilson pipette (200µl or 1000µl) with the BIAcore X and is automatic loading in the BIAcore 3000. It also has two outlet ports one for the sample out and the other for the flow cell out. Waste from these runs directly into a waste beaker under the connector port in both models.

The IFC (Figure 5.6) consists of a series of channels and pneumatic valves controlling delivery of liquids to the sensor chip. The chip is in direct contact with the connector block and the inlet and outlet ports are in parallel. The IFC and the flow cell together, form two flow cells in the BIAcore X and 4 in the BIAcore 3000, which can be used for multichannel analysis of samples. Serial flow can enable two or more of the same samples to be tested at the same time on two different surfaces or for in line reference measurements or used to determine different analytes. Two basic operating modes exist in the IFC due to pneumatic values. The load allows buffer to flow through the flow cells bypassing the sample loops. The injection allows buffer to be prepared through the sample loop and to the flow cells. The IFC operates in load mode at all other times when a sample is not being injected.

Sensor chips commonly used in the BIAcore X and 3000 are CM5 (Figure 5.7), which are carboxymethylated dextran coated. Biomolecules can be adhered to this surface with general chemical methods (Section 5.4). Other sensorchips include SA (Figure 5.7), which has a dextran matrix and a streptavidin covalently immobilised for use with high affinity capturing of biotinylated ligands. This allows rapid immobilization of biotinylated ligands and due to the strength of biotin-streptavidin bonds permits harsh regeneration without the loss of reproducibility.

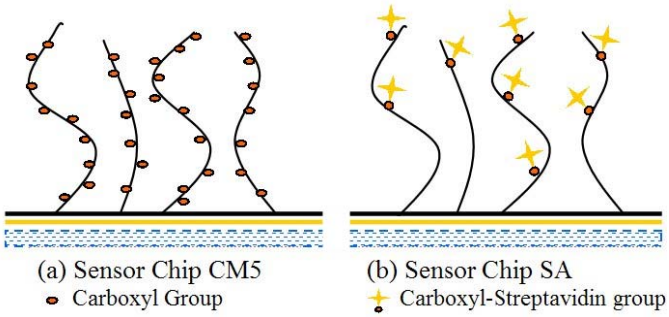


Figure 5.7 Drawing of the Sensor chip surface (adapted from www.BIAcore.com). (a) Sensor Chip CM5, showing carboxymethylated dextran matrix. (b) Sensor Chip SA, showing the Streptavidin bound to the carboxymethylated dextran matrix.

NTA chips have a nickel-chelating mechanism for the capturing of poly-his tagged recombinant products. And HPA chips are used for the analysis of membrane related interactions due to their hydrophobic surface which allows the creation of lipid

monolayers on the surface of the chip. A sensor chip can be used 50-100 times for repeated measurement. It can be docked in the processing unit for up to 1 week. The shelf life of a chip however, depends on the immobilised ligand.

The flow cell is contained within the optical system. The outside wall of the flow cell consists of glass which is pressed up against a glass prism in the optical unit. The silicone opto-interface between the chip and the prism is matched in refractive index and means a good optical coupling between the prism and removable sensor chip is achieved. Light is forced through the prism via a light emitting diode; this hits the sensor chip in a wedge shaped beam to give a wide range of incident light. The reflected light from the sensor chip is then monitored by an array of light sensitive diodes that are linear to the range of incident light rays. The SPR angle can be monitored accurately in real time without the movement of the light source, chip, or detector. Surface plasmon resonance measurements are sensitive to temperature changes. The temperature is monitored via Peltier elements which ensure a constant temperature change. The air temperature when the BIAcore is operating, must not deviate 10^{o}C above or below the operating temperature range of the BIAcore system.

A microcomputer controls the operations of the processing unit within the BIAcore. It's job is to record and process raw data from the detection unit. The microcomputer is then controlled by a PC within the lab; all data can then be tabulated or printed as sensorgrams, (BIAcore; Sensor Surface Handbook, 2003).

5.5.5 BIAcore 3000 Maintenance

Training was provided by Università degli Studi di Firenze (Italy). A Kinetic and Affinity Analysis Level 1 training course was provided by BIAcore UK.

Throughout all experiments, and all conditioning of sensor chips and maintenance of the Biacore machine the flow rate was 5µl per minute. Before and after each experiment a Desorb was run (in accordance with IBST/SOP RQ1037). The BIAcore was a busy

instrument with several users therefore a desorb was run at the start of each users turn both for machine maintenance and to ensure a constant environment was retained. BIAdesorb solution 1 (0.5% (w/v) sodium dodecyl sulphate) and solution 2 (50mM glycine pH 9.5) (BIAcore, Sweden) were used. All buffers were filtered using 0.2µm syringe filers and degassed for 10 minutes before use. All buffers were made from fresh stock each day and the stocks made fresh monthly. When changing buffers the Prime tool was used followed by the Flush tool to completely remove any of the previous buffer that was remaining. This sequence was repeated if the refractive index signal was unstable or if air bubbles were disrupting the signal.

5.5.6 General Reagents

All buffer recipes can be found in Appendix G.

During the preconditioning of the SA sensors and the immobilization of Streptavidin on the CM5 chips HBS-EP buffer (BIAcore, Sweden) was used as the running buffer. To immobilize Streptavidin onto CM5 chips the surface was activated with an amine coupling kit (Appendix G). Streptavidin was purchased from Sigma-Aldrich Ltd, from *Streptomyces avidinii.* A solution of Ethanolamine (98% purity) (Biacore, Sweden) was prepared to deactivate any unbound NHS-esters.

The SA sensor chips were preconditioned with a preconditioning solution (Appendix G). This preconditioning solution was injected three times for one minute each time. The biotinylated anti-Lysozyme aptamer was purchased from MWG (UK). The aptamer buffer (Appendix G) was used as the running buffer during the aptamer immobilisation. The negative control was a custom made biotinylated 80mer oligo (Sigma-Genosys, UK). The Poly-T buffer (Appendix G) was used as the running buffer during the Poly-T immobilisation, and was used to make Poly-T dilutions. Biotin (Sigma-Aldrich Ltd, UK) was used to block unbound Streptavidin sites on the SA chips (Appendix G).

5.5.7 Regeneration Solutions

Several regeneration solutions were tried to remove analyte bound (Appendix G).

5.5.8 Aptamer Preparation

The anti-lysozyme aptamer was synthesised by MWG. The aptamer was biotinylated at the 5' and stored in the recommended buffer (Appendix G).

5.5.9 Analyte Preparation

Human Lysozyme (Sigma-Aldrich Ltd, UK) was dissolved in Aptamer buffer. The lysozyme was stored at -20°C. On the day of use the lysozyme aliquot was defrosted by leaving it on the bench for 30 minutes. Dilutions were made using the Aptamer buffer.

5.5.10 Serum Preparation

All serum was centrifuged using Vivaspin centrifugal filters 30kDa (Fisher, UK), at 1200rpm for 10 minutes, to remove all albumin from the sample. The residue containing the albumin was discarded and the filtrate was then used for all dilutions and injection onto the sensor surface.

5.6 Final Lysozyme Biosensor Method

5.6.1 Sensor Chip Preparation

The SA sensor chip was docked and HBS-EP buffer was allowed to flow for about 5-10 minutes to stabilise the signal. Flow cells were then pre-conditioned separately using the preconditioning solution; 3 injections of 5µl at a flow rate of 5µl/min. Signal changes were recorded.

5.6.2 Control Application

After preconditioning the buffer was changed to the Poly-T buffer and primed for 6 minutes to remove any residual HBS-EP buffer. The sensorgram was started and the signal allowed to stabilise at a flow rate of 5µl/min. An injection of 75µl of 0.1µM Poly-T was carried out. The signal was recorded before and after the injection.

5.6.3 Ligand Application

The poly-T buffer was replaced with Aptamer buffer and the system primed for 6 minutes. The sensorgram was restarted and the new running buffer allowed to stabilise for several minutes. During this time the Aptamer was prepared for injection. The

$2x10^{-3}$pmol aptamer was heated using a Bunsen burner, for 3 minutes at 95^{o}C. It was then removed from the heat and injected 75µl. The signal was recoded at the start and end of the injection.

5.6.4 Analyte Preparation & Use

The Lysozyme was stored at -20^{o}C until use. On the day of use the aliquot was defrosted at room temperature for 30 minutes then diluted using aptamer buffer. An injection volume of 75µl was used at a flow rate of 5µl/min. The baseline was recoded before and after the injection.

5.6.5 Serum Preparation & Use

The serum was divided into aliquots of 150µl on the day of collection and stored at -20^{o}C. On the day of use an aliquot was defrosted at room temperature. All serum was spin-filtered and the filtrate used for dilution and use in the BIAcore. The dilution of serum samples was made using aptamer buffer. Lysozyme spiked serum samples were used in the development of the method. Here, a lysozyme negative serum sample was used from non hospitalised patients. A $^{1}/_{100}$ dilution of serum was produced in aptamer buffer and spiked with various concentrations of lysozyme.

5.6.6 Regeneration Using Heat Changes Protocol

After the analyte injection had finished the signal was allowed to stabilise for several minutes (Cycle 1). The baseline was then recorded and the sensorgram stopped. The new temperature was set and allowed to reach temperature over several minutes until stable. The sensorgram was then restarted (Cycle 2) and the loss in baseline recorded. The sensorgram was again stopped and the temperature reduced back to 25^{o}C. Once the BIAcore temperature was stable, the sensorgram was restarted (Cycle 3) and the baseline recorded. All fluctuations in baseline were recorded.

5.7 Lysozyme Biosensor Method Optimisation Results

This section presents the development and optimisation of the lysozyme biosensor. This included testing aptamer and control concentrations, sample preparation, non-specific binding using control analytes, and types of regeneration solution. The Response Units (RU) represents an arbitrary measurement used by BIAcore to measure changes within the baseline.

5.7.1 Ligand Binding

Figure 5.8 and Table 5.1 show successful aptamer binding onto an SA sensor chip. Table 5.1 shows the different concentrations of aptamer that were tried to reach an amount which was stable and also produced a response similar to that achieved with the control immobilisation. The final Aptamer concentration chosen was $2x10^{-3}$pM. During these trials, the aptamer was immobilised after treatment and without treatment. The treatment consisted of heating the aptamer to 95^{o}C for 3 minutes. The results showed that heating the aptamer had a positive effect on binding.

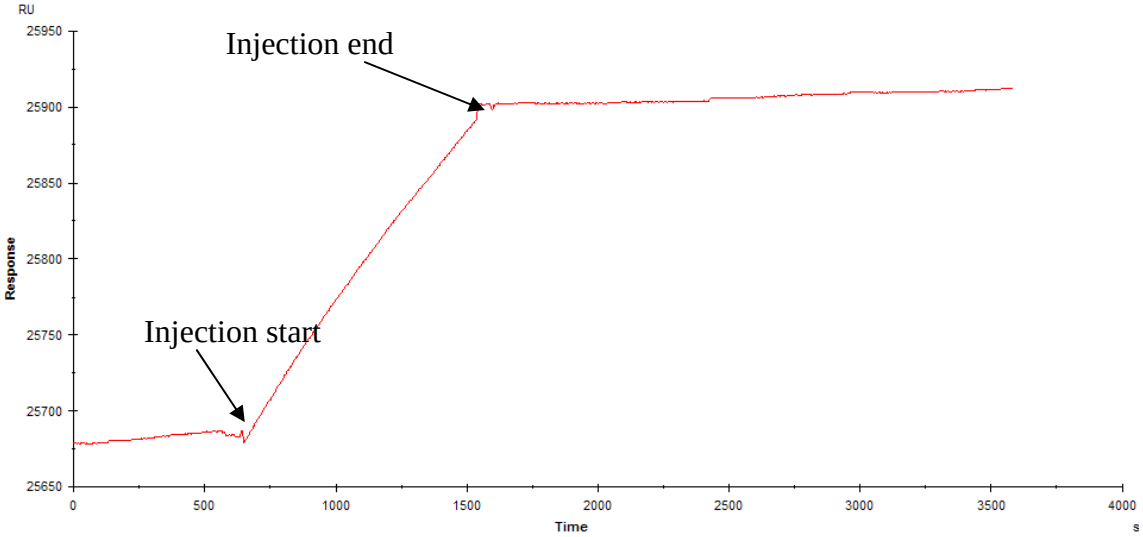


Figure 5.8 Aptamer binding ($2x10^{-3}$pM); A stable signal after the injection has ended showing final bound concentration of aptamer of ~250RU.

Table 5.1 Selection of aptamer concentrations immobilised on to Carboxymethylated dextran sensor chips (CM5) and sensor chips which have a Streptavidin covalently bonded to the carboxymethylated dextran (SA); $2x10^{-3}$pM on SA used.

Concentration of Aptamer	Preparation (Treated*)	Sensor Chip Type	Volume	Response (RU)
$2x10^{-6}pM$	Treated	CM5	100µl	407.1
$2x10^{-12}pM$	Untreated	CM5	100µl	15
$2x10^{-12}pM$	Treated	CM5	100µl	55.6
$4x10^{-11}pM$	Treated	CM5	100µl	600
$2x10^{-6}pM$	Treated	SA, no preconditioning	100µl	719
$2x10^{-6}pM$	Treated	SA, preconditioning	75µl	233
$2x10^{-3}pM$	Treated	SA, preconditioning	75µl	250

*Treated=Heated to 95^{o}C for 3 minutes

The sensor chips initially used were CM5 (dextran immobilised), but SA (streptavidin immobilised) chips were used later to speed up the assay. The use of SA chips also ensured that the immobilisation surface was constant. With the CM5 chips the surface was produced by immobilising streptavidin, and the biotinylated aptamer. However, the streptavidin did not distribute evenly over the surface of the 4 flow cells, producing a higher degree of binding onto flow cells 1 and 2 and much less on 3 and 4. This obviously affected the amount of aptamer which could potentially bind. The SA chips eliminated this uncertainty, producing a sensor chip surface which evenly distributed with streptavidin.

5.7.2 Control Ligand Binding

Figure 5.9 shows the immobilisation of the Poly-T ligand. The graph shows a stable immobilisation at the end of the injection. Table 5.2 shows the concentrations of Poly-T control that were tried. The injected volume was set at 75µl purely by mistake, but the result showed that the original 100µl volume was far too much ligand to inject. Subsequently the volume of aptamer was also changed to 75µl and again the immobilisation was much improved.

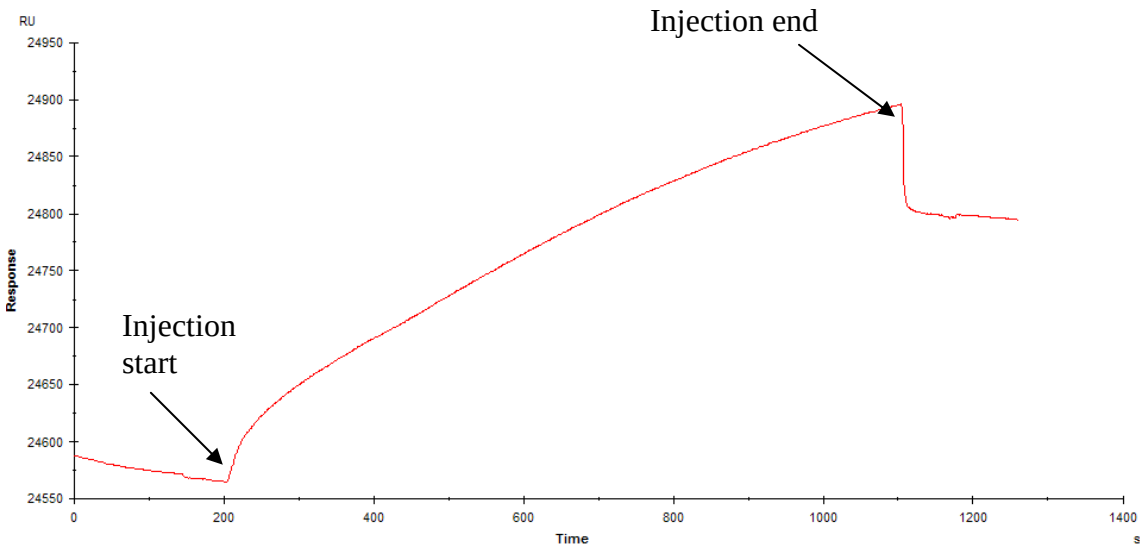


Figure 5.9 Poly-T Binding (1µM) and the stable signal after the injection had ended showing final bound concentration of ~245RU.

Table 5.2 Results from the Poly-T immobilisation. Various concentrations were tried, along with two types of sensor chips to achieve a stable immobilisation of the control.

Concentration of Control (Poly-T)	**Sensor Chip Type**	**Volume**	**Response (RU)**
1µM	CM5	100µl	1071
0.1µM	CM5	100µl	104
0.1µM	SA, preconditioned	75µl	240

When the sensor chip was changed for the aptamer immobilisation, the control chip was also changed. Again the immobilisation of the control was more reliable with the SA chips, due to the uneven binding of the streptavidin on the CM5 chips.

5.7.3 Non-Specific binding of Poly-T

Figure 5.10 shows the injection of Poly-T onto a CM5 sensor chip. The sensor chip was not activated with the NHS-EDC solution nor was it immobilised with streptavidin. The two injections of Poly-T here show no binding. After the first injection, the baseline increases slightly, but then after the second injection the baseline decreased, indicating no binding has taken place. This therefore shows that Poly-T is not non-specifically binding to the sensor chip surface and is in fact binding to the streptavidin.

This graph does show some binding due to the buffer at 5000 seconds onwards. However, the Poly-T buffer was only used during the immobilisation of the control and during serum injections. As fresh buffer was made on each new day, and because the amount of binding was quite small this was ignored.

Figure 5.10 Sensorgram showing two injections of 1µM poly-T. No binding was present with either injection.

5.7.4 Non-Specific Binding Tests with Analytes

Figure 5.11 shows the injection of lysozyme onto an immobilised SA sensor chip. Two injections of lysozyme were injected onto a sensor chip which had no ligand immobilised and had under gone no preconditioning. The lysozyme had not bound in either injection showing that lysozyme does not non-specifically bind to the sensor surface.

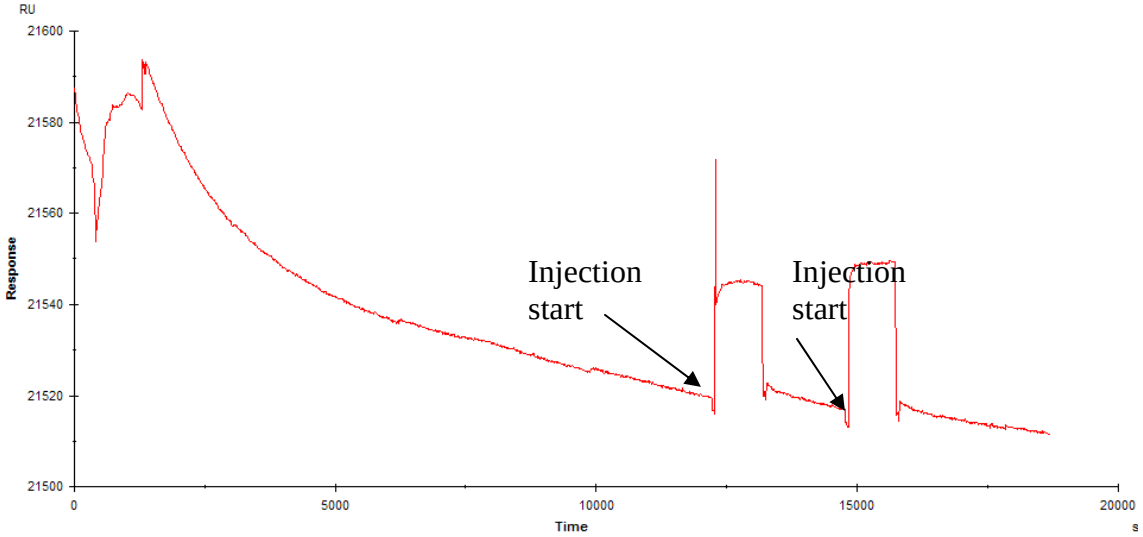


Figure 5.11 Test for non-specific binding. Lysozyme was injected on to an unmodified SA sensor chip; no binding was present.

Figure 5.12 below the use of biotin to block any unbound sites on the sensor surface. The idea behind this experiment was to prevent non-specific binding of lysozyme and any other analyte to these unbound sites. However, the biotin did not bind, so was not applied again in future experiments.

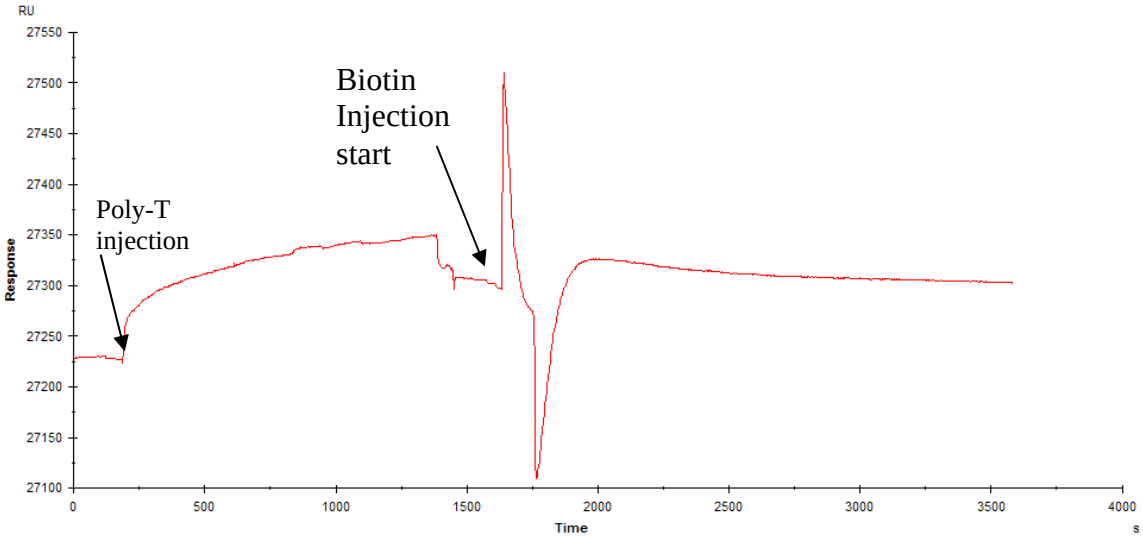


Figure 5.12 Sensorgram showing the injection and binding of Poly-T and the injection of biotin. Biotin did not bind as the signal slowly returned to the pre-biotin level.

The use of a negative control was used in Figure 5.13 to prove that lysozyme was specific to the anti-lysozyme aptamer. BSA was used as the negative control because it was readily available in the lab. The BSA did bind to the sensor surface as shown by

the dips on the graph; this is not binding but simply a change in the refractive index. The BSA also caused the aptamer to change in confirmation in someway so it did not bind to the serum injection or the buffered lysozyme injection.

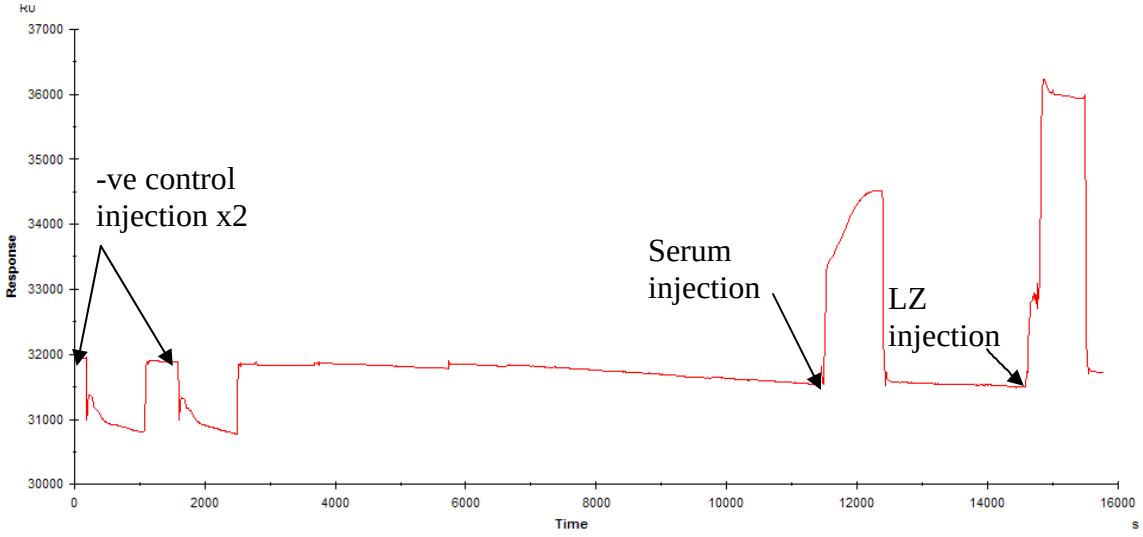


Figure 5.13 Application of a negative control. BSA showed some binding and interfered with subsequent binding of serum samples and buffered lysozyme injections.

Myoglobin from equine heart was the second negative control to be used as shown in Figure 5.14. This analyte was chosen because it had a similar molecular weight to lysozyme. The dotted lines on the graph illustrates how the baseline has increased slightly after the injection, indicating that the myoglobin has bound to both the aptamer and poly-T flow cells. An explanation of why this may have occurred is due to the charges of the molecules. If lysozyme and myoglobin have the same charge then both would be attracted and bound to the poly-T and the aptamer.

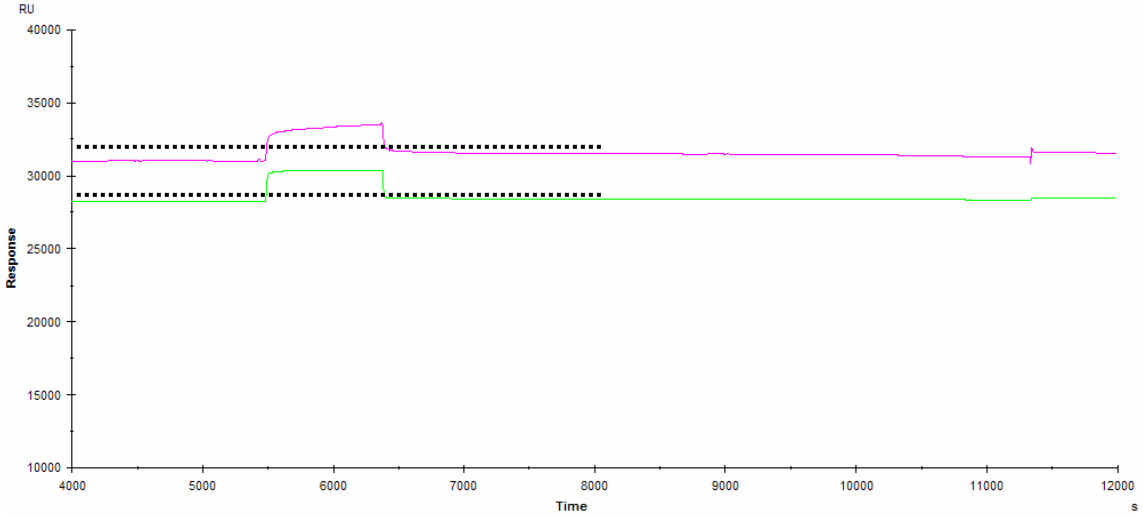


Figure 5.14 Injection of second negative control Myoglobin from Equine Heart. The dotted line shows increase in baseline indicating binding.

5.7.5 Regeneration

Table 5.3 shows the solutions that were used to regenerate the sensor chip. During regeneration the ideal situation is where the ligand is freed of all analyte and is left in an active state to bind to further injections of analyte. The only regeneration solution which came close to achieving this ideal situation was the ethanol and sodium hydroxide solution. All others either increased the baseline indicating binding of the regeneration solution or decreased the baseline destroying the ligands.

Table 5.3 Table shows the Results from the Regeneration Scouting; table lists the regeneration solutions tried, along with concentrations, volumes and the responses.

Regeneration Solution	**Concentration & Volume of Regeneration Solution**	**Response**
Hydrochloric Acid	1,5, 10 & 20mM 5-2µl	HCl bound to sensor surface and increased baseline. No regeneration achieved
Ethanol & Sodium Hydroxide	25-100% (25mM) 5-40µl	Baseline decreased. A degree of regeneration success was achieved but was unpredictable. Some results varied as the solution was unstable after preparation (see table

		5.4).
Sodium Citrate	0.1 & 1M 10 & 50µl	Signal increased. Sodium citrate bound to the sensor surface.
Sodium Hydroxide	1mM &1M 5µl	The baseline decreased, destroying bound ligand. No regeneration achieved.
Sodium Chloride	1 & 10mM 5µl	No regeneration was achieved. Binding took place on the aptamer flow cell and the signal decreased on the control flow cell.
Magnesium Chloride	1M 10µl	No regeneration achieved. Binding took place on the aptamer immobilised flow cell and the signal decreased on the control.

The ethanol and sodium hydroxide was tried in various percentage concentrations and several volumes were injected. The volume and percentage concentration was dependent on the amount of analyte binding; if a lot of analyte had bound then a stronger and larger concentration was injected. A degree of regeneration success was achieved with this solution. However, the search for a regeneration solution/process which produced better results was still required.

Table 5.4 shows the instability of the ethanol and sodium hydroxide regeneration solution. The "fresh 100%" indicated a solution made fresh on the day of use and without dilution. An "old 25%" solution indicates a solution not made fresh on the day of use and diluted ¼. Even though the solution was stored over night in a sealed container and stored at 4^{o}C, the ethanol was still evaporating, making the effects of the regeneration solution unpredictable. In some cases the regeneration would not only take off the analyte but also remove bound ligand. This begs the question of whether it was better to regenerate to a point where a small amount of analyte was still bound to the ligand, or whether it was better to remove a small amount of the ligand to ensure all analyte was completely removed. As it was felt that removing ligand may cause more

damage to ligand that remained bound, a decision was taken to regenerate using this solution to a point where some (>10RU) was still bound. This ensured that free ligand still bound, was not deactivated.

Table 5.4 Table showing the Instability of the Regeneration Solution Ethanol & Sodium Hydroxide (EtOH+NaOH)

Percentage Concentration of 25mM EtOH+NaOH	**Baseline reading at the Mid Injection of Regeneration Solution**	**Baseline Reading at the End Injection of Regeneration Solution**
Fresh 100% (no dilution)	234	137
Fresh 50%	1156	230
Fresh 25%	1481	264
Old 25%	1248	38

Table 5.4 above shows an experiment carried out to determine the effects of day-old and freshly prepared ethanol and sodium hydroxide solution. The results showed that the day-old solution had very little regeneration effect compared to solutions made fresh on the day. This suggests that the ethanol was evaporating overnight and reducing the effectiveness of the solution.

The freshly made solutions of ethanol and sodium hydroxide produced unexpected results. As the concentration of ethanol and sodium hydroxide reduced the effect, on reducing the baseline was stronger. The results suggest that the regeneration solutions used were unreliable at regenerating a sensing surface and produce random effects regardless of concentration. This is the main reason why the search for appropriate regeneration conditions continued.

5.7.6 Thermo-Regeneration

The experiment from Table 5.5 shows the sensor chip was prepared in the usual way with preconditioning of 3x 1 minute injections of 50mM Sodium Hydroxide + 1M Sodium Chloride, and then injection of both aptamer and control. A concentration of 0.1µg/ml lysozyme was injected and the temperature was increased by a known amount and the decrease in bound analyte recorded. The temperature was then reset at 25^{o}C and

further ligand and analyte was injected. This cycle was repeated several times until the temperature of the BIAcore had reached its upper limit (40^{o}C). The experiment showed that as the internal BIAcore temperature increased, the amount of bound analyte removed also increased. This was the most effective regeneration regime used. Not only was analyte removed, but the ligands remained attached and active. There was also a high degree of control over the amount of regeneration. The temperature could be tailored to how much regeneration was required simply by using the data shown in Figure 5.15 below and determining the temperature the BIAcore needed to be set at to achieve adequate regeneration.

Table 5.5 Table showing the results from the thermo-regeneration method. The baseline decreased with increasing temperature showing the sensor surface was being cleaned of immobilised analyte.

Temp Change (ºC)	**Baseline Before Temp Change**		**Baseline After Temp Change**		**Difference in Baselines**	
	FC1	FC2	FC1	FC2	FC1	FC2
25→25.2	27172	27331	27172	27329	0	-2
25→25.3	27172	27331	27165	27322	-7	-10
25→25.4	27172	27331	27128	27285	-44	-46
25→25.5	27184	27215	27119	27151	-65	-64
25→26	27170	27106	27033	26972	-137	-134
25→30	27146	27395	26444	26684	-703	-711
25→35	27102	27558	25708	26067	-1394	-1490
25→40	27108	27539	24961	25381	-2147	-2158

The problem of how much regeneration to expose the ligand to was still present. Whether to leave a certain amount of analyte bound or to remove a small amount of ligand ensuring the analyte was completely removed was still a matter for consideration. Again the amount of analyte left bound was >10RU, this was sufficient to ensure that all remaining free ligand was active.

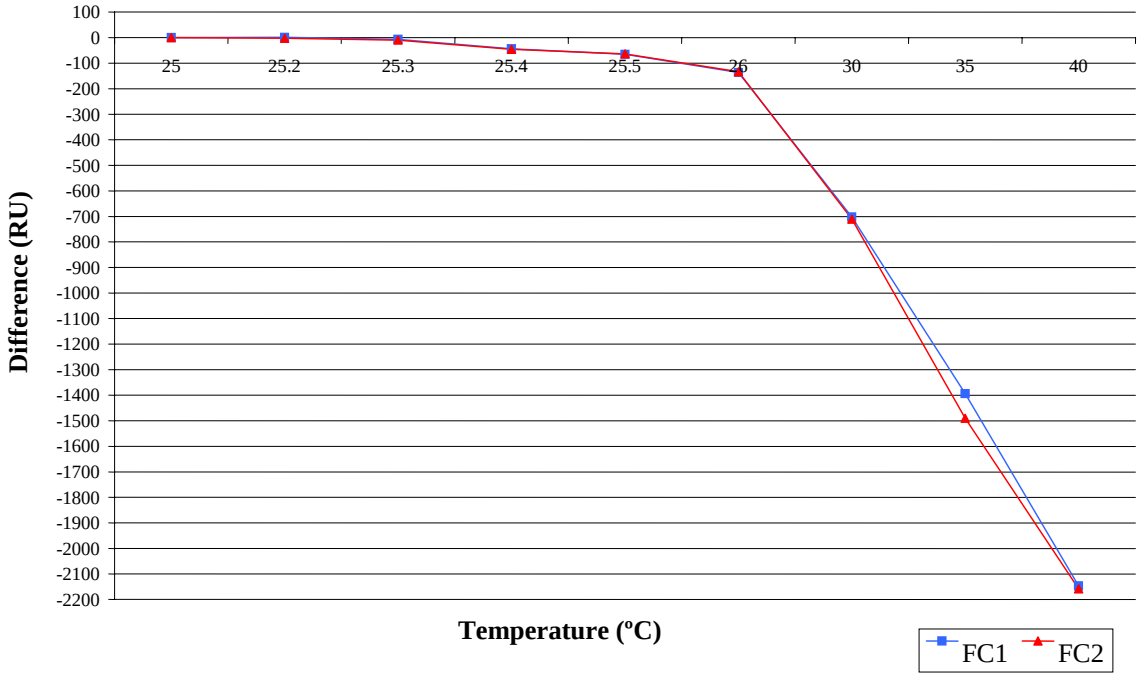


Figure 5.15 Graph showing the degree of regeneration achieved when the BIAcore temperature was increased.

5.7.7 Serum Preparation

Table 5.6 shows the results from various dilutions of the same concentration of serum spiked lysozyme. The dilution of $^{1}/_{100}$ was chosen for all future serum samples as this was the lowest dilution producing viable results.

Table 5.6 Results showing dilution scouting to determine the dilution to be used for all Serum samples.

Lysozyme Dilution (0.2µg/ml)	**Response Units**		**Description**
	FC3	**FC4**	
$^{1}/_{400}$	-4.2	-2.0	No binding on either FCs
$^{1}/_{200}$	-3.8	-4.5	No binding on either FCs
$^{1}/_{100}$	1.1	10	Binding on both FCs
$^{1}/_{50}$	1.0	18.5	Binding Increased in FC4
$^{1}/_{25}$	-1.2	29.6	Further binding increase on FC4, no binding on FC3.

5.8 Lysozyme Biosensor Results

This section describes the results gained from testing the biosensor. Tests included standard lysozyme in buffer, spiked lysozyme in serum, clinical serum samples and regeneration using the thermo-regeneration technique.

5.8.1 Lysozyme in Buffer

Figure 5.16 shows the results gained from a variety of lysozyme injections. The results show no binding of the lysozyme on the control (Poly-T) ligand. All results for the control ligand were negative showing lysozyme to be specific to the anti-lysozyme aptamer. The results for the aptamer show an increase in the amount of binding as the concentration of lysozyme increases. This is true up until the final injection of 0.5µg/ml lysozyme at which point the binding decreased and presumably the ligand is saturated with lysozyme. It should be noted that no regeneration was carried out in-between each lysozyme injection. The lysozyme concentrations were simply repeatedly injected until the limit was reached at 0.5µg/ml.

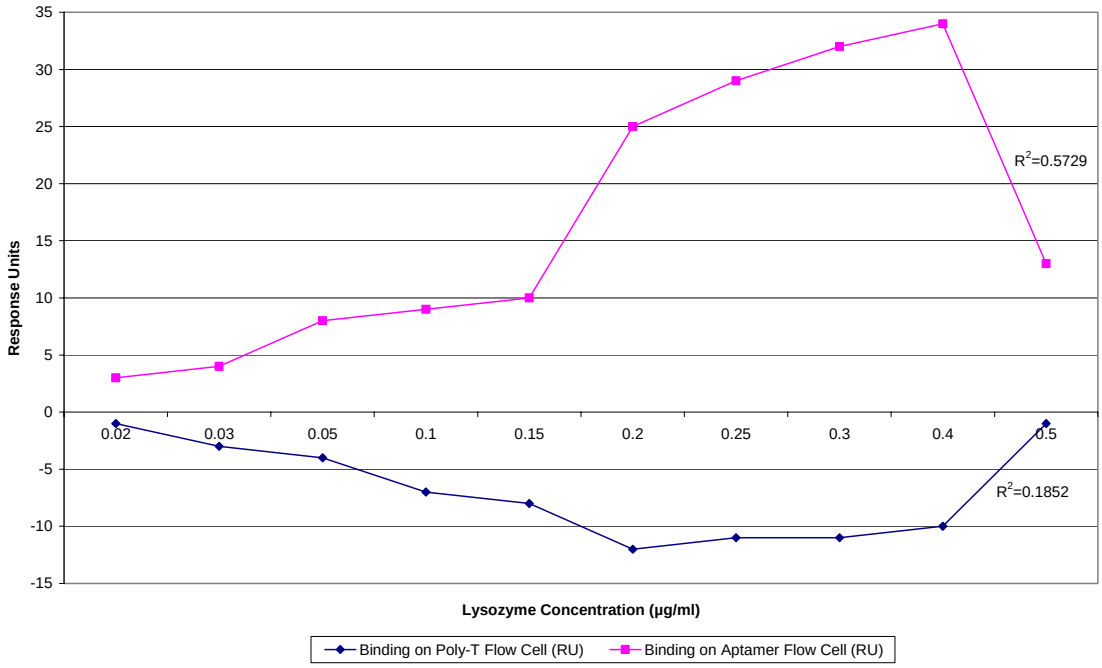


Figure 5.16 Response Units (RU) from the control and Aptamer surface, for various concentrations of Lysozyme in Buffer

5.8.2 Calibration Curve Construction

The experiment for Figure 5.17 was carried out due to the unreliability of the Poly-T control; in some experiments lysozyme bound to the Poly-T. In this experiment the Poly-T was not immobilised. Instead the sensor chip was preconditioned as usual using the 3x 1 minute injections of the preconditioning solution (Appendix G). It was then used without a ligand present.

The low concentrations of lysozyme (0.05-0.25µg/ml) did not bind to the blank control surface, but the final concentrations of 0.3-0.5µg/ml did. This could have been due to small amounts of the lysozyme from previous injections binding and changing the charge of the surface so it became attractive to subsequent lysozyme injections and thus causing the binding of lysozyme at the high concentrations. The lysozyme binding on the aptamer showed a consistent pattern. As the lysozyme concentration increased so did the amount of lysozyme bound. The only exception was with 0.25µg/ml and the final lysozyme concentration 0.5µg/ml. As found with the previously (Figure 5.16), the limit of ligand binding was reached at 0.5µg/ml.

This experiment also incorporated the thermo-regeneration regime. Figure 5.17 shows that the regeneration is working and the ligand is being freed from analyte for subsequent analyte binding. This is also why the lysozyme was binding to such a greater extent than compared to Figure 5.16.

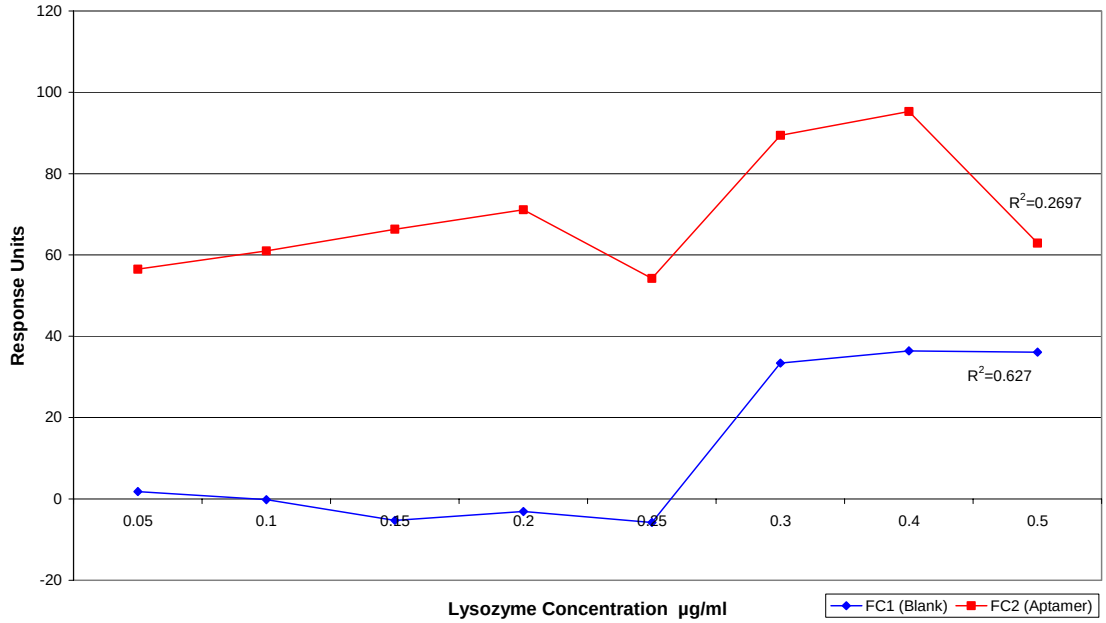


Figure 5.17 Calibration Curve of Various Buffered Lysozyme Concentrations injected onto both an Aptamer immobilised flow cell and a blank flow cell acting as control.

5.8.3 Thermo-Regeneration Using Lysozyme-Spiked Serum Samples

Table 5.7 shows the results from the spiked serum samples. Again with this experiment the Poly-T was attracted to the serum and produced high RU's with all dilutions of lysozyme tried. This can both be attributed to the unreliability of the control and also the stickiness of the serum. Even though the serum was centrifuged to remove the albumin, it still contained unknown contaminants, possibly bacteria, or remnants of bacterial cells, which were attracted to the ligands. These unknown contaminants made regeneration via temperature change more difficult, requiring a higher temperature, but still manageable.

Table 5.7 The Response of Lysozyme Spiked Serum Samples regenerated via temperature change

SLZ Conc.	LZ Bound (RU)	Temp Increase for Regeneration	RU loss due to Temp Increase	Difference in Start & End Baselines (RU)
0.05µg/ml	FC1 15	25.2°C	5.2	
	FC2 27		8.2	
		25.4°C	55.1	-1.6
			55.8	+8.5
0.1µg/ml	FC1 51	25.2°C	11.8	
	FC2 46		13.0	
		25.3°C	27.6	
			27.8	
		25.4°C	59.2	-4.5
			58.3	-7.3
0.2µg/ml	FC1 28	25.3°C	5.7	
	FC2 31		8.2	
		25.4°C	45.6	+7.3
			43.2	+7.4

*Values in Red are obtained from FC1; values in Blue are obtained from FC2.

The lysozyme binding was again affected by the regeneration. After 0.05µg/ml was regenerated, 8.5RU was still left bound. As less than 10RU remained bound it was thought that this was small enough to not effect any subsequent injections. However, the remaining bound analyte did have the effect of increasing the RU for the 0.1µg/ml injection.

5.8.4 Validation Study

This validation study (Table 5.8) was carried out to test the quality of samples after they had been spin filtered. Before injection into the BIAcore each serum sample was spin filtered using Vivaspin centrifugal filters which removed all molecules larger than 30kDa. Before centrifuging, each sample was spiked with 0.25µg/ml lysozyme. In theory the lysozyme should remain in the sample after centrifugation as its molecular weight is 15kDa and the albumen in the serum (MW 60kDa) should be removed. Once spun, the samples were then diluted and assayed with the ELISA protocol and also injected into the BIAcore. The lysozyme content was then recorded.

Table 5.8 Validation study; results were compared from two spiked serum samples assayed with the ELISA and then injected into the BIAcore.

Sample ID	BIAcore Response Units		ELISA (µg/ml)
	FC1 (Control)	FC2 (Aptamer)	
VN1	4.3	34.8	0
VN2	14.4	37.9	0

The results from the BIAcore experiment seemed promising. Lysozyme was seemingly detected in both samples and there was a difference between anti-lysozyme aptamer detection and poly-T control detection. However, when the samples were assayed via the ELISA protocol, a system which has a proven record of good lysozyme detection, no lysozyme could be detected in either sample. This experiment suggested that using the centrifugal preparation method with serum samples, resulted in total removed of all lysozyme in the sample. All previous results using this technique are therefore brought into question. The removal of lysozyme maybe due to its attraction to albumen or other molecules in the serum, such as bacteria, drugs and plasma proteins. If the lysozyme binds to any of these molecules, and is then trapped on the membrane and disallowed through the pores, the lysozyme will be removed from the sample.

5.8.5 Serum Sample Trial

Figure 5.18 shows the effects of injection of a non-centrifuged spiked serum sample. The serum sample was spiked with 0.05µg/ml lysozyme. The sample produced a huge response, with FC1 3101.9RU and FC2 2458.7RU. As such a small amount of lysozyme was used to inoculate this sample the huge response can only be attributed to the albumin, bacteria and unknown contaminants of the serum. Regardless of the increase in internal BIAcore temperature, the ligand could not be regenerated.

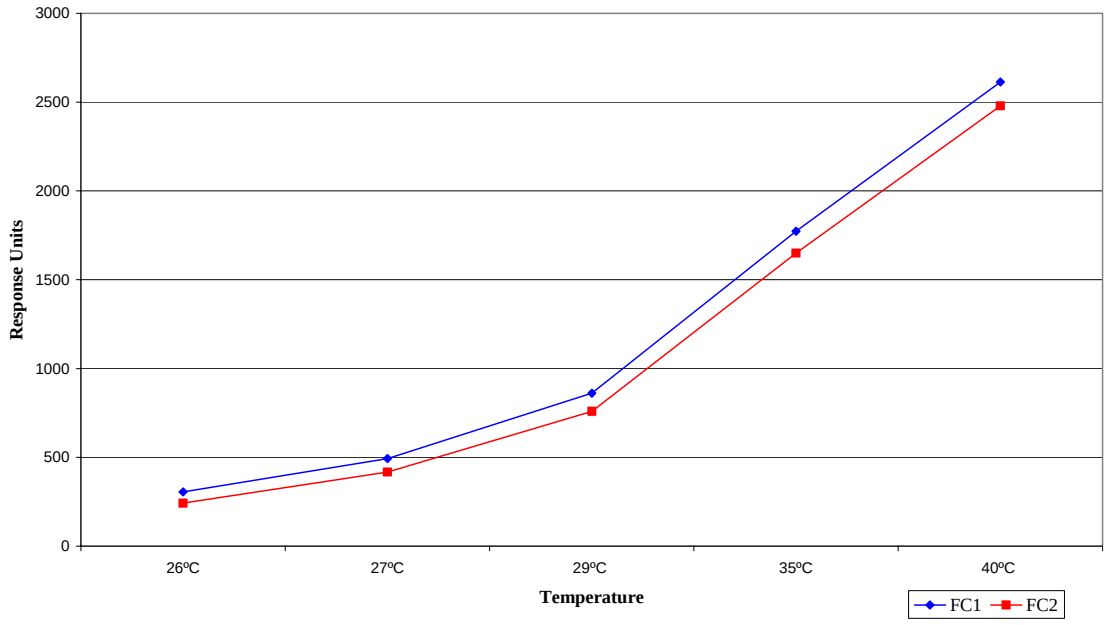


Figure 5.18 Graph showing the loss in baseline due to temperature regeneration method using an un-spun lysozyme spiked serum sample.

This experiment showed the importance of the centrifugal process in the serum sample preparation. It additionally showed that the serum could pass through the micro-tubing of the microfluidic system. As previous experiments had shown, a compromise between not centrifuging the sample at all and centrifuging using the spin filters needed to be found, before adequate lysozyme detection could be achieved using this method. The solution may simply lie in using a spin filter with a greater pore size.

5.8.6 Clinical Samples

For the final experiment (Figure 5.19) to test the Lysozyme Biosensor, 10 clinical samples were randomly chosen from those assayed using the ELISA protocol. The samples were chosen because of their high lysozyme content, as shown by the ELISA. The BIAcore results were produced from serum samples which were diluted $^{1}/_{100}$ and compared to those shown in Figure 5.17. Figure 5.19 shows that there is no correlation between the samples processed using the Lysozyme Biosensor and those from the ELISA.

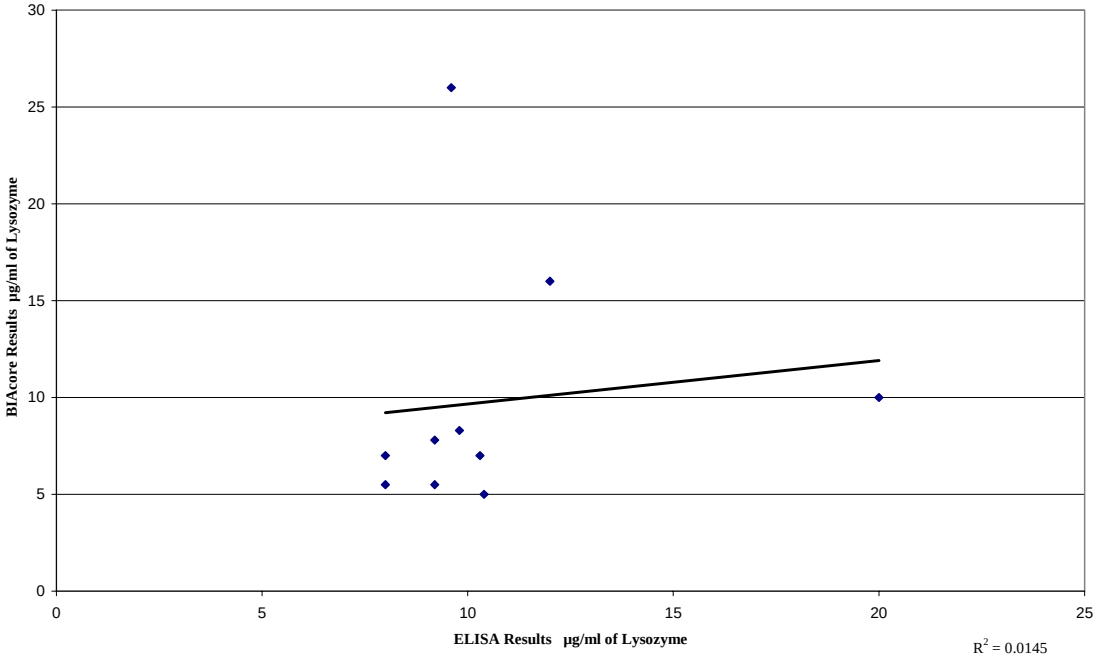


Figure 5.19 Clinical Samples; the figure shows the lysozyme content of a set of serum samples donated from of a selection of ill patients all with known high lysozyme levels. The samples were assayed using the ELISA system and the Lysozyme-Biosensor method.

Eight of the samples (D4, D22, D52, D55, D46, N26, A15, & A17) yielded results that were lower than the results gained from the ELISA. These results do tally with the results from the Validation Study, which showed samples that were centrifuged using spin filters contained no lysozyme. The clinical samples used with the lysozyme biosensor could be influenced by bacterial presence in the samples. The conclusion therefore is that another method needs to be sought after to prevent the loss of lysozyme when serum samples are being prepared for use in the BIAcore.

5.9 Discussion

This section discusses the results from sections 5.7 and 5.8. Further detailed analysis is collated in Appendix M.

5.9.1 Ligand & Analyte Binding

The final concentration of anti-lysozyme aptamer which was used for clinical testing was $2x10^{-3}$pM. This was shown to be stable after injection as well as being active. Preconditioning of the SA sensor chip helped make the aptamer stable and the binding of the aptamer reproducible. The pre-treatment of the aptamer at 95^{o}C for 3 minutes also assisted in the binding of the aptamer securely. The aptamer preparation was prescribed in the paper by Liss *et al.* (2002). The theory behind the heating of the aptamer before injection was to unfold the structure. The aptamer was then chilled on ice after heating to preserve the intra-molecular folding and then immediately injected. The binding of the control ligand was achieved with 0.1µM concentration. This concentration was the same as the IgE control used in Appendix B. The control binding and aptamer ligand binding were tailored to the same RU values. This was to ensure an equivalent test was carried out, with the same ligand coverage on the chip surface.

5.9.2 Non-Specific Binding

Non-specific binding was tested using several different methods. Poly-T was injected onto an unmodified CM5 sensor chip. The poly-T did not bind showing that it was not non-specifically binding to the surface, but in fact binding via the streptavidin-biotin bond. Streptavidin is a homotetrameric protein with a single biotin binding site in each subunit. It is to be expected that the biotinylated ligand binds to the streptavidin because of the exclusively strong bond between the two. The dissociation constant of a streptavidin and biotin complex is estimated at $4x10^{-14}$M (Qureshi *et al*., 2001).

Lysozyme was injected onto a blank SA sensor chip. This was to test for non-specific binding of the analyte. Again no binding was seen showing the binding of lysozyme to the ligand was specific to the aptamer. This was to be expected as the lysozyme was specific only to the anti-lysozyme aptamer. The aptamer buffer may have also helped as non-specific binding is limited by the salt content in the running buffer.

The problem of non-specific binding of the lysozyme on the control ligand surface was tackled by immobilising biotin on to the free streptavidin molecules. This idea was taken from a paper by Schneider *et al.* (2000), where biotin was used to control the surface chemistry of an optical sensor chip for the detection of human chorionic gonadotropin (hCG). In theory, the biotin should have bound to the free streptavidin rather than the ligand (poly-T) and thus eliminated any non-specific binding of lysozyme. However, no biotin bound and therefore a solution to this problem was not found. An explanation of why the biotin did not bind could have been that instead of it working as an allosteric activator and binding to the allosteric (other) site on the streptavidin, it worked as an allosteric inhibitor in that it decreased the activity of the streptavidin (Huang *et al.*, 2002).

5.9.3 Negative Control Analytes

The molecular charges of the ligand and analyte have an important bearing on their binding ability. This is also true for the negative control analytes which were used to test the ligand. Bovine serum albumin (BSA) was chosen due to its availability in the laboratory. On injection, the BSA coated the surface of the sensor chip and destroyed the ligand completely. There are two explanations for this, either the charge of the BSA was opposite to that of the ligand and an attraction was formed, or the BSA could have been too sticky and viscous and simply covered the surface. The more likely effect is the charge of the molecules. An article by Krieger (2003) explains that molecules with the same charge can "snuggle up" if the ions in solution are oppositely charged. These oppositely charged ions nullify the repulsion between the larger molecules which have the same charge.

The second negative control analyte to be tried was myoglobin from equine heart. This was chosen because of its similar molecular weight to lysozyme. However, the myoglobin again coated the surface of the sensor chip. As such a weak solution was prepared and filtered rigorously, the myoglobin's coverage onto the chip was put down to charge attraction. As explained above the ions in solution could have acted to nullify the repulsion of the myoglobin and caused an attraction to the surface of the sensor.

5.9.4 Biosensor Regeneration

The initial search for a regeneration solution began with examples from the work on the IgE biosensor (Appendix B). These solutions were tested first as they were known to have an affect on the IgE aptamer. There was little information in the literature that was directly relevant since sources such as the "Biacore Sensor Surface Handbook" (2003) lacks information on aptamer regenerations due to their novelty. Ideally regeneration should simply remove the bound analyte from the ligand leaving the ligand intact, undistorted and reusable. The analyte should be removed and the original pre-analyte-injection baseline should be reached. In cases where the ligand is lost during regeneration it can be assumed that the surface modification of the senor chip has also been damaged and the life of the chip is effectively ended. The nature of and strength of association and dissociation of the analyte and ligand binding can determine whether regeneration will work properly.

The Hydrochloric acid and Ethanol & sodium hydroxide solution did not have an affect on the aptamer ligand. The hydrochloric acid did not decrease the baseline and the ethanol & sodium hydroxide was very unstable. The "Biacore Sensor Surface Handbook" (2003) also suggested sodium hydroxide, sodium chloride and magnesium chloride as potential regeneration solutions, but again none had the desired effects. Many of the regeneration solutions were tried because they were known to reduce or increase the pH on the surface of the sensor chip; such as hydrochloric acid and sodium hydroxide. Others were tested because they increased the ionic strength on the sensor surface; such as magnesium chloride and sodium chloride. As none had favourable effects other regeneration resolutions were sort after.

The question as to whether to regenerate to a point of some ligand loss or to leave some analyte bound was apparent with all regenerations. The theoretical R_{max} equation showed that the anti-lysozyme aptamer binding capacity was 149RU. The maximum analyte capacity was never reached by any of the lysozyme concentrations shown in sections 5.8.1-4. Not even the highest dilutions of lysozyme or where lysozyme was repeatedly injected and not regenerated produced a response that reached the R_{max}. This according to the BIAcore "Sensor Surface Handbook" (2003) is normal. R_{max} is simply

an estimation of the maximum amount of binding possible; and is rarely obtainable due to ligand accessibility, dissociation of ligand after immobilisation and regenerations with harsh solutions. Therefore a decision was made to leave some analyte bound when regenerations could not be fully achieved. A limit of analyte bound was arbitrarily set at 10RU. Leaving 10RU of analyte bound ensured that the ligand remained intact and active.

5.9.5 New Regeneration Regime

As the stability and ultimately the effectiveness of the regeneration solution ethanol & sodium hydroxide was deemed unreliable, a new regeneration regime was sought after. The idea for the thermo-regeneration came from a consideration of the aptamer confirmation change during a temperature increase, which is known to be useful to help immobilise the aptamer. It follows then that the aptamer's confirmation could be changed to release analyte.

The thermo-regeneration was both inexpensive to perform and most importantly did not harm the ligand. The only limitation of this regeneration is the time it takes to perform. The BIAcore 3000 can only increase the internal temperature by $5^{o}C$ per hour. Therefore, when processing large numbers of clinical samples, the regeneration time may have to be taken into consideration or the instrument redesigned. A solution to this lengthy regeneration process might be to transfer the method over to a newer BIAcore system. The BIAcore T100, allows 384 samples to be analysed per run. Incorporating the lysozyme biosensor method, with the thermo-regeneration and the T100 would justify the lengthy regeneration time.

A further limitation with the thermo-regeneration was with the fine-tuning. Whether to regenerate to a point of ligand loss or to a point of some analyte remaining was still a problem even with this regeneration regime. The same pragmatic solution was applied to this regime and a limit of 10RU of analyte remaining was adopted. This allowed the ligand to remain intact and as the R_{max} was never reached, for the analyte to continue binding.

5.9.6 Serum Dilution

A range of serum dilutions were tried but $^{1}/_{100}$ was chosen because of its positive binding response. Published work has described serum dilutions as low as $^{1}/_{40}$ (Jongerius-Gortemaker *et al.*, 2002). Even though other published methods use different serum dilutions, the method here responded better to the $^{1}/_{100}$ dilution. This is perhaps due to the spin filters used, which other published methods have omitted.

5.9.7 Lysozyme in Buffer Calibration Curve

The lysozyme calibration curve (Figure 5.16) showed specificity of the lysozyme to the aptamer as no binding was seen on the control ligand. The amount of lysozyme binding increased with increasing concentration, up until the final dilution (0.5µg/ml). With this dilution and subsequent higher dilutions binding decreased. Regeneration was not carried out in between each lysozyme concentration when building this calibration curve. The ligand was simply being saturated over time and saturation was reached at 0.5µg/ml. There is the possibility that some active ligand sites were blocked, but the R_{max} was reached as the following explains.

The R_{max} determines the capacity of the ligand. Here the R_{max} is 149RU. However, this is a rough estimate of the ligand capacity; the exact R_{max} is unattainable due to the nature of this work, as the ligand and analyte are not under complete control possibly due to active sites on the ligand being blocked or allosteric effects taking place. By taking the figures from Figure 5.16 and adding them a saturation of 154RU is calculated. This shows that the R_{max} has been reached and explains why no further binding takes place after 0.4µg/ml.

5.9.8 Thermo-Regeneration Using Lysozyme-Spiked Serum Samples

The regeneration in this experiment using serum samples (Table 5.7) was far more difficult compared to the regeneration of buffered lysozyme as shown in Figure 5.19. This work showed that the preparation of the serum samples was not adequate for the detection of lysozyme. Bacteria, remnants of bacteria or unknown factors were still present and attracted to both ligands. This resulted in the regeneration behaving very differently to other trials, in that higher temperatures were required.

A method which may improve the lysozyme measurement from serum was proposed by Hansen & Andersen (1973). Their work tested the quality of lysozyme from blood plasma and found that solublisation with *n*-butanol was the best method for releasing lysozyme for the measurement. One millilitre of *n*-butanol was added to the plasma and mixed vigorously for 30 minutes. The suspension was then centrifuged for 10 minutes at 3000g and the sample was then ready for use. Hansen & Andersen (1973) suggest that during the spinning of serum samples, without this pre-treatment there is destruction and fracturing of the cells which release lysozyme. This suggests that lysozyme itself may also be inactivated or destroyed. This method could be used with the lysozyme biosensor method and may increase the lysozyme content of the serum samples, leading to a more accurate measurement.

5.9.9 Validation Study

This study was carried out to establish whether there was any lysozyme present in the spiked serum samples. Two samples were assayed using the ELISA and also injected into the BIAcore for measurement. This study showed that no lysozyme was present after centrifugation. Possible reasons for this might include the lysozyme being broken down during this process, leading to sedimentation and ultimately no detection. A further reason might be that the bacteria, drugs and other plasma proteins in the sample may be breaking down and the fragments are then being separated into the working serum portion and being injected into the BIAcore and interfering with the ligand. Either of these reasons are plausible. The "Biacore Sensor Surface Handbook" (2003) recommends the use of salt in the running buffer to reduce non-specific electrostatic interactions; however, the aptamer buffer did contain salt and this did not solve the problem.

A limitation of the ELISA was that the anti-lysozyme aptamer was not used in this method. Future work may include the development of an aptamer based ELISA known as an Enzyme Linked Oligonucleotide Assay (ELONA). Serum samples in the ELISA were not subjected to spin filtering so any bacteria, drugs etc in the samples were shown not to be interfering with the ELISA results. Further work needs to be carried out to explore this aptamer based ELISA system.

The answer to the Lysozyme biosensor problem may lie not actually in filtering the serum further but in treating the serum sample prior to injection into the lysozyme biosensor. The "Biacore Sensor Surface Handbook" (2003) recommends "ligand mimics", which actively compete with the analyte in the serum for ligand on the sensor surface. This may limit the need for rigorous filtering of the serum and may therefore preserve the lysozyme in the serum.

5.9.10 Un-spun Serum Sample Trial

Figure 5.18 shows the response from injection of an un-spun serum sample. A serum sample was spiked with a known amount of lysozyme and immediately injected into the BIAcore. No centrifugation was carried out on this sample, so the response was purely due to the lysozyme and the other contaminants of the serum sample. The result was that the sensor could not be regenerated. As we know from previous experiments, a centrifuged spiked lysozyme sample can be regenerated and a buffered lysozyme sample can also be regenerated. This leads to the conclusion that the contaminants of the serum sample which are not being removed by the centrifugation are interfering with and binding to the ligand. This shows that the preparation method for the serum samples is not sufficient for injection into the BIAcore. A further method for the preparation of serum needs to be investigated.

5.9.11 Serum Sample Trial

Despite the problems with spiked serum samples, an initial experiment was carried out using 10 clinical samples, which were injected onto the lysozyme biosensor. The majority of the results produced from the BIAcore revealed lower amounts of lysozyme in the samples than those produced by the ELISA (Pearson r^2=0.0145). The conclusion here still stands, due to time constraints this was not addressed, but a new preparation method for the serum, which removes contaminants such as albumin and bacteria, but leaves lysozyme intact, needs to be established before this system can be used as part of routine clinical detection of lysozyme.

5.9.12 Lysozyme Biosensor Synopsis

The lysozyme biosensor has huge potential, but not enough time was available to explore this further. Once established, the biosensor would require very little training by an operator. A further advantage would be if the method was transferred to the T100 BIAcore, which could process more than 300 samples per run.

The only downfall of the BIAcore method is the initial cost of the system and the expense of the sensor chips which would be beyond the scope of a hospital. A way to overcome this problem is to use an inexpensive system such as the Spreeta (Texas Instruments, USA), which has small initial costs, is inexpensive to run and analyse samples. The lysozyme biosensor method could potentially be transferred over to the Spreeta (Appendix L) and would still produce a real-time detection system. However, there would be some loss of sensitivity with these simpler instruments. The only way the BIAcore system would be cost effective for a hospital is if it were to be used to analyse more than one clinical parameter.

Currently the lysozyme biosensor gives good results for lysozyme in buffer, where it can be successfully regenerated. Pearson r^2=0.5729 for lysozyme in buffer and clinical samples r^2=0.0145. Further development is needed on the serum preparation method. Serum contains proteins such as albumen and globulins with a MW66kDa. Serum from septic patients, also contain bacteria and prescription drugs which also have a high MW. Therefore spin filters (30kDa pore) should remove all albumen and bacteria from the serum, leaving free lysozyme to be detected. But this was not the case. Remnants of bacteria, drugs, plasma proteins, sedimentation of disturbed lysozyme, lysozyme sticking to albumen could all prevent suitable filtering and explain why lysozyme was not found in serum. The serum samples for the ELISA and BIAcore were all prepared in the same way, in that they were all centrifuged at the hospital and frozen for delivery. No spin filtering took place. A serum sample which undertook no spin filtering was injected into the BIAcore but coated the surface of the chip. A spin-filtered serum sample was also injected into the BIAcore, but no lysozyme could be detected. Therefore a method for removing these interfering particles, which leaves the lysozyme free and detectable, is needed.

A simple test to illuminate the problem of bacteria with the lysozyme would be to culture a spun and an unspun serum sample. This would elucidate one of the possible causes of lysozyme loss. Ideas for lysozyme extraction will be discussed in the Future Work section. This may then allow the lysozyme biosensor to detect lysozyme in serum samples and create a test with huge possibilities for future diagnosis of sepsis.

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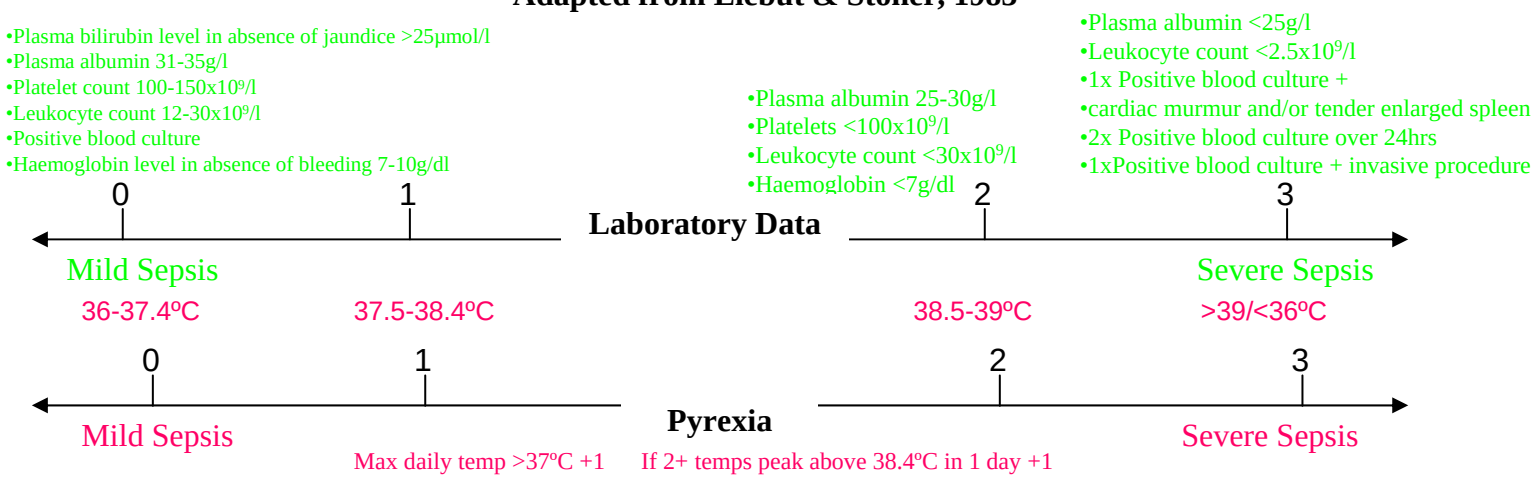
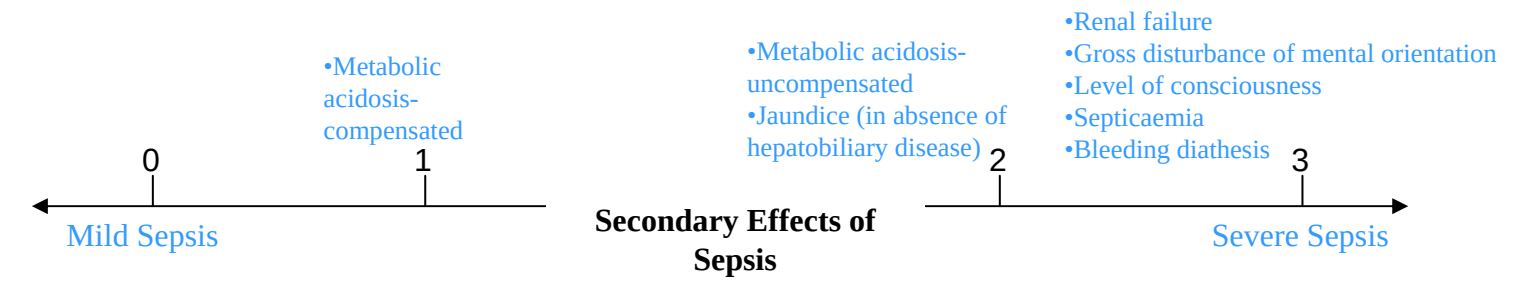
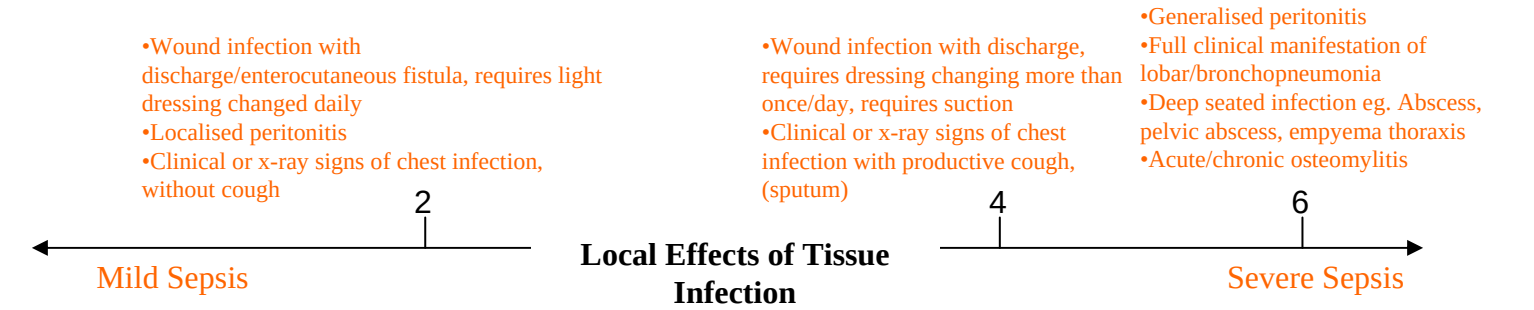
APPENDIX A -Sepsis Grading

Adapted from Elebut & Stoner, 1983







Appendix A

APPENDIX B - IgE BIAcore Protocol & Discussion of Results

Objective

This part of the project took place at the Universita Degli Studi Di Firenze in Italy. The main objective of this work was to produce a model system using Immunoglobulin E as the target using the BIAcore X. The working system would then be considered for future use for the determination of lysozyme using the BIAcore 3000 at Cranfield University. At the time of this work, the BIAcore X was malfunctioning so the IgE Spreeta work was carried out first, therefore several of the buffers used for the Spreeta work were also used in the BIAcore X method. These can be found in Appendix L.

Materials & Methods

Many of the materials used for the Spreeta experiments (Appendix L) were also used for the BIAcore IgE biosensor. Changes are shown below.

Sensor Chips

The Sensor chips used with the BIAcore experiments were CM5 (Research Grade) (Biacore, Sweden). These sensor chips were immobilized with Dextrin.

Control Ligand

The non specific DNA used for the control sensor chip with the Biacore was poly-T_57_B purchased from MWG (the IgE aptamer has 45 base pairs so the poly-T was a good comparison). No heat-treatment was required for the preparation of this.

Buffers

A buffer recommended for use with the BIAcore was used, this was HSP-EP buffer with added detergents (Tween 20). Spreeta buffer was also used with these experiments.

BIAcore X

The BIAcore flow rate for all injections was set at 5µl/min.

Before use the BIAcore was cleaned for 72hours with reverse osmosis water in continuous flow. The water was then changed for degassed Spreeta buffer. The cleaning sensor chip was undocked and refrigerated.

Sensor Chip Docking

After initial preparations were carried out the CM5 sensor chip was inserted into the BIAcore X and primed for 3 minutes.

Continuous flow of the buffer was carried out for a further 12 hours.

CM5 Activation

The CM5 sensor chip was activated using a NHS+EDAC solution. A volume of 55µl was injected at a flow rate of 5µl/min.

Streptavidin Immobilization

Streptavidin was prepared (200ppm) as of Appendix G and 50µl injected. A blocking solution of ethanolamine was used (35µl), to block unbound sites on the chip.

Control Ligand Immobilisation

On flow cell 1 a non specific DNA molecule was used. This flow cell became the control. The non specific DNA was poly-T (57 bases); this was injected for 20 minutes, (100µl) onto flow cell 1 only.

Aptamer Ligand Immobilisation

On flow cell 2 the anti-IgE aptamer was immobilized. Pre-treatment was carried out as with the Spreeta (Appendix L) and a 200ppm dilution was injected over 20minutes, (100µl).

Analyte Injection

All IgE antigen dilutions were prepared in the Spreeta buffer (Appendix L). The running buffer of the BIAcore X was the BIAcore Specific buffer HBS-EP, but was changed to the Spreeta buffer during the analyte injections.

Control Analyte

IgG was used as a control analyte and were prepared in the Spreeta buffer.

After injection of IgE & G sample, buffer was allowed to flow for several minutes to stabilize the signal.

Regeneration

The regeneration buffers included 1mM hydrochloric acid, 5mM hydrochloric acid, 10mM hydrochloric acid, and NaOH+EtOH 50%.

BIAcore IgE Results

Figure B1 shows the association between IgE and aptamer at various concentrations. Some concentrations could only be tested once due to the limited supply of the IgE antigen. However, the results from 30ppm-50ppm were in proportion with all other concentrations, even though they were only trialed once.

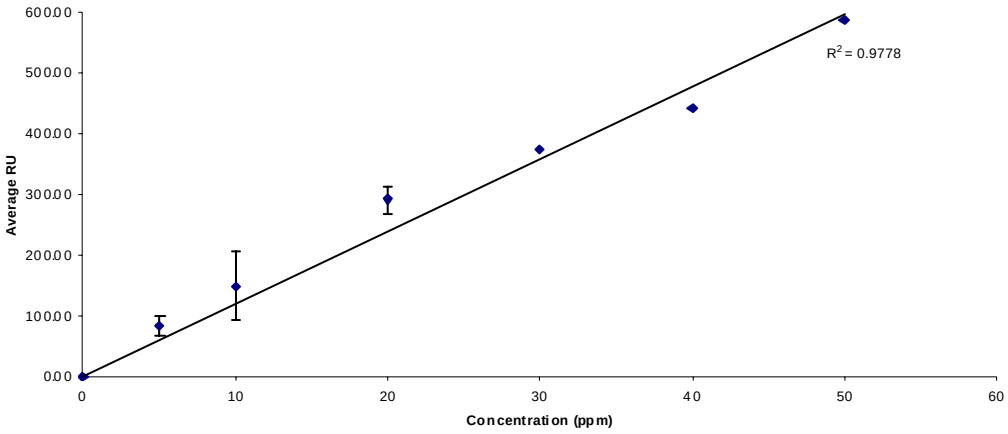


Figure B1 Shows the concentration of IgE and the respective RU value obtained.

Running Buffer

Initially the running buffer and the buffer used to make dilutions of IgE and G was not the same. All IgE and G dilutions were made using the spreeta buffer and the running buffer was the BIAcore specific HBS-EP. No signal could be seen with this, so the running buffer was changed to the spreeta buffer. The baseline increased but stabilized quickly and produced positive results.

Control Analyte

Double negative controls were carried out as seen in the raw data (Table 2) using IgG and flow cell 1 with the non specific poly-T immobilisation. Binding was only seen on flow cell 2 with IgG once, giving a value of 5.9 RU. However, the IgG 20ppm was trailed twice more shown on 21/07/04 giving negative results both times (Table 2). An IgG 50ppm was also injected onto flow cell 2 also showing no binding.

IgE Binding

Insignificant binding of IgE was seen on flow cell 1 (control). Where binding was seen it could be attributed to the IgE being from an old aliquot.

The IgE was purchased at the start of the experiments. All aliquots of this were made on the same day using fresh spreeta buffer and all were frozen at $-20^{o}C$ at the same time. However, specific dilutions were made at various times during the experiments. A new sample would therefore be the first dilution made from a fresh aliquot from the freezer and all dilutions there after would be considered old. Where an old sample is noted on the raw biacore results a significant decrease was seen in the binding of the IgE and aptamer results. This led to significantly large error bars on Figure B1, for 10ppm especially.

The only conclusion to be drawn from this is that the IgE is very unstable in the spreeta buffer. We can hypothesis that when the initial aliquots of IgE were made the freezing process stopped the denaturation of the IgE. However, upon defrosting denaturation began, thus resulting in low RU results as the aliquots aged. This meant that the concentrations of IgE were not reproducible unless fresh aliquots were used with every dilution.

This hypothesis is given credibility by the high dissociation rate as seen in Figure B2. Figure B2 shows the dissociation rate in seconds when the sample injection stops. The dissociation rate shows a decrease constant for all concentrations, with an R^2 value of 0.9778. Compared to the results seen in the paper by Liss *et al.* (2002) this dissociation is ~100 times faster than that seen in their experiments. This is perhaps due to the degradation of IgE which only temporarily binds to the aptamer then quickly released

leaving only a small amount securely bound. The RU value after the dissociation for 5ppm was less than 10, meaning that any lower concentrations would probably not have been detectable. Liss *et al.* (2002) reported a detectable limit of 100µg/l IgE were as the detection limit here is $5x10^3$µg/l; a considerable difference. However, their aptamer was used in solution and not immobilized to a solid surface, which could explain our aptamers disappointing performance when compared to the work by Liss *et al.* (2002). This degeneration of IgE was also seen in the regeneration phase. Hydrochloric acid 1, 5 and 10mM were all tried without success. A harsher solution of sodium chloride and ethanol was used to remove the IgE from the aptamer. If the IgE was broken down into fragments of immunoglobulins then some were remaining intact and binding securely, which therefore warranted a harsh regeneration solution.

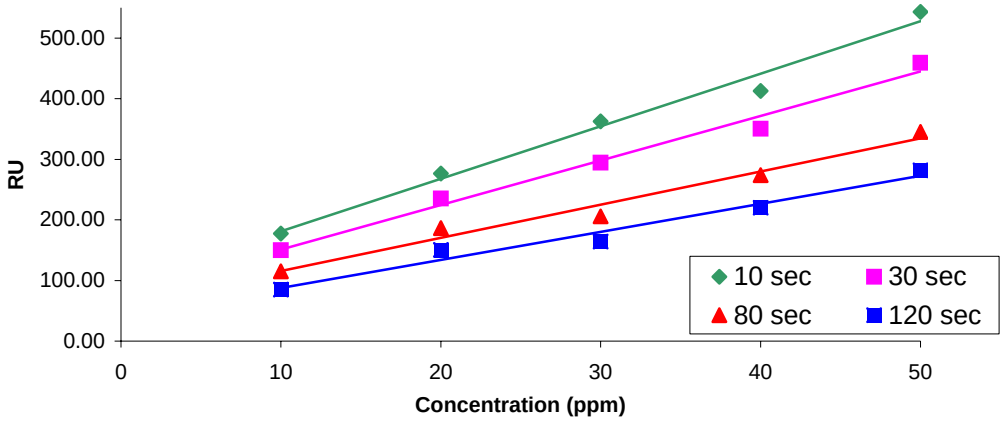


Figure B2 shows the dissociation for various IgE concentrations.

Due to this harsh regeneration solution the viability of the sensor chip surface was brought into question. After each regeneration phase the baseline would always be lower than the pre-IgE injection baseline. In conjunction, was the declining signal with increased use of the sensor chip. This may indicate that the regeneration solution is degrading or removing aptamers from the surface with increased use; thus limiting the life of the sensor.

APPENDIX B –IgE Raw BIAcore Results

Immobilization Phase								
			Plateau Result		Post Dissociation Result (RU)			
Date	Analyte	Dilution	Cell 1- Control	Cell 2- Aptamer	Cell 1- Control	Cell 2- Aptamer	Injection volume/time, if different from 75µl/15mins	Comments
14/07/2004	NHS+EDC				271.9	236.1	35/7	All dilutions made in Spreeta buffer. Running buffer was HBS-EP
	Streptavidin	200ppm			4555.2	4093.4	50/10	
	Ethanolamine				-37.7	-3.1	35/7	
	Poly-T	25µM			2004.7	-22.1	125/25	
	Aptamer				-112.3	1108.4	100/20	
IgE Testing Phase								
			Plateau Result		Post Dissociation Result (RU)			
Date	Analyte	Dilution	Cell 1- Control	Cell 2- Aptamer	Cell 1- Control	Cell 2- Aptamer	Injection volume/time, if different from 75µl/15mins	Comments
14/07/2004	IgE	0.1ppm			-34.6	-35		Dilutions made in Spreeta buffer, running buffer was HBS-EP.
15/07/2004	IgE	1ppm			-12.1	-6.9		Reg. HCl 1mM 4µl
	IgE	1ppm			-3	-6		Reg. HCl 1mM 4µl
	IgE	2ppm			-1.3	-4.1		Reg. HCl 1mM 4µl
	IgG	2ppm			1	-2.7		Reg. HCl 1mM 4µl
16/07/2004	IgE	5ppm			-2.8	2.4		Reg. HCl 1mM 4µl
	IgE	5ppm			-1.7	-1.5		Reg. HCl 1mM 4µl
	IgE	10ppm			-0.7	1.3		Reg. HCl 1mM 4µl
	IgE	20ppm			-6	6		Reg. HCl 1mM 4µl

	IgG	20ppm			9.3	5.9		Reg. HCl 1mM 4µl, 5mM 4µl, NaOH+EtOH 4µl
	IgE	10ppm	31.5	32.7	6.9	5.6		Reg. HCl 1mM 4µl, NaOH+EtOH 50% 4µl
19/07/2004	IgE	20ppm	34.9	340.5	-0.7	57.8		Reg. HCl 5mM 4µl, 10mM 4µl, NaOH+EtOH 50% 4µl
	IgE	30ppm	50405.3	405.3	-5.9	65.9		Reg. HCl 10mM 4µl, NaOH+EtOH 50% 4µl
	IgE	40ppm	7.4	467.4	-3.1	94.5		Reg.NaOH+EtOH 50% 4µl
						50		
	IgE	20ppm	32.5	309.1	0.6	60.5		Reg.NaOH+EtOH 50% 4µl
20/07/2004	IgE	10ppm	13.4	12.2	-0.2	-1.1		Old IgE used. Reg.5mM HCl 4µl, NaOH+EtOH 50% 4µl
	IgG	40ppm			0.9	-4.2		
	IgG	50ppm			10.3	-2.3		Old IgG used. Reg.NaOH+EtOH 50% 4µl
	IgG	50ppm			-12.6	-10.5		Old IgG used. Reg.NaOH+EtOH 50% 4µl
						10		
	IgE	10ppm	10.1	202.6	14.8	33.2	70/14	New IgE used. Reg.NaOH+EtOH 50% 4µl
	IgE	10ppm	-2.3	171.8	-13.9	18.8	70/14	New IgE used. Reg.NaOH+EtOH 50% 4µl
	IgE	5ppm	-10.5	86.8	-4.3	10.6	70/14	New IgE used. Reg.NaOH+EtOH 50% 4µl
	IgE	5ppm	-10.6	68.9	-0.8	8	70/14	New IgE used. Reg.NaOH+EtOH 50% 4µl
	IgE	10ppm	24.1	105.9	-0.7	7.5	70/14	Reg.NaOH+EtOH 50% 4µl
						5p		
	IgE	20ppm	-8.1	254.9	4.5	56.2	70/14	Reg.NaOH+EtOH 50% 4µl
	IgE	5ppm	-7.1	59.9	-3.7	14.4	70/14	Reg.NaOH+EtOH 50% 4µl
21/07/2004	IgE	10ppm	63.6	73.8	7.8	7.5	70/14	Reg.NaOH+EtOH 50% 4µl
	IgG	20ppm			-1.6	-6.2	70/14	/
	IgG	20ppm			0.6	-7.5	70/14	/
						50		

Appendix C- Ethical Approval

Swindon Research Ethics Committee (REC)
Kennet and North Wiltshire Primary Care Trust
Southgate House
Pans Lane
Devizes
Wiltshire
SN10 5EQ

Direct Line: 01380 733767
Fax: 01380 733779

GF kp SW 87/2003

22 April 2004

Mr Philip Burgess
Consultant Surgeon
Department of Surgery
Great Western Hospital
Swindon
Wilts
SN3 6BB

Dear Mr Burgess

SW 87/2003
Evaluation of a novel technique for detection of pyogenic sepsis based on bacterolytic properties of serum

At its meeting on 13 April 2004 Swindon Research Ethics Committee received your letter dated 7 March 2003 addressing the issue raised by the committee in respect of an adequate sample number which was noted. This study may now proceed.

Any changes or extensions to the protocol, or additional investigators, should be notified to the Committee for approval. Adverse events should also be reported to the Committee. May we remind you of the Data Protection Act 1998, and the need to conduct the trial in accordance with the Good Clinical Practice guidelines.

The Committee is required to audit progress of research and to produce a yearly report to the Avon Gloucestershire and Wiltshire Strategic Health Authority and Department of Health. You are therefore required to provide a brief yearly report and a short final report.

The Swindon Research Ethics Committee is fully compliant with the International Conference on Harmonisation/Good Clinical Practice (ICH) Guidelines for the Conduct of Trials Involving the Participation of Human Subjects and undertakes to adhere to the relevant clauses of the guidelines for clinical practice adopted by the European Union in January 1997.

Yours sincerely

Kirsten Peck

pp **Godfrey Fowler (Mr)**
Chairman
Swindon Research Ethics Committee

APPENDIX D – Collection of Normal Blood Samples

Analysis of Serum Samples from Healthy Donors to Establish a Reference Range for Lysozyme

Aim of Study -This study will be used to determine normal serum lysozyme levels. These can then be used as a reference range for clinical samples provided by the Great Western Hospital. This study will also validate the ELISA test currently used for blood samples.

Background -My project is involved with the development of a diagnostic test for lysozyme which can be used to indicate intra abdominal sepsis. There is currently no standard reference range for lysozyme which is universal across hospitals. This study will be used to determine the normal level for lysozyme in healthy individuals, to be used as a guide for clinical samples provided by the Great Western Hospital.

Samples Required -Human blood samples from healthy individuals are required. 20 volunteers will be recruited and blood samples taken by the campus nurse.

Exceptions -Samples will only be taken from those people who deem them selves well enough, who have no known infections or have had recent surgery.

Protocol

1. Volunteers will be recruited from within the Institute of Bioscience and Technology via verbal invitation.
2. An explanation of the study will be given and the volunteer will receive an information sheet and consent form to sign.
3. No personal information will be taken from the volunteers. Each sample taken will be given a unique lab number.
4. A blood sample of >10ml will be taken by the campus nurse.
5. The sample will be centrifuged and the serum extracted and stored at -20^{o}C. When all samples have been collected an ELISA will be carried out and the lysozyme level determined.
6. When the test is complete the volunteers will not be given the lysozyme level results.
7. All information collected from the samples will be stored in a locked filing cabinet and the samples put in long term storage at -80^{o}C.

You are being invited to take part in a research study. Before you sign up it is important that you understand why the research is being carried out and what will be expected of you.

Consumers for Ethics Research (CERES) publish a leaflet entitled Medical Research and You. A copy of this may be obtained from CERES PO BOX 1365, London N16 0BW if you require any further information regarding medical research.

Purpose of the Study -The purpose of this study is to establish a normal level of lysozyme in serum. Lysozyme is an enzyme in blood, released during infection. It is therefore a good indicator for Intra-abdominal Abscess. Intra-abdominal abscess causes multiple organ failure in severe cases with a mortality rate exceeding 90%. The current tests for lysozyme both lack reliability and sensitivity and are therefore not relied on in hospitals. Signs, symptoms, and white blood cell counts are used instead which are not conclusive. A need for a clinical test which provides a definite response for the diagnosis of sepsis is required. However, in order to do this a target needs to be set. This involves establishing the normal lysozyme range in disease free volunteers.

Do I have to take Part? -If you do decide to take part you will be asked to sign a consent form with the understanding that you are free to withdraw from participating at any point. You should not take part in this study if you have had had a surgical procedure or an infection recently.

What will be Required From You? -A 10ml blood sample will be taken by the medical centre nurse.

Possible Risks -You may suffer from discomfort while the blood is being taken and slight bruising in the region where the blood was taken from. If you are harmed during the procedure due to negligence then you may have grounds for legal action.

At the End of the Study -All samples will be destroyed at the end of the project and no information regarding results will be relayed to the volunteers.

Confidentiality -All personal information including any results gained will remain confidential. No results will be given you any volunteer.

Study Reviewers -The Cranfield Biomedical Ethics Committee has deemed this study fit for execution.

Contact for More Information -If you have any further questions, please contact:
n.j.lawton.s02@cranfield.ac.uk or s.morgan@cranfield.ac.uk

Volunteer Consent Form:

Analysis of Serum Samples from Healthy Donors to Establish a Reference Range for Lysozyme

Please initial all boxes:

1. I confirm that I have read and understood the information sheet for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time. ☐
3. I understand that I should not take part in the study if I have had a recent infection or surgery. ☐
4. I agree to take part in the above study. ☐

Name of Volunteer .....

Signature ..... Date .....

Name of Campus Nurse .....

Signature ..... Date .....

APPENDIX E –Blood Count Data

Sample No.	M/F	Age	Date	LZ µg/ml	Com. Kit ng/ml	Hb	Platelets	WBC	Neut %	Lym %	ESR	CRP	Diagnosis	Diag. Ref.
A29	M	51	-	5.58	0.0179	9.2	116	6.6	31	66		38	Leukaemia, cellulites, cerotic	A
A33	M	51	-	5.2	0.0148								Leukaemia, cellulites, cerotic	A
A34	M	51	-	5.5	0.0183								Leukaemia, cellulites, cerotic	A
D34	M	66	28/11	4.8	0	8.6	16	0.8	8.7	83.4		33	Leukaemia, no sepsis	A
D35	F	64	29/11	6.8	0	10.4	1248	28.2	87	5		132	Leukaemia, no sepsis, died	A
D4	M	90	30/11	10.3	0.55	8.3	778	89.7	15	14		26.1	Leukaemia, septic, died	A
D57	M	43	5/12	9.4	0	5.7	261	9	84	7		20	Chronic myeloid Leukaemia, non sepsis	A
N12	M	58	30/11	3.7	0.2	11.2	195	1.9	39	37			Leukaemia, non-Hodgkin's lymphoma	A
A11	M	79	13/9/04	8	0.0183	10.1	31	0.6	51	45		122	Died neutropenic, lymphopenic, pneumonic, leukaemia	Ab
A13	F	89	-	5.5	0.0183	12.4	373	10	60.6	26.8		70	Pneumonia	B
A15	M	95	-	9.2	0.0195	9.2	292	14.9	81	7.9			Pneumonia, septicaemia, died	B
A17	F	66	12/9/04	8	0.0195	12.1	209	9.9	65.5	18.4		105	Pneumonia, died	B
A2	M	67	11/9/04	3.9	0.0148	7.2	650	17.7	95	2.6			Pneumonia	B
A21	F	75	-	5.7	0.0195	7.2	26	1.5	61	34		62	Pneumonia, died	B
A23	M	95	-	6.3	0.0195								Pneumonia, septicaemia, died	B
A30	F	76	-	7.2	0.015	9.4	433	8.9	54	29		100	Septic pneumonia	B
A31	M	84	-	7.8	0.0195	9	443	14.8	92	5.9		194	Pneumonia, pseudo +ve, died	B
A32	F	75	-	5.7	0.0195								Pneumonia, died	B
A36	F	59	-	5.6	0.0195	8	621	11.7	74	16		153	Peripheral vascular disease, pneumonia, +ve dipthroid, died	B
A5	M	67	11/9/04	5.2	0.018	7.2	650	17.7	95	2.6			Pneumonia, septicaemia	B
C15	F	68	3/11	5.6	0	11.8	295	12.5	77	8.8		63.9	Died, stroke, pneumonia, bilary tumour	B
C20	F	72	8/11	4.5	0.25	11.7	422	11	76.5	13	51	71	Recovery from pneumonia, non septic	B
D1	F	90	4/12	5.6	0	10.6	336	7.6	86.1	7.7			Fractured neck of femur, pneumonia, died	B
D13	F	75	26/11	8.2	0	9.6	57	4.8	11.8	18.3		240	Pneumonia, died	B
D23	F	61	1/12	6.4	0	10.9	276	11.8	86	6.8			Infected hip, pneumonia	B
D28	F	61	6/12	7.5	0	6	144	11.9	91	4.3		55	Infected hip, pneumonia	B
D51	F	61	28/11	9.8	0	5	91	10	90	9			Infected hip, pneumonia	B

D55	F	75	27/11	20	0.5	8.6	33	4.6	22.6	20		240	Pneumonia, died	B
D58	F	87	6/12	12	0	6.4	163	9.7	81	12		41	Pneumonia, pulmonary embolism=septic.	B
D9	F	61	30/11	4	0	10.3	186	10	60	8			Infected hip, pneumonia	B
E13	M	98	11/1	7.1	0.22	13	141	7.9	72	14		106.4	pneumonia	B
F4	M	83	10/2	1.5	0.13	6.5	360	12.7	83	9.3			Pneumonia	B
N25	M	81	30/11	8	0.37	11.8	219	8.4	61	25			pneumonia	B
C6	F	61	5/11	8	0	8.3	203	7.1	61	29			Wound infection, staph +ve, pneumonia, septicaemia, CRF	Bde
A25	F	84	8/9/04	5.3	0.0148	11.1	345	4.4	56	36		1	Died, MRSA +ve, fractured neck femur, pneumonia	Be
C17	M	87	2/11	6.2	0	9.4	263	19.7	85	7		143.1	Died, chest infection, anaemia, pneumonia	Be
E14	F	25	11/1	6.2	0.24	12	267	9.5	75	18			No sepsis, pregnant	C
E27	F	23	11/1	6.9	0.29	13.7	278	9.4	70	21	3		No sepsis, pregnant	C
E7	F	34	11/1	5.5	1.94	11.7	214	5.7	60	31			No sepsis, pregnant	C
F14	F	28	10/2	5.2	0.28								No sepsis, Pregnant	C
A26	F	59	11/1/04	3.4	0.0196	11.3	456	14.4	84	6.5			C. renal failure, died	D
N9	F	80	30/11	5.3	0.42								CRF, no sepsis	D
F32	F	80	10/2	4.4	0.15	9.5	287	4.9	75	19			+ve blood culture, CRF, myeloma	DG
A1	F	56	-	3.8	0.015	13.4	194	4.1	90.6	8.6		47	Sepsis URTI, UTI	E
A18	M	33	9/9/04	5.8	0.0075	13	271	15	63	21	16	50	Septic arthritis, knee trauma, no sepsis	E
A27	F	90	-	5.7	0.0183	12.4	278	8.8	66	19			Infected hip prosthesis, died	E
A3	M	48	11/9/04	3.8	0.0148	8.5	306	13.4	52	22		104	GM-ve septic, diabetic	E
A35	M	48	8/9/04	5.58	0.015	11.6	87	13.7	89	11			GM-ve septic, diabetic	E
A4	M	48	9/9/04	3.1	0.017	11.6	87	13.7	89	11			GM-ve septic, diabetic	E
A6	M	55	13/9/04	5.6	0.015	14.9	254	11.3	77	14			+ve lactose fermenting, coli forms in urine	E
A7	M	78	-	6.32	0.0168	9.8	367	6.6	58.7	24.3		48	Pretibial abscess Ostiomylitis	E
A8	F	48	10/9/04	3.5	0.015	13.4	261	10.0	66	27.7		20	Groin abscess, diabetic +ve culture	E
A9	F	66	14/9/04	5.5	0.0195	6.7	162	14.9	85	6.3		80.1	Died, septic, +ve staph, septicaemia, MRSA	E
C12	F	50	2/11	5.2	0	11.8	740	13.4	72	19			Wound infection, MRSA, UTI, sepsis	E
C13	F	77	2/11	4.7	0	7	255	16.4	81	11		348.5	Chemotherapy , UTI sepsis	E

C2	F	55	4/11	4.5	0	13	239	7	72	17		22.4	Wound infection, β-haemolytic strep +ve, abscess on foot	E
C3	F	56	5/11	6	0	13.6	193	18.2	86	6.3	14	229.6	Died, staph in line, infected elbow, septicaemia	E
C7	M	42	5/11	4.6	0	14.5	282	11	70	22	4	11	+ve staph culture, septic arthritis	E
D12	F	61	3/12	3.7	0	9.4	383	16.3	87	8.4		48	Infected Hip, septicaemia	E
D14	F	64	6/12	3.8	0	11	158	6.2	89	1.6		3.7	Sepsis, pneumonia, died	E
D15	M	40	5/12	5.6	0	9.2	462	11.8	76	14		44.8	Inflammation of testes, +ve coli form	E
D18	F	75	6/12	5.7	0	8.3	218	16.7	17	12		127	Coli forms in sputum, heart failure, chest infection, died	E
D20	F	88	2/12	5.6	0	10.8	520	8.1	73	16		131	UTI, coli forms in urine, died	E
D21	F	75	20/11	7.2	0	7.4	209	12.5	68	14		66	Coli forms in sputum, heart failure, chest infection, died	E
D22	M	64	5/12	9.2	0	13	165	30	81	7		63	Wound & chest infection, septic	E
D29	F	77	4/12	8.8	0	10.5	361	17.4	78	12			MRSA, septic	E
D3	F	29	5/12	5.7	0	13.6	242	12.8	79	15			UTI, coli form in urine	E
D30	M	89	6/12	8.9	0	13	415	19	87	5		125	C-difficile infection in bowel	E
D32	F	64	5/12	5.5	0.33	12.2	162	6.1	88	2		307	Sepsis, pneumonia, died	E
D37	F	75	1/12	5.3	0	7.8	241	28.2	75	10.6			Coli forms in sputum, heart failure, chest infection, died	E
D41	F	60	26/11	7.5	0	5.2	195	7.9	75	19		13	Cyst abscess	E
D46	F	32	6/12	8.4	0	6.2	384	16.2	70.6	15		11.7	Pelvic inflame disease, UTI	E
D47	M	72	30/11	6.6	0.55	6.2	697	15	82	8.9		93	Staph. in urine, N/S	E
D49	F	58	3/12	7.6	0	6.8	334	12.7	74	14		194.8	Obstructed renal system, UTI	E
D5	M	25	1/12	4.5	0	15.2	198	11.4	58	27			Renal colic, UTI	E
D53	M	64	6/12	13	0	6.5	112	22.7	87	3.5			Wound & chest infection, sepsis	E
D56	M	85	26/11	10.4	0	4.8	59	13	70	11		181	C-difficile, septic	E
D8	M	65	29/11	4.6	0	11.2	397	8.7	77	14		14	Peritoneal abscess	E
E26	F	77	11/1	7.2	0.18	11.8	262	6.5	80	14			THR, UTI, +ve blood cultures	E
F1	M	67	11/2	4.8	0.14	6.8	52	16.5	86	7			Died, septicaemia, sepsis, MRSA, CRF	E
C4	F	54	3/11	5.6	0	12.9	419	8.8	64	22		21.9	Rectal cancer, sepsis, neutropenic	Eg
A28	F	59	-	6.4	0.0168								No sepsis	F
A10	F	76	10/9/04	2.6	0.0199	9.4		8.9	54	29		100	epilepsy	F
A14	F	53	13/9/04	3.7	0.0143								No sepsis	F

A19	F	83	13/9/04	3.5	0.0179	12.3	154	18.1	89	4.5		205	Heart failure, died	F
A20	M	84	-	3.8	0.0195	8.8	346	18.5	91	6		198	Heart failure, died	F
A22	M	62	7/9/04	6.3	0.0112	13.8	200	5.3	50	34		42	No Sepsis, died	F
A24	M	33	7/9/04	6.4	0.0183	15	369	11.2	54	33			Non septic	F
C10	M	61	5/11	5.5	0	10.3	526	7.1	63	23	32	14.7	Out patient, no sepsis	F
C11	F	41	5/11	4.4	0.22	16.3	321	5.4	61	27.8		2.2	Gall stones, no sepsis	F
C14	F	79	2/11	4.8	0	7.9	699	15.1	74	12		37.2	Asthmatic, anaemic, no sepsis	F
C18	F	45	3/11	4.4	0	10.9	52	5.1	57	32			Non septic	F
C21	F	69	2/11	4.8	0	14.8	202	5.9	56	33.8			Non septic	F
C23	M	61	8/11	4.2	0	9.2	31	33.9	59	22		186.3	Head ache, non septic	F
C8	F	74	5/11	4.7	0.13	13.2	306	8.6	87	24			No sepsis	F
D10	M	75	1/12	5.7	0	13.7	266	41	90	3		179	Perforated gall bladder, died	F
D11	M	73	6/12	3.8	0	10.3	130	7	81.8	9.8		17	Diabetic, leg ulcer, non septic	F
D16	M	87	28/11	5	0	9.8	387	8	62	23		57	Non septic	F
D17	M	87	2/12	4.6	0	9.8	384	7.2	64	20			Non septic	F
D26	M	32	6/12	6.4	0	11.8	295	7	60	28	18	4	Non septic	F
D27	M	87	26/11	6.8	0	8.3	289	8.5	71	16.2		57	Non septic	F
D33	F	75	3/12	4.6	0	9.6	249	25	71	15			Heart failure, died	F
D36	F	17	26/11	5.5	0	8.5	458	10	67	24			Over dose, no sepsis	F
D38	M	78	5/12	6	0	12	350	10	81	9		101	Non sepsis, died	F
D42	F	30	28/11	7.4	0	5.5	345	10	62	27		0.5	Non septic	F
D44	F	58	2/12	8.2	0	6.8	360	14.7	83	11			anaemia	F
D45	F	58	6/12	8.7	0	7	457	10	62	21			anaemia	F
D48	F	66	6/12	8.4	0	6.2	267	6.8	50.9	37		36.4	Chrones disease, N/S	F
D50	M	84	26/11	9.2	1.1	8.6	432	24.5	80	6.1		115	MI, died	F
D7	F	80	29/11	3.1	0	15.4	159	40.4	96	3			Trauma, no infection, died	F
E1	F	79	11/1	5.7	1.28	11.5		12.1					No sepsis	F
E10	M	51	11/1	6.4	0								No sepsis, diabetic	F
E11	M	35	11/1	5.2	0								No sepsis	F
E12	F	56	11/1	6.6	0	13.9	253	6	69	18			No sepsis	F
E15	F	80	10/1	5.7	0.38								No sepsis, varicose ulcer	F

E16	F	36	11/1	6.3	0.24								No sepsis, diabetic	F
E17	F	49	11/1	5.3	0.3	11	196	5.1	84	10		6.3	No sepsis, chrone's disease	F
E19	M	75	11/1	6.3	0.25								No sepsis	F
E2	F	69	11/1	5.8	1.2								No sepsis	F
E20	F	91	10/1	7.1	0.23	9.3	179	3.5	46	43			No sepsis	F
E21	M	84	11/1	4.6	0.23								No sepsis	F
E22	F	84	10/1	6	0.24								No sepsis, abdo pain	F
E23	F	33	11/1	6.6	0.3	11	356	10	82	12			No sepsis	F
E24	F	37	10/1	6.9	0.24								No sepsis	F
E28	M	82	11/1	7.2	0.3								No sepsis	F
E29	M	48	11/1	6.2	0.3	15	197	7.5	45	40			No sepsis, chest pain	F
E3	M	55	11/1	6	0.82								No sepsis	F
E30	M	72	11/1	6.5	0.25						3	0.5	No sepsis	F
E5	M	43	11/1	6.9	0.92	14.2	358	11.5	60	19			No sepsis	F
E8	F	77	11/1	8	1.47						4	2.4	No sepsis	F
E9	F	40	11/1	6.7	0.56	11.4	184	6.3	74	16			No sepsis	F
F10	F	81	10/2	5.8	0.22	12.7	292	7.5	74	15	11	2.8	+ve blood culture, no sepsis	F
F12	M	65	10/2	5.2	0.3								No sepsis	F
F13	M	72	10/2	7.6	0.35	12.7	201	7.2	76	14			No sepsis	F
F15	M	82	10/2	5.2	0.2	11	245	6	58	24		4	No sepsis	F
F16	M	65	10/2	4.6	0.3	14.5	328	8.8	57	30	5		No sepsis	F
F17	M	61	10/2	5.5	0.3	12.1	241	7.5	62	28			No sepsis	F
F18	F	68	10/2	5.4	0.3								No sepsis	F
F19	F	61	10/2	5.7	0.25	12.7	240	7	65	25			No sepsis	F
F2	M	55	10/2	5	0.3	15.4	207	7	52	35			Diabetic, no sepsis	F
F21	M	73	10/2	6.2	0.16								Diabetic, no sepsis	F
F23	F	66	10/2	6.3	0.25								No sepsis	F
F24	M	33	10/2	5.6	0.15								Diabetic, no sepsis	F
F25	M	65	10/2	6.5	0.22	14.2	254	7	52	34			No sepsis, chron's disease	F
F26	F	70	10/2	6.2	0.29	12.4	298	6.8	63	27			no sepsis	F
F27	M	49	11/2	6.2	0.2	11.1	347	7.5	38	45			Chest pain, no sepsis	F

F28	F	64	10/2	5.7	0.17	10.6	265	9.3	74	17			THR, no sepsis	F
F29	F	52	10/2	5.7	0.22								No sepsis	F
F3	M	34	10/2	4.7	0.12								No sepsis	F
F30	M	54	10/2	5.68	0.23								No sepsis	F
F31	M	72	10/2	6.4	0.24								Diabetic, No sepsis	F
F33	M	33	10/2	6.1	0.23	14.5	255	6.8	77	14	9		No sepsis	F
F34	M	56	10/2	5.9	0.25	12.7	295	5.6	61	26			No sepsis	F
F35	F	50	10/2	5.7	0.22								No sepsis	F
F36	F	54	10/2	5.7	0.24								Diabetic, No sepsis	F
F37	M	57	10/2	5.9	0.3	14.5	195	5.9	61	27			No sepsis	F
F38	M	42	9/2	5.7	0.29								Diabetic, No sepsis	F
F39	F	66	10/2	6.6	0.22								No sepsis	F
F40	F	53	10/2	6.1	0.25	12.8	259	6.5	72	16		18.6	No sepsis	F
F5	F	54	10/2	5.4	0.12								No sepsis	F
F6	F	68	10/2	4.9	0.18								No sepsis	F
F7	M	40	10/2	5.2	0.1								No sepsis	F
F8	F	62	10/2	5.1	0.24								No sepsis	F
F9	M	78	11/2	1.4	0.12	9.7	84	5.3	70.9	12.4		17	Non septic, died	F
N1	M	60	30/11	4	0.42	13.9	208	5.8	62	26			Non septic	F
N11	M	30	30/11	4.5	0.22	15.8	219	12.7	69	20	12		Seizures, no sepsis	F
N13	F	65	30/11	4.5	0.42	12.4	267	8.8	50	39			Gynaecological, non sepsis	F
N14	M	70	30/11	3.4	0.22	13.9	201	8.5	48	35		3.3	Non septic	F
N15	M	69	1/12	4.2	0.2								Non septic	F
N16	F	51	30/11	4.6	0.29								Non septic	F
N18	M	70	30/11	4.7	0.2								Non septic	F
N19	M	69	30/11	5.5	0.22								Non septic	F
N2	F	41	30/11	5	0.28								Non septic, high cholesterol	F
N20	F	79	1/12	6.2	0.35								gout	F
N21	M	60	30/11	6.9	0.3	13	326	10	62	27	33	12.4	Divaticulitis, inflammation of the colon, non sepsis	F
N23	M	57	30/11	7.7	0.42	15.1	242	5.8	55	35			GP referral , Non septic	F
N26	M	71	30/11	8	0.15	9.8	308	3.6	74.3	17		16.9	Chest complaint, Vegas syndrome, no	F

													sepsis	
N27	M	69	30/11	7.5	0.25								Non septic	F
N28	M	63	30/11	6.6	0.2								Non septic	F
N29	M	51	30/11	8.2	0.16	15.5	243	5.7	53	35			GP referral, normal	F
N3	F	47	30/11	3.9	0.29	12.7	233	4.5	65	17		6.1	Liver disease	F
N30	F	43	30/11	7.1	0.16	13.1	421	12.4	64	20	8	1.5	Non septic	F
N4	F	70	30/11	4.5	0.2	14.4	198	5.5	56	31	3		Non septic	F
N5	F	62	30/11	4.6	0.03	13.9	164	7.8	71	21			Non septic	F
N6	F	30	30/11	5.4	0.35	11.8	134	10.3	76	14.7		69	Non septic	F
N7	M	35	30/11	5.2	0.69								Non septic, GP referral	F
N8	F	91	30/11	5.2	0.36								died	F
V1	F	26	Dec 04	3.7	0.15								Healthy	F
V2	M	25	Dec 04	2	0.15								Healthy	F
V3	F	42	Dec 04	3.8	0.16								Healthy	F
V4	F	28	Dec 04	4.6	0.15								Healthy	F
V5	F	26	Dec 04	5.8	0.18								Healthy	F
V6	M	27	Dec 04	5.7	0.15								Healthy	F
VN1	M	27	Aug 05	3.7									Healthy	F
VN2	F	26	Aug 05	4.4									Healthy	F
VN3	F	29	Aug 05	3.8									Healthy	F
VN4	M	31	Aug 05	5.4									Healthy	F
VN5	F	40	Aug 05	5.4									Healthy	F
VN6	F	45	Aug 05	5.4									Healthy	F
VN7	F	40	Aug 05	3.8									Healthy	F
VN8	M	37	Aug 05	3.7									Healthy	F
D6	F	51	-	5.2	0								Appendicitis, Non septic, abdo pain	Fh
E4	F	33	11/1	5.4	1.26	12.3	391	13.2	79	15			No sepsis, pregnant	FC
N24	F	32	30/11	7.4	0.24	11.9	231	8.3	60	30.3	7		pregnant	FC
C9	F	81	4/11	4.5	0.18	12.5	343	8.6	43	29			Renal cancer removal, pre-op	G
D19	M	82	26/11	4.6	0	10.9	337	19.6	89	3		9	Bladder tumour	G
F11	F	68	10/2	5.2	0.12	13.3	420	12.9	81	14	48		Breast cancer, No sepsis	G

F20	M	75	10/2	6.2	0.25	11.1	434	5.2	62	25			Tumour, chemo, No sepsis	G
N10	M	71	30/11	6.3	0.4	11.8	175	4.7	60	24			Chemotherapy, colon tumour, no sepsis	G
N17	M	53	30/11	4.6	0.25	14	178	10	90	6.7			Lymphoma patient	G
N22	M	50	30/11	7.4	0.13	13	201	4.3	66	20	5		Lymphoma, Hodgkin's disease, no sepsis	G
D52	F	64	2/12	9.6	0	4.4	358	8.7	76	14	40	9	Pancreatic tumour, stroke, chest infection, died	Ge
A12	F	13	10/9/04	5.2	0.0183								NS abdo pain	H
C1	F	45	7/11	5.2	0	13.9	271	9.7	86	7		119.2	Abdo pain	H
C16	M	30	4/11	4.6	0	13.4	219	4.9	49	35		6.9	Abdo pain	H
C5	F	20	4/11	5.3	0	13.4	381	12.3	75	15			Ovarian cyst, abdo pain	H
D25	M	22	5/12	6.8	0	13	203	5	6	23		41	Abdo pain	H
D31	F	48	28/11	5.8	0	10.6	348	5	65	21			N/S abdo pain	H
D39	F	44	6/12	5.7	0	11	501	16.9	83	9.2		175	N/S abdo pain	H
D40	F	43	3/12	5.4	0	5.2	494	27.3	89	4.4			N/S abdo pain	H
D43	F	58	4/12	7.4	0.15	7	428	8	64	20		107	Abdo pain	H
D54	F	78	23/11	9.8	0	4.9	292	5	49	35			N/S abdo pain, GP results	H
E18	M	53	11/1	6.5	0.2	13	237	12	87	7		0.3	Abdo pain, No sepsis	H
E25	F	26	11/1	6.5	0.24	13.2	397	9.8	60	29		9.8	No sepsis, abdo pain	H
E6	F	63	11/1	5.6	0.98	14.1	236	7.1	57	33	69	23	Abdo pain, No sepsis	H
F22	F	43	10/2	6.9	0.25	12.7	333	9.8	55	35	16	5	Abdo pain	H
A16	F	46	11/9/04	7.7	0.015	12.9	294	19.2	92	5		47	Perf. Appendix	I
C19	M	16	4/11	4.6	0.35	13.7	296	16.7	79	7.4		258.6	Gangrenous appendix	I
C22	F	42	5/11	5.3	0	8.9	233	9.8	47	48		38.4	Wound infection, staphylococcus +ve, appendectomy	I
D2	M	17	3/12	5	0	16.2	241	17.4	76	11		66	Acute gangrenous appendix	I
D24	F	45	29/11	6.6	0	13	312	17	79	8		258	Appendectomy, +ve wound culture	I

*Diagnosis Reference =A-leukaemia, B-pneumonia, C-pregnant, D-CRF, E-septicaemia, MRSA, UTI, URTI,
F-no sepsis, C. difficile, G-tumour, H-abdo pain, I-appendicitis

Appendix F Concentration Comparison Assays

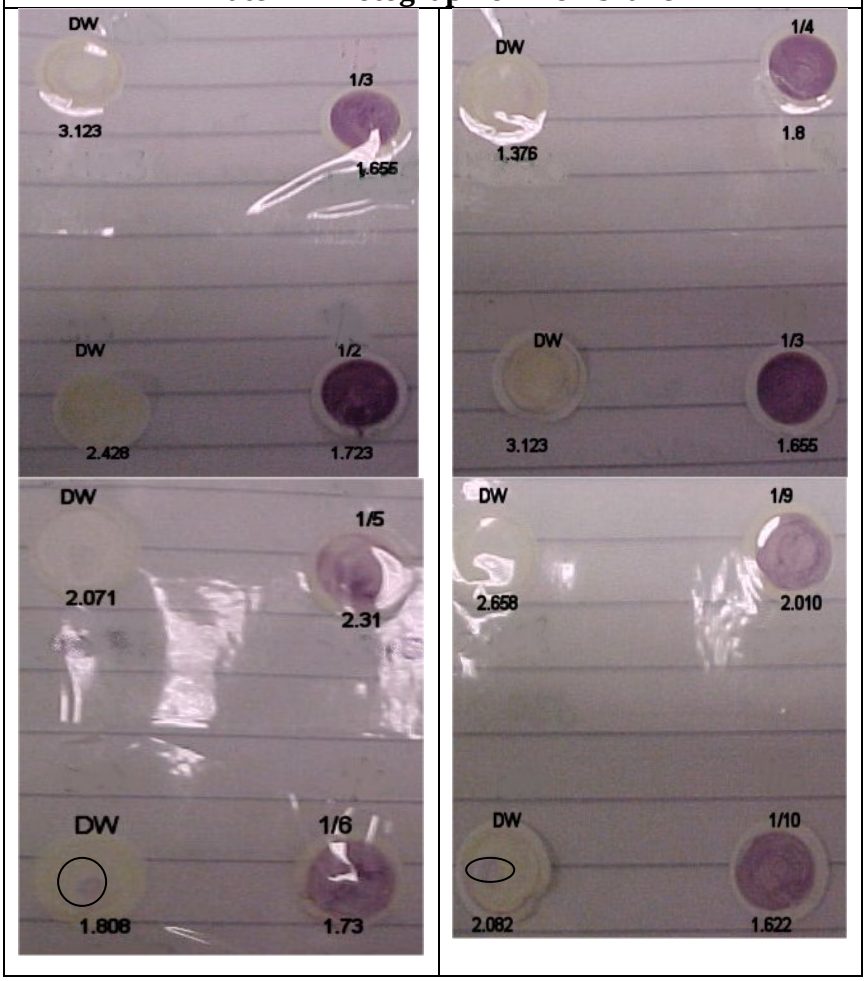
Membrane Descriptions from Plate F1

The discolouration on ½ was far greater than on sample $^1/_3$, as expected. The OD of ½ was also higher than the filtrate of $^1/_3$. Neither control membrane shown as discolouration, and both filtrate OD's were greater than their respective samples. The control OD's were very different to each other.

The discolouration on $^1/_5$ is slightly lighter then on $^1/_6$ membrane. The control membrane for $^1/_6$ has a small amount of binding present as highlighted. The control filtrate OD for was higher than the $^1/_6$ filtrate OD. However, the OD of the control for $^1/_5$ was not greater than $^1/_5$.

Plates F1 Comparison of membrane discolouration for several bacteria samples, showing. The OD for each sample is underneath the membrane. The concentration of each sample is above each membrane.

Plate F1 Photograph of Membrane



The discolouration on $^{1}/_{3}$ is clearly darker than the ¼ membrane. No discolouration was seen on either the controls, and only the $^{1}/_{3}$ control OD was greater than the sample.
There is a slight difference between the discolouration on $^{1}/_{9}$ and $^{1}/_{10}$ with $^{1}/_{10}$ being slightly darker. The control for $^{1}/_{10}$ also showed a small amount of binding as highlighted above. Both control filtrate OD's were greater than the sample OD.

APPENDIX G -Solution Recipe Index

Table G1 -ELISA Solutions

Coating Buffer	Tris (Hydroximethyl) aminomethone (Fisher, UK) 25mM pH8 dissolved in Reverse Osmosis (RO) water.
Wash Buffer	0.1% BSA, 0.05% Tween 20 solution in Tris pH8 25mM.
Blocking Agent	250µl of a 1% Bovine Serum Albumin (BSA grade V, Sigma-Aldrich Ltd, UK) and Tris pH 8 25mM
TMB cocktail	1:1 dilution of TMB (3,3',5,' – Tetramthylbenzidine), (Sigma-Aldrich, UK) containing chromogen & hydrogen peroxide with Tris pH8 25mM

Table G2 -ELIFA Solutions

Wash Solution	Tris pH7.5 25mM, 0.1% BSA and 0.05% Tween 20
Blocking Agent	1% Casein in Tris pH7.5 25mM
Coating Buffer	Tris (Hydroximethyl) aminomethone (Fisher, UK) 25mM pH8 dissolved in Reverse Osmosis (RO) water.
WBTC	Tris pH7.5 25mM, 0.1% casein, 0.05% Tween 20 and 1% BSA.

Table G3 -Mini-ELIFA Solutions

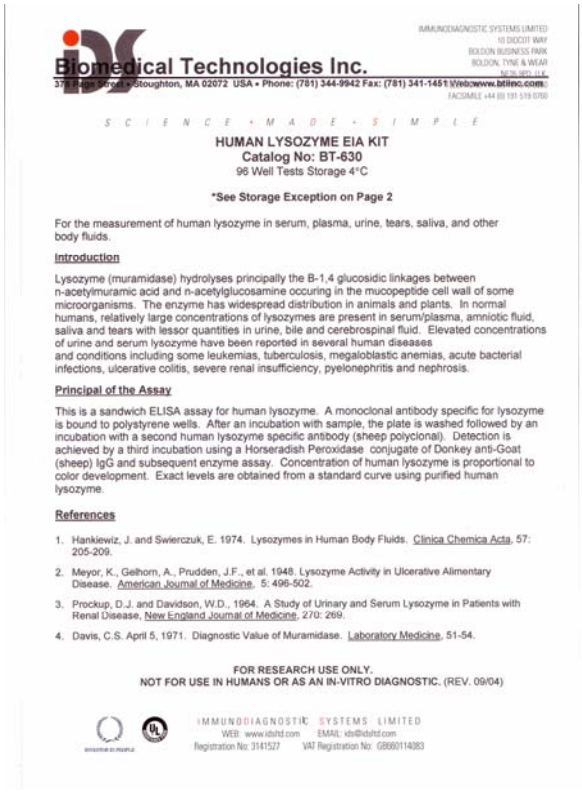
Wash Solution	Tris pH7.5 25mM, 0.1% BSA and 0.05% Tween 20
Blocking Agent	1% Casein in Tris pH7.5 25mM
Coating Buffer	Tris (Hydroximethyl) aminomethone (Fisher, UK) 25mM pH8 dissolved in Reverse Osmosis (RO) water.
WBTC	Tris pH7.5 25mM, 0.1% casein, 0.05% Tween 20 and 1% BSA.
Liquid Broth	0.1% Bacto Tryptone (The microbiological Supply Company, UK), 0.05% Bacto Yeast Extract (Oxoid, UK) and 0.05% Sodium Chloride (Fisher, UK) in 1 litre reverse osmosis (RO) water.
Agar	15g/litre of agar (The microbiological Supply Company, UK) added to Liquid Broth *E. coli* JM83 K12 derivative was used from the laboratory stock. A loop of *E. coli* from the laboratory stock was incubated and shaken in 50ml LB broth for 24 hours at 37°C. Cotton wool was placed inside the neck of the conical flask and silver foil and autoclave tape were used to seal the flask during incubation. The contents of the conical flask were then transferred to a centrifuge tube and spun for 10 minutes at 14000g-force. The supernatant was poured off and disposed of and replaced by an equal volume of PBS. The tube was vortexed to mix and resuspend the pellet.

Table G4 -BIAcore Solutions

Aptamer buffer	Tris 20mM pH 7.5, 10mM Sodium Chloride and 5mM Magnesium Chloride (Sigma-Aldrich Ltd, UK).
Poly-T Buffer	10mM Tris pH 7.5 and 1mM Ethylenediamiretracetic Acid 99% (EDTA) (Sigma-Aldrich Ltd, UK).
Anti-lysozyme	Tris 20mM pH 7.5, 100mM NaCl and 5mM $MgCl_2$ (Fisher, UK).

aptamer buffer	
Amine Coupling Kit	N-hydroxysuccinimide (≥97% purity) and 1-Ethyl-3-(3-Dimethylamin propyl) Carbondiimide (Biacore, UK)
Streptomyces avidinii	Stock solution of *Streptavidin avidinii* diluted in acetate buffer 10mM pH5
Ethanolamine	1M pH8.6 (Biacore, UK)
Preconditioning Solution	50mM Sodium Hydroxide and 1M Sodium Chloride (Fisher, UK)
Regeneration Solutions	0.1-1M Sodium Citrate (Sigma-Aldrich Ltd, UK); 1, 5 and 10mM Hydrochloric Acid (Fisher, UK); 1M Magnesium Chloride (Sigma-Aldrich Ltd, UK) and a 50% solution of 2.5% Ethanol with 25mM Sodium Hydroxide (Fisher, UK)
Biotin	1mM 10µl dilution on Poly-T buffer

APPENDIX H –Commercial Kit Instructions



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***See Storage Exception on Page 2**

For the measurement of human lysozyme in serum, plasma, urine, tears, saliva, and other body fluids.

Introduction

Lysozyme (muramidase) hydrolyses principally the B-1,4 glucosidic linkages between n-acetylmuramic acid and n-acetylglucosamine occuring in the mucopeptide cell wall of some microorganisms. The enzyme has widespread distribution in animals and plants. In normal humans, relatively large concentrations of lysozymes are present in serum/plasma, amniotic fluid, saliva and tears with lessor quantities in urine, bile and cerebrospinal fluid. Elevated concentrations of urine and serum lysozyme have been reported in several human diseases and conditions including some leukemias, tuberculosis, megaloblastic anemias, acute bacterial infections, ulcerative colitis, severe renal insufficiency, pyelonephritis and nephrosis.

Principal of the Assay

This is a sandwich ELISA assay for human lysozyme. A monoclonal antibody specific for lysozyme is bound to polystyrene wells. After an incubation with sample, the plate is washed followed by an incubation with a second human lysozyme specific antibody (sheep polyclonal). Detection is achieved by a third incubation using a Horseradish Peroxidase conjugate of Donkey anti-Goat (sheep) IgG and subsequent enzyme assay. Concentration of human lysozyme is proportional to color development. Exact levels are obtained from a standard curve using purified human lysozyme.

References

1. Hankiewiz, J. and Swierczuk, E. 1974. Lysozymes in Human Body Fluids. Clinica Chemica Acta, 57: 205-209.
2. Meyor, K., Gelhorn, A., Prudden, J.F., et al. 1948. Lysozyme Activity in Ulcerative Alimentary Disease. American Journal of Medicine, 5: 496-502.
3. Prockup, D.J. and Davidson, W.D., 1964. A Study of Urinary and Serum Lysozyme in Patients with Renal Disease, New England Journal of Medicine, 270: 269.
4. Davis, C.S. April 5, 1971. Diagnostic Value of Muramidase. Laboratory Medicine, 51-54.

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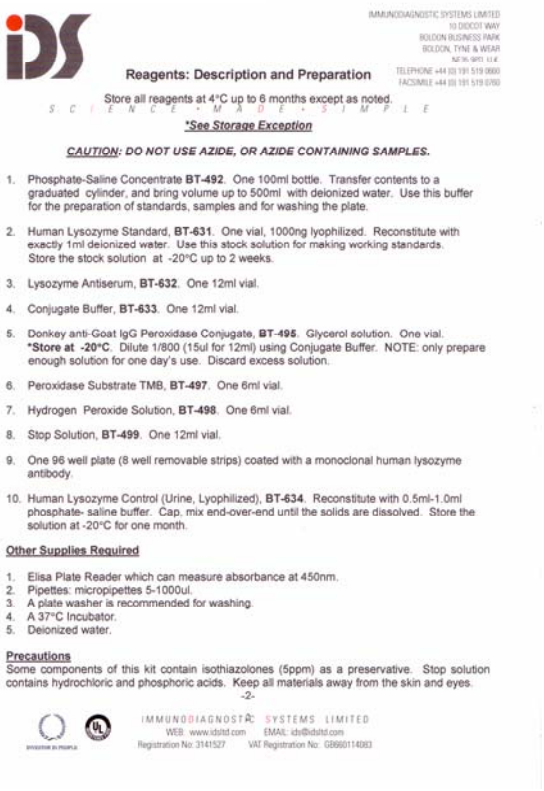
NOT FOR USE IN HUMANS OR AS AN IN-VITRO DIAGNOSTIC. (REV. 09/04)



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Reagents: Description and Preparation

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Store all reagents at 4°C up to 6 months except as noted.
See Storage Exception

CAUTION: DO NOT USE AZIDE, OR AZIDE CONTAINING SAMPLES.

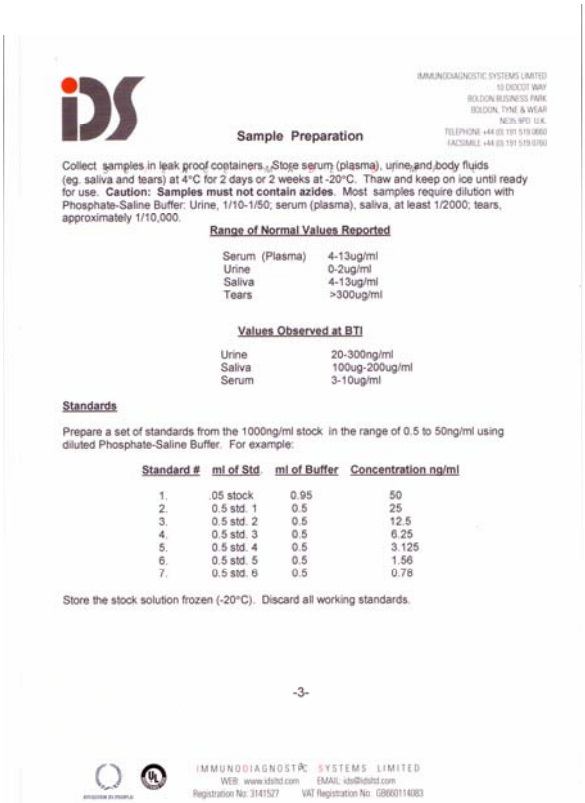
1. Phosphate-Saline Concentrate **BT-492**. One 100ml bottle. Transfer contents to a graduated cylinder, and bring volume up to 500ml with deionized water. Use this buffer for the preparation of standards, samples and for washing the plate.
2. Human Lysozyme Standard, **BT-631**. One vial, 1000ng lyophilized. Reconstitute with exactly 1ml deionized water. Use this stock solution for making working standards. Store the stock solution at -20°C up to 2 weeks.
3. Lysozyme Antiserum, **BT-632**. One 12ml vial.
4. Conjugate Buffer, **BT-633**. One 12ml vial.
5. Donkey anti-Goat IgG Peroxidase Conjugate, **BT-495**. Glycerol solution. One vial. ***Store at -20°C**. Dilute 1/800 (15ul for 12ml) using Conjugate Buffer. NOTE: only prepare enough solution for one day's use. Discard excess solution.
6. Peroxidase Substrate TMB, **BT-497**. One 6ml vial.
7. Hydrogen Peroxide Solution, **BT-498**. One 6ml vial.
8. Stop Solution, **BT-499**. One 12ml vial.
9. One 96 well plate (8 well removable strips) coated with a monoclonal human lysozyme antibody.
10. Human Lysozyme Control (Urine, Lyophilized), **BT-634**. Reconstitute with 0.5ml-1.0ml phosphate- saline buffer. Cap, mix end-over-end until the solids are dissolved. Store the solution at -20°C for one month.

Other Supplies Required

1. Elisa Plate Reader which can measure absorbance at 450nm.
2. Pipettes: micropipettes 5-1000ul.
3. A plate washer is recommended for washing.
4. A 37°C Incubator.
5. Deionized water.

Precautions

Some components of this kit contain isothiazolones (5ppm) as a preservative. Stop solution contains hydrochloric and phosphoric acids. Keep all materials away from the skin and eyes.



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Sample Preparation

Collect samples in leak proof containers. Store serum (plasma), urine and body fluids (eg. saliva and tears) at 4°C for 2 days or 2 weeks at -20°C. Thaw and keep on ice until ready for use. **Caution: Samples must not contain azides**. Most samples require dilution with Phosphate-Saline Buffer: Urine, 1/10-1/50; serum (plasma), saliva, at least 1/2000; tears, approximately 1/10,000.

Range of Normal Values Reported

Serum (Plasma)	4-13ug/ml
Urine	0-2ug/ml
Saliva	4-13ug/ml
Tears	>300ug/ml

Values Observed at BTI

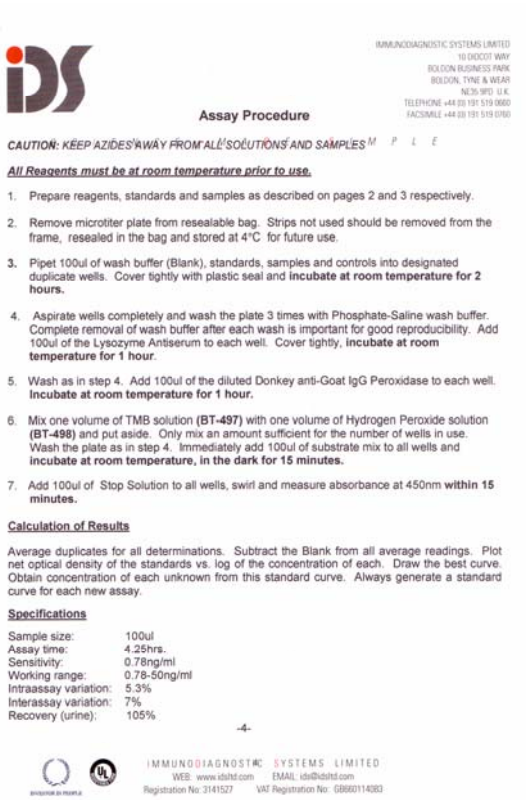
Urine	20-300ng/ml
Saliva	100ug-200ug/ml
Serum	3-10ug/ml

Standards

Prepare a set of standards from the 1000ng/ml stock in the range of 0.5 to 50ng/ml using diluted Phosphate-Saline Buffer. For example:

Standard #	ml of Std.	ml of Buffer	Concentration ng/ml
1.	.05 stock	0.95	50
2.	0.5 std. 1	0.5	25
3.	0.5 std. 2	0.5	12.5
4.	0.5 std. 3	0.5	6.25
5.	0.5 std. 4	0.5	3.125
6.	0.5 std. 5	0.5	1.56
7.	0.5 std. 6	0.5	0.78

Store the stock solution frozen (-20°C). Discard all working standards.



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Assay Procedure

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CAUTION: KEEP AZIDES AWAY FROM ALL SOLUTIONS AND SAMPLES

All Reagents must be at room temperature prior to use.

1. Prepare reagents, standards and samples as described on pages 2 and 3 respectively.
2. Remove microtiter plate from resealable bag. Strips not used should be removed from the frame, resealed in the bag and stored at 4°C for future use.
3. Pipet 100ul of wash buffer (Blank), standards, samples and controls into designated duplicate wells. Cover tightly with plastic seal and **incubate at room temperature for 2 hours.**
4. Aspirate wells completely and wash the plate 3 times with Phosphate-Saline wash buffer. Complete removal of wash buffer after each wash is important for good reproducibility. Add 100ul of the Lysozyme Antiserum to each well. Cover tightly, **incubate at room temperature for 1 hour**.
5. Wash as in step 4. Add 100ul of the diluted Donkey anti-Goat IgG Peroxidase to each well. **Incubate at room temperature for 1 hour.**
6. Mix one volume of TMB solution **(BT-497)** with one volume of Hydrogen Peroxide solution **(BT-498)** and put aside. Only mix an amount sufficient for the number of wells in use. Wash the plate as in step 4. Immediately add 100ul of substrate mix to all wells and **incubate at room temperature, in the dark for 15 minutes.**
7. Add 100ul of Stop Solution to all wells, swirl and measure absorbance at 450nm **within 15 minutes.**

Calculation of Results

Average duplicates for all determinations. Subtract the Blank from all average readings. Plot net optical density of the standards vs. log of the concentration of each. Draw the best curve. Obtain concentration of each unknown from this standard curve. Always generate a standard curve for each new assay.

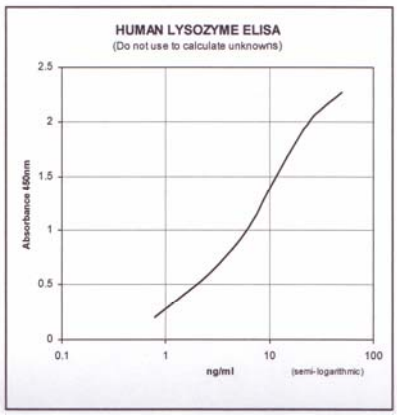
Specifications

Sample size:	100ul
Assay time:	4.25hrs.
Sensitivity:	0.78ng/ml
Working range:	0.78-50ng/ml
Intraassay variation:	5.3%
Interassay variation:	7%
Recovery (urine):	105%

Typical Data (Do not use for determination of Unknowns)

ID	A 450nm	Average-Blank
Blank, 0ng/ml	.213	
Blank, 0ng/ml	.202	
0.78ng/ml	.393	
0.78ng/ml	.424	.201
1.5ng/ml	.656	
1.5ng/ml	.566	.404
3.12ng/ml	.889	
3.12ng/ml	.874	.674
6.25ng/ml	1.234	
6.25ng/ml	1.238	1.029
12.5ng/ml	1.778	
12.5ng/ml	1.739	1.555
25ng/ml	2.176	
25ng/ml	2.285	2.023
50ng/ml	2.400	
50ng/ml	2.546	2.266

Typical Standard Curve



Appendix I - Additional ELISA Clinical Data Analysis

This section details the suggestive relationship between the patient's condition and the results from the blood analysis provided by the Great Western Hospital and the lysozyme analysis from the ELISA. The results from the commercial kit are also shown on each graph, however, no correlation could be found for any value.

Clinical Sample Results -Abdominal Pain & Appendicitis

Figure I1 shows the haemoglobin levels of patients suffering from abdominal pain. The graph shows the haemoglobin levels increasing with increasing lysozyme. Compared to the reference range, the lysozyme levels are high for this group of patients but the haemoglobin levels are within range (Appendix J).

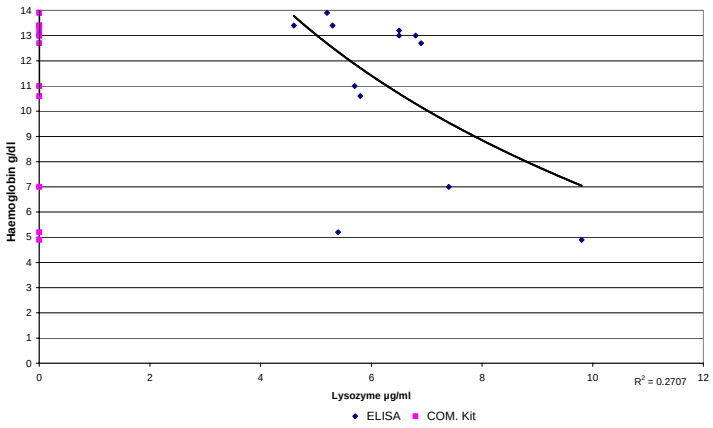


Figure I1 Comparison of Lysozyme and Haemoglobin levels in patients with abdominal pain; graph showing lysozyme levels lowering with lower haemoglobin.

Figure I2 shows the neutrophil levels of these patients against lysozyme levels. The neutrophil levels are decreasing as the lysozyme levels increase. The neutrophil levels for this group are at the top end of the reference range, where as the lysozyme levels range from normal to higher than normal.

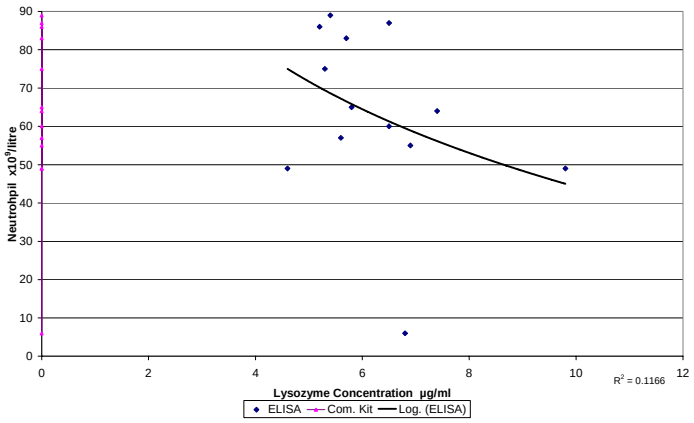


Figure I2 Comparison of lysozyme and neutrophil levels in patients with abdominal pain; graph indicates neutrophil levels decreasing with lysozyme concentration.

Figure I3 shows the lymphocyte levels. The lymphocyte levels for the abdominal pain patients varied enormously, increasing with increasing lysozyme levels. The white blood cell counts of Figure I4 also covered the entire reference range from 4-11x10^9/µl. The white blood cells appeared to decrease with increasing levels of lysozyme.

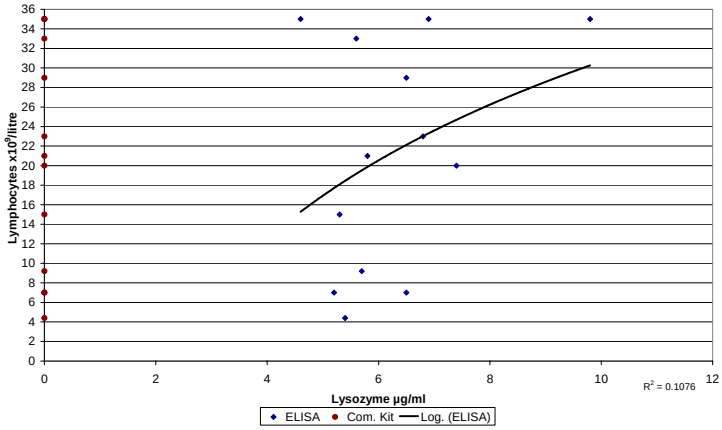


Figure I3 Comparison of lysozyme and lymphocytes in patients with abdominal pain; graph indicates lymphocyte levels increasing with lysozyme increase.

For the appendicitis patients the only correlation in the results which could be found was between lymphocytes and lysozyme. The lymphocytes appeared to decrease as lysozyme increased. During the aftermath of surgery the body goes through a stressful period and many changes in the leukocyte family occur. This happens due to the stress of surgery which impairs the lymphocyte function causing a defect on antigen recognition, proliferation response and a decrease in antibody production.

The lysozyme levels from Figure I5 are higher than the normal reference range; this is due to lysozyme release from neutrophils at the infection site.

No correlation could be seen when comparing the haemoglobin levels with lysozyme in the appendicitis patients.

The white blood cells counts from Figure I4 are raised in some causes and this may be an indication of the start of a more serious infection.

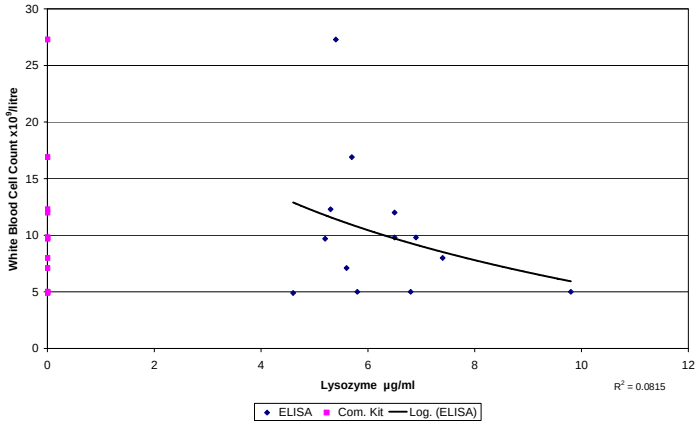


Figure I4 Comparison of lysozyme and white blood cells in patients with abdominal pain; figure indicates decreasing white blood cells with increasing lysozyme levels

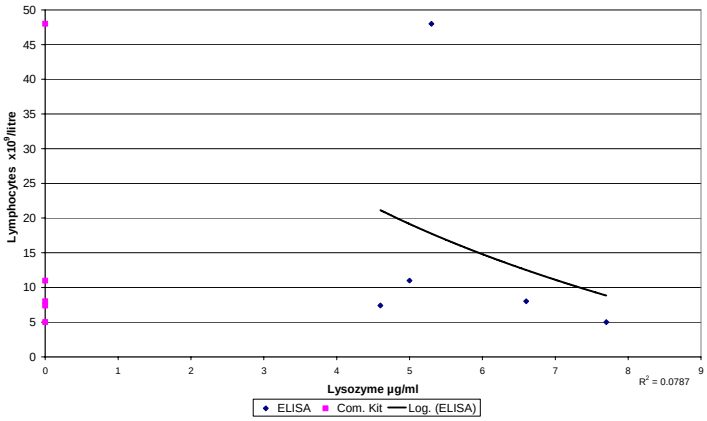


Figure I5 Comparison of lysozyme and lymphocytes in patients with appendicitis; Figure I5 indicates decreasing lymphocytes with increasing lysozyme levels.

Clinical Sample Results –Chronic Renal Failure

Chronic renal failure is the progressive deterioration of renal function, leading to complete renal failure. Figure I6 shows the decreased haemoglobin levels from the

chronic renal failure patients sampled. Sixty to eighty percent of renal failure patients suffer from anaemia (lack of haemoglobin) (www.medlineplus.com, 2005). This graph illustrates the symptoms of this disease.

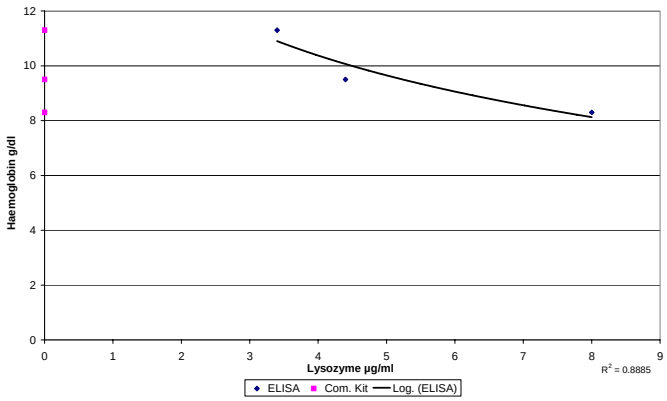


Figure I6 Comparison of lysozyme and haemoglobin in patients with chronic renal failure; Figure shows decreasing haemoglobin levels with increasing lysozyme levels.

Figure I7 shows the platelets levels in the chronic renal patients. Normally in chronic renal failure platelet levels are decreased.

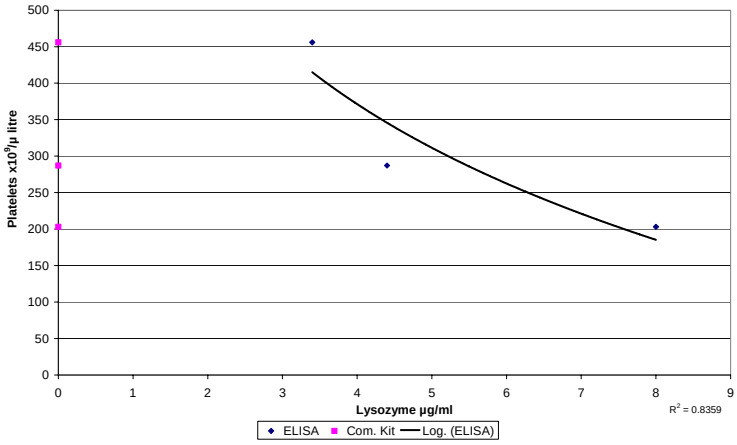


Figure I7 Comparison of lysozyme and platelets in patients with chronic renal failure; graph shows decreasing platelet levels with increasing lysozyme levels.

The lysozyme levels in these patients are raised due to the kidneys lack of ability to excrete waste products. Also during chronic renal failure the renal mass can reduce drastically and this can also increase the serum lysozyme levels, (Davis, 1971).

Very often infections are a result of renal failure for this reason and the increase in lymphocytes in Figure I8 shows this.

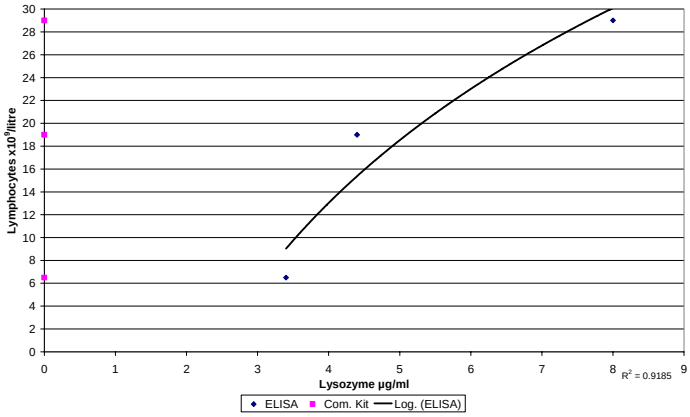


Figure I8 Comparison of lysozyme and lymphocytes in patients with chronic renal failure; graph shows increasing lymphocyte levels with increasing lysozyme levels.

Acute renal failure precedes the chronic stage of the disease. This stage is not as severe and the neutrophil counts of these patients are generally raised. In the chronic form of the disease the neutrophil counts are depressed due to the stress of the disease on the immune system. Figure I9 shows the depression of the neutrophil count as the secondary infection is succeeding, due to the development of chronic renal failure in these patients.

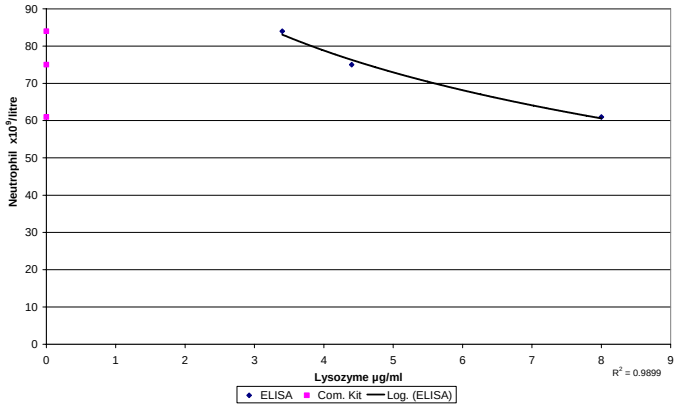


Figure I9 Comparison of lysozyme and neutrophils in patients with chronic renal failure, Figure shows decreasing neutrophil levels with increasing lysozyme levels.

Clinical Sample Results – Leukaemia

Leukaemia is a form of cancer involving the lymphocytes of the bone marrow (www.cancer.org, 2005). Leukaemia is known to cause to be associated with anaemia. Figure I10 shows a decreased haemoglobin levels in these patients and an increased

lysozyme level. Lysozyme levels are increased in leukaemic patients due to the high WBC count and increased turnover which is indicated in Figure I11, (Davis, 1971).

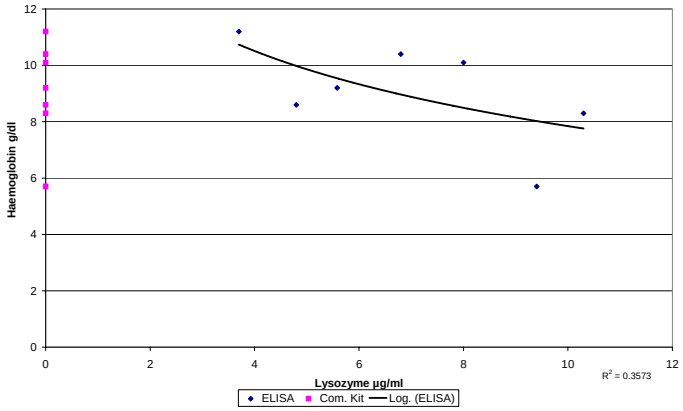


Figure I10 Comparison of lysozyme and haemoglobin in patients with leukaemia; Figure indicates decreasing haemoglobin levels with increasing lysozyme levels.

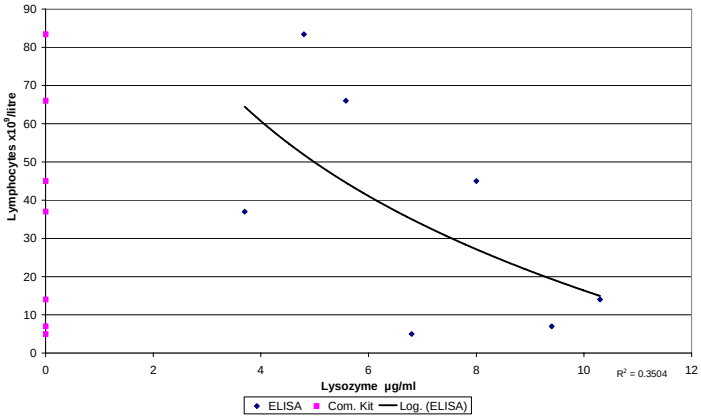


Figure I11 Comparison of lysozyme and lymphocytes in patients with leukaemia; graph shows decreasing lymphocytes levels with increasing lysozyme levels.

Figure I12 shows normal levels of platelets in the leukaemia patients which are not expected. Due to the increased prevalence of anaemia the platelet levels should have been decreased; however Figure I12 does not show this.

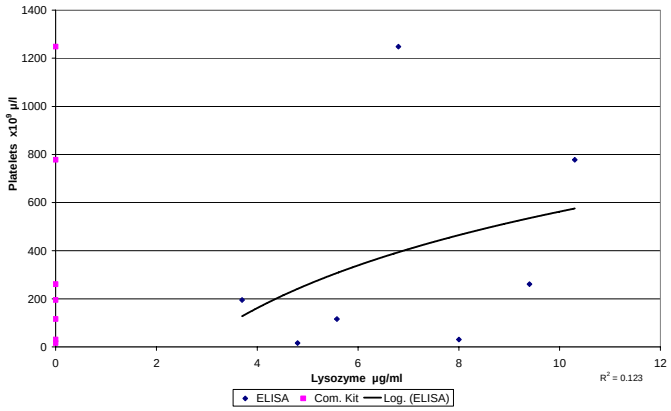


Figure I12 Comparison of lysozyme and platelets in patients with leukaemia; graph indicates increasing platelets levels with increasing lysozyme levels.

The neutrophil count of these patients is shown in Figure I13. Myeloid leukaemia is cancer of the granulocyte white blood cells, (e.g. neutrophils) and lymphocyte leukaemia is cancer affecting the lymphocytes of the body. The clinical information provided, in most cases did not specify which type of leukaemia these patients were suffering from. Therefore it can only be assumed from the results that most patients were suffering from lymphocytic leukaemia as the lymphocytes in this sample group were lower than normal values. The neutrophils were higher as seen in Figure I13.

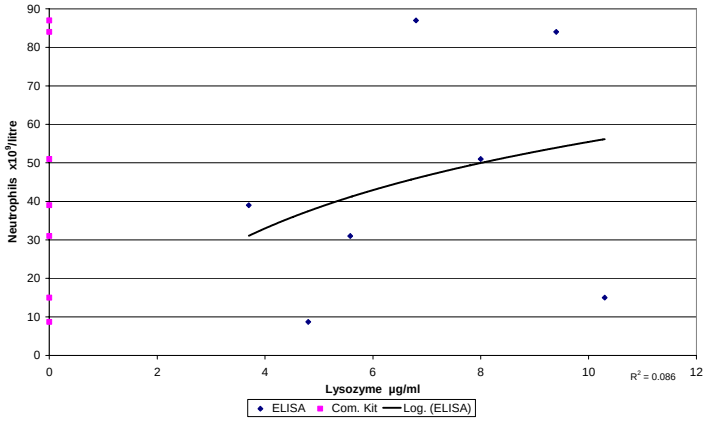


Figure I13 Comparison of lysozyme and neutrophils in patients with leukaemia; graph indicates increasing neutrophils levels with increasing lysozyme levels.

The white blood cell count is a total count of all leukocytes, including neutrophils, monocytes, lymphocytes, and eosinophils. In Figure I14 the graph shows the white cell count to be vastly increased due to the leukaemia. Leukaemia like many cancers involves uncontrollable cell growth.

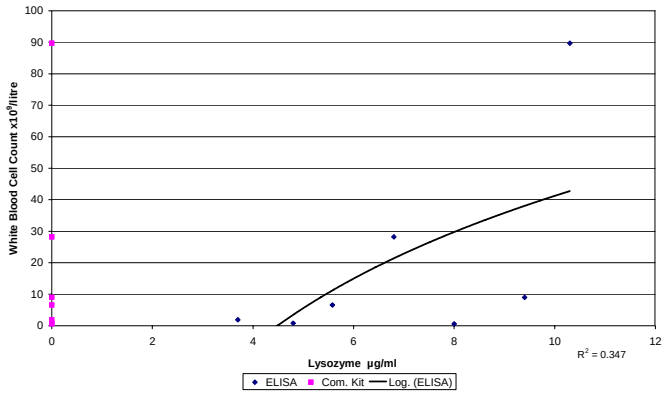


Figure I14 Comparison of lysozyme and white blood cells in patients with leukaemia; graph indicates increasing white blood cells levels with increasing lysozyme levels.

Clinical Sample Results – Pneumonia

Pneumonia is the inflammation of the lung tissue caused by infection. Figure I15 shows the platelet counts from the pneumonia patients. This graph shows the decrease in platelets as lysozyme is released.

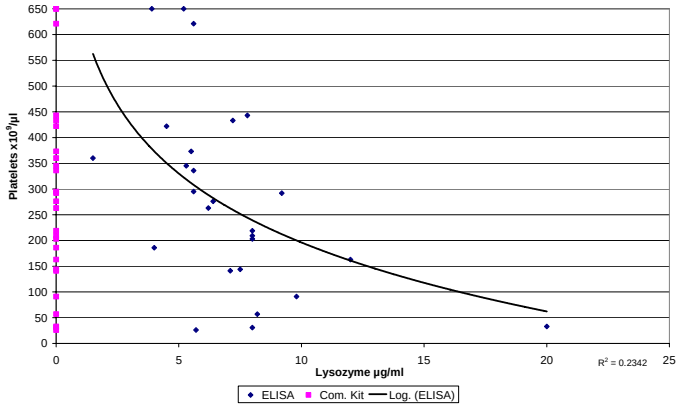


Figure I15 Comparison of lysozyme and platelets in patients with pneumonia; Figure shows decreasing platelets levels with increasing lysozyme levels.

Figure I16 shows the C-reactive protein levels in the pneumonia patients. Levels of CRP are usually increased in pneumonia patients as CRP is released by the liver in response to inflammation. Therefore as lysozyme is released and more neutrophils are present at the site of infection the higher the CRP level will become. Figure I16 shows this increase in CRP with increasing lysozyme.

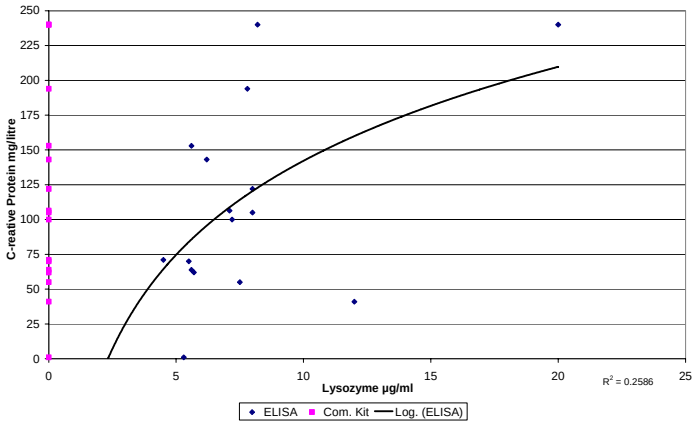


Figure I16 Comparison of lysozyme and C-reactive protein in patients with pneumonia; graph shows increasing C-reactive protein levels with increasing lysozyme levels.

Clinical Sample Results – Pregnant

During pregnancy it is normal to have a slightly low platelet count due to the body producing more blood plasma which makes the platelets more dilute. Figure I17 shows a normal amount of platelets in the patient's samples. As the graph shows a fall in platelets as lysozyme is released it indicates that these patients are suffering from some kind of infection.

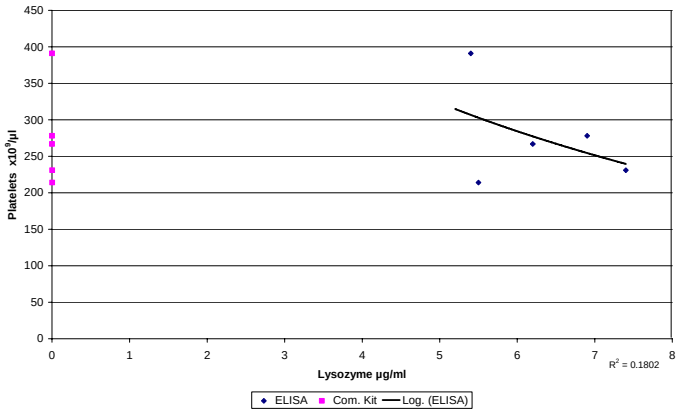


Figure I17 Comparison of lysozyme and platelets in patients who are pregnant; shows decreasing platelets levels with increasing lysozyme levels.

Figure I18 shows the increased neutrophil count along with a decrease in neutrophils when lysozyme increases. During pregnancy leukocytosis occurs; this is the increase of neutrophils with a slight increase in lymphocytes.

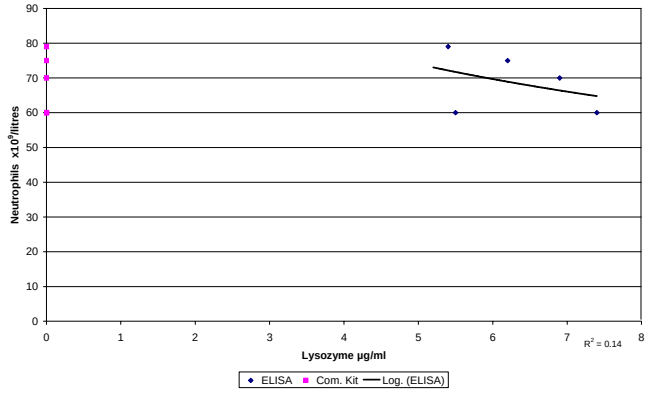


Figure I18 Comparison of lysozyme and neutrophils in patients who are pregnant; graph shows decreasing neutrophils levels with increasing lysozyme levels.

Clinical Sample Results – Tumours

Anaemia is very common in cancer patients. It was not certain whether the patients in this study had malignant tumours but the low haemoglobin levels in Figure I19 suggest anaemia in these patients. Anaemia can be a result of bleeding or a lack of red blood cells being produced in the bone marrow. Either reason could be linked to the patients underlining condition.

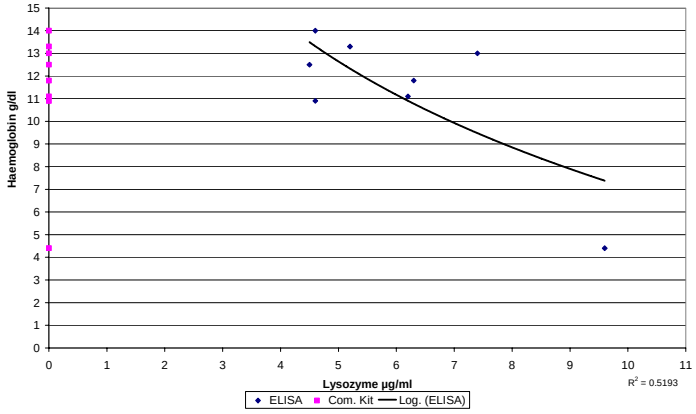


Figure I19 Comparison of lysozyme and haemoglobin in patients with tumours; graph shows decreasing haemoglobin levels with increasing lysozyme levels.

Figure I20 shows the white cell count of the patients with tumours. As many of these patients had suspected cancerous tumours it would be expected that their white cell count be depressed. During chemotherapy to treat malignant tumours the white blood cells numbers fall and sepsis is very likely due to the body's lack of defence. Figure I20 shows normal white blood cell counts for these patients but it also shows the white cell count decreasing as lysozyme increases. The lysozyme levels in these patients are

increased either due to infection or increased white blood cell turnover. As mentioned previously, the white blood cell count measures the collective amount of neutrophils, band cells, T-lymphocytes, B-lymphocytes, monocytes, eosinophils, and basophils (www.medlineplus.com, 2005). Therefore, as the lysozyme levels are increased it suggests that some of the patients in this group may also have infections or that the patients cells are being destroyed and the lysozyme is freed from the cells.

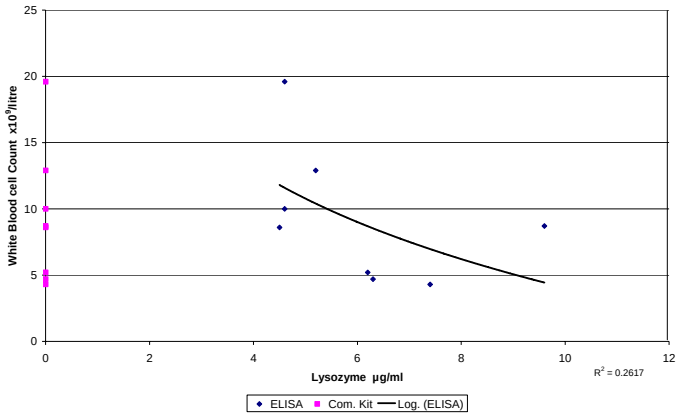


Figure I20 Comparison of lysozyme and white blood cells in patients with tumours; graph shows decreasing white blood cells levels with increasing lysozyme levels.

Clinical Sample Results – Age & Sex Distribution

Groups	Sample Number	Sex Ratio M:F	Mean age (years)	Age range (years)
Controls	14	5:9	32	25-45
Tumours	10	1:1	67.8	50-82
Bacteraemia	42	8:13	62.1	25-90
Pregnant	6	0:6	29.2	23-34
Non Septic	109	54:55	60	17-91
Pneumonia	28	5:9	76.1	59-98
Leukaemia	9	8:1	61	43-90
Deceased	37	12:25	75.5	56-95
CRF	4	0:4	60	59-80
Appendicitis	5	2:3	33	16-46
Abdo Pain	15	1:4	42	13-78
Totals	279	23:33	54	13-98

The groups of patients studied were well matched for age and sex. Lysozyme levels were compared between male and female patients who were non septic to determine if there were sex related differences. No correlations between lysozyme and sex could be established.

Clinical Sample Results – Sex Related Variation of Lysozyme Concentrations in all Non-Septic Patients

	Mean Age (years)	Range (years)	Mean Lysozyme	Standard Deviation
Male	58.6	25-87	5.6	1.42
Female	55.6	17-91	5.5	1.28

In non septic patients lysozyme levels did not differ significantly between male and females.

Appendix J -Reference Ranges

Table J1 - Ranges used at Swindon's Great Western Hospital, 2005

Blood Cell	Normal Range
Haemoglobin	Male 13-17.0g.dl^{-1} Female 11.5-16.5g.dl^{-1}
Platelets	150-400x10^{9}/µL
White Blood Cell Count	4-11 x 10^{9}/L
Neutrophil	2-7.5x10^{9}/L
Lymphocyte	1.0-4x10^{9}/L
C-Reactive Protein (CRP)	<6mg/L
Erythrocyte Sedimentation Rate (ESR)	0-35mm in 1hr

Table J2 - Cited Lysozyme Reference Range

Reference	Method	Patients Condition	Biological Fluid	LZ Range (µg/ml)*
Medlab Analytic	Undisclosed	Control	Serum	3-9
Taylor et al., 1992	Direct Immunoassay	Control	Serum	0.54±0.15
Giles et al., 1990	Turbidimetric	Control	Serum	3.38±7.35
Borgen et al., 1977	Turbidimetric	Control	Serum	2.1
Reitamo et al., 1981	Lysoplate	Control	Serum	10-25
	Turbidimetric	Control	Serum	4-12
	Nephelometirc	Control	Serum	5-11
Francina et al., 1986	Non-Competitive Avidin-Biotin Assay	Control	Serum	0.95-2.45mg/ml
Maron et al., 1971	Turbidimetric	Control	Serum	10
Johansson et al., 1971	Electrophoresis	Control	Serum	2.8±0.8
Mörsky, 1983	Turbidimetric	Control	Serum	0.9-2.1mg/l
Klass et al., 1971	Lysoplate	Control	Serum	22.4±1.4
	Turbidimetric	Control	Serum	4.54±0.36
	Fluorimetric	Control	Serum	11.1±0.63
Brouwer et al., 1984	Immunoassay	Control	Serum	0.95-2.45
Burgess, 1973	Undisclosed	Control	Serum	M 6.4±0.8
				F 5.8±0.5
Hankiewicz et al., 1974	Undisclosed	Control	Serum	9.8±2.9mg/l
Hayes et al., 1972	Turbidimetric	Control	Serum	3.7±2
Harrison et al., 1971	Turbidimetric	Control	Serum	5-14
Catovsky et al., 1971	Turbidimetric	Control	Serum	27.7±5.6

*M=Males, F=Females

Table J3 - Other Reference Ranges for Diseased Patients

Reference	Method	Patients Condition	Biological Fluid	LZ Range (µg/ml)*
Syrjanen et al., 1983	Lysoplate	Rheumatoid Arthritis	Serum	5.7±3.2
Klass et al., 1971	Lysoplate	Crohn's disease	Serum	34.5±3.3
	Turbidimetric	Crohn's disease	Serum	8.79±0.47
	Fluorimetric	Crohn's disease	Serum	18.4±1.5
	Fluorimetric	Ulcerative colitis	Serum	17.6±2.5
	Turbidmetirc	Ulcerative colitis	Serum	7.24±0.28

APPENDIX K -Expected ELIFA Results

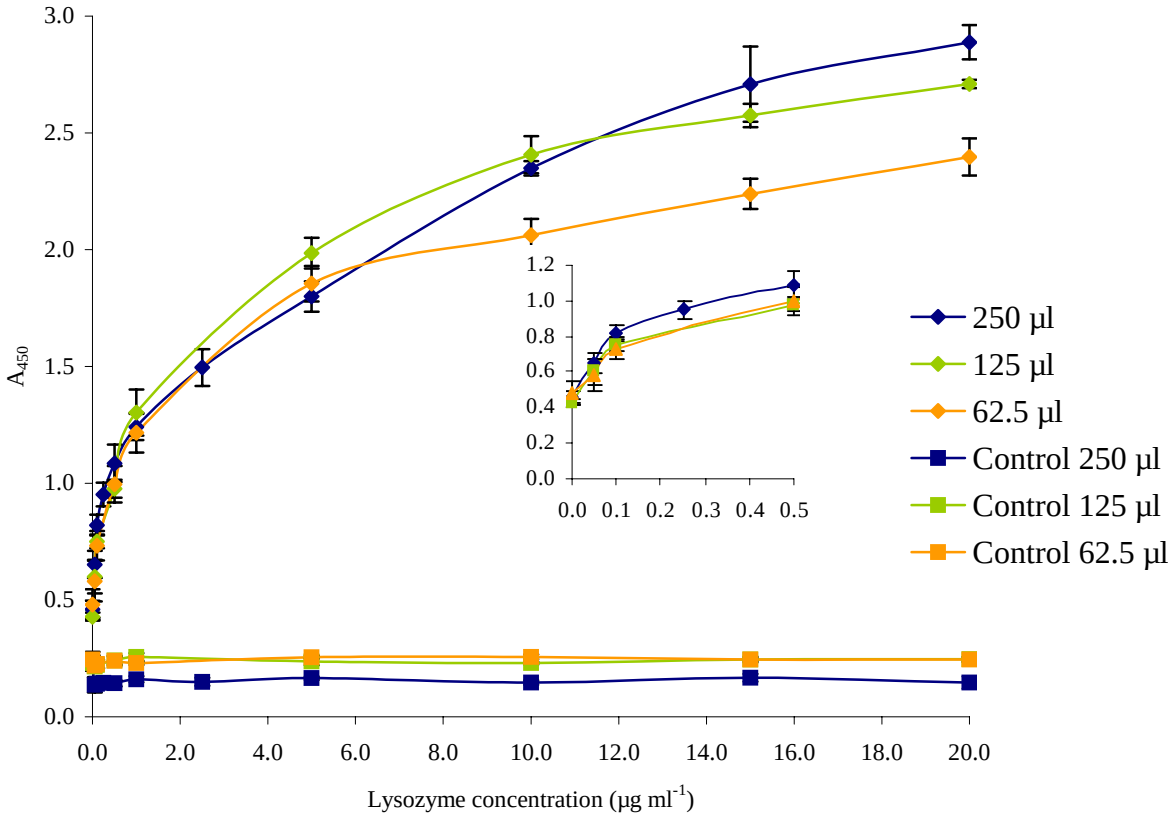


Figure K1 - Effect of the volume of lysozyme solution upon signal size.
ELIFA's were performed with lysozyme ranging from 0.05 to 20.00 µg ml^{-1} and three different volumes (62.5, 125 and 250 µl) of each concentration were passed through nylon membranes. Controls lacked primary antibody. The inset enlarges results obtained over the lysozyme range 0.05-0.5 µg ml^{-1}. Results are presented as mean values±SD; n=3. Faedda A, 2003.

APPENDIX L - IgE Spreeta Protocol & Discussion of Results

Objectives

With the ELISA method established the next step was to produce a test which met the initial requirements of the project; to produce a test which could be carried out at the patient's bedside. The Spreeta technology meets this criterion, as it is a portable, compact device with the potential for near patient execution. This part of the project took place at the Universitå Degli Studi Di Firenze in Italy. The work shows a technique which has the potential for a near patient test. If the lysozyme-Biosensor assay can be transferred to the spreeta it would produce a cost effective, rapid near patient test with huge potential.

Materials & Methods

General Reagents

The Spreeta running buffer consisted of 60mM $NaHPO_4$, 40mM $NaHPO_4$, 100mM sodium chloride, 1mM Magnesium chloride made up to 1 litre in reverse osmosis water. The pH was adjusted with sodium chloride to pH7.4.

Spreeta Preparation & Construction

The Integrated Flow Cell (IFC) (SPR-EVM-BT, Texas Instruments, USA) was opened by loosening the screw and the black nitrile silicon gasket ($^1/_{32}$" thick 1560 type) was fitted inside the IFC, (Figure L1). The Spreeta sensor chip was washed with ethanol and a Kimwipe to remove surface debris. The Spreeta sensor was inserted with the gold facing the flow cell. The lid was closed and the gasket checked to ensure the inlet and outlet are still visible. The lid was screwed tight. The tubing was attached to the inlet and outlet holes and the inlet tube is connected to the peristaltic pump and the outlet to the waste container. The BNC-Ext cable was connected to the two pins on the face of the flow cell. The Interface box is also connected using a 15-pin cable and printed circuit board. This contains the electrons needed to interface between the PC and the Spreeta sensor.

Supelco (Dorset, UK) TFE Teflon tubing 0.5mm IDX 1.5mm OD was connected to the inlet and outlet. The outlet tubing was then connected to solvent flex tubing (ELKAY, Ireland) via a peristaltic pump (Gilson, Italy). The flow rate was set at 30µl/min.

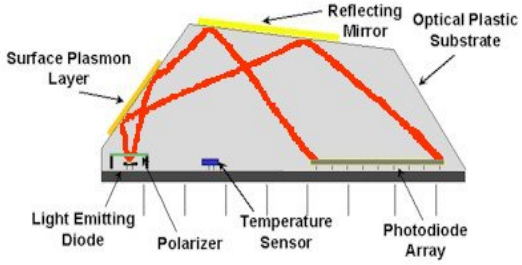


Figure L1 - Cross section of the Spreeta Sensor Chip, adapted from Earp & Dessy, 1996.

System Tests

A calibration of the sensor with the software in air was carried out and the reference file saved. A calibration in water was also carried out. Water was pumped through the Spreeta until a stabile signal was obtained.

Sensor Chip Preparation

The thiol solution consisted of 11-mercaptoundecanoic acid 95% (Sigma-Aldrich, UK) 2mg in 10ml Ethanol. This was pumped though the Spreeta for 15 minutes then left stationary for 1 hour. Ethanol was pumped past the sensor surface to remove unbound thiol until the signal stabilized. The thiol activation solution consisted of N-hydroxysuccinimide (≥97% purity) (Sigma-Aldrich, UK), 5.8mg and 1-Ethyl-3-(3-Dimethylamin propyl) Carbondiimide (Sigma-Aldrich, UK) 38.3mg and water (1ml). This solution was left in continuous flow for 5 minutes.

A stock solution of Streptavidin (Sigma-Aldrich, UK) from *Streptomyces avidinii* 1000ppm was made and diluted to 200ppm for use in acetate buffer 10mM pH5.
This was pumped though the Spreeta for ~10 minutes until the volume was depleted.

Blocking Solution

Ethanolamine (pH 8.6) was used to block unbound sites on the sensor. A 1M solution (98% purity) (Sigma-Aldrich, UK) was pumped passed the sensor surface continuously for 20minutes. After the blocking phase, buffer was pumped through the sensor to stabilize the signal.

Ligand Immobilisation

The IgE Aptamer (D17.4ext) (MWG Biotech, IT) was diluted using the Spreeta buffer to 200ppm and stored at –20^{o}C until use. The IgE aptamer was pretreated before the immobilization by heating to 95^{o}C for 3 minutes in a water bath. The Aptamer was pumped across the Spreeta sensor for 10mintes in continuous flow and left stationary for 50minutes. Running buffer was pumped through the Spreeta to stabilize the signal and to wash unbound aptamer from the sensor surface. The sensor was then ready for testing.

Analyte Preparation

All IgE antigen (Biogenesis, UK) dilutions were prepared using the Spreeta running buffer. Each IgE antigen dilution pumped through the spreeta for 15 minutes. Buffer was then used to wash the unbound protein from the surface.

Control Antigen

The control antigen was IgG (Biogenesis, UK) was tested using the same procedure as the IgE analyte.

Regeneration of the Sensor

Various solutions were used to regenerate the sensor. These included 1mM hydrochloric acid, 10mM hydrochloric acid, 50mM EDTA, 6M Urea, and Sodium hydroxide 25mM with Ethanol 2.5%. Each solution was pumped through the Spreeta for 1 minute. Buffer was always applied to stabilize the signal after regeneration.

Results

Preamble to Results

This section described all the results gained from the spreeta experiments. As previously mentioned, this work was carried out to demonstrate a method that could be used as a model system for future lysozyme detection. The results here are therefore generated using the IgE target.

Streptavidin & Aptamer Immobilisation

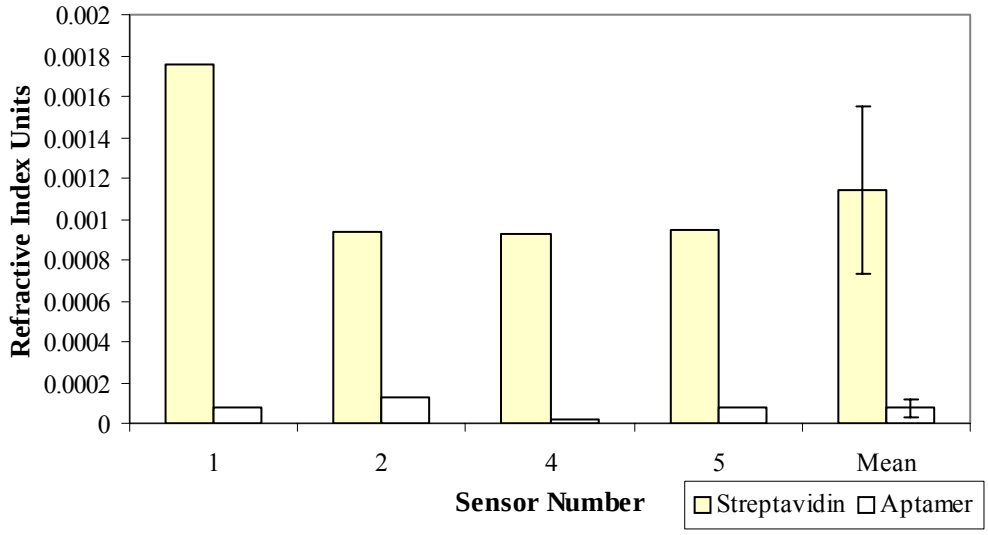


Figure L2 Streptavidin and aptamer immobilisation for various different sensors.

Figure L2 shows the streptavidin and aptamer immobilised on each sensor. Sensor 1 had the highest amount of streptavidin immobilised but this did not result in the highest amount of aptamer immobilised. An even amount of streptavidin was achieved on sensors 2, 4 and 5 but the aptamer immobilisation was not reproducible.

Analyte Binding

Figure L3 shows the amount of IgE binding on all sensors including the controls used. The control sensors were blank gold surfaces without the ligand bound; therefore no IgE should have bound. On both control surfaces IgE bound. On the ligand bound spreeta's very similar IgE binding of 1ppm was seen on all sensors tried. However, 10ppm did not to the same consistency, with different levels of binding seen on sensors 1 and 2.

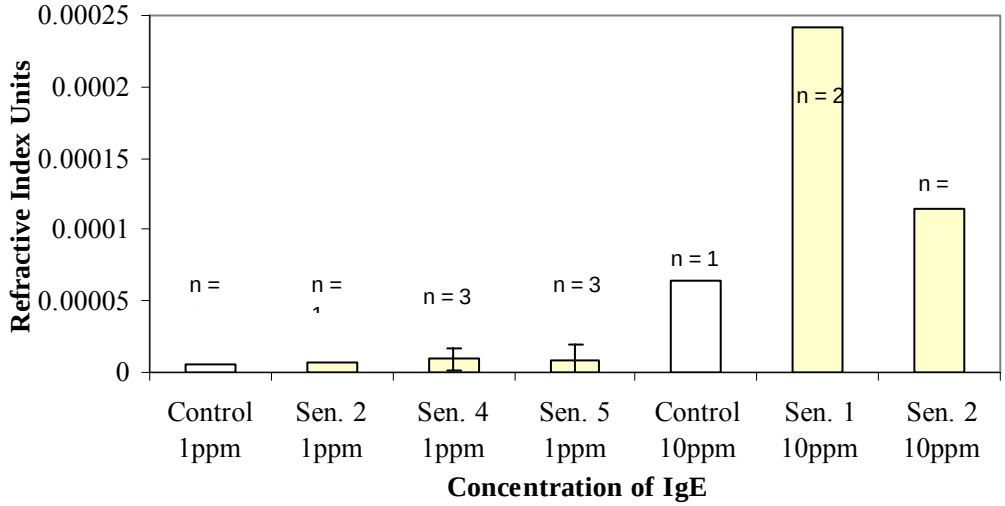


Figure L3 Comparison of IgE binding on control sensors and ligand bound sensors.

Analyte & Control Analyte Binding

Figure L4 shows the levels of binding of IgE and IgG; the control analyte. The IgE levels of binding look promising, as the amount of IgE binding increases with increasing IgE concentration. However, when the control analyte was injected the binding patterns were irregular, showing no correlation indicating non-specific binding.

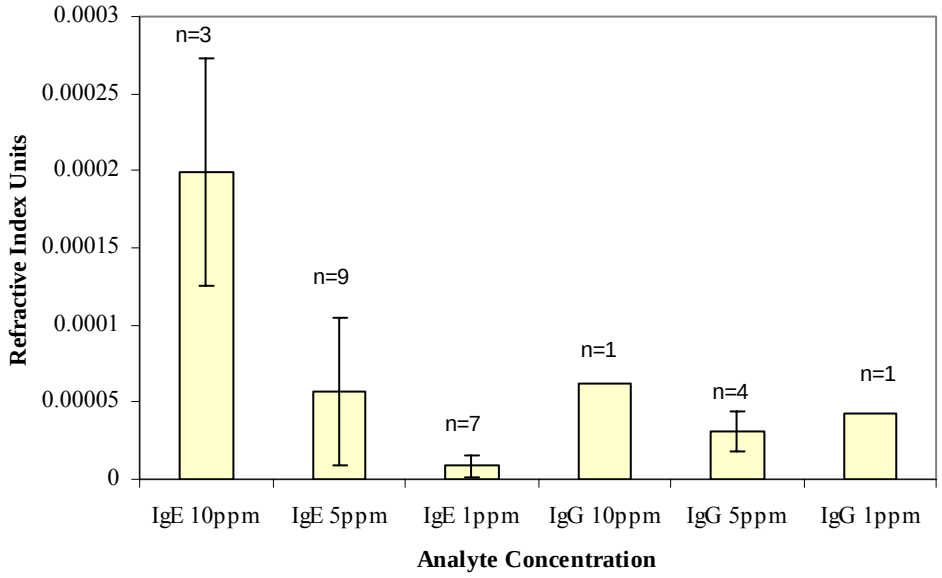


Figure L4 Comparison of IgE and IgG binding at various concentrations.

Reproducibility Testing

Figure L5 shows the binding responses of several IgE solutions (5ppm) repeatedly injected on to sensor 4. As the sensor was regenerated between IgE injections the same number of ligands should be available for further IgE binding; this should produce a graph which shows a reproducible amount of binding. However, as no pattern of binding is seen it can only suggest the analyte is binding none specifically. If the number of binding sites reduced with repeated regenerations then the levels of IgE binding should be decreasing with increasing numbers of injections; but this is not indicated.

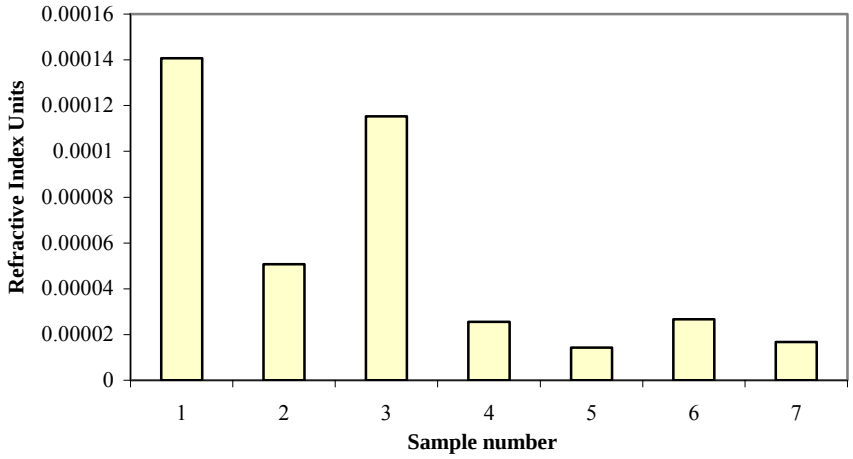


Figure L5 Various IgE 5ppm samples injected on to sensor 4.

Discussion

Preliminary Testing

Sensor 0 was the first sensor to be used. The immobilization technique as described in Miller *et al.* (1991) stated that the sensor should be left immersed in the thiol solution for 2 days. After 2 days the plastic coating of the spreeta sensor had degraded and the thiol solution had evaporated. The sensor was inserted into the IFC but no minimum value for the baseline could be obtained. This resulted in the conclusion that the organic solvent in the thiol solution had also damaged the gold surface, as well as the plastic coating around the sensor. For the remaining sensors all steps of the immobilization procedure were carried out with the sensor inside the IFC. All solutions

were pumped by the peristaltic pump passed the surface of the sensor. This eliminated degradation and over exposure of the gold to the solvents.

Streptavidin Immobilisation

All 5 sensors (1-5) had the exact same concentration and volume of the thiol, thiol-activation, and streptavidin used. However, different running conditions were employed. Figure L2 shows the streptavidin RI is much higher in sensor 1 than any other sensor. In the method of sensor 1, the spreeta was left stationary in the thiol for 2.5 hours, whereas sensors 2, 3, 4, and 5 all had three hours. This could explain the increase. A reduced time of 2.5 hours of thiol incubation may be the optimum incubation time. Perhaps after this period the gold is either saturated with thiol or the gold begins to degrade as with sensor 0 from over exposure with an organic solvent in the thiol mixture. Another explanation is the pump rate. In sensor 2, 3, 4, and 5 the pump rate was altered to be in conjunction with the IgE BIAcore method Appendix B, of 30µl/min (pump number 0.33). However, the immobilization phase of sensor 1 was carried out using pump number 2.4 a much faster speed (~240µl/min). This could have caused the decrease in the streptavidin binding on all other sensors.

A further change was the duration of the streptavidin incubation. With sensor 1 the streptavidin sample was pumped through the spreeta, and then left for 20 minutes stationary. This procedure was the same as that used for all other sensors however, with spreeta sensor 1 the signal had not stabilized after 20 minutes. It was then left a further 10 minutes stationary. This would also explain the huge difference in streptavidin binding between sensor 1 and sensors 2, 4, and 5 as shown on Figure L2, because the longer the incubation time the more binding of the streptavidin will occur until saturation.

Aptamer Immobilisation

All sensors (1, 2, 4, and 5) had the same volume and concentration of aptamer passed through the spreeta. However, differences can be seen on Figure L2 in the aptamer immobilization RI value. Sensor 2 showed the greatest amount of binding of aptamer. The method here involved a 20 minute stationary phase with ethanolamine then a 15

minute continuous flow of aptamer followed by 60 minutes stationary phase (where the pump flow was stopped for 60 minutes and the sensor surface was exposed for the duration without a flowing solution). This method was identical to that used in sensor 1.

The method for sensor 4 and 5 was different. Here the ethanolamine was pumped through the spreeta for 20 minutes continuously and no stationary phase was used. Sensor 4 had a 15minute continuous flow of aptamer then a 60minute stationary phase. Sensor 5 had 10 minutes continuous flow and then 50 minutes stationary of aptamer (as seen in the raw date results). Of the two sensors, sensor 4 yielded the lowest aptamer binding result. But overall sensors 4 and 5 had lower results compared to sensors 1 and 2. This may be attributed to the continuous flow of the ethanolamine which did not effectively bind to the COOH groups of the streptavidin as well as when it was left in contact with the sensor in stationary phase as shown in sensor 1 and 2.

A further reason why sensor 4 results may be low could be due to the cooling phase of the aptamer after the pre treatment. The aptamer of all sensors are "unfolded" with 3 minutes in a water bath at 95^{o}C. The aptamer is biotinylated and during the refrigeration the aptamer forms a secondary structure, which traps the biotinylate inside. The thermo-treatment unfolds the aptamer and releases the biotinylate so it can bind to the streptavidin. Therefore if the aptamer after the thermo-treatment was left on ice for too long before injecting into the spreeta for the immobilization on the gold, it could refold into this secondary structure again and thus not bind properly to the gold, resulting in low aptamer binding.

IgE Binding

Figure L3 shows the results gained for 1ppm and 10ppm IgE for the control sensor. It also shows the same concentrations for other sensors. As the control had no aptamer immobilized, no binding of the IgE antigen was expected. However, non specific binding (NSB) did occur. This is shown in Figure L4. IgE where 1ppm was applied to sensor 2, 4, and 5 yielding very similar results. However, these were also similar to the 1ppm IgE's on the control sensor, showing that NSB was taking place. This was further

shown where 10ppm IgE was applied to the Spreeta, as the binding increased on the control sensor at this concentration. When comparing the results from sensor 2 10ppm with sensor 1 10ppm and the control, the results show that this concentration was not reproducible across the sensors.

Figure L4 shows a comparison between IgE and IgG binding. The overall graph shows that NSB is a problem with this immobilization procedure. As IgG concentration decreased the NSB did not decrease as expected. The NSB does not show a trend and is therefore not reproducible because of the nature of the NSB interactions.
A trend can be seen in the IgE concentrations. As IgE concentration decreases the RI also decreases. However, as the amount of NSB is high and due to the degree of error also being high the reproducibility of the results is very low.

Figure L5 shows all IgE 5ppm results on sensor 4 in the order they were applied to the sensor. Samples 1 and 3 show a reasonable amount of binding, but as the sample numbers increase and the use of the sensor increases, the 5ppm RI decreased dramatically. This shows firstly that the binding of IgE is not reproducible. It also shows that the more the sensor is used the less binding of IgE takes place. This may be due to the regeneration phase. It was noted that with increased use of the sensor the number of regeneration phases also increased to reduce the signal back to the baseline; this trend did not correlate with the concentration of the sample. Various regeneration solutions were tried in accordance with Liss *et al.* (2002). This may indicate that with increased exposure to regeneration solutions (sensor 4 used only 10mM HCl) the aptamer is denatured thus reducing the IgE binding.

Limitations of Methods

Air bubbles in the spreeta system disrupt the signal both during the sample injection and during running buffer intervals. Air bubbles were introduced by two methods; via the sample itself, via the inlet and outlet tubing not forming a complete seal as well as the change of solutions. Regardless of the method of introduction, air bubbles caused many problems in the spreeta data collection. Several responses were seen when an air bubble was introduced into the spreeta. The signal would either produce peaks within the

signal, which would reduce back to the baseline when the bubble had passed through the system. Alternatively the baseline signal would slowly decrease as the air was trapped inside the spreeta. The only way to remove this was to discontinue sensing and remove the sensor chip from the Spreeta, thus losing the original baseline. Performing a fast flush by altering the peristaltic pump speed had no bearing on the removal of the air bubbles.

A further limitation of the Spreeta technique is the software. The Spreeta software has 2 options for viewing the refractive index signal; either a zoomed-in view of a particular point in the signal or a rescaled view showing the whole recorded signal in one window. The problem here was the signal peaks never remained at their correct proportion. This means a minor peak when zoomed-in to show a single point of the signal measured, became a significant peak when the signal view was rescaled to show the entire recorded signal. This was because when the signal window was rescaled to fit all the peaks and dips of the baseline into 1 small window, it would be compressed; thus making minor peaks into significant ones. This therefore relies on the operators experience and judgment to dictate whether regeneration was needed and whether the baseline in fact stable; thus leading to potential errors. This was a particular problem when air bubbles disrupted the signal. The BIAcore 3000 software does not have this problem. This software produces a graph that is continuously expanding with time used. Here the proportion of the peaks always remains constant.

Another limitation with the Spreeta was the stabilization of the baseline. At the start of a days work, whether reusing a sensor or using a new one, the baseline would take over an hour to stabilize. This dramatically decreased the amount of work that could be done in 1 day. An explanation of why the signal was so erratic at the start of each day can only be the lack of continuous flow of buffer overnight. This may suggest that deposits of the Spreeta buffer maybe adhering to the sensor surface, and during the initial run of buffer at the start of the day these deposits are washed free, showing an erratic signal. A solution to this problem therefore is to leave the Spreeta in a state of continuous flow of running buffer.

Conclusions

The full potential of the Spreeta method, was not realised in these limited experiments. The time available to work on this piece of kit was very limited. This meant the method was only partially developed and the capability of the equipment not fully explored. With more time to focus on this method, optimisation and refinement could have produced more promising results both for IgE detection and eventually lysozyme.

The Spreeta has advantages over other methods such as the Biacore 3000. Its portability, inexpensive sensor chips and real time detection should not be over looked as a possible future diagnostic instrument for lysozyme.

Appendix L –IgE Raw Spreeta Results

	Before		Standard Dev.	After		Standard Dev.	Difference	Comments
	Interval	RIU		Interval	RIU		RIU	
Sensor 1 Results								
H_2O 080604a	1100	1.334540486	3.77-06					
Thiol 080604a	1800	1.362212	5.95-06	/	1.362862	7.25-06	0.000650391	
Streptavidin 090604a	/	1.332752834	8.32824-06	/	1.33450880	6.24636-06	0.001756	
Aptamer 090604a	/	1.337744398	6.54735-06	/	1.33782483	6.33167-06	0.0000804	
IgE 10ppm 1st 100604a	400	1.337690507	5.63592-06	1700	1.33791779	6.73931-06	0.000227	
IgE 10ppm 2nd 100604c	300	1.339888184	1.04259-05	1640	1.34014589	1.08024-05	0.000255	
IgG 10ppm 100604b	200	1.340192696	6.59391-06	1500	1.34025489	7.59934-06	0.0000622	
Sensor 2 Results								
H_2O 170604d	300	1.33282831	5.7398-06					
Thiol 170604d	/	1.360005021	6.49978-06	/	1.36091772	6.06148-06	0.000913	
Streptavidin 180604a	2500	1.332961628	4.12236-06	4552	1.33389855	6.70201-06	0.000937	
Aptamer 180604b	7150	1.336829131	6.83416-06	13100	1.336959	6.64-06	0.00013	
IgE 1ppm 210604a	5300	1.336192338	1.93953-06	6400	1.33619863	4.26939-06	0.00000629	
IgE 10ppm 210604b	200	1.336297097	3.81347-06	2000	1.33641166	3.04485-06	0.000115	
IgE 5ppm 1st 220604a	650	1.33646917	4.0527-06	2500	1.33656468	4.32638-06	0.0000955	
IgE 5ppm 2nd 220604b	4200	1.336485451	4.16703-06	6000	1.33651232	7.00693-06	0.000026868	
IgG 1ppm 210604a	600	1.33619904	3.36914-06	2100	1.33624151	1.92108-05	0.00004246	
IgG 5ppm 220604b	300	1.336483873	4.72041-06	2000	1.33651232	4.19748-06	0.0000284	
Sensor 3= Control								
IgE 5ppm 010704a	190	1.337612835	4.29872-06	4000	1.3376092	3.2845-06	-0.000003635	
IgE 10ppm 010704c	200	1.337600975	3.80857-06	3500	1.33766582	3.42184-06	0.000064844	
IgE 1ppm 010704c	100	1.337600531	3.50728-06	1500	1.33760666	3.29915-06	0.000006129	
Sensor 4 Results								
Thiol 020704b&c	3200	1.358349315	4.6183-06	6500	1.35847775	5.6026-06	0.0004278	
Streptavidin 050704a	320	1.331659328	2.64384-06	2300	1.33258821	5.9297-06	0.000928884	

Aptamer 050704a	4200	1.335834699	4.48428-06	8000	1.33581208	5.78416-06	0.000022616	
IgE 5ppm 1st 050704b	1390	1.335777238	6.994-06	3600	1.3359131	5.7385-06	0.000140719	
IgE 5ppm 2nd 050704b	7700	1.335916745	3.75013-06	10300	1.33596746	3.31001-06	0.000050719	
IgE 5ppm 3rd 050704c	400	1.335923467	3.83416-06	2400	1.3360388	3.60285-06	0.000115328	
IgE 5ppm 4th 060704a	5200	1.336286256	4.34647-06	8200	1.33631178	4.84709-06	0.000025522	
IgG 5ppm 1st 060704b	300	1.336280218	4.66378-06	1700	1.33629511	4.21854-06	0.000014892	
IgG 5ppm 2nd 060704b	3000	1.36266674	5.10356-06	5500	1.3363015	4.29111-06	0.000034826	
IgG 5ppm 3rd 060704c	540	1.33649559	3.59807-06	1700	1.3365412	5.19323-06	0.000045609	
IgE 1ppm 1st 070704a	8000	1.336692267	4.10104-06	9800	1.33670486	2.89472-06	0.000012597	
IgE 1ppm 2nd 070704a	10800	1.33668269	4.21473-06	12500	1.33668336	4.72579-06	0.00000067	very little signal was seen
IgE 1ppm 3rd 070704b	1600	1.336820994	4.84408-06	5700	1.33675658	3.79686-06	-0.000064415	hardly any signal was seen so repeated (4th)
IgE 1ppm 4th 070704b	5700	1.336756579	3.79686-06	7200	1.33677097	4.54203-06	0.000014388	
IgE 5ppm 1st 070704a	300	1.336767035	3.27052-06	2200	1.33678124	3.81754-06	0.000014205	
IgE 5ppm 2nd 070704a	4000	1.336764859	5.15096-06	5700	1.33679148	3.23245-06	0.000026622	
IgE 5ppm 3rd 080704a								no signal seen
IgE 5ppm 4th 080704a	9600	1.337221233	4.32227-06	11600	1.33723805	4.64445-06	0.000016813	
IgE 10ppm 1st								no buffer run after sample so no results gained
Sensor 5 Results								
Thiol 090704a	1550	1.358698107	3.53553-06	13300	1.35924995	5.08218-06	0.000016813	
Streptavidin 090704b	2000	1.33114825	3.5617-06	2800	1.33209664	5.6137-06	0.00094839	
Aptamer 130704a	2300	1.336974067	6.06415-06	6300	1.33705158	4.80408-06	0.000077512	
IgE 1ppm 1st 130704a	6300	1.337051579	4.80408-06	9700	1.33707209	5.35053-06	0.000020511	
IgE 1ppm 2nd 130704a/b	13300	1.33702903	3.96855-06	1800	1.33699811	4.04928-06	-0.00003091	very little binding seen plus an initial dip at start of IgE sample
IgE 1ppm 3rd 130704c	130	1.336978489	4.69078-06	1400	1.33698121	4.43842-06	0.000002722	
IgE 1ppm 4th 130704c	2500	1.336979409	3.696-06	4000	1.33698333	4.16179-06	0.000003916	
IgE 2ppm 1st 140704a	3000	1.337662342	5.08527-06	5700	1.33761564	5.11062-06	-0.000046702	
IgE 2ppm 2nd 140704a	7500	1.337595212	5.71825-06	9400	1.33759346	4.48803-06	-0.00000175	

APPENDIX M –Lysozyme BIAcore Sensor Chip Analysis

Sensor No. & Type	Aptamer Binding (RU)	Control Binding (RU)	Binding Events	Experiment Aims
1 CM5 FC1234	No thermo-treatment used.=very little binding FC1 241, FC2 218, FC3 326, FC4 364.		The RB was HBS-EP at a flow rate of 5µl/min. NHS+EDC-11 minutes, 35µl, FC1-4, -4.5, -4.1, -1.6, -3.2 50µl streptavidin injection, FC1-4, 122, 101, 205, 278 Followed by 35µl ethanolamine, FC1-4, 243, 212, 234, 372 Used Ap buffer for activation and AP immobilisation 2x10-6pMol Ap injected. 75µg/ml LZ sample tried	To test the preconditioning regime & compare the results from IT.
2 CM5 FC1234	Thermo-treated 95^0C 3mins (PCR) FC1 157, FC2 125, FC3 360, FC4 211.		Chip conditioned as above with NHS+EDC, strep, ethanolamine NHS-EDC, FC1-4, -4.2, -3.9, -3, -2.8 Strep FC1-4, 41, 31, 73, -124 Ethanolamine FC1-4, 262, 217, 428, 233 2x10-6pMol Ap injected.	To test the preconditioning regime & compare the results from IT.
3 CM5 FC1234	Thermo-treated Ap (PCR) FC1 977, FC2 704, FC3 6.4, FC4 -68. 2^{nd} Ap injected, untreated, FC1 964, FC2 676, FC3 -84, FC4 -176 3^{rd} Ap injection, FC1 85, FC2 98, FC3 63, FC4 80		Conditioning carried out using HBS-EP buffer, then buffer changed to Ap buffer. NHS-EDC, FC1-4, 378, 263, 24, 158 Strep FC1-4, 969, 715, 4.8, 59 Ethanolamine FC1-4, 977, 8.3, 368, 511 Immobilisation of the strep is uneven across the chip. Need to do separate immobilisations running one FC at a time. 2x10-6pMol Ap injected. 75µg/ml lysozyme injected (volume too small for 4 flow cells) FC1 943, FC2 297, FC3 228, FC4 276 Regeneration=10mM HCl= Not successful. FC1 317.4 RU↓27.2 FC2 378.3 RU↓-66.3 FC3 -156.6 RU↓118.5 FC4 485.8 RU↓-73.1	Test the preconditioning regime using HBS-EP buffer
4 CM5 FC1234	Thermo-treated Ap (PCR) FC 3+4, -33, -27 Thermo-treated Ap (PCR) FC2, 0	Poly-T FC1 50, 2^{nd} 60	Bought Poly-T control, 80mer 1µM in Poly-T buffer NHS-EDC FC1-4, 393, 578, 103, -45 Strep FC1-4 4128, 4464, 3190, 3067 Ethanolamine FC1-4, 4120, 4548, 3379, 3291 Poly-t was injected twice due to such a small amount of binding need to change RB before injection. Did not change buffer for Ap injection. RB changed for injection onto FC2. PCR machine has a cooling phase at end of heating cycle so need to change treatment method. 1µM Ap injected. 75µg/ml LZ injected 75µl x4 volume- no binding	Immobilisation of Control - Poly-T Volume modifications
5 CM5 FC1234	Treated BH Ap FC3+4, 41,-9.2	Poly-T FC1 174.8-no	All injected fluids were increased in volume by 4 to cover all 4 FC's. NHS-EDC FC1-4, 694, 1094, 1138, 341 Strep FC1-4 3975, 4320, 3913, 3690	Volume modifications for combined FC Strep Immobilisation

		binding overall	Ethanolamine FC1-4 -16, -28, 7.7, 62 Before Poly-T Immobilisation the RB was changed to Ap buffer from HBS-EP- no binding overall, need to change RB to poly-t buffer just for immobilisation. Ap buffer=RB 1µM Ap injected x4 volume. 75µl of 75µg/ml LZ x4.5 volume injected. FC1-4 89.2, 135, 134, 146. Regeneration10mM HC x3. no poly-t binding to LZ. Regen. unsuccessful.	
6 CM5 FC1234	BH treated Ap FC2 -8.3,33.6	Poly-T FC1 0 binding	Reverted back to standard method as the 3000 model was explained. These volumes are the standard method for single FC immobilisation. NHS-EDC FC1-4, 391, 238, -4.3, -23.7 Strep FC1-4 680, 624, 180, 163 Ethanolamine FC1-4 695, 654, 248, 252 Poly-T 1µM injected while in Poly-t RB. 1µM Ap injected yielding no result, tried 2µM small amount of binding. 0.05µg/ml LZ injected. FC1-4 5, 18.5, -1.3, 4 Regen. 10mM HCl x3 without success. EtOH+NaOH 50% 20µl injected with out success.	To get the running buffers right for each immobilisation.
7 CM5 FC1+2	BH treated Ap FC2 1618.5	Poly-T FC1-8.2 no binding	Buffers were changed according to the immobilisation. NHS-EDC FC1-2, 404, 284 Strep FC1-2397, 409 Ethanolamine FC1-2 36.9, 51 Poly-T 5µM injected Ap 5µM injected, slow association but an actual binding event. 0.1µg/ml LZ injected. This was diluted in Tris which caused a change in the RI. FC1-2, 293.8, -59.7. 0.2µg/ml LZ FC1-2, 38.8, 83.8	To increase the immobilised Ap & control
7 CM5 FC3+4	BH treated Ap 5µM 853.2 reducing to 168.9	Poly-T FC3 -83.9, 2nd -247.5	NHS-EDC FC3+4 380, 307 Strep FC3+4 417, 357.5 Ethanolamine FC3+4 369, 317 50µM Poly-t injected on to FC3. Poly-T buffer used for RB. 5µM Aptamer injected. Not much binding took place, that that did dissociated. A degree of aptamer had bound but the signal refused to stabilise.	To increase the Poly-T binding
8 SA FC1	BH treated Ap Fc1 41 2nd untreated Ap407.1		No preconditioning with 3x 1 minute injections of 1M NaCl + 50mM NaOH as this was a free chip from a biacore rep and came without instructions. Used Ap buffer for RB, injected 1µM Ap. A binding did take place but then after a few hours of the buffer being run the signal was very unstable and the Ap may have dissociated. A 2nd 1µM Ap was injected showing a binding signal of 407.1RU but again after running the buffer reduced to 218.9RU.	Trial of an SA sensor chip
8 SA FC2	Untreated Ap FC2 4µM 40.3RU?		Preconditioned the chip with 3x 1 minute injections of 1M NaCl + 50mM NaOH with HBS-EP RB. Change RB to Ap buffer for the Ap injection. The signal dropped drastically. Suspected binding of 40.3RU. But air bubbles hamper the actual signal diagnostic.	Trial of SA chip after preconditioning
8 SA FC3	Untreated Ap FC3 24.3		Fresh Ap buffers were made. FC3 was not preconditioned to test the necessity of the preconditioning treatment. $2x10^{-12}$µM Ap injected. Some binding was present which remained stable.	Testing the requirement of the Preconditioning of the SA chip with my Aptamer
8 SA	BH treated Ap FC4 -3.6		Engineers visit, new IFC. Repeat of 8 SA FC2 with extra sonication of the fluids to prevent further air bubbles interfering with the signal.	Preconditioned SA chip-repeat of 8 SA FC2

FC4	2nd Ap 15 3rd Ap 55.6		HBS-EP RB, preconditioned chip. The baseline signal was very unstable with both the HBS-EP buffer and the Ap buffer. May need to make up fresh every day to prevent impurities forming. Injected $2x10^{-12}$µM Ap. No signal seen. A second Ap injection was made $2x10^{-12}$µM. A third Ap injection of $2x10^{-12}$µM. No saturation of the chip reached. LZ sample made in Ap buffer, 0.05µg/ml. Response of 1.7RU but the signal decreased over a couple of hours by 1000RU due to an air bubble. 0.1µg/ml LZ injected, another air bubble disrupted the signal so no result. 2nd LZ injection 20.9RU.	
9 SA FC1+2	BH treated Ap FC1+2 620, 547	Poly-T FC3 1070.8	Preconditions FC. Injected $4x10^{-11}$µM Ap, good binding which remained stable after injection ended. Poly-T injection of 1µM. 1070.8RU produced but was obviously an air bubble disrupting the signal. FC4 had nothing immobilised-double negative control. 0.01µg/ml LZ. FC1-4, 235.9, 194, 1.9, 4.9. Regenerations, 1mM HCl 5µl, 5mM HCl 5µl, 10mM HClx3 5/10/20 µl, 20mM HClx2 5/10 µl, 50% EtOH+NaOH x5 5/10/20/30/40 µl. after regenerations the LZ remaining was FC1-4, 52.4, 6.7, -4.7, 93.7. FC4 bound to the regen. solutions. (*Left to run in buffer over night) The flow path was changed and FC1 was regenerated alone with 50% EtOH+NaOH 20µl. This final regeneration was not actually required. The starting baseline of FC1 was 25834.9RU, the final baseline from the previous day (*) was 25916.7RU. After this final regeneration the baseline was 25772.7RU; some Ap was lost due to this. Regeneration appears to take place with prolonged buffer exposure. 0.02µg/ml LZ injected. FC1-4, -725.9, -613.7, -271.4, 22.9. Again these results are due to air bubbles. 2nd injection of 0.02µg/ml LZ FC1-4 1.8, 6.8, 19.6, 3.2.Regeneration 50% EtOH+NaOH 10µl. Further immobilised ligand was lost from all FC's. 3rd injection of 0.03µg/ml LZ. FC1-4 -27.1, -29.4, 1, -35.6. No LZ was binding.	Increased concentration of Ap Trial of regeneration solutions & exposure limits
10 SA FC1+2	BH treated Ap FC1 491.5	FC2 Poly-T 176.5	Preconditions FC 1+2. Injected $2x10^{-15}$ used to lower the signal. (Conflicting ideas here, wasn't sure who much Ap to immobilise. At this point thought 50-100 was enough, hence the reduced amount of Ap immobilised.) The difference between this result and the 9SAFC1+2 is that this was only injected onto 1 flow cell. The sensorgram was ended for the Poly-T injection. When restarted the baseline had fallen by 291.4RU. This may show a loss in Ap bound. Injected 0.1µM poly-T. Good binding seen but an air bubble disrupted the signal. 0.01µg/ml LZ injected. FC1+2, 97.8, 186 Regen. 5mM HCl x3, 20µl, 10mM HCl x3 10µl, 50% EtOH+NaOH x2 10µl. Regeneration successful on FC1 but not on FC2. Left in RB overnight. 0.02µg/ml LZ injected. FC1 dissociated gradually over several hours but FC2 remained stable & bound. FC1+2, 8.8, 119.9. Regen, 10mM HCl x2 10µl. Regeneration caused a small amount of AP loss & no reduction on FC2/ Injection of 0.03µl showed the AP was deactivated as no binding took place.	To run experiment in full altering concentrations of Poly-T & Aptamer

10 SA FC3+4	BH treated Ap 15.3RU	Poly-T FC3 104	Preconditioned FC3+4 together. FC4 does not get the thorough cleaning as FC3; need to precondition separately. RB Poly-T, 0.1µM injected. Good binding seen. Injected $2x10^{-12}$ Ap. Not much binding evident. Injected 0.01µg/ml LZ. FC3+4 83.4, 45.9. Regeneration 0.1M C6H8O7 (sodium citrate) x2 10µl, 50µl, 1M C6H8O7 10µl, 50% EtOH+NaOH x2 10µl, 5µl. Regen successful on FC4 Ap but not on FC3. Injected 0.01µg/ml LZ FC3+4 377.4, 26.8. Regen. 50% EtOH+NaOH x3 10µl. Regen successful on FC4. 0.01µg/ml LZ injected, FC3+4 181.8, -65.2. Ap activity was reducing with each regeneration until it finally deactivated.	Try to get equal binding (in RU regardless of true conc.) of Poly-T & Ap Exploration of different regeneration solutions
11 SA FC1+2	BH treated Ap FC2 719.7	Poly-T FC1 - 113.6	Preconditioning took place on individual flow cells. Poly-T injected $2x10^{-6}$µM no binding present possibly due to air bubbles in the system again. $2x10^{-6}$µM Ap injected. Good binding curve seen. 0.01µg/ml LZ injected. FC1+2 61.8, 127.2. Regen. 50% EtOH+NaOH x4, 10µl, 5µl, 10µl x2. Fc1 was regenerated to -2.7, Fc2 regenerated to 10.6 (not completely but hard to judge how much regen solution to use to prevent loss of Ap bound.) 0.01µg/ml LZ injected. Fc1+2 40.3, 84.2 Regen 50% EtOH+NaOH x2 10µl Regeneration not successful. Fc1 -99.8, Fc2 130.2. Ended sensorgram at this point and flushed to relieve air bubble. Restarted with 0.01µg/ml LZ. FC1+2 28.9, 115.8 Regen. 50% EtOH+NaOH x7 10µl, 20µl x3, 10µl x3*. lost -5.4 Ap with this final injection. *These injections were reduced due to a refill of the vial with fresh regen solution. With exposure to air the ethanol evaporates making the regen solution weaker. RB overnight. Injected 0.02µg/ml LZ. FC1+2 -1.6, 45.7. Regen 50% EtOH+NaOH 10µl. total loss of poly-T & Ap. FC1+2 -354.9, -220.7	Use of the same concentration of Poly-T & Ap
12 CM5 FC1		Poly-T FC1 FC1 - 30.7. 2^{nd} Poly-T FC1 -30.2	Used an unmodified blank CM5 to test for NSB of Poly-T. Poly-T 0.1µl injected. No binding 2^{nd} 0.1µl Poly-T injected. No binding No binding took place showing Poly-T is not non-specifically binding to the chip.	Test for Poly-T Non-Specific Binding
13 SA FC1+2	BH treated Ap FC2 232.7		Preconditioned FC's separately. FC1=poly-T absent FC2 $2x10^{-6}$µM injected. Not a good binding. Think the RB had not been flushed through properly before it was changed to the Ap buffer. 0.01µg/ml LZ injected FC1+2, 135.3, 151.1. Regeneration using 1M $MgCl_2$ 10µl-No major effect seen. 50% EtOH+NaOH 10µl x2, 5µl x2. 0.1µg/ml LZ. FC1+2, 95.5, 93. Regen, 50% EtOH+NaOH x3, 10µl x2, 5µl. Lost -21.7 Ap on FC2.	A blank chip used as a control instead of Poly-T to test for NSB of LZ Also regeneration scouting continued.

			0.01µg/ml LZ injected. FC1+2 87.3, 50.2 Regen. 50% EtOH+NaOH 10µl FC1+2 83.9, -28.7. Aptamer loss. This work showed the difficulty in accurately regenerating the chip surface without loses bound aptamer.	
13 SA FC3+4	BH treated Ap FC4 10.9 2nd injection 47.7		Preconditioned FC's separately. No Poly-T immobilised, to test for NSB of LZ. AP 2x10-6 injected FC4. Not much binding took place, question the viability of the aptamer? Or could have been day old buffer. Need to remake buffer every week and change daily. 0.01µg/ml LZ injected. FC3+4 204.1, 189.7. This shows NSB is taking place on FC3. Regen 50% EtOH+NaOH x4 10µl x2, 5µl x2. FC3+4 132.4, 12.8. 0.01µg/ml LZ injected. FC3+4 182.9, 60.2. reduced amount of LZ-Ap binding. Regen. 50% EtOH+NaOH x3, 10µl x2, 5µl. FC3+4 155.3, 11. Even without loss of Ap from the surface the small amount of remaining binding reduces the amount of potential binding. RB overnight. Huge difference in baselines, FC3+4 279.7, 182RU due to overnight soak in RB. 0.01µg/ml LZ injected. FC3+4 142.9, 107.1. An overnight soak has removed the remaining LZ from the Ap of the previous day, rejuvenating the Ap's binding potential. Regen. 50% EtOH+NaOH x7 10µl, 5ul x4, 10µl, 5µl. FC3+4 had 59.1, 10.5 LZ left bound. 0.02µg/ml LZ injected. FC3+4 58.9, 9.5. After several regenerations the aptamer is no longer viable.	Test to prevent Ap loss during regeneration. Which is better a small amount of loss during regeneration or a little analyte still left bound??
14 SA FC1+2		Poly-T FC1 30.6	Preconditioned FC's separately. FC1 Poly-T injected $2x10^{-6}$. No binding took place after 2 injections so increased the concentration. 0.001µM injected. Good binding produced. $2x10^{-6}$ Ap injected FC2 48.4, 2nd injection 98.5. This could show that layering is taking place, where aptamer is binding to the streptavidin, and then when a 2nd injection of Ap takes place the Ap is simply binding to the primary Ap layer. RB HBS-EP. 0.01µg/ml LZ injected. No binding took place. The RI changed but after several minutes after the end of the injection the baseline retuned to the preinjection level. This showed that binding is very buffer-sensitive. RB Ap buffer. Injected 0.01µg/ml LZ- no binding took place. Flushed the system as the baseline appeared to be increased. Injected 0.01µg/ml LZ. FC1+2, 1.4, 10.6. Regen. 50% EtOH+NaOH x3 5µl x2, 5µl (fresh aliquot). FC1+2 -75.3, -62.3 Not convinced Ap was bound properly here so not a fair test.	Replacement of the Ap running buffer used throughout all LZ injections with HBS-EP
14 SA FC3+4	Ap untreated FC4 308.7	Poly-T FC3 80RU	Preconditioned separetly FC3+4 FC3 poly-t injected 100µl 0.1µM- good binding seen. Biotin used to block unbound strep sites, 1mM 10µl- this reduced bound poly-t slightly. Untreated Ap injected, 100µl 0.002pmol. the refractive index changed even though the Ap and RB were the same. Suspect Ap degradation at this point. Although good binding seen. LZ trial, 75µl 0.01µg/ml- not much binding, FC3+4 6.9, 7.3 Regeneration 50% EtOH+NaOH, lost a lot of ap and poly-t binding, FC3+4 -53.9, -81.9	Test to see if Biotin blocking on the Control FC reduces NSB

			Left in RB over lunch. Tried a 2nd 0.01µg/ml FC3+4 4.1, 5.7. As the binding in such a small amount did not want to risk losing any more Ap of poly-t so did not regenerate. 3rd LZ 0.01µg/ml FC3+4 10.1, 8 Did not regenerate again as binding was so small. 4th LZ 0.05µg.ml FC3+4 12.8, 6.6. Again not much binding so no regeneration. 5th LZ 0.05µg/ml FC3+4 18.2, 8.8. Did not regenerate just left in standby mode overnight. It seems that the LZ is binding non specifically to the chip because even though a large amount of Ap and Poly-t was lost the LZ still bound. After another overnight soak in RB a further LZ injection was made to properly make sure the Ap was dead. 0.01µg/ml LZ injected. 2RU bound on Ap but nothing on Poly-T. The Ap must have been denatured.	
15 CM5 FC1			RB=HBS-EP NHS+EDC FC1+2, 480, 456 Strep FC1+2, 629, 664 Ethanolamine FC1+2, 570, 594.7 Changed RB to Ap buffer, baseline to ages to stabilise. FC1 0.75µg/ml LZ injected. FC1-0 binding 2nd LZ injected FC1-0 binding 3rd LZ injection FC1-0 binding. Therefore the LZ is specifically binding to the AP and poly-t. Perhaps because of charge.	Test to see if LZ is non specifically binding to strep
15 CM5 FC2&1 (FC1 reused as no binding)	Ap FC2 untreated 15.7RU		Immobilised $2x10^{-6}$pMol Ap Tested 0.05µg/ml LZ on to FC1+2, no binding on either. Suggesting that heating the Ap is needed or the Ap aliquots have denatured over time.	Test to see if the CM5 chip (above) is working by immobilising Ap
15 CM5 FC3+4			Primed with HBS-EP buffer. NHS+EDC FC3+4, 386, 383.5 Strep FC3+4, 817, 833 Ethanolamine FC3+4, 854, 872.4 Changed buffer, primed, FC4 Poly-t 0.1µM immobilised 18RU. Baseline 22312.8 Changed buffer, primed FC3 $2x10^{-6}$pMol 12RU. Baseline 23147.5 Left in RB for several hours (Computer crashed, restated and primed with Ap buffer) Baseline after several hours of RB FC3+4 23218.2, 21585.5 – Shows that some binding is lost over several hours, but the priming may also have caused a loss. 0.02µg/ml 75µl injected LZ FC3+4, 11.3, 3.1. Left in buffer for several hours to see if binding dissociated over time. FC3+4 13.6, 0.7 FC3+4 14.7, -1.6 FC3+4 59.7, 10.8 LZ stayed bound to the Ap but released from the poly-t. after several hours though binding of the buffer increased on both FC's.	To test whether the Ap and Poly-t are staying immobilised after prolonged exposure of RB Test with LZ to see if the Ap & Poly-t left bound are active

16 SA FC1+2	BH treated Ap $2x10^{-3}$ pMol – 250RU	Poly-t FC1 242RU	Forgot to precondition the chip. Fresh poly-t buffer, primed, flushed x2. 0.1µM injected, only 75µl injected by mistake. Left in RB for a couple of hours-remained stabile. Primed with Ap buffer. Only injected 75µl. but got good binding that remained stabile over several hours. Made several LZ injections with binding increasing with the increasing concentration of LZ (but only on FC2). FC1, 2 0.02µg/ml- -0.7, 3 FC1, 2 0.05µg/ml- -1.3, 4.2 FC1, 2 0.1µg/ml- -2.7, 5.8 FC1, 2 0.15µg/ml- -2.9, 9.6 FC1, 2 0.2µg/ml- -5, 11.3 FC1, 2 0.25µg/ml- -7.5, 19.7 (left over night) FC1, 2 0.3µg/ml- 5.3, 15.7 FC1, 2 0.4µg/ml- 4.8, 30.5 (flush) FC1, 2 0.5µg/ml- -0.6, 13 calibration curve constructed. Overnight, primed, flushed with new buffer due to computer crash FC1, 2 0.01µg/ml- 10.4, 18.4 FC1, 2 0.02µg/ml- 22.1, 39.5 FC1, 2 0.03µg/ml- 54.3, 37.8 FC1, 2 0.05µg/ml- 37.8, 67.6 Regenerated with EtOH+NaOH (25mM 50%) FC1, 2 0.01µg/ml- -69.4, -26.5= no binding Ap and poly-t lost off surface. Need to make regen solution up fresh each day as the ethanol seems to be evaporating.	Test for stability of immobilised ligands. Test to see if a calibration curve could be produced (without regenerations inbetween).
16 SA FC3+4	BH treated Ap $2x10^{-3}$ pMol –80RU	Poly-t FC3 85RU	Again forgot to precondition chip. Poly-t buffer, primed, flushed x2, 0.1µM poly-t injected, 75µl FC4 Ap buffer primed, flushed x2 75µl Ap 2x10-3 injected FC3, 4 0.02µg/ml- 12, 11.9 FC3, 4 0.05µg/ml- 41.7, 31.1 FC3, 4 0.1µg/ml- 83.5, 60.4 FC3, 4 0.15µg/ml- 21, 15 FC3, 4 0.2µg/ml- 42.4, 30.2 FC3, 4 0.25µg/ml- 66.5, 45.5 FC3, 4 0.3µg/ml- 89.9, 61.7 FC3, 4 0.4µg/ml- 105.3, 72.2 FC3, 4 0.5µg/ml- 128.4, 87.8 Regenerated, fresh 25mM 25% EtOH+NaOH, 5µl, 10µlx2, 5µl- successful regeneration back to baseline from previous day.	Repeat calibration curve with regenerations
			Ap RB X2 0.05µg/ml, FC3+4 12, 10.6, 27.5, 23.1 Regenerated fresh 25mM 25% EtOH+NaOH but lost all binding of ligand. 0.05µg/ml, FC3+4 -116.6, -41.5	Show reproducibility of LZ binding after regeneration.

			Reused this damaged chip. All regeneration solutions were 25mM EtOH+NaOH, and various percentages of these. All were carried out on the Ap surface. Old=25mM 25% EtOH+NaOH New=25mM 25%, 50% & 100% EtOH+NaOH Mid End Old 25% 1248.3 38.1 New 100% 233.5 137 New 50% 1155.5 230 New 25% 1481.1 264.4 This showed that regeneration is different depending on the surface as the poly-t surface gave much different results. Will try different percentages of 10mM.	Tested the strength of regeneration solutions comparing old v newly made.
17 SA FC1+2	Ap FC2 HB treated 262.3RU	Poly-t FC1 255RU	Preconditioned FC1, changed buffer to poly-t T RB, primed. Injected 75µl 0.1µM poly-t-good binding. Flushed with HBS-EP buffer, Preconditioned FC2. Changed buffer to ap RB, primed. Injected 75µM $2x10^{-3}$pMol HB treated-good binding but did not save graph. 0.02µg/ml FC1+2= -1.2, -1.1 0.03µg/ml FC1+2=-2.7, 2.5 0.05µg/ml FC1+2=-3.8, 8.2 0.1µg/ml FC1+2=-6.9, 9.4 0.15µg/ml FC1+2=-7.7, 10 0.2µg/ml FC1+2=-12, 25 0.25µg/ml FC1+2=-11.4, 28.7 0.3µg/ml FC1+2=-10.7, 31.7 0.4µg/ml FC1+2=-10.4, 34 0.5µg/ml FC1+2=-1, 13.2 As binding was increasing, tried to regenerate with 25% 10mM EtOH+NaOH but lost all bound.	Repeat of calibration curve
17 SA FC3+4	Ap FC4 BH treated 111RU	Poly-t FC3 36.5RU	Preconditioned FC3+4, Used week old poly-t buffer which limited binding of poly-t (pH must be affected in the tris, which affects the binding affinity). Used fresh ap buffer for analyte immobilisation. A negative analyte was used, BSA dilution. Used because it was easily available, however MW was 66kDa 0.01µM BSA (in water) x2 both removed aptamer/poly-t 0.01µM Serum LZ (200µl of LZ dilution + 1µl serum=1/200) 0.1µM Serum LZ = both serum dilutions removed aptamer.	Trial of a negative analyte BSA and serum sample
18 SA 1+2	Ap FC42BH treated 259.8RU	Poly-t FC1 228.7RU	Preconditioned FC1+2. Injected 75µl 0.1µM poly-t-good binding Injected 75µl 2x10-3pmol Ap, good binding 0.01microg/ml LZ, Fc1+2 1.4, 4.1 0.01microg/ml SLZ Fc1+2 14.4, 22.8 0.01microg/ml LZ Fc1+2, 14.7, 22.7 0.1microg/ml LZ Fc1+2, 16.3 29.1 0.1microg/ml SLZ Fc1+2 17 32.9 1mM HCl Fc1+2, 21.9 99.3	To repeat the injection of serum samples and try regeneration

			10mM HCl Fc1+2, 32 131.2 1M NaCl2 Fc1+2 -15 156.2 1M NaCl2 Fc1+2 -17 156.8 1M NaOH Fc1+2 -128.9 -284.5 The final regeneration did not reduce the amount of analyte bound but it reduced the signal too far destroying the receptor. Will try a lower concentration in future.	
18 SA 3+4	Ap FC4 156RU	Poly-t FC3 91.7RU (but falling)	Preconditioned chip, changed buffer to poly-t and primed. 0.1µM poly-t injected (this was old buffer which showed the poly-t slowly unbinding over time.) Changed buffer to Ap, primed. Injected $2x10^{-3}$ Ap. Again old buffer used so limited binding. Injected 0.1µg/ml LZ FC3+4, 16.5, 5.8 Signal was unstable to flushed. Injected 0.1µg/ml LZ FC3+4, 20.5, 16.3 5µl 1mM NaOH, -148, -113.8 -much binding lost. Injected 0.1µg/ml LZ FC3+4, -128.3, -98 NaOH destroyed chip	Repeat of calibration curve with new regeneration solution
19 SA FC1+2	Ap FC2 205RU	Poly-t FC1 237RU	No preconditioning. All fresh buffers used. FC1 primed with poly-t buffer, flushed, 0.1µM poly-t injected. FC2 Ap buffer primed, 75µl $2x10^{-3}$ Ap injected. Injected 0.1µg/ml LZ FC1+2, 4.9, 5. regenerated itself over 15 minutes of running buffer. Injected 0.2µg/ml LZ FC1+2, 15.4, 1.9, left over lunch and baseline increased due to air bubble. Injected SLZ 1/200 FC3+4, not enough sample, so stopped injection. 19µl injected of SLZ FC1+2, 566.7, 329.1 Regenerated with 50% EtOH+NaOH 25mM- regeneration successful but the chip was unusable afterwards. Injected 0.1µg/ml LZ FC1+2, 5.7, -12.5. Need to try reducing the concentration of regen solution as its too harsh for the aptamer.	Trial of serum LZ samples with regeneration
19 SA FC3+4	Ap FC4 195.9RU Ap FC3 173.1RU	Poly-t FC3 253RU Poly-t FC3 91.5RU	Ran unclog, as FC3 was producing a poor signal output. Preconditioned chip Changed buffer to poly-t, primed, injected poly-t 0.1µM Changed buffer, primed, injected $2x10^{-3}$ pMol Ap Injected 0.01µg/ml LZ FC3+4, 9.5, 3.3- strange binding pattern-baseline set Injected 0.02µg/ml LZ FC3+4, 12.8, 6.2 Regeneration using 25mM EtOH+NaOH 25%, ~50RU lost on both FC's-set baseline Injected 0.01µg/ml LZ FC3+4, -10.1, 0.3 Injected 0.01µg/ml LZ FC3+4, 6.5, 4.6- binding does take place after the regeneration which suggests that the aptamer and poly-t might not be being removed. New sensorgram as signal was increasing. Injected 0.01µg/ml LZ FC3+4- no binding Reinjected Ap on to FC3 and then changed buffer and reinjected poly-t. – good amount of binding of both. Injected 0.01µg/ml LZ FC3+4, 4.9, 8.7-binding still took place be was not a clean as before as more binding was taking place on the control than before, but less compared to the Ap.	Test for regeneration between analyte injections & whether a second injection of aptamer can be made after regeneration has failed.

20 SA FC1+2	Ap FC1 128.9RU	Poly-t FC2 110RU	Preconditioned chip Changed buffer to poly-t, primed, injected poly-t 0.1µM Changed buffer, primed, injected $2x10^{-3}$ pMol Ap Injected 0.1µg/ml –ve analyte FC1+2, 194, 138RU huge amount of binding present. Several regenerations carried out, 25% EtOH+NaOH, 5µlx2, 10µlx2, 20µlx6, 10µl, 5µl-regeneration successful. Injected 0.2µg/ml LZ FC1+2, 5.5, 3.3 hardly any binding Regeneration 25% EtOH+NaOH 5µlx2. no regeneration successful.	Test for –ve analyte binding-Myoglobin from Equine Heart
20 SA FC3+4	Ap FC4 115.2RU	Poly-t FC3 123.9RU	Preconditioned chip Changed buffer to poly-t, primed, injected poly-t 0.1µM Changed buffer, primed, injected $2x10^{-3}$ pMol Ap Injected 0.1µg/ml LZ FC3+4 11.1, 2.8 Injected 0.1µg/ml LZ FC3+4 21.8, 4.8- regeneration not needed as after a while the LZ was freed. Injected 0.1µg/ml myoglobin FC3+4, 243.4, 148.5-several regenerations carried out with 25% EtPH+NaOH 10µl, 20µlx4, 10µl, 20µl regen successful. Injected 0.1µg/ml LZ FC3+4-no binding.	Test for –ve analyte binding
21 SA FC1+2	Ap FC2 226.4 RU Ap FC2-no binding	Poly-t FC1 180.6 RU	Preconditioned chip separately. Changed buffer to poly-t, primed, injected poly-t 0.1µM Changed buffer, primed, injected $2x10^{-3}$ pMol Ap Injected 0.1µg/ml LZ FC1+2, binding on both. Left in continuous flow over lunch. As binding was falling off, increased flow rate to 10µl. left to see if ap and poly-t would regenerate. (Buffer needs to be left on a magnetic stirrer. No matter how long its sonicated for bubbles still appear.) Signal did not reach the baseline as air bubbles increased the signal. On FC1 binding was present but returned to the baseline after continuous flow. Ap binding was stable at end of injection. Stopped sensor-gram and flushed, when restarted the baseline was not restored. Injected 0.1µg/ml LZ FC1+2, 6.6, 7.3 regenerated with 25% EtOH+NaOH, regen successful. Injected 0.1µM myoglobin FC1+2, 2295.7, 427.7. Again left in continuous flow to see how much dissociation would occur. Binding on FC1 was very fast, lots of binding taking place where as the binding on the Ap was much slower. As dissociation was occurring the flow rate was increased to 50µl. Dissociation stabilised after a few hours. Stopped sensorgram and flushed. End of sensorgram FC1, 27277.2, FC2, 26715.3 Beginning of new sensorgram FC1, 27268.5, FC2, 26840.3 Next day, Made fresh buffer. Injected 0.1µg/ml LZ FC1+2, 4.6, 22.2 regenerated with 5µl 25% EtOH+NaOH, regen successful. Injected 0.1µg/ml LZ FC1+2, 5.9, 19.9 regenerated with 5µlx2 and 10µlx2 25% EtOH+NaOH, regen not achieved. Preconditioned FC2 and reapplied aptamer. (Did not change buffer for preconditioning.) Injected 0.1µg/ml LZ FC1+2, -1.7, 6.3 some binding taking place but is reduced. 5µl 25% EtOH+NaOH, regen successful.	Regeneration with continuous buffer & 25% EtOH+NaOH To test how many times LZ will bind and regenerate and test secondary Ap binding.

			Injected 0.1µg/ml LZ FC1+2, 3.7, 9.1. 5µl 25% EtOH+NaOH, regen not successful.	
21 SA FC4	Ap FC4-66RU		No preconditioning of chip surface. Injected Ap. Strange binding, huge difference in refractive index even though the Ap was in the same buffer. Injected 0.1µg/ml LZ FC4, 14RU Stopped sensorgram 26948.0 Changed temperature to 40^{o}C Restarted sensorgram 24931.9 Difference of 2016.1RU, suggesting the Ap and LZ have both been removed. Need to try adding more Ap to surface to check if it's still in tact. Then try a smaller temperature change.	Does changing the temperature regenerate the chip?
22 SA FC1+2	FC2 Ap 56.6RU FC2 Ap 92RU FC2 Ap 37.3RU	FC1 Poly-T 201.2RU	Queried the quality of the preconditioning step when both FC's were preconditioned together. Preconditioned FC's separately. Changed buffer, primed + flushed. Injected 0.1µM poly-t Changed buffer, primed, flushed. Injected 2x10^{-3} Ap. Binding wasn't great with the Ap. No explanation for it. (Engineers visit) Injected a 2nd round of Ap, not much bound again. Suspect that the aliquot in the small PCR tubes is hampering the binding. The aliquot, once defrosted is pipetted into a second epindorf, is the Ap stuck to the plastic? Injected 0.1µg/ml LZ FC1+2, 42.5, 59.5. 25% EtOH+NaOH regeneration. Regenerated too far. Changed flow path and injected Ap 3rd round. Again not much bound, will try not changing the epindorf just in case the oligo is attracted to the plastic. Injected 0.1µg/ml LZ FC1+2, 27.6, 4.6. Chip obviously not working, possibly because its one SN gave me and was sitting in ambient temperature for too long.	Testing the quality of the preconditioning; does preconditioning the FC's separately create a chip which binds to the ligand more affectively?
22 SA FC3+4	FC4 Ap 111.8RU FC4 Ap 136.7RU	FC3 Poly-T 334.7RU	Preconditioned FC's separately. Changed buffer, primed + flushed. Injected 0.1µM poly-t Changed buffer, primed, flushed. Injected 2x10^{-3} Ap. Binding was better than before the preconditioning step was changed. Injected 0.1µg/ml LZ FC3+4, -41.1, 22.0RU Regenerated with 25% EtOH+NaOH (Change the flow path as only FC4 needed regenerating) Regen too far. Injected 0.1µg/ml LZ FC4, 3.8RU No binding took place on the FC4. Changed flow path back to FC3+4 Injected 0.2µg/ml LZ FC3+4, -33.7, 14.3 Allowed to dissociate over lunch, no dissociation took place. Regenerated with 25% EtOH+NaOH Fc3+4, -109.6, -11.5-remade fresh regen solution Changed flow path to FC4 only, Injected 2nd Ap. Good Ap binding. Injected 0.1µg/ml LZ FC3+4 20.9, 11.5RU Injected 0.2µg/ml LZ FC3+4, 17.9, 11.5RU Injected 0.3µg/ml LZ FC3+4, 19.0, 10.9	

	FC4 Ap 147RU FC4 Ap 76.9RU		Next day. Changed buffer, primed. Injected 3rd Ap. Good binding. Injected 0.1µg/ml LZ FC3+4, 39.1, 0.3 Regenerated with 25% EtOH+NaOH Fc3+4, -10.2, -82.4 Injected 0.1µg/ml LZ FC3+4, 57.0, 35.4RU Regenerated with 25% EtOH+NaOH Fc3+4, 27.7, 6.0RU Injected 0.1µg/ml LZ FC3+4, 42.2, 22.5RU Regenerated with 25% EtOH+NaOH Fc3+4, 48.3, 5.7 Injected 0.1µg/ml LZ FC3+4, 1.6, 2.1 Regenerated with 25% EtOH+NaOH Fc3+4, -16.7, -13.7 Injected 0.1µg/ml LZ FC3+4, 2.5, 2.2 Regenerated with 25% EtOH+NaOH Fc3+4,-13.6, -13.9RU Made mistake and forgot to change flow path, injected onto both flow cells Stopped sensorgram. Next day. Injected fresh Ap onto FC4. Hardly any binding. Stopped sensorgram.	
23 CM5 FC1			Docked CM5 chip. NHS+EDC 35µl, FC1, 560 50µl streptavidin injection, FC1, 1113.4 Followed by 35µl ethanolamine, FC1, 1064.6 Injected 0.1µM Myoglobin, FC1 47.4RU. Test showed that myoglobin is very sticky and binds to the surface.	Test for non specific binding of Myoglobin onto a mock SA chip
24 SA FC1+2			Preconditioned the FC's separately. Changed buffer to poly-t and primed. Injected 0.1µM poly-t. Changed buffer, injected $2x10^{-3}$ Ap. Injected 0.1µg/ml LZ FC1+2 Stopped sensorgram. Increased temp. This procedure was carried out for each temperature change. (Temperature was very unstable when changed by 0.05. the Biacore tended to settle for temperature that were almost right. All experiments were carried out only when the temperature was stable and set.)	Change of temperature experiement. Temp increased by 5^oC to test for regeneration properties.

Temp Change ºC	**Ap FC2**	**Poly-t FC1**	**LZ**		**Baseline Before Temp Change**		**Baseline After Temp Change**		**Difference in Baselines**	
			FC1	FC2	FC1	FC2	FC1	FC2	FC1	FC2
25-25.2	151	84.2	21	4.8	27171.8	27331.2	27171.9	27328.9	1	-2.3
25-25.3	151	84.2	21	4.8	27171.8	27331.2	27164.8	27321.5	-7	-9.7
25-25.4	151	84.2	21	4.8	27171.8	27331.2	27128.1	27285.2	-43.7	-46
25-25.5	124	94.7	17	10	27183.8	27214.6	27119.0	27150.9	-64.8	-63.7
25-26	79	222	39	26.6	27169.8	27105.9	27033.1	26972.2	-136.7	-133.7
25-30	152.4	202	40	24	27146.3	27395	26443.7	26684	-702.6	-711
25-35	176.1	87	-3.7	33.1	27101.7	27557.5	25708.2	26067.2	-1393.5	-1490.3
25-40	109.2	61.2	8.5	19	27108.1	27539.2	24961.4	25381.0	-2146.7	-2158.2

24 SA FC3+4	FC4 Ap 176.5RU 2nd Ap 131.8RU	FC3 poly-t 66RU	Preconditioned FC3 and 4 separately. Injected 0.1µg/ml LZ- signal kept decreasing-rinsed, but signal still kept decreasing. Flushed Injected 0.1µg/ml, FC3+4, -1.2, -6.6 Injected 0.25µg/ml FC3+4, 0.1, -0.3 Changed flow path and injected a second Ap. Injected 0.1µg/ml-no binding, ended chip. (this chip was undocked and left in the fridge over the weekend in its original sealed packet. The exposure to buffer, undocking, drying out, then exposure to more buffer damages the chip in some why which inactivated its surface for further use. Chips therefore need to be fully exhausted of use before undocked.)	Incorporation of regeneration by thermodynamics with protocol. Try creating a calibration curve.
25 SA FC1+2	FC1 Ap 188.6RU	FC1 poly-t 276.1RU	Preconditioned FC1 and 2 separately. Made fresh buffer.	Repeat of thermo regenerations using buffered LZ samples to produce a calibration curve.

Starting Baseline (FC1+2)	LZ Bound (FC1+2 RU)	Baseline after LZ Bound	Baseline after Change of Temp (25.3°C)	RU loss due to Temp Change	Baseline after Temp is returned to 25°C	Difference in Start & End Baselines (RU)
27521.8	0.1µg/ml 14.6	27536.4	27493.3	43.1	27518.9	-2.9
27772.1	26.7	27798.8	27753.6	45	27780.4	-8.3
27518.9	0.15µg/ml 17.9	27536.8	27496.6,	40.2	27512.4	-6.5
27780.4	27.1	27807.5	27764.2	43.3	27780.7	-0.3
27523.6	0.15µg/ml 14.6	27538.2	27503	35.2	27530.5	-6.9
27793.2	22.7	27815.9	27792.8	23.1	27822.2	-29
27530.5	0.2µg/ml 11.5	27541.9	27500	41.9	27535	-4.5
27822.2	14.6	27836.8	27794.9	41.9	27831.6	-10.1
27535	0.25µg/ml 10.6	27545.7	27514	31.7	27540.3	5.4
27831.6	14.8	27846.4	27818.6	27.8	27847	-0.6

(*where a loss of -1.2 is seem, the baseline has increased by 1.2RU)

25 SA FC1	Previously immobilised		Mass transfer=the ease in which the analyte moves out of solution and binds to the surface layer. Flow rates of 5,15 and 75µl/min are used. All signals produced from the injection should give the same result showing mass transfer is not influenced by flow rate. The results here show mass transfer is not having an affect as all signals coincide with each other. Results for 75µl/min show a spike of air. So this will be repeated.	Mass Transfer, Is Mass Transfer having an effect on analyte binding
25 SA FC3+4	FC4 148.6RU	FC3 313.4RU	Preconditioned FC3 and 4 separately.	Continuation of regeneration by temp changes to construct a calibration curve.

Starting Baseline (FC1+2)	LZ Bound (FC1+2 RU)	Baseline after LZ Bound	Baseline after Change of Temp (25.3°C)	Amount of RU loss due to Temp Change	Baseline after Temp is returned to	Difference in Start & End Baselines (RU)

					25°C	
28116.7 27854.9	0.05µg/ml 1.2 7.1	28117.9 27861.9	25.2°C 28085 27825.5	32.9 36.4	28087.1 27827.2	29.6 27.7
28087.1 27827.2	0.1µg/ml 25.6 30.5	28112.6 27857.7	28081.9 27826.5	30.7 31.2	28085 27828.8	27.6 28.9
28085 27828.8	0.15µg/ml 34.7 46.9	28119.8 27875.7	28117.7 27873.3	2.1 2.4	28119 27876.5	-34 -47.7
28119 27876.5	0.2µg/ml 5 12	28124.1 27888.5	28089.6 27853.9	34.5 34.6	28090.3 27854.6	28.7 21.9
28090.3 27854.6	0.25µg/ml 36 44.6	28127.2 27899.2	28098.9 27869.8	28.3 29.4	28100.3 27869.7	-10 -15.1
28100.3 27869.7	0.2µg/ml 31 37.2	28131.3 27907	28129.1 27903.5	2.2 3.5	28127.1 27901.3	-26.8 -31.6
28127.1 27901.3	0.15µg/ml 9.7 15	28136.9 27916.2	28131.7 27909.6	5.2 6.6	28127.6 27904.1	-0.1 -2.8
28189.8 28035.7	0.1µg/ml 15.9 16	28205.6 28052.3	28200.2 28043.6	5.4 8.7	28200.3 28041.6	-10.5 -5.9
28066.1 28223.9	19.6 21.1	28085.7 28245	28050.2 28207	35.5 38	28051.3 28210.2	14.8 13.7

26 FC1&2	FC2 225RU	FC1 200RU	Made fresh buffers. Docked new chip and preconditioned flow cells separately. Injected 0.1µg/ml SLZ -11.4, 2.4 Changed buffer to poly-t to stop any nucleases in the serum from destroying the aptamer and poly-t	Calibration curve of serum spiked samples with heat regen.

Starting Baseline (FC1+2)	**LZ Bound (FC1+2 RU)**	**Baseline after LZ Bound**	**Baseline after Change of Temp (25.3ºC)**	**Amount of RU loss due to Temp Change**	**Baseline after Temp is returned to 25ºC**	**Difference in Start & End Baselines (RU)**
25075.6 24954.1	0.1µg/ml 50.9 46	25126.1 24996.3	25.2 °C 25114.3 24983.3 25.3 °C 25098.5 24968.5 25.4 °C 25066.9 24938	11.8 13 27.6 27.8 59.2 58.3	25071.1 24946.8	-4.5 -7.3
25087.1 24963.2	0.2µg/ml 28 31	25115.1 24994.2	25.3 °C 25109.4 24986 25.4 °C 25063.8 24942.8	5.7 8.2 45.6 43.2	25094.4 24970.6	+7.3 +7.4
25094.4 24970.6	0.05µg/ml 14.6 26.5	25109 24997.1	25.3 °C 23103.8 24988.9 25053.9 24941.3	5.2 8.2 55.1 55.8	25092.8 24979.1	-1.6 +8.5

SA26 FC3&4	Ap FC4 57.3RU	Poly-t FC3 29RU	Preconditioned chip. Poor binding, no explanation for it. Running buffer poly-T buffer to reduce nuclease damage to Ap and poly-T. Injected 1/200 0.2µg/ml SLZ FC3&4, -3.8, -4.5 Injected 1/100 0.2µg/ml SLZ FC3&4, 1.1, 10 Injected 1/50 0.2µg/ml SLZ FC3&4, 1.0, 18.5 Injected 1/25 0.2µg/ml SLZ FC3&4, -1.2, 29.6 Left over night in buffer Injected 1/400 0.2µg/ml SLZ FC3&4, -4.2, -2.0	Experiment to test which is the best dilution of serum to use.

SA 27 FC1&2	FC2 138.1RU	FC1 71RU	Preconditioned Fc's separately. FC1 poly t unusually low binding again. FC2 ap, slightly lower than usual but not too bad. Baseline FC1&2, 25739.8, 25705.9 Injected 0.2µg/ml SLZ 1/100, 0.7, 25740.6, -7.0, 25699 -hardly any binding Injected 0.25µg/ml SLZ 1/100, 3.2, 25744.3, 2.4, 25698.9 Injected 0.25µg/ml SLZ 1/100, -5.9, 6.3 Try applying LZ, 0.2µg/ml -5.0, -4.1 Injected 0.25µg/ml SLZ 1/100, 0.7, 1.6 Injected 0.25µg/ml SLZ 1/100, 0.7, 3 Gave up as nothing was binding- no explanation. Serum samples, unspiked, naturally containing high lysozyme levels Defrosted samples, D4 10.3, D22 9.2, D54 9.8, D52 9.6, D55 20, D56 10.4, D58 12, N26 8, A15 9.2, A17 8. All samples were diluted 5/500 and spun. Injected 75µl unspiked serum. Massive change in refractive index. Positive samples must contain more crap/bacteria than a negative serum sample. A17 FC1&2, -0.7, 1.8.	Continuation of calibration curve Lysozyme positive samples tested with the biacore method
SA 27 FC3&4	FC4 81Ap	FC3 33.3polyt	Preconditioned both FC's separately. Low binding again, no explanation as buffers were fresh. Injected unspun 0.1µg/ml SLZ FC3&, 0.5, -4.7	

Starting Baseline (FC1+2)	LZ Bound (FC1+2 RU)	Baseline after LZ Bound	Baseline after Change of Temp (25.3°C)	Amount of RU loss due to Temp Change
25653.5	0.05µg/ml SLZ 3101.9	28761.2	26°C 28465.3 27501.4	304.9 242.1
25283.7	2458.7	27743.5	27°C 28268.6 27326.8	492.6 416.7
			29°C 27899.9 26985.1	861.3 758.4
			35°C 26988.6 26093.6	1772.6 1649.9
			40°C 26147.8 25264.2	2613.4 2479.3

SA28 FC1&2	FC2 Ap 200.8	FC1 Polyt 239.1	Preconditioned chip and used fresh buffers. Also ELISA result and Actual BIAcore result	Repeat of clinical samples

Result	*ELISA*	*Final Biacore*
D4 FC3&4, 9.6, 21	10.3	7
D22 FC3&4, 10.6, 22.9	9.2	7.8
D52 FC3&4, 11.3, 25	9.8	8.3
D54 FC3&4, 10.7, 58.1	9.6	26
D55 FC3&4, 8, 23.8	20	10
D46 FC3&4, 7.4, 14.5	10.4	5
D58 FC3&4, 10.5, 32.8	12	16
N26 FC3&4, 6.6, 17.6	8	7
A15 FC3&4, 12.3, 20	9.2	5.5
A17 FC3&4, 3.7, 11.4	8	5.5

SA 29 FC 1&2	FC2 209RU Ap	Blank	Calibration curve using a blank surface as the control. (No poly-t used) 0.05µg/ml 1.8, 56.5	Calibration curve for buffered LZ

			0.1µg/ml -0.2, 61.0 0.15µg/ml -5.3, 66.3 0.2µg/ml -3.1, 71.1 0.25µg/ml -5.8, 54.2 0.3µg/ml 33.4, 89.4 0.4µg/ml 36.4, 95.3 0.5µg/ml 36.1, 62.9 1.0µg/ml air disrupted signal-possibly reached limit. Serum was diluted 1/200 and spin filtered in the usual way. The filtrate was then used both with the ELISA and with injected onto a sensor chip for comparison. Sample V1 FC1&2, 14.4, 37.9 ELISA result 0.063=0LZ Sample V2 FC1&2, 4.3, 34.8 ELISA result 0.068=0LZ	Cross reference of spiked clinical samples with ELISA

6.0 Final Discussion & Conclusions

The focus of this project was to produce a diagnostic test for the determination and quantification of lysozyme in clinical samples. Currently diagnosis involves collation of signs and symptoms from the patient, along with blood analysis and blood cultures for bacterial presence. Current high mortality rates for sepsis show that the diagnostic and management tools used need immediate improvement.

6.1.1 ELISA (Chapter 2)

The aim of the ELISA work was to produce and optimise an ELISA for the quantification of lysozyme in serum samples. A reference range for lysozyme in buffer was also produced for use with the ELISA and the clinical samples. The reference range is test-specific, but proved to be reliable when compared to clinical samples wherein the highest lysozyme levels tallied with diagnosis of sepsis.

The freeze-thaw experiments showed no detrimental effects were being caused by the storage and the repeated thawing of the samples. This therefore should not have caused any ill effects when the clinical samples were processed with a commercially available kit. The commercially available kit was purchased and compared with the ELISA in terms of sensitivity, ease of use and cost. The commercial kit did not have the degree of sensitivity of the ELISA, could not compete on price and was far more complex to execute.

Serum samples were provided by septic patients at the Great Western Hospital and these were compared with both hospitalised non-septic samples and non-hospitalised healthy volunteer samples (total = 274). The lysozyme levels from the ELISA assay were also compared with the results from routine blood analysis and overall diagnosis carried out at the hospital. The results showed a statistically significant difference between healthy patients' lysozyme levels and septic patients.

The advantage of the ELISA system is that is uses very small blood samples. This is particularly useful with patients who are reluctant to give blood, who are anaemic or

who have to have several blood samples taken daily. At present this assay is working and could be incorporated into the routine work-up of a patient suspected of sepsis.

6.1.2 ELIFA (Chapter 3)

The aim of the ELIFA work was to develop on the ELISA success and to use the basic assay format to produce a faster, cheaper, and easier to use detection system for lysozyme. A method for quantifying the results produced from the ELIFA was also sought. The total ELIFA time was reduced to 40 minutes and the reusable flow cells made this assay far faster and cheaper to run compared to the ELISA.

The initial aim when the ELIFA was up and running, was to produce a calibration curve of lysozyme in buffer. The intention was to process the clinical samples used in the ELISA. Production of a calibration curve using the ELIFA was attempted, but due to the problem of non-specific binding was unsuccessful. Consideration of the exact nature of the problem suggests that the intense washing of the flow cells may have heightened the problem. However, these problems were tackled with success in Chapter 4.

6.1.3 Mini-ELIFA (Chapter 4)

The main aim of the Mini-ELIFA work was to build on the progress made on the ELIFA system and to correct the problems faced with the non-specific binding. The Swinnex filters were purchased because of their ability to be autoclaved. This eliminated the problem of non-specific binding and the problem of the harsh wash solutions degrading the filter plastic. Another aim was to produce a calibration curve of lysozyme/bacteria in buffer and to establish a method of measuring the results produced.

The bacterial mini-ELIFA produced a series of membranes which showed discolouration dependent on the concentration of bacteria in the sample. These membranes could then form a colour chart which could be used for unknown samples, as the discolouration was very distinguishable. The lysozyme mini-ELIFA however, did not produce staining to such a degree. A possible reason for this was a result of a

conformational change in the lysozyme when it bound to the membrane. The deactivated lysozyme simply did not bind and therefore did not produce reliable results. The bacterial mini-ELIFA did show promising results and with more work the lysozyme mini-ELIFA also has the potential to become a fast test for lysozyme determination.

6.1.4 Lysozyme Biosensor (Chapter 5)

The main aim of the biosensor work was to develop a further detection system that was a real time assessment, with greater through-put and a greater limit of detection. A further aim of this chapter was to incorporate the use of aptamers as an artificial receptor into the biosensor. The biosensor is a working system for the detection of lysozyme in buffer. The anti-lysozyme aptamer was incorporated with success. The aptamer was also successfully regenerated using a heat change within the BIAcore. This allowed the aptamer to be reused for other lysozyme in buffer determination. Control of non-specific binding was also successful with experiments showing that the lysozyme in buffer was specifically binding to the aptamer.

The final objective of this work was to test the clinical samples with the lysozyme biosensor. The serum posed a specific problem with contaminants within the serum binding to the sensor chip surface. This meant that the lysozyme was not detectable in serum. A better method for extracting the lysozyme prior to injection is needed for the biosensor to be viable.

The advantage of the lysozyme biosensor is that no radioactivity is used during the testing and once established the test is a single step, reagentless assay. This suggests the test would be very simple to use, requiring very little training for the operator. The BIAcore 3000 produces 2 replicates of each sample, but a better system such as the T100 model would allow multiple testing of many samples, making it usable in a hospital environment.

6.2 Conclusions & Significance of the Project

The main focus of this project was to produce a test to indicate the onset of sepsis which would be used to aid in the management of treatment and recovery of patients. The lysozyme tests in this project would not be used on their own to indicate the positive presence of sepsis, but the aim was to provide more information for the clinician and increase diagnostic certainty with the hope of lowering mortality rates. The test would provide additional information, which along with signs and symptoms and blood analysis, would be used for patients who were too ill for routine imaging investigation and would confirm or refute the need for surgery. Time, in the case of sepsis, is not in abundance, therefore during this project the need for a test which could be carried out in real-time, ideally at the patient's bedside was of great importance. Throughout the development of each test (ELISA, ELIFA, Mini-ELIFA and the Biosensor) the remit always involved a reduction in the total test time. The biosensor has the potential to become a real-time test for lysozyme, which would enable multiple testing for each patient during the course of their illness so their lysozyme levels could be directly monitored. An objective within the ELISA work was to further the knowledge of lysozyme involvement in sepsis. This was done by collecting the data from routine blood analysis and making comparisons with the lysozyme levels from the ELISA assay. Adding lysozyme determination to the usual work-up of critically ill patient's, using techniques from this project, would give greater confidence in diagnosis.

6.3 Future Work

This project still has huge potential. If more time was allowed several additional experiments would have been included to both extend work executed in Chapters 2, 3, 4 and 5 and also to explore other avenues in the hope of producing better tests. Some key areas are as follows:

- Aptamers Incorporated in the ELISA – This has the potential of creating a reusable assay. The benefits of aptamers described in section 5.4.6.2 would be harnessed and incorporated producing a very reliable, reusable and stable assay.
- Using Lysozyme to Distinguish between forms of Sepsis – The blood samples provided in this project were randomly chosen from a range of patients during their stay in hospital. If more time was available, then several blood samples

could be taken from patients throughout the course of their illness. Samples would be specifically taken from septic patients at the start of their stay in hospital and the same patients would be monitored until they improved, left hospital or died. The analysis of these samples could show a difference in lysozyme levels, distinguishing between the progressive forms of sepsis; i.e. septic shock, severe sepsis and therefore help in the diagnosis and prognosis of the patient and also improve understanding of lysozyme further.

- Mini-ELIFA Clinical Sample Testing – The mini-ELIFA should be tested with clinical samples. The bacterial mini-ELIFA has the potential of becoming a very fast, novel test for *E. coli* in urine samples. If more time was available urine samples from both healthy controls and septic patients would be tested.
- Mini-ELIFA for UTI Bacteria – Further work in this area would include developing the test for more than one UTI causing bacterium. The test would have to be converted to a sandwich immunoassay format where several different bacterial antigens were seeded onto a membrane and actively captured bacteria from the urine sample i.e. the membrane divided into 4 segments and a different antigen adhered to each quarter. The degree of staining on each quarter of the membrane would indicate the quantities of each bacterium present. This would then produce a fast, multi-bacterial test useable in GP surgeries or at the hospital bedside.
- Lysozyme Mini-ELIFA – This test needs further work to improve sensitivity to become a viable assay in the management of sepsis. Speculation over the lysozyme binding to the membrane in this test may be overcome by changing the format to a sandwich immunoassay. This would mean an anti-lysozyme molecule being adhered to the membrane to actively capture the lysozyme from the sample. Due to the speed of the assay, when working this test has the potential to become a bedside test for better management of sepsis.
- Lysozyme Biosensor – More serum samples needed to be tested with the lysozyme biosensor, to produce a test which can be used in a clinical environment. A method for extracting the lysozyme from the serum is also required. This may involve affinity column purification prior to injection onto the biosensor. Hansen & Andersen (1973) also mention in their study of

lysozyme extraction methods using n-Butanol. This may eliminate all other factors, such as residual bacteria in the serum which cannot be spin-filtered out, from interfering with the ligand so accurate serum lysozyme measurements can be achieved.

- Comparison between an Aptamer and an Antibody Biosensor - An anti-lysozyme antibody could have been immobilised onto the surface of a CM5 sensor chip and the effectiveness in terms of binding ability of lysozyme could be compared with the aptamer biosensor. This would have given first hand evidence to back up the claims mentioned in section 5.4.6.2 on the advantages of aptamers over antibodies.
- Lysozyme Biosensor Spreeta Modification – Ideally the lysozyme biosensor method would be transferred to the Spreeta (Appendix L). The Spreeta system is a low cost, miniature biosensor. Transferring and adapting the lysozyme biosensor method to this system would produce a technique which was inexpensive to develop and run, making it a realistic system for hospital use, have the speed of the BIAcore system and be miniaturised for bedside/GP surgery use.
- A final thought for lysozyme detection would include coupling the technology of aptamers with cantilevers. A cantilever sensor has two sensing arms which cantilever together in response to changes in surface stress which is caused by adsorption of biomolecules. Cantilevers have the advantage of high-throughput label-free detection and differential sensing whereby disturbances from non-specific molecules are suppressed directly during the measurement of the analyte. Combining the advantages of aptamers (high specificity, *in vitro* production, resistance to denaturation and long shelf life) with the benefits of cantilevers would produce an easy to engineer and highly advanced sensor for lysozyme.

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