A novel application of affinity biosensor technology to detect antibodies to mycolic acid in tuberculosis patients

by

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Submitted in partial fulfillment of the requirements for the

MSc. Degree in Biochemistry

in the Faculty of Natural and Agricultural Sciences

University of Pretoria

October 2003

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to the following people and institutions for their support during my MSc. studies:

My promoter Prof. J.A. Verschoor for his assistance, support and encouragement throughout my studies.

My co-promoter Dr. Gilbert Siko for teaching me the IAsys biosensor, advice, valuable input, encouragement and friendship throughout.

Sandra van Wyngaardt for her continual assistance, advice and for being friendly all the time.

Dr. Annemieke Ten Bokum and Dr. Anton Stoltz for their valuable input.

Yvonne Vermaak and Pieter Vrey for their scientific input in this project.

Members of the TB team for the best teamwork we had all the time.

My friends for being there for me always, during good and bad times.

My parents for all they have sacrificed in order for me to continue this far, and their loving and valuable support.

Financial support from National Research Foundation (NRF), Medical Research Council and Adcock Ingram Limited is gratefully acknowledged.

"I can do all things through Christ who strengthens me".

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List of Abbreviations

AFB	Acid-fast bacilli
AG	Arabinogalactan
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
ASI	Artificial sensing instrument
BCG	Bacillus Calmette-Guerin
CMD	Carboxymethyl dextran
CO_2	Carbon dioxide
CPC	Cetyl pyridinium chloride
DNA	Deoxyribonucleic acid
EDC	Ethyl-dimethylaminopropyl carbodiimide
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
F	Frequency
FET	Field effect transistor
HCl	Hydrochloric acid
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
IAsys	Interaction analysis system
IDL	Intermediate density lipoprotein
IgG	Immunoglobulin G

KCl	Potassium chloride
kDa	Kilodalton
KOH	Potassium hydroxide
LAM	Lipoarabinomannan
LAPS	Light addressable potentiometric sensor
LDL	Low density lipoprotein
LED	Light emitting diode
LM	Lipomannan
MA	Mycolic acids
mAGP	Mycolyl-arabinogalactan peptidoglycan
MDR	Multi drug resistance
MHC	Major histocompatibility complex
MS	Mass spectroscopy
M.tuberculosis	*Mycobacterium tuberculosis*
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NHS	*N*-hydroxy-succinimide
NTA	Nickel chelating surface
PBS/AE	Phosphate buffered saline azide EDTA
PCR	Polymerase chain reaction
PEG	Polyethylene glycerol
PIM	Phosphatidyl inositol mannosides
PPD	Purified protein derivative
RNA	Ribonucleic acids
rRNA	Ribosomal ribonucleic acids
RU	Resonance units

SPR	Surface plasmon resonance
TB	Tuberculosis
TDM	Trehalose dimycolate
TIR	Total internal reflection
TMM	Trehalose monomycolate
VLDL	Very low-density lipoprotein

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CHAPTER 1

General Introduction

1.1 Introduction

Tuberculosis (TB) is a chronic pulmonary disease caused by infection with *Mycobacterium tuberculosis* (*M. tuberculosis*). It is a major scourge in developing countries as well as an increasing problem in many developed areas of the world, with about 8 million new cases and 3 million deaths each year (Hendrickson *et al.*, 2000; Drobniewski *et al.*, 2003). *M. tuberculosis* is spread primarily through aerosolized infectious particles generated from coughing and sneezing by individuals with TB (Dillon *et al.*, 2000). Macrophages are the host cells for *M. tuberculosis* infection and constitute the first line of defense against spread of infection. After being inhaled, the bacilli are able to reach the terminal pulmonary airways and alveoli of uninfected individuals (Lawn *et al.*, 2002). After entry into the host, the *M. tuberculosis* is phagocytosed by macrophages. Such phagocytosed material is normally degraded in lysosomes, but *M. tuberculosis* can resist lysosome degradation and survive, were they multiply within macrophage phagosomes (Pieters, 2001).

Although tuberculosis is a curable disease that responds well to antibiotics it has re-emanated as a growing global health problem because of the development of drug-resistant strains. Human immunodeficiency virus (HIV) is a major risk factor in developing tuberculosis. HIV infected individuals have a weakened immune system and as a result, they have a much greater chance of developing active tuberculosis either by the activation of the latent infection or by becoming newly infected (Small and Selcer, 1999).

The long duration of the so-called short course antibiotic therapy (6-9 months) enlarges the risk of non-compliance and subsequent development of drug resistant strains of *M. tuberculosis* during resurgent infection. It is therefore important that a reliable and fast

serodiagnostic assay be developed to establish resurgence of disease in patients undergoing therapy so as to stop the development of multi drug resistance (MDR) in its early tracks. Tuberculosis still continues its ravenous journey across the world (i.e., parts of North America, Latin America, Western Europe, sub-Saharan Africa, South Asia and the Russian Federation) killing young and middle-aged adults faster than any other disease other than AIDS (Khasnobis *et al.*, 2002). It is believed that one in every three individuals on the planet harbours the causative microorganism, *M. tuberculosis*. A resurgence of TB, largely due to the emergence of drug resistant strains of *M. tuberculosis* and the increased risk for TB in HIV infected persons, has magnified the need for rapid, inexpensive, and accurate methods for the diagnosis of TB (Cheon *et al.*, 2002). Therefore, simple diagnostic assays that do not require highly trained personnel or a complex technological infrastructure are essential for global control of TB (Foulds and O'Brien, 1998; Fatkenhever *et al.*, 1999).

1.2 Diagnosis of Tuberculosis

The rise of TB is due to drug-resistant strains of *M. tuberculosis* and to more incidences of co-infections with HIV, particularly in sub-Saharan Africa (Houghton *et al.*, 2002). The basis for effective treatment and cure of patients is the rapid diagnosis of the disease and its causative agent, which is founded on the analysis of the clinical symptoms coupled with laboratory tests (Reischl, 1996). Basically, there are five different possibilities for laboratory diagnosis of infections:

- Measurement of hypersensitivity to mycobacterial antigens (e.g. tuberculin skin test)
- Direct detection of the pathogens (e.g. microscopy and /or culture)
- Detection of protein components of the pathogens with the help of specific antibodies
- Specific detection of antibodies directed against a given pathogen and changes in their corresponding titer.
- Specific detection of nucleic acids (e.g., PCR) of the pathogens.

1.2.1 Tuberculin Skin Test

The tuberculin skin test is currently the most generally used method for identifying TB infection. The technique is based on the injection of 0.1 ml of a solution of tuberculin, a purified protein derivative (PPD), intradermally into the volar or dorsal surface of the forearm. If positive, this produces a discrete, pale elevation of the skin, 6 mm to 10 mm in diameter after 48 to 72 hours of injection (Charnace and Delacourt, 2001). The reading is based on a measurement of swelling. The tuberculin skin test is a valuable tool, but it is not perfect. As PPD contains many antigens widely shared among mycobacteria, the specificity is low. Some persons may react to the tuberculin skin test though they are not infected with *M. tuberculosis* (Doherty *et al.*, 2002). Others may not react to the tuberculin skin test even though they are truly infected with *M. tuberculosis*. These false negative reactions can occur in several circumstances:

- When skin testing persons who are recently infected with *M. tuberculosis*. These persons may have a false negative reaction because developing an immune response to tuberculin can take 2-10 weeks after infection
- When skin testing persons who are anergic, i.e. unable to react to the skin test because of a weakened immune system. Anergy is often caused by HIV infection, but it can also be caused by other medical conditions (Fatkenheuer *et al.*, 1999).

Several studies have demonstrated that PPD cannot reliably distinguish between previous *Mycobacterium bovis* BCG vaccination, exposure to environmental mycobacteria, or infection with *M. tuberculosis* (Chan *et al.*, 2000; Charnace and Delacourt, 2001). The PPD skin test remains in use despite these limitations, reveals the urgent need for better diagnostic tests for TB (Doherty *et al.*, 2002).

1.2.2 Direct microscopy

The detection of mycobacteria by microscopic examination after staining of the mycobacteria according to Ziehl-Neelsen is a simple technique and the cornerstone for the diagnosis of TB in developing countries. The technique can be used for sputum,

lymph nodes, pleural fluid, urine, cerebrospinal fluid and biopsies and is amenable to refinement (Fig.1.1). The presence of acid-fast bacilli (AFB) on a sputum smear often indicates tuberculosis. Acid-fast microscopy is easy and quick, but it doesn't confirm a diagnosis of TB because some acid-fast bacilli are not *M. tuberculosis* (Hamasur *et al.*, 2001).

The direct microscopy of sputum for AFB is reliable for pulmonary tuberculosis, but is not very sensitive. It may give false negative results and require a high degree of bacillary load of 10000 bacilli/ml of sputum (Mitarai *et al.*, 2001). Direct microscopy is not valuable for diagnosing extrapulmonary tuberculosis or child tuberculosis (Charnace and Delacourt, 2001). Unfortunately, this technique can't identify the mycobacterial species. It is therefore recommended that the result of microscopy of the smear be confirmed by culture.

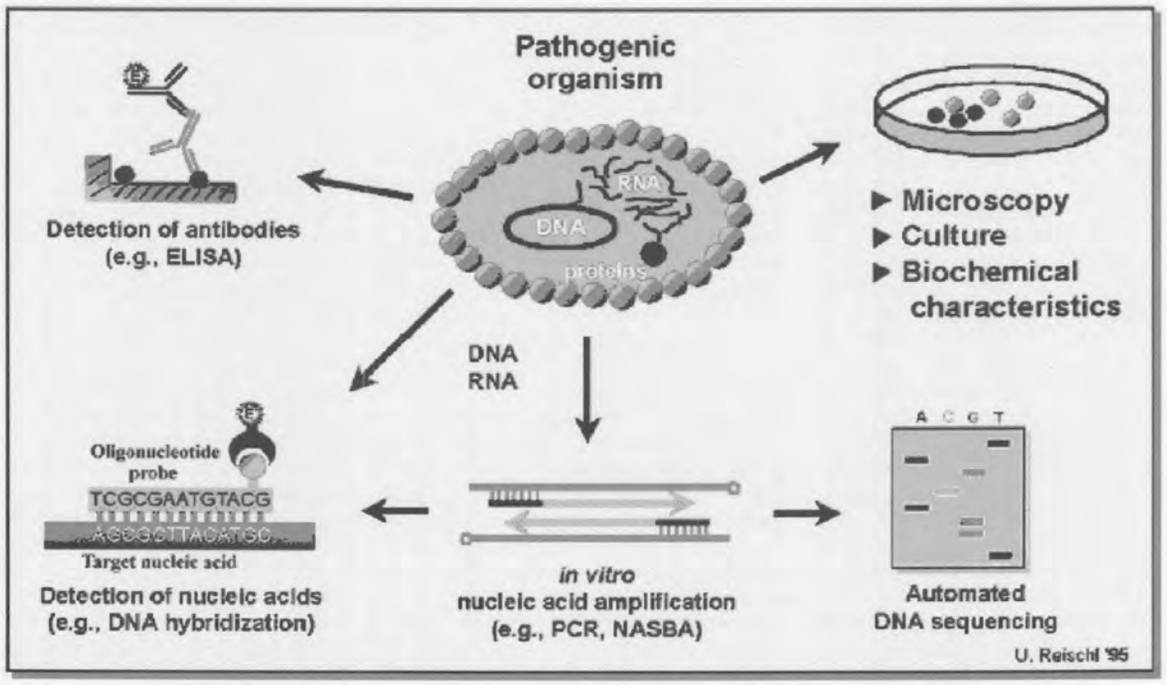


Figure 1.1: Modern laboratory diagnosis of infections, based on refined traditional methods like microscopy and culture, specific detection by antibodies and, more recently, on the specific and highly sensitive detection methods for detection of nucleic acids (Reischl, 1996).

1.2.3 Culture

Culture is the gold standard for the diagnosis of tuberculosis. The technique is very sensitive, such that even a few mycobacteria can be detected. However, primarily due to the slow growth of the bacteria, this method usually requires 4 to 8 weeks for completion (Samanich *et al.*, 2000) and is subjects to contamination. This often results in delayed diagnosis, adversely affecting patient care and TB control and allows for the spread of infection (Reischl, 1996).

M. tuberculosis is a slow-growing organism with a doubling time of 20 – 22 hours. The bacilli can be cultured in liquid medium or agar medium. Cultures may be positive after 12 days when using the BACTEC system that measures growth by radio-active CO_2 released from the cultures. Visual colonies may be observed only after 14 days when using solid medium after concentration of the isolates. Using conventional methods, identification of the species to which the cultured mycobacterial strain belongs, may require an additional 2 – 4 weeks (Verbon, 1992; Samanich *et al.*, 2000). Diagnostic procedures that shorten the time required for identification of *M. tuberculosis* are therefore needed.

1.2.4 Enzyme Linked Immunosorbent Assays (ELISA)

Serological tests using an ELISA to detect antibodies to *M. tuberculosis* are relatively simple and inexpensive. However, most studies performed in adults have yielded poor sensitivity and specificity. Most assays developed for enzyme linked immunosorbent assay (ELISA) are based on the detection of antibodies in serum or antigen in sputum and other body fluids from patients (Fig.1.1). Many variations in the methodology of the ELISA have evolved since its development in the 1960s, but the basic concept is still the immunological detection and quantitation of single or multiple antigen or antibody in a patient sample, usually serum (Lyashchenko *et al.*, 2000; Charnace and Delacourt, 2001). ELISA has become a fundamental tool for drug discovery, animal studies, and clinical trials in the pharmaceutical industry because of its ability to screen large numbers of samples. A serologic test, using ELISA, is a simple and inexpensive alternative to other

TB diagnosis methods (Simonney *et al.*, 1996; Maron, 2001). The disadvantage of ELISA is that it detects only the high affinity antibodies to the antigen.

Several studies have addressed the problems of detecting *M. tuberculosis*-specific antibodies in TB patients co-infected with HIV (Hendrickson *et al.* 2000; Lawn *et al.*, 2002; Schleicher *et al.*, 2002; Stavri *et al.*, 2003). For example, in a field test in Mexico, an ELISA based on the mycobacterial 30-kDa protein antigen had a sensitivity of 70 % in patients with culture-positive or smear-positive pulmonary TB and a specificity of 100 % in 125 control donors. The same test was evaluated with HIV-positive and negative patients in Uganda. Although the sensitivity and specificity in HIV–negative donors were the same as in the Mexico test, the ELISA was positive for only 28 % of 128 sera from HIV-positive donors (Hendrickson *et al.,* 2000).

1.2.5 End-point Polymerase Chain Reaction

The polymerase chain reaction (PCR) has created new possibilities for the rapid diagnosis of mycobacterial infections. With this technique target nucleic acid sequences are exponentially replicated in repeated cycles of DNA synthesis using specific oligonucleotide primers (Fig.1.1). The amplified DNA is usually detected by hybridization with radiolabelled probes and characterized by direct sequencing. A common strategy for detecting and speciating microorganisms is the specific gene-probe hybridization and PCR targeted to the 16rRNA subunit, which is coded in mycobacteria by multiple gene copies (10 genes for 16S RNA) per cell (Kox *et al.*, 1995; Singh *et al.*, 2000). The 16S subunit contains sequences that have highly conserved regions virtually identical in all bacteria, interspersed with other sequences found to be genus- and/or species-specific (Kox *et al.*, 1995). The PCR has been utilized in the rapid diagnosis of mycobacterial infection (De Wit *et al.*, 1990; Shankar *et al.*, 1990; Waleria-Aleixo *et al.*, 2000). Its high degree of sensitivity in clinical samples afforded by DNA amplification have been described by various investigators (Shankar *et al.*, 1990; Chakravorty and Tyagi, 2001). Direct diagnosis by PCR is not always possible and may require 10–14 days of culture followed by probe-based diagnostics (Versalovic and Lupski, 2002).

Many end-point PCR techniques that are currently employed for the isolation of mycobacterial DNA from clinical samples suffer from one or many drawbacks that include long processing times, use of organic solvents and enzymes and multiple steps resulting in DNA loss or in the inefficient removal of PCR inhibitors. These multi-step methods increase false positive results due to the risk of cross-contamination and also augment false negative, due to losses in DNA recovery (Jordan, 2000). Many studies have shown the limitations of detecting *M. tuberculosis* with mutations by PCR. (Torres *et al.*, 2003; Miller *et al.*, 2002).

Bacterial staining, culturing, or PCR can accomplish direct detection of acid-fast bacilli in sputum. Drawbacks to these approaches include difficulty in obtaining sputum from children, slow growth of the bacteria as well as the overall low sensitivity, particularly for extrapulmonary TB (Stavri *et al.*, 2003). In spite of new technologies such as PCR, no reliable and affordable tests have been accepted in the market for the diagnosis of TB (Ahmad *et al.*, 1998).

1.3 Real-time detection of biomolecules using biosensors

Biosensor technology enables researchers to detect molecules with low affinity in a biological medium. This new technology makes it possible to visualize on a computer screen the progress of binding of biomolecules as a function of time, in terms of changes in mass accumulation occurring on a sensor surface. Biosensor instruments make it possible to determine how fast and how strongly molecules interact and what the binding stochiometry is (Van Regenmortel, 1999). The time dependent measurements provide kinetic information that has been difficult to obtain with other methods. The lack of labeling requirements, low sample consumption and ease of use has made optical biosensors an essential component of both academic and commercial laboratories (Myszka, 1999). The biosensor technology offers sensitive detection of surface adsorption, but all adsorbed molecules are detected, there by putting very high demands on the surface properties to avoid unwanted interactions (Malmqvist, 1999).

Conventional methods for the detection and identification of bacteria mainly rely on specific microbiological and biochemical identification, while biosensors methods can be sensitive, inexpensive and give both qualitative and quantitative information on the number and the nature of the microorganisms tested (Leonard *et al.*, 2002). While conventional methods of pathogen detection require time-consuming steps to arrive at a useable measurement (Jongerius-Gortermaker *et al.*, 2002; He and Zhang, 2002), biosensor technology can significantly reduce the time as well as detect even smaller amounts of pathogens with fewer false positives.

Since Leland C. Clark (1962) fabricated the first enzyme electrode, biosensors have become attractive and popular objects of research (Dong and Chen, 2002). A biosensor is an analytical tool consisting of biologically active material used in close conjunction with a device that will convert a biochemical signal into a quantifiable electrical signal (Kumar, 2000). Such devices are based on incorporating some kind of biological element in a sensing layer (enzymes, antibodies, and lipid layers) intimately connected with a transducer. Due to its simplicity, high sensitivity and potential ability for real-time and on-site analysis, biosensors have been widely applied in various fields (Dong and Chen, 2002).

Biosensors have many applications, especially in health and medical fields (Frostell-Karlsson *et al.*, 2000, Rogers, 2000). They have become increasingly popular for determining the affinity and kinetics of interactions of biological macromolecules (Schuck, 1996; Myszka *et al.*, 1996; Markgren *et al.*, 2000). The optical biosensors that measure refractive index changes caused by bound macromolecules permit one to monitor the time dependence of the binding of label-free macromolecules to receptors immobilized on a surface (Malmqvist, 1999; Van Regenmortel, 1999). They are used to study binding in a number of different applications, e.g., antigen-antibody interactions, protein-protein interactions, protein-DNA interactions, and in interaction of HIV-1 protease with inhibitors (Schuck, 1996; Markgren *et al.*, 2000; Scheller *et al.*, 2001). Additional uses include epitope mapping, ligand fishing and small molecule screening (Muller *et al.*, 1998; Myszka, 1999).

Significant advances in biosensors have been achieved over the past few years, such as rapid growth of DNA sensors, introduction of advanced sensing materials, application of quartz-based piezoelectric oscillators, evanescent field and surface acoustic wave detectors. All of the currently available real-time detection systems come with the necessary software for data analysis.

1.3.1 Potentiometric sensors

A potentiometric biosensor consists of a perm-selective outer layer and a bioactive material, usually an enzyme. The enzyme-catalyzed reaction generates or consumes a species, which is detected by an ion selective electrode. Potentiometry provides logarithmic concentration dependence (Leonard *et al.*, 2002). A field effect transistor (FET) is a device where the transistor amplifier is adapted to be a miniature transducer for the detection and measurement of the potentiometric signal produced by a sensor process at the gate of the FET. It consists of an n-type silicon semiconductor-based sensor and an insulating layer that is in contact with an aqueous solution where a bio-reaction takes place. Changes in potential at the silicon-interface are detected by the difference in charge distribution between the surface of the insulator and the FET (Leonard *et al.*, 2002).

The recently developed light addressable potentiometric sensor (LAPS), based on the FET has proved to be suitable for detection of microbial contamination. A LAPS measures an alternating photocurrent generated by a light source, such as a light emitting diode (LED), so that changes in potential can be transduced into voltage per time differentials.

The United States Department of Defence has introduced a biological integrated detection system for providing detection of airborne biological threats. One of the instruments incorporated in this is the Threshold Immunoassay System (Fig.1.2). This system is capable of detecting eight agents simultaneously within 15 minutes (Rogers, 2000; Leonard *et al.*, 2002). A potentiometric biosensor, LAPS, exploits the natural

affinity between streptavidin and biotin. An immunocomplex is formed in the solution phase between sample (antigen), a labeled antibody, biotinylated antibody and streptavidin, which is then captured onto solid phase biotinylated filter (Fig. 1.2). Upon addition of substrate the filter is brought in contact with the silicon semiconductor and the enzyme generates a potentiometric signal (Leonard *et al.*, 2002).

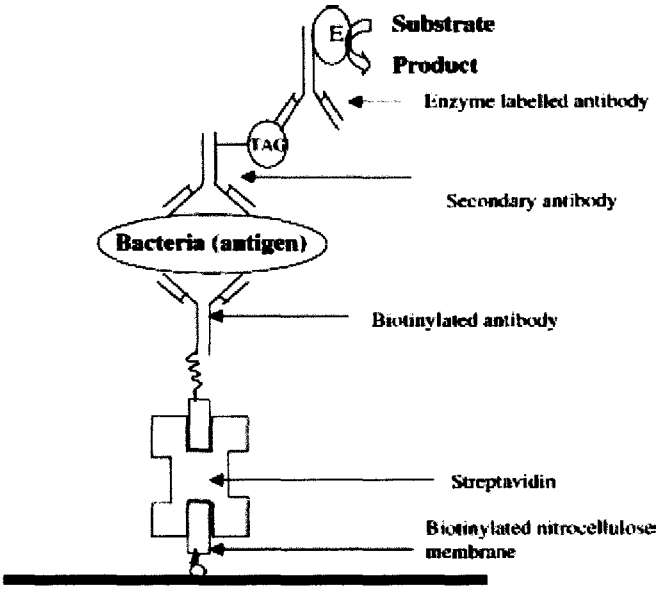


Figure 1.2: Schematic representation of immunocomplex formed in the Threshold Immunoassay System (Leonard *et al.*, 2002).

1.3.2 Piezoelectric Immunosensor

The use of piezoelectric devices as potential sensors is attributed to the Sauerbray equation, which describes the relationship between the resonant frequency of a crystal and mass deposited on its electrodes; $\Delta F = -2.3 \times 10^6 F^2 \Delta M/A$, where ΔF is the change in frequency, F the resonant frequency of the crystal, ΔM the deposited mass and A the electrode area. By keeping F and A constant, ΔF has a linear relationship with ΔM. The piezo-immunosensor is based upon a measurement of the mass change caused by the binding of an antigen to an antibody. Therefore, for more bindings of the antigen to the antibody, a greater frequency shift occurred (He and Zhang, 2002). Non-specific binding

results are limited by using a control antibody, which is irrelevant to tuberculosis. By comparing different changes between the control and experimental groups, the presence of foreign antigens, such as those associated with tuberculosis can be detected. This is a promising method, which can be easily set up in every public health laboratory for the diagnosis of tuberculosis (He and Zhang, 2002).

When a piezoelectric quartz crystal is used as a biosensor, the crystal surface is treated with an antibody coating that will capture a specific pathogen. A sufficient amount of biomolecules should be immobilized on the piezoelectric surface without loss of the biological activity. The increase of mass and viscosity caused by the capture of the target pathogen changes the resonant behaviour of the crystal, including a decrease in the resonant frequency (Kumar, 2000; Kim *et al.*, 2003).

The application of a mass or non-mass sensitive piezoelectric quartz crystal sensor is subjected to a variety of errors, since any variation in the experimental conditions, even a small change in the position of the quartz crystal and in the distance between the leading wires, would alter the response of the sensors (Su *et al.*, 1998). The piezoelectric sensor is also limited by the need of purified biomolecules to be introduced onto the surface of the crystals and the requirements of relatively large molecular weights of the analytes to be detected. Direct binding of small molecules such as drugs and vitamins cannot produce sufficient measurable frequency changes, and an indirect assay is required to overcome these limitations.

1.3.3 Optical biosensors

The introduction of optical biosensors in 1990, based on the phenomenon of surface plasmon resonance (SPR), has revolutionized the measurement of binding interactions in biochemistry (Malmqvist and Karlsson, 1997). Most optical biosensors rely upon a phenomenon called the evanescent field to monitor changes in refractive index occurring within a few hundred nanometers of the sensor surface. Such changes are generated as a result of the binding of a molecule to a surface immobilized receptor (or the subsequent

dissociation of this complex), and real-time monitoring of these effects allows binding constants to be derived (Cush *et al.*, 1993). Optical biosensors can be used to provide qualitative information, such as whether two molecules interact, and quantitative information, such as kinetic and equilibrium constants for complex formation, for a wide range of biological systems. Thus the result in the form of a response versus time diagram contains much more relevant information about the interactions of molecules than can be obtained by end-point determinations such as ELISA.

A limited number of commercial instruments are available; for example, BIAcore (Uppsala, Sweden), Affinity Sensors (Cambridge, UK), and Artificial Sensing Instruments (ASI) (Zurich, Switzerland) (Leatherbarrow and Edwards, 1999). The instruments differ in the method used to generate the evanescent field.

1.3.3.1 Biacore

The Biacore instrument utilizes surface plasmon resonance (SPR) laser light that shines onto a glass prism in contact with a gold surface (Myszka, 1999; Malmqvist, 1999). The incident light is introduced at moving angles. At the critical angle, the light excites the metal surface electrons (plasmons), thereby generating the evanescent field and causing a dip in intensity of the reflected light (Fig. 1.3). The critical angle is sensitive to refractive index changes occurring close to the sensor surface and thus, by monitoring change in critical angle with time, details of the events at the surface can be probed.

Changes in the SPR signal are expressed in resonance units (RU), where one RU is equivalent to one picogram of protein per square millimeter on the sensor surface. In general, the refractive index change for a given change of mass concentration at the surface layer, is practically the same for all proteins and peptides, and is similar for glycoproteins, lipids and nucleic acids (Myszka and Rich, 2000).

The Biacore system is equipped with a continuous flow system in which different channels may be coupled in series (Fig. 1.4). It has an automated sample needle to deliver

buffer and sample to the sensor chip surface (BIAcore website, 2003). The continuous flow ensures that no change in analyte concentration occurs during the measurement.

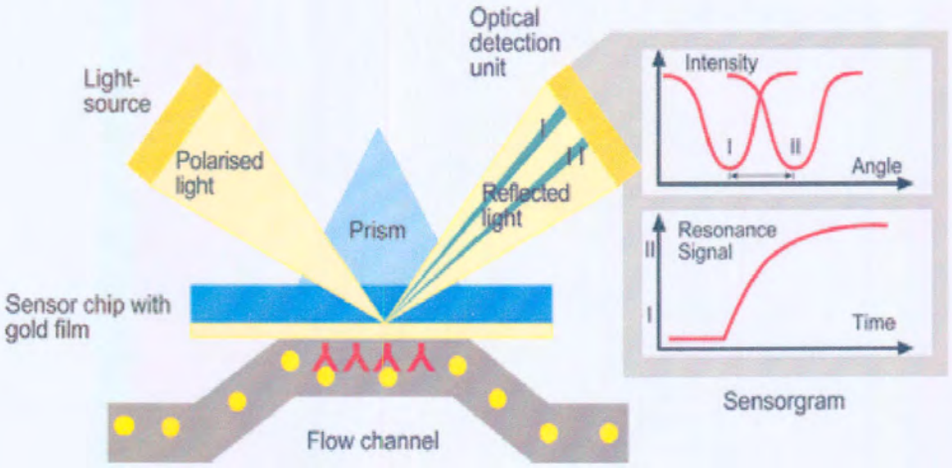


Figure 1.3: The Biacore's integrated system (BIAcore website, 2003).

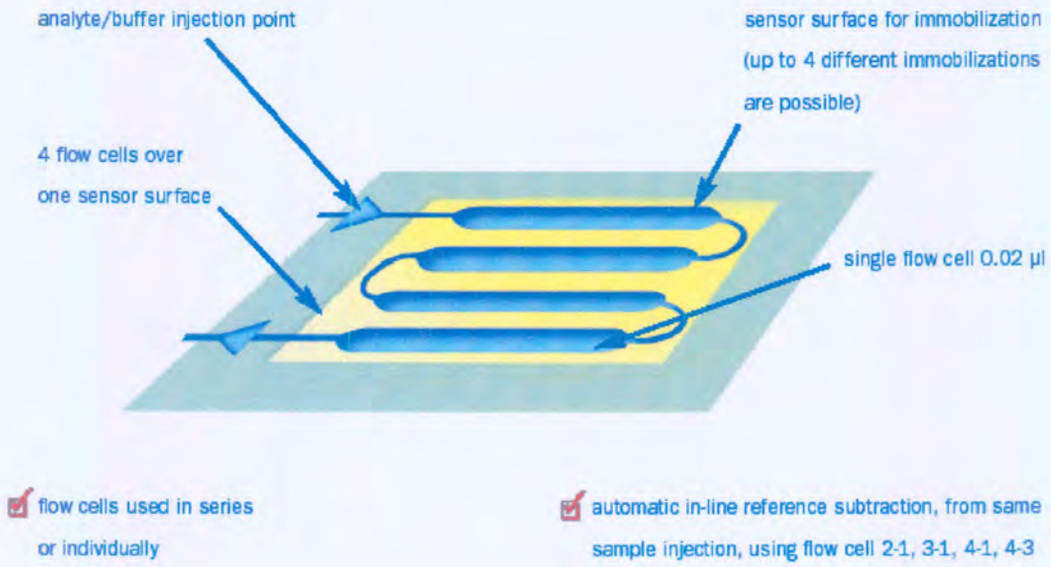


Figure 1.4: Microfluidic system of Biacore 300 housed in a temperature-controlled environment. Four sensorgrams (one for each cell) and three reference-subtracted curves can be displayed per single run. Flow cells 1-2 and 3-4 can be used as separate pairs for increased efficiency (BIAcore website, 2003).

1.3.3.2 IAsys Biosensor

The IAsys biosensor from Affinity Sensors also uses the evanescent field to probe surface changes but generate it in a different manner using waveguiding techniques (Cush *et al.*, 1993). The resonant mirror biosensor is aimed at producing a sensor, which combines the enhanced sensitivity of waveguiding devices with the simple construction and use of SPR sensors (Buckle *et al.*, 1993; Cush *et al.*, 1993). In construction it is similar to SPR devices. Light is totally internally reflected from the sensing surface by means of a prism (Fig. 1.5). The operation of these is based on the optical properties of the films with high refractive index deposited on a glass surface. Incident laser light is totally internally reflected, and at a certain angle of incidence the excitation of resonance in the film produces intensity and phase changes in the reflected beam (Fig. 1.5). For the measurement one exploits the fact that the resonance characteristic are very sensitive to changes of the refractive index induced by the evanescent field, which extends with exponential decay into the space above the sensor surface (Schuck, 1996).

Applications of the IAsys biosensor require different sensor surfaces for immobilization of ligands. In addition to the widely used carboxymethyl dextran (CMD), the following surfaces are also commercially available; planar surfaces (carboxylate, biotin, amino), nickel chelating surfaces (NTA) and streptavidin coated dextran surfaces (Myszka, 1999).

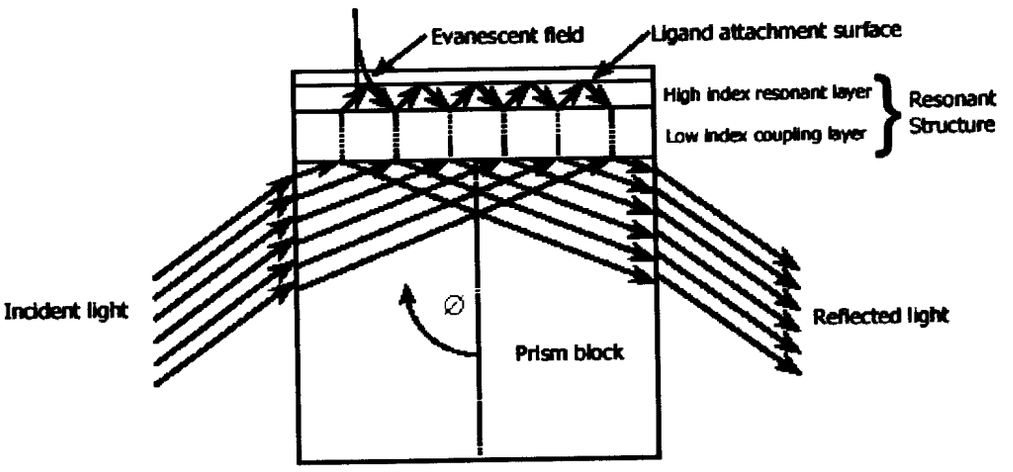


Figure 1.5: Schematic representation of the IAsys resonant mirror-sensing device (Cush *et al.*, 1993).

The IAsys CMD cuvette has been used in a very wide range of interaction analyses including those between proteins, nucleic acids and carbohydrates. It is hydrophilic and charged with derivatizable carboxylate groups that allow the unique feature of efficient electrostatic binding prior to covalent immobilization (Morgan *et al.*, 1998). Planar surfaces provide enhanced sensitivity for exploring and comparing biomolecular interactions using alternative immobilization chemistry. It allows ligate interaction to take place close to the biosensor surface where the evanescent field is most intense. Both amino and carboxylate surfaces can be useful for the analysis of high molecular weight ligates or particulates which may be unable to enter the CMD matrix. The biotinylated planar surface is ideal for rapid, convenient and well controlled capture of biotinylated ligands including proteins, lipids, nucleic acids and glycoproteins with streptavidin

linking the ligands to the surface. The hydrophobic surface enables hydrophobic binding of biomolecules, such as lipid monolayers and proteins (Altin *et al.*, 2001). The non-derivatized surface offers an alternative to the hydrophobic cuvette for simple immobilization of lipids and carbohydrates.

1.4 Antigens of the cell envelope of *Mycobacterium tuberculosis*

The cell wall of mycobacteria has several unique features, which distinguishes it from all other prokaryotes thereby qualifying as an ideal target for diagnosis of infection (Khasnobis *et al.*, 2002). It consists of a plasma membrane surrounded by a lipid and carbohydrate rich shell, which in turn is encircled by a capsule of polysaccharides, proteins and lipids. The insoluble matrix is composed of covalently attached macromolecules, i.e. peptidoglycan, arabinogalactan and mycolic acid (Fig. 1.6).

Most bacteria contain both a cell membrane and a peptidoglycan layer, but mycobacteria also contain a hydrophobic layer of mycolic acids, which are long branched chain, β-hydroxyl fatty acids. They are attached covalently to the cell wall of the bacteria or non-covalently in the form of trehalose dimycolate (TDM) or trehalose monomycolate (TMM). The cell envelope is also decorated with diverse lipids and glycolipids, such as lipoarabinomannan, trehalose dimycolate, phthiocerol dimycocerosate. Each of these, especially mycolic acid, contributes to *M. tuberculosis* extreme hydrophobic nature. While the structures of many of these molecules have been defined in exquisite chemical detail, their role in pathogenesis remains unclear.

The insoluble cell wall fraction formed after the removal of soluble proteins, lipids and carbohydrates is chemically composed of three covalently linked macromolecules: highly cross-linked peptidoglycan, arabinogalactan (AG), and mycolic acids (Chatterjee, 1997). A recent model of the mycobacterial cell wall, based on structural knowledge and various interpretations of the physical organization of cell envelope constituents is shown in Fig. 1.6 (Khasnobis *et al.*, 2002). The cell envelope contains a number of unusual lipid molecules, ranging from waxes to biologically active glycolipids. A number of these have

been isolated characterized and tested in immunoassays to assess their suitability as antigens for the detection of patient antibodies as surrogate markers of infection (Munoz *et al.*, 1997).

Several serological tests for tuberculosis using antigens from the soluble part of the cell wall have been developed (Chatterjee, 1997; Barry *et al.*, 1998). The development of a serological test for TB has been hampered by the limited specificity and sensitivity of antibodies to the available cell wall antigens (Hendrickson *et al.*; 2000). Some of the proteins of mycobacteria that are associated with the cell wall are powerful immunogens. These proteins are useful as antigens in serological assays (Maekura *et al.*, 2001), but virtually all of the proteins isolated from *M. tuberculosis* appear to possess cross-reactive parts that limit their potential as diagnostic antigens. Whereas carbohydrate antigens generally evoke IgM antibodies, which are of low affinity and specificity, glycolipid antigens have been evaluated with varying results in standard serological tests for tuberculosis (Simonney *et al.*, 1996).

A cell wall lipid that showed much potential as antigen in serodiagnostic assay was mycolic acid. Mycolic acids are very long branched chain fatty acids in nature. Their long alkyl chains are extremely hydrophobic, which makes them very different from hydrophilic antigens, such as proteins or carbohydrate molecules. Due to this, mycolic acid is not plausible as an antigenic molecule. It is therefore surprising that such wax-like structures of mycolic acid can be recognized by host cellular immune systems (Beckman *et al.*, 1994) and that antibody against mycolic acids are produced. Pan *et al.* (1999) suggested that the presence of anti-mycolic acids antibodies in the sera of subjects might be surrogate markers for *Mycobacterium tuberculosis* infection.

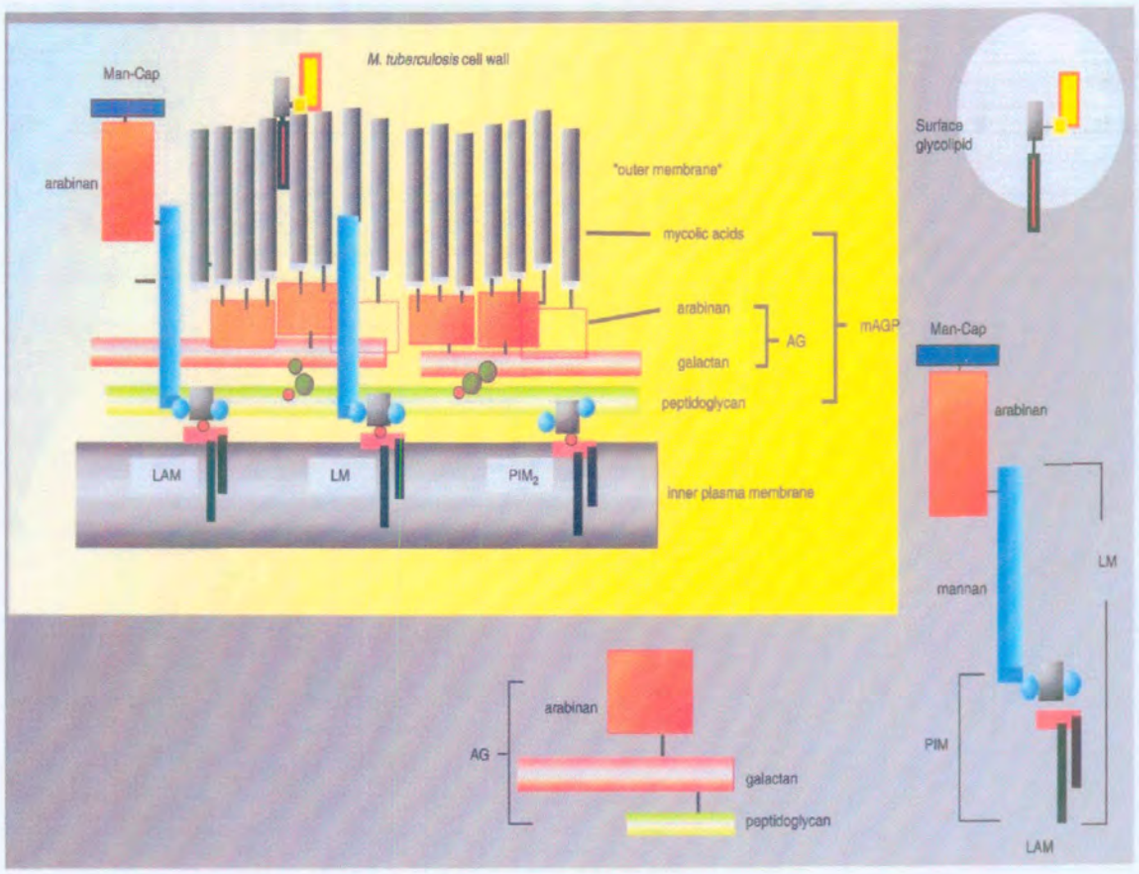


Figure 1.6: The cell envelope of mycobacteria. The three major macromolecules mycolic acids, arabinogalactan and peptidoglycan are covalently linked forming an insoluble complex (mAGP). AG: Arabinogalactan; LAM: Lipoarabinomannan; LM: Lipomannan; mAGP: Mycolyl-arabinogalactan peptidoglycan; PIM: Phosphatidyl inositol mannosides (Khasnobis *et al.*, 2002).

1.5 The prevalence of antibodies to mycolic acids as surrogate marker for TB

Mycolic acids are unique 60-90 carbon length branched α-alkyl, β-hydroxy fatty acids, which form an outer waxy lipid layer around the mycobacteria (Barry *et al.*, 1998). Three families of mycolic acids are known; α-mycolic acids without any oxygenated functional groups and the two oxygenated types (Fig.1.7) that differ primarily in the presence and nature of oxygenated-containing substituents in the distal portion of the meromycolate branch (Chatterjee, 1997; Yuan *et al.*, 1998). The methoxymycolate series has a methoxy group adjacent to a methyl branch, in addition to a cyclopropane in the proximal position.

The arabinan of arabinogalactan (AG) provides the anchoring point for the outer mycolyl lipid layer and links it to the underlying peptidoglycan layer via a galactan chain to form the mycolyl-arabinogalactan peptidoglycan (mAGP) complex, which is the essence of the mycobacterial cell wall (Fig. 1.6).

Previously, Kato (1972) reported that anti-cord factor antibodies produced in experimental animals recognized the trehalose sugar part of the molecule. They succeeded in inducing the production of antibodies to cord factor in rabbits and mice, and investigated the characteristics of the IgM antibody. However, they couldn't detect antibody reactive against cord factor in tuberculosis patients sera, possibly due to the limitations on immunological techniques. It was later reported that the anti-cord factor IgG antibody was detectable by ELISA in active and inactive TB patients and was useful for the early clinical diagnosis of TB (Pan *et al.*, 1999). Pretorius (1999) has also shown the existence of antibodies against mycolic acid in human tuberculosis patients by using ELISA.

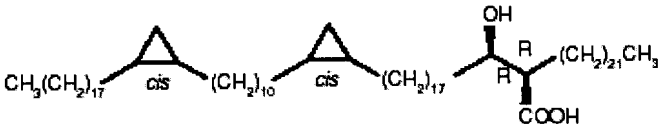
Cord factor is a unique and ubiquitous surface molecule in mycobacteria, which may contact with the host phagocytic cells at the early step of infection. Therefore, cord factor may induce the immune response against host animals quickly. Cord factor is a very hydrophobic molecule containing the very long branched chain fatty acid, mycolic acid, that make up the exact antigenic epitope or binding site recognized by the antibody (Fujiwara *et al.*, 1999). Pan *et al.* (1999) also showed that the anti-cord factor IgG antibody recognized the structure of the mycolic acid element. Among the three subclasses (alpha, methoxy and keto) of mycolic acids, tuberculosis patient's sera reacted most prominently against methoxy mycolic acid.

Serodiagnosis, if it can be made to work, is a fast, affordable and efficient way to manage disease. Thus, many mycobacterial antigens have been investigated for this purpose; such as cellular extracts, proteins and glycolipids molecules from the mycobacterial cell wall. The lipoarabinomanan antigen and the acylated trehalose family have been frequently investigated among the group of mycobacterial glycolipids. They are 2,3- di-

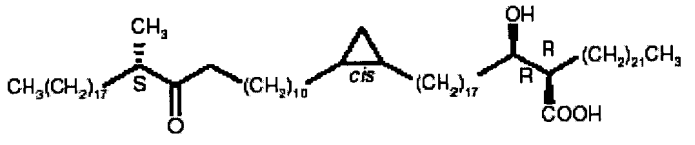
acyltrehalose; 2,3,6- triacyltrehalose; 2,3,6,6- tetraacyl trehalose 2'-sulfate (sulfolipid I); and trehalose-6,6- dimycolate (cord factor). By using the enzyme-linked immunosorbent assay technique, several studies were performed with these antigens. An extensive variability in immunoglobulin G (IgG) or IgM titers was obtained. Julian *et al.* (2002) has also shown that TB patients have a specific IgA response against glycolipids.

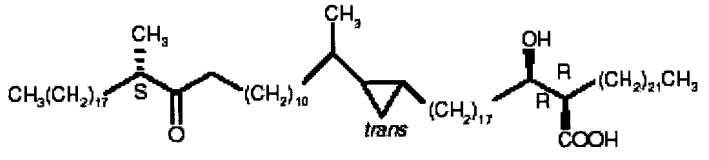
Maekura *et al.* (2001) demonstrated that the glycolipid antigen, trehalose 6,6'-dimycolate (TDM) purified from *M. tuberculosis* H37Rv could be used as an antigen for detecting anti-tuberculosis immunoglobulins and claimed that this glycolipid could be an effective antigen for serodiagnosis of TB. Siko (2002) has developed an assay to detect antibodies to mycolic acids in human TB serum on the IAsys biosensor. This study aimed at assessing the potential of detecting anti-mycolic acid antibodies on IAsys affinity biosensor in the serum of patients with active pulmonary tuberculosis as a surrogate marker for tuberculosis infection.

(a) Alpha Mycolic acids

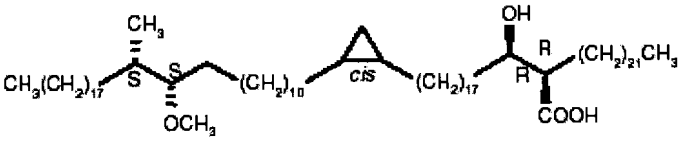


(b) Keto Mycolic acids





(c) Methoxy Mycolic acids



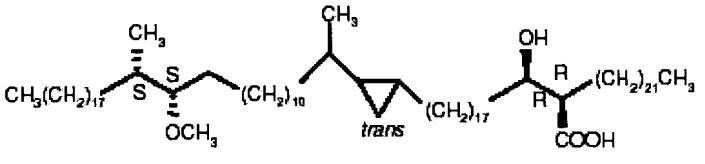


Figure 1.7: Structures of mycolic acids from *M. tuberculosis* (Chatterjee, 1997).

1.6 Hypothesis

The presence of anti-mycolic acids antibodies detectable with the IAsys resonant mirror biosensor may serve as surrogate marker for tuberculosis infection.

1.7 Aims

- To assess the IAsys affinity Biosensor application developed by Siko and Verschoor (Siko, 2002) in different patients and controls for its ability to detect antibodies to mycolic acid.

- Enhance the predictiveness of the test by competitive inhibition of antibody in patients with phosphatidylcholine, cholesterol and mycolic acids containing liposomes.

CHAPTER 2

Setting up the IAsys Biosensor to detect anti-Mycolic antibodies

2.1 The IAsys biosensor

2.1.1 Principle of action

Interaction analysis system (IAsys) is an optical biosensor system, which incorporates a stirred, micro-cuvette for studying biomolecular interactions in real-time. It allows binding reactions to be observed and measured as they happen, so revealing the dynamics as well as the strength of binding. Analysis is carried out rapidly and conveniently, using small amounts of material and without the need for labels or steps to separate the bound species from the free (Cush *et al.*, 1993; Myszka, 1999).

IAsys uses the well-established, optical phenomenon of an evanescent field. This occurs when light undergoes total internal reflection (TIR) and is enhanced within a patented waveguide structure called the resonant mirror as described in chapter 1 (Fig.1.5). This is because it forms a resonant cavity (or waveguide) and is an almost perfect reflector of light (Cush *et al.*, 1993; Schuck, 1996).

The IAsys biosensor can monitor and quantify bio-recognition processes, by detecting changes in refractive index in the vicinity of the immobilized biomolecules, because of the binding of the interacting analyte. The changes in refractive index values are proportional to the change in the adsorbed mass, thus the analysis allows the monitoring of the interaction process in real-time. By immobilizing a ligand to the sensor surface, it is possible to measure only those molecules (ligates) that bind to or dissociate from the ligand (Cush *et al.*, 1993; Buckle *et al.,* 1993).

The resonant mirror is a simple structure of two dielectric layers of glass, which leads to a reproducible and robust performance. The device consists of a high index waveguide

separated from a high index prism block by an intervening, low index coupling layer (Fig. 2.1) (Cush *et al.*, 1993). Changes in refractive index due to the interaction of ligand-analyte at the surface of the device (the biological layer) changes the angle at which light can be made to propagate in the waveguide. At the resonance angle, light of a high intensity passes from the prism, through the coupling layer, to propagate in the waveguide as a surface evanescent wave. The light returns through the coupling layer, emerging to strike the detector, which is then monitored in real-time as the binding of molecules occurs (Cush *et al.*, 1993; Schuck, 1996).

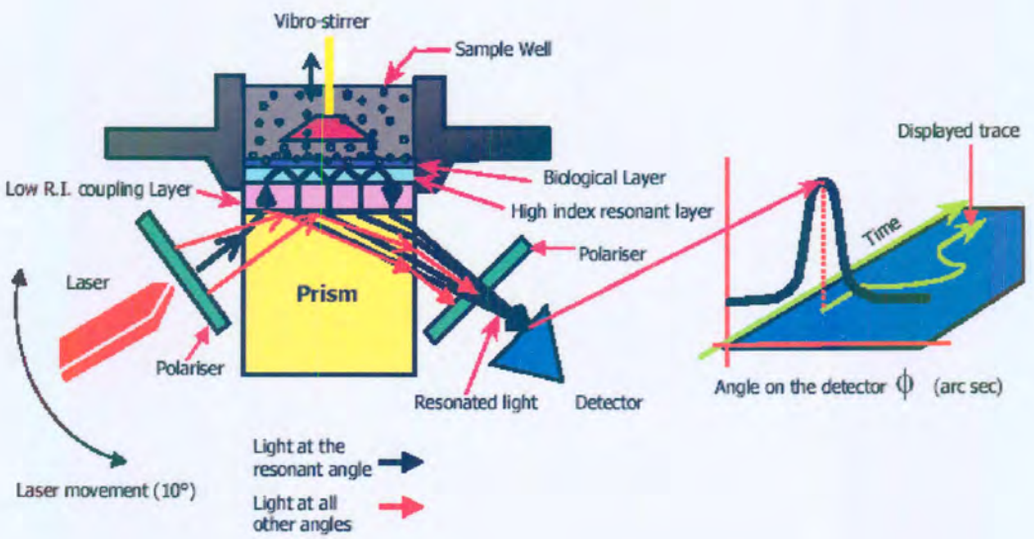


Figure 2.1: Cross section of the IAsys Affinity Biosensor cuvette and how the resonant mirror works (IAsys technical manual).

2.1.2 Factors affecting device sensitivity

The design of the device can be optimized to produce maximum overall sensitivity. Two aspects need to be considered, i.e. the sensitivity of the resonance angle to changes in the sensing layer and the resolution in resonance angle (Cush *et al.*, 1993). The sensitivity of the resonance angle is directly proportional to the fraction of the resonant mode that lies within the sensing layer. Although SPR sensors and waveguide construction and

operation are very different from each other, the resonant mirror balances the properties of both of these sensors into a highly sensitive, yet simple device (Myszka, 1999).

2.1.3 Advantages of the IAsys Biosensor

Optical biosensors represent a relatively new approach to study biomelecular interaction, with the two most common configurations of the technique being the surface plasmon resonance biosensor and the resonant mirror biosensor (Altin *et al.*, 2001). The present study employed an IAsys resonant mirror biosensor with twin-cell non-derivatized cuvettes to study the specificity and sensitivity of binding of antibodies to mycolic acids. There are many advantages of the IAsys biosensor over other popularly used biosensors. These include their compatibility with the use of particulates, high viscosity buffers and organic solvents, their better mass transport characteristics in some cases, true independent wells compared to the sequential flow path in flow based systems, easy and efficient recovery of samples from the cuvette and flexibility to allow additions to an already equilibrated system (equilibrium titration, co-factor experiments) (Cush *et al.*, 1993).

The amount of both ligand and analyte needed to obtain informative results is low and the time required to perform an assay is very short. Another advantage is that the cuvette can be reused many times. This indeed lowers the costs with the only limitation being the repeated verification of the stability of the immobilized ligand (Bertucci *et al.*, 2003). The IAsys biosensor ensures rapid sample mixing and complete solution homogeneity throughout the cuvette by means of a vibro-stirrer, which is of extreme importance in order to minimize mass-transport effects. The IAsys cuvettes are available with one or two cells. The two cells offer an advantage since one cell could be used as control in comparative measurements. The internal aspirators are used to remove solutions from the cells without removing the cuvette from the system, which makes the addition of the solution easy and fast. Another experimental advantage of the biosensor is that there is no need to label the antigen that is being used, since that could interfere with the binding sites of the antibodies to the antigen. Gonzales *et al.* (2002) reported that the use of

radiolable to the molecule under investigation on the SPR affects the binding of antibodies to the molecule and this compromised the accuracy of the assay.

The IAsys affinity biosensor requires about one tenth (5µl) of the amount of patients' sera that is required for ELISA and other standard serological tests (Siko, 2002). Since a patient's serum is a limited resource, the ability to use a minimal amount of serum could make the IAsys affinity biosensor an instrument of choice for the detection of anti-mycobacterial antibodies in patients infected with *M. tuberculosis*.

2.1.4 Applications of the biosensor using immobilized ligands

Most applications of the biosensor are based on the immobilization of the ligand on the sensor surface followed by the interaction of these molecules with the ligate. Immobilization is a critical step in the use of the biosensor, because of the risk of blocking the active site that is required for interaction with other molecules to be investigated (Stein and Gerisch, 1996). The techniques for immobilization of ligand include physical entrapment by an inert membrane, physical or chemical adsorption and entrapment in an activated surface or membrane. The interaction is then detected in real-time and directly registered. This then offers the possibility to calculate parameters for interactions, such as association and dissociation rate constants. The ability to detect and quantify bio-specific interactions from complex mixtures makes biosensors a useful tool for identifying specific ligands from crude samples (Schuck, 1996).

Biosensors can be used for characterizing and identifying protein-protein interactions from complex mixtures and also from purified samples (Williams and Addona, 2000). The SPR biosensor can also be used in proteome analysis, to confirm and quantify specific binding events to a target and to act as a micro-purification support for further analysis. The value of the combined use of the biosensor (SPR) and mass spectroscopy (MS) technology has been shown in ligand fishing studies in which the analyte is captured from a complex biological mixture onto the sensor surface and then identified using MS analysis (Malmqvist and Karlsson, 1997).

2.1.4.1 Immobilized antibodies

Antibodies play an important role in the body's immune system, and are powerful diagnostic and research tools (Pei *et al.,* 2000). There is increasing research interest in antibodies because they are useful reagents in diagnostic, therapeutic, and medical applications. A requirement for a ligand immobilized on the sensor surface is that its functional interaction with the binding molecule is retained. Antibodies are amenable to immobilization, due to their inherent resistance to denaturation (Kuby, 1997).

Leornard *et al.* (2002) described that there is no single immobilization method that has proven to be optimal for all biosensors. This suggests that a suitable immobilization method needs to be established for each biological reagent and possibly each format. For example, Babacan *et al.* (2000) have shown that immobilization of anti-*Salmonella* antibodies onto a gold electrode of piezoelectric quartz crystal through protein A has proven more reproducible and more stable than coupling of antibody with polyethylenimine. Gomes and Andreu (2002) immobilized monoclonal antibodies on the SPR (carboxymethylated dextran) sensor by chemical activation of the surface carboxyl groups with *N*-ethyl-*N'*-dimethylaminopropyl-carbodiimide (EDC)/*N*-hydroxy-succinimide (NHS) and subsequent covalent binding to the monoclonal antibodies' primary amino groups (Gomes *et al.*, 2000). A plethora of other coupling methods exist.

2.1.4.2 Immobilized protein antigens

A large number of techniques have been described for introducing proteins onto the biosensor surface. Immobilization protocols that require the protein to be present at a pH below its isoelectric point for efficient coupling to the sensor matrix may result in denaturation of acidic proteins. The strong binding between biotin and avidin provides an alternative immobilization method, but the risk of blocking the active site of the protein by direct labeling with biotin remains problem (Stein and Gerisch, 1996). The most widely used immobilization chemistry for proteins is the coupling between the carboxylate group of carboxy methyl dextran and the protein amino groups with EDC /NHS chemistry. The primary amino groups are then charged, hydrophilic and usually

projecting from the surface of the protein. After immobilization, an addition of antibody follows in order to monitor the association process (Gonzales *et al.*, 2002; Dmitriev *et al.*, 2002). The advantages of using the dextran matrix are prevention of proteins from coming into contact with metal surface, minimizing non-specific adsorption, providing better accessibility of immobilized protein to the other molecule and increased sensitivity (Schuck, 1996).

2.1.4.3 Immobilized non-protein antigens

The biosensor surface can also provide a pseudo-membrane environment that can be used to study the interaction of proteins, drugs, and receptors within a lipid bilayer. Methods for the immobilization of receptors on sensor surfaces are covalent immobilization in polymerized lipids and non-covalent adsorption or immobilizations in lipid bilayers. (Klee *et al.*, 1995). Examples of lipid-anchored receptors are glycolipids, peripheral and integral membrane proteins. The creation of the lipid bilayers can be used to analyze how proteins interact with specific phospholipid headgroups on a membrane surface (Myszka, 1999; Homola *et al.*, 2001). In order to perform studies on the receptors, they must be incorporated into the lipid layer. This layer must be firmly attached and stable on a sensor surface and the receptor must be in the correct orientation for the binding interactions to occur. Membrane fluidity can also affect the activity of the biosensor surface and is particularly important when an active biomolecule requires multiple binding sites. For example, cholera toxin contains a pentamer of the B chains that will bind to five molecules of the ganglioside GM1 on a cell surface and so the membrane must be fluid enough to allow movement of the GM1 into the correct positions. If the membrane fluidity is too high, activity may be lost due to detachment of the receptor, from the membrane lipids on the surface (Fisher and Tjarnhage, 2000).

2.1.4.4 Mycolic acids as immobilized antigens

Fisher and Tjarnhage (2000) studied different methods of liposome immobilization that are suitable for membrane formation on the resonant mirror surface. One immobilization technique used fluid liposomes containing lower phase transition temperature lipids and a small percentage of a biotinylated lipid. The liposomes were then captured and fused on a streptavidin surface to produce a bilayer above a hydrated protein layer on the biosensor. Another approached involved a direct fusion of liposomes onto an underivatized silicon nitride surface, producing a bilayer in direct contact with the biosensor surface. They showed that immobilization of lipid layers onto a resonant mirror surface enables the measurement of molecular interactions in real time.

Siko (2002) reported a novel method whereby liposomes carrying mycolic acids could be immobilized on the non-derivatized IAsys biosensor cuvettes and then used for monitoring the binding of anti-mycolic acids antibodies for the development of a serodiagnostic method for tuberculosis. He initially immobilized liposomes containing both mycolic acids and cholesterol onto the surface of the hydrophobic cuvette, but found that the coated surface was not stable. By firstly activating the surface of a non-derivatized cuvette with a cationic detergent, cetyl pyridinium chloride (CPC), the hydrophilic surface was made hydrophobic and could be stably coated with mycolic acids and cholesterol containing liposomes. A neutral surfactant, saponin, was used to further stabilize the surface and also to block the non-specific binding to immobilized liposomes. Saponins are highly soluble in water and show a typical surfactant behaviour, i.e. forming colloid solutions that easily generate foam at low concentrations. The saponins are known as biologically highly active substances. Their interaction with biological membranes is based on their affinity for membrane bound sterols. The specific interaction with cholesterol within membranes result in formation of aggregates, which build pores, thus allowing permeability even of large molecules (Karabaliev and Kochev, 2003). The surface was regenerated with 0.1M HCl, 50mM NaOH, 96% ethanol and 12.5M KOH (Siko, 2002). This approach was followed in this study to set up an IAsys biosensor configuration for the development of an assay that detects anti-mycolic acid antibodies in tuberculosis patient sera.

2.2 Aims

To optimize an existing approach for the immobilization of mycolic acids antigens on a non-derivatized IAsys biosensor cuvette in respect of the following:

(a) To determine the reproducibility of mycolic acid liposomes coating on the same surface of a single cuvette after regenerations.

(b) To determine the effect of different batches of saponin as blocking agent on a surface immobilized with liposomes containing mycolic acids and cholesterol.

(c) To optimize the concentration of saponin as a blocking agent before antibody binding studies.

2.3 MATERIALS

2.3.1 Mycolic acids

Mycobacterial mycolic acids were isolated from a culture of *M. tuberculosis* H37Rv (American Type Culture Collection 27294) as described by Goodrum *et al.* (2001).

2.3.2 The resonant mirror biosensor apparatus

The IAsys resonant mirror biosensor system and non-derivatized cuvettes were from Affinity Sensors (Cambridge, United Kingdom).

2.3.3 Reagents

Cetyl-pyridinium chloride (1-hexadecylpyridinium chloride), cholesterol (5-cholesterol-3β-ol), L-α-phosphatidylcholine (L-α-Lecithin), batches of saponin and ethylene diamine tetra-acetic acid (EDTA) were from Sigma (St Louis, MO). Sterile double distilled water was used throughout for the preparations of aqueous solutions. Sodium chloride (NaCl), Potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), and sodium hydrogen phosphate (Na_2HPO_4) were from Merck (NT laboratories, SA). Chloroform, potassium hydroxide (KO) and ethanol (98 %) were from Saarchem (SA).

2.3.4 Buffer

Phosphate buffered saline (PBS) azide EDTA buffer (PBS/AE): 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.05 g Na_2HPO_4 per liter ultra-pure, distilled water with 1 mM EDTA and 0.025% (m/v) sodium azide, adjusted to pH 7.4.

2.4 METHODS

2.4.1 Preparations of solutions

Stock solutions of cholesterol and phosphatidylcholine (100 mg/ml) were prepared by dissolving the weighed amounts in chloroform. Cetyl-pyridinium chloride (0.02 mg/ml) and saponin (1 mg/ml) were prepared in PBS/AE. Saline (0.9 %, sodium chloride) was prepared in double distilled water.

2.4.2 Preparations of liposomes

Cholesterol containing liposomes were prepared by combining 30 µl cholesterol and 60 µl phophatidylcholine stock solutions. Mycolic acids containing liposomes were prepared by adding 90 µl phosphatidylcholine stock to 1 mg dried mycolic acids. Empty liposomes, i.e. without cholesterol and mycolic acids, were prepared by taking 90 µl phosphatidylcholine of stock solution. During pipetting, everything was kept on ice to avoid evaporation of chloroform. The liposome ingredients were dried with nitrogen gas in a heat block at 85 ^{0}C for about 10 minutes. Liposome formation was induced by addition of 2 ml saline (0.9 % NaCl) and placing in a heat block at 85 ^{0}C for 20 minutes, with vortexing every 5 minutes. The liposomes were then sonicated for 2 minutes at 30 % duty cycle at an output of 3 % with the Model B-30 Branson sonifier (Sonifier Power Company, USA). The sonicator tip was thoroughly washed with chloroform and rinsed with distilled water before and after use. The liposomes (200 µl) were aliquoted into 10 tubes and kept at –20 ^{0}C overnight before freeze-drying. After freeze-drying, 2 ml of PBS/AE was added to each tube containing liposomes. The tubes were placed in a heat block for 20 minutes and sonicated as before.

2.4.3 Immobilization of liposomes on IAsys non-derivatized cuvettes

In this study, twin-cell non-derivatized micro-cuvettes were used for all experiments. The cells were rinsed three times prior to use with ethanol (98 %) followed by extensive

washing with PBS/AE. All the samples that were used in this study were prepared in PBS/AE, to prevent non-specific bulk refractive index jumps taking place upon the addition of different buffers to one another. IAsys software was used to set the device at a data-sampling interval of 0.4 s, temperature of 25 ^{0}C and stirring rate of 75 % for all experiments on the IAsys affinity biosensor. The data obtained were analyzed using the FASTplot program (Fisons Applied Sensor Technology). The programme allows the user to select areas of the graph (analysis regions) covering the baseline, association and dissociation of the reaction. The resonance scan was checked frequently as a quality indicator of the cuvette surface.

A 60 µl volume of PBS/AE was pipetted into each cell of the cuvette to obtain a stable baseline for 1 minute. The PBS/AE was subsequently aspirated and the surface activated with 50 µl of cetyl-pyridinium chloride (CPC) for 10 minutes. This was followed by 5 times washing with 60 µl PBS/AE and then substituting with 25 µl PBS/AE for a new baseline before immobilization of cholesterol or mycolic acids containing liposomes to the surface for 20 minutes. The immobilized liposomes were then finally washed 5 times with 60 µl PBS/AE and substituted with 50 µl of saponin, incubated for 10 minutes. This latter step was to avoid non-specific binding of other molecules on the surface of the cuvette during the subsequent binding events. The surface was finally washed 5 times with 60 µl PBS/AE, and substituted with 25 µl PBS/AE for a baseline. The cuvette was regenerated by 3 washes with 50 µl ethanol (98 %). After ethanol washing, the cuvette was extensively washed with several volumes of 70 µl PBS/AE.

2.5 RESULTS

2.5.1 Immobilization of liposomes on non-derivatized cuvettes

The results obtained in this study indicate that the mycolic acid liposomes could be immobilized several times on the same surface of a non-derivatized cuvette. The hydrophilic surface of the non-derivatized cuvette was made hydrophobic by activation with cetyl-pyridinium chloride (CPC). The excess CPC was removed with a 5 times wash with PBS/AE, which brought the signal back to nearly baseline. It was subsequently coated with mycolic acids carried in liposomes. From the results presented in Fig. 2.2, a binding capacity of approximately 2500 arc seconds was repeatedly achieved. The binding of liposomes to the surface was stable, since it was only marginally affected by washing with PBS/AE, indicated by the dent on the top of the binding curves in Fig. 2.2.

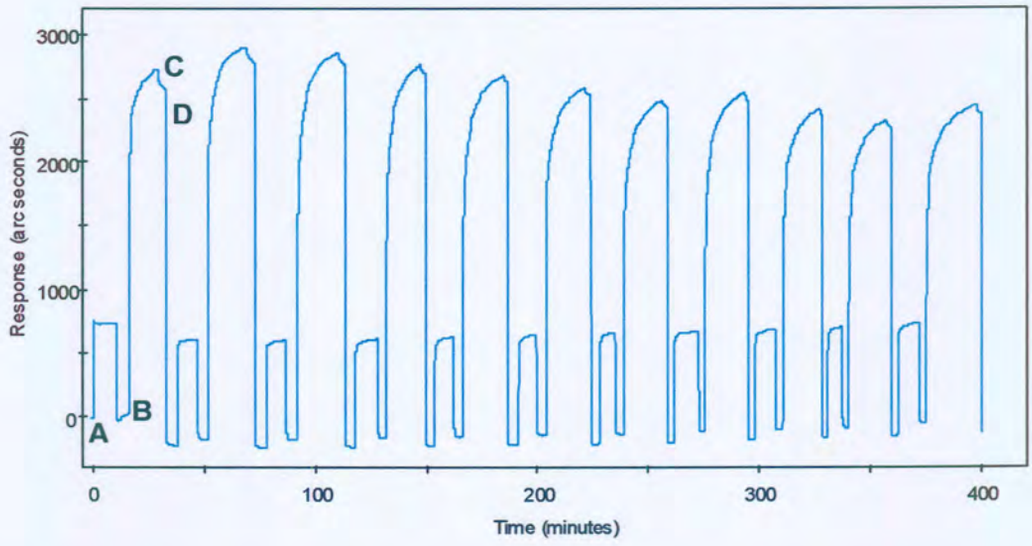


Figure 2.2: Consecutive immobilization of mycolic acids-containing liposomes to a non-derivatized surface, measured in a resonant mirror system (IAsys). The surface was activated with CPC (A), coated with liposomes (B), washed with PBS/AE (C) and regenerated (D) with ethanol (98 %) after each cycle of immobilization.

To determine if mycolic acids liposomes could be reproducible coated on the same surface of a single cuvette, regeneration of the surface was effected by 98 % ethanol wash and the cycle of activation and liposomes immobilization repeated eleven times. An exposure of the cuvette surface for 30 seconds in ethanol was adequate to remove mycolic acids containing liposomes from the surface. The regeneration procedure resulted in a decrease of the baseline to below zero only after the first cycle. For all the consecutive cycles performed, a similar, albeit slightly declining response of liposomes binding to the surface of the cuvette after activation with CPC was obtained. The repeated immobilization of mycolic acids liposomes therefore appears to be reproducible on the surface of a single cuvette. However long incubation of mycolic acids and cholesterol containing liposomes on a cuvette surface sometimes results in a decrease in response.

The IAsys instrument allows a quality check of the coated surface at any time during the process by performing a resonance scan. The prepared mycolic acids and cholesterol containing liposomes, when immobilized on the IAsys cuvette surface, always gave the expected symmetrical resonance scan, indicating that the liposome coating was uniform. The resonance scan was checked frequently, and if irregularities were observed, typically after many regenerations, the cuvette was retired.

2.5.2 Effects of saponin on liposomes

Cholesterol liposomes coated the CPC activated cuvette surface similar to mycolic acid liposomes. As indicated in Fig. 2.4, an approximately 2500-3000 arc seconds binding response of cholesterol liposomes was obtained, compared to 2500 arc seconds obtained with mycolic acids containing liposomes (Fig. 2.3). Experiments were performed to determine if saponin could have any detrimental effect on the immobilized liposomes. Various dilutions of saponin (0.2 mg/ml – 1 mg/ml) were tested. An analysis of the immobilized liposomes on the surface after different concentrations of saponin addition was made using the Fast plot program. As shown in Fig. 2.3, it was found that saponin removes some of the mycolic acids liposomes from the surface of the cuvette in a

concentration dependent manner. The phenomenon was much more pronounced when the cuvette surface was coated with cholesterol liposomes (Curve B, Fig. 2.4).

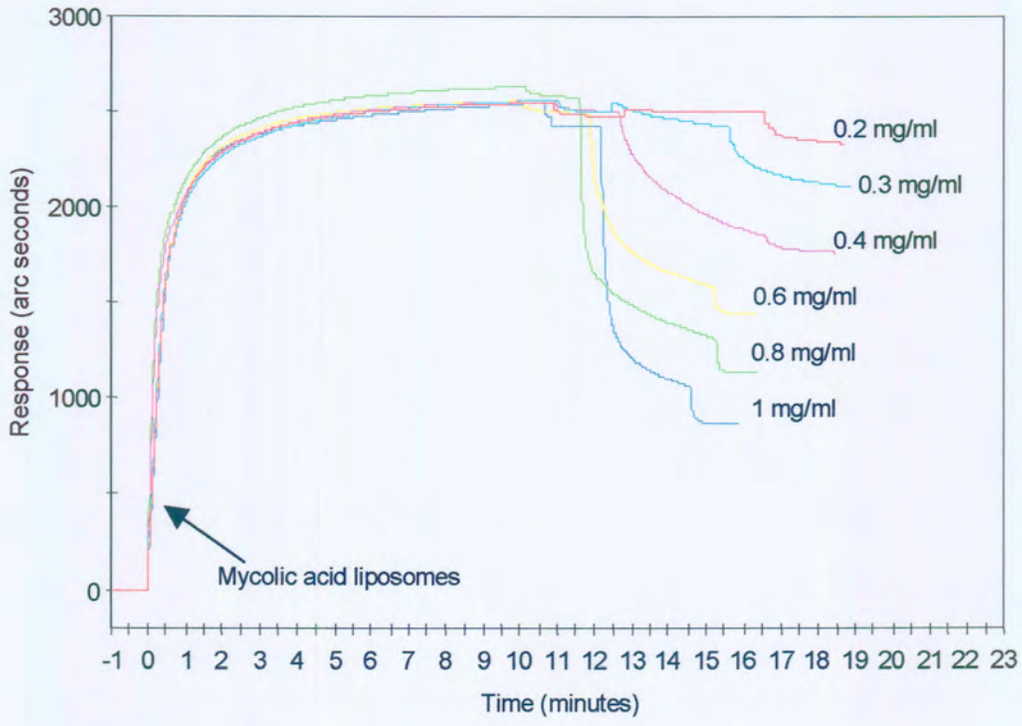


Figure 2.3: Different concentrations (1 mg/ml – 0.2 mg/ml) of saponin tested for their effect on the biosensor cuvette surface coated with mycolic acids containing liposomes.

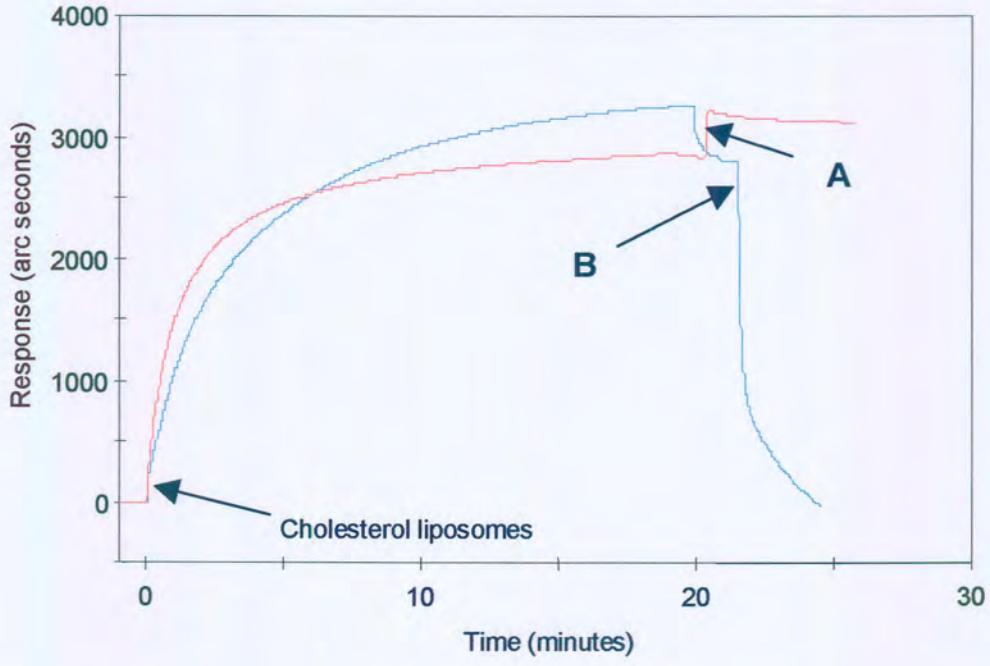


Figure 2.4: Effect of first (A) and second (B) batches of saponin (1 mg/ml) on a cholesterol-liposome coated biosensor cuvette surface.

A next experiment determined the variation between different batches of saponin. A concentration of 1 mg/ml of one batch of saponin dissociated less mycolic acids liposomes than a second batch of saponin at the same concentration (Fig.2.4). Therefore different concentrations of the second batch of saponin had to be titrated with the aim of finding a response that was similar to the first batch of saponin after its addition. The results in Fig. 2.5 show that 0.3 mg/ml of the second batch of saponin had the equivalent strength of 1 mg/ml of the first batch of saponin. From the results obtained in this study it was concluded that an effective concentration of 0.3 mg/ml of second batch of saponin was efficient to block cholesterol or mycolic acids containing liposomes immobilized on the non-derivatized cuvette, but that the concentration of each batch of saponin that is used for blocking, needed to be titrated first.

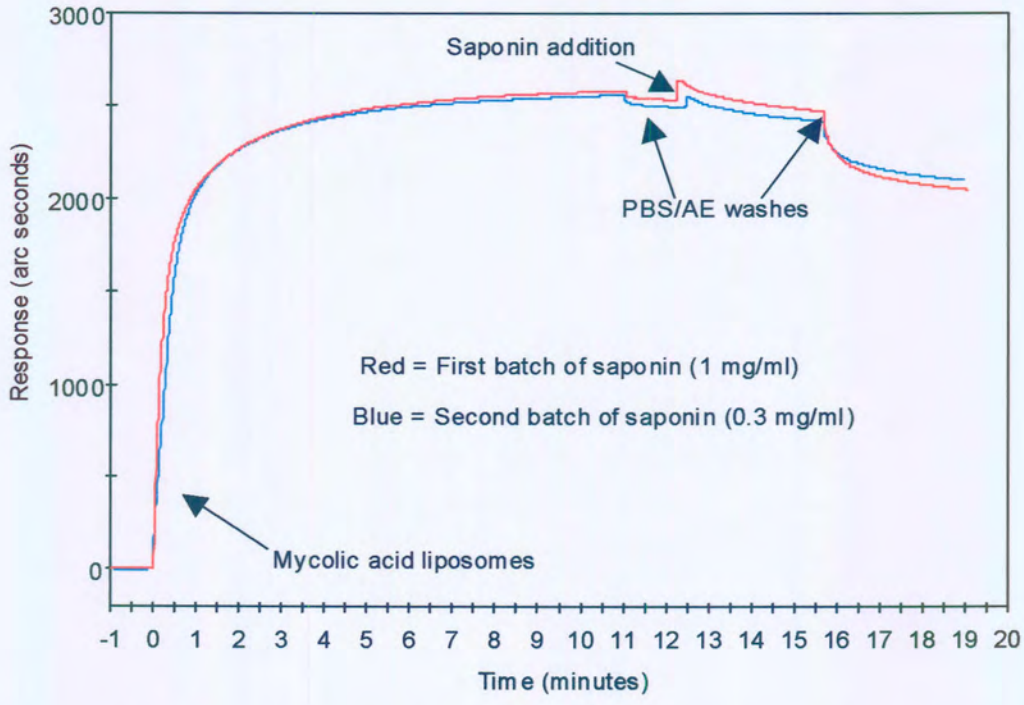


Figure 2.5: Effects of different saponin batches on immobilized mycolic acids liposomes. Addition of 1 mg/ml and 0.3 mg/ml of first (red) and second (blue) batches of saponin respectively are shown to be equivalent in their activity.

2.6 DISCUSSION

The immobilization of liposomes containing mycolic acids and cholesterol on the non-derivatized cuvettes after CPC activation is a novel application for the biosensor (Siko, 2002). He showed that there was less binding of liposomes on hydrophobic or non-activated non-derivatized cuvettes. Adequate binding of liposomes containing mycolic acid and cholesterol occurred after activation of the non-derivatized hydrophilic surface with the cationic detergent CPC. Here it was shown that mycolic acid or cholesterol-containing liposomes can be coated reproducibly on the same surface of the regenerated non-derivatized cuvette. This reduces the number of cuvettes to be used for serodiagnosis, to make the procedure more economical. Regeneration with ethanol (98 %) seemed effective for at least 11 regenerations since the baseline went below zero after each cycle.

Different batches of saponins were also tested for their effectivity as blocking agents on immobilized mycolic acid and cholesterol liposomes. Siko (2002) showed that an optimum concentration of saponin to stabilize the immobilized surface and to block non-specific binding serum components to be 1 mg/ml. In this study, it was found that when different batches of saponins were used at the same concentration they gave different results. This shows that an optimum concentration needs to be determined on the mycolic acids and cholesterol surface for each batch of saponin that is obtained. The difference in effect of the saponin batches on to the immobilized mycolic acids and cholesterol liposomes could be due to the difference in age of the particular saponin batch or that they were extracted from different raw materials.

The work in this chapter was done to improve the biosensor system for its application as a serodiagnostic device for tuberculosis. This involves detection of antibodies against mycolic acid in human patient sera. For such a test, the specificity of binding needs to be determined by inhibiting the binding signal with antigen. Both cells of a cuvette are then required to compare inhibited and non-inhibited sera. The next chapter concerns itself with these issues, to determine the feasibility of serodiagnosis of tuberculosis with

mycolic acid antigen. After blocking the mycolic acid and cholesterol immobilized surface with saponin, comparability of the two cells was determined by addition of much diluted human serum in PBS/AE, followed by further addition, without aspiration, of human serum at higher concentration, pre-incubated with mycolic acid, empty phosphatidylcholine liposomes, or liposomes containing cholesterol or mycolic acids to determine if the antibodies in serum are specific to mycolic acids.

CHAPTER 3

Differentiation between TB patient and control sera

3.1 Introduction

Schleicher *et al.* (2002) showed with ELISA that there is a higher anti-mycolic acid antibody level in TB positive than in TB negative patients. They investigated the diagnostic potential of an ELISA, based on the detection of antibodies to *Mycobacterium tuberculosis* mycolic acids in sera of HIV seropositive and HIV seronegative tuberculosis patients, in a population with a high prevalence of TB. Pan *et al.* (1999) reported the results in which the anti-mycolic acid antibody signals from TB patients sera were compared only with healthy controls. A more realistic assessment was made by Schleicher *et al.* (2002) who selected control sera from non-TB patients that had various medical conditions, including non-mycobacterial infections. Although they did observe a higher signal of antibody to mycolic acids in TB positive patients than in TB negative patients, they also found quite a number of false positive and false negative results. From their studies, they then concluded that the ELISA has poor sensitivity and specificity to detect anti-mycolic acid antibody and is therefore not suitable as a reliable serodiagnostic assay for the diagnosis of pulmonary TB.

3.1.1 A possible mimicry between mycolic acids and cholesterol

The discovery of a cross-reactivity of binding of TB patient sera antibodies between mycolic acids and cholesterol on the IAsys affinity biosensor was reported by Siko (2002). From the results based on the observations made on the IAsys biosensor, ELISA and molecular modelling it was hypothesized that the methoxy-mycolic acid mimics the structure of cholesterol by folding up twice in the distal part of the long hydrocarbon chain, away from the carboxy group. It is in this part that the keto- or methoxy- groups

reside (Fig.3.1). In this way, methoxy-mycolic acid could assume a folded structure in which structural mimicry with cholesterol could be envisaged, with the methoxy-group of mycolic acid corresponding to the 1- hydroxyl position of cholesterol. The folded structure clustered all the oxygenated groups on one side of the molecule by means of the two hairpin bends induced by the two cyclopropane moieties in the long hydrocarbon chain of mycolic acids (Siko, 2002).

The hypothetical structural mimicry between mycolic acids and cholesterol may explain the poor specificity of the ELISA test for anti-mycolic acid antibodies of Schleicher *et al.* (2002). It has been shown that naturally occurring IgM and IgG antibodies to cholesterol are found in humans and in many animals (Alving and Wassef, 1999; Shoji *et al.*, 2000). These antibodies are present ubiquitously in normal human serum and bind to low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and intermediate density protein (IDL), but not to high-density lipoprotein (HDL). Antibodies to cholesterol have been induced in rabbits by injection of cultured *Mycoplasm* cells, several species of which are common inhabitants of the human oral cavity and lower urogenital tract (Watanable *et al.*, 2001). Siko (2002) also described a strong affinity of binding of cholesterol to mycolic acid. He showed that an addition of cholesterol liposomes to a mycolic acid liposomes coated surface led to the accumulation of cholesterol onto the surface. The pre-incubation of empty phosphatidylcholine liposomes on a mycolic acid liposomes coated surface showed no accumulation onto the mycolic acid coat. When analysing sera on a mycolic acid coated surface, the mycolic acid may become covered with serum cholesterol, thereby obliterating the specificity of binding of the anti-mycolic acid antibodies.

An inhibition step was added to the biosensor assay to detect anti-mycolic acids antibodies to determine the relative specificity of the antibodies, i.e. to determine whether the cross-reactivity between mycolic acids and cholesterol would render the test inadequate for the diagnosis of tuberculosis. Here I show that pre-incubation of TB positive serum with mycolic acid liposomes results in an inhibition of antibodies, while no inhibition was observed after pre-incubation with cholesterol liposomes. This shows

that the antibodies that are found in TB positive patients have a stronger affinity for mycolic acids than cholesterol, and that this difference can be measured with the biosensor to provide a basis for a serodiagnostic test for tuberculosis.

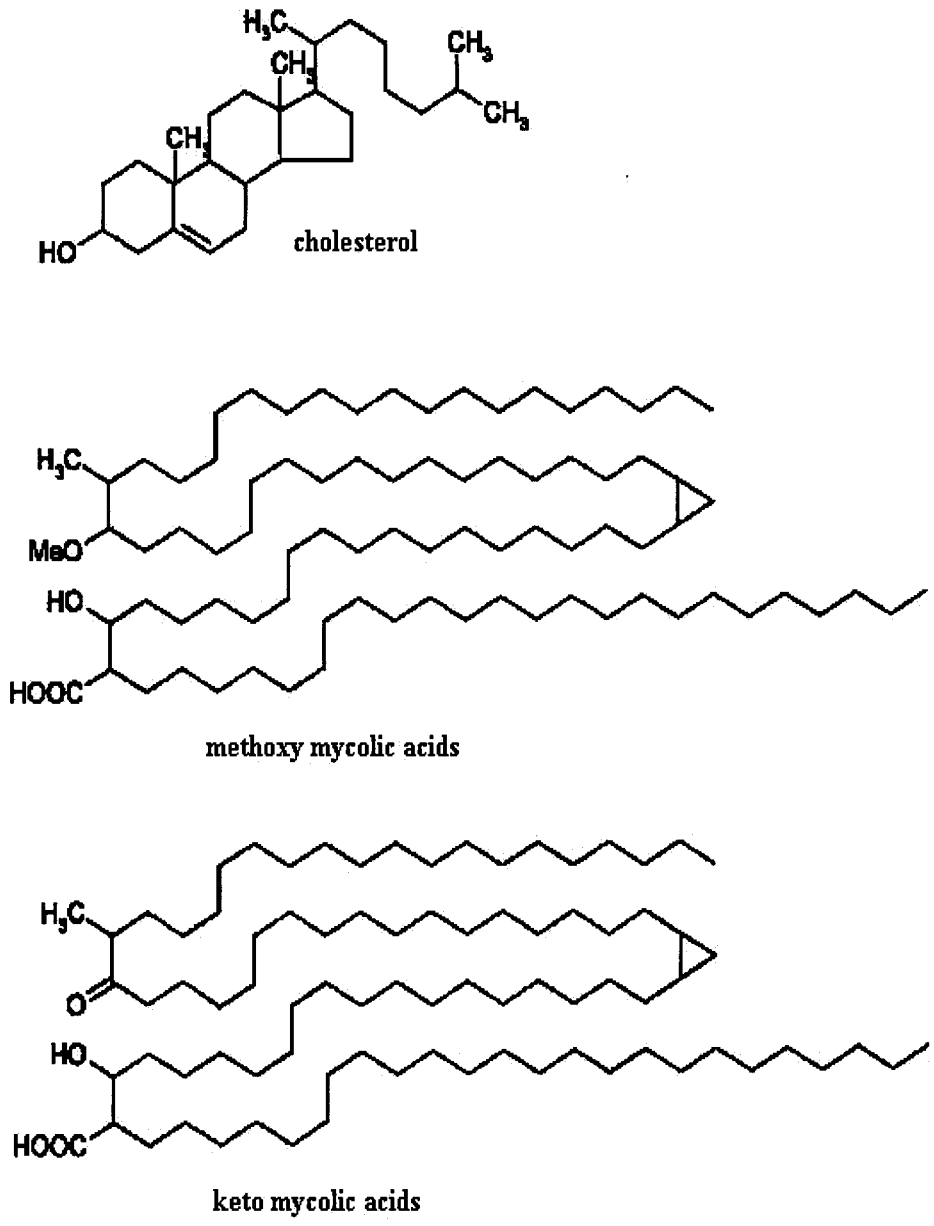


Figure 3.1: The proposed molecular mimicry between the methoxy- and keto- mycolic acids and cholesterol (Siko, 2002).

3.2 Aim

The main aim was to investigate the application of a commercial biosensor instrument (IAsys biosensor, Cambridge, UK) for the detection of antibodies against mycolic acids in human serum samples as surrogate markers for infection.

More specifically, the following questions were addressed:

(a) Will the apparent cross-reactivity between mycolic acids and cholesterol negatively affect the biosensor assay to detect anti-MA antibodies in TB patient sera?

(b) Does the biosensor assay improve the positive and negative predictiveness of the test for anti-MA antibodies when compared to ELISA?

3.3 MATERIALS

3.3.1 Enzyme Linked Immunosorbent Assay (ELISA)

Serowell ELISA plates: flat-bottom 96-well plates; disposable pipette tips; Sterile, disposable 50 ml centrifuge tubes and disposable pipettes were from Bibby Sterilin Ltd, Stone, UK.

Goat anti-human IgG (Heavy and Light chain) antibody conjugated to peroxidase was obtained from Sigma, St Louis, MO, USA.

Carbohydrate- and fatty acid free casein was from Calbiochem, La Jolla, CA and hydrogen peroxide from Merck (Darmstadt, BRD).

o-Phenylenediamine and Polyethylene glycerol (PEG) were from Sigma, St Louis, MO, USA.

3.3.2 Buffers

PBS buffer: 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 (anhydrous) and 1.05 g Na_2HPO_4 (anhydrous) per 1 liter distilled water, adjusted to pH 7.4 using 0.1M HCl and 1 M NaOH.

Neutralisation buffer: K_2HPO_4 (1 M in dddH_2O) adjusted to pH 9.0 with H_2KPO_4 (1 M) if necessary.

Acidification buffer: Glycine HCl (0,2 M, pH 2.8).

Diluting buffer: 0.5% (m/v) carbohydrate- and fatty acid free casein in PBS buffer adjusted to pH 7.4 was used for diluting the sera and the immunoreagents.

3.3.3 Human sera

Serum samples were randomly selected from 17 patients (aged between 18 and 65) collected for another study by Schleicher *et al.* (2002), who were admitted to the general medical wards of the Helen Joseph Hospital; Johannesburg, (South Africa), including a number with active pulmonary tuberculosis. The sera were collected from this hospital between August and December 2000. The Institutional Ethics Committee (University of Witwatersrand) approved the study and informed consent was obtained from all patients prior to enrolment.

The study population consisted of a tuberculosis-positive (TB^+) group and a control tuberculosis-negative (TB^-) group. The TB^+ group consisted of patients with newly diagnosed smear-positive pulmonary tuberculosis of which some were HIV-seropositive. Expectorated sputum samples were stained with Ziel-Neelsen stain and microscopy performed for acid-fast bacilli (AFB). All sputum samples that were considered positive for AFB were submitted for mycobacterium culture by inoculation into appropriate broth culture media and analysed radiometrically (BACTEC-460 TB Hood, Becton Dickinson and Co., USA). All positive mycobacterium specimens were further identified and confirmed as *M. tuberculosis*.

The TB^- patients that were used for control had medical conditions other than TB and were recruited from the general medical wards. They had no clinical, radiological or microbiological evidence of active infection with *M. tuberculosis*. These control subjects were matched with the TB^+ group as best as possible for gender, age and race.

All patients had routine sputum analyses, haematological, biochemical, and serological blood tests (including testing for HIV infection), as well as chest X-rays performed as part of the usual clinical practice.

3.4 METHODS

3.4.1 Serum preparations for ELISA

The patient and control sera were aliquoted into empty sterile tubes. Aliquots of 25 µl of sera were diluted in 475 µl diluting buffer.

3.4.2 Preparation of serum precipitates

Patient serum precipitates were prepared by the following method: A mixture of undiluted serum (100 µl) and 4 % PEG in 0.01 M PBS pH 7.4 (100 µl) was left overnight at 4 ^{0}C. The precipitate was collected by centrifugation at 4 ^{0}C for 30 minutes at 4500 rpm. The precipitates were kept and the supernatants discarded. The precipitates were washed twice with 100 µl of 4 % PEG by centrifuging for 30 minutes at 4 ^{0}C at 4500 rpm. The washing buffer 4 % PEG was aspirated and the precipitate dissolved in 100 µl PBS (0.154 M, pH 7.4). To release the antibodies from the immune complexes, acidification buffer, 50 µl glycine HCl (0.2 M, pH 2.8) was added, and left on ice for 15 minutes. Neutralisation buffer, 25 µl K_2HPO_4, was added, followed by addition of double distilled water (25 µl) and casein PBS (1800 µl) to obtain a final volume of 2 ml, corresponding to a 1: 20 dilution of serum.

3.4.3 Immobilization of antigen for ELISA

Mycobacterial mycolic acids were isolated from a culture of *M. tuberculosis* as described by Goodrum *et al.* (2001). Mycolic acids (250 µg) were dissolved in 4 ml hot phosphate-buffered saline (PBS, pH 7.4) for 20 minutes at 85 ^{0}C and sonicated at 20 % duty cycle and optimal output level for 1 minute. The solution was kept at 85 ^{0}C during pipetting into ELISA plates, after which the plates were placed in plastic bags and incubated overnight at 4 ^{0}C. The final antigen load was approximately 3 µg/well. Control wells were coated with hot PBS only. After overnight incubation, the ELISA plates were flicked out and the wells blocked with 0.5 % (m/v) carbohydrate- and fatty acid-free casein in PBS for 2 hours at room temperature. The solution was flicked out, filled with 50 µl serum or serum precipitate dilutions in triplicate and incubated for 1 hour at room

temperature, flicked out and washed three times with PBS/0.5% casein. The wells were aspirated to remove all the bubbles left during washing. The plates were incubated for 30 minutes at room temperature with peroxidase-conjugated anti-human IgG diluted 1: 1000 in PBS/0.5% casein, flicked out and followed by three washes with PBS/0.5% casein. The wells were aspirated to remove all the bubbles left during washing. Peroxidase activity was revealed using 50 µl of hydrogen peroxide (40 mg) and *o*-phenylenediamine (50 mg) in 50 ml 0.1M citrate buffer (pH = 4.5). The reaction product was measured after 30 minutes at 450 nm using a SLT 340 ATC photometer (Thermo-Labsystems, Finland).

3.4.4 Liposome preparations for IAsys biosensor

The liposomes containing either cholesterol or mycolic acids in phosphatidylcholine were prepared as described in 2.4.2.

3.4.5 Detection of anti-mycolic acids in human sera on IAsys affinity biosensor

Immobilization on non-derivatized cuvettes of either mycolic acids or cholesterol liposomes was done as described in 2.4.3. After saponin addition to avoid non-specific binding, the surface was then washed 5 times with PBS/AE and each cell content was substituted with 25 µl of PBS/AE and allowed to equilibrate for about 5 - 10 minutes to achieve a stable baseline. Inhibition studies were performed using patient's serum that was first placed at room temperature to thaw completely. After obtaining a stable baseline, a 1:1000 dilution of serum antibodies (10 µl) in PBS/AE was added, to compare the response of two cells over 10 minutes. A pre-incubation of 1:500 dilutions of serum antibodies in liposomes containing either mycolic acids or cholesterol, and empty liposomes (phosphatidylcholine alone) were allowed for 20 minutes. These were then added (10 µl) for inhibition studies in different cells, one with mycolic acids or cholesterol liposomes and the other with phosphatidylcholine as a control, and allowed to bind for 10 minutes. Finally, dissociation of antibodies was effected with 3 times PBS/AE washing and measurement of the response for 5 minutes.

3.4.6 Regeneration of non-derivatized cuvettes

The regeneration of the cuvettes surface was performed with 5 times washing with 50 µl of hydrochloric acids (0.1 M) for 1 minute, 7 times washing with 70 µl PBS/AE, 5 times washing with sodium hydroxide (10 mM) for 1 minute, and then 7 times wash with 70 µl PBS/AE for 1 minute. This was then followed by 3 times washing with 50 µl ethanol (98 %) for 30 seconds, and 7 times washing with PBS/AE washing for 1 minute. The surface was then finally washed with 50 µl of guanidine thiocynate salt (3.5 M) for 1 minute and followed by 7 times with 70 µl PBS/AE for 1 minute.

Alternatively, regeneration was effected by initial 3 times washing with ethanol (98 %) for one minute, followed by 7 times washing with 70 µl PBS/AE for 1 minute. The surface was then finally treated with 50 µl potassium hydroxide (12.5 M) for 2 minutes and followed by 7 times washing with 70 µl PBS/AE for 1 minute.

3.5 RESULTS

Fig. 3.2 shows the six main stages involved to measure the binding of specific antibodies to lipid antigens in liposomes in real time: (A) the activation of the surface with CPC, (B) immobilization of the liposomes containing cholesterol or mycolic acids to the surface, (C) blocking with saponin to prevent non-specific protein binding, (D) binding (association) of antibodies from a high dilution of serum to calibrate the signal of the two cells of the cuvette, (E) the binding and dissociation of inhibited patient sera at lesser dilution, and finally (F) surface regeneration.

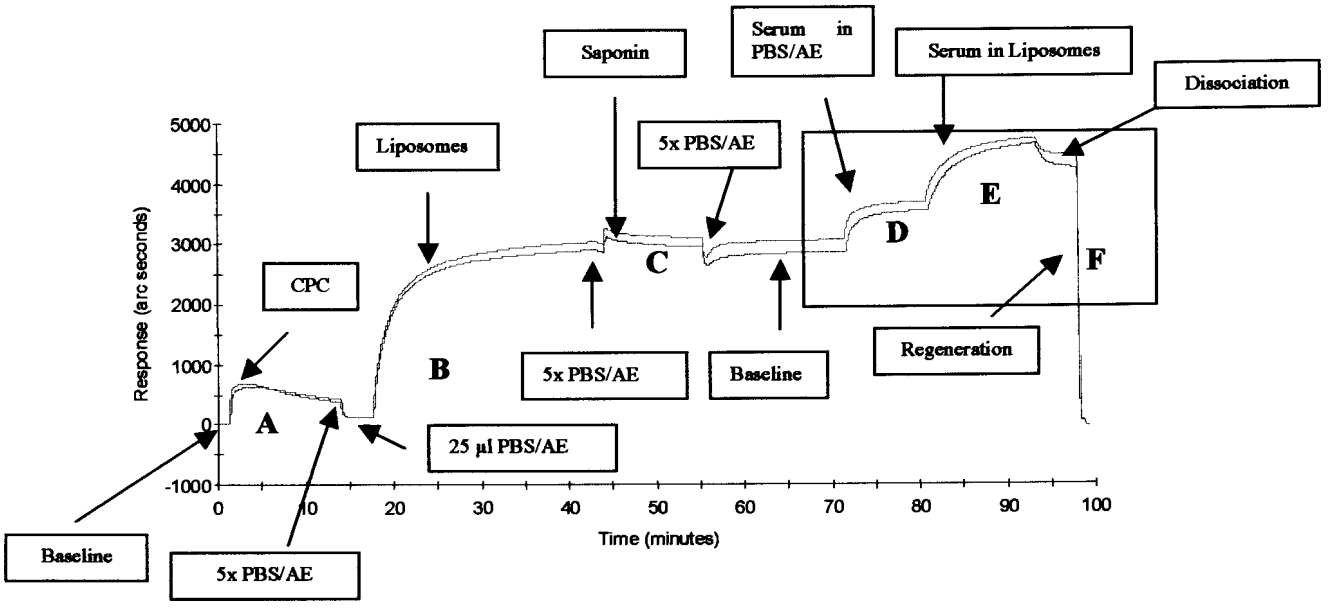


Figure 3.2: A typical graph that summarises the process of measuring antibody binding or inhibition of binding by cholesterol or mycolic acid and phosphatidylcholine liposomes, on an IAsys biosensor cuvette surface coated with mycolic acid or cholesterol liposomes. The surface was activated with CPC (A), coated with mycolic acids or cholesterol liposomes (B), blocked with saponin (C), calibrated with a high dilution of serum (D), applied to measure the binding and dissociation of inhibited sera at lesser dilution (E), and regenerated with KOH and ethanol (F).

The surface of the non-derivatized cuvette was first activated with CPC after a PBS/AE wash. It gave a response of about 500 arc seconds followed by a gradual decline, probably due to the driving off of water from the surface. After a PBS/AE wash the cuvette content was then substituted with a 25 µl PBS/AE and left to obtain a stable baseline. Coating with cholesterol or mycolic acid liposomes gave a response of ± 2500 arc second after 20 minutes. Unbound liposomes were removed by 5 times washing with PBS/AE. Fig. 3.2 shows an example where the immobilization of liposomes containing cholesterol or mycolic acids was stable, since dissociation with PBS/AE showed only a slight decrease in response. Addition of saponin at the previously optimised concentration resulted in a slight increase of response that gradually decreased over 10 minutes of incubation. Unbound or loosely attached saponin was removed with PBS/AE washing, followed by incubation with PBS/AE for a baseline.

A high dilution (1:1000) of patient serum was first added to both cells of the biosensor cuvette in order to determine the comparability of the two cells before binding and inhibition of binding studies with a higher concentration of serum. When the difference between responses of the cells in one cuvette was found not to approach identity, the test was aborted and the cuvette regenerated or another cuvette was employed.

3.5.1 Comparability of paired cells in the IAsys biosensor non-derivatized cuvettes

Fig. 3.3 summarises the success frequency of using the non-derivatized cuvettes for the antibody binding studies that requires identical responsiveness of the two cells. Many experiments were rejected due to the two cells not being comparable. The numbers on top of each bar indicate the number of times each cuvette was used in anticipation of obtaining comparable responsiveness of the two cells. The cuvette numbers in Fig. 3.3 are shown in the order in which they were used starting from 1C3 up to 8C3. One cuvette (1C4) never gave comparability of the two cells, even after eight experiments were tried. The best cuvette was 8C1, which gave 80 % successful mycolic acids or cholesterol liposome coats with comparable responses, resulting in successful binding assays with sera.

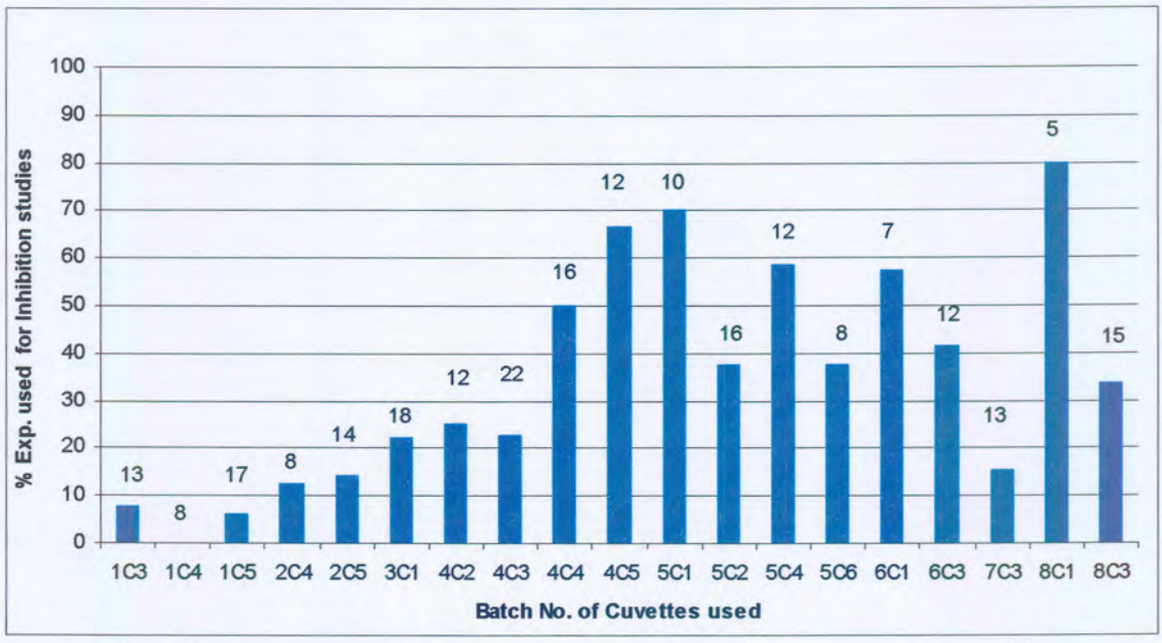


Figure 3.3: Frequency of successful use of non-derivatized cuvettes coated with either mycolic acids or cholesterol containing liposomes for differential analysis of inhibited or placebo-inhibited patient sera. Numbers above the bars indicate the number of attempts to use the cuvettes. Cuvettes were used in the order at which they are shown, starting with 1C3.

3.5.2 Regeneration of non-derivatized cuvettes

Regeneration of the IAsys biosensor cuvette surface is essential for affordable use of the biosensor with large numbers of samples. Two regeneration methods have been used in this study that were optimised by Mr Pieter Vrey, a member of the research team. The first one involved the use of GSCN, hydrochloric acid, sodium hydroxide, and ethanol. The method was found to be too mild, since it was not possible to obtain a stable baseline at the origin after an experiment was performed. Some of the bound cholesterol or mycolic acids liposomes, antibodies and other molecules in patients' sera were apparently not fully removed during regeneration. Therefore a harsher regeneration method was applied that involved the use of potassium hydroxide (12.5 M) and ethanol (98 %) only, and this method was found to be more effective.

The repeated regeneration with KOH and ethanol was noted to contribute to gradual cuvette surface deterioration. Each IAsys cuvette could be re-used for about 10 – 30 times in separate experiments, provided that freshly prepared liposomes were used and provided that each regeneration step was performed with separate washes using ethanol, PBS/AE, KOH and an extensive final wash with PBS/AE.

3.5.3 Detection of anti-mycolic acids antibodies in human sera

Patient sera selected from the collection of Schleicher *et al.* (2002) were used to detect antibodies against mycolic acids or cholesterol on the optical IAsys biosensor. The small box in Fig. 3.2 (D and E) indicates the binding of antibodies to a surface immobilized with mycolic acids or cholesterol liposomes. Since it was proposed that there could be a mimicry between cholesterol and mycolic acids structures (Siko, 2002) it was important to test the specificity of binding to determine if the antibodies directed to mycolic acids could also bind cholesterol. This was determined by pre-incubating test serum with either mycolic acids- or cholesterol- containing liposomes and applying these on cuvettes coated with mycolic acids. In the control experiments sera were pre-incubated with empty liposomes (phosphatidylcholine only) containing neither mycolic acids, nor cholesterol. With true cross-reactivity, an inhibition of antibody binding to either immobilized mycolic acids or cholesterol should be effected by pre-incubation of the sera with either mycolic acids- or cholesterol-containing liposomes. After completion of each experiment, the results such as those in the small box in Fig. 3.2 (D and E) were analyzed using a FAST-plot program.

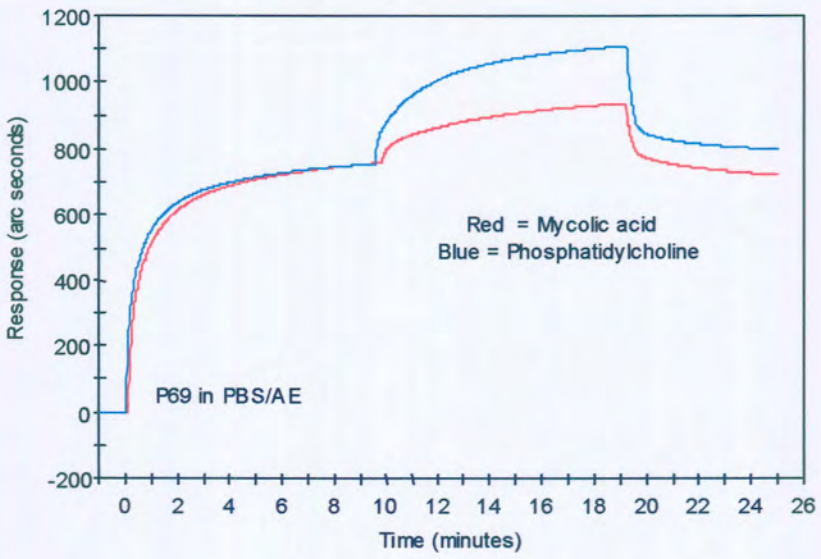
The pre-incubation of a sputum positive TB patient serum with mycolic acids liposomes resulted in an inhibition of antibody binding on a surface coated with mycolic acids when compared to the signal generated by the same serum pre-incubated with phosphatidylcholine liposomes (Fig. 3.4A). This confirmed the specificity of binding of antibodies to mycolic acids in sputum positive TB patient's sera. There was no apparent inhibition of antibody binding when the same patient serum was pre-incubated with cholesterol liposomes (Fig. 3.4B). The results suggest that the anti-mycolic acids

antibodies in tuberculosis patients may have a much higher affinity for mycolic acids than for cholesterol.

In some cases, there was a rapid increase in signal after immobilization of the surface with mycolic acids or cholesterol liposomes and also after addition of sera in PBS/AE as a control and in pre-incubation of patient sera with liposomes, which was mainly due to refractive index change in the bulk solution, but after an extended period of incubation with identical additions, the two cells gave the same response.

No inhibition of binding was observed when a sputum negative control serum (HIV^-TB^-) was pre-incubated with liposomes containing either mycolic acids (Fig. 3.5A) or cholesterol (Fig.3.5B) and tested on the biosensor to determine binding of antibodies to mycolic acids. This shows that specific anti-mycolic acids antibodies may only be demonstrated in TB^+ patients, but not in TB^- controls after pre-incubation of serum with mycolic acids.

A



B

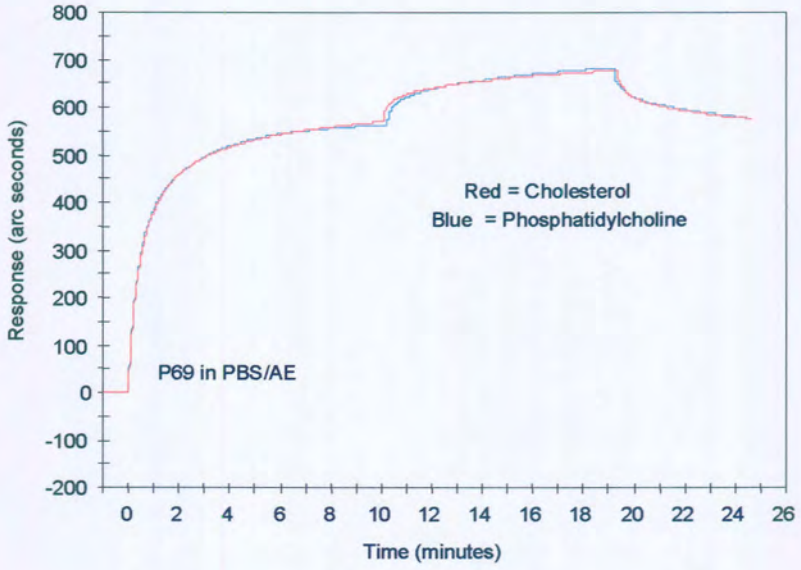
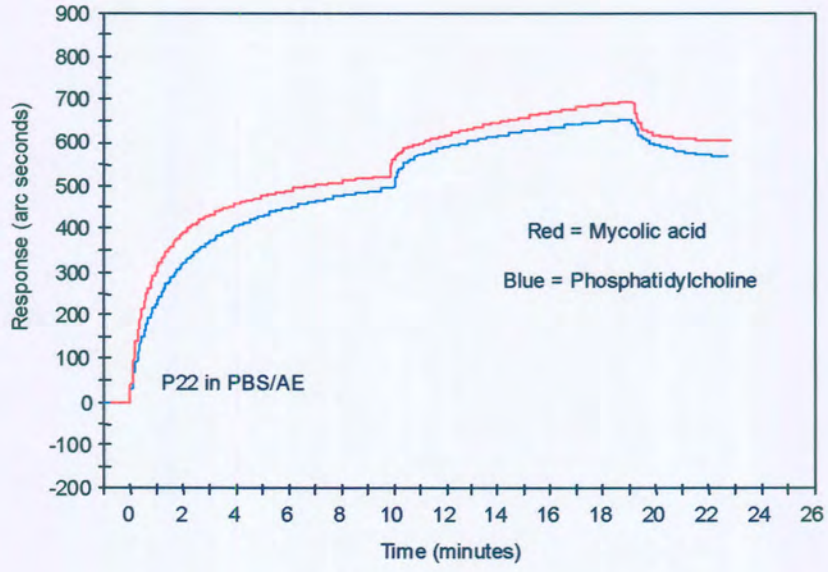


Figure 3.4: Inhibition of human TB^+ patient (P69) serum antibody binding on a mycolic acids immobilized surface of the IAsys cuvette. For the first 10 minutes, a 1:1000 dilution of serum in PBS/AE was incubated in both cells. For inhibition studies, the pre-incubated serum in a dilution of 1:500 was then added. (A) Inhibition with mycolic acids or phosphatidylcholine and (B) inhibition with cholesterol or phosphatidylcholine.

A



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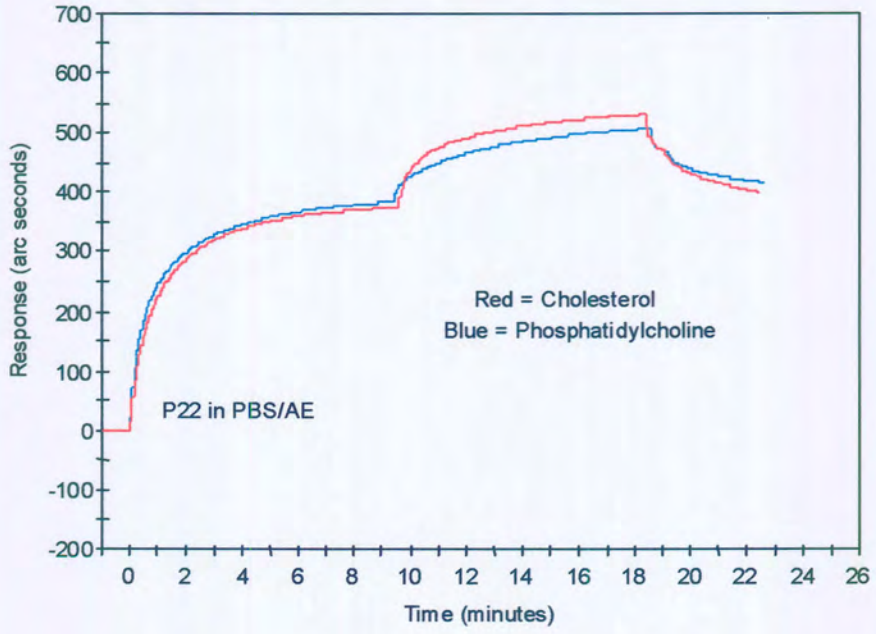


Figure 3.5: Inhibition of human TB$^-$ patient (P22) serum antibody binding on a mycolic acids immobilized surface of the IAsys cuvette. For the first 10 minutes, a 1:1000 dilution of serum in PBS/AE was incubated in both cells. For inhibition studies, the pre-incubated serum in a dilution of 1:500 was then added. (A) Inhibition with mycolic acids or phosphatidylcholine and (B) inhibition with cholesterol or phosphatidylcholine.

After analysing a number of sera on the IAsys biosensor, an ELISA experiment was done on mycolic acids coated plates to correlate the results. Table 3.1 summarises all TB patient and control sera that were analysed on the ELISA and IAsys biosensor. Those TB patient sera that gave a signal to background value of above 2 from ELISA after normalization with control sera were considered positive on ELISA and those below 2 were false negative. The normalised ELISA signals of sera from patient 57 (HIV^+TB^+) and 22 (HIV^-TB^-) were obtained from Schleicher *et al.* (2002), since there were no sera left to redo the ELISA experiments.

For ELISA, sera were also treated with PEG to precipitate all immunoglobulin and immunoglobulin-antigen complexes. This was then dissolved in acid to break up the immune complexes, neutralised and was then rapidly added to the mycolic acids coated ELISA plates. Serum precipitates from P17 (HIV^-TB^-), P3 (HIV^-TB^-), P67 (HIV^+TB^-), P65 (HIV^+TB^-), P41 (HIV^+TB^-) and P21 (HIV^-TB^-) were false positive when tested on ELISA, but registered truly negative on the biosensor. Likewise, serum precipitates from patients 60 and 48 that were HIV^+/TB^+ gave false negative results when analysed on the ELISA but registered truly positive on the biosensor, by showing a clear inhibition of antibodies after a pre-incubation of serum with mycolic acids.

The TB^+ patients (P56, 69, 63 and 42) that showed truly positive responses of antibodies to mycolic acids on ELISA also tested truly positive on biosensor. From eight TB^+ sera selected (inhibition on the biosensor greater than 30 %), significant differences were obtained in binding signals between mycolic acids and phosphatidylcholine-inhibited samples (Fig. 3.6). In contrast, of nine TB^- sera selected, seven showed no significant differences (less than 20 % inhibition). Only sera from patients 75 and 77 (HIV^+TB^-) tested false positive on both ELISA and the biosensor (Fig. 3.6 and Table 3.1). It is noteworthy that both these patients were HIV^+. One explanation for these results could be that the sputum test for TB was in error, as it is known to be more difficult to detect the *M. tuberculosis* from sputum in HIV and TB co-infected patients (Dr. Stoltz A.C, personal communication).

Table 3.1: Correlation between ELISA and IAsys biosensor responses of anti-mycolic acid antibody binding.

Patients/ Controls sera	Type	ELISA signal to background values on MA (Serum)	ELISA signal to background values on MA (Precipitate)	Biosensor signal on MA coated surface, inhibited by pre-incubation with MA-liposomes (% Inhibition)
P57	HIV^+TB^+	7.6*	6.2*	√ (44)
P60	HIV^+TB^+	1.7	1.9	√ (33.3)
P71	HIV^+TB^+	1.6	2.8	√ (33.3)
P69	HIV^+TB^+	2.8	4.4	√ (50)
P48	HIV^+TB^+	1.1	1.9	√ (56.2)
P56	HIV^+TB^+	8.4	8.3	√ (54)
P67	HIV^+TB^-	2.5	3.8	X (14.7)
P75	HIV^+TB^-	2.8	2.6	√(38.2)
P65	HIV^+TB^-	2.1	3.2	X (13.2)
P41	HIV^+TB^-	2.2	2.4	X (5.3)
P77	HIV^+TB^-	2.1	1.9	√ (50)
P22	HIV^-TB^-	1.5*	1.1*	X (1.6)
P17	HIV^-TB^-	1.5	2.3	X (2.1)
P3	HIV^-TB^-	3.9	3.6	X (18.2)
P21	HIV^-TB^-	3.0	2.9	X (3.2)
P63	HIV^-TB^+	1.7	2.0	√ (61.9)
P42	HIV^-TB^+	2.1	2.2	√ (48.4)

X = No Inhibition (less than 20%), √ = Inhibition (more than 30 %), * = ELISA signals obtained from Schleicher *et al.* (2000).

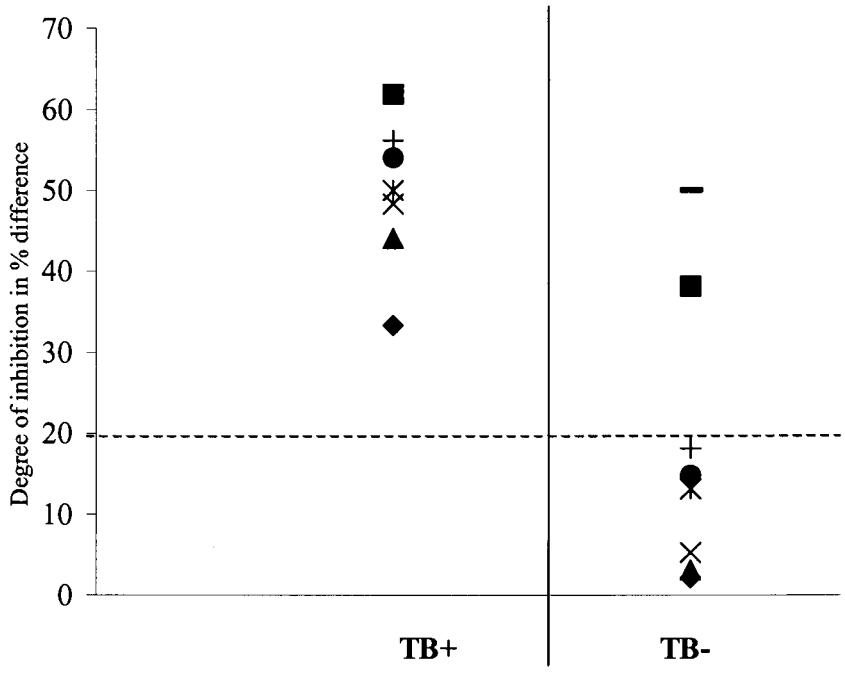


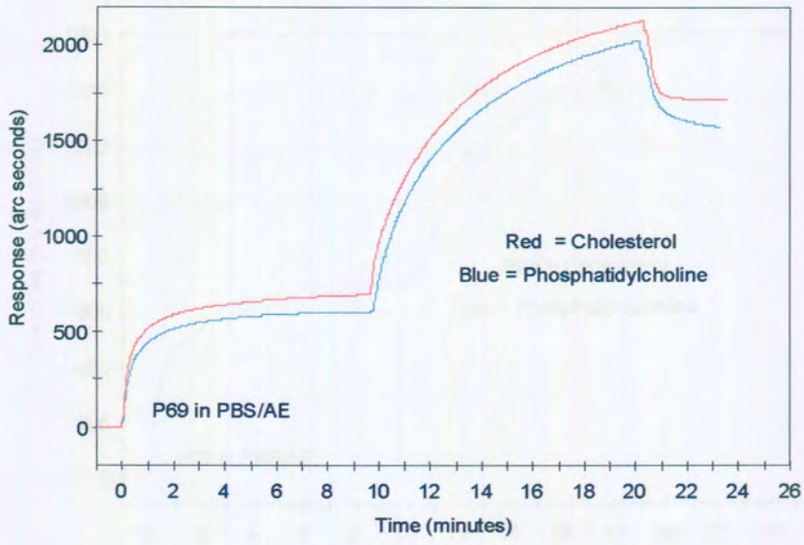
Figure 3.6: The percentage of inhibition of binding of biosensor signal in TB^+ patients and TB^- controls after pre-incubation of sera with mycolic acids and phosphatidylcholine liposomes before testing on mycolic acids coated cuvettes.

3.5.4 Biosensor experiments with immobilized cholesterol liposomes

In section 3.5.3 it was shown that mycolic acid appears to be a much stronger antigen for binding to TB^+ patient antibodies than cholesterol, despite a previous report on cross-reactivity and molecular mimicry between these two structures. Here the surface of the IAsys biosensor cuvette was now coated with cholesterol liposomes, in an attempt to test if those antibodies directed to mycolic acids will also bind specifically to cholesterol. There was no inhibition of antibody binding after pre-incubation of patient serum with cholesterol liposomes and applied to a cholesterol-coated cuvette in all 17 TB^+ and TB^- patients analysed (Fig.3.7A and 3.8A). When patient's sera were pre-incubated with mycolic acids liposomes and binding measured on a surface coated with cholesterol liposomes, variable and inconsistent degrees of inhibition were observed (Fig. 3.7B, 3.8B and 3.9). It was not possible to state that the pre-incubation of patient sera with mycolic acids liposomes on cholesterol-coated surface resulted in an inhibition of antibodies since some patients showed inhibition (Fig. 3.8B), but others resulted in a huge decrease of signal after dissociation of unbound or loosely bound antibodies with PBS/AE (Fig. 3.9).

The inability of cholesterol liposomes to inhibit antibody binding in TB^+ patient sera against immobilized cholesterol on the cuvette surface argues against a straightforward molecular mimicry between mycolic acid and cholesterol. Mycolic acid appears to be a stronger antigen than cholesterol for the detection of antibodies in serum. Since there was no inhibition of binding to the cholesterol coated cuvette when the serum was pre-incubated with cholesterol, the saponin-cholesterol complex on the cuvette surface may provide a stronger antigen or the saponin could penetrate the liposomes, thereby washing away phosphatidylcholine, and leaving a more concentrated cholesterol coat on the binding surface to out-compete the soluble cholesterol during the antibody binding event in the cuvette. This bodes well for the avoidance of false positive outcomes in the use of the biosensor to detect anti-mycolic acids antibodies as surrogate marker for TB.

A



B

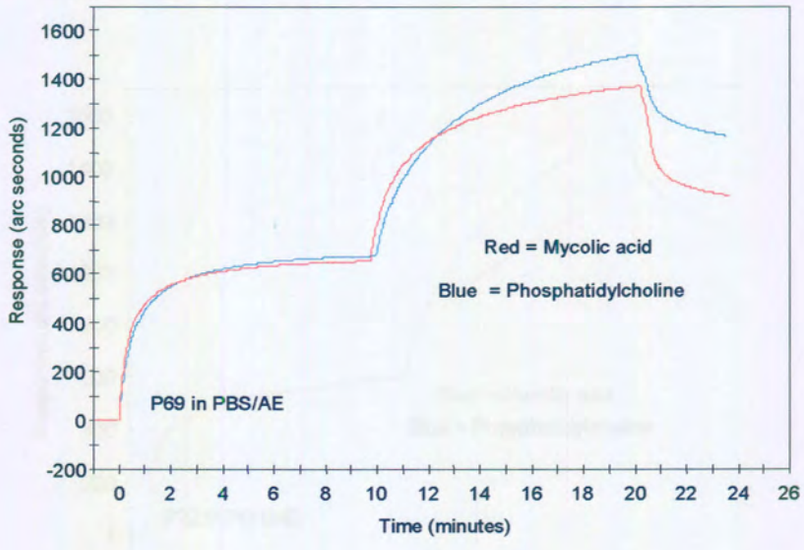
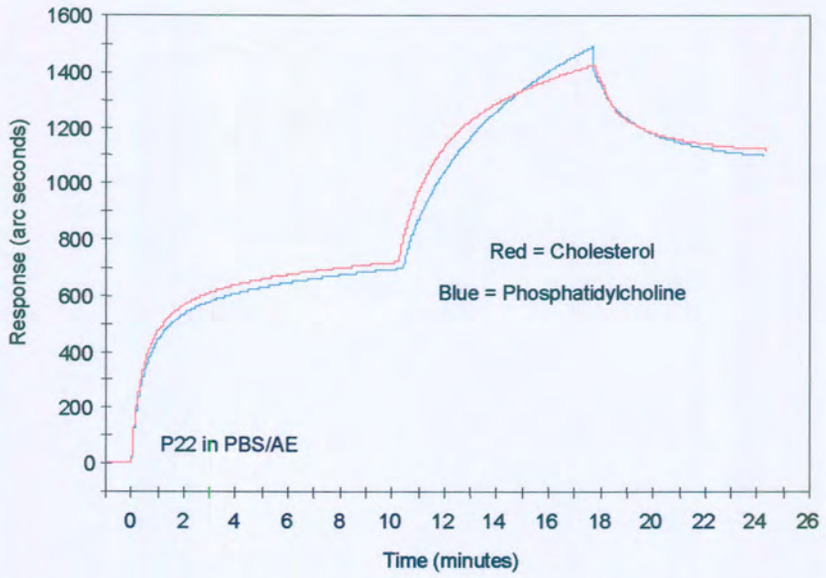


Figure 3.7: Inhibition of human TB^{+} patient (P69) serum antibody binding on a cholesterol-coated surface of the IAsys cuvette. For the first 10 minutes, a 1:1000 dilution of serum in PBS/AE was incubated in both cells. For inhibition studies, the pre-incubated serum in a dilution of 1:500 was then added. (A) Inhibition with cholesterol or phosphatidylcholine and (B) inhibition with mycolic acids or phosphatidylcholine.

A



B

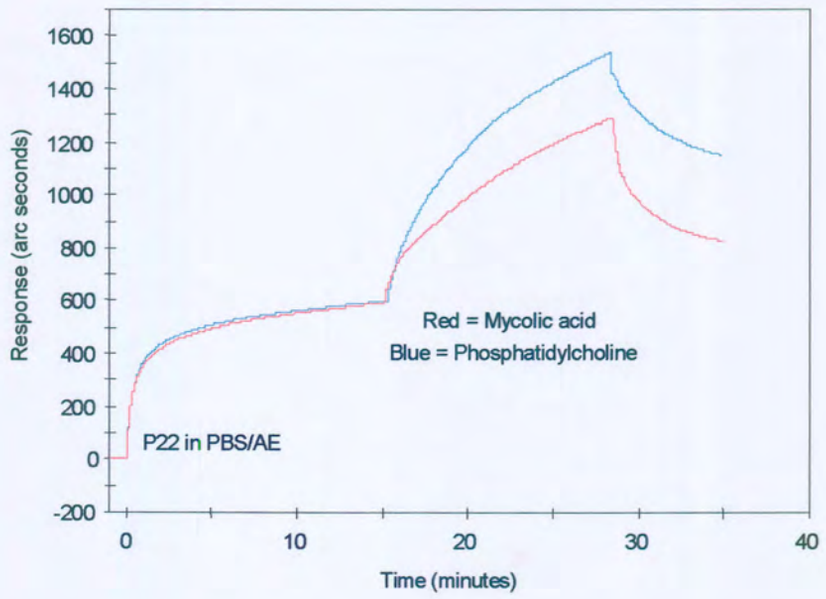


Figure 3.8: Inhibition of human TB$^-$ patient (P22) serum antibody binding on a cholesterol-coated surface of the IAsys cuvette. For the first 10 minutes, a 1:1000 dilution of serum in PBS/AE was incubated in both cells. For inhibition studies, the pre-incubated serum in a dilution of 1:500 was then added. (A) Inhibition with cholesterol or phosphatidylcholine and (B) inhibition with mycolic acids or phosphatidylcholine.

The results imply that mycolic acid liposomes cannot be used as inhibitor on cholesterol coated cuvettes, as mycolic acid appears to affect the cholesterol coat. This is especially evident in Fig. 3.9, since the curve went down to below zero after dissociation of mycolic acids pre-incubated serum. The cholesterol-coated surface was therefore not used for further studies to characterize the binding specificity of the anti-MA antibodies.

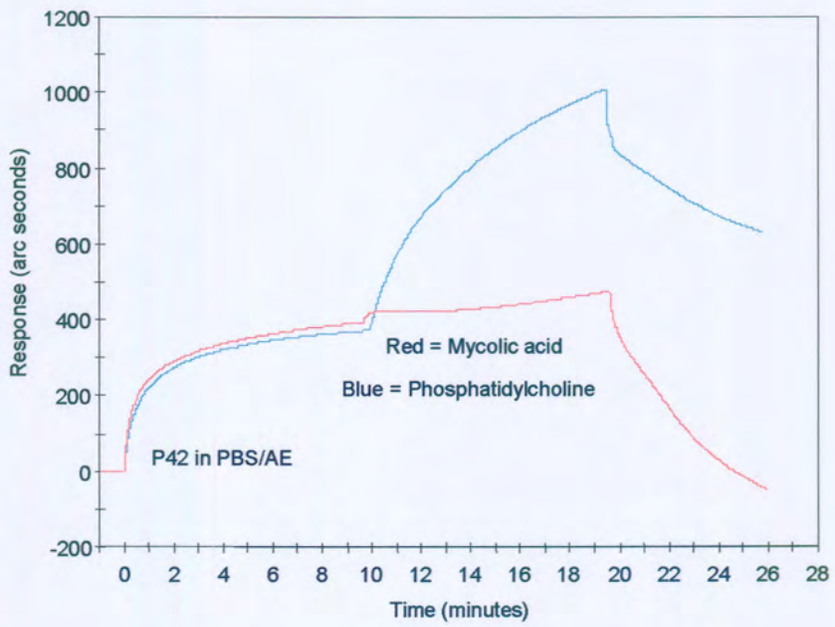


Figure 3.9: Inhibition of human TB^+ patient (P42) serum antibody binding on a cholesterol-immobilized surface of the IAsys cuvette.

3.6 DISCUSSION

Although, no serodiagnostic assay for tuberculosis has been accepted until now, much progress has been reported in studies of antibodies to *M. tuberculosis* in the serum of patients with TB using various antigens (Pan *et al.*, 1999; Pottunarthy *et al.*, 2000; Julian *et al*, 2002; Schleicher *et al.*, 2002; Lopez-Marin *et al.*, 2003). This study shows that, in comparison to the ELISA procedure that was used to detect anti-mycolic acids antibodies in human sera, the resonant mirror biosensor is a much more powerful tool for diagnosis of *M. tuberculosis* infection in patients. The IAsys affinity biosensor was able to detect low affinity antibody binding to mycolic acids, which the conventional methods cannot achieve. The reason for this was demonstrated with the biosensor, showing that antibodies clearly bound to the mycolic acids coated surface, but were washed away during dissociation. In an ELISA these antibodies would have been washed away before the final step and the patient would have tested false negative. The success of the biosensor therefore lies in its reliable detection of antibodies of even low affinity to mycolic acids. The associated determination of the specificity of binding, by binding inhibition after pre-incubation with antigen is also especially amenable to low affinity antibodies.

Antunes *et al.* (2002) described the MycoDot serological assay for tuberculosis that is based on the detection of specific IgG antibodies against the lipoarabinomannan (LAM) antigen, fixed onto a solid support consisting of a plastic comb designed to fit into the wells of a microtiter plate. The sensitivity values observed were definitely lower in cases of TB associated with HIV, which refuted the usefulness of the test in regions where HIV is highly endemic. They concluded that LAM as an antigen is only satisfactory in the serodiagnosis of TB as long as HIV is not highly prevalent in the population. The sensitivity of the MycoDot assay has been shown not to be sufficient to confirm the diagnosis with respect to the Mantoux reaction ($\geq$10 mm considered as recent primary infections), showing that it is inappropriate for diagnosis of newly acquired TB. In this study, all TB patients that were co-infected with HIV remained positive on the surface coated with mycolic acid after pre-incubation of serum antibodies with mycolic acid.

This shows that the IAsys biosensor could be more efficient for diagnosing TB patients than MycoDot.

Pan *et al.* (1999) indicated that the anti-mycolic acid antibodies (IgG) in TB patients specifically recognized mycolic acid structure, especially methoxy mycolic acid. Mycolic acid is presented by antigen-presenting cells (APC) through a mechanism that does not involve MHC-class I or MHC-class II molecules. Mycolic acid is a CD1 restricted antigen with the ability to induce proliferation of a T-cell line (Beckman *et al.*, 1994). The human CD1 protein is known to mediate T-cell responses by presenting at least the three classes of mycobacterial lipids, i.e. free mycolates, glycosylated mycolates and diacylglycerol based glyco-phospholipids such lipoarabinomannan (Beckman *et al.*, 1994; Moody *et al.*, 1997). The alkyl chains of the mycolic acid antigen has been proposed to bind directly within the hydrophobic groove of CD1 resulting in presentation of the hydrophilic caps to the T-cell's antigen receptor (Porcelli *et al.*, 1996; Moody *et al.*, 1999). The CD1-restricted lipid antigen presentation pathway could probably be the reason why the antibody response to mycolic acids is preserved in HIV-seropositive patients despite a declining CD4 T-lymphocyte count (Schleicher *et al.*, 2002). There is no data available that presentation of cholesterol may also occur by the CD1 molecule. Alving and Wassef (1999) measured the anti-cholesterol antibodies in healthy individuals and described that almost every healthy individual has various amounts of IgM and IgG anti-cholesterol antibodies partly present in complexed form with LDL and VLDL (Dijkstra *et al.*, 1996; Horvath *et al.*, 2001; Shoji *et al.*, 2000). Horvath and Boro (2003) showed cholesterol concentration to be higher in HIV patients than in HIV-seronegative controls. This could also explain the false positive results in HIV patients, without TB, obtained in this study. The level of anti-cholesterol antibodies may also be high in these patients. These antibodies could then be inhibited with mycolic acid due to the presumed molecular mimicry between mycolic acid and cholesterol.

This study shows that even though the anti-mycolic acid IgG antibodies in human serum that may recognize both cholesterol and mycolic acid, the antibodies are more specific to mycolic acid and can be distinguished with the biosensor from non-specific binding. The

number of samples tested was too small to accurately assess whether the presence of anti-cholesterol antibodies will interfere in the predictiveness of the test.

Pei *et al.* (2000) described that chemical reagents such as acid, base, or salt with high ion strength could be used to desorb antigens or antibodies and other molecules on a sensor surface. In this study, one sensor surface was regenerated and used up to 22 times after regenerations with KOH and ethanol. The reproducibility of mycolic acid liposomes immobilization for several times after regeneration on the same surface of a cuvette showed that a single cuvette could be used many times which could then reduce the number of cuvettes required to detect anti-mycolic acid antibodies for the serodiagnosis of TB. Automated pipetting may prove the comparability of the two cells in the biosensor cuvettes and extend the active shelf life and frequency of use of the cuvettes.

For further experiments in developing an improved serodiagnostic assay for TB, antibodies should be separated from serum and tested against mycolic acid for their specificity on the biosensor, since there are many antibodies against antigens of glycolipids other than mycolic acid in the serum of TB patients or other molecules in serum that could interfere with the binding of anti-mycolic acid antibodies. Since only seventeen patients were analyzed with the biosensor, more patient and control sera will have to be analyzed to statistically account for the preliminary finding that the resonant mirror biosensor can be used as a reliable technique to determine anti-mycolic acids antibodies as surrogate markers for tuberculosis in patients infected with *M. tuberculosis*.

The detection of anti-mycolic acids antibodies with the IAsys affinity biosensor appears to be technically feasible, quick and may also be made affordable by further optimisation and innovation of the biosensor hardware. Moreover, the biosensor assay may even prove to be more sensitive than the sputum assay, as was shown here with two serum samples from HIV^+ patients that tested positive with the biosensor, but negative with the sputum assay.

CHAPTER 4

CONCLUDING DISCUSSION

An early diagnostic assay that can detect patients with TB is urgently needed to control and prevent the spread of TB in the community. A rapid and reliable test for infection with *M. tuberculosis* would be a suitable instrument for first line screening of suspected cases, especially in resource-poor countries where access to diagnostic laboratories are limited. Although culture of bacteria is the reference standard in diagnosis and follow-up of disease, it can take up to 6-8 weeks to grow and identify *M. tuberculosis* and false negative culture results may be obtained (Raqib *et al.*, 2003). Correct anti-tuberculosis therapy could have been initiated several weeks before culture results were returned. A reliable, rapid test for diagnosis of *M. tuberculosis* infection should be time saving and cost effective compared to this standard assay based on culture of mycobacteria.

It is generally known that TB patients produce antibodies against more than one antigen. Consequently, a wide spectrum of humoral responses exists in these patients that may be exploited for the purpose of serodiagnosis (Julian *et al.*, 2002; Lyashchenko *et al.*, 1998a). Whereas serological tests that are based on the mycobacterial antigens to detect circulating antibodies may be faster and more cost-effective, they are hampered by low sensitivity and cross-reactivity with other mycobacteria, or have been found to be of poor positive predictiveness in the diagnosis of TB in countries where HIV is endemic.

McConkey *et al.* (2002) reported that the sensitivity of serologic tests for TB depended on the origin of the sample and the clinical spectrum of the disease groups prevalent in that area. Therefore, each new serodiagnostic test should be validated with cases and control specimens from the countries/regions in which it will be used. It has been reported that most of the patients that have pulmonary TB are also HIV positive in sub-Saharan Africa. HIV/TB co-infected patients have an increased chance of developing extrapulmonary disease. The serodiagnosis of individuals with extrapulmonary *M. tuberculosis* and those co-infected with HIV and *M. tuberculosis*

is specifically complicated by a decreased cell-mediated response to some *M. tuberculosis* antigens. Liang (1999) suggested that atypical or delayed presentations of hospital-acquired infections such as TB, together with the co-existence of other infections (HIV), could make the diagnosis of hospital-acquired infection a difficult one.

In this study, a novel technique on the IAsys biosensor that identifies anti-mycolic acid antibodies in human patient's sera (Siko, 2002) was assessed. Siko (2002) compared the serum of a patient infected with TB to that of a healthy individual and found a significant difference in the binding signal on the non-derivatized cuvette coated with mycolic acid liposomes. This study on the IAsys biosensor confirmed the results of Schleicher *et al.* (2002) that there are anti-mycolic acid antibodies in TB patients, even in those co-infected with HIV. The IAsys biosensor method has shown an improved sensitivity and specificity relative to the ELISA method. Four patients diagnosed clinically as having tuberculosis whose sera gave false negative results on the ELISA, tested truly positive on the IAsys biosensor, thereby demonstrating improved sensitivity. Three of these were co-infected with HIV. Six TB negative patient sera that tested positive on the ELISA tested negative on the biosensor demonstrating the improved specificity. Several studies have addressed the problems of detecting *M. tuberculosis* in TB patients co-infected with HIV when using different antigens (Antunes *et al.*, 2002; Hendrickson *et al.*, 2000; Schleicher *et al.*, 2002). In this study, mycolic acid appears to be a highly specific and sensitive antigen for the serodiagnosis of TB.

The biosensor assay may be more sensitive than the sputum assay. Mitarai *et al.* (2001) showed that a low number of mycobacterial bacilli in sputum specimens often result in negative sputum smear. It is estimated that 10, 000 mycobacteria per ml sputum are required for AFB smear positive results. In their study they showed that approximately half of the patients studied who had pulmonary mycobacterial infection, especially pulmonary tuberculosis, gave negative sputum smears. This could also be the case with some of the patients that were infected with HIV, but apparently without TB according to sputum analysis, that gave "false" positive results on ELISA and biosensor. So when these patients' sera were analysed on the ELISA and biosensor they showed positive due to the presence of antibodies to mycobacterial

mycolic acids. The serological diagnosis of TB may prove superior in cases where patients are unable to produce adequate sputum, which appear to be more common in HIV-positive patients. This is due to decreased cavitary tissue damage in the lungs of HIV positive co-infected patients that limits the release of *M. tuberculosis* bacilli in sputum (Crook and Mir, 1999; Lucas *et al.*, 1994). Foulds and O'Brien (1998) indicated that the problem of diagnosis of smear-negative tuberculosis has become urgent because the number of patients with bacillary pulmonary tuberculosis in countries with an HIV epidemic is increasing rapidly.

Chiang *et al.* (1997) described that although the diagnostic value of a given test in clinical practice depends on its positive and negative predictive values, these values vary markedly with the prevalence of the disease in a given community. A high positive predictive value of a test would make a positive test result useful in strengthening the clinical suspicion due to the specificity of the test, but a low positive predictiveness would be less useful. The poor negative predictive value of serologic assays for TB based on patient antibody detection of protein and glycolipid antigents in HIV positive patients make them unreliable (Chan *et al.*, 2000). The fact that mycolic acid was shown to bind antibodies specifically on the IAsys biosensor in all TB patient sera with or without co-infection with HIV, some of which previously tested false negative on the ELISA, indicates the importance of mycolic acids as an antigen in the design of a serodiagnostic assay of TB in an HIV endemic country.

Lyashchenko *et al.* (1998b) indicated that the IgG antibody responses against protein antigens are CD4 T-cell dependent. It has been indicated that the presentation of mycolic acid by the CD1 molecule to $CD4^-$, $CD8^-$ double negative T cells could be the reason why anti-mycolic acids antibodies are maintained in HIV positive patients. Samanich *et al.* (2000) showed that serum antibodies from HIV-infected TB patients poorly recognized the 38-kDa and Ag 85C antigens on ELISA. Several studies on the serological response in TB infected individuals have revealed that non-protein antigens gave more sensitive serologic tests for TB in HIV co-infected individuals (Lopez-Marin *et al.*, 2003; Simonney *et al.*, 1996). Hendrickson *et al.* (2000) showed that the use of a 38-kDa protein antigen as diagnostic marker lacks sufficient sensitivity, especially for smear-negative TB positive individuals.

Alifano *et al.* (1997) showed a low sensitivity of the protein antigen P-90 based test in the early stage of TB. They observed the production of anti-P-90 IgA in some healthy individuals, leading to false positive results. In their studies they found that patients with non-TB lung disease showed a more frequent incidence of false-positive results, due to this disease causing some immune interferences, possibly owing to disorders in immune response occurring in the course of these diseases. They also indicated that false positive results could be due to infection with other mycobacteria, showing that P-90 is not species specific. Antunes *et al.* (2002) reported that the presence of leprosy contributed to a rise in false positive frequency on the MycoDot assay based on the detection of antibodies against LAM, indicating the cross-reactivity of this antigen in mycobacteria. Stavri *et al.* (2003) also found some false positive results from patients with lung cancer, basal bronchiectasis and chronic pneumonia in rapid dot sputum and serum assay based on the detection of antibodies against culture filtrate proteins. It was described that patients who have these diseases could have physical lung or bronchial damages, which could permit the presence of tubercle bacilli into the already damaged tissues. Julian *et al.* (2002) found a low specificity (false positives) when sera from patients with non-TB pneumonia were analyzed on ELISA coated with 2,3-di-acyltrehalose; 2,3,6-triacyltrehalose; 2,3,6,6-tetraacyltrehalose-2'-sulfate and trehalose-6-6'-dimycolate (cord factor). The present study, based on the detection of anti-mycolic acid antibodies on IAsys biosensor, overcome all these limitations by showing high specificity in hospitalized patients that were infected with other diseases.

A recently developed biosensor approach to detect *M. tuberculosis* was based on the growth of *M. tuberculosis* (He *et al.*, 2003). The conductivity of culture medium was changed by the growth of *M. tuberculosis* that was monitored by the bulk acoustic wave impedance biosensor through the frequency response curve as *M. tuberculosis* assimilated glutamate, hydrogen phosphate, magnesium ion and other strong electrolytes. This caused the decrease of the conductivity and the concomitant increase in frequency. In the frequency response curve, the time at which the signal changes is defined as the frequency detection time. In comparison with the present method used to detect anti-mycolic acid antibodies that takes less than an hour, their method takes about 42 hours to analyze a single patient. In their studies they did not include mycobacterial species, so mycobacteria other than *M. tuberculosis* could also

produce those essential signals resulting in frequency change, showing that their method may lack specificity compared to the present study.

The present study showed that the IAsys biosensor is potentially useful for detecting anti-mycolic acid antibody in TB patients' sera. More TB patients, healthy controls and patients co-infected with TB and other diseases should be analyzed with the biosensor to make a statistically accountable statement on the improved sensitivity and specificity before it may be further developed into a useful assay for diagnosis of TB.

Based on the findings of the current study, it was demonstrated that the IAsys affinity biosensor based on the detection of anti-mycolic acid antibodies in patient serum could be a valuable alternative method for detection of *M. tuberculosis*, even among patients co-infected with HIV and other diseases. If subsequent studies confirm these findings, then automated microfluidics and some innovative laser technology may convert the discovered concept into a simple, accurate, rapid, affordable, sensitive and specific serodiagnostic assay. Field-testing of such a biosensor-based device in sub-Saharan Africa, where there is high prevalence of TB and co-infection with HIV, may turn out to be the first practical solution to the monitoring of the epidemic. Accurate and rapid monitoring of disease is a prerequisite for proper control of the sub-Saharan TB epidemic.

SUMMARY

Tuberculosis has re-emerged as a global health problem due to co-infection with HIV and the emergence of drug resistant strains of *Mycobacterium tuberculosis*. There is a need for a reliable and fast serodiagnostic assay to reduce the time required for test results from weeks to hours, in order to better control the spread of the disease.

Previous studies have shown that TB patients contain antibodies against *M. tuberculosis* mycolic acids. In standard immunoassays such as ELISA, an unacceptable number of false positive and negative test results were obtained. This study aimed at assessing the potential of detecting anti-mycolic acids antibodies in TB patient sera on a biosensor as surrogate marker for TB infection. Mycolic acid liposomes were immobilized reproducibly on a non-derivatized biosensor cuvette and blocked with saponin. A high dilution of serum in PBS/AE was used to calibrate the signal of the two cells, followed by binding of patient sera inhibited with either mycolic acid, cholesterol or placebo phosphatidylcholine liposomes at a lesser dilution. The inhibition was done to confirm the specificity of the binding response.

There was no inhibition of binding when a sputum negative control serum (HIV^-TB^-) was pre-incubated with either cholesterol or mycolic acids on the biosensor coated with mycolic acid liposomes. The antibodies that are specific to mycolic acid were demonstrated in all TB positive patients on mycolic acids coated cuvette cell surfaces after pre-incubation of serum with mycolic acids. The patient sera that were false positive and false negative on ELISA tested negative and positive respectively on the biosensor. Only sera from two patients, both HIV positive, tested false positive on both ELISA and biosensor. The biosensor was able to detect anti-mycolic acids antibodies of even low affinity. In ELISA, these antibodies were washed away. No inhibition of antibody binding on cholesterol-coated cuvettes was found after pre-incubation of serum with mycolic acids or cholesterol liposomes. The cholesterol surface became unstable during pre-incubation of serum with mycolic acids. Mycolic acid appeared to be a stronger antigen than cholesterol. The anti-mycolic acids antibodies were specific and sensitive for

diagnosis of TB on the biosensor. More sera should be analyzed on the biosensor to make a statistically accountable statement on whether the improved sensitivity and specificity is adequate for a simple, rapid, sensitive and accurate biosensor-based serodiagnostic assay.

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