An assessment of two evanescent field biosensors in the development of an immunoassay for tuberculosis

by

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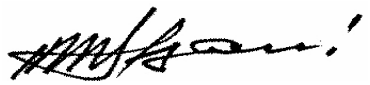
SIGNATURE : 

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

SUMMARY

Title: An assessment of two evanescent field biosensors in the development of an immunoassay for tuberculosis
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Accurate diagnosis of active tuberculosis is required to improve treatment, reduce transmission of the disease and control the emergence of drug resistance. A rapid and reliable test would make a considerable contribution to the management of the TB epidemic, especially in HIV-burdened and resource-poor countries where access to diagnostic laboratories are limited. Surrogate marker antibody detection to mycobacterial lipid cell wall antigens gave promising results, in particular with cord factor. The specific advantage of using mycolic acids as lipid antigens in comparison to protein antigens is that mycolic acid is a CD1 restricted antigen with the ability to induce proliferation of CD4/CD8 double negative T-cells, which may explain the sustained antibody production in AIDS patients. Traditional end-point assays to detect anti-MA antibodies showed an unacceptable number of false positive and negative test results. Here a much improved biosensor method (the MARTI-assay, i.e. Mycolic acid Antibody Real-Time Inhibition assay) was developed to detect antibodies to mycolic acid in patient sera as surrogate markers of active tuberculosis. The test was assessed on an IAsys optical biosensor and gave an accuracy of 82%. The technology was transferred to an SPR (ESPRIT) biosensor to economise and simplify the assay. Mycolic acid containing liposomes were immobilized on the SPR gold surface pre-coated with octadecanethiol. The following parameters were optimized on the ESPRIT biosensor to enable reliable TB diagnosis: effect of degassed buffer, saponin blocking, first exposure to serum at low concentration and second exposure to antigen inhibited serum at high concentration. The IAsys biosensor system has a weakness in the double channel cuvette system, in which the channels often do not give matching results, while being ten times more expensive than the gold discs provided for the

ESPRIT biosensor. The ESPRIT biosensor is provided with an adjustable laser setting to compensate for differences in the channel readings as well as an automated fluidic system that reduces variance from one sample to the next. First indications are that the test can also be used for prognosis of TB during treatment. It is hoped that the ESPRIT biosensor will improve the accuracy of the test to more than 90%. If the MARTI-assay technology could be made amenable for high throughput screening, it may provide the solution to the serodiagnosis of tuberculosis and monitoring of progress during TB treatment both in adult and children, thereby reducing the spread of TB within the communities.

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List of Abbreviations

AFB	Acid-fast bacilli
AFM	Atomic force microscopy
AG	Arabinogalactan
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
ART	Antiretroviral therapy
ARV	Antiretroviral
ASI	Artificial sensing instrument
Au	Gold
BCG	Bacillus Calmette-Guerin
CMD	Carboxymethyl dextran
CO_2	Carbon dioxide
CPC	Cetyl pyridinium chloride
CFP	Culture filtrate protein
CIC	Circulating immune complex
CV	Cyclic voltammetry
DAT	2,3-diacyl trehalose
DIBA	Dot immunobinding assay
DNA	Deoxyribonucleic acid
ESAT	Early secretory antigenic target
EDC	Ethyl-dimethylaminopropyl carbodiimide
EDCTP	European and Developing Countries Clinical Trials Partnership
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot

F	Frequency
FET	Field effect transistor
FIND	Foundation for Innovative New Diagnostics
hr	Hour
HCl	Hydrochloric acid
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
IAsys	Interaction analysis system
IDL	Intermediate density lipoprotein
IgG	Immunoglobulin G
IFN- γ	Interferon gamma
INH	Isoniazid
IRIS	Immune reconstitution inflammatory syndrome
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
MARTI	Mycolic acid Antibody Real-Time Inihibition
MDR	Multi-drug resistance

MHC	Major histocompatibility complex
Min	Minute
MS	Mass spectroscopy
M.tb	*Mycobacterium tuberculosis*
MTBDR	*Mycobacterium tuberculosis* drug resistance
NaCl	Sodium chloride
NaOH	Sodium hydroxide
neg	Negative
NHS	*N*-hydroxy-succinimide
NTA	Nickel chelating surface
NTM	Non-tuberculosis mycobacteria
ODT	Octadecanethiol
PBS/AE	Phosphate buffered saline azide EDTA
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGL	Glycolipid
PIM	Phosphatidyl inositol mannosides
pos	Positive
PPD	Purified protein derivative
QCM	Quartz crystal microbalance
RIfS	Reflectometric interference spectroscopy
RNA	Ribonucleic acids
rRNA	Ribosomal ribonucleic acids
RU	Resonance units
SAM	Self-assembled monolayer

SDS	Sodium dodecylsulphate
SEM	Standard error of the mean
SPR	Surface plasmon resonance
TAT	2,3,6-triacyl trehalose
TB	Tuberculosis
TDM	Trehalose dimycolate
TIR	Total internal reflection
TMM	Trehalose monomycolate
TST	Tuberculin skin test
VLDL	Very low-density lipoprotein
WHO	World Health Organization
XDR	Extensively drug-resistant

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CHAPTER 1

General Introduction

1.1 Tuberculosis

Tuberculosis (TB) mainly presents as a pulmonary disease caused by infection with *Mycobacterium tuberculosis* (*M. tuberculosis*). Other mycobacteria such as *M. avium* and *M. kansasii* may cause a pulmonary disease resembling TB in patients with immune disorders. *Mycobacterium bovis* (*M. bovis*) also causes tuberculosis and the clinical features are indistinguishable from that caused by *M. tuberculosis*. However *M. bovis* is more likely to cause non-pulmonary disease due to different routes of infection and treatment is usually based on a short course of anti-TB regimens, as compared to 6-9 months of therapy for TB caused by *M. tuberculosis* (Grange, 2001; Piersimoni and Scarparo, 2008). Tuberculosis is a major scourge in developing countries as well as an increasing problem in many developed areas of the world, registering about 8 million new cases and claiming 3 million deaths each year (Hendrickson *et al.*, 2000). Dye *et al.* (2005) reported that much of the observed increase in the incidence of global TB since 1980 can be attributed to the spread of human immunodeficiency virus (HIV) in Africa. Although tuberculosis is a curable disease that responds well to antibiotics it has re-emerged as a growing global health problem because of the development of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains. Another complicating factor is the increased risk for TB in HIV infected persons and also in children. Mycobacterioses became particularly relevant in relation to the global emergence of HIV/AIDS. *Mycobacterium tuberculosis* is a pathogen capable of producing both progressive disease and latent infection after inhaling an aerosol containing tubercle bacilli (Hugget *et al*., 2003). The initial infection usually occurs in the lungs and in most cases is controlled by the immune system. Even after successful control of primary TB infection, some bacilli remain in a non- or slowly replicating state, termed latent TB infection. Latently infected individuals have a 10% risk of developing the disease in their lifetime, which constitutes a huge global reservoir of infection and a continuous threat of disease transmission. However HIV infected people are more likely to develop TB. Most active TB cases arise as a result of relapse after previous treatment, reinfection or

reactivation of latent infection (WHO, 2006; Huggett *et al*., 2003; Palomino *et al*., 2007).

Bacille Calmette-Guerin (BCG), a live vaccine derived from an attenuated strain of *M. bovis,* is currently still the only vaccine available for prevention of TB in humans. BCG is usually given at birth in most countries, has an overall efficacy that ranges from a negative value to around 80% for preventing TB. This highly variable efficacy of BCG could be due to the presence of environmental, non-pathogenic mycobacteria, genetic factors and also the type of BCG strain used. The diagnosis of individuals with tuberculin skin test who received BCG vaccine is controversial as a positive result can indicate either active disease, infection in the past, or BCG vaccination (Valadas and Antunes, 2005).

Infection with HIV may alter the clinical presentation of active pulmonary tuberculosis. During early HIV infection when immune function is relatively intact, sputum smear-positive TB predominates. However, patients with advanced HIV disease and significant immunosuppression often present with sputum smear-negative and disseminated TB (Mwandumba *et al*., 2008). TB patients infected with HIV and receiving antiretroviral therapy (ART) for immune restoration may experience temporary exacerbation or worsening of symptoms of TB disease. This phenomenon is described as *paradoxical reaction* or *immune reconstitution inflammatory syndrome* (IRIS). This occurs in various forms of TB within a few weeks of ART (Manosuthi *et al*., 2006; Buckingham *et al*., 2004; Lawn *et al*., 2005). Manosuthi *et al*. (2006) indicated that the factors that contribute to TB associated IRIS with the initiation of ART include anti-TB drug resistance and non-compliance with TB treatment. A rapid and reliable diagnostic assay for TB that can detect the early emergence of multi-drug resistant strains and monitor the prognosis of TB during treatment may allow clinicians to lessen the risk of IRIS before commencing with ART chemotherapy in HIV infected patients. It is urgently required to control the spread of the disease and lessen the misery that is associated with HIV infection.

1.2 History of tuberculosis diagnosis

The diagnosis of mycobacterial infections remained practically unchanged for many decades and probably would not have progressed at all without the unexpected resurgence of TB (Palomino *et al*., 2007). Clinical management of TB cases in developing countries is being hampered by the inadequacies of current diagnostic assays for tuberculosis. Correct diagnosis of TB is required to initiate treatment, reduce transmission of the disease and control the emergence of drug resistance (Huggett *et al*., 2003; Guillerm *et al*., 2006). Inadequate case detection and cure rates, among children and adults, have been identified as reasons for a mounting global tuberculosis burden (Siddiqi *et al*., 2003). Culture of *M. tuberculosis* from sputum has been the gold standard for the diagnosis of tuberculosis, but can take up to six weeks before certainty is acquired to exclude the possibility of TB in a patient. This often results in delayed diagnosis, adversely affecting patient care and TB control and allows for the spread of infection (Reischl, 1996). This limits the usefulness of culture as a first-line diagnostic test (Siddiqi *et al*., 2003).

The cornerstone of the diagnosis of pulmonary TB in adults is based on the demonstrations of *M. tuberculosis* by means of microbiological or molecular methods. Paediatric TB on the other hand, is usually considered a paucibacillary disease, which makes bacteriological diagnosis of TB extremely challenging, because of difficulty in isolating *M. tuberculosis* from the sputum samples. Most tests such as TST, chest x-ray and direct microscopy give low sensitivity and specificity. This is often due to HIV co-infection, BCG vaccination or other infection with other mycobacterium. HIV infection contributes significantly to an increase in the world incidence of TB - it is the single most important risk factor for TB (Valadas and Antunes, 2005). There is clear synergy between *M. tuberculosis* and HIV and active TB increases HIV-related immunodeficiency and mortality. Tuberculosis remains the largest attributable cause of death of HIV infected individuals, being responsible for 32% of the deaths of HIV infected individuals in Africa (Palomino *et al*., 2007; Toossi *et al*., 2001). The increased incidence of TB has stimulated the development of sensitive, rapid and direct detection methods for the laboratory diagnosis of *M. tuberculosis* (Albay *et al*., 2003). The World Health Organization recommended that the test should give better than 80% sensitivity and 90% specificity for its application to detect TB (WHO, 2001).

An important aspect of preventing tuberculosis is an early diagnosis followed by an appropriate treatment (Taci *et al*., 2003). A simple diagnostic assay that does not require highly trained personnel or complex technological infrastructures would be ideal to assist in the global control of TB (Foulds and O'Brien, 1998). A serological test, such as ELISA, is a simple and inexpensive alternative to other TB diagnosis methods (Simonney *et al*., 1996; Moran *et al*., 2001). The disadvantage of ELISA is that it detects only the high affinity antibodies to the antigen. Irrespective of the antigen(s) used, no single ELISA test has hitherto succeeded as a reliable test to confirm or exclude tuberculosis in a patient. New diagnostic tests that are simple and robust enough to be used in the field, accurate enough to confirm or exclude TB correctly, able to identify drug resistance of *M. tuberculosis* and responsive enough to monitor the efficiency of treatment programmes are desperately required (Guillerm *et al*., 2006).

1.2.1 Tests that can't distinguish between latent and active TB

One third of the total world's population, two billion people, is believed to be latently infected with *M. tuberculosis. Mycobacterium tuberculosis* is sometimes difficult to culture from patients with active TB and impossible to culture from latently infected healthy people. It is therefore important to have efficient tools for diagnosis of active TB and latent infection. It is necessary to differentiate between *M. tuberculosis* and other environmental mycobacteria in order to know the prevalence and distribution of human TB due to other mycobacteria (Palomino *et al*., 2007; Morrison *et al*., 2008). The HIV/AIDS epidemic has produced a devastating effect on TB control worldwide. One out of ten immunocompetent people infected with latent *M. tuberculosis* will fall sick in their lifetimes, and among those with HIV infection, one in ten per year will develop active TB (Palomino *et al*., 2007). Immunosuppressed individuals are more likely to develop active tuberculosis after infection by other mycobacteria such as *M. bovis* (Grange, 2001).

One of the first lines in establishing the diagnosis of latent tuberculosis has been the tuberculin skin test (TST), also known as the intradermal Mantoux- or the purified protein derivative test (PPD). Despite its longevity, the TST has several important

disadvantages, such as giving false positive results due to a reaction produced to non-pathogenic mycobacterial infections or by previous vaccination with BCG (Farris and Branda, 2007). A number of alternative testing strategies, such as interferon gamma (IFN-γ) release assays, have been developed in order to address some of the TST's disadvantages (Farris and Branda, 2007).

1.2.1.1 Tuberculin skin test

The tuberculin skin test which is based on the intradermal injection of mycobacterial purified protein derivative (PPD), a crude mixture of *M. tuberculosis* proteins widely shared among *M. tuberculosis*, *M. bovis* BCG, and most environmental mycobacteria. It is 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, 48 to 72 hours after injection (Charnace and Delacourt, 2001). The reading is based on a measurement of swelling. The specificity is low as purified protein derivative contains many antigens widely shared among mycobacteria. Some persons may react to the tuberculin skin test though they are not infected with *M. tuberculosis* (Doherty *et al*., 2002; Anderson *et al*., 2000). Several studies have demonstrated that PPD cannot reliably distinguish between previous *Mycobacterium bovis* BCG vaccination, exposure to environmental mycobacteria, or infection with *M. tuberculosis* (Charnace and Delacourt, 2001; Chan *et al*., 2000; Ewer *et al*., 2003). It is currently estimated that almost one third of people positive to tuberculin skin test (TST) do not actually have TB infection. The sensitivity of the skin test is estimated to be around 70% of alternatively confirmed active TB cases. The sensitivity decreases to as low as 30% in immunocompromised people (Palomino *et al*., 2007). The TST is useful for proving infection, but not necessarily the disease. A positive test only suggests prior exposure to the antigen, not active infection. If the patient is in an immunosupressed state, a negative test does not rule out TB infection (Hornum *et al*., 2008). The TST can give false positive results leading to inappropriate initiation of chemotherapy, which can be a waste of health care resources (Kunst, 2006) and a discomfort to the patient.

1.2.1.2 Interferon gamma assay

Besides the TST, a newer type of *in vitro* T-cell based assay has been assessed to detect *M. tuberculosis* in patients (Tufariello *et al.,* 2003; Hornum *et al*., 2008) (Fig. 1.1). The IFN-γ assays are based on the principle that T cells of individuals sensitised with tuberculosis antigens produce IFN-γ when they re-encounter the antigen of *Mycobacterium tuberculosis* (Tufariello *et al.,* 2003; Ruhwald *et al*., 2007). A high level of interferon-γ production is presumed to be indicative of tuberculosis infection. The IFN-γ assays that are now commercially available include the enzyme-linked immunospot (ELISPOT) T SPOT-TB assay and QuantiFERON-TB Gold assay (Pai *et al*., 2004; Farris and Branda, 2007; Palomino *et al*., 2007). Both tests measure cell-mediated immunity by measuring IFN-γ released from T-cells in response to tuberculosis antigens, using ELISA and enzyme-linked immunospot (ELISPOT) technology, thereby enabling a clear distinction between TB infection and BCG vaccination (Hornum *et al*., 2008; Veenstra *et al*., 2007; Ruhwald *et al*., 2007) (Fig. 1.2). The IFN-γ assays have been quite successful in detecting latent TB infection.

The QuantiFERON-TB is a whole blood assay based on the detection of INF-γ released by T cells in response to *M. tuberculosis*- specific antigens. The test uses three antigens encoded by a unique genomic segment that is present in *M. tuberculosis*. These proteins, early secretory antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP10) and a peptide from *M. tuberculosis* specific antigen (TB7.7) are major targets for INF-γ-secreting T lymphocytes in *M. tuberculosis* infected individuals. The test has operational advantages over the TST because results can be available 24 hours after testing (Hornum *et al*., 2008; Harada *et al*., 2008). The T SPOT-TB assay, which uses peripheral blood mononuclear cells, also uses ESAT-6; TB7.7 and CFP10 and detects the number of T cells producing IFN-γ using ELISPOT. The incubation periods used for T SPOT-TB is 5-6 days (Pai *et al*., 2004; Farris and Branda, 2007; Ruhwald *et al*., 2007).

The use of patients with advanced disease or who have completed treatment creates potential problems for the estimation of sensitivity, because IFN-γ results can be influenced by disease severity and treatment. These can have unpredictable and dissimilar effects on the estimates on the sensitivity of the test (Pai *et al*., 2004). A negative IFN-γ test does not exclude tuberculosis disease in immunocompromised

patients, since the magnitude of IFN-γ release is correlated with the level of CD4 cells (Ruhwald *et al*., 2007; Hornum *et al*., 2008). The major drawback is that the assay detects latent infection, which may make it of limited value for the identification of contagious tuberculosis in high endemic countries (Andersen *et al*., 2000; Higuchi *et al*., 2008). Veenstra *et al*. (2007) showed no significant difference between IFN-γ production at diagnosis or at any points during anti-TB chemotherapy.

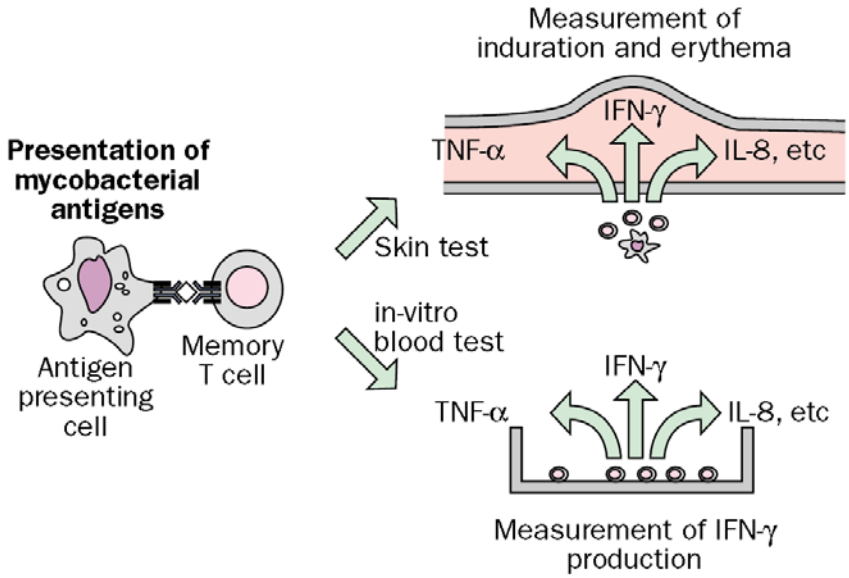


Figure 1.1: *In vivo* and *in vitro* diagnostic tests for tuberculosis. Both *in vivo* (skin test) and *in vitro* (blood test) depend on the elaboration of inflammatory cytokines by T cells previously sensitised to mycobacterial antigens (Anderson *et al*., 2000).

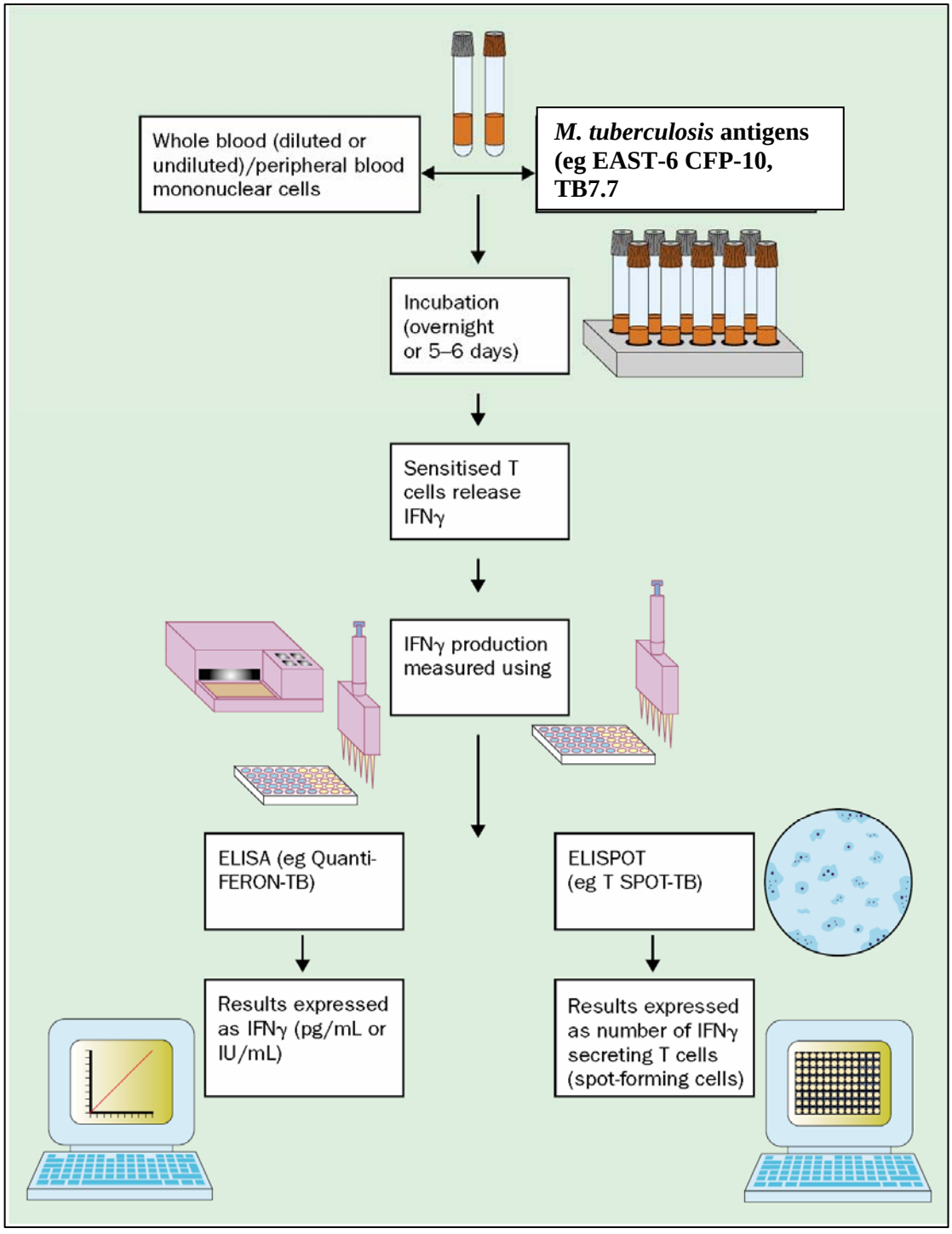


Figure 1.2: An overview of the interferon γ assay technology. The test uses the protein antigens early secretory antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP10) and a peptide from *M. tuberculosis* specific antigen (TB7.7) that are major targets for INF-γ-secreting T lymphocytes in *M. tuberculosis* infected individuals. The tests measure cell-mediated immunity by measuring IFN-γ released from T-cells in response to tuberculosis antigens, using ELISA and enzyme-linked immunospot (ELISPOT) assay (Pai *et al*., 2004).

1.2.2 Tests for active TB diagnosis

Active tuberculosis is diagnosed by detecting *Mycobacterium tuberculosis* complex bacilli in specimens from the respiratory tract (pulmonary TB) or in specimens from other body sites (extrapulmonary TB). Accurate detection is the rate-limiting step in TB control (Palomino *et al*., 2007; Perkins and Kritski, 2002). In developed countries, it is fairly easy to distinguish latent TB from active TB disease. TB infection is normally characterized by the presence of a positive TST in the absence of symptoms or progressive lesions consistent with TB disease. This classification is useful for control strategies in areas of low prevalence of infection and low incidence of new cases. However the application of such strategies is very difficult to implement in low and middle resource countries with high rates of infection, high incidences of new infectious TB cases and positive results due to BCG vaccination (Palomino *et al*., 2007). It is therefore very important to have access to an assay that can distinguish latent from active TB disease.

Many new diagnostic techniques are never accepted into routine practice, usually because they are too labour intensive and expensive (Fawley and Wilcox, 2005). Although there have been many diagnostic assays developed in the past decades, acid fast bacilli (AFB) smear microscopy and culture based assays are the gold standards for the diagnosis of active disease (Palomino *et al*., 2007). Though new assays may theoretically be more sensitive than traditional culture based methods, problems with specificity and reproducibility from country to country are usually significant, especially in HIV epidemic areas. Many efforts are being made to standardize methodology and to identify and eliminate factors responsible for low sensitivity and specificity (Fawley and Wilcox, 2005). The accuracy of most of the newly developed diagnostic assays is validated using conventional different testing methods such as culture based assays, AFB microscopy and chest X-rays (Albay *et al*., 2003; Harada *et al*., 2008; Hornum *et al*., 2008).

1.2.2.1 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 biopsy samples and is amenable to refinement. The presence of acid-fast bacilli (AFB) on a sputum smear often indicates tuberculosis. Acid-fast microscopy is inexpensive, relatively easy to perform and quick, but it doesn't necessarily 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 requires a high degree of bacillary load - of up to 10 000 bacilli/ml of sputum (Mitarai *et al*., 2001). Direct microscopy is not valid for diagnosing extrapulmonary tuberculosis or child tuberculosis (Charnace and Delacourt, 2001; Tiwari *et al*., 2007). Unfortunately, this technique can't distinguish among the various possible mycobacterial species. It is therefore standard protocol that the result of microscopy of the smear be confirmed by culture.

1.2.2.2 Chest X-rays

Chest radiography is fast and it provides some clues, but the radiographic analysis is often ambiguous and not very specific for tuberculosis (Sao *et al*., 1992). Patients co-infected with HIV may further complicate the classical radiographic analysis of the lesions in pulmonary tuberculosis. The degree of immunodeficiency in patients with HIV also affects the chest x-ray manifestations of TB. The chest x-ray of a TB patient with advanced AIDS may look normal. Interpretation of the radiographic findings is often prone to inter-observer variations (Tiwari *et al*., 2007).

1.2.2.3 Culture based method

This remains the gold standard for both diagnosis and drug sensitivity testing. The technique is very sensitive, such that even a few mycobacteria can be detected. Culture can be performed on a variety of specimen sources, including sputum, bronchial lavage and non-pulmonary samples like blood and urine. However, culture using solid media techniques usually requires 4 to 8 weeks for completion, due to the slow growth of *M. tuberculosis* and is subject to contamination with other microbiological growth (Huggett *et al*., 2003; Samanich *et al*., 2000). There has been

a considerably improvement of the culture methodology and the application of liquid culture media systems since the first BACTEC system was introduced (Morgan *et al*., 1983; Huggett *et al*., 2003). The new systems improved the time to test positively for *M. tuberculosis* to as little as 10 days, with fully automated and continuous monitoring of growth utilizing oxygen quenching fluorescent sensor technology (Kanchana *et al*., 2000; Laverdiere *et al*., 2000). Development of culture systems for detection of mycobacteria from clinical samples aim to be faster and more accurate, allowing optimal patient treatment and effective epidemiology control (Scarparo *et al*., 2002). Culture assay can confirm TB in about 2 weeks, but requires at least 8 weeks to exclude the possibility of TB. Even though culture-based assays are sensitive and specific, they are still unsuitable to implement in the field, because they require dedicated facilities and staff with specific requirements for training, quality assurance, biosafety, infrastructure and equipment, which can take time and significant local resources to set up. The sensitivity of culture is limited by the need to have bacilli present in the sample to be cultured. HIV positive patients and children have difficulty in producing sputum and sputum culture will not detect extrapulmonary forms of TB. Extrapulmonary TB is very common in HIV positive patients and is rapidly fatal, because of the risk factor of IRIS development in such patients. Even in patients with active pulmonary TB the bacilli may be protected in lung cavities or be absent from a particular sputum sample, or may be lost in the decontamination treatment required to process sputum for mycobacterial culture (Guillerm *et al*., 2006).

1.2.2.4 Fast techniques

Rapid detection of *M. tuberculosis* strains is one of the most important factors to minimize the spread of contagion (Albay *et al*., 2003). The use of x-ray and acid-fast microscopy is easy and quick, but it does not accurately confirm a diagnosis of TB (Hamasur *et al*., 2001; Tiwari *et al*., 2007; Sao *et al*., 1992). This shows that there is a need for reliable and rapid assays that can be used to detect TB within few hours. Recently, nucleic acid amplification techniques such as PCR were introduced as an alternative approach in the rapid detection of *M. tuberculosis* (Scarparo *et al*., 2000; Shibuya *et al*., 2000; Vadrot *et al*., 2004).

1.2.2.4.1 Polymerase chain reaction assays for TB

A number of amplification-based techniques have been developed to speed up detection and increase the sensitivity of TB detection. The majority of the molecular assays for TB detection are based on the Polymerase chain reaction (PCR) (Huggett *et al*. 2003; Shankar *et al*., 1990). PCR targets DNA, insertion and repetitive elements and various protein-encoding genes. Most strains belonging to *M. tuberculosis* complex carry multiple copies of the insertion element IS6110. The most commonly used sources for detecting DNA include sputum, bronchoalveolar lavage, cerebrospinal fluid, blood, lymph node, urine and tissue samples. The PCR amplification process can be completed in 2 – 4 hours after obtaining the processed clinical sample. The PCR technique is powerful and capable of detecting very low numbers of the DNA targets, but the down side is that very low levels of contamination can lead to false positivity. False positive results are usually derived from laboratory contamination (Huggett *et al*., 2003; Trinker *et al*., 1996; Doucet-Populaire *et al*., 1996; Tiwari *et al*., 2007). Trinker *et al*. (1996) showed that although PCR assays are highly specific and sensitive for the detection of mycobacterial DNA, the results should be interpreted only in conjunction with clinical information in order to avoid inappropriate treatment.

1.2.2.4.2 FASTPlaque TB test

This rapid test utilizes bacteriophage amplification technology for the detection of viable *M. tuberculosis* in clinical specimens. Bacteriophages replicate hundreds of times faster than bacteria. If amplified in a suitable bacterial host a single bacteriophage will reach detectable levels in 3-4 h. By adding target specific bacteriophage to a decontaminated sputum sample, all the target bacteria are rapidly infected. After phage infection, a virucidal solution is added which destroys all phage that have not infected the tubercle bacilli (Fig. 1.3). The newly infecting phages are amplified by the addition of a non-pathogenic rapid growing mycobacterial host (*M. smegmatis*), and can be visualized as plaques (Albay *et al*., 2003; McNerney *et al*., 1998). Phage-based assays are technically complex to perform, and they require a well functioning bacteriology laboratory, a strict incubation protocol and well-trained technicians. They are very labour intensive and some studies also report a high rate of contamination, making the test and its results both difficult to perform and to

interpret. FASTPlaque cannot be used for children or HIV-positive patients as it needs sputum, as a source of *Mycobacterium tuberculosis*.

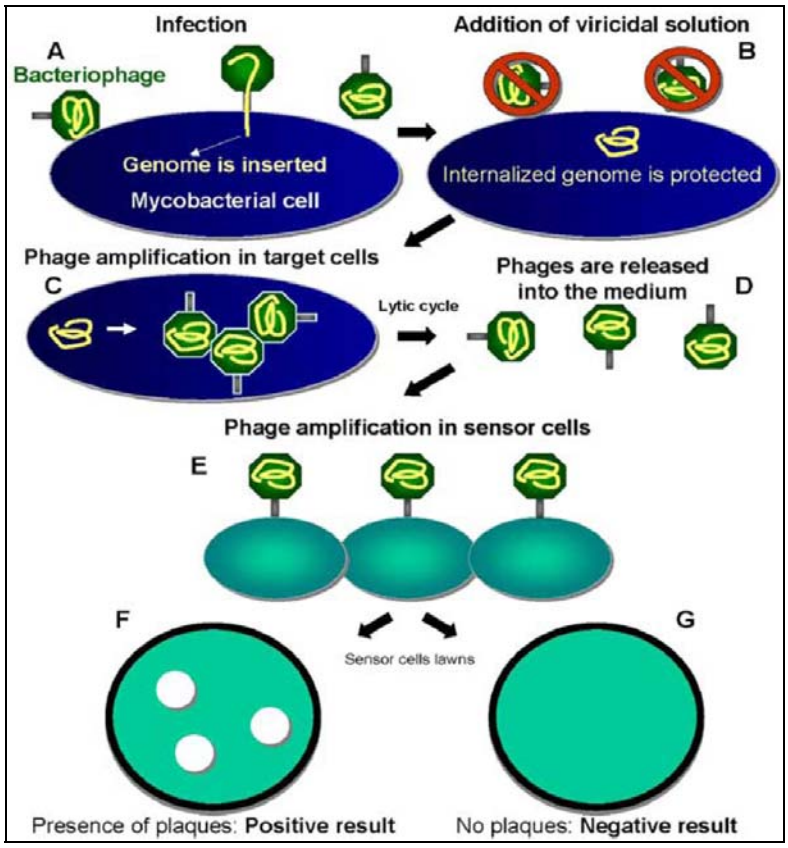


Figure 1.3: An overview of the phage amplification assay (Hazbon, 2004).

The FAST-plaque assay is normally used to detect *M. tuberculosis* strains that are multi-drug resistant. Zaki and Goda (2007) showed a high sensitivity and specificity of 100% and 97.2%, with an accuracy of 97.6% for the detection of rifampicin resistance after primary culture and the results were available within 10 to 12 days. Although phage assays for rifampicin resistance are usually performed after primary isolation of *M. tuberculosis*, their reasonably high accuracy has greater clinical implications if they can be directly applied to sputum specimens. Because culture assays are not easy to obtain in resource limited areas where TB burden is high, Bellen *et al*. (2003) reported a low sensitivity and specificity of 31.1% and 86.1% respectively due to poor quality for sputum samples obtained from Philippines. Bacteriophages can replicate in non-tuberculosis mycobacteria as well as *M.*

tuberculosis, so there is always a potential for false positive results when phage assays are directly applied to sputum specimens (Pai *et al*., 2005). Rifampicin resistance may not be a perfect surrogate marker of MDR-TB in all settings; therefore this assay will give false positive results. To minimize the false positive results, a second confirmatory test may be required to confirm and validate all positive results (Pai *et al*., 2005).

1.3 Mycobacterial antigens for serodiagnosis of TB

Serology for the diagnosis of TB has been explored since 1898, when crude cell preparations containing carbohydrates, lipids, and proteins from *M. tuberculosis* or *M. bovis* BCG were used as antigen. Most of these antigens make for lack of sensitivity and specificity, which makes the assays not applicable for routine diagnosis of TB (Uma Devi *et al*., 2003). Most serologic methods use ELISA to detect antibodies in *M. tuberculosis* infected individuals (Tiwari *et al*., 2007). Modern developments in the purification of antigens, generation of monoclonal antibodies and chromatographic techniques have led to a considerable improvement in specificity (Palomino *et al*., 2007). Serological assays have been regarded for a long time as attractive tools for the rapid diagnosis of TB due to their simplicity, rapidity and low cost (Daniel and Debanne, 1987; Palomino *et al*., 2007; Starvi *et al*., 2003). It is well known that the results of any serological study in infectious diseases depend on the quality of the antigen used.

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; Chatterjee *et al*., 1997). 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. 1.4). Despite its low sensitivity and specificity, a large number of native and recombinant antigens of *M. tuberculosis* such as purified protein derivative, acylated trehalose family and 38 kDa respectively have been assessed, showing substantial progress for the serodiagnosis of TB (Antunes *et al*., 2002; Thanyani, 2003; Verma and Jain, 2007; Palomino *et al*., 2007).

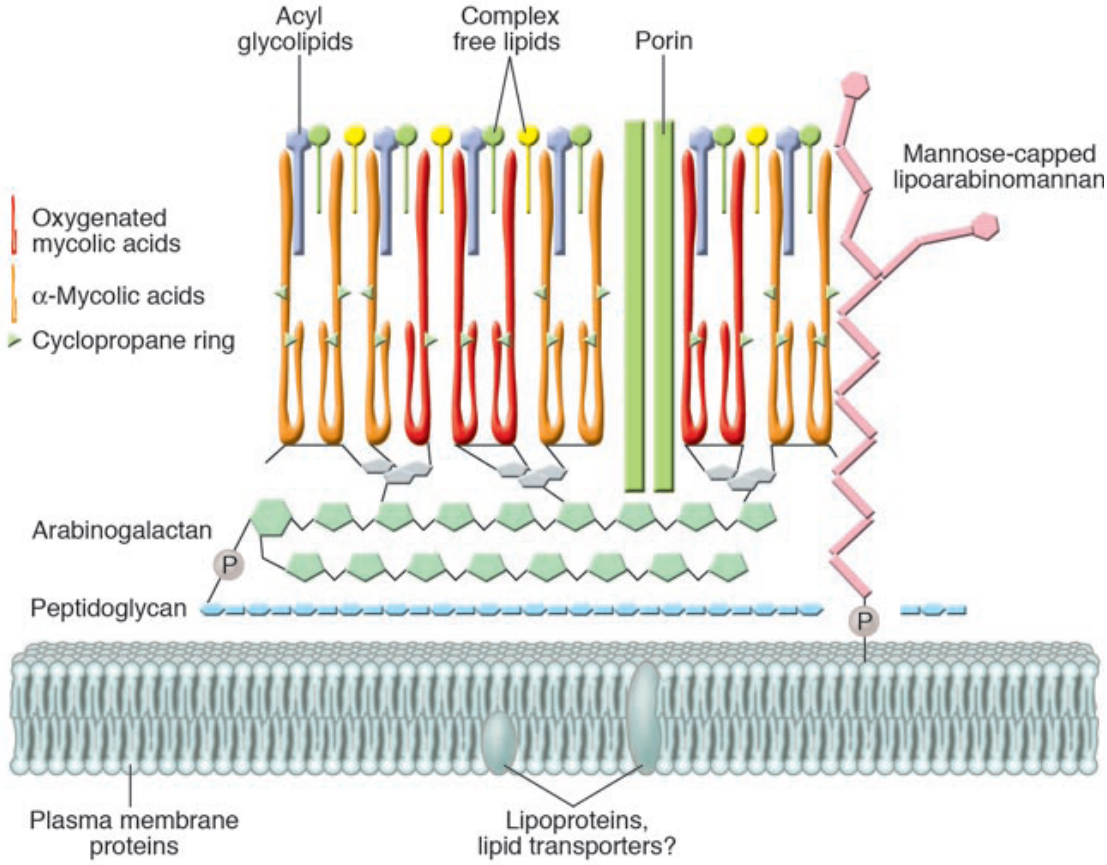


Figure 1.4: Schematic representation of *Mycobacterium tuberculosis* cell envelope (Riley, 2006).

1.3.1 38 kDa antigen

The 38 kDa antigen is a lipo-glycoprotein antigen of *M. tuberculosis* (Wilkinson *et al*., 1997). This antigen induces B- and T-cell responses with high specificity for tuberculosis and is considered a prime candidate for the development of new diagnostic assays for TB. An antibody to 38 kDa antigen occurs in a high percentage of TB patients, and is the serological antigen most studied (Wilkinson *et al*., 1997; Uma Devi *et al*., 2003; Uma Devi *et al*., 2001; Pottumarthy *et al*., 2000; Kulshrestha *et al*., 2005; Raja *et al*., 2008). Anti-38 kDa antibody ELISA was also found to be a useful tool for monitoring the efficacy of chemotherapy and for differentiating between active and treated cases of TB (Ahmad *et al*., 1998). Serological sensitivity have been shown that ranged from 16% to 94% and specificity from 93% to 100%, depending upon the AFB smear results of patients and selection of patient population

in different studies (Wilkinson *et al*., 1997; Pottumarthy *et al*., 2000; Chan *et al*., 2000).

1.3.2 Lipoarabinomannan antigen

Lipoarabinomanna (LAM) is a polysaccharide antigen present in cell wall of all mycobacteria. It constitutes 25 – 40% of the cell walls of *M. tuberculosis* (Patil *et al*., 1995). Purified LAM from *M. tuberculosis* in its native acylated state was first used for serodiagnosis of leprosy (Hunter *et al*., 1986; Levis *et al*., 1987). Sada *et al*. (1990) later reported that LAM of *M. tuberculosis* is a potentially useful antigen in its acylated state for the serodiagnosis of tuberculosis. They measured anti-LAM IgG antibodies in the sera of patients with pulmonary, miliary and pleural tuberculosis using ELISA. They observed a high degree of specificity (91%) and sensitivity (72%) and found no significant difference in the levels of antibodies between patients with pulmonary, miliary or pleural TB. Tessema *et al.* (2002) investigated anti-LAM antibody response in the sera of patients with pulmonary tuberculosis and reported a sensitivity and specificity of 50.5% and 78.3%, respectively. A commercially available test (MycoDot; Genelabs Switzerland) specific for *M. tuberculosis* which detects IgG antibodies to lipoarabinomannan antigen was evaluated by several workers (Julian *et al*., 1997; Lawn *et al*., 1997; Sousa *et al*., 2000; Antunes *et al*., 2002). The assay proved to have a high degree of specificity (84 – 100%) but the sensitivity was low (16 – 56%). A low degree of sensitivity was mostly seen in patients infected with HIV (Lawn *et al*., 1997). This low sensitivity therefore doesn't support its use in the diagnosis of TB, especially in HIV infected patients (Verma and Jain, 2007).

Hamasur *et al*. (2001) demonstrated with a dipstick test that LAM is excreted in the urine of patients with active TB. Their studies showed sensitivity of 81% and specificity of 87%. However further studies are required to determine the pattern of excretion of LAM over time in patients with different clinical types of infection.

1.3.3 Acylated trehalose antigen

Antigens of the acylated trehalose family have been the most frequently investigated group of glycolipids (Verma and Jain, 2007; Simonney *et al*., 2007). They are 2,3-diacyl trehalose (DAT); 2,3,6-triacyl trehalose (TAT), 2,3,6,6 tetraacyl trehalose 2'-sulphate (Sulfolipid, SL-1), and trehalose 6,6-dimycolate (cord factor). Different IgG or IgM titres were obtained when these antigens were investigated on ELISA (Julian *et al*., 2002, Maekura *et al*., 1993). Cord factor is a key molecule for pathogenesis and immunity in tuberculosis within the mycobacterial cell wall (Fujita *et al*., 2005b). Julian *et al.* (2001) reported that glycolipids are physico-chemically quite stable on microplate ELISA. Cord factor antigen assays showed better stability, reproducibility and low cross-reactivity compared to protein antigens (Maekura *et al*., 2001; Fujita *et al*., 2005a). The structure of the mycolyl moiety of cord factor varies widely among mycobacterial species and may seriously affect their detection by antibodies. The studies by Fujiwara *et al.* (1999) and Pan *et al.* (1999) showed that anticord-factor IgG antibody recognizes the mycolic acid subclasses as an epitope. Pan *et al*. (1999) indicated that the anti-mycolic acid antibodies (IgG) in TB patients specifically recognized mycolic acid methyl ester structures, especially methoxy mycolic acid ester.

1.3.4 Mycolic acid antigen

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.

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 (Dobson *et al.*, 1985). Three families of mycolic acids are known; α-mycolic acids without any

oxygenated functional groups and the two oxygenated types that differ primarily in the presence and nature of oxygenated-containing substituents in the distal portion of the meromycolate branch (Khasnobis *et al*., 2002; Yuan *et al*., 1998) (Fig. 1.5). The methoxymycolate series have a methoxy group adjacent to a methyl branch, in addition to a cyclopropane in the proximal position. Among the three subclasses (alpha, methoxy and keto) of mycolic acids, tuberculosis patients' sera reacted most prominently against methoxy mycolic acid (Pan *et al*., 1999). Our previous study on ELISA and IAsys biosensor also showed the presence of anti-mycolic acid antibodies in TB patients, irrespective of co-infection with HIV (Schleicher *et al*., 2002; Thanyani, 2003).

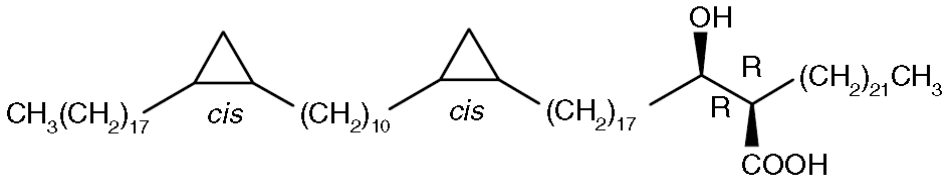
Mycolic acid is presented by antigen-presenting cells (APC) through a mechanism that does not involve MHC-class I or MHC-class II molecules. Mycolic acid is a CD1 restricted antigen with the ability to induce proliferation of specialized T-cells of low abundance in the blood (Beckman *et al*., 1994). 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 have 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 mycobacterial lipid antigens is preserved in HIV-seropositive patients, despite a declining CD4 T-lymphocyte count (Schleicher *et al*., 2002; Simonney *et al*., 2007).

Schleicher *et al*. (2002) showed with ELISA that there is a significantly 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 *M. tuberculosis* mycolic acids in sera of HIV seropositive and HIV seronegative tuberculosis patients, in a population with a high prevalence of TB. 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

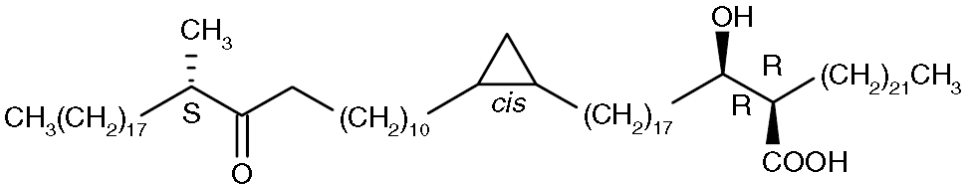
sensitivity and specificity to detect anti-mycolic acid antibody and is therefore not suitable as a reliable serodiagnostic assay for the diagnosis of pulmonary TB.

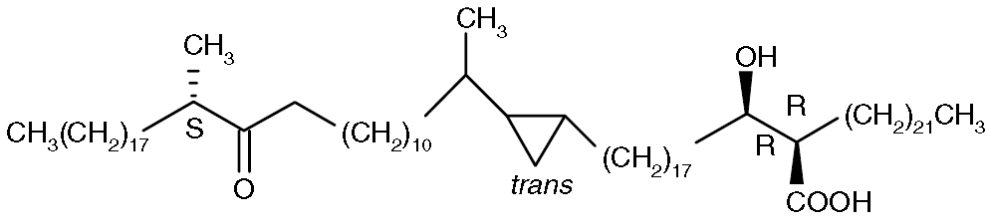
Our previous study on an IAsys biosensor showed its potential to detect antibodies to mycolic acids in active TB patient sera (Thanyani, 2003; Siko, 2002). The current study will focus on the validation of the MARTI (Mycolic acid Antibody Real Time Inhibition)-assay on IAsys biosensor and its further application on the surface plasmon resonance based ESPRIT biosensor.

(a) Alpha Mycolic acids

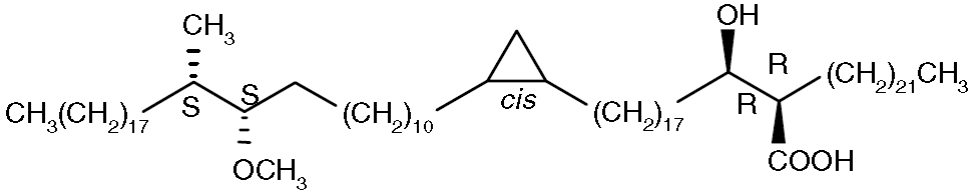


(b) Keto Mycolic acids





(c) Methoxy Mycolic acids



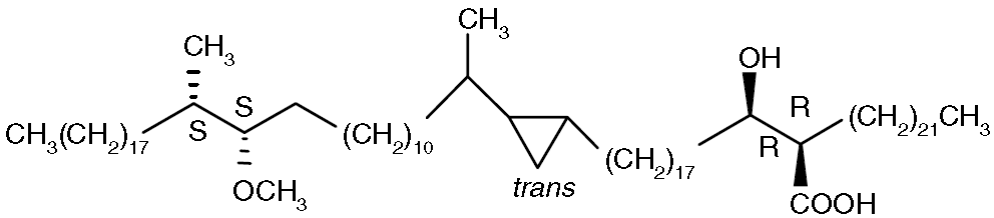


Figure 1.5: Structures of mycolic acids from *M. tuberculosis* (Khasnobis *et al*., 2002).

1.4 Modern alternative tests for serodiagnosis of TB

In spite of new technologies such as PCR, no reliable and affordable tests have been generally accepted in the market for the diagnosis of TB (Ahmad *et al*., 1998). Our preliminary study on IAsys affinity biosensor showed the detection of anti-mycolic acids antibody in human TB patient sera (Thanyani, 2003). 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; Marcheini *et al*., 2007). 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). 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 (Fig. 1.6). Different chemicals can be used to regenerate the surface for re-use when molecules are immobilized on the surface.

Optical biosensors are most popularly used in bioanalysis, due to selectivity and sensitivity (Lazcka *et al*., 2007). Recent progress in optics technology suggests that the optical biosensor may become a powerful tool in the imminent future for the real-time and remote detection of infectious diseases (Pejcic *et al*., 2006).

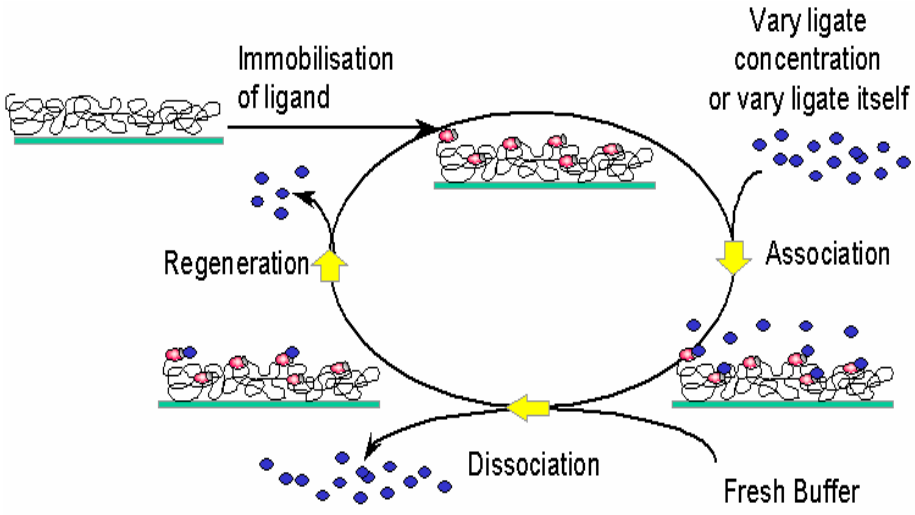


Figure 1.6: An experimental cycle of a sensor surface after regeneration (IAsys Manual).

A biosensor is a device that incorporates a biological recognition (sensing) element in close proximity or integrated with the signal transducer, to give an electronic response that reports the specific binding of a ligand to a target compound (analyte). Transducers are the physical components of the sensor that react to a signal due to the interaction between the biological sensing element and the target analyte. Biosensing occurs only when the analyte is recognized specifically by the biological element. Biosensors are usually classified into various groups according to the signal transduction and to the biorecognition principles. On the basis of the transducing element, biosensor can be categorized as electrochemical, optical, piezoelectric, and thermal sensors.

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). They provide rich information on the specificity, affinity, and kinetics of biomolecular interactions and the concentration levels of an analyte of interest from a complex sample (Shankaran *et al*., 2007). The independence from labeling requirements and low sample

consumption have 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, thereby putting very high demands on the measures 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 fast, sensitive, relatively affordable and able to generate 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-Gortemaker *et al*., 2002; He and Zhang, 2002), biosensor technology can significantly reduce the time as well as detect trace amounts of pathogens with fewer false positives. However conventional methods are being used despite their long turnover times because of their high selectivity and sensitivity. Biosensors have the potential to shorten the time span between sample uptake and results, but their future lies in reaching selectivities and sensitivities comparable to established methods, but at a fraction of the cost (Lazcka *et al*., 2007).

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*., 1999; Markgren *et al*., 2000). Most of the commercially available biosensor systems are applied in the clinical and pharmaceutical markets (Rodriguez-Mozaz *et al*., 2004). 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 the rapid growth in the application of DNA sensors, introduction of advanced sensing materials, and 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.

Nagel *et al.* (2007) showed the detection of anti-tuberculosis antibodies in blood serum using three label-free optical biosensors on a sensor surface coated with a recombinant 30-kDa antigen (Fig. 1.7). The three biosensors, a grating coupler in the reflection mode, an interferometric biosensor and a reflectometric interference spectroscopy (RIfS) device, use glass surfaces (Ta_2O_5 and SiO_2). The grating coupler and the interferometric biosensor determine changes of the effective refractive index at the sensor surface within an evanescent field. Both devices work in a refractometric mode. In their study, they showed that the use of these three biosensors systems for serodiagnosis of TB gave comparable performance.

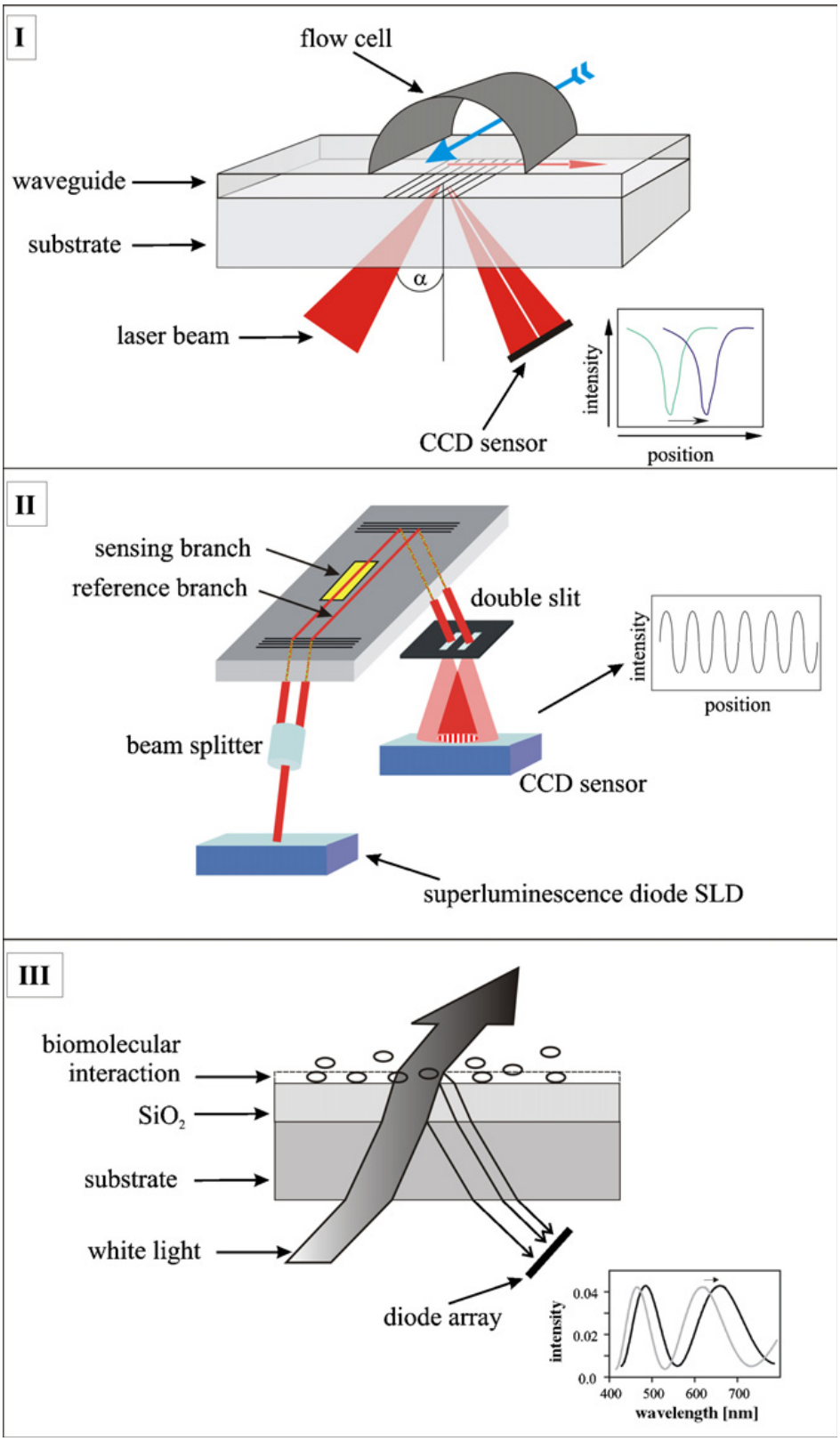


Figure 1.7: Configurations of the three used optical label free devices to diagnose TB: grating coupler (I), interferometric biosensor (II) and the RIfs system (III), (Nagel *et al.*, 2007).

The medical application of diagnosis using a biosensor can be conceived by coating appropriate antigens or antibodies against a target analyte in a sample. Usually, the samples that are used for diagnosis include urine, saliva, serum, and plasma. However serum is most frequently used for medical diagnosis of infectious diseases. This is a very complicated protein mixture for the direct application to a biosensor (Chung *et al*., 2005).

A limited number of commercial optical biosensor instruments are available; for example, BIAcore (Uppsala, Sweden), Affinity Sensors (Cambridge, UK), Artificial Sensing Instruments (ASI) (Zurich, Switzerland) (Leatherbarrow and Edwards, 1999) and ESPRIT (Eco Chemie, The Netherlands). The instruments differ in the method used to generate the evanescent field. The main aim of this study is to investigate the application of both IAsys and ESPRIT biosensor for the detection of anti-mycolic acid antibodies in human TB patient's sera as surrogate marker for active TB.

1.4.1 IAsys biosensor

Interaction analysis system (IAsys) is an optical biosensor system that 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).

The IAsys biosensor can monitor and quantify bio-recognition processes, by detecting changes in 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. The device consists of a high refractive index waveguide separated from a high refractive index

prism block by an intervening, low refractive index coupling layer (Fig. 1.8) (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).

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).

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. In our previous studies, we showed how the IAsys technology could be applied in the detection of anti-mycolic acid antibodies as surrogate markers for active TB on a non-derivatized cuvette coated with mycolic acid liposomes (Thanyani, 2003). 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. In the current study, an IAsys biosensor was used to validate the mycolic acid antibody real-time inhibition (MARTI)-assay for its application to detect anti-mycolic acid antibodies in human serum samples from patients suffering from active tuberculosis due to infection with *M. tuberculosis*.

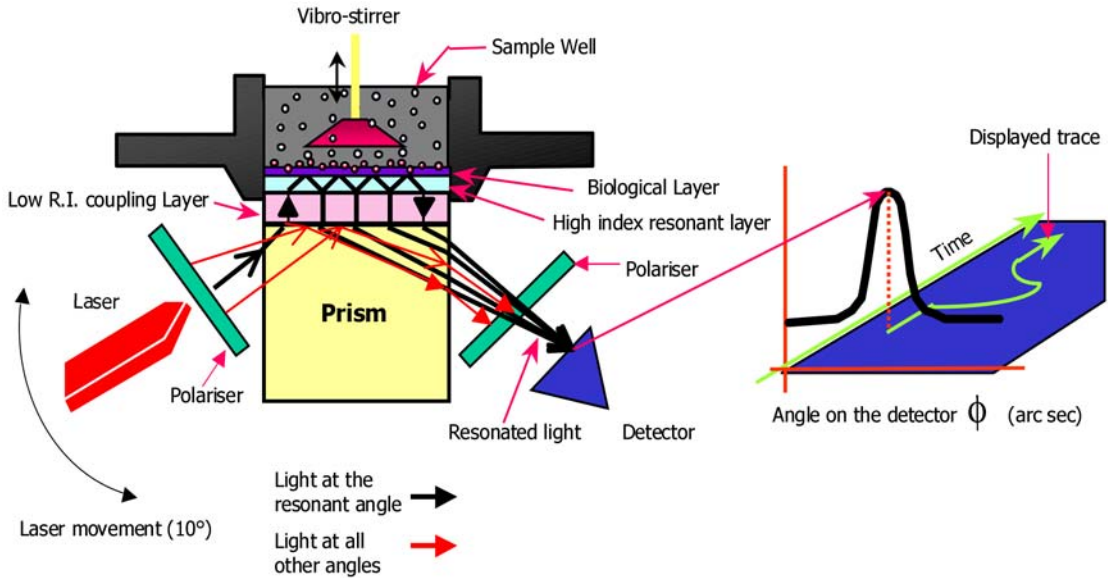


Figure 1.8: Cross section of the IAsys Affinity Biosensor cuvette and how the resonant mirror works (IAsys technical manual).

1.4.2 ESPRIT biosensor

There are several companies manufacturing Surface Plasmon Resonance (SPR) instruments for studying biomolecular interactions, eg. Biacore, Windsor scientific, Quantech, Moritex and ESPRIT (Shankaran *et al*., 2007). Each company produces different SPR systems equipped with a variety of options usable for specific applications. The SPR can be simply described as follows: when light is irradiated on to the underside of a thin film of metal having a thickness of several to hundreds of nm so that total reflection occurs, evanescent waves are generated on the metallic film

side (Fig. 1.9). At the metallic surface in contact with a dielectric space, surface plasmons are simultaneously generated. When the wave numbers and frequencies of these two kinds of waves match, resonance occurs, which attenuates the reflected light. This phenomenon is known as SPR. The dielectric constant of a dielectric material influences the evanescent waves. Thus, interactions between substances occurring on the surface of the sensor chip cause differences in the dielectric constant.

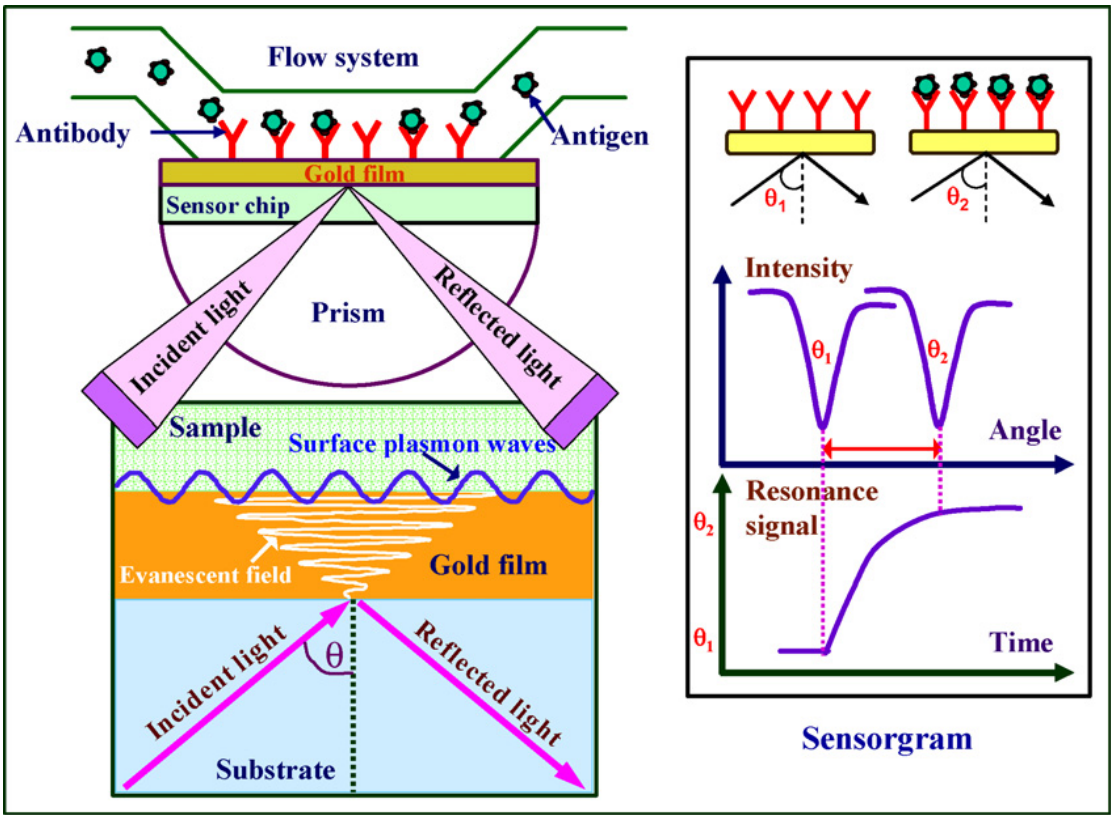


Figure 1.9: Schematic view of the surface plasmon resonance immunoassay technique (Shankaran *et al*., 2007).

These differences, which in turn influence the surface plasmons, can be detected as changes in resonance (Fig. 1.9). Biosensors based on SPR exploit this phenomenon and read the changes in the dielectric constant that occurs as a result of biomolecular interactions on the surface of a metallic thin film or changes in refractive index, by the attenuation of reflected light (Matsushita *et al*., 2008). In the current study, the MARTI assay will be transferred from waveguide technology (IAsys affinity biosensor) to surface plasmon resonance (ESPRIT biosensor). This involves

optimization of the method and its application in detecting anti-mycolic acids in TB patients before and during anti-TB chemotherapy.

1.5 Application of biosensors as immunosensors

The analysis of the interaction between biomolecules is a key aspect to understand biological systems and has been carried out with several different techniques in the past years. The specificity of the molecular recognition of antigens by antibodies to form a stable complex is the basis of both the analytical immunoassay in solution and the immunosensor on solid-state interfaces (Luppa *et al.*, 2001). The biosensor technology is an advantageous tool for biological analysis and is currently under intensive development for a wide range of applications (Sun *et al.*, 2007). Pathogen detection is of the utmost importance primarily for health and safety reasons. These include food industry, water and environmental quality control, and clinical diagnosis (Lazcka *et al.*, 2007). Currently, biosensors that use highly specific antigen-antibody reactions are being developed in a wide range of applications such as food, industry, environmental monitoring and clinical diagnostics. Most established immunoassay techniques, such as radio-immunoassay, fluorescence labelled antibody assays and ELISA are widely used. However these assays are expensive, time-consuming and require complex sample pre-treatment procedures (Wong *et al.*, 2002). The immunosensor is now considered as a major development in the immunochemical field. Despite extensive studies being done in this field, there are only few commercial applications of immunosensors in clinical diagnostics. This is because of the unresolved fundamental questions relating to ligand surface immobilization, orientation and specificity properties of the antibodies and antigens on the transducer. An ideal immunosensor for a routine analysis must be simple, fully automated and capable of performing rapid measurements with turnaround times of less than 1 hour (Luppa *et al.*, 2001).

Miura *et al.* (2003) developed an assay using an indirect competitive inhibition principle, showing the detection of methamphetamine in human urine. It was shown that this molecule could be detected down to 0.02 ppm level using quartz crystal microbalance technique (Miura *et al.*, 2003). A simple scheme of the principle of indirect competitive immunoassay is shown in Fig. 1.10. The antigen is normally

mixed with the relevant antibody containing sample and introduced over the antigen immobilized surface. The concentration of the antibody is kept constant so that the response variations are proportional to the amount of the antigen mixed with antibody. An increase in the resonance angle occurs when the antibody binds with the conjugate immobilized on the surface. However, when an equilibrium mixture of antibody and antigen is added onto the conjugate, only the unbound antibody in the equilibrium mixture can be available for binding to the conjugate surface, hence a decrease in the resonance angle is observed. Because of its promising advantage for highly sensitive detection of small molecules, there is a rapid growth in the use of indirect competitive inhibition based SPR immunosensors in a variety of applications (Shankaran *et al*., 2007). A similar approach of an indirect competitive inhibition study was performed on the ESPRIT biosensor to detect anti-mycolic acid antibodies in TB human sera.

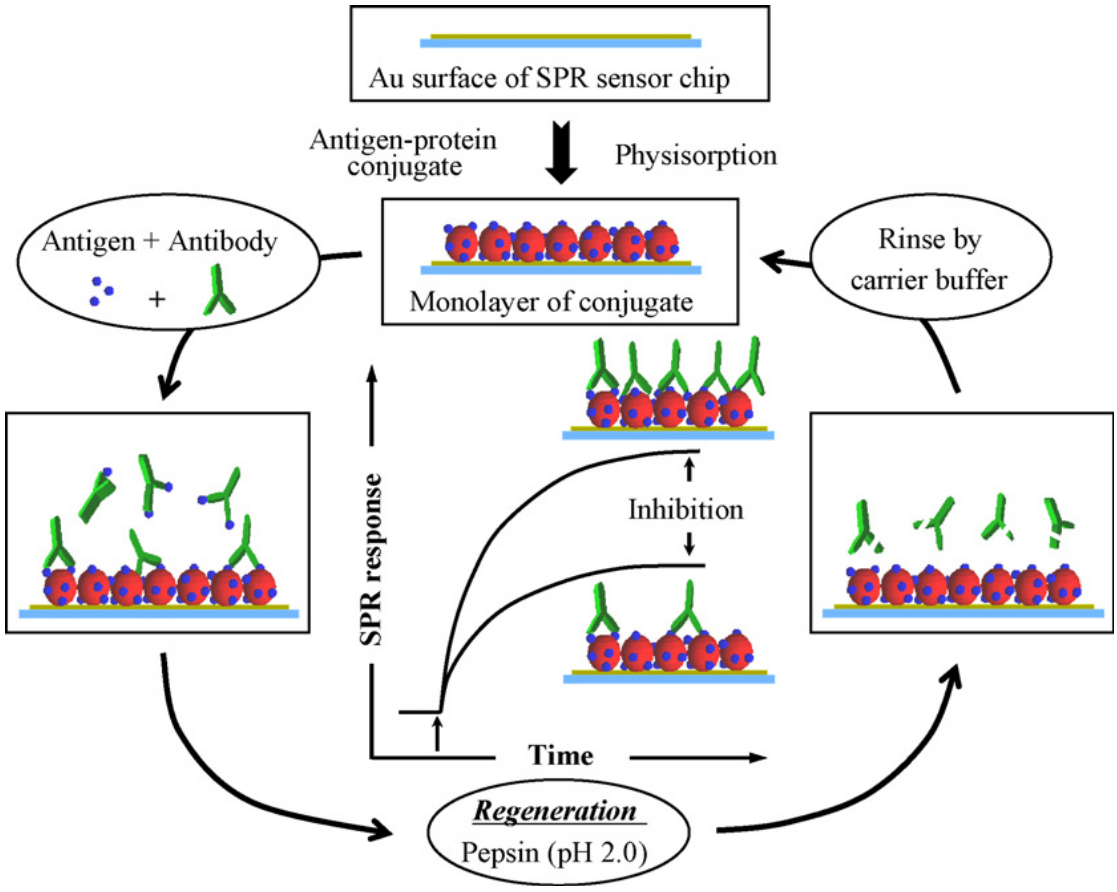


Figure 1.10: Schematic view of the indirect competitive inhibition immunoassay (Shankaran *et al*., 2007).

1.6 Advantages of biosensors in immunoassays

Biosensors offer several advantages as compared to various other transduction techniques for application as a high throughput tool into a variety of applications: Labeling of reagents is not required when they are used. It has been realized that fluorescent labeling or radio labeling of reagents involved with expensive or hazardous labeling procedure can be laborious and time consuming. Labeling of proteins may alter the reactivity or specificity of the biomolecules, thereby reducing both qualitative (detectability, specificity, selectivity, etc.) and quantitative (kinetic and thermodynamic parameters, concentration analysis) information of the biological assays (Shankaran *et al*., 2007; Ayela *et al.*, 2007). The hydrophobic nature of the fluorescence compounds tends to cause background binding, which may result in false positive signals. Biosensors are capable of producing continuous real-time responses to biomolecular interactions occurring at the interface, leading to a rapid evaluation of the analytical systems. The active sensor surface could be regenerated for repeated multiple use of a same sensor chip by an effective regeneration protocol, while monitoring carefully the reactivation process. Most significantly, it is the application of biosensors in the monitoring of small molecules with enhanced sensitivity that has greatly increased the utility in drug screening (Shankaran *et al*., 2007).

1.7 Hypothesis

Evanescent field biosensors (IAsys and ESPRIT) can support an effective and fast serodiagnostic assay for tuberculosis based on the detection of anti-mycolic acid antibodies as surrogate markers of active tuberculosis.

1.8 Aims

- To validate the mycolic acid antibody real-time inhibition (MARTI)-assay on an IAsys biosensor for its application to detect anti-mycolic acid antibodies in human serum samples from patients suffering from active tuberculosis due to infection with *M. tuberculosis*.

- To transfer the technology from IAsys to the ESPRIT biosensor:
  - Immobilization of mycolic acids onto a gold surface coated with octadecanethiol
  - Optimization of the MARTI-conditions on the ESPRIT biosensor
  - Optimization of the regeneration protocol of the ESPRIT gold disc after inhibition studies

- To prepare and analyze serum from blood samples collected at Pretoria Academic hospital by Prof. A.C. Stoltz (Foundation for Professional Development, Pretoria) from HIV positive patients who were clinically assessed to confirm their TB status.

- MARTI-analysis of serum samples that were collected at University of Stellenbosch as a subcontract of a European and Developing Countries Clinical Trials Partnership (EDCTP) on surrogate markers for tuberculosis. Patients donated samples before treatment and several times after commencement of chemotherapy, in order to determine the immune memory of antibodies to mycolic acids in TB patients and also to monitor the progression of the disease during chemotherapy.

CHAPTER 2

Validation of the MARTI-assay on IAsys biosensor

2.1 Introduction

Active tuberculosis is diagnosed by detecting *Mycobacterium tuberculosis* complex bacilli in specimens from the respiratory tract (pulmonary TB) or in specimens from other sites of the body (extrapulmonary TB). Although many new diagnostic methods have been developed, acid fast bacilli (AFB) smear microscopy and culture are still the gold standards for diagnostic of active TB, especially in low resource countries (Palomino *et al*., 2007). Microscopic identification and culture of *Mycobacterium* species in sputum are the most common methods for diagnosis of pulmonary disease, but the detection of extrapulmonary TB is often more difficult. In the search for rapid and cost-effective diagnostic methods for TB, immunodiagnosis is considered an attractive option, because it uses the specific humoral and cellular immune responses of the host to infer the presence of infection or disease; thereby avoiding the problem of sensitivity of detection of traces of the infectious agent itself. A wide variety of serological tests for the detection of antibodies in individuals suspected to have TB have been evaluated to detect active disease (Chan *et al*., 2001; Schleicher *et al*., 2002; Pan *et al*., 1999). Serology has additional advantages in situations where the patient is unable to produce adequate sputum, where TB is extrapulmonary and where sputum smear and culture results are negative (Palomino *et al.*, 2007).

Our previous studies (Schleicher *et al*., 2002) and that of others (eg. Pan *et al*., 1999) have shown the prevalence of anti-mycolic acid antibody in TB patients with ELISA. Schleicher *et al.* investigated the diagnostic potential of an ELISA, based on the detection of antibodies to *M. tuberculosis* mycolic acids in sera of HIV seropositive and HIV seronegative tuberculosis patients, in a population with a high prevalence of HIV. Although they observed 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 concluded that the ELISA has inadequate 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.

Our previous study on the IAsys biosensor showed good potential in detecting anti-mycolic acid antibodies in seventeen active TB patient serum samples (Thanyani, 2003). A recent study done by Nagel *et al*. (2007) reported a specificity of 100% and a sensitivity of 75% for detecting antibodies as surrogate markers of active TB using immobilized 30-kDa antigen from *M. tuberculosis* and contacting it with undiluted blood serum samples when optical biosensors were utilized. However, the dilution of the samples decreased the sensitivity of the assay (Nagel *et al*., 2007). Their results are probably not universally applicable, as many studies have shown a decrease in specificity and sensitivity of immunoassay when the 30-kDa is used in an HIV burdened population (Daniel *et al*., 1994; Hendrickson *et al.,* 2000). It is known that the production of antibodies to protein antigens generally depends on the help of $CD4^+$ T cells and the infection with HIV results in depletion of $CD4^+$ T cells and inhibition of function of the remaining T cells (Price *et al.*, 2001). The reason why the antibody response to glycolipid antigens such as mycolic acid is preserved in HIV seropositive TB patients, despite declining CD4 T lymphocyte counts, has been reported to be due to the novel CD1-restricted lipid antigen presentation pathway (Moody *et al.*, 1997; Schleicher *et al*., 2002; Simonney *et al*., 2007). However, the lipid antigen presentation to B-cells in humoral immune responses has not yet been reported. It is believed that mycobacterial lipid antigen epitopes may be presented by novel mechanisms different from the classical MHC class I or class II restricted proteins antigens (Fujita *et al*., 2005a).

2.1.1 Prevalence of HIV in TB

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. World Health Organization (2006) has reported that, in countries with the highest HIV prevalence, more than 75 % of cases of tuberculosis are HIV-associated. Individuals with HIV infection are at increased risk for TB infection and more serious disease due

to their weak immune system when compared to those without HIV infection. Strategies that are normally effective in healthy populations cannot be transferred directly to control TB in persons with HIV infection (Frothingham *et al*., 2005). It is estimated that 50% of the population of sub-Saharan Africa is latently infected with TB. Once infected with *M. tuberculosis*, progressive deterioration of cell mediated immunity caused by HIV infection increases the risk of TB disease by a hundred fold or more (Frothingham *et al*., 2005). In Africa, TB is often the first manifestation of HIV infection, and it is the leading cause of death among HIV-infected patients. Corbett *et al.* (2006) state that every opportunity should be taken to screen HIV-infected patients for active TB, just as every patient with TB should be screened for HIV. The timing of the initiation of antiretroviral therapy in patients with HIV and TB co-infection is also difficult, due to quick immune deterioration in such patients (Frothingham *et al*., 2005). WHO (2006) guidelines suggest starting antiretroviral drugs within two months of tuberculosis treatment. Since patients who start antiretroviral drugs early in their TB treatment can be predisposed to immune reconstitution syndrome, which is frequent, have symptoms overlapping with worsening TB and can be life threatening to the patients (Lawn *et al*. 2005). Only a rapid and reliable diagnostic assay can reduce the TB infection, especially to those co-infected with HIV because they are more likely to develop drug resistant TB (Frothingham *et al*., 2005). Previous studies done by Schleicher *et al.* (2002) has shown that anti-mycolic acid antibodies can be shown also in HIV infected patients. Our previous studied on the IAsys biosensor has shown a potential development of a serodiagnosis assay in TB patients co-infected with HIV (Thanyani, 2003). 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).

2.1.2 Advantages of the IAsys Biosensor

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 and Cimitan, 2003). The IAsys biosensor ensures 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.

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*.

2.1.3 Immobilization of mycolic acids antigen on IAsys biosensor

The immobilization of mycolic acids was first reported by Siko (2002). This was followed up by Thanyani (2003) to give a proof of principle of the MARTI (Mycolic acid Antibody Real-Time Inhibition)-assay, 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. Siko (2002) 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. Altin *et al*. (2001) also showed that immobilization of lipid membranes on IAsys hydrophobic cuvettes from a solution of lipids in organic solvents did not always produce consistent results, even when the procedure was carried out according to IAsys protocols manual. Siko (2002) firstly activated the surface of a non-derivatized cuvette with a cationic detergent, cetyl pyridinium chloride (CPC) to make the hydrophilic surface hydrophobic and could then stably coat with mycolic acids and cholesterol containing liposomes. In their studies Siko (2002) and Thanyani (2003) showed adequate binding of mycolic acid and cholesterol containing liposomes occurred after activation of the non-derivatized hydrophilic surface with the cationic detergent CPC. 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 colloidal solutions that easily generate foam at low concentrations. The saponins are known as biologically highly active substances. Previous studies (Thanyani, 2003) showed that an optimum concentration needs to be determined on the mycolic acids and cholesterol surface for each batch of saponin that is obtained. The same approach was followed in this study to validate the MARTI-assay on IAsys biosensor using tuberculosis patient sera collected by Schleicher *et al.* (2002).

2.3 Aims

To validate the mycolic acid antibody real-time inhibition (MARTI)-assay on an IAsys biosensor for its application to detect anti-mycolic acid antibodies in human serum samples from patients suffering from active tuberculosis due to infection with *M. tuberculosis*.

2.4 Materials and Methods

2.4.1 Materials

2.4.1.1 General reagents

Cetyl-pyridinium chloride (1-hexadecylpyridinium chloride), L-α-phosphatidylcholine (L-α-Lecithin, 99%), 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 (KOH) and ethanol (98%) were from Saarchem (SA).

2.4.1.2 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 glycol (PEG) from Sigma, St Louis, MO, USA.

2.4.1.3 ELISA 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.

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.

2.4.1.4 Resonant mirror biosensor apparatus

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

2.4.1.5 Human sera

Serum samples were selected from 101 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 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. The TB^- patients that were used for control had medical conditions other than TB and were recruited from the general medical wards. None of the TB^+ patients were on anti-TB chemotherapy at the time of serum collection.

2.4.1.6 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).

2.4.1.7 Biosensor 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.

2.4.2 Methods

2.4.2.1 Preparations of liposomes

Stock solution of phosphatidylcholine (100 mg/ml) (Sigma, St Louis, MO) was prepared by dissolving the weighed amounts in chloroform. Mycolic acids containing liposomes were prepared by adding 90 µl phosphatidylcholine stock to 1 mg dried mycolic acids. Empty liposomes, i.e. with no mycolic acids, were prepared by using 90 µl of phosphatidylcholine stock solution only. 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 °C for about 10 min. Liposome formation was induced by addition of 2 ml saline (0.9% NaCl) and placing in a heat block at 85 °C for 20 min, with vortexing every 5 min. The liposomes were then sonicated for 2 min at 30% duty cycle at an output of 3% with a Model B-30 Branson sonifier (Sonifier Power Company, USA). The sonicator tip was washed with chloroform and rinsed with distilled water before and after use. The liposomes (200 µl) were aliquoted into ten tubes and kept at –20 °C overnight before freeze-drying. After freeze-drying, 2 ml of phosphate buffered saline (PBS) azide EDTA (Sigma, St Louis, MO) buffer (PBS/AE, pH = 7.4) was added to each tube containing liposomes. The tubes containing liposomes were placed in a heat block for 20 min. and sonicated as before.

2.4.2.2 ELISA of patient sera

Mycobacterial mycolic acids were isolated from a culture of *M. tuberculosis* H37Rv (American Type Culture Collection 27294) 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 min at 85 °C and sonicated (Virsonic 600, United Scientific, USA) at 20% duty cycle and optimal output level for 1 min. The solution was kept at 85 °C during pipetting into ELISA plates (Sero-Well®, Bibby Sterilin Ltd, UK), after which

the plates were placed in plastic bags and incubated overnight at 4 °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 h at room temperature. The solution was flicked out, filled with 50 µl serum or serum precipitate in triplicate and incubated for 1 h at room temperature, flicked out and washed three times with PBS/0.5% casein. The wells were aspirated to remove proteinaceous froth. The plates were incubated for 30 min. at room temperature with peroxidase-conjugated goat anti-human IgG (whole molecule, Sigma) diluted 1/1000 in PBS/0.5% casein, flicked out, washed three times with PBS/0.5% casein and aspirated. The presence of antibody was revealed using 50 µl/well of hydrogen peroxide (40 mg) and *o*-phenylenediamine (50 mg) in 50 ml 0.1 M citrate buffer (pH = 4.5). Measurement of the yellow colour was done after 30 min at 450 nm using a Multiskan Ascent photometer (Thermo-Labsystems, Finland). To correct for background binding in serum, the signal generated with those samples in PBS coated wells was subtracted from that generated in mycolic acid coated wells.

2.4.2.3 Detection of anti-mycolic acids antibody with IAsys affinity biosensor

The IAsys resonant mirror biosensor sensor was set for a data-sampling interval of 0.4 sec, temperature of 25 °C and stirring rate of 75% for all experiments. The cells were rinsed three times prior to use with 96% ethanol (Saarchem, SA), followed by extensive washing with PBS/AE. A 60 µl volume of PBS/AE was pipetted into each cell of the cuvette to obtain a stable baseline for 1 min. The PBS/AE was subsequently aspirated and the surface activated with 50 µl of 0.02 mg/ml CPC, which was freshly prepared every week, for 10 min. This was followed by five times washing with 60 µl PBS/AE and then substituting with 25 µl PBS/AE for a new baseline before immobilization of mycolic acids containing liposomes to the surface for 20 min. The immobilized liposomes were then finally washed five times with 60 µl PBS/AE, substituted with 50 µl of saponin and incubated for 10 min. This latter step was to avoid non-specific binding on the surface of the cuvette during the subsequent binding events. The cells were then washed five times with PBS/AE, the content of each cell substituted with 25 µl of PBS/AE and left for about 5 - 10 min 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 in each cell, to compare the responses of the two cells over 10 min. A pre-incubation of 1/500 dilutions of serum with solutions of liposomes containing mycolic acids and empty liposomes (phosphatidylcholine alone) were allowed for 20 min. These were then added (10 µl) for binding inhibition studies in different cells, one with mycolic acids liposomes and the other with empty liposomes as a control, and allowed to bind for 10 min. Finally, dissociation of antibodies was effected with three times PBS/AE washing and measurement of the response for 5 min.

2.4.2.4 Regeneration of non-derivatized cuvettes

Regeneration was effected by initial three times washing with 96% ethanol for 1 min, followed by seven times washing with 70 µl PBS/AE for 1 min. The surface was then finally treated with 50 µl potassium hydroxide (12.5 M) for 2 min followed by seven times washing with 70 µl PBS/AE for 1 min.

2.5 Results

2.5.1 Biosensor criteria applied for validation

In this study, 102 patients were analysed on the IAsys biosensor to identify anti-mycolic acid antibodies in human patient's sera, to determine the specificity and sensitivity of the MARTI-assay. After analyzing all the patient sera, the profiles were then analyzed for accuracy of measurement and the following criteria were applied for the acceptance of the data point: the cuvette cell calibration curves of the high dilution serum in the two cells of one cuvette had to fall within 90 – 100% identity in terms of the relative response amplitudes, calibration curve profiles had to be similar by eye, and the amplitude of binding of the calibration curves had to be at least the average of all 102 samples analyzed minus one standard deviation. This translated into 480 – 145 = 335 arc.seconds as minimum response amplitude required for the calibration curve. Figure 2.1 indicates one of the sensorgrams that was not accepted due to the difference in response of the two channels. Of the 102 sera that were analyzed, 61 met the criteria above. These were divided into 32 TB positive, 11 TB negative and 18 $HIV^{+}TB^{-}$ samples.

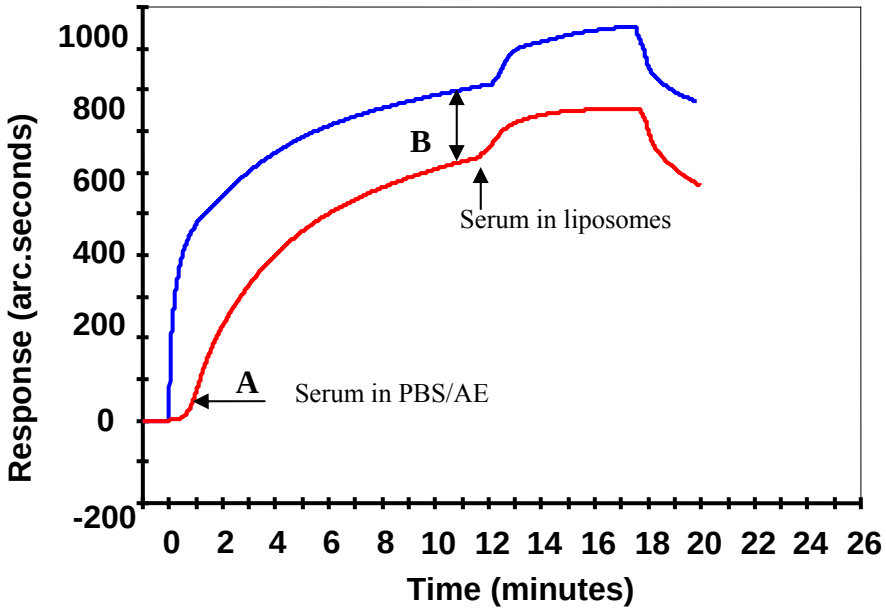


Figure 2.1: A typical inhibition binding profile on the IAsys biosensor that was not accepted due to channel differences in binding response. A and B show two manifestations of retardation in the initial binding response when the same volumes of serum in PBS/AE were added in the two cells.

2.5.2 Detection of anti-mycolic acids antibodies in human sera

The six main stages involved to measure the binding of specific antibodies to lipid antigens in liposomes in real time on the biosensor are: (A) the activation of the non-derivatised cuvette surface with CPC, (B) immobilization of the liposomes containing 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 higher concentration, and finally (F) surface regeneration (Fig. 2.2). The dilutions of sera used were estimated from a dilution range of one positive and one negative serum sample and are not necessarily optimal for all sera. The cuvette cell calibration curves of the high dilution serum in the two cells of one cuvette had to fall within 90 – 100% identity in terms of the relative response amplitudes in order to be accepted. A limitation of the IAsys system was found to be the difference in quality from one cuvette to another when using liposomes as antigen

coat. In rare cases, new cuvettes were not usable at all. Usually, new cuvettes were found to be reliable only after a succession of regenerations, while in other rare cases, new cuvettes could be reliably applied after a single regeneration cycle. The results (those in the rectangle, Fig. 2.2) were aligned using the Fastplot programme from IAsys.

Patient sera selected from the collection of Schleicher *et al*. (2002) were used to detect antibodies against mycolic acids on the optical IAsys biosensor. The ELISA experiments were performed as described in Schleicher *et al*. (2002). Out of the 61 patient sera that were analyzed on the IAsys biosensor, 17 were re-analyzed on ELISA to confirm that the original antibody activity as reported by Schleicher *et al.* (2002) was still intact and to compare them with the results found on the IAsys biosensor during the same period of assessment. The inhibition studies on the IAsys were determined by pre-incubating test serum with mycolic acids-containing liposomes and applying these on biosensor cuvettes coated with mycolic acids. In the control experiments, sera were pre-incubated with empty liposomes. The pre-incubation of a sputum positive TB patient serum with mycolic acids liposomes resulted in inhibition of antibody binding to mycolic acids when compared to the signal generated by the same serum pre-incubated with empty liposomes (Fig. 2.3A). This confirmed the specificity of binding of antibodies to mycolic acids in sputum positive TB patients' sera.

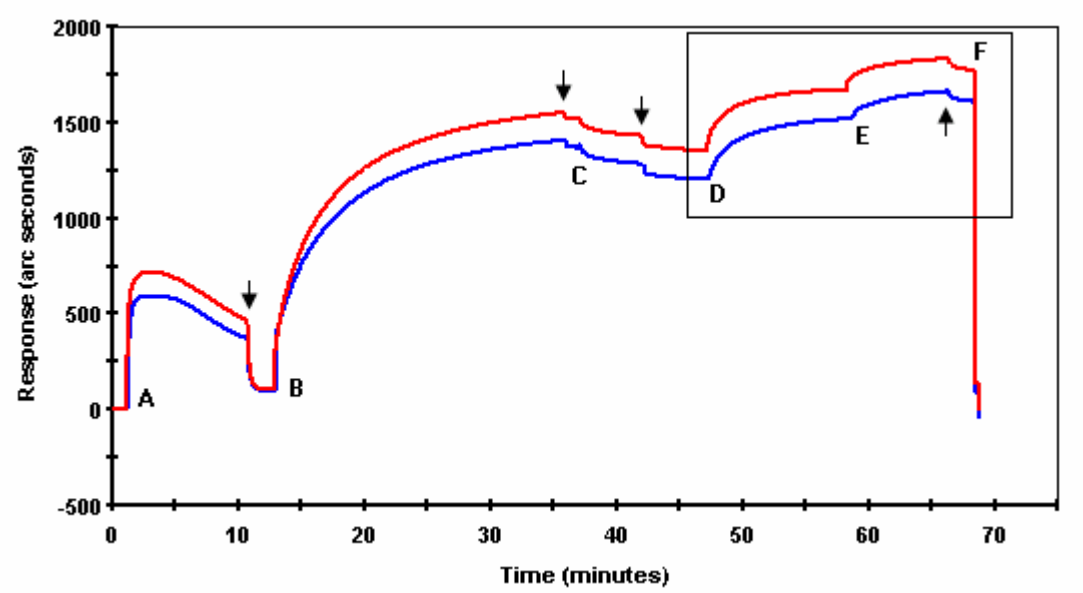


Figure 2.2: A typical graph summarizing the process of measuring antibody binding or inhibition of binding by mycolic acid and empty (phosphatidylcholine only) liposomes, in the two cells of an IAsys biosensor cuvette surface coated with mycolic acid liposomes. The surface was activated with cetyl-pyridinium chloride (A), coated with mycolic acids 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 potassium hydroxide (12.5 M) and 96% ethanol (F). The arrows indicate washing with PBS/AE and the response from the two cells are differentiated by red lines (channel 1, upper curve) and blue lines (channel 2, lower curve).

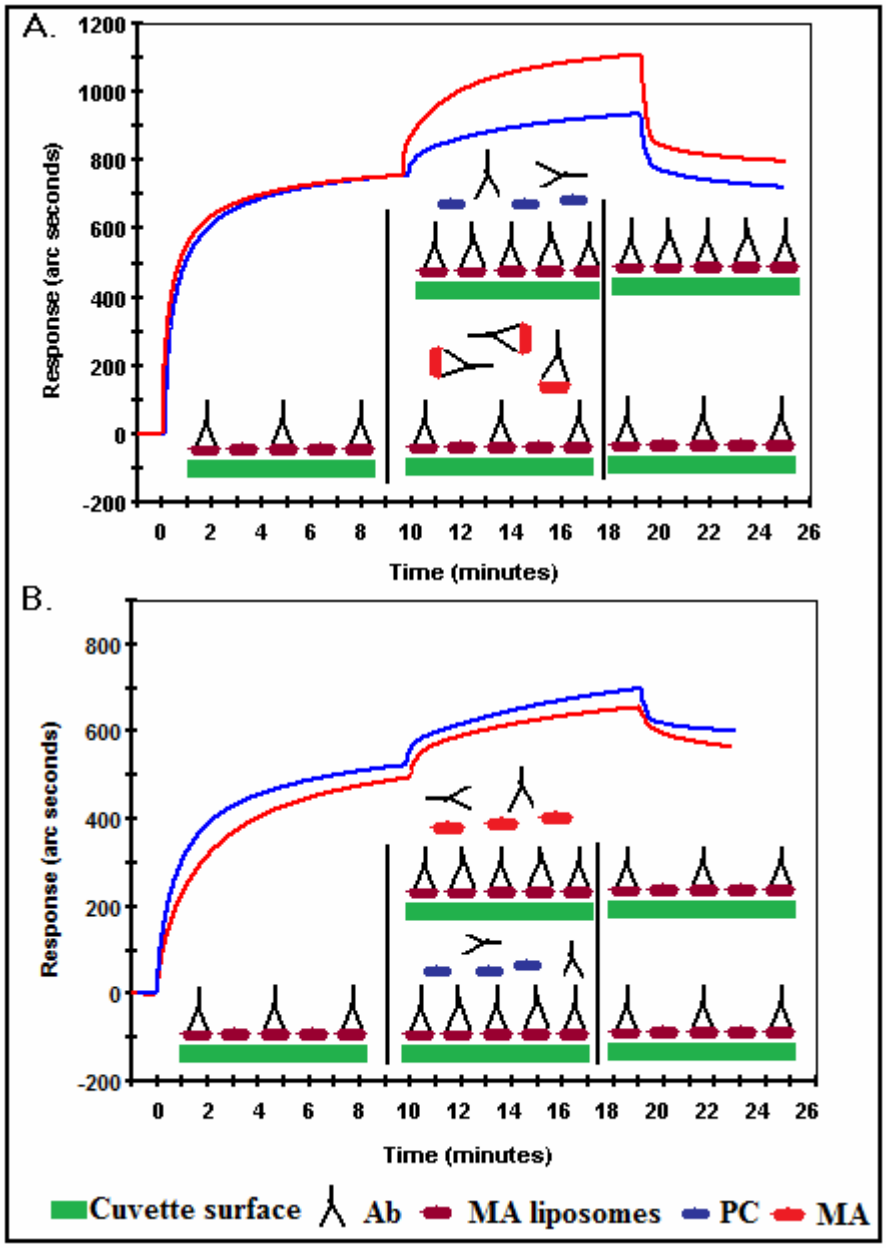


Figure 2.3: Inhibition of human TB^+ (A) and TB^- (B) patient serum antibody binding with mycolic acids or empty liposomes on an IAsys cuvette surface coated with immobilized mycolic acids liposomes. For the first 10 min, a 1/1000 dilution of serum in PBS/AE was incubated in both cells. For inhibition studies, the pre-incubated serum in a dilution of 1/500 was then added with the blue channels (A = lower curve and B = upper curve) representing the binding response of serum in mycolic acids and the red channels (A = upper curve and B = lower curve) representing that of serum in empty liposomes as control.

There was no inhibition of binding observed when a sputum negative control serum (TB^-HIV^-) was pre-incubated with liposomes containing mycolic acids and tested on the biosensor to determine binding of antibodies to mycolic acids (Fig. 2.3B). This shows that specific anti-mycolic acids antibodies can be demonstrated in TB^+ patients, after pre-incubation of serum with mycolic acids. TB negative sera from patients infected with HIV tested negative on the IAsys biosensor, with inhibition values of less than 20% (Fig. 2.4).

From 23 TB^+HIV^+ patient sera selected, two sera samples tested false negative on the biosensor (Fig. 2.4). Thirteen TB^-HIV^+ patients' sera tested "false" positive, showing an inhibition of greater than 20% on the biosensor (Fig. 2.4). It is noteworthy that these patients were HIV positive. Some patient sera that were false negative (eg. Fig. 2.5A) and false positive (eg. Fig. 2.5B) on the ELISA tested positive and negative respectively on the biosensor. The normalized signals on ELISA that were above 2 were regarded as positive and below two as negative. The TB^+ and TB^- patients that showed truly positive and negative responses of antibodies to mycolic acids on ELISA also tested truly positive and negative on IAsys biosensor respectively (Fig. 2.6). Our previous studies have also addressed the problems of detecting *M. tuberculosis*-specific antibodies to mycolic acid in TB patients co-infected with HIV on ELISA (Schleicher *et al*., 2002). Three of the patient sera tested from the thirteen HIV^-TB^- tested false positive on the biosensor, and only two serum samples tested false negative in TB^+ HIV^- population (Table 2.1). An apparently lower specificity (27.8%) was observed in TB^-HIV^+ subgroups. However, all these patients were hospitalized with diseases other than TB with the prevailing diagnostic methods. The low specificity obtained amongst the HIV^+ population could reflect true positive results, since it is known that the sputum culture assay is not sensitive enough to detect TB in HIV positive patients (Frieden *et al*., 2003). This may reflect the better ability of the serum test to detect TB in HIV^+ patients. The IAsys affinity biosensor was found to be more sensitive (91.3%) in detecting TB amongst the TB positive patients co-infected with HIV. The overall specificity and sensitivity of the assay after analyzing 61 patient sera was 48.4% (15/31) and 86.7% (26/30) respectively.

It is known that the gold standard of sputum growth of mycobacteria does not measure accurately in the TB^-HIV^+ cohort (Table 2.1). As the serum collection was

actually made for an earlier study, follow-up data were not available to determine the true TB status of the TB^-HIV^+ cohort tested here. When the 18 TB^-HIV^+ sera were omitted in the calculation of the performance parameters of the test based on the 61 data points, accuracy of the assay was found to be 83.7% (36/43). The sensitivity (86.7%, 26/30) remained the same after exclusion of the TB^-HIV^+ population, and the specificity was 76.9% (10/13). The assay showed a high sensitivity (91.3%) in sera from patients who were TB positive and co-infected with HIV. It is known that HIV-positive patients generally have lower levels of *Mycobacterium tuberculosis*-specific antibodies to protein and certain lipid antigens than HIV-negative patients. This shows that the IAsys biosensor can detect anti-mycolic acids antibodies in an HIV endemic population.

Table 2.1: Specificity and sensitivity of the IAsys affinity biosensor assay for detecting anti-mycolic antibody in pulmonary TB and negative control patient sera.

Patient group	*n*	False +	False -	Specificity	Sensitivity
TB^+HIV^+	23	-	2	-	91.3% (21/23)
TB^+HIV^-	7	-	2	-	71.4% (5/7)
TB^-HIV^+	18	13	-	27.8% (5/18)	-
TB^-HIV^-	13	3	-	76.9% (10/13)	-
TOTAL	**61**	**16**	**4**	**48.4% (15/31)***	**86.7% (26/30)***

(+ = positive, - = negative, and *n* = number of patients)

***Accuracy = 81.8%** (the data for specificity of the TB^-HIV^+ group is omitted because of its known underestimation of TB positiveness by standard culture growth assays)

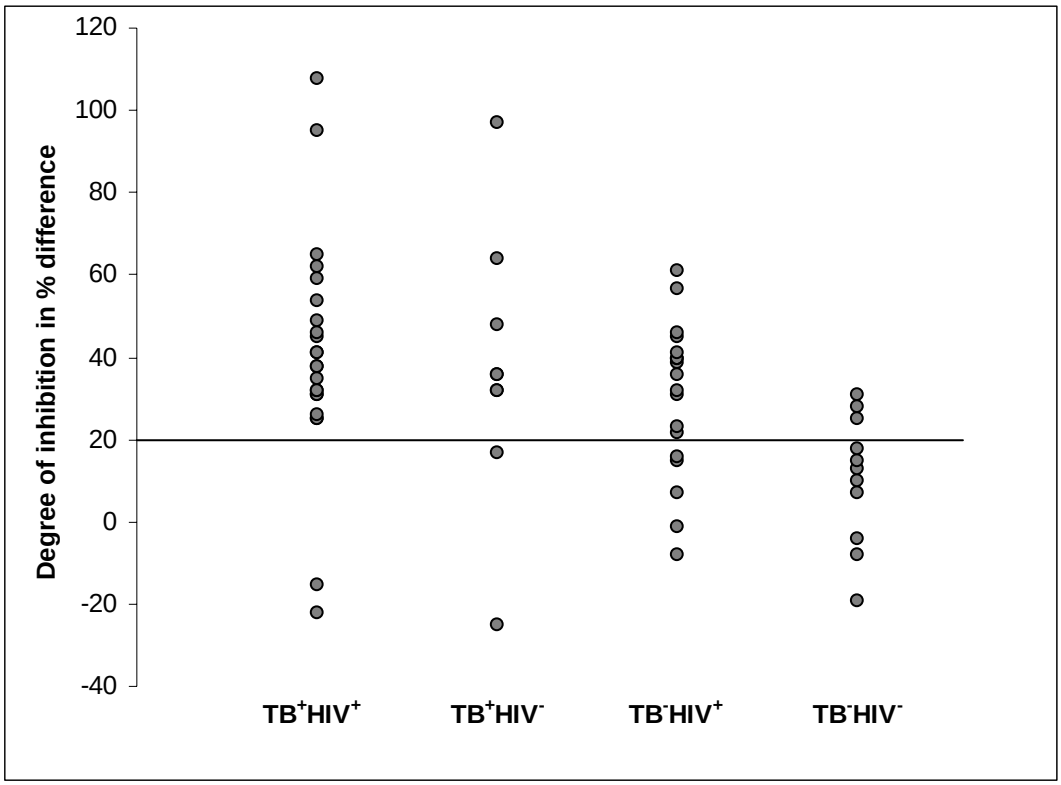


Figure 2.4: The percentage of inhibition of binding of biosensor signal for the 61 patient sera of TB^+ and TB^- controls after pre-incubation of sera with mycolic acids and empty liposomes before testing on mycolic acids coated cuvettes.

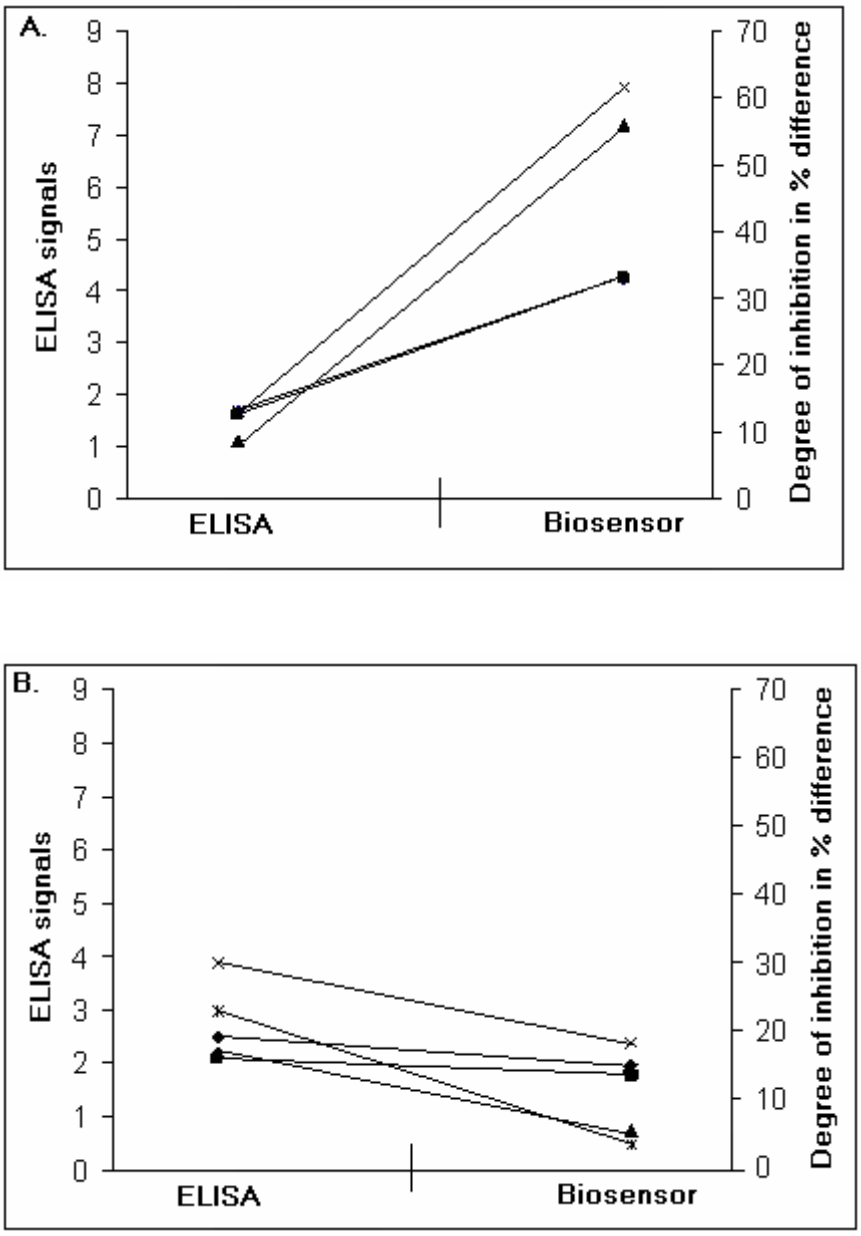


Figure 2.5: Normalized ELISA signals and the percentage of inhibition of binding of biosensor signal of false negative (A) and false positive (B) patients on ELISA who tested correctly on the biosensor.

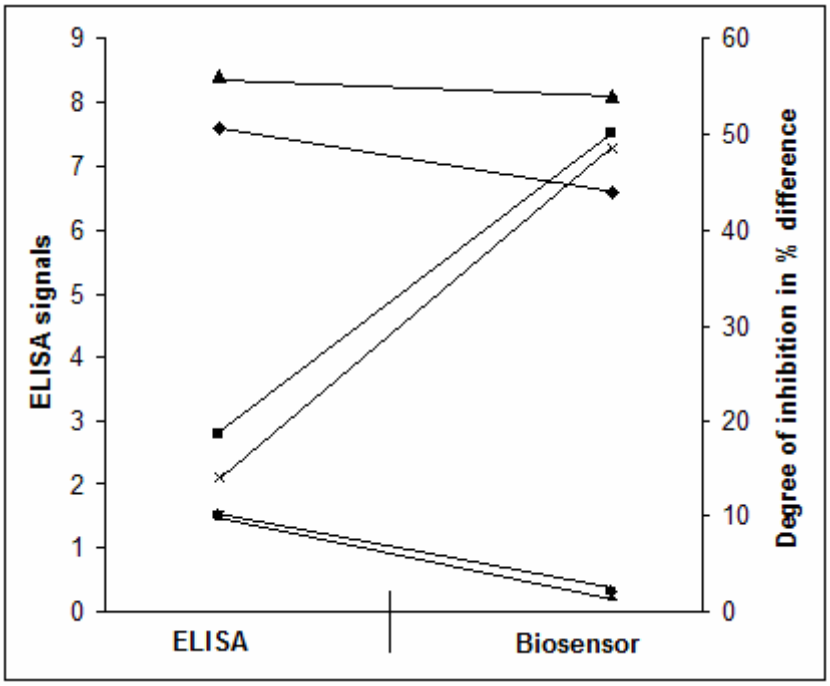


Figure 2.6: Normalized ELISA signals and the percentage of inhibition of binding of biosensor signal of true negative and true positive patients who tested correctly on ELISA and biosensor.

2.6 Discussion

In Africa, TB is often the first manifestation of HIV infection: it is the leading cause of death among HIV-infected patients. Corbett *et al.* (2006) stated that every opportunity should be taken to screen HIV-infected patients for active TB in order to prevent rapid death when both diseases manifest themselves in an individual, and to safely provide antiretroviral (ARV) treatment. The shorter the time from sampling to the diagnostic result, the more lives will be saved. Serodiagnosis with mycolic acids as antigen provides such an opportunity (Verschoor and Onyebujoh, 1999).

Pan *et al*. (1999) indicated that the anti-cord factor antibodies (IgG) in TB patients specifically recognized mycolic acid structure, especially methoxy mycolic acid methyl esters. Mycolic acid is presented by antigen-presenting cells (APC) through a mechanism that does not involve major histocompatibility complex (MHC)-class I or MHC-class II molecules (Moody *et al*., 1999). The anti-mycolic acid immune response could therefore be independent from the participation of $CD8^+$- or $CD4^+$-T cells that respond to antigen that is respectively presented on MHC I and MHC II surface proteins. Other than the MHC-presented protein antigens, mycolic acid is presented on CD1, with the ability to induce proliferation of T-cell lines, with or without the CD4 or CD8 molecules (Beckman *et al*., 1994, Goodrum *et al*., 2001). The production of antibodies to protein antigens generally depends on the help of $CD4^+$ T cells. It is known that infection with HIV results in depletion of $CD4^+$ T cells and inhibition of function of the remaining T cells (Price *et al.*, 2001). Thus, Hendrickson *et al.* (2000) showed a decreased antibody specificity and sensitivity to a mycobacterial 30-kDa protein antigen with ELISA when screening patients in a population that had a high prevalence of HIV. Ratanasuwan *et al*. (1997) showed that when a lipoarabinomannan (LAM) was used in serological tests on HIV-negative and TB positive patients, it showed sensitivities varying from 21% to 89%, but only 7% to 40% in the HIV-positive patients. Antunes *et al*. (2002) described the MycoDot serological assay for tuberculosis that is based on the detection of specific IgG antibodies against the 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. Daniel *et al*. (1994) performed a field test in Mexico, and showed that the 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 similar to the results of the Mexico test, the ELISA gave a sensitivity of 28% of 128 sera from HIV-positive donors. However, the immune response to mycolic acid could in principle proceed independently of the $CD4^+$/$CD8^+$ T cells. 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. The alkyl chains of the mycolic acid antigen have 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 provide a possible explanation why the antibody response to mycolic acids is maintained in HIV-seropositive patients, despite a declining CD4 T-lymphocyte count (Schleicher *et al*., 2002). Simonney *et al.* (2007) also suggested that the CD1-restricted lipid antigen presentation pathway is the likely mechanism accounting for the perseverance of high circulating antibody responses to PGL-Tb1 antigen in HIV infected patients with TB. Simmonney *et al*. (2007) showed that about half of HIV-positive individuals produce specific anti-glycolipid antibody several months before a diagnosis of TB disease can be made.

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). Siko (2002) previously reported a discovery of a cross-reactivity of binding of TB patient sera antibodies between mycolic acids and cholesterol on the IAsys affinity biosensor. This work was followed up by Benadie *et al*. (2008), see Appendix B for detail. Horvath and Biro (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. However our previous study on IAsys biosensor 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 (Thanyani, 2003).

Here, a significant increase in sensitivity and specificity was shown for the anti-mycolic acid antibody detection in patient sera with the inhibition assay on biosensor, as compared to that reported in our previous study using an ELISA (Schleicher *et al*., 2002). The false positive results observed amongst the $TB^{-}HIV^{+}$ population could show that the patients were true positive on the IAsys biosensor, since a sputum culture was used as gold standard method for confirming their TB status. However, it is known that sputum culture of HIV-infected patients need more incubation time than that of patients without HIV infection, which is consistent with the lower bacillary load seen in the sputum of HIV infected patients (Brindle *et al*., 1993). The culture requires 10 – 100 viable *Mycobacterium tuberculosis* per millilitre of sputum to give positive results (Colebunders and Bastian,, 2000). It has also been shown that 15 – 20% of adults with pulmonary TB whose diagnosis has been based on clinical, radiographic, and histopathological findings and response to anti-TB treatment have negative sputum cultures (Frieden *et al*., 2003).

The IAsys affinity biosensor was able to detect low affinity antibody binding to mycolic acids, in addition to high affinity antibody, which the conventional methods cannot generally achieve. In an ELISA these antibodies would have been washed away before the final step and the patient would have tested false negative. The advantage of the biosensor lies in its real-time detection of antibody binding, without the need for prior washing away of the unbound antibody excess. In addition, the inhibition of binding as an endpoint eliminates much non-specific binding interference, which adds to the increased specificity of the biosensor assay. A disadvantage of the biosensor is that it is blind to the identity of the binding ligand from the serum sample. The binding of IgG to mycolic acids was confirmed by showing that its binding inhibition could be reproduced with purified IgG from the same serum sample. The IgG experiments were co-ordinated with one the co-authors (Vanessa Roberts) of the recently published work (Appendix A). The inhibition

signals of isolated IgG from patient serum and the IgG negative control were measured on different channels of the IAsys biosensor. A significant difference in the ability of IgG positive and IgG negative to be inhibited by mycolic acid liposomes was shown. These results correlate with data obtained using whole serum and confirm that it is the IgG fraction of serum that is inhibited from binding by pre-incubation with the mycolic acid antigen (Appendix A).

The few false negative results that still remain with the biosensor analysis are probably due to the inhibition of antibody activity by circulating mycolic acid antigen in the circulation. Should this be the case, one can envisage that a duplicate test be run that is spiked with a stable source of anti-mycolic acid antibodies, such as monoclonal antibodies. A true negative will then return the spike signal, whereas a false negative will consume the signal. False positive results pose a more daunting technical challenge, but may be due to the cross-reactivity of antibodies to mycolic acids of non-tuberculous pathogenic mycobacteria, eg *M. avium*, which do occur at low frequency in especially HIV positive patients. More work is required to manage the specificity of the assay by, for instance, screening sera from patients that are TB negative, but test positive for *M. avium* disease. This work is currently underway.

Many serological assays have been developed for specific antibody detection to lipid cell wall antigens in tuberculosis patients (Lyashchenko *et al*., 1998; Pan *et al*., 1999; Pottunarthy *et al*., 2000; Julian *et al.*, 2002; Schleicher *et al*., 2002; Lopez-Marin *et al*., 2003; Fujita *et al*., 2005a), but generally they do not meet the requirements on specificity and sensitivity (Attallah *et al*., 2005). The biosensor approach may improve that by means of its unique benefits reported here. However, the technique is technically quite difficult to perform in the laboratory and the technology is not yet amenable to large scale screening of patients.

Since only 61 patients were analyzed with the biosensor in this study, more patient and control sera will have to be analyzed to properly validate it as a reliable technique to determine anti-mycolic acids antibodies as surrogate markers for active tuberculosis. However, 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 microbiological sputum growth assay, as was suggested here with some serum samples from HIV^+ patients that tested positive with the biosensor, but negative with the sputum assay.

CHAPTER 3

Technology transfer from waveguide to surface plasmon resonance biosensors

3.1 Introduction

A major challenge with immunological diagnosis of tuberculosis is to distinguish between prior TB exposure, latent TB infection, mild disease and severe disease (Pai *et al*., 2006). Other factors that affect the performance of immune based assays include BCG vaccination, exposure to non-tuberculosis mycobacteria, or HIV co-infection. It has been stated that a good immunological test must distinguish between the various states of TB and other mycobacterial exposures, while retaining sensitivity and specificity in patients co-infected with HIV (Pai *et al.,* 2006). Schleicher *et al*. (2002) investigated the diagnostic potential of an ELISA, based on the detection of antibodies to *Mycobacterium tuberculosis* mycolic acids in a South African population with a high prevalence of both TB and HIV. They 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.

A previous study found the IAsys affinity biosensor a better technique for the detection of anti-mycolic acid antibodies in patient serum as surrogate marker of active TB. The test is called the MARTI-test, short for Mycolic Acid Real-Time Inhibition-test. It registered false positives mainly in the HIV pos TB neg population, of which TB was excluded merely on the basis of best clinical assessment and a negative TB culture from sputum. It is well established that these diagnostic techniques underestimate TB positiveness in the HIV-pos population due to the effect of HIV on the quality of the sputum sample and the suppression of typical TB symptoms by the altered immunological state of the patient (Mwandumba *et al*., 2008; Albay *et al*., 2003; Hornum *et al*., 2008; Manosuthi *et al*., 2006). The false positive results obtained with the MARTI-test could therefore actually be true positives, since there was no adequate standard to confirm the TB status of the HIV-infected patients unequivocally. However, the waveguide technology (IAsys biosensor) is now out-

dated and has largely been replaced by surface plasmon resonance (SPR) based devices (Cush *et al*., 1993).

The principle of the SPR biosensor is based on the change in the refractive index on a thin gold film surface modified with various materials (Lee *et al*., 2005). The ESPRIT biosensor that uses the SPR technology will be used in this study to detect antibodies to mycolic acid in human patient sera. Both IAsys and ESPRIT biosensors use a cuvette system and they rely upon a phenomenon called the evanescent field to monitor changes in refractive index occurring within a few hundred nanometers of the sensor surface. The light is totally internally reflected from the sensing surface by means of a prism in both biosensors. The operation of the IAsys is based on the optical properties of the films with high refractive index deposited on a glass surface as compared to the ESPRIT that uses a gold surface. The advantage of ESPRIT biosensor is its auto-pippetting of samples into the cuvette as compare to the manual pipetting on IAsys. The SPR biosensor will be used in the current study, to show if the MARTI-assay can be even better applied for the diagnosis or progression of tuberculosis or as a criterion to determine whether the patient should end or change the anti-TB chemotherapy, eg when drug resistance becomes evident. In order to investigate this, positive pulmonary tuberculosis patient serum samples under treatment that were collected from University of Stellenbosch will be used to determine the immune memory of antibodies to mycolic acids in TB patients and also to monitor the progression of the disease during TB chemotherapy. This programme was funded by European and Development Countries Trials and Partnership (EDCTP) to search for surrogate biomarkers for chemotherapeutic cure of tuberculosis in order to shorten drug trials and treatment, since there are currently no such markers. The MARTI-assay on ESPRIT biosensor will also give an indication as to whether the antibody to mycolic acids production are of long or short immune memory once the infectious agent has been therapeutically cleared after chemotherapy. The EDCTP serum samples will be analyzed blinded on the MARTI-assay and the patient data will then be released only after submission of the results to the project coordinator.

3.1.1 Immune memory in TB

Drowart *et al*. (1991) indicated that many studies are focusing on the design of early serodiagnosis of tuberculosis or other mycobacterial diseases. In their studies, they showed the detection of antibody level to whole culture filtrate and purified P32 antigens during ant-TB chemotherapy. The mechanisms involved in TB persistence during therapy are not well understood, as there are no satisfactory models to study this phenomenon *in vitro*. However, it is generally believed that most actively replicating bacilli are killed early in therapy and that prolonged treatment is required to eradicate persisting *M. tuberculosis* exhibiting reduced or altered metabolism (Wallis *et al*., 1998). Many studies have shown that the IgG antibody levels against mycobacterial antigens in TB patients' sera varied greatly depending on the stages of the disease after initiation of the anti-TB chemotherapy (Drowart *et al*., 1991; Sousa *et al*., 2000; Fujita *et al*., 2005a). The notion that anti-mycolic acid antibodies may be used as surrogate markers for active tuberculosis was first claimed as a preferred embodiment in a patent application by our group (Verschoor *et al*., 2005). This was corroborated in a report by Fujita *et al*. (2005a) who showed that IgG antibodies to mycobacterial lipid antigens are of short immune memory in active TB. Fujita *et al*. (2005a) showed that the levels of anti-TDM (trehalose 6,6'-dimycolate) antibodies either decreased immediately, or were first elevated for a few weeks and then decreased sharply towards to the normal healthy control level after 3-4 months of anti-TB therapy when the elimination of bacilli was complete. Thus, the serodiagnostic assay based on anti-TDM antibodies could be useful for monitoring the progression of the disease as a criterion to determine whether the patient may end or should change the anti-TB chemotherapy (Fujita *et al*. 2005a).

Culture and Acid-fast bacilli (AFB) detection by smear microscopy can also be used to monitor the effectiveness of treatment and can help to determine when a patient is less likely to be infectious, despite their limitations (Palomino *et al*., 2007). Culture assay is very sensitive, however due to the slow growth of the bacteria; this method usually requires 4 to 8 weeks for completion (Samanich *et al*., 2000). This often results in delayed diagnosis, adversely affecting patient care and TB control and allows for the spread of infection (Reischl, 1996). AFB 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). It may give false negative results, especially in

children and HIV positive patients, because it requires a high degree of bacillary load of 10^3 bacilli/ml of sputum (Mitarai *et al*., 2001). This shows that there is a need to develop a fast assay that can easily diagnose TB while patients are on chemotherapy.

The study that was performed by Simonney *et al*. (2007) showed that the clearance of anti-PGL-Tb1 IgG antibody from humans is a lengthy process in HIV-positive patients co-infected with TB, after successful treatment of the TB. They found that significant levels of anti-PGL-Tb1 antibody levels remained prevalent in patients with inactive TB for a lengthy period of up to 18 months after stopping TB treatment. They concluded that the decline in the circulating free anti PGL-Tb1 antibody levels cannot be used as a short-term surrogate marker for TB to determine anti-TB treatment success in HIV-positive patients.

The World Health Organization recommended that TB diagnostic tools for general use should have a sensitivity of over 80% and specificity of over 95% (WHO, 1997). The MARTI-assay on the IAsys biosensor gave 76.9% and 86.7% specificity and sensitivity respectively for the detection of anti-mycolic acids antibody in TB patients, when TB^-HIV^+ were excluded since a sputum culture was used as a gold standard assay for TB diagnosis (Chapter 2). We hope to improve the sensitivity of the assay by analyzing serum on the ESPRIT technology. There was no guarantee on the MARTI-assay on IAsys biosensor that the sample could be analyzed in a single day due to the deviation of the two channels of the IAsys cuvette. One had to repeat until one became lucky. This could have been due to cuvette manufacture, difficulty to effect a comparable mycolic acid liposomes coat or differences bought about manual addition of samples into the cuvette system. The current study of the MARTI-assay on the ESPRIT biosensor focussed on the development of alternative coating approaches and optimization of all subsequent steps to achieve the same or better results with ESPRIT than we obtained before on IAsys biosensor. A secondary benefit with the EDCTP samples arose from the way they were collected. This allowed one to also determine the duration of antibody immune memory to mycolic acid antigen that could maybe indicate the feasibility of applying MARTI-assay for monitoring TB prognosis during treatment.

3.1.2 Principle of Surface Plasmon Resonance

Surface plasmon resonance (SPR) is a physical process, which happens when light hits a metal under a special angle position during total internal reflection conditions. SPR signal/wave is related to the refractive index close to the sensor surface and is therefore related to the amount of macromolecules bound to the sensor surface. SPR is created by a consistent longitudinal charge fluctuation at the surface of a metal that have their induced magnetic field intensity maximum on the surface, from where it decays exponentially in a perpendicular direction. The literature contains numerous examples of novel SPR biosensor designs that improve upon the traditional and popular prism-coupled SPR, called the Kretchmann's configuration (Hoa *et al*., 2007) (Fig. 3.1). Currently, much of the development of SPR is directed towards providing an integrated, low cost and sensitive biosensor with reusable SPR sensor surfaces (Hoa *et al*., 2007).

An SPR immunosensor is comprised of several important components such as a light source, detector, prism with transducer surface (usually a gold film on which, biomolecules such as antibody or antigen is immobilized) and flow system (Shankaran *et al.*, 2007). The transduction surface is usually a gold film (50 – 100 nm) on a glass slide optically coupled to the glass prism through refractive index matching oil. Besides gold, other metals can also be used, such as silver, copper and aluminium. However, gold is preferred due to its chemical stability and free electron behaviour. Plane polarized light is directed through a glass prism to the gold over a wide range of incident angles and the intensity of the resulting reflected light is measured against the incident light angle with a detector. At certain incident light wavelength and angles, a minimum in the reflectivity is observed at which the energy of the light waves can be absorbed by the gold in order to activate electrons for oscillation of surface plasmons at the gold interface. The angle at which the minimum in reflectivity occurs is denoted as an SPR angle (Fig. 3.1). This critical angle is very sensitive to the dielectric properties of the medium adjacent to the transducer surface apart from its dependence on the wavelength and polarization state of the incident light. In particular, the resonance condition is extremely sensitive to the refractive index of the sample in contact with the metal surface to within a depth of ~200 nm; because the optical induced electric fields are localized to within ~ 250 nm from the gold surface (Shankaran *et al*., 2007).

The resonance conditions are influenced by the biomolecules immobilized on the gold layer. When the molecules interact, the change in the interfacial refractive index can be detected as a shift in the resonance angle. These changes are monitored over time and converted into a sensorgram, from which the kinetics and affinity constants of the interaction can be determined.

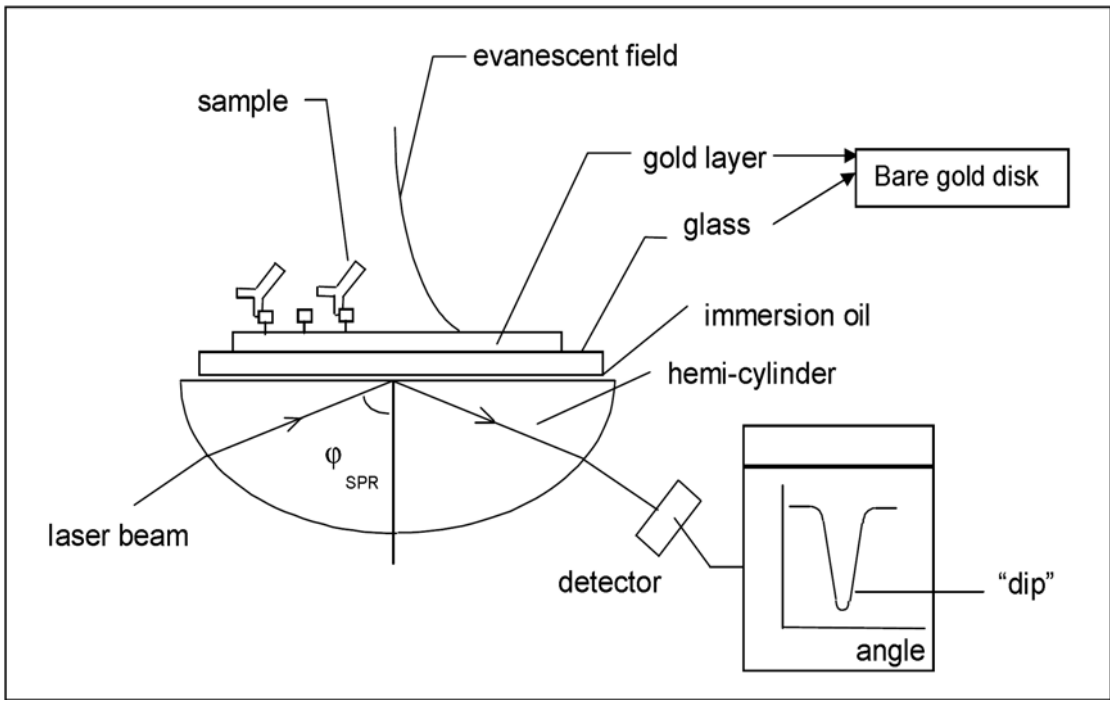


Figure 3.1: The Kretschmann configuration of a surface plasmon resonance biosensor (Eco Chemie B.V., Autolab ESPRIT manual).

3.1.3 The Autolab ESPRIT biosensor based on SPR

The Autolab ESPRIT is an optical biosensor that detects real-time binding events on a solid phase by means of surface plasmon resonance induced by a laser source with an adjustable light path. It is a modular-set up that enables easy access to all the important components and allows flexibity in the design of experiments (Fig. 3.1 and Fig. 3.2). Interaction plots will show binding curves of macromolecule interactions and baseline shifts due to changes in refractive indices of sample solutions. SPR occurs under certain conditions when a thin film of metal (gold or silver) is placed inside the laser beam. When the incoming light is monochromatic and p-polarized (i.e. the electric vector component is parallel to the plane of incidence), the free electrons

of the metal will oscillate and absorb energy at a certain angle of incident light (Fig. 3.2). The angle of incidence at which SPR occurs is called the SPR angle. SPR is detected by measurement of the intensity of the reflected light. At the SPR angle a sharp decrease or dip intensity is measured. The position of the SPR angle depends on the refractive index in the substance with a low-refractive index, i.e. the sensing surface. The refractive index of the sensor surface changes upon binding of macromolecules to the surface. As a result, the SPR wave will change and therefore the angle will change proportionally to the amount of bound macromolecules. There is a linear relationship between the amount of bound material and shift in SPR angle. The SPR angle shift in millidegrees is used as a response unit to quantify the binding of macromolecules to the sensor surface. The response also depends on the refractive index of the bulk solution (Eco Chemie B.V., ESPRIT biosensor manual).

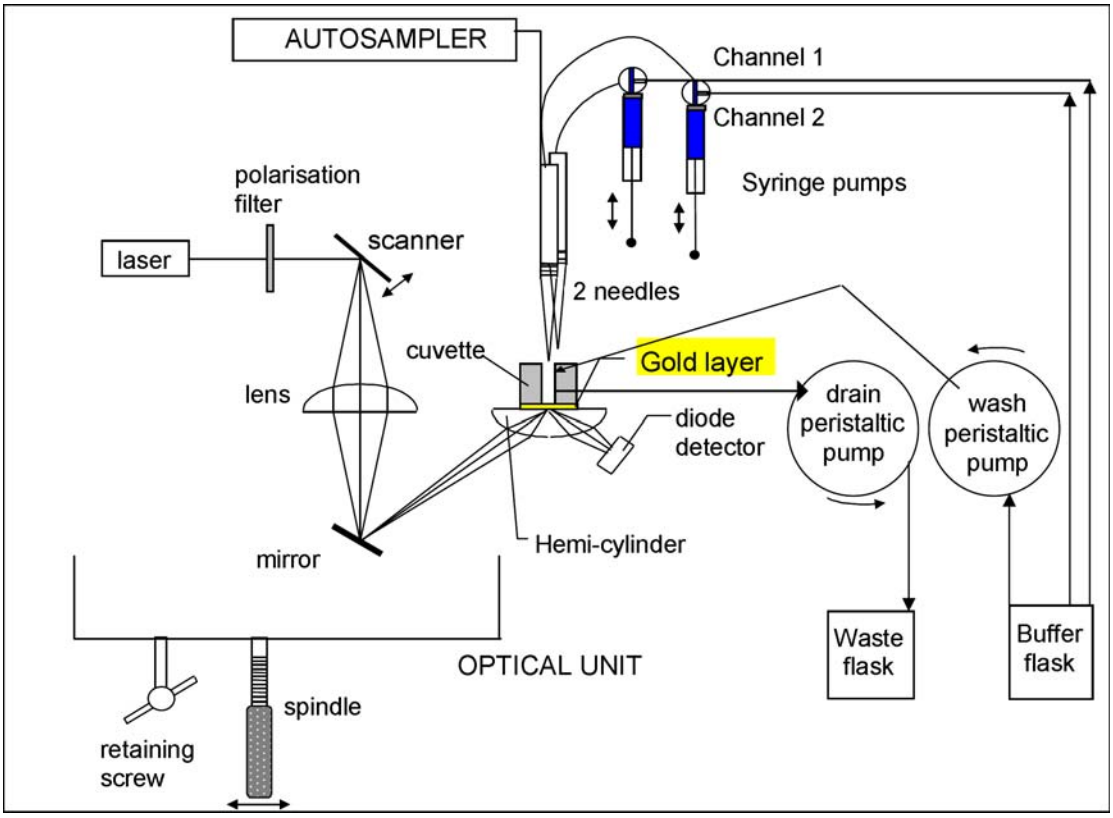


Figure 3.2: Schematic picture of the ESPR configuration (Eco Chemie B.V., ESPRIT biosensor manual).

ESPRIT measurements can be performed using different sensor surfaces, of which the most general are the many options of modified gold layers. Desirable features of the sensor surface for the study of macromolecule interactions are; rapid, simple and

reproducible immobilization technique; stability and retained biological activity of the immobilized biomolecules and low non-specific interaction. A modified gold layer disk can be bought, but also made with help of an Autolab spincoater.

3.1.4 Immobilization of biomolecules onto the Au surface of ESPRIT sensor disks

The present level of research on new biosensors as well as the development of currently available biosensors has increased dramatically over the past decade. There has been considerable progress in the development of new methods of immobilizing biological recognition elements onto transducer sensor surfaces (Zhang *et al.*, 2000), a key step in the development of biosensors. The immobilization methods that are mostly used include physical adsorption, cross-linking between molecules, covalent binding to the surface, entrapment within a membrane, surfactant matrix, polymer or microcapsule and self-assembly membranes (Rodriguez-Mozaz *et al*., 2004). The sensitivity of the biosensor is highly dependent on the surface preparation (Pejcic *et al*., 2006). The use of self-assembled mono- and multi-layers (SAMs) is increasing rapidly in various fields of research, and this applies especially to the construction of biosensors (Zhang *et al*., 2000; Zhang *et al.*, 2008). The main driving force for this enhanced research activity is the booming demand for miniaturized biosensors, particularly for diagnostic applications (Chaki and Vijayamohanan, 2002). SAM offer several attractive features for these kinds of application due to various reasons. More important, the uncomplicated procedure for SAM formation and compatibility with metal substrates such as gold for electrochemical measurements enable special benefits for biosensor applications involving current or potential measurements. The term self-assembly, involves the arrangement of atoms and molecules into an ordered stable form or even aggregate of functional entities without the intervention of a human hand (Tecilla *et al*., 1990). For example, the highly ordered and dense nature of the long chain alkane thiols of SAMs mimic the cellular microenvironment of lipid bilayer structures, thereby providing novel substrates for immobilized biomolecules (Fig. 3.3).

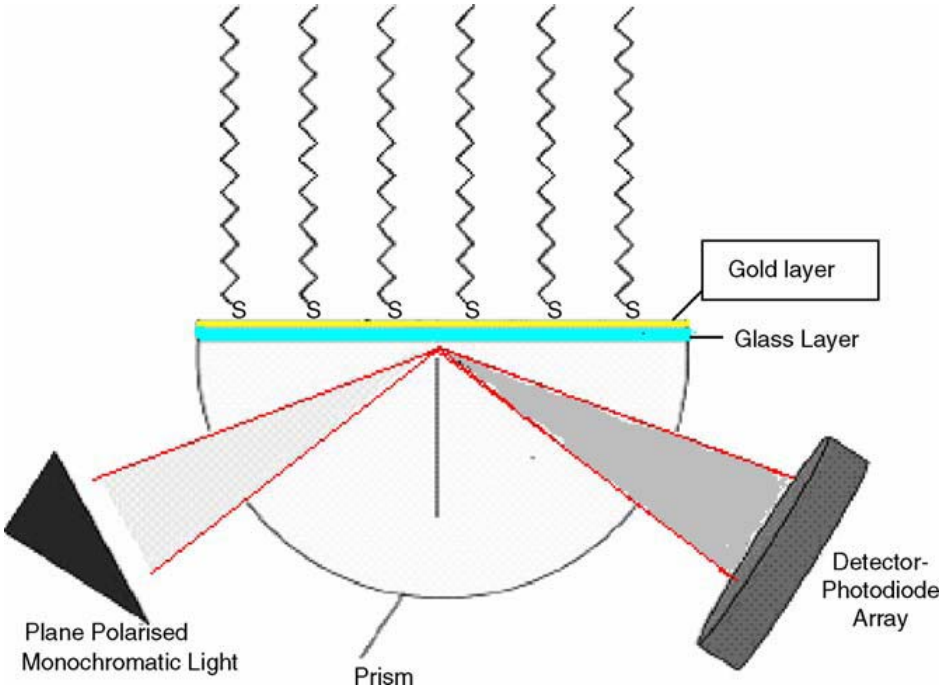


Figure 3.3: A schematic presentation of a hydrophobic SPR surface where a gold disk is coated with ODT (Arya *et al*., 2006).

The molecular self-assembly of long chain alkanethiol on gold has drawn considerable attention during the past decade, since self-assembled monolayers (SAMs) have strong adhesion to a substrate, high degree of thermal and chemical stability and mechanical strength (Kim *et al*., 2001). The stability of the SAMs of the alkanethiol molecules formed on the gold depends on the strength of Au-S bond and the Van der Waals force between a thiol molecule and its surrounding molecules (Han *et al*., 2004). Many recent reports on the alkanethiol monolayer adsorbed on the gold surface have been focused on their structure and properties of X-ray diffraction and scanning tunnelling microscope measurements revealed that these organic films form a specific monolayer structure on gold surfaces. SAMs can be used as interface layers upon which almost all types of biological components, including proteins, enzymes, antibodies and their receptors can be loaded (Zhang *et al*., 2000). The current study involves the preparation of octadecanethiol in absolute ethanol to form a SAM that was characterized using cyclic voltammetry and applied for the measurement of binding, or inhibition of binding of patient serum antibodies to mycolic acids that were immobilized as liposomes onto the alkanethiol coated ESPRIT biosensor surface.

3.2 Aims

To transfer the MARTI-test for TB serodiagnosis from IAsys to ESPRIT biosensor technology by

- Coating the ESPRIT gold disc with octadecanethiol and characterize the formation of the self assembled monolayer with cyclic voltammetry.
- Determining if mycolic acid liposomes can be immobilized on the octadecanethiol coated gold disc
- Determining the inhibition of binding of antibodies to mycolic acid on the immobilized mycolic acid liposomes.
- Regenerating the gold disc.

To determine the reproducibility of the MARTI-assay on ESPRIT by using a TB negative control serum from Schleicher *et al*. (2002) with a TB positive control on the ESPRIT biosensor from serum samples collected from HIV positive patients who were clinically assessed to confirm their TB co-infection status at Pretoria Academic hospital by Prof. A.C. Stoltz (Foundation for Professional Development, Pretoria).

To determine the immune memory of antibodies to mycolic acids in TB patients and also to monitor the progression of the disease during TB chemotherapy of the serum samples from a subcontract of a European and Developing Countries Clinical Trials Partnership (EDCTP) project with Prof Paul van Helden (University of Stellenbosch).

3.3 Materials and Methods

3.3.1 Materials

3.3.1.1 ESPRIT biosensor

The Autolab ESPRIT instrument was obtained from Eco Chemie B.V. (Utrecht, The Netherlands) and the gold discs from Metrohm (Gauteng, SA).

3.3.1.2 Cyclic voltammetry

Cyclic voltammetry (CV) experiments were carried out using an Autolab potentiostat PGSTAT 30 from Eco Chemie (Utrecht, The Netherlands) driven by the General Purpose Electrochemical Systems data processing software (GPES, software version 4.9).

3.3.1.3 Reagents

Sodium dodecylsulphate (SDS) and absolute ethanol (analytical grade) were obtained from Merck (Gauteng, SA). Octadecanethiol, ferricyanide [$K_3Fe(CN)_6$], ferrocyanide [$K_4Fe(CN)_6$], potassium chloride (KCl) and urea, all analytical grade, were obtained from Sigma-Aldrich (St. Louis, USA). Acetic acid (analytical grade), sodium bicarbonate ($NaHCO_3$), isopropanol (chemically pure), sodium hydroxide (NaOH) were obtained from Saarchem (Gauteng, SA).

3.3.2 Methods

3.3.2.1 Preparations of solutions

Octadecanethiol (10 mM) was dissolved in absolute ethanol using a water bath sonifier (Ultrasonic cleaner, Optima Scientific CC, Model: DC150H) for 30 minutes. Sodium bicarbonate (0.2 M), SDS (0.5%), sodium hydroxide (50 mM), 1 mM ferrocyaninde/ferricyanide, 1 M potassium chloride and urea (6 M) were prepared in sterile double distilled water.

3.3.2.2 Preparation of serum from HIV positive patient

Dr. A.C. Stoltz, from Foundation for Professional Development, Pretoria, collaborates on this project and was responsible for collecting serum samples from HIV patients who are screened for TB before commencement of ARV treatment. He goes to extremes of clinical assessment and pathology to determine tuberculosis in these patients as explained below. The blood collected from HIV positive patients by Dr Anton Stoltz at Pretoria Academic Hospital was delivered fresh to the laboratory for serum preparation. Blood was withdrawn from patients in sterile Vacutainer tubes (with brown lids, Aquila Health Care, Pinegowrie, SA). Some of the patients were TB positive and others TB negative, but they were all HIV positive. Samples were stored at 15°C before they were processed. After the blood clotted (2 – 4 hours after sampling), serum was removed from the blood clot with plastic pipettes to 1.5 ml Eppendorf tubes. The serum samples were then centrifuged in a microfuge (362 g, 5 minutes, 4 °C). This was done to remove any red blood cells that were still in the serum. The serum samples were then aliquoted in 500 µl portions into 1.8 ml cryo tubes (NUNC^{TM} Brand products, Nunc international, Denmark) and stored at – 70 °C. These samples were thawed and then γ-irradiated (30 Gy for 5 minutes on each side of the box, Pretoria Academic Hospital) as an additional safety precaution (Vermaak, 2004).

3.3.2.3 Preparation of liposomes with Branson and Virsonic sonicators

The mycolic acids and phosphatidylcholine liposomes were prepared as described in chapter 2. The liposomes were prepared using either a Branson (Mobel B-30, USA) sonicator as described before in chapter 2 or a Virsonic 600 sonicator (United scientific, USA). The liposomes were sonicated for 4 minutes at a maximum output of 10 with the Virsonic 600 sonicator after addition of 2 ml saline (0.9%). The liposomes (200 µl) were aliquoted into 10 tubes and kept at –70 ^{0}C for an hour 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 above on the Virsonic 600, before they were used on the ESPRIT biosensor.

3.3.2.4 Serum samples

Two series of patient sera samples from a collection made at University of Stellenbosch for the purpose of a European and Developing Country Clinical Trials Programme (EDCTP) research contract were taken at diagnosis before initiation of TB treatment and at different weeks after start of anti-TB drug treatment. The patient serum samples received in our study were excluded from study done at Stellenbonsch University because some of them were MDR TB during treatment, HIV positive, infected with NTM, had any disease or medication known to affect the immune system, had previous TB or had a lung condition, similar to TB, or became lost to follow-up. In our laboratory, serum samples were first γ-irradiated to prevent viral or bacterial infection (as described in 3.3.2.2). The EDCTP serum samples were irradiated, but special safety precautions rules were followed to avoid any hazard. The serum samples were stored at – 70 °C until use. Some of the Schleicher *et al.* (2002) patient sera were used as control in this study. The details of the Schleicher *et al.* (2002) patient sera were discussed in chapter 2.

3.3.2.5 Coating of a SPR gold disc with octadecanethiol

The gold disc was first rinsed with absolute ethanol before it was immersed in 10 mM octadecanethiol dissolved in absolute ethanol for 16 hours at room temperature. The gold disc was then washed with absolute ethanol and PBS/AE. The disc was then inserted into the biosensor on a droplet of special refractive index oil, after wiping the glass bottom surface with lens tissue. The PBS/AE, as prepared in chapter 2, was filtered through a 0.2 μm particle retention membrane and degassed with helium for 30 minutes before they were used.

3.3.2.6 Cyclic voltammetry measurements

The gold disc coated with ODT was analysed with cyclic voltammetry to confirm if there was a formation of a self assembled monolayer (SAM). The coated disc was immersed in a solution of 1 mM ferrocyaninde/ferricyanide containing 1 M potassium chloride at a scan rate of 25 mV/s and 50 mV/s at a potential window of -0.1 to 0.5v.

3.3.2.7 Immobilization of mycolic acids on ESPRIT gold disc

After the formation of the octadecanethiol SAM, the coated gold disc was then inserted in the instrument. An automated programme sequence was created to control the addition of all the samples and liquids into the cuvette, as it was done manually on the IAsys biosensor, described in chapter 2. Quality of the surfaces were monitored by determining the SPR dips (Appendix D) after cleaning the Au ODT coated surface with 96% ethanol and a mixture of isopropanol and 50 mM sodium hydroxide (2:3, v/v). The samples were aspirated by the needles from a 384 multi-well plate (Bibby Sterilin Ltd, Stone, UK) to the cuvette surface. First the baseline of the ESPRIT biosensor was set with 10 µl PBS/AE, followed by addition of 50 µl MA liposomes on the disc for 20 minutes. The immobilized liposomes were then finally washed five times with 100 µl PBS/AE, substituted with 50 µl of saponin (0.25 – 0.5 mg/ml) and incubated for eight minutes. This latter step was to avoid non-specific binding on the surface of the cuvette during the subsequent binding events. The cells were then washed five times with 100 µl PBS/AE, the content of each cell substituted with 50 µl of PBS/AE and left 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 1/500 dilution of serum sample (10 µl) in PBS/AE was added in each cell, to compare the responses of the two cells over ten minutes. A pre-incubation of 1/250 dilutions of serum with solutions of liposomes containing mycolic acids and empty liposomes (phosphatidylcholine alone) were allowed for 20 minutes at room temperature. These were then added (10 µl) for binding inhibition studies in different cells, one with mycolic acids liposomes and the other with empty liposomes as a control. Finally, dissociation of antibodies was effected with 5 times PBS/AE washing and measurement of the response for 5 minutes. A full-automated sequence was created to control the addition of all the samples into the cuvette. In this study, only the incubation of the gold disc for ODT coating was done outside the instrument, all the other steps were performed in situ and with the built-in autodispenser of the ESPRIT biosensor (Appendix C).

3.3.2.8 Regeneration of ESPRIT gold disc

After dissociation of the unbound serum antibodies from mycolic acids (3.3.2.7), the surface was regenerated with 100 µl mixture of isopropanol and 50 mM NaOH (2:3,

v/v) for 2 minutes and finally washed with 100 µl of 99% absolute ethanol. The surface was washed 5 times with 100 µl of PBS/AE after each regeneration step to prepare it for a next round of liposome coating on the stable ODT layer (Appendix D).

3.3.2.9 Cleaning of cuvette and needles

A flow wash sequence (Appendix E) was used to clean the needles, after analyzing approximately 30 sample runs, in a sequential way with 0.5% sodium dodecylsulphate (SDS), 6 M urea, 1% acetic acid, 0.2 M sodium bicarbonate ($NaHCO_3$) and ddd H_2O in order to improve the SPR dips taken during measurements.

3.3.2.10 Statistical analysis

A student's t-test, two tailed, assuming unequal variance was used for statistical comparison of the results on the ESPRIT biosensor to determine if the MARTI-assay can show a significant difference between TB positive and negative patient sera.

3.4 Results

The main aim of this study was to transfer the MARTI-test for TB diagnosis from IAsys wave guide technology to the ESPRIT SPR biosensor. Several aspects required attention in order to achieve this, of which the different sensor surfaces provided the first challenge.

3.4.1 Preparation of MA-liposome coated ESPRIT gold discs

The waveguide IAsys cuvette provided a hafnium oxide surface of which the properties approximated glass that could be made hydrophobic by a treatment with cationic detergent (cetyl pyridinium chloride, CPC). The underivatised Au disc surface of the ESPRIT biosensor was first treated with CPC to demonstrate that it could not activate the gold surface for liposome binding. Subsequently, standard procedure was followed to activate the gold layer to a hydrophobic, liposome binding surface using octadecanethiol to effect a covalent binding of a layer of octadecane to the gold.

The coating of the gold disc could not be performed in real-time, since the octadecanethiol was dissolved in absolute ethanol that generates too large jumps in the sensor signals when alternated with PBS/AE due to the large differences in refractive index. The underivatised Au disc was then incubated for 16 hours at room temperature in 10 mM octadecanethiol. The formation of the SAM or coverage of the gold surface with octadecanethiol after 16 hours incubation of the gold disc was investigated with cyclic voltammetry to determine the efficiency of the coating. This was not done routinely for every gold disc that was prepared. The results in Fig. 3.4 show that there was no redox peak current observed of the ODT coated disc, in comparison to the uncoated gold disc. The significant drop in background current is assumed to be due to the formation of a stable self assembled monolayer (SAM) of octadecanethiol, formed by covalent S-Au bonds on the surface of the gold disc. The stability of the SAM was subsequently tested by exposure to regeneration solutions, absolute ethanol and a mixture of 50 mM NaOH with isopropanol (2:3, v/v). Fig. 3.4 indicated that full coverage of the surface with the alkane thiol was effected only after 16 hours of incubation. The SAM was maintained after the exposure of the coated surface in regeneration solutions (Fig. 3.4). The ODT coated disc was then inserted

into the ESPRIT biosensor to monitor the binding of mycolic acids liposomes (Fig. 3.5). The difficulty in obtaining an initial SPR dip using PBS/AE was resolved by flushing the cuvette with 500 µl ethanol (96%) using the automatic dispenser with simultaneous draining, followed by brief (~ 60 s) flow-washing with PBS/AE. If the SPR dip goes above 10%, air bubbles are present in most cases. The SPR dips verify the quality of the sensor disk, how the disk matches with the hemi-cylinder and whether the optical path is clean or not.

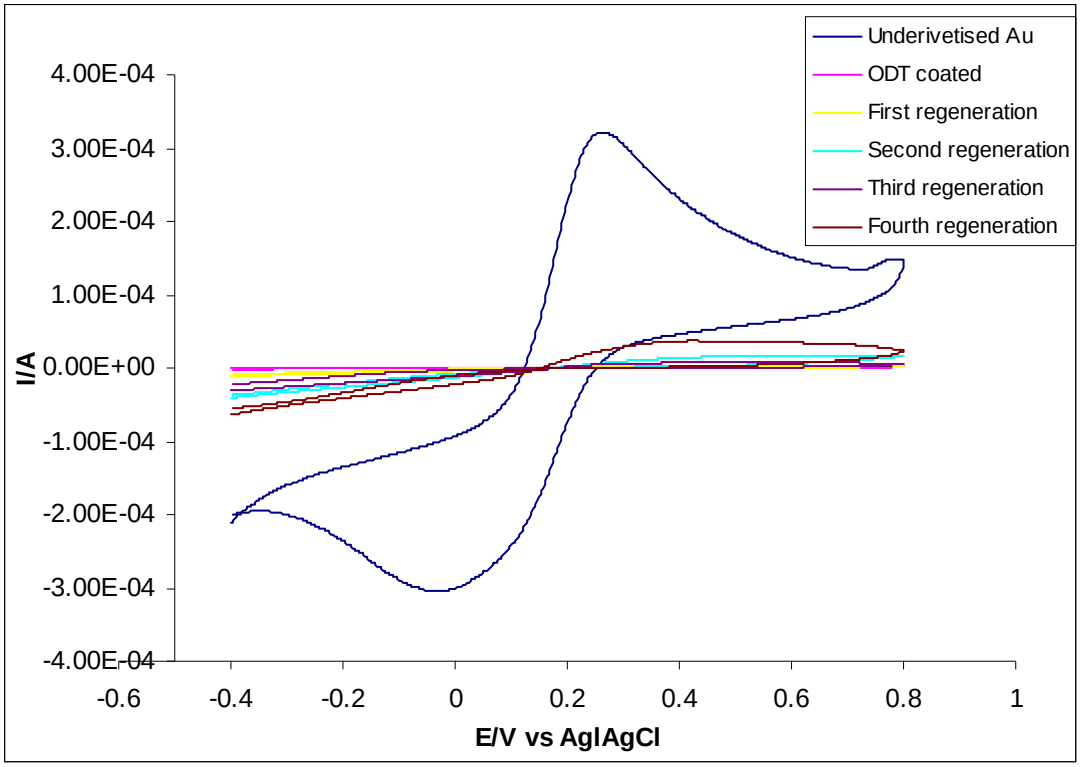


Figure 3.4: Testing of the octadecanethiol coated ESPRIT biosensor gold surface against sequential times of regeneration with a mixture of isopropanol and 50 mM NaOH (2/3, v/v) using cyclic voltammetry.

The results (Fig. 3.4) showed that a stable formation of octadecanethiol self-assembled monolayer on the gold disc occurred. A response signal of about 250 millidegree was obtained after immobilization of mycolic acids liposomes onto the octadecanethiol-coated gold disc (Fig. 3.5). The washing of the unbound mycolic acids liposomes from the octadecanethiol did not significantly alter the binding signal. This shows that the mycolic acids liposomes were adequately bound to the octadecanethiol surface. The surface plasmon resonance on the ESPRIT measures

angle versus time, and there is a linear relationship between the amount of bound molecules on the gold surface coated with octadecanethiol and shift in SPR angle. Upon binding, the SPR dip will shift to the right as shown in Fig. 3.5.

The regeneration of the gold disc was effective with a mixture of 50 mM sodium hydroxide and isopropanol (2:3, v/v) followed by 99% ethanol. The results obtained in this study indicate that the mycolic acid liposomes could be immobilized several times on the same octadecanethiol-coated surface after regeneration (Fig. 3.4). The binding of liposomes was not affected by up to four regeneration steps, since the same cyclic voltammetry profile was obtained after recoating with MA-liposomes and the same binding response (± 250 milli-degrees, Fig 3.5 and 3.6) of mycolic acids liposomes to the octadecanethiol-coated surface was obtained with the biosensor on the same spot of a disc.

3.4.2 Detection of anti-MA antibodies in TB negative and TB positive sera

A TB negative patient serum selected from Schleicher *et al*. (2002) and a confirmed TB positive sample from the 2006 collection of Prof. A.C. Stoltz were used to determine the reproducibility of the ESPRIT biosensor assay. The experiments were repeated ten times and the average and standard deviation values for the percentage inhibition of binding for TB negative were 10±12, and for TB positive, 64±18.5 (Fig. 3.5 and Fig. 3.6). There was a significant difference (P value < 0.05) between the TB positive and the TB negative sera (Fig. 3.7).

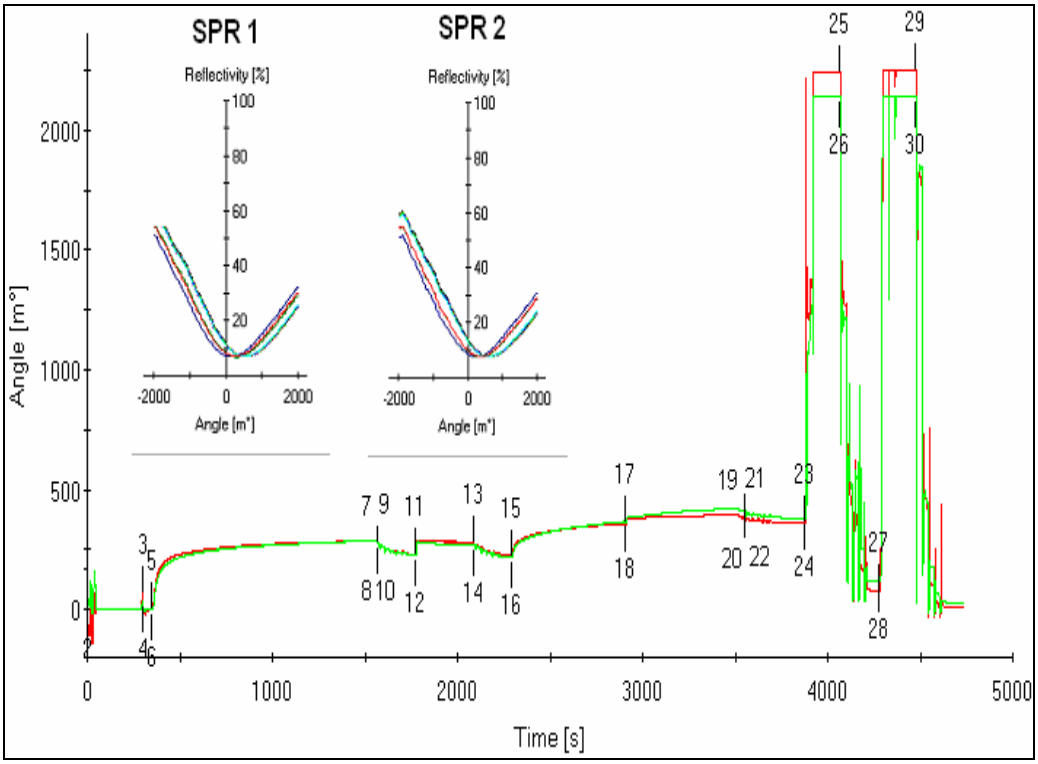


Figure 3.5: A representative ESPRIT sensorgram showing the full sequence of events to measure the inhibition of binding of human TB pos patient serum ($HIV^{+}TB^{+}$) antibodies to immobilized MA-liposomes on the ESPRIT biosensor. The event markers on the graphs are represented as follows: 3 – 4 baseline setting, 5 – 6 mycolic acids immobilization, 11 – 12 saponin blocking, 15 – 16 first exposure of serum in PBS/AE (1:500), 17 – 18 second exposure to serum (1:250), pre-incubated with mycolic acids liposomes (red) and and empty liposomes (phosphatidylcholine only, green), 23 – 24 regeneration with mixture of isopropanol and 50 mM NaOH, and finally 27 – 28 ethanol (99%). All the steps were followed by PBS/AE wash.

The ESPRIT instrument allows a quality check of the coated surface at any time during the process by performing an SPR dip (Fig. 3.5, SPR 1 and SPR 2). The prepared mycolic acids containing liposomes always gave the expected symmetrical SPR dips when immobilized on the octadecanethiol coated gold disc, indicating that the addition of mycolic acids liposomes, saponin, inhibition studies and regeneration did not disturb the uniformity of the sensor surface. The SPR dip was checked frequently. If irregularities were observed an experiment was stopped and repeated after regeneration with 96% ethanol and a mixture of isopropanol and 50 mM sodium hydroxide. The regeneration procedure resulted in a decrease of the baseline to below zero after PBS/AE wash.

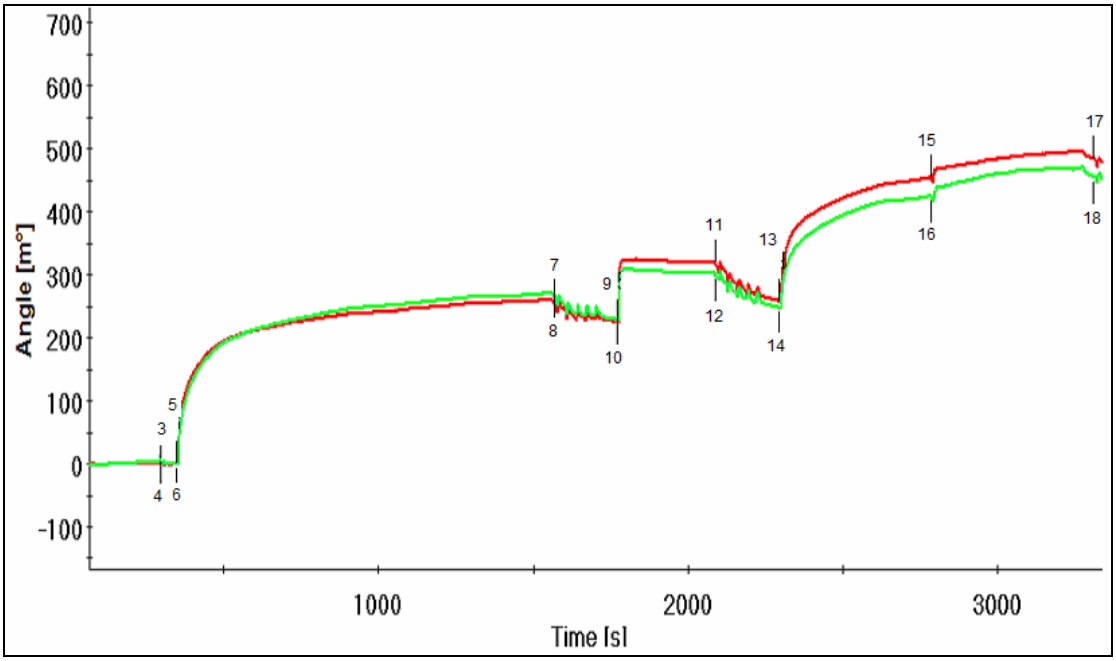


Figure 3.6: A representative ESPRIT sensorgram showing the full sequence of events to measure the inhibition of binding of human TB neg patient serum (HIV^-TB^-) antibodies to immobilized MA-liposomes on the ESPRIT biosensor. The event markers on the graphs are represented as follows: 3 – 4 baseline setting, 5 – 6 mycolic acids immobilization, 9 – 10 saponin blocking, 13 – 14 first exposure of serum in PBS/AE (1:500), 15 – 16 second exposure to serum (1:250), pre-incubated with mycolic acids liposomes (red) and empty liposomes (phosphatidylcholine only, green). All the steps were followed by PBS/AE wash.

This study shows that the MARTI-test can be performed on the Autolab ESPRIT biosensor, but with different coating strategies than was applied for the IAsys biosensor. A cause for concern is the relatively high deviation (standard error) of the results, approximating 30% of the value of the average of the TB positive sample.

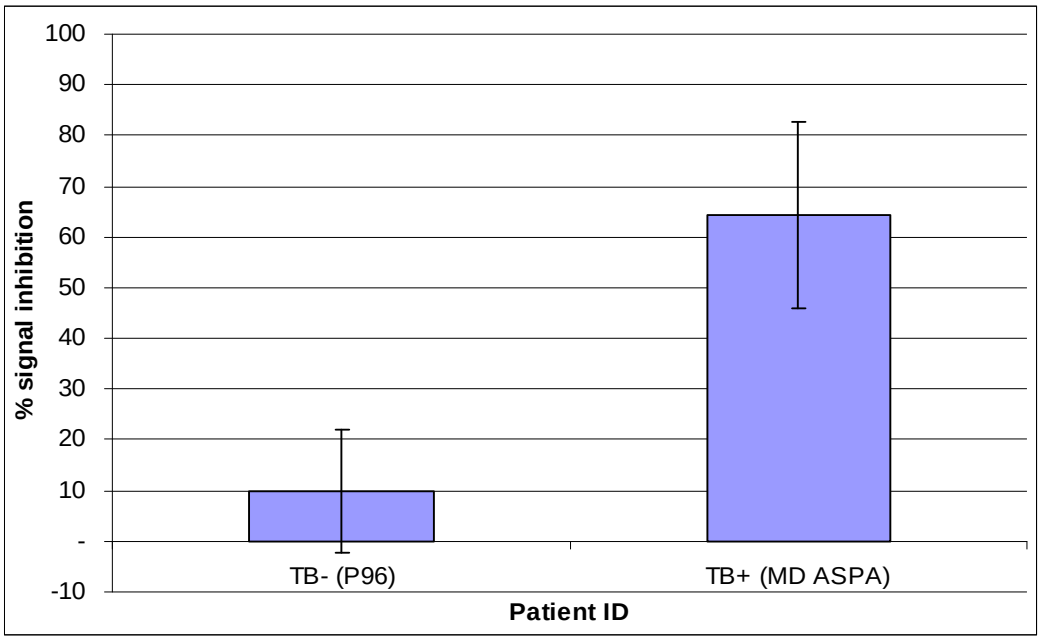


Figure 3.7: Inhibition of human serum antibody with mycolic acids in a TB negative (P96) and a TB positive (MD ASPA) patient serum on the ESPRIT biosensor, $p < 0.05$ ($n = 10$).

3.4.3 Detection of anti-MA antibody in TB patients during chemotherapy

In order to determine whether the immune memory of anti- mycolic acids antibodies is sufficiently short to allow monitoring of progress of tuberculosis patients during treatment, serial collections of sera were made from patients who were diagnosed with TB and then put on therapy. These sera were part of a bigger collection made for an international research programme under the management of Prof. Paul van Helden at University of Stellenbosch, to identify surrogate markers of TB in humans. The MARTI-test was applied to each of the serial samples of two patients using the ESPRIT biosensor. Fig. 3.8 shows the percentage inhibition signal of antibody to mycolic acid for each of the serum samples of the two patients that were taken before and after initiation of anti-TB chemotherapy. The first patient P5121 was diagnosed TB positive (an average percentage inhibition of > 20%) at week zero on the ESPRIT biosensor assay (Fig. 3.8A). The same criteria in describing the positive and negative status of a patient serum on the MARTI-assay on IAsys biosensor (chapter 2) was also used on ESPRIT biosensor. Some of the patient serum samples (week 2 and 6) were only done in duplicate, due to instrument failure during operation. There was no

significant change in the antibody signal response after initiation of anti-TB chemotherapy at week one as compared to week zero. After 6 months of receiving anti-TB chemotherapy, the patient remained TB positive and there was no significant decrease in response after 12 months. The second patient P3897 tested false negative for active TB on the ESPRIT biosensor before receiving anti-TB chemotherapy (Fig. 3.8B). After two weeks on TB treatment the patient tested TB positive. The anti-mycolic acids antibody gradually decreased during treatment and the patient appeared cured after 12 months. After analyzing the samples on the ESPRIT biosensor, the data were then submitted to University of Stellenbosch for assessment. Patient P5121 became multi-drug resistant (MDR), but survived and remained positive up to 12 months during treatment as determined by best clinical assessment and pathology. Patient P3897 was first determined TB positive in the clinic, but was cured due to the successful drug treatment. The clinical assessment matched the results of the MARTI-test very well for both patients (Fig. 3.8A – P5121 and Fig. 3.8B – P3897), but the concern on the standard error of the measurements remains with deviation of values still around 30% of the average of three measurements.

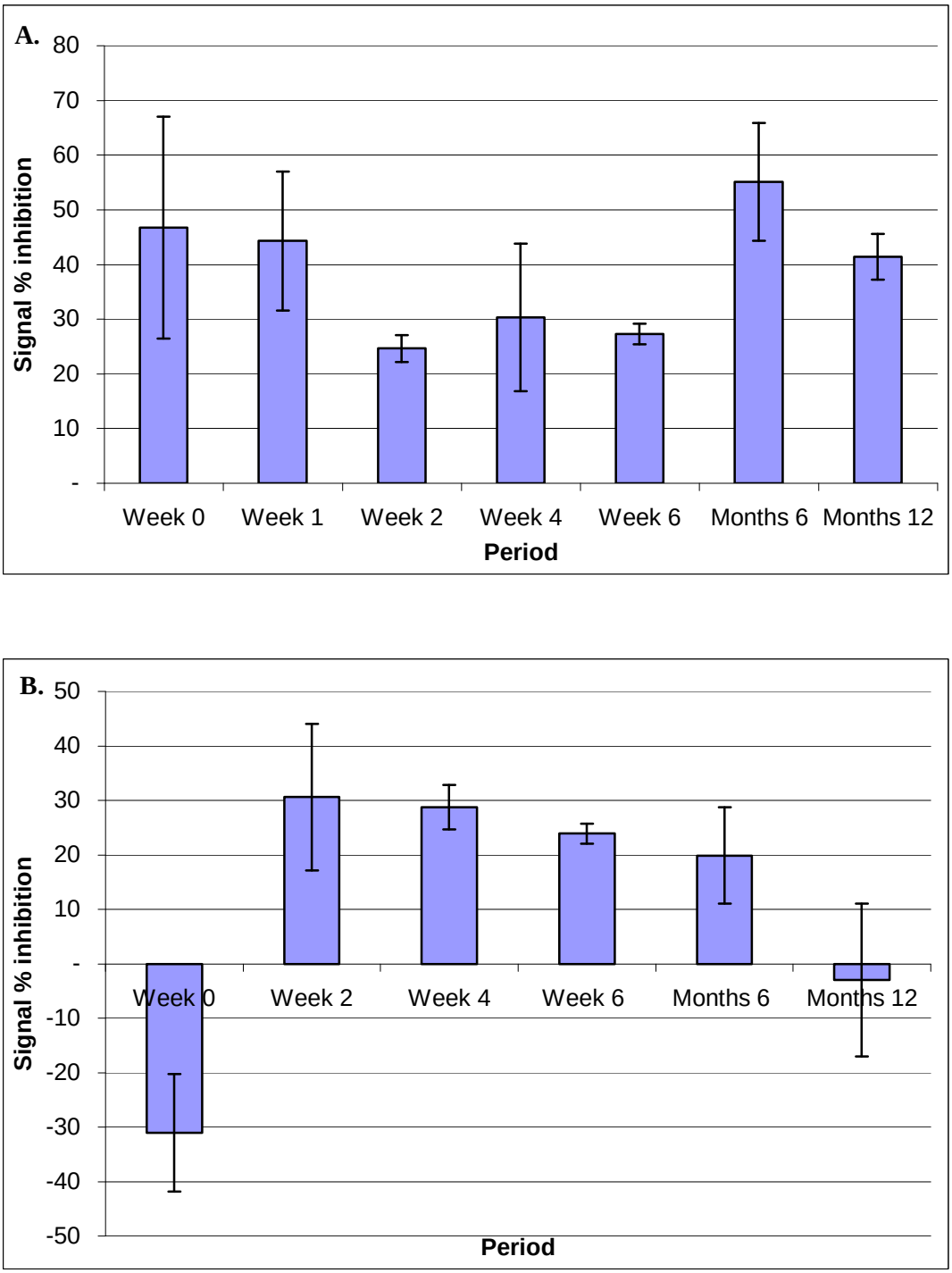


Figure 3.8: ESPRIT- MARTI test results of inhibition of human serum antibody binding to mycolic acids in TB patients (A = P5121 and B = P3897) before and during anti-TB chemotherapy on the ESPRIT biosensor ($n \geq 3$, except for P5121 in week 2 and 6, $n = 2$).

3.4.4 False negatives: ESPRIT compared to the validated IAsys biosensor

The false negative result that was obtained with patient P3897 at week 0 before commencement of therapy was a concern. Because the IAsys version of the MARTI-assay tested quite accurate (Chapter 2), there was an opportunity to determine whether the ESPRIT instrument or the way that technology was transferred to it was to blame for the result. The IAsys biosensor was therefore used to get a MARTI-result on the same sample. The patient that tested false negative on the ESPRIT biosensor also tested false negative on IAsys biosensor (Fig. 3.9), but both the error (value below zero inhibition) and the standard deviation was considerably bigger with the ESPRIT than with the IAsys biosensor.

It is concluded that the ESPRIT biosensor can be applied with the MARTI-test to come to more or less the same results as with the IAsys biosensor, but that it is of weaker reliability and accuracy compared to IAsys.

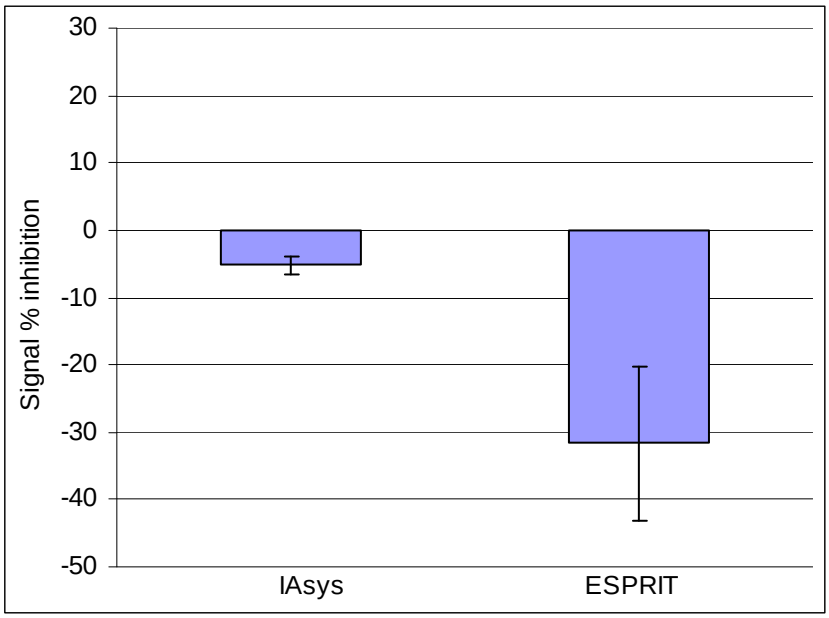


Figure 3.9: Comparison between IAsys and ESPRIT biosensor to determine the source of the false negative MARTI-test outcome of patient P3897 (week 0) on ESPRIT ($n = 3$).

3.4.4 Sources of error of the ESPRIT biosensor

The ESPRIT biosensor exhibited considerably more error in its MARTI measurement values than was obtained with the IAsys instrument. This may seriously affect the outcome of the validation of the MARTI-test on the ESPRIT biosensor and it can already be predicted that it will not achieve the required 80% accuracy to make it a serious consideration for the market. Two possible sources of error are inherent in the design differences between the two instruments: First, the ESPRIT biosensor does not have its optical path protected from dust, while the IAsys optical path is integrated into an enclosed space with glass covered windows as openings for the incident and reflected laser light. Second, the ESPRIT is equipped with an automated liquid dispenser fitted with two metal needles to serve each of the two cells in the cuvette. These can possibly accumulate dirt and lipid residues.

It can be seen from Fig. 3.10 that the presence of dust results in unsymmetrical, non-smooth SPR resonance dips during experimental measurements. The instrument was serviced when it became difficult to obtain smooth resonance dips. After service, clear symmetrical dips were maintained throughout the experimental procedure (Fig. 3.11). The two channels were also comparable during measurements throughout the experiment. The high standard deviation seen in some of the patient sera could be due to the dust sticking to the optics, thereby reducing the intensity of the laser light to monitor the interaction on the Au surface. This is evident from the following properties of the profile in Fig 3.10 that was obtained just before the instrument was serviced. The two channels were not comparable in Fig. 3.10 during measurements of mycolic acids immobilized on the Au-ODT coated surface. The red channel gave an unstable profile during PBS/AE wash. After saponin blocking in Fig. 3.10, the baseline (event markers 9 – 11, and 10 – 12) was not stable before first exposure of serum in PBS/AE. After dissociation of the unbound serum antibody, the signals remained unstable in both channels (Fig. 3.10, step 17 – 19 and 18 – 20). Different solutions that were used to clean the needles and tubing system were also effective to eliminate the accumulation of particles within the tubes and needles. The unwanted particles normally fell into the cuvette system during measurements, leading to loss of the SPR dips (> 10% reflectivity).

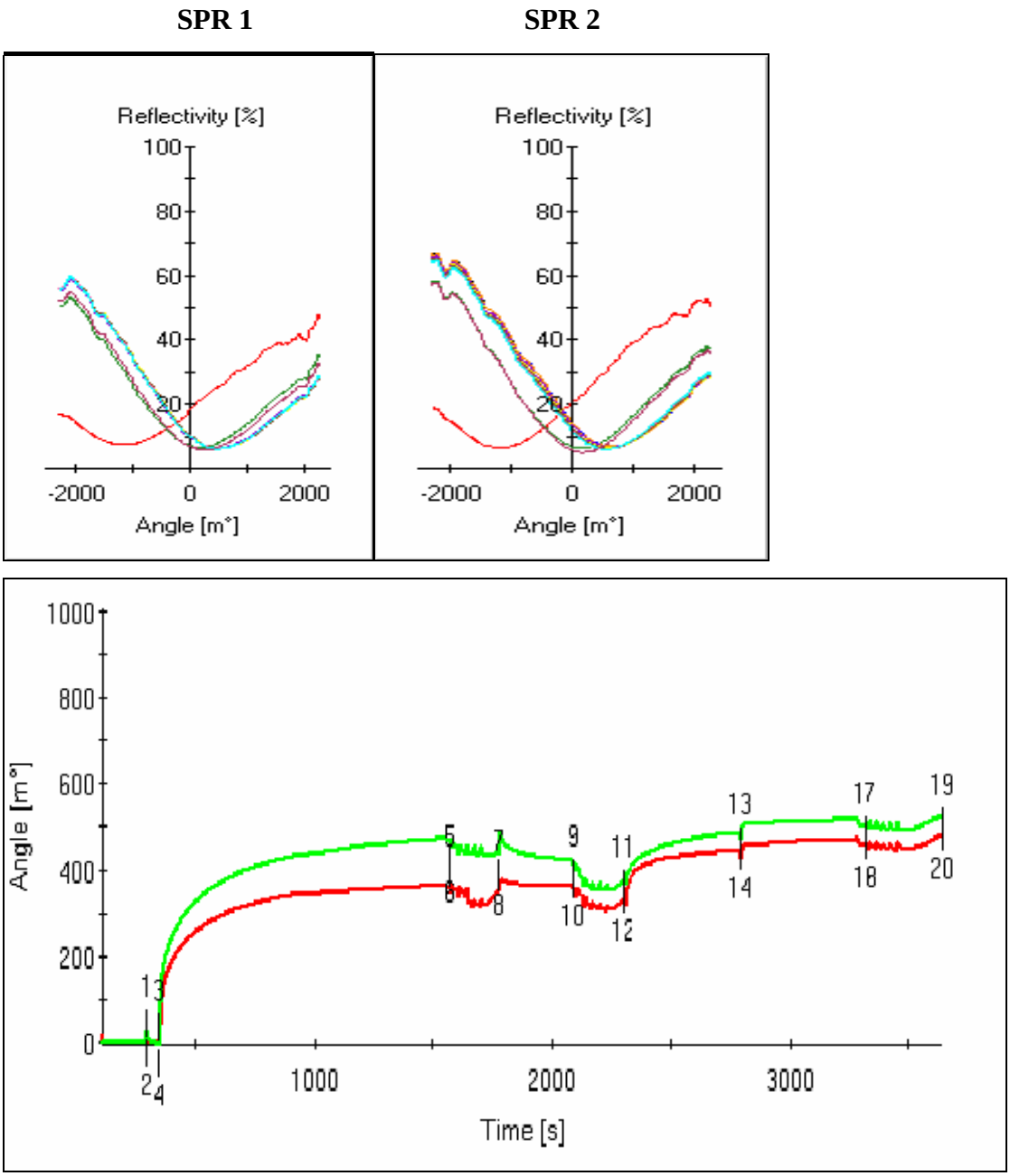


Figure 3.10: A representative ESPRIT sensorgram showing the full sequence of events to measure the inhibition of binding of human TB neg patient serum (HIV^-TB^-) antibodies to immobilized MA-liposomes on the ESPRIT biosensor before service. The unsymmetrical SPR dips (1 and 2) indicate the accumulation of dust on the optics. The event markers on the graphs are represented as follows: 1 – 2 baseline setting, 3 – 4 mycolic acids immobilization, 7– 8 saponin blocking, 11 – 12 first exposure of serum in PBS/AE (1:500), 13 – 14 second exposure to serum (1:250), pre-incubated with mycolic acids liposomes (red) and empty liposomes (phosphatidylcholine only, green). All the steps were followed by PBS/AE wash.

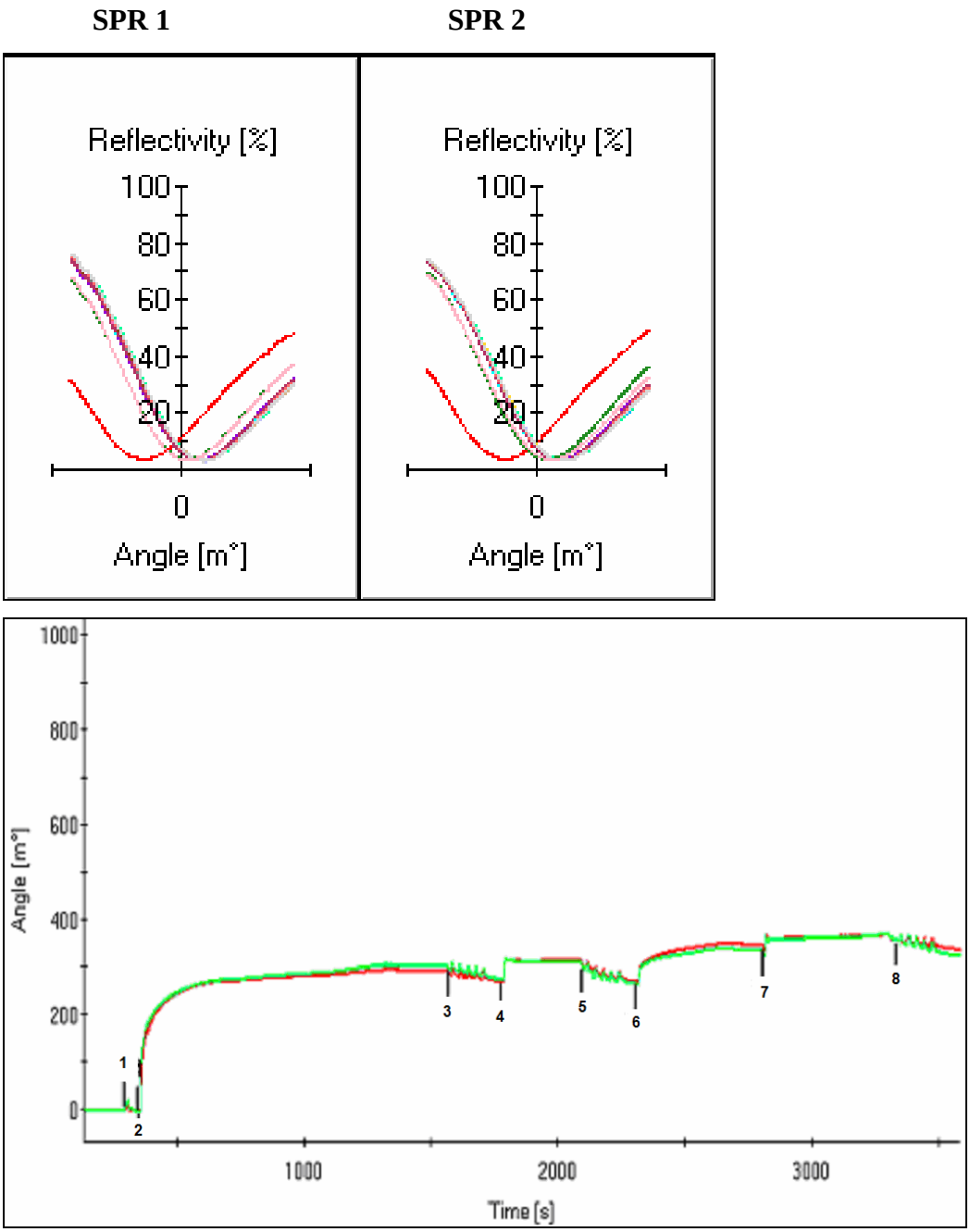


Figure 3.11: A representative ESPRIT sensorgram showing the full sequence of events to measure the inhibition of binding of human TB neg patient serum (HIV^-TB^-) antibodies to immobilized MA-liposomes on the ESPRIT after service. The symmetrical SPR dips (1 and 2) indicate free from dust optics. The event markers on the graphs are represented as follows: 1 baseline setting, 2 mycolic acids immobilization, 4 saponin blocking, 6 first exposure of serum in PBS/AE (1:500), 7 second exposure to serum (1:250) pre-incubated with mycolic acids liposomes (red) and empty liposomes (phosphatidylcholine only, green). All the steps were followed by PBS/AE wash.

After servicing the ESPRIT biosensor, it was possible to obtain proper binding profiles as indicated in Fig. 3.11. However the MARTI-assay on the ESPRIT biosensor still gave high variations as compared to the IAsys biosensor (Fig. 9). It was observed that there was instability of the baseline after PBS/AE wash with some of the experiments, after blocking the surface with 0.05% saponin (Fig. 3.12). This could also be the reason why a variation in signal percentage inhibition binding values with most experiment obtained. This shows that the MARTI-assay on ESPRIT biosensor was not yet ready to commence its validation using the EDCTP sample sera, since the sensorgrams were still not of the required quality (Fig. 3.12).

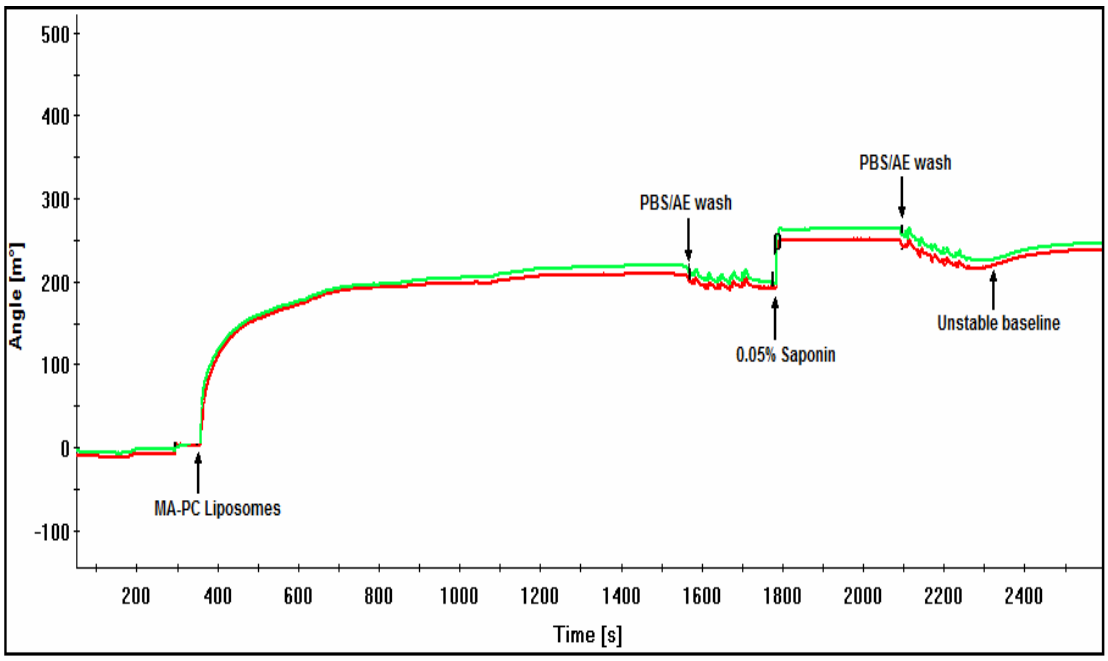


Figure 3.12: Effect of saponin (0.05%) on mycolic acid liposomes immobilized on the ESPRIT gold surface coated with octadecanethiol.

3.5 Discussion

The immersion of a clean gold disc in a solution of octadecanethiol results in the formation of a self-assembled monolayer. The low solubility of octadecanethiol in ethanol is preferred to form the SAMs (Kim *et al*., 2001). Radler *et al*. (2000) showed the formation of a lipid monolayer on hydrophobic SAMs of alkylthiols on the SPR biosensor and also demonstrated the AFM image of SAMs. Kim *et al*. (2001) demonstrated that the adsorption rate of alkanethiol onto clean gold when using a quartz crystal microbalance (QCM) biosensor depends on the thiol concentration, temperature and solvent used. In our study, a full coverage of the underivatised Au surface was observed when 10 mM of octadecanethiol was used. This was proven by a strongly hindered redox reaction when the surface was characterized with a cyclic voltammetry instrument. A low immobilization signal of mycolic acid liposomes in some experiments was also observed. This could be due to the different formation of the SAMs on the gold surfaces. Kim *et al*. (2001) reported that partial octadecanethiol multilayers on the gold surface could be formed via the formation of disulfides, since thiols are oxidized to disulfides in the presence of oxygen and the solubility of disulfides in ethanol is much less than that of thiols. If a solution of octadecanethiol in ethanol is exposed to oxygen and oxidized to disulfide, the oxidized disulfide can be precipitated onto the monolayer (Kim *et al*., 2001). In the current study, the solution of octadecanethiol in absolute ethanol was covered with parafilm to avoid oxygen exposure.

A number of critical technical parameters were optimized on ESPRIT biosensor, including the use of degassed buffers, prevention of dust accumulation on the mirrors, temperature control and regeneration steps. The creation of an auto-pipetting sequence for sample addition and re-usability of the gold disc after 15 regenerations contributed to better results.The degassing of all solutions helped to minimize formation of air bubbles on the gold surface coated with octadecanethiol and within the tubing system during mixing to prevent the loss of SPR dips (0-10%, reflectivity) and interrupted operation of the pumps. However, a recent study by Eastoe and Ellis (2007) showed that exposure of lipids to degassed buffers resulted in a detergent effect that destabilised the lipids. This problem is addressed in the next chapter. Here, however, the degassed buffer was still used throughout to determine if the MARTI-assay could distinguish between patient serum with and without TB.

In the current study, the anti-mycolic acids antibodies in human patient serum could be detected on the octadecanethiol coated gold surface with immobilized mycolic acids. The MARTI-assay could clearly distinguish between a TB positive patient co-infected with HIV and a TB negative patient without HIV. However, more sera need to be analyzed to confirm the reproducibility of the assay among the HIV positive population, since many studies reported low sensitivity and specificity with HIV positive samples (Schleicher *et al*., 2002; Antunes *et al*., 2002; Hendrickson *et al.,* 2000).

In the current study, the MARTI-assay was also applied to determine the progression of the disease during anti-TB treatment. A patient serum, P3897, which initially tested false negative on MARTI, tested TB positive after two weeks of anti-TB chemotherapy. Subsequently, the level of serum anti-mycolic acid antibody in the patient declined to that of a cured patient after 12 months of anti-TB treatment. In a different patient, P5121, the anti-mycolic acid antibody could still be detected after 12 months of anti-TB chemotherapy. This was subsequently confirmed to be due to multi drug resistance that developed in this particular patient. The results obtained with the MARTI assay were therefore confirmed in accordance to the clinical history of both patients after the MARTI-test results were submitted to Stellenbosch University. Inadequate treatment is the main cause for relapse (Lambert *et al*., 2003). Relapse of tuberculosis can be due to true recurrence or, more commonly where ongoing tuberculosis transmission is high, to exogenous re-infection (Sonnenberg *et al*., 2001). Most treatment failure occurs in patients whose first TB episode was caused by a multi-drug resistant strain, which causes a disease that is unlikely to be fully cured by a standard six months treatment regimen. The MARTI-test results gave an indication that antibody to mycolic acids are of short immune memory. Fujita *et al*. (2005a) indicated that IgG antibody to mycobacterial lipid antigens are of short immune memory in active TB, a prerequisite for a successful diagnostic assay. They found that the IgG antibody levels against lipid antigens in TB patients' sera correlated well with the stages of the disease after initiation of the anti-TB chemotherapy. Our study focused on the response of antibody to free mycolic acids in TB patients, while Fujita *et al*. (2005a) looked at the anti-cord factor response such as TDM. Fujita *et al*. (2005a) indicated that after the initiation of anti-TB chemotherapy, the IgG antibody

titer of active TB patient sera against mycobacterial lipid antigen decreased either immediately, or after a period of elevation for a few weeks. There was a sharp decrease down to normal healthy control levels after 3-4 months, when the presence of bacilli could no longer be detected in sputum sample analysis.

Simonney *et al*. (2007) evaluated an ELISA assay that uses one specific glycolipid antigen (PGL-Tb1) for the diagnosis and monitoring of prognosis of tuberculosis in HIV positive patients compared to HIV negative patients. In their studies, they showed that one patient that was TB positive according to culture growth assay, tested negative at enrolment with ELISA serodiagnosis on PGL-Tb1 coated on ELISA plates and remained ELISA negative during the observation period. This indicates that the PGL-Tb1 ELISA assay is not sensitive enough to detect TB in some of the patients who are on TB chemotherapy. Their studies also provided evidence that the clearance of anti-PGL-Tb1 IgG antibody is a lengthy process in HIV-positive patients with TB after successful chemotherapy. Significant anti-PGL-Tb1 antibody levels have been detected in patients with inactive TB for a period of time, as long as 18 months after treatment. Therefore the decline in circulating free antibody levels cannot be used as a short-term surrogate marker for treatment success in HIV positive patients using PGL-Tb1 as antigen in ELISA (Simonney *et al*., 2007). The preserved CD-1 restricted lipid antigen presentation pathway is the likely mechanism accounting for the high circulating antibody responses to PGL-Tb1 in HIV infected patients with TB (Simonney *et al*., 2007). In contrast to this, Fujita *et al*. (2005a) have found that the IgG antibody levels against trehalose 6,6'-dimycolate (TDM) in TB patients' sera tended to be of short immune memory in active TB patients under chemotherapy, but this was not so clear with other mycobacterial lipid antigens. In the current study with the MARTI-assay on the ESPRIT biosensor, we showed that the antibodies to mycolic acids are of short immune memory after analyzing two patient sera. However more patient sera need to be analyzed to confirm this study.

For future studies, the TB patient (P3897) that tested false negative with MARTI-assay during diagnosis and tested positive after initiation of anti-TB treatment needs to be re-analyzed to determine if the spiking with TB positive IgG could result in the consumption of the anti-MA spike antibodies, thereby indicating the source of the false-negativeness as being due to an excess of circulating free mycolic acids in the

serum. This would improve the MARTI-assay by eliminating the false negative results. In another approach, patients who appear TB positive by best clinical assessment, but test negative on MARTI, can be put on prophylactic isoniazid (INH) treatment and tested again after a week or two. The removal of replicating mycobacteria by INH reduces the circulating MA antigen, because P3897 tested MARTI-positive after two weeks of treatment. In these ways, false negative testing with MARTI may be completely eliminated. More patient sera need to be analyzed to determine the reliability of the MARTI-assay in its application to monitor the progression of tuberculosis and compliance of TB treatment.

The current study showed that the MARTI-assay could distinguish between a cured and multi-drug resistant patient, but with high deviation. In addition, it was hard to get reproducible data from the biosensor in subsequent tries. It appeared as if the coated liposome layer lost its stability, as binding signal was often lost in the last stages of the experimental procedure. Upon learning about the detergent-like properties of degassed water/buffers, we came to suspect the role of de-gassed buffer on the stability of the coated liposomes. The next chapter will focus on the optimization of the MARTI-assay using non-degassed buffer in the last stages of the test procedure.

CHAPTER 4

The optimal MARTI-assay with ESPRIT biosensor

4.1 Introduction

The opportunity to diagnose active TB by means of the detection of anti-mycolic acid (MA) antibodies as surrogate markers was first realised in a patent application from our group (Verschoor *et al.,* 1998) and later by a publication from a Japanese group (Pan *et al.* 1999). Standard immunoassay such as ELISA was found to be inadequate to meet the sensitivity and specificity for a practical laboratory test at a central laboratory or at point of care. Subsequently the idea of an inhibition immunoassay with real time binding measurement of antibodies was developed, using an IAsys biosensor. This increased the accuracy of the test from 54% (Schleicher *et al*., 2002) to 82% (Chapter 2), but could not be applied rigorously, due to the imperfections in the two channel system of the waveguide biosensor, without the benefit of laser adjustment to compensate for channel differences. When the IAsys Company went out of business, transfer of technology was necessitated to a different biosensor (ESPRIT, from Eco Chemie B.V., The Netherlands) that worked on the principle of surface plasmon resonance. This machine is equipped with an adjustable laser that solves the problem of channel comparability, but required an altogether different method for immobilisation of the mycolic acid antigens in liposomes. Whereas the IAsys biosensor surfaces were never exposed to air on a dry hydrophobic surface, this was necessitated in the ESPRIT biosensor where an octadecanethiol layer first had to be prepared in ethanol on a gold surface, leaving a dry hydrophobic surface before the immobilisation with liposomes. To prevent the dissolution of air into the hydrophobic layer that would destroy the plasmon resonance activity, liposomes were added in degassed buffer solution to the dry surface. Helium saturated buffer was used as a functional degassed medium for liposome coating in the ESPRIT, which was subsequently maintained in all subsequent steps on the biosensor surface, because of its convenience to prevent the possibility of air-bubbles developing in the automated fluid dispensing system. The ESPRIT system worked, but never in a rigorous way that could guarantee a reproducible outcome of sample analysis within a day. This problem was addressed in the current report and a solution found.

Eastoe and Ellis (2007) recently showed that exposure of lipids to degassed buffers resulted in a detergent effect that solubilized the lipids. This aspect was recently patented as a new approach to degrease surfaces without leaving a detergent residue (Pashley, 2005). It was therefore necessary to investigate the effect of helium degassed PBS/AE on the immobilized mycolic acid liposomes. The main focus of this current study was to fully optimize the MARTI-assay before its application in validation and clinical trials to detect anti-mycolic acid antibody in patient sera as surrogate marker for active TB. Our first priority was to reintroduce non-degassed buffers in the ESPRIT system after the liposome coating, followed by re-optimisation of every subsequent step to effect a rigorous MARTI-assay.

4.2 Aim

To optimize the different aspects of the MARTI-assay for its commercial application to detect anti-mycolic acid antibody in human sera:

- Demonstrate the effect of degassed and non-degassed PBS/AE on the immobilized mycolic acid liposome layer.

- Optimize saponin concentration for blocking the liposome layer towards non-specific hydrophobic binding.

- Determine the optimal concentrations of first and second serum exposures to antigen in PBS/AE that will give a proper binding profile on ESPRIT biosensor.

4.3 Materials and Methods

4.3.1 Effect of degassed PBS/AE on immobilized MA-liposomes

A bare gold disc was incubated for 16 hours at room temperature in a 10 mM solution of octadecanethiol (ODT) that was dissolved in absolute ethanol. The gold disc was then washed with absolute ethanol and PBS/AE, before it was inserted into the ESPRIT biosensor. The liposomes containing mycolic acids were immobilized on gold sensor discs coated with ODT for 20 minutes. The liposomes were washed 5 times with degassed or non-degassed PBS/AE, and left for 5 minutes with mixing to achieve a baseline. This procedure was repeated 3 times. Degassed buffer was achieved by bubbling helium gas through the buffer solution for 30 minutes.

4.3.2 Optimization of saponin concentration

Different concentrations of saponin prepared in PBS/AE (0.1%, 0.05%, 0.025%, 0.0125%, and 0.00625%) were tested to block the hydrophobic sites of the MA-liposome layer. The stock saponin concentration was 0.1% and the subsequent dilutions were prepared from this stock solution.

4.3.3 Optimization of first serum exposure dilution in PBS/AE

The liposomes were immobilized as described above and the surface was blocked with 0.0125% saponin to avoid non-specific binding. After saponin wash, 50 µl of PBS/AE was left for 5 minutes to effect a stable baseline. This was followed by addition of 35 µl of either 1/500, 1/1000, 1/2000, or 1/4000 dilutions of serum in PBS/AE. For the assessment of the optimal dilution of the first serum exposure, a second exposure of serum pre-incubated in mycolic-acids-containing or empty liposomes was kept constant at 1/250 in all the experiments.

All patient sera used in this study were selected from the collection reported in Schleicher *et al*. (2002). Two TB positive patient sera without HIV (P129, P96) and one co-infected with HIV (P135), and a control serum (P94) with no TB, nor HIV infection were used.

4.3.4 Optimization of second serum exposure dilution in liposomes

Different dilutions (1/250, 1/500, 1/1000 and 1/2000) of pre-incubated serum in mycolic acid and phosphatidylcholine liposomes were applied by 35 µl addition to either 1/4000 or 1/2000 of first serum exposure in PBS/AE, after 10 minutes of incubation. This was followed by washing away of the unbound antibody with 5 times 100 µl PBS/AE.

4.3.5 Regeneration of the ODT coated gold discs

After dissociation of the unbound antibodies to mycolic acids, the surface was regenerated with a mixture of isopropanol and 1 M NaOH (2:3, v/v) for 2 minutes, followed by washing first with 96% ethanol (AnalaR, Merck) and then exhaustively with PBS/AE (Appendix D).

4.4 Results and Discussion

4.4.1 Effect of degassed buffer on immobilized MA liposomes

The use of degassed buffer during the coating of the octadecane layered gold surfaces of the sensor discs is essential to prevent an air pocket forming between the hydrophobic surface and the liposome coat that destroys the surface plasmon resonance signal. The cuvette of the ESPRIT biosensor is of a design that can easily allow the surface to become exposed to air during substitution of cell contents, with subsequent reduction of reflectivity and quality of the signal. Our feedback of this problem to the Eco Chemie B.V. Company that supplies the ESPRIT biosensor, prompted them to redesign the cuvette to prevent this from occurring, but this was not yet available for this study. Here, the risk of exposure to air had to be compensated for by the continued use of degassed buffers that removed any air from the surface that might have formed during content substitutions. Alternatively, the volumes of aspiration and refilling could be carefully programmed to ensure that exposure to air would not occur. This required more washing steps to ensure the removal of content before refilling of the cuvette cells with analyte solution.

Recent work done by Eastoe and Ellis (2007) showed an unexpected property of degassed aqueous solvents: degassed water obtained by repeated freeze, pump and thaw treatments act as a detergent until it has been resaturated with either lipids or with gas. To fully optimize the method for the validation of the MARTI-assay with the ESPRIT biosensor, it was necessary to compare the degassed and non-degassed buffers after liposome coating to convincingly demonstrate the effect of degassed buffers on mycolic acid liposome stability when immobilized on the gold surface coated with octadecanethiol.

Two experiments were performed in order to determine if the continued use of degassed buffer after liposome coating will cause destabilization of the coat. Figure 4.1A demonstrates how the baseline is affected during movement of degassed PBS/AE over the liposome coat; compared to when buffer was used that was not degassed. A stable baseline was obtained only when a non-degassed PBS/AE was used (Fig. 4.1B). The degassed buffer was also kept in the washing bottle to avoid the formation of bubble in the pumps during mixing, which stops the operation of the

pumps. The rest of the procedure in the MARTI-assay was subsequently done with buffer that was not degassed, taking special care that air bubbles did not develop in the fluid lines that could affect the working of the pumps.

From the results in Figure 4.1, it is clear that a subtle destabilisation occurs with the use of degassed buffers that negatively affects the quality of the baseline that is achieved.

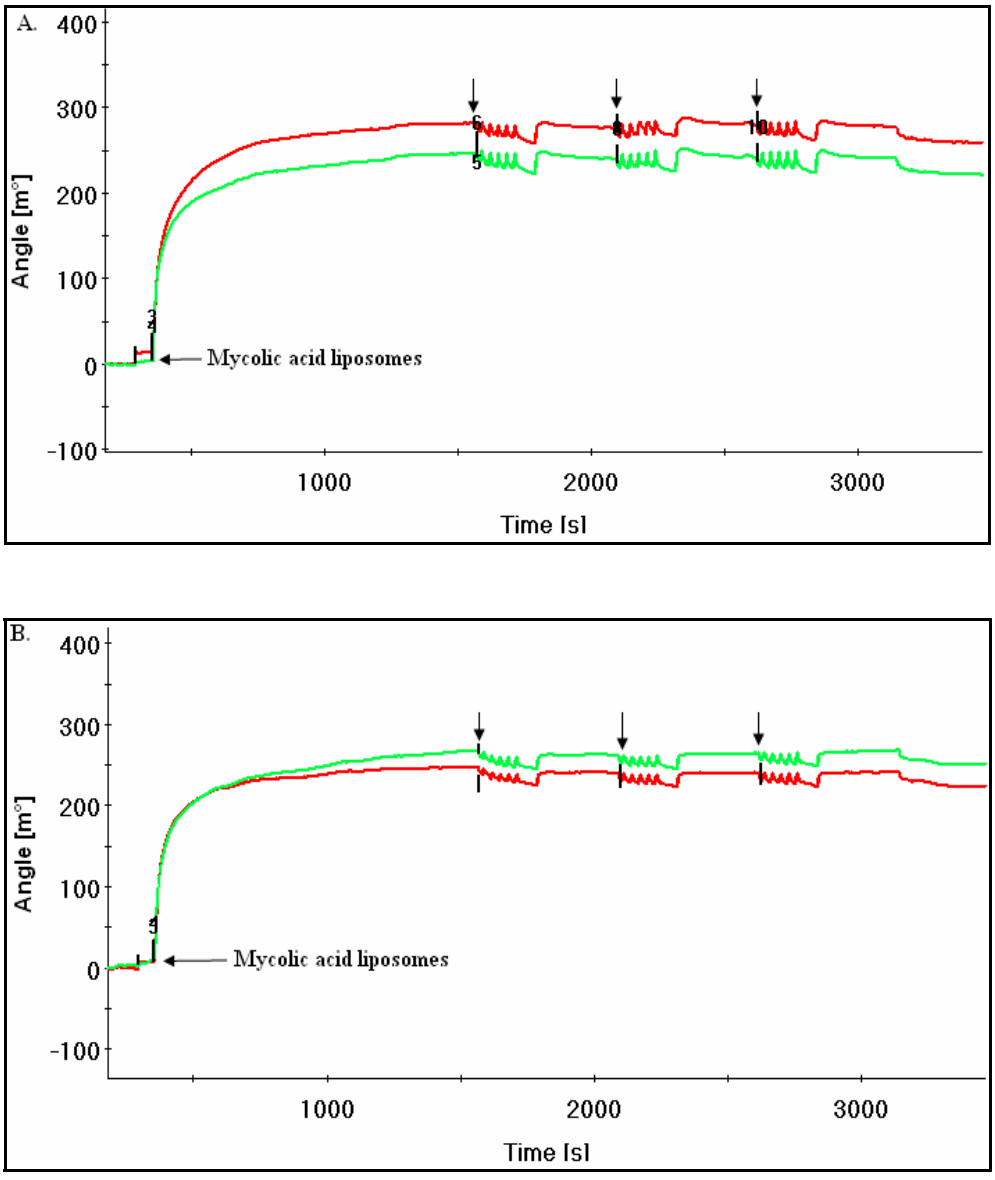


Figure 4.1: Effect of degassed (A) and non-degassed (B) buffer on immobilized mycolic acids liposomes in the ESPRIT biosensor. The arrows indicate where washing cycles with PBS/AE were introduced before allowing a baseline to be reached with mixing before substitution of cell content.

4.4.2 Optimization of saponin concentration

After correcting the instability of immobilized liposomes on the gold surface coated with ODT by the use of non-degassed buffers, it was necessary to re-optimise the concentration of saponin to avoid non-specific binding. From the results obtained (Fig. 4.2A), there was a tendency of an increase in saponin accumulation onto mycolic acid liposomes immobilized on an ODT coated gold surface, as the saponin concentration was increased from 0.00625% to 0.05%.

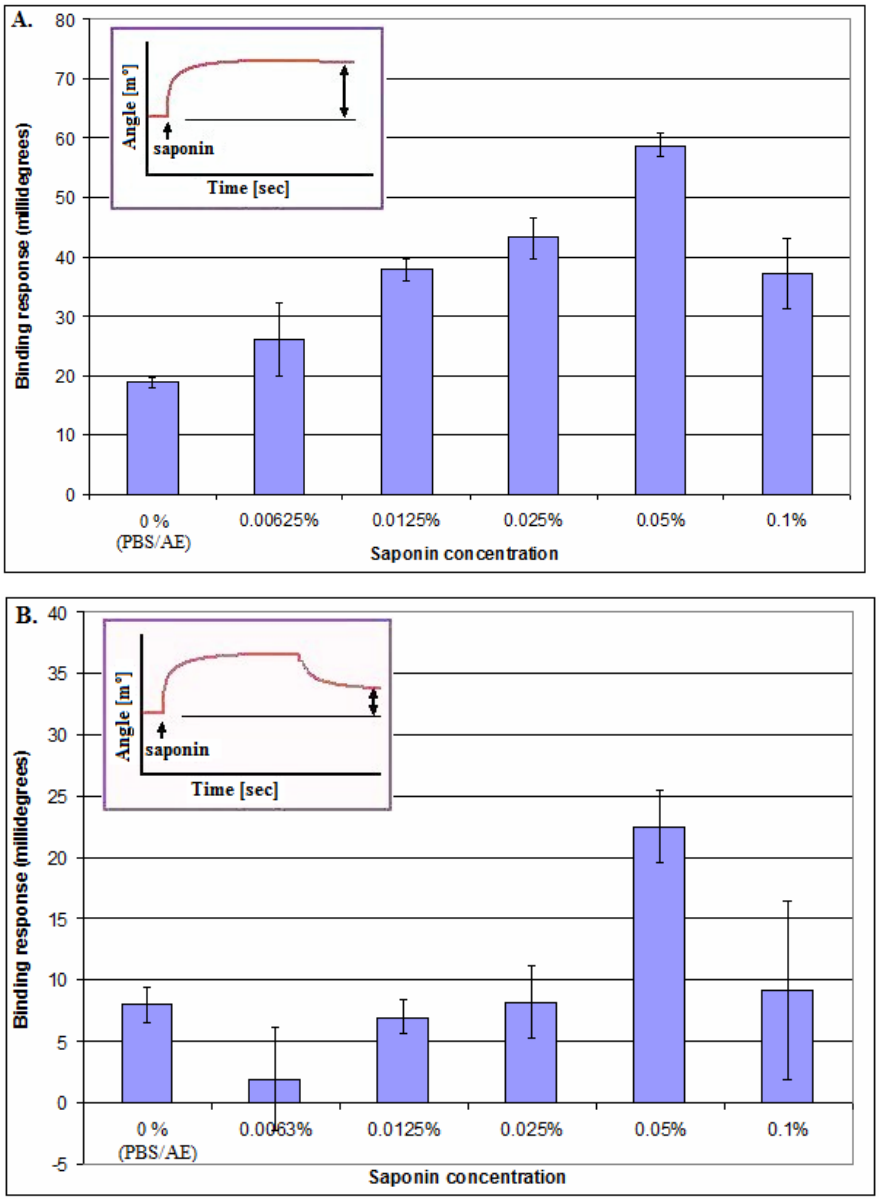


Figure 4.2: Optimization of saponin concentration to avoid non-specific binding on immobilized mycolic acids on the Au surface coated with octadecanethiol. Accumulation of saponin was performed for 5 minutes (A) and washed with PBS/AE (B). The error bars indicate the standard error of the mean (SEM) and $n = 3$.

At a saponin concentration of 0.05% there was an amount of net saponin accumulation after PBS/AE buffer wash (Fig. 4.2B). This could explain the inconsistency of the results obtained when such a high concentration of saponin was used previously. An unstable baseline was also obtained when 0.05% saponin was used. A saponin concentration of 0.0125% was chosen as optimal, because it gave a stable baseline and acceptable variation after PBS/AE wash (Fig. 4.2B) as compared to 0.0063% and 0.025%. Another reason why this concentration was selected was that previous results on IAsys biosensor showed that saponin never gave a nett binding response after PBS/AE wash (Thanyani, 2003). The differences in optimal saponin concentration used on the IAsys (0.03%) and current ESPRIT biosensors (0.0125%) could be due to different batches of saponin (noted before), or that the CPC and ODT activation before immobilization of the mycolic acid produced different surface properties.

4.4.3 The optimized MARTI-assay

With the lesson learnt of avoiding degassed buffers after coating and the conditions optimised for the blocking of the liposome layer with saponin, titrations of the optimal dilutions for first exposures to serum and second exposure to antigen inhibited serum dilutions were done. It was concluded that best results were obtained with 1:4000 dilution of serum at first exposure and 1:500 dilution of serum at second exposure. In the second exposure, the serum was pre-incubated with antigen in order to effect an inhibition of binding signal, as graphically explained in Fig. 4.3.

From the results obtained, a pre-incubation dilution of 1/500 serum in liposomes appears to be optimal, after a first serum exposure dilution of 1/4000. At these serum dilutions, good sensorgram profiles were obtained, as indicated in Fig. 4.3. Figure 4.4 shows the excellent SPR dips at 0 – 10% reflectivities that were associated with the binding profiles indicated in Fig. 4.3, proving that the sensor surfaces remained intact and fully activated during the run of the experiments.

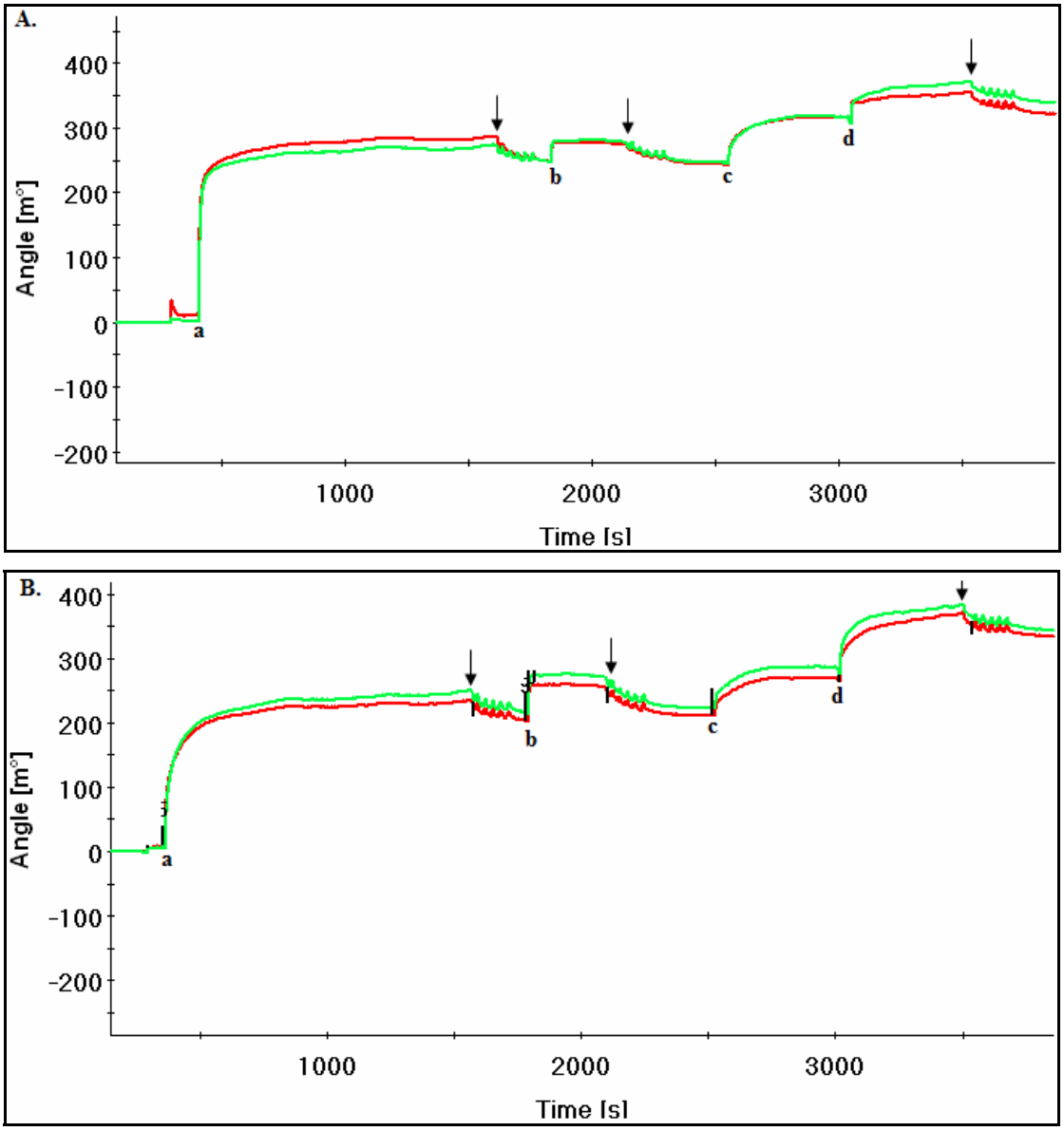


Figure 4.3: Typical sensorgrams summarizing the process of measuring serum antibody (A = TB positive P129 and B = TB negative P94) binding or inhibition of binding by mycolic acid containing and empty liposomes, on an ESPRIT biosensor with ODT coated gold surface and immobilized mycolic acid liposomes. Mycolic acids liposomes were immobilized on the ESPRIT biosensor surface (a), blocked with saponin (b), calibrated with a 1/4000 first exposure of serum (c), and applied to measure the binding and dissociation of 1/500 diluted sera inhibited with phosphatidylcholine (green) or mycolic acid (red) liposomes at lesser dilution (d). The arrows indicate washing with PBS/AE.

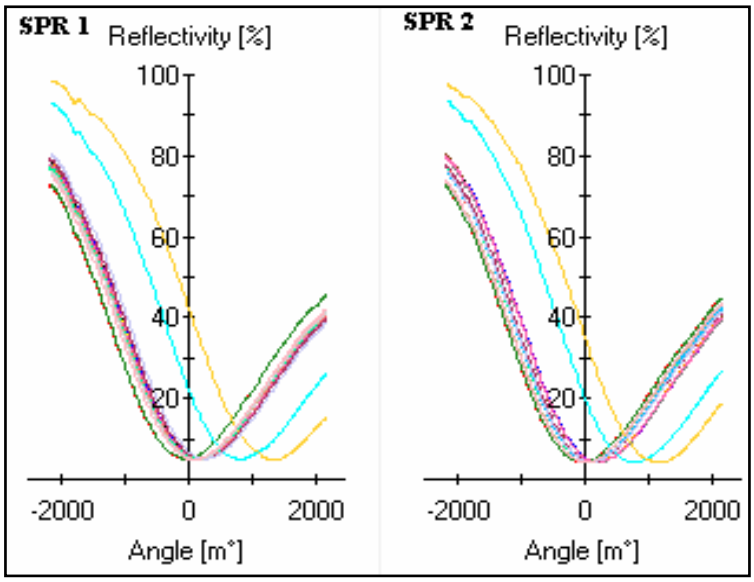


Figure 4.4: SPR dips reflecting the reliability of binding profiles during the experimental data acquisition period of the optimized MARTI-assay.

Using this optimised protocol (Appendix C), four serum samples were selected from the Schleicher *et al.* (2002) collection and assessed for the presence of anti-MA antibodies. In table 4.1, the MARTI-assay results are presented and compared with that obtained on ELISA by Schleicher *et al*. (2002).

Table 4.1: MARTI and ELISA analysis compared for their ability to detect antibody to MA in four selected human sera

Patient no.	TB/HIV status	ELISA-assay*	MARTI-assay[#]
P135	$TB^{+}HIV^{+}$	2.16	-21.51
P129	$TB^{+}HIV^{-}$	1.59	25.09
P96	$TB^{+}HIV^{-}$	1.05	29.53
P94	$TB^{-}HIV^{-}$	1.69	-0.23

* Signal to background value of absorbance at 450 nm. Values higher than 2 are taken as positive

[#] % inhibition of antibody binding to MA liposomes. Values higher than 20% are taken as positive.

4.4.3.1 First serum exposure

After optimization of saponin concentration, the next step was to determine which concentration of serum is optimal for the MARTI-assay in the first exposure to antigen. Different dilutions of a TB/HIV double positive patient serum (P135) in PBS/AE (1/4000 to 1/500) were tested. The end-point was determined with a second serum dilution at 1/250, pre-incubated with empty, or mycolic acid containing liposomes for inhibition. The results obtained in this study showed that the chosen serum dilution range of 1/4000 to 1/500 responded in an almost linear positive correlation between antibody binding signal and serum concentration with a slight running out at 1/4000 that indicates that the lower limit of the serum concentration is reached. The results obtained in Fig. 4.5 gave a correlation coefficient (r^2) of 0.9749. This shows that there is a positive linear correlation between the serum concentrations and their signal binding response over the range measured, which is a requirement for a successful MARTI-assay.

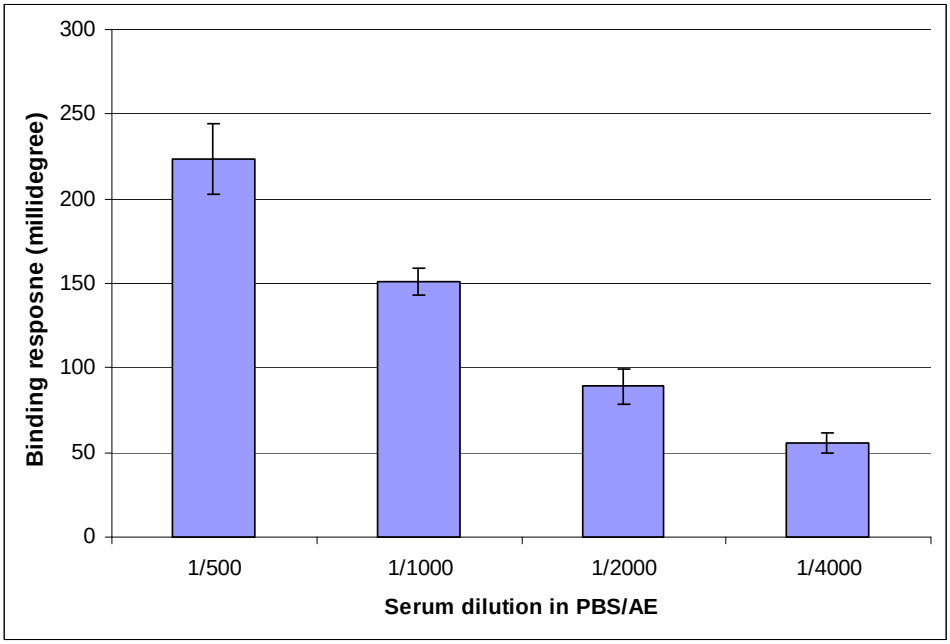


Figure 4.5: Optimization of the dilution of serum (P135) for the first exposure to antigen in the MARTI-assay, after 0.0125% saponin blocking of the mycolic acid liposome coat of the ESPRIT biosensor. The error bars indicate the standard deviation. Correlation co-efficient (r^2) = 0.9749, $n \geq 3$.

The 1/4000 and 1/2000 dilutions appear useful for the first serum exposure at high dilution in PBS/AE (Fig. 4.5), leaving enough room for a positive binding event at second serum exposure. The addition of serum at higher concentration (1/250), pre-incubated in either liposomes containing mycolic acid or empty liposomes, resulted in a good binding profile following on first serum exposures at dilutions of 1/4000 and 1/2000, but gave no inhibition of antibodies to mycolic acids, indicating the sample to be false negative (Table 4.1). Therefore a next experiment was done with a true positive serum sample to optimize the liposomes-pre-incubated serum dilution for best inhibition of serum antibody to mycolic acids.

4.4.3.2 Second serum exposure with liposome pre-incubation

Patient serum P135 (TB false negative on MARTI-assay) and P129 (TB positive) were used to optimize the dilution of the second exposure to pre-incubated serum in mycolic acid containing PC or empty PC liposomes for inhibition studies, following on a first serum exposure to immobilized antigen at a dilution of 1/4000. The first exposure should avoid the saturation of antigen with antibody before the addition of pre-incubated serum. There was no inhibition of antibody to mycolic acids after testing a range of lower dilutions (1/250 to 1/2000) of serum (P135) in liposomes (Fig. 4.6A) as the patient turned out to be false negative. The TB positive patient P129 showed a significant decrease of signal when the serum was pre-incubated in liposomes containing mycolic acid as compared to empty liposomes over a range of 1/250, 1/500 or 1/1000 dilution (Fig. 4.6B). There was no inhibition of antibody by mycolic acid pre-incubation observed when 1/2000 dilution of serum was used and binding response signals were also too low (Fig. 4.6B). This shows that the lower limit of serum concentration was reached to measure the inhibition of anti-MA antibody binding.

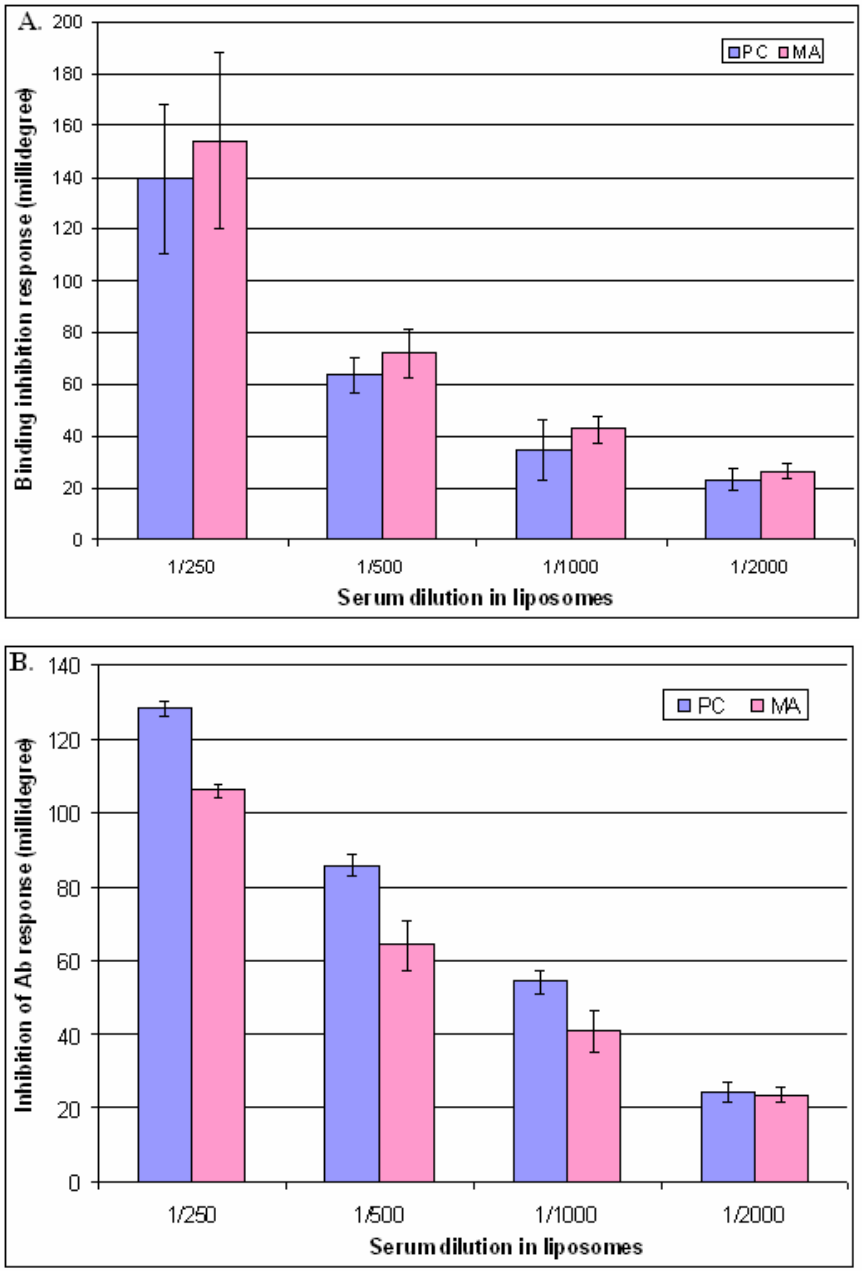


Figure 4.6: MARTI-antibody binding inhibition response of pre-incubated serum dilutions inhibited with mycolic acids (MA) and phosphatidylcholine (PC) after first serum exposure of 1/4000 on immobilized mycolic acids liposomes. A = results with TB false neg. P135 serum. B = results with TB pos. P129 serum. The error bars indicate the standard deviation. P135 gave no statistical difference between MA and PC-inhibition at all dilutions with *P*-values > 0.05. In contrast, P129 showed significant MA inhibition signals at 1/250, 1/500 and 1/1000 serum dilutions, with *P*-values of 0.00014, 0.01411 and 0.0393 respectively, but no significant inhibition at

1/2000 serum dilution (*P*-value of 0.7857). A 95% (0.05) confidence limit was used, *n* = 3.

After demonstrating the inhibition of antibody to mycolic in Fig. 4.6B when using serum at 1/4000 dilution as a first exposure to antigen, the next experiment was to determine if 1/2000 dilution of first exposure serum could give a better inhibition value of antibody with mycolic acid when the same TB patient serum was used (Fig. 4.7).

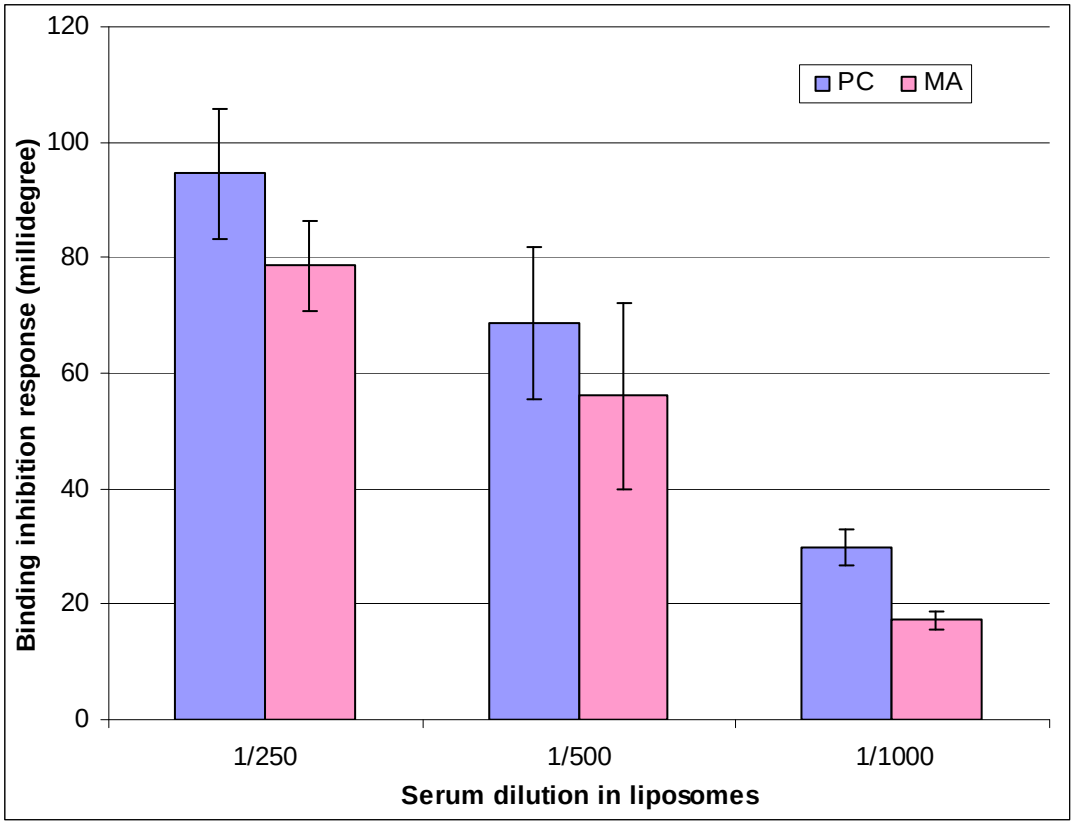


Figure 4.7: MARTI-binding inhibition response of various dilutions of pre-incubated TB positive patient serum (P129) with mycolic acids (MA) and phosphatidylcholine (PC) after first exposure serum dilution of 1/2000 to surface immobilized mycolic acids liposomes. The error bars indicate the standard deviation. No statistical difference (at 95% confidence limit) was obtained at 1/250 and 1/500 dilutions between MA- and sham inhibited serum, with *P*-values of 0.116 and 0.356 respectively, while a significant inhibition was observed at 1/1000 (*P*-value of 0.0086) *n* = 3.

The results in Fig. 4.7 indicate that inhibition values of 16.58%, 19.22% and 41.47% were obtained at 1/250, 1/500 and 1/1000 dilutions of serum in liposome solution respectively. At first sight, it appeared that a better value was obtained by using a first serum exposure of 1/2000 dilution, followed by a second, antigen pre-incubated serum dilution at 1/1000 dilution (numerical difference: 12.50 millidegrees). However, when looking at the numerical signal difference between MA-inhibited and empty liposome inhibited serum, then the 1/4000 dilution of first serum exposure followed by second serum exposure at 1/500 still gave the best value (21.53 millidegrees). In addition, the significance of the difference between antibody binding inhibition with MA-liposomes and empty liposomes was significant at 1/250, 1/500 and 1/1000 dilution of serum after first exposure at 1/4000 dilution, while only the 1/1000 dilution of inhibited serum produced significant difference after a first serum exposure of 1/2000 dilution (*P*-value limit of 0.05). The 1/2000 dilution of first serum exposure appears therefore to provide a much narrower workable range of serum dilutions at the second critical serum exposure that provides the inhibition end-result. This was confirmed when another TB positive - HIV negative serum (P96) was tested and for which a good inhibition response was obtained at the preferred serum dilutions of 1/4000 and 1/500 for first and second serum exposures respectively, while first exposure at 1/2000 did not give the expected result at 1/1000 dilution of second exposure serum, but moved the window of responsiveness to 1/500 (Appendix F). At the preferred serum dilutions of exposure, the TB negative - HIV negative serum P94 gave the expected zero inhibition value, with a *P*-value of 0.9863 (Appendix F).

From Table 4.1, it can be seen that ELISA gave an accuracy of 50% (two samples predicted correctly) and MARTI-assay 75% (three samples predicted correctly) with the selected four sera analyzed. These results of MARTI-assay on the ESPRIT biosensor confirm our results obtained previously on the IAsys biosensor (Chapter 2), although the current experiment was done with a far too small population of patient sera to allow proper statistical assessment. Table 4.1 showed that P135 tested false negative on ESPRIT biosensor and true positive on ELISA. However P129 and P96 tested false negative on ELISA, and true positive on ESPRIT biosensor. P94 tested false positive on ELISA and true negative on ESPRIT biosensor, as it was previously shown on IAsys (Table 4.1). Our previous results on IAsys biosensor also showed false negative results amongst the HIV positive population (Chapter 2). In chapter 3,

we noted that false negatives may occur in MARTI-assay of sera drawn from patients when they are first admitted to the clinic and before commencement of therapy, probably due to antigen excess in the circulation, which already inhibits much of the anti-mycolic aid antibody activity. The patient sera then tested positive after 1 week of antibiotics treatment that should have reduced the antigen load in the circulation. The false negative (P135) in the MARTI-assay could therefore also be due to the antigen overload in the serum. We are currently investigating the possibility of finding a monoclonal antibody against mycolic acids that could be used to spike serum samples. Should the monoclonal antibody spike disappear, then the antigen in the serum will have overwhelmed it and the serum could be identified as a false negative. Should the spike remain detectable, then the negative signal in MARTI cannot be explained by an antigen overload, and the result will provide a statistical event against the accuracy of the MARTI-assay. If successful, this will improve the accuracy of the assay to more than 90%, since all the false negative results would be correctly identified as being mycolic acid antigen carriers and therefore TB positive samples. The MARTI-assay could then meet the standard requirements for the World Health Organization (WHO).

Chung *et al*. (2005) indicated that serum should be diluted to minimize the non-specific binding to the biosensor surface. Serum is a complicated protein mixture for direct application to a biosensor surface. The optimization of first serum exposure dilution was previously done on IAsys biosensor to provide a practical working dilution that did not fully saturate the antigen coat, but was still concentrated enough to give a measurable signal that could be significantly increased by a two-fold higher concentration, both for TB negative and TB positive serum samples (Siko, 2003). One TB positive patient and a healthy TB negative control serum sample that gave a high and a low ELISA antibody signal respectively, were used to determine the difference of antibody binding in second serum exposure diluted from 1/400 to 1/50. It was shown that there is an increase in binding relative to the increase in concentration over the whole range, but with better resolution at the lower concentration end for both sera. For the IAsys biosensor therefore a 1/1000 dilution was chosen for the first serum exposure that would not saturate the surface but would be able to provide a good indication of surface stability, before a 1/500 dilution was added to detect the inhibition of antibody binding (Chapter 2). Because we expected the antibody titres to

differ among samples, we were more concerned to obtain sensitivity and specificity values for this educated guess of serum dilution to a workable protocol, than to statistically weigh the best dilution from a series of positive and negative serum samples. A similar approach on the ESPRIT biosensor was taken to optimize the first exposure of serum in PBS/AE, followed by the higher concentration of serum pre-incubated with mycolic acids or phosphatidylcholine liposomes. These results are shown in Appendix F. Some of the dilutions used for the first exposure of serum antibody in PBS/AE gave a high signal binding profile, which then hindered the binding of second serum antibody pre-incubated in mycolic acid or phosphatidylcholine to the surface. This could be due to the fact that the surface was already saturated with antibody to mycolic acid after an exposure of the first high dilution serum in PBS/AE. At the preferred dilutions however, the MARTI-assay on the ESPRIT biosensor can reliably distinguish between a TB positive and TB negative patient sera, even better and more reliable than on the IAsys biosensor and ELISA.

In chapter 2, the MARTI-assay on IAsys biosensor was successfully validated to an accuracy of 82% for the serodiagnosis of active pulmonary TB. The IAsys biosensor system, however, has a weakness in the double channel cuvette system, in which the channels often do not give matching results, while being ten times more expensive than the gold discs provided for the ESPRIT biosensor. The ESPRIT biosensor is provided with an adjustable laser setting to compensate for differences in the channel readings as well as an automated fluidic system that reduces variance from one sample to the next.

The MARTI-assay as applied in the ESPRIT biosensor has now reached the stage where a result of sample analysis can be guaranteed within 4 hours of receipt of the serum. This is the first time that such reliability has been achieved. However only four patient sera from Schleicher *et al.* (2002) collection were used to obtained an optimal MARTI-assay on the ESPRIT biosensor. Therefore the assay is now ready for proper validation with blind samples of patient serum and eventual development into clinical trials and the market. In order to come to a clear conclusion on the potential of the MARTI-assay to comply to the WHO standards for a commercial TB diagnostic assay, validation with 110 double blind serum samples from an EDCTP project anchored in Stellenbosch is currently undertaken.

CHAPTER 5

Concluding Discussion

HIV infection is thought to be a major contributor to the increase in TB incidence across the world (Dye *et al*., 2005). An estimated 9% of adults globally with newly diagnosed TB are HIV positive, but this rate is 31% in Africa. Therefore, HIV co-infection with TB presents challenges to effective diagnosis of TB and diagnosis can also be more difficult in children. The rapid rise of drug-resistant (DR) and extreme drug-resistant (XDR) TB has further complicated TB diagnosis. Therefore a new diagnostic assay that is fast and accurate enough to diagnose all infected individuals, and able to identify drug resistance strains of *M. tuberculosis*, which would effectively contribute to monitor treatment programmes, is urgently needed (Guillerm *et al*., 2006).

An effective means of preventing tuberculosis is an early diagnosis followed by an appropriate treatment, with an assay that could also follow the prognosis. Despite a large number of studies being done in the past decades to develop a serodiagnosis of TB, none has found a place in the routine diagnosis, even though antibody tests are rapid and do not require specimens from the site of infection (Palomino *et al*., 2007). A recent study by Steingart *et al*. (2007) indicated that commercial antibody detection assays produce inconsistent estimates of accuracy and none of them perform well enough to replace sputum smear microscopy. This indicates that there is a need for a rapid and accurate serodiagnostic assay that could be used to reduce the spread of tuberculosis. Most of the antibody assays give low specificity and sensitivity, which make them difficult to be applied for routine analysis. The factors that affect the sensitivity and specificity of the assays include the antigen used, prior BCG vaccination, exposure to non-tuberculosis mycobacteria strains, and the particular manifestation of TB disease (pulmonary or extrapulmonary) with HIV co-infection or child-TB. In HIV/TB co-infection and child TB the gold standard used for the diagnosis of TB is also problematic. Although culture of bacteria is the reference standard in diagnosis and follow-up of disease, it can take up to 4 - 8 weeks to grow and identify *M. tuberculosis.* False negative culture results may be obtained (Raqib *et*

al., 2003) especially in children and HIV-infected patients from whom it is difficult to obtain sputum. Sputum culture will not detect extrapulmonary forms of TB.

The current study showed that the MARTI-assay on IAsys and ESPRIT biosensor can be used to detect antibodies in TB positive patients co-infected with HIV and also in TB patients that are undergoing treatment, on the same day that sampling is done. The MARTI-assay on IAsys biosensor gave an accuracy of 82% in an HIV epidemic population in which most TB assays fail. The accuracy was obtained by exclusion of $HIV^{+}TB^{-}$ patient sample results, since the gold standard used to identify the patients as TB positive or TB negative was a sputum culture assay, which is known to underestimate the TB positiveness in HIV-infected populations (Brindle *et al*., 1993; Colebunders *et al*., 2000; Frieden *et al*., 2003). Therefore the results obtained with the MARTI-assay in the HIV positive population could be true positive. According to the recommendations by WHO (1997), a serological test should possess sensitivities of more than 80% and test specificities of more than 95% to replace the gold standard culture. The MARTI-assay on the IAsys biosensor showed a significant increase in sensitivity and specificity, as compared to that reported in our previous study using an ELISA (Schleicher *et al*., 2002). However the IAsys biosensor could detect low affinity antibody to immobilized mycolic acid as compared to ELISA. The identity of the binding antibodies to mycolic acid as being of the IgG isotype was also confirmed (Appendix A). The problem on IAsys biosensor was that reproducibility of the cell calibration curves of the high dilution serum in the two cells of one cuvette was difficult to fall within the required relative response amplitudes, as the calibration curve profiles had to be similar by eye. It was believed that if this problem could be resolved, the accuracy of the MARTI-assay could be achieved to more than 90%. However the IAsys biosensor was outdated and the technology was transferred first to the ESPRIT biosensor, which uses a gold surface, instead of hafniumoxide.

In the current study, it was demonstrated that the MARTI-assay on the ESPRIT biosensor could detect anti-mycolic acid antibodies, thereby differentiating between TB positive and negative patients. Our preliminary study with two patient's sera showed that the MARTI-assay on ESPRTI biosensor could also be used to monitor the prognosis of the disease during anti-TB chemotherapy. It could distinguish between a cured and multi-drug resistant TB patient. Sousa *et al.* (2000) showed that

the levels of circulating IgG antibody against several *M. tuberculosis* antigens such as 38 kDa, LAM, diacyltrehalose (DAT) detected by ELISA in the whole serum, varied depending on the antigen used. Their results confirmed the lack of predictive power of serological tests in solving the treatment monitoring of TB in patients. Fujita *et al*. (2005a) confirmed that the IgG antibody levels against lipid antigens in TB patients' sera varied greatly depending on the stages of the disease, but found that TDM antigens detected antibodies of which the immune memory appeared to be short, therefore related directly to active TB disease. TDM is a mycolate derivative, meaning that this antigenic determinant may be the important one for monitoring the prognosis of TB disease during treatment. An advantage of using lipid antigens such as mycolic acids is that the humoral response is unique in comparison to protein antigens (Palma-Nicolása and Bocanegra-Garcíab, 2007). Protein antigens show long-lasting positive results, or the disappearance of signal when immune compromise weakens the specific antibody responses, such as in AIDS patients. It is known that mycolic acid is a CD1 restricted antigen with the ability to induce proliferation of CD4/CD8 double negative T-cells (Beckman *et al*., 1994). A recent study by Simonney *et al*. (2007) showed that the use of non-protein antigens, such as glycolipids, for immunodiagnosis of tuberculosis gives improved specificity and sensitivity especially in TB patients co-infected with HIV. However, the use of MARTI-assay in monitoring anti-TB treatment need to be validated with more patient sera undergoing TB chemotherapy before it can be recommended for use. If the MARTI-assay proves itself in its validation, it could be used to monitor if the patients comply with their treatment regime, are cured after months of taking anti-TB chemotherapy, or if they developed drug resistance, as in XDR or MDR TB.

A recent study by Margot (2008a) on the state of TB in South Africa and reported at the 1st TB conference in Durban, disclosed that XDR-TB infected individuals were prone to die within a few weeks after presenting themselves at the hospital, even though they were not co-infected with HIV. An earlier report from *South African health news* (http://www.health-e.org.za/news, 2008) quoted Margot (2008b) saying that "Tugela Ferry…renewed the interest in TB that has been lacking. So, things like new diagnostic methods…quicker diagnostic methods…shorter treatment regimens, better drugs to manage it - it's renewed the interest in finding these…which was starting to wane and going along very slowly… So in a way, although it's not good

news, it helped us". Margot emphasized that a fast diagnostic assay is required to alert the clinicians to place the patients on anti-TB treatment before they spread the XDR TB to the community. According to the WHO (2008) report, South Africa has a policy of hospitalizing all patients with MDR-TB or XDR-TB for at least six months, thereby decreasing the infection rate within the community. The MARTI-assay, which only takes a few hours to diagnose TB, could be used to alert the clinicians to put TB patients on treatment timeously. A recent report by WHO (2008) indicated that an early diagnosis of TB can lead to improved treatment statistics.

A rapid and reliable test for infection with *M. tuberculosis* would make a considerable contribution to the management of the TB epidemic, especially in HIV-burdened and resource-poor countries where access to diagnostic laboratories is limited (Raqib *et al*., 2003). Before starting anti-retroviral roll out, patients with HIV require careful screening for subclinical TB infection, including careful clinical review, routine blood analyses, chest radiology and examination of induced sputum specimens and culture of blood for mycobacteria (Lawn *et al.,* 2005). A recent report by WHO (2008) suggested that TB patients should also be tested for HIV. This could help the clinicians to decide as to whether to place the patient on ARV or anti-TB chemotherapy in order to avoid immune reconstitution inflammatory syndrome (IRIS). Longer duration of TB treatment, about two months, before initiating ARV may lead to the lower bacterial load of *M. tuberculosis.* Extra-pulmonary TB patients are more likely to develop subsequent IRIS, which may cause respiratory failure and death (Manosuthi *et al*., 2006). If the MARTI-test on the ESPRIT biosensor can detect anti-mycolic acid antibody in HIV-infected individuals, it can be used as an efficient initial test to screen TB. The ESPRIT biosensor now appears to be able to meet this challenge. This study on the ESPRIT biosensor confirmed the results previously obtained with the IAsys biosensor that there are anti-mycolic acid antibodies in TB patients. It is hoped that the ESPRIT biosensor will improve the accuracy of the test to more than 90% after analyzing all (110) of the EDCTP patient sera on the ESPRIT biosensor. This expectation is based on the observed improved sensitivity and reproducibility of the ESPRIT biosensor compared to IAsys. On the ESPRIT biosensor higher diluted serum during the first and second exposures of antibody to mycolic acid brought the test into range as compared to IAsys. Spiking of suspect false negative sera with monoclonal antibodies may increase the accuracy even further.

The MARTI-assay needs to be validated to determine its application to diagnose pulmonary and extra-pulmonary TB in population that has a high incidence of HIV both in adults and children. Simonney *et al*. (2000) showed the detection of immune complexes and free antibodies against glycolipid antigens is useful for the serodiagnosis of children with a high probability of pulmonary TB. This bodes well for the detection of anti-mycolic acid antibodies in children with the MARTI-assay to solve this bottle-neck of TB diagnosis.

Although wave guide and SPR assays are currently still experimentally cumbersome, there is considerable technological development in this field that allows one to realistically expect that the prevailing technical challenges can be overcome to make the MARTI-assay amenable for a routine diagnostic laboratory in the not too distant future. It is believed that the cost of the MARTI-assay could be expensive, however re-engineering of the biosensor device with the latest laser scanning, automated pipetting and micro-array technology to allow miniaturisation and high throughput screening at low cost could make the MARTI-test more affordable to the public. In recent years, there has been intensive research effort towards increasing the number of sensing channels to introduce benefits of SPR biosensor technology to multi-analyte detection and highly parallelized biomolecular interaction analysis. Numerous approaches in multi-channel SPR sensor development have been demonstrated to date (Dostalek *et al*., 2005; Kim *et al*., 2007). Dostalek *et al*. (2005) have shown a development of an eight channel SPR sensor. Kim *et al*. (2007) demonstrated an application of a miniature multi-microchannel (eight channels) SPR sensor for the detection of environmental toxins. If the validation of the MARTI-assay confirms its usefulness for TB serodiagnosis, it would lead to further development of a multi-channel SPR sensor for high throughput screening of patient sera, thereby reducing the cost per test. The MARTI-assay in its current state of development will be applicable to reference and peripheral labs, but can be developed in later years to serve the needs of resource-poor areas where access to diagnostic laboratories is limited.

The cost of the MARTI-assay on the ESPRIT biosensor could be reasonably affordable given the fact that it is rapid. There has been an improvement in terms of the cost of the assay, since the price of the gold disk for ESPRIT (Metrohm, South

Africa, www.metrohm.co.za) is 7 times less as compared to the IAsys cuvette (www.farfield-group.com). If the MARTI-assay could be validated, it can potentially replace most of the available assay used to diagnose TB and especially as a first screening of patients suspected of suffering from tuberculosis. Other current techniques that could compete with the MARTI-assay include the GenoType MTBDR (Hain Lifescience, 2007) assay that is based on the PCR amplification of 16-23S ribosomal DNA products with 16 specific oligonucleotide probes (www.hain-lifescience.de). Its advantages are that it can be used to confirm TB infection and detect drug resistance to rifampicin and isoniazid at the same time. Preliminary data suggest that the GenoType MTBDR test can detect at least 90% of MDR-TB cases in a few hours (de Luna *et al*., 2006). However the assay still requires sputum for testing and is therefore of little use in child-TB and HIV burdened populations. The MARTI-test has the potential to meet this challenge. The advantage of the GenoType MTBDR, however, is that it can simultaneously detect drug-resistance TB, which the MARTI-assay can not do. The GeneXpert diagnostic system assay (www.cepheid.com) that completely automates sample preparation, amplification of extracted DNA and detection of a target gene sequence could replace most of the conventional methods used for TB diagnosis, were it not for the fact that it still relied on sputum samples. Most commercial molecular tests with prices typically higher than conventional assays (typically R200 – R300, i.e. the price of one ESPRIT gold disc) are popular in resource-rich settings. However, the most resource-constrained countries tend to have the highest burden of TB or X/MDR-TB cases and are least likely to benefit from expensive technologies because of high costs and lack of appropriate laboratories (Perkins *et al*., 2006). Because of this, several groups such as WHO, Foundation for Innovative New Diagnostics (FIND, 2007) have launched initiatives to improve global laboratory capacity and to make new diagnostics affordable (www.finddiagnostic.org). Such initiatives might also be required in a later development stage of the MARTI-assay.

The attachment of mycolic acids covalently or non-covalently onto the underivatized gold disc could simplify and make the MARTI-assay more affordable, instead of incorporation of mycolic acids into the liposomes. The ongoing studies by our group in collaboration with University of Bangor in Wales (UK) that focuses on the synthesis of mycolic acids could improve the accuracy of the MARTI-assay (Al

Dulayymi *et al*., 2005; Al Dulayymi *et al*., 2007; Koza and Baird, 2007; Deysel, 2008). Al Dulayymi *et al*. (2007) have reported the syntheses of three stereoisomers of a complete methoxy mycolic acid corresponding to the major component of the one isolated from *M. tuberculosis*, thereby demonstrating that the technology is available to synthetize any mycolic acid sub-type in a stereo-controlled way. The synthetic mycolic acid would then be covalently linked to the gold and investigated for its antigenicity. The attachment of synthetic alpha, methoxy and keto mycolic acid subclasses could improve the specificity of the MARTI-assay.

The specificity of the MARTI-assay needs to be assessed in individuals infected with other mycobacteria to determine if the false positive results could be due to cross-reaction of the patient antibodies to mycolic acid from other species such as *M. bovis* or *M avium*. In addition, it is necessary to determine if serum IgG antibodies from confirmed TB patients infected only with *M. tuberculosis* are specific enough to distinguish TB from non-TB mycobacterial diseases in the MARTI-test with immobilized synthetic mycolic acids representing those from other *Mycobacterium* species. This is particularly important in HIV patients where the immune system is compromised thereby making a patient susceptible to co-infection with *M. avium* and other mycobacterial species (Manosuthi *et al*., 2006).

Even though the cross-reactivity of antibody with mycolic acid and cholesterol in TB patients was previously shown with ELISA (Siko, 2003), this was not apparent in the MARTI-test results. The inhibited IgG antibodies that make up the positive signal in MARTI-testing are more specific to mycolic acid and could be distinguished unequivocally with the biosensor from anti-cholesterol binding (Thanyani, 2003; Vermaak, 2004). The MARTI-assay appears therefore to be suitable for TB diagnosis in an HIV epidemic population, despite the observation by Horvath and Biro (2003) that there is a higher level of anti-cholesterol antibodies in HIV patients than in HIV-seronegative controls.

A new design of the ESPRIT cuvette system by Eco Chemie B.V. (Utrecht, The Netherlands) that retains a certain volume of solution on the ODT coated surface might increase the accuracy of the MARTI-assay on the ESPRIT biosensor. This will decrease the formation of air bubbles by the needles during washing steps, since a

flow wash system could be used without leaving the surface dry. Eco Chemie is currently producing this type of cuvette to suit the MARTI-test; which is expected to increase the rate of sample throughput of the MARTI-assay.

The current study gives proof of principle of a totally novel way to diagnose TB from serum samples. It was named the MARTI-assay, was patented and subsequently brought into the public domain by publication. If confirmed by validation then MARTI is the first and only TB-test to date that can diagnose TB accurately in an HIV epidemic population. It may also be used as a tool to monitor the progression of the disease during anti-TB chemotherapy. The MARTI-assay could therefore give an indication of developing drug resistance in TB patients, thereby saving millions of lives by curbing the spread of XDR and MDR TB to the communities that harbour the patients, while allowing timeous chemotherapy aimed at a cure. If the MARTI-assay could also diagnose extra-pulmonary and child TB, it will be regarded as a global health solution to control the transmission of TB. It stands on the threshold of changing the way that TB is managed in HIV burdened and TB-drug resistant populations, by providing results within a day. An immediate activity flowing from this research is the development of a business plan to develop the test to the market, starting in South Africa. There is much scope to improve the MARTI-test in terms of its affordability and to make it amenable for high throughput screening.

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APPENDIXES

Appendix A: Proof of principle of MARTI-assay on IAsys biosensor

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Research paper

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

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Abstract

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*. HIV co-infection introduced a 30% underestimation in TB diagnosis based on sputum analysis, calling for a reliable and fast serodiagnostic assay to assist in the management of TB in HIV-burdened populations. Serodiagnosis with mycobacterial lipid cell wall antigens gave promising results, in particular with LAM and cord factor. Free mycolic acids have also been considered because they are unique in structure to each species of *Mycobacterium* and can be economically extracted and purified. In a standard immunoassay such as ELISA, however, an unacceptable number of false positive and false negative test results were obtained. Here we report a much improved biosensor method to detect antibodies to mycolic acids in patient serum as surrogate markers of active tuberculosis. Mycolic acid (MA) liposomes were immobilized on a non-derivatized twin-celled biosensor cuvette and blocked with saponin. A high dilution of serum was used to calibrate the binding signal of the two cells, followed by contact with patient serum at a lesser dilution, but pre-incubated with either antigen-carrying, or empty liposomes. The serum, or the protein A purified IgG thereof, from sputum-positive tuberculosis patients could be inhibited from binding to the MA in the biosensor by prior incubation with MA-containing liposomes. The accuracy of the inhibition test was 84% if HIV-positive patients for whom a negative TB sputum analyses could not be relied upon to serve as a reference standard were excluded. If biosensor technology could be made suitable for high throughput screening, then it may provide the solution to the serodiagnosis of tuberculosis against a background of HIV.

Keywords: Antibodies; Mycolic acids; Biosensor; *Mycobacterium tuberculosis*; Serodiagnosis

1. Introduction

Tuberculosis (TB) is a chronic pulmonary disease caused by infection with *Mycobacterium 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). Although tuberculosis is a curable disease that responds well to antibiotics, it has re-emerged as a growing, global health problem because of the development of drug-resistant strains. The resurgence of TB and the increased risk for TB in HIV-infected persons has magnified the need for rapid, inexpensive and accurate methods for the

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diagnosis of TB (Cheon et al., 2002). People with TB that are co-infected with HIV have a 5–15% risk yearly of developing active tuberculosis (Winkler et al., 2005). World Health Organization (WHO) guidelines suggest starting antiretroviral drugs within 2 months of tuberculosis treatment. Patients who start antiretroviral drugs too early in their TB treatment can be predisposed to immune reconstitution syndrome (IRS). Immune reconstitution syndrome has symptoms overlapping with worsening TB and can be life threatening to the patients (Lawn et al., 2005). A major challenge with immunological diagnosis of tuberculosis is to distinguish between mere physical exposure to TB, latent infection and chronic or severe active disease (Pai et al., 2006). Other factors that affect the performance of immune-based assays include previous BCG vaccination, exposure to non-tuberculous mycobacteria and co-infection with *M. tuberculosis* and HIV.

The tuberculin skin test, based on the cellular response to protein antigen, is currently the most generally used method of identifying TB infection. The specificity is low as purified protein derivative (PPD) contains many antigens widely shared among mycobacteria. Several studies have demonstrated that PPD cannot reliably distinguish between previous *Mycobacterium bovis* BCG vaccination, exposure to environmental mycobacteria, or infection with *M. tuberculosis* (Doherty et al., 2002; Chan et al., 2000). Sputum analysis for the presence of live *M. tuberculosis* is done in a variety of ways and is generally employed for the diagnosis of TB. Acid-fast microscopy is quick and easy, but needs confirmation by other tests, as some acid-fast bacilli are not *M. tuberculosis* (Hamasur et al., 2001). Culture of *M. tuberculosis* from sputum 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 4–8 weeks for completion (Samanich et al., 2000) and is subject to contamination. This often results in delayed diagnosis, adversely affecting patient care and TB control and allows for the spread of infection. In addition, HIV-infected patients often cannot produce sputum of the desired quality for TB detection (Frieden et al., 2003). PCR DNA detection kits are faster, more sensitive and accurate, but also require a sputum sample and a sophisticated laboratory with matching staff competency. Samples from other sources, in particular blood, showed much less sensitivity with PCR amplification tests (Saltini, 1998).

A simple diagnostic assay that does not require highly trained personnel or a complex technological infrastructure is essential for global control of TB (Foulds and O'Brien, 1998). A serologic test, such as ELISA, is a simple and inexpensive alternative to other TB diagnostic methods (Simonney et al., 1996; Moran et al., 2001). The disadvantage of ELISA is that it detects only the high affinity antibodies to the antigen, due to the need for washing steps after contact between the antigen and serum antibodies. Irrespective of the antigen(s) used, no single ELISA test has hitherto succeeded as a reliable test to confirm tuberculosis. Due to the limitations of conventional TB diagnostic tests, there is a widely felt need for a novel approach to develop a diagnostic test that is accurate and convenient to use, especially in HIV patients (Pavlou et al., 2004; Reid et al., 2006).

Mycolic acids are unique, 60–90 carbons long, branched α-alkyl, β-hydroxy fatty acids, which form an outer waxy lipid layer around the mycobacteria (Barry et al., 1998). Our previous studies (Schleicher et al., 2002) and those of others (e.g. Pan et al., 1999) have shown the prevalence of anti-mycolic acid antibody in TB patients with ELISA. Schleicher et al. (2002) investigated the diagnostic potential of an ELISA, based on the detection of antibodies to *M. tuberculosis* mycolic acids in the serum of HIV-seropositive and HIV-seronegative tuberculosis patients, in a population with a high prevalence of HIV. Although they observed 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 concluded that the ELISA has inadequate 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.

This study aimed to assess the potential of detecting anti-mycolic acid antibodies as a surrogate marker for tuberculosis with an IAsys affinity biosensor in the serum of patients with active pulmonary tuberculosis. The IAsys biosensor can monitor and quantify the binding of interacting analytes in real time, by detecting changes in refractive index in the vicinity of the immobilized ligand. The changes in refractive index values are proportional to the change in the accumulated mass (Cush et al., 1993; Buckle et al., 1993). The benefit of not having to label any of the reagents, combined with low sample consumption has made the optical biosensor a useful instrument in both research and commercial laboratories (Myszka, 1999). Although wave guide and SPR assays are currently still experimentally cumbersome, there is considerable technological development in this field such that one can realistically expect that the prevailing technical challenges can be overcome to

make such tests suitable for a routine diagnostic lab in the not too distant future.

Here, we describe how the technology could be applied in the detection of anti-mycolic acid antibodies as surrogate markers for active TB. To confirm that the active binding agents in the human sera were of antibody nature, the IgG antibodies were isolated from a healthy control and a TB-positive patient using protein A and tested in the biosensor assay. The advantages of protein A-based affinity chromatography (Verdoliva et al., 2002) were exploited to confirm that binding to immobilized mycolic acid on the IAsys cuvette surface was due to IgG from patient serum.

2. Materials and methods

2.1. Human sera

Serum samples collected for another study (Schleicher et al., 2002) were used; they were obtained from 61 patients (aged between 18 and 65 years), who were admitted to the general medical wards of the Helen Joseph Hospital, Johannesburg, South Africa, including a number with active pulmonary tuberculosis. The study population consisted of 32 tuberculosis-positive (TB^+) and 29 control tuberculosis-negative (TB^-) patients. The TB^+ group consisted of patients with newly diagnosed smear-positive pulmonary tuberculosis of which some were HIV-seropositive (23 patients). The TB^- patients that were used as controls had medical conditions other than TB and were recruited from the general medical wards. None of the TB^+ patients were on anti-TB chemotherapy at the time of serum collection.

For the purpose of IgG isolation and testing, a TB^+ patient serum was selected from a collection of sera supplied by the Medical Research Council (MRC) (Clinical and Biomedical Research Unit, King George V Hospital, Durban, 1994). Healthy volunteer sera were used as negative controls for IgG isolation.

2.2. ELISA of patient sera

Mycobacterial mycolic acids were isolated from a culture of *M. tuberculosis* H37Rv (American Type Culture Collection 27294) as described by Goodrum et al. (2001). Mycolic acids (250 µg) were dissolved in 4 ml of hot phosphate-buffered saline (PBS, pH 7.4) for 20 min at 85 °C and sonicated (Virsonic 600, United Scientific, USA) at 20% duty cycle and optimal output level for 1 min. The solution was kept at 85 °C during pipetting into ELISA plates (Sero-Well®, Bibby sterilin Ltd., UK), after which the plates were placed in plastic bags and incubated overnight at 4 °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 h at room temperature. The solution was flicked out, the wells were filled with 50 µl of serum, serum precipitate in triplicate and incubated for 1 h at room temperature, flicked out and washed three times with PBS/0.5% casein. The wells were aspirated to remove proteinaceous froth. The plates were incubated for 30 min at room temperature with peroxidase-conjugated goat anti-human IgG (whole molecule, Sigma) diluted 1/1000 in PBS/0.5% casein, flicked out, washed three times with PBS/0.5% casein and aspirated. The presence of antibody was revealed using 50 µl/well of hydrogen peroxide (40 mg) and *o*-phenylenediamine (50 mg) in 50 ml of 0.1 M citrate buffer (pH 4.5). Measurement of the yellow colour was done after 30 min at 450 nm using a Multiskan Ascent photometer (Thermo-Labsystems, Finland). To correct for background binding in the serum, the signal generated for the samples in PBS-coated wells was subtracted from that generated in mycolic acid-coated wells.

2.3. Preparation of liposomes

Stock solution of phosphatidylcholine (100 mg/ml) (Sigma, St Louis, MO) was prepared by dissolving the weighed amounts in chloroform. Mycolic acids-containing liposomes were prepared by adding 90 µl of phosphatidylcholine stock to 1 mg of dried mycolic acids. Empty liposomes, i.e. with no mycolic acids, were prepared by using 90 µl of phosphatidylcholine stock solution only. 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 °C for about 10 min. Liposome formation was induced by addition of 2 ml of saline (0.9% NaCl) and placing in a heat block at 85 °C for 20 min, with vortexing every 5 min. The liposomes were then sonicated for 2 min at 30% duty cycle at an output of 3% with a Model B-30 Branson sonifier (Sonifer Power Company, USA). The sonicator tip was washed with chloroform and rinsed with distilled water before and after use. The liposomes (200 µl) were aliquoted into ten tubes and kept at −20 °C overnight before freeze-drying. After freeze-drying, 2 ml of phosphate-buffered saline (PBS) azide EDTA (Sigma, St Louis, MO) buffer (PBS/AE, pH 7.4) was added to each tube containing liposomes. The tubes were placed in a heat block for 20 min and sonicated as before.

2.4. Isolation of IgG using protein A Sepharose

A TB-positive patient serum was selected from a collection of sera supplied by the Medical Research Council (MRC) (Clinical and Biomedical Research Unit, King George V Hospital, Durban, 1994). The only criterion for selecting this serum for isolation was that it had previously given good responses to mycolic acid on ELISA (unpublished results). TB-negative samples, which consisted of pooled sera from healthy students working in the Biochemistry Department of the University of Pretoria, were used as negative controls. A modified method for IgG purification was used compared to that given in the literature (Seppala et al., 1990). Protein A, immobilized on Sepharose (3 ml; Fluka, BioChemika) was transferred to a 50-ml tube. The Sepharose was washed twice by centrifuging at 3000 rpm for 5 min, the supernatant removed by pipette and 10 mM Tris–HCl buffer (pH 8.08) added until the final volume in the tube was 20 ml. After the last washing step, the supernatant was removed, leaving the Sepharose just covered in buffer. Serum aliquots (500 µl each) were thawed at 37 °C for 30 min. Two aliquots (1 ml) were added to the Sepharose beads and the volume was adjusted to 20 ml with 10 mM Tris–HCl to effect a final serum dilution of 1/20. The serum was incubated with the Sepharose at room temperature with rotation for 2 h and then left to stand for the Sepharose to settle out. The supernatant was removed by pipette and transferred to a labelled 50-ml tube. This represented the IgG-depleted serum fraction.

The remaining bound IgG was removed from the protein A beads by washing with 100 mM Tris–HCl, keeping the final volume in each tube at 20 ml and then removing the supernatant after gentle mixing. This was repeated twice, after which the buffer was changed to 10 mM Tris–HCl for another three washes. The supernatant of the last washing step was measured at 280 nm against a suitable blank for the efficiency of washing. The Sepharose in the tube was finally washed once more with 10 mM Tris.

IgG was eluted with 0.1 M glycine (3 ml, pH 2.7) into tubes containing 1 M Tris–HCl (200 µl) to neutralize the acid. This process was repeated seven times using 0.1 M glycine (2 ml) and 2 min incubation before removing the next IgG supernatant. The efficiency of elution of protein from the beads was monitored at 280 nm against a suitable blank. The IgG fractions were pooled and dialyzed against 1.5 l of PBS with two exchanges after 12 h and 18 h. The IgG-depleted serum fraction isolated earlier was also dialyzed in the same manner. After dialysis, the solutions were transferred to 50 ml tubes, and 100 µl were removed for protein concentration determination. To the IgG (12 ml) and depleted serum fractions (12 ml), 99% glycerol (8 ml, 40% final concentration) was added and filtered through a 0.2-µm filter (Ministart Plus, CA-membrane and GF-prefilter) into sterile 50-ml tubes that were subsequently stored at 4 °C. This isolation process was repeated for the TB-negative serum sample. The protein A Sepharose beads were regenerated by washing twice with 0.1 M glycine (5 ml) and four times with 100 mM Tris–HCl (10 ml), until the pH was above 7. The Sepharose was stored in 100 mM Tris–HCl solution (10 ml, containing 1% benzyl alcohol).

2.5. Removal of glycerol for biosensor

To prepare the IgG and depleted serum fractions for IAsys biosensor analysis, the glycerol had to be removed. VetaSpin Micro columns (400 µl, Whatman) were pre-washed with PBS (400 µl) by centrifuging at 10 000 × *g* for 15 min. The glycerol-containing samples (400 µl) were then transferred to the spin columns and centrifuged at 10 000 × *g* for 40 min. The filtrate was removed and 200 µl of PBS added to the retentate and again centrifuged. This process was repeated another five times for each glycerol-containing sample. The final volume was reconstituted to the original sample volume (400 µl) with PBS. The concentration of IgG in the purified samples was determined by absorbance measurement at 280 nm using a Shimadzu spectrophotometer.

2.6. SDS-PAGE of isolated IgG and depleted serum

The protein concentration of the IgG fractions was determined using the absorbance at 280 nm and an extinction coefficient of 1.4, whereas the concentration of depleted serum and other protein fractions was determined using the Bradford assay. An amount corresponding to 10 µg per sample was aliquoted into Eppendorf tubes and four volumes of acetone added. The samples were then left to precipitate overnight at −70 °C. The next day the samples were centrifuged at 14 000 × *g* for 1 h at 4 °C. The supernatant was discarded and the samples dried under vacuum for 20 min. Deionized water (8 µl) was added to re-suspend the protein pellet and non-reducing sample buffer (8 µl) was added. The samples were boiled at 100 °C for 5 min. SDS-PAGE analysis of purified IgG fractions and depleted sera was performed according to Ey et al. (1978) in a MINIVE complete vertical electrophoresis system (Amersham Pharmacia Biotech AB, USA). Approximately 10 µg of protein was loaded per lane. The gel

consisted of a 10% separating gel and a 4% stacking gel. The low molecular weight markers contained 97, 66, 45, 30, 20.1 and 14.4 kDa standards. The loaded gel was first run at 60 V for 1 h and then at 120 V for 3 h until the indicator was 0.5 cm from the end of the gel.

2.7. Detection of anti-mycolic acid antibody (IgG) with the IAsys affinity biosensor

The PBS/AE buffer (8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.05 g Na_2HPO_4 per litre, 1 mM EDTA and 0.025% (m/v) sodium azide) was prepared in double-distilled water and adjusted to pH 7.4. Cetyl-pyridinium chloride (CPC, 0.02 mg/ml) and saponin (1 mg/ml) were prepared in PBS/AE. The IAsys resonant mirror biosensor system and twin-cell non-derivatized cuvettes were from Affinity Sensors (Cambridge, UK). The sensor was set to a data-sampling interval of 0.4 s, a temperature of 25 °C and a stirring rate of 75% for all experiments. The cells were rinsed three times prior to use with 96% ethanol (Saarchem, SA), followed by extensive washing with PBS/AE. A 60-µl volume of PBS/AE was pipetted into each cell of the cuvette to obtain a stable baseline for 1 min. The PBS/AE was subsequently aspirated and the surface activated with 50 µl of CPC for 10 min. This was followed by washing five times with 60 µl of PBS/AE and then substituting with 25 µl of PBS/AE for a new baseline before immobilization of mycolic acids-containing liposomes to the surface for 20 min. The immobilized liposomes were then finally washed five times with 60 µl of PBS/AE, substituted with 50 µl of saponin and incubated for 10 min. This latter step was to avoid non-specific binding on the surface of the cuvette during the subsequent binding events. The cells were then washed five times with PBS/AE, the content of each cell substituted with 25 µl of PBS/AE and left for about 5–10 min to achieve a stable baseline. Inhibition studies were performed using patient serum that was first left 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 each cell, to compare the responses of the two cells over 10 min. A pre-incubation of 1/500 dilutions of serum with solutions of liposomes containing mycolic acids and empty liposomes (phosphatidylcholine alone) were allowed for 20 min. These were then added (10 µl) for binding inhibition studies in different cells, one with mycolic acid liposomes and the other with empty liposomes as a control, and allowed to bind for 10 min. Inhibition studies using IgG antibody were also performed, but differently. After obtaining a stable baseline, 10 µl of IgG solution (0.04 mg/ml) in PBS/AE were added to both cells and the response measured over 10 min as above. Subsequently, 10 µl of 0.08 mg/ml IgG diluted with either mycolic acids or phosphatidylcholine liposomes were added and the response measured for 10 min. Finally, dissociation of antibodies was effected by washing three times with PBS/AE and measurement of the response for 5 min.

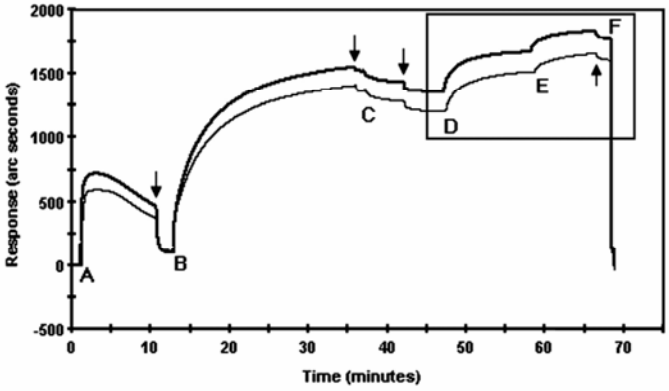

Fig. 1. A typical graph summarizing the process of measuring antibody binding or inhibition of binding by mycolic acid and empty (phosphatidylcholine only) liposomes, in the two cells of an IAsys biosensor cuvette surface coated with mycolic acid liposomes. The surface was activated with cetyl-pyridinium chloride (A), coated with mycolic acid 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 potassium hydroxide (12.5 M) and 96% ethanol (F). The arrows indicate washing with PBS/AE and the response from the two cells are differentiated by thick lines (channel 1, upper curve) and thin lines (channel 2, lower curve).

2.8. Regeneration of non-derivatized cuvettes

Regeneration was effected by three initial washes with 96% ethanol for 1 min, followed by washing seven times with 70 µl of PBS/AE for 1 min. The surface was then finally treated with 50 µl of potassium hydroxide (12.5 M) for 2 min followed by seven washes with 70 µl of PBS/AE for 1 min.

3. Results

The six main stages involved in measuring the binding of specific antibodies to lipid antigens in liposomes in real time on the biosensor are: (A) activation of the non-derivatized cuvette surface with CPC; (B) immobilization of the liposomes containing 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) binding and dissociation of inhibited patient serum at lesser dilution; and finally (F) surface regeneration (Fig. 1). The dilutions of serum used were estimated from a dilution range of one positive and one negative serum sample and are not necessarily optimal for all sera. The cuvette cell calibration curves for the high dilution serum in the two cells of one cuvette had to

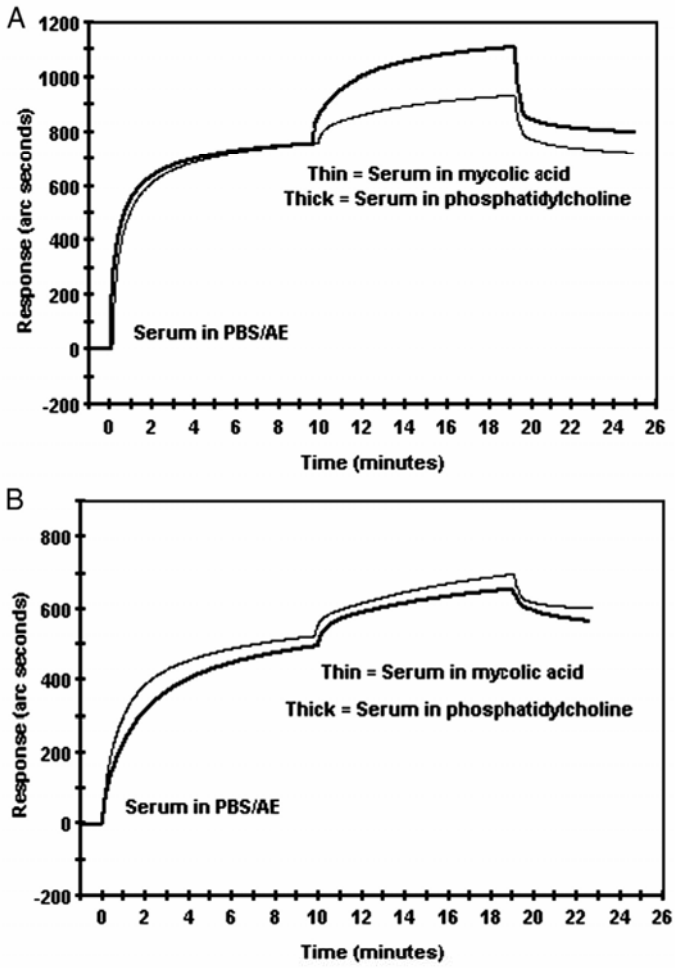

Fig. 2. Inhibition of human TB^+ (A) and TB^- (B) patient serum antibody binding with mycolic acid liposomes or empty liposomes on an IAsys cuvette surface coated with immobilized mycolic acid liposomes. For the first 10 min, a 1/1000 dilution of serum in PBS/AE was incubated in both cells. For inhibition studies, the pre-incubated serum in a dilution of 1/500 was then added with the thin line (A, lower curve; B, upper curve) representing the binding response of serum in mycolic acids and the thick line (A, upper curve; B, lower curve) representing that of serum in empty liposomes as control.

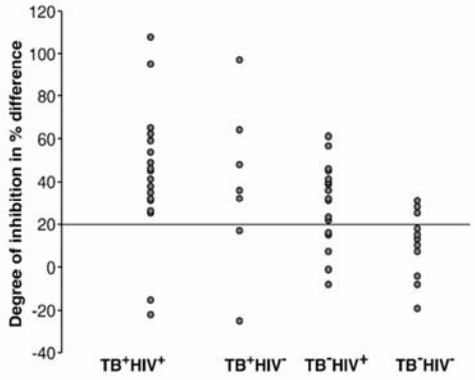

Fig. 3. The percentage of inhibition of binding of the biosensor signal for the 61 patient sera of TB^+ and TB^- controls after pre-incubation of sera with mycolic acid liposomes and empty liposomes before testing on mycolic acid-coated cuvettes.

fall within 90–100% identity in terms of the relative response amplitudes in order to be accepted. A limitation of the IAsys system was found to be the difference in quality from one cuvette to another when using liposomes as antigen coat. In rare cases, new cuvettes were not usable at all. Usually, new cuvettes were found to be reliable only after a succession of regenerations, while in other rare cases, new cuvettes could be reliably applied after a single regeneration cycle. The results (those in the rectangle, Fig. 1) were aligned using the Fastplot programme from IAsys.

3.1. Detection of anti-mycolic acid antibodies in human serum

Patient sera selected from the collection of Schleicher et al. (2002) were used to detect antibodies against mycolic acids on the optical IAsys biosensor. The ELISA experiments were performed as described in Schleicher et al. (2002). Of the 61 patient sera that were analyzed on the IAsys biosensor, 17 were re-analyzed on ELISA to confirm that the original antibody activity as reported by Schleicher et al. (2002) was still intact and to compare them with the results found on the IAsys biosensor during the same period of assessment. The inhibition studies on the IAsys were determined by pre-incubating test serum with mycolic acids-containing liposomes and applying these on biosensor cuvettes coated with mycolic acids. In the control experiments, sera were pre-incubated with empty liposomes. The pre-incubation of a sputum-positive TB patient serum with mycolic acid liposomes resulted in inhibition of antibody binding to mycolic acids when compared to the signal generated by the same serum pre-incubated with empty liposomes (Fig. 2A). This confirmed the specificity of binding of antibodies to mycolic acids in sputum-positive TB patient sera.

There was no inhibition of binding observed when a sputum-negative control serum (TB^-HIV^-) was pre-incubated with liposomes containing mycolic acids and tested on the biosensor to determine binding of antibodies to mycolic acids (Fig. 2B). This shows that specific anti-mycolic acid antibodies can be demonstrated in TB^+ patients, after pre-incubation of serum with mycolic acids. TB-negative sera from patients infected with HIV tested negative on the IAsys biosensor, with inhibition values of less than 20% (Fig. 3).

From 23 TB^+HIV^+ patient sera selected, two serum samples tested false negative on the biosensor (Fig. 3). Thirteen TB^-HIV^+ patient sera tested false positive, showing an inhibition of greater than 20% on the biosensor (Fig. 3). It is noteworthy that these patients were HIV-positive. Some patient sera that were false negative (e.g. Fig. 4A) and false positive (e.g. Fig. 4B) on the ELISA tested positive and negative respectively on the biosensor. The normalized signals on ELISA that were above two were regarded as positive and below two as

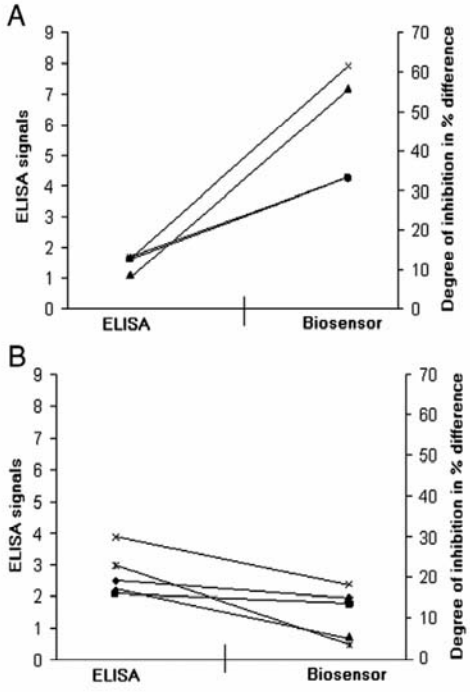

Fig. 4. Normalized ELISA signals and the percentage of inhibition of binding of the biosensor signal of false negative (A) and false positive (B) patients on ELISA who tested correctly on the biosensor.

negative. The TB^+ patients who showed truly positive responses of antibodies to mycolic acids on ELISA also tested truly positive on the biosensor. Our previous studies have also addressed the problems of detecting *M. tuberculosis*-specific antibodies to mycolic acid in TB patients co-infected with HIV on ELISA (Schleicher et al., 2002). Three of the patient sera tested from the 13 HIV^-TB^- tested false positive on the biosensor, and only two serum samples tested false negative in the TB^+HIV^- population (Table 1). An apparently lower specificity (27.8%) was observed in the TB^-HIV^+ sub-groups. However, all these patients were hospitalized with diseases other than TB based on the prevailing diagnostic methods. The low specificity obtained amongst the HIV^+ population could reflect true positive results, since it is known that the sputum culture assay is not sensitive enough to detect TB in HIV-positive patients (Frieden et al., 2003). This may reflect the fact that the serum test is better able to detect TB in HIV^+ patients. The IAsys affinity biosensor was found to be more sensitive (91.3%) in detecting TB amongst the TB-positive patients co-infected with HIV. The overall specificity and sensitivity of the assay after analyzing 61 patient sera was 48.4% (15/31) and 86.7% (26/30), respectively.

It is known that the gold standard of sputum growth of mycobacteria does not measure accurately in the TB^-HIV^+ cohort (Table 1). As the serum collection was actually made for an earlier study, follow-up data were not available to determine the true TB status of the TB^-HIV^+ cohort tested here. When the 18 TB^-HIV^+ sera were omitted in the calculation of the performance parameters of the test based on the 61 data points, the accuracy of the assay was found to be 83.7% (36/43). The sensitivity (86.7%, 26/30) remained the same after exclusion of the TB^-HIV^+ population, and the specificity was 76.9% (10/13). The assay showed a high sensitivity (91.3%) in sera from patients who were TB-positive and co-infected with HIV. It is known that HIV-positive patients generally have lower levels of *M. tuberculosis*-specific antibodies to protein and certain lipid antigens than HIV-negative patients. This shows that the IAsys biosensor can detect anti-mycolic acid antibodies in an HIV endemic population.

Table 1
Specificity and sensitivity of the IAsys affinity biosensor assay for detecting anti-mycolic antibody in pulmonary TB and negative control patient sera

Patient group	No. of patients	False positive	False negative	Specificity (%)	Sensitivity
TB^+HIV^+	23	–	2	–	91.3 (21/23)
TB^+HIV^-	7	–	2	–	71.4 (5/7)
TB^-HIV^+	18	13	–	27.8 (5/18)	–
TB^-HIV^-	13	3	–	76.9 (10/13)	–
Total	61	16	4	48.4 (15/31)[a]	86.7 (26/30)[a]

[a] Accuracy=81.8% (the data for the specificity of the TB^-HIV^+ group is omitted because of the known underestimation of TB positives by standard culture growth assays).

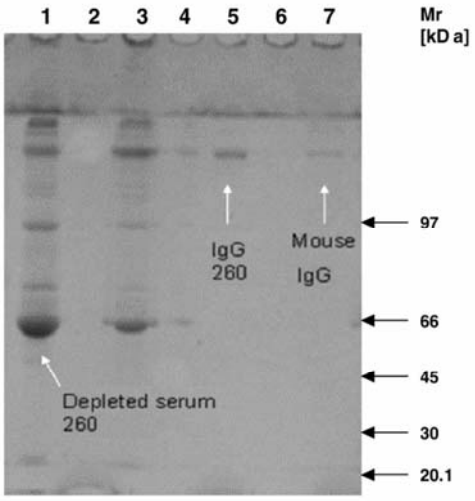

Fig. 5. SDS-PAGE gel indicating the purity of the IgG fraction in relation to a standard mouse IgG sample. The depleted serum fraction in lane 1 still contains IgG along with albumin at 66 kDa and other unidentified proteins. Samples were loaded in non-reducing buffer bovine serum albumin (BSA): (1) depleted serum no. 260, (2) blank, (3) whole serum no. 260, (4) blank, (5) IgG fraction from patient no. 260, (6) blank, (7) mouse IgG.

3.2. Isolation of IgG

To assess whether the inhibited signal on the IAsys affinity biosensor was due to the IgG in human sera, isolation of IgG was done using protein A Sepharose beads, which are specific for IgG but not for the other classes of immunoglobulin. The serum was not fully depleted of IgG as is evident from Fig. 5. The IgG fractions were expected to represent a repertoire of antigen binding specificities found in serum. The eluted IgG fractions were tested to bind mycolic acid on the IAsys biosensor.

3.3. Analysis of IgG on the IAsys biosensor

The isolated IgG from TB-positive serum no. 260 and TB-negative serum was analysed on the IAsys resonant mirror biosensor to confirm that the binding of

antibody to mycolic acid is due to the IgG. The liposomes containing mycolic acids were immobilized on non-derivatized cuvettes after activation with CPC as described previously. The response of IgG in PBS/AE to mycolic acid liposomes was measured in arc seconds at an IgG concentration of 0.04 mg/ml to detect high affinity binding. When comparing the binding of IgG from the TB-positive serum and IgG from negative serum to mycolic acid liposomes, there was a significant difference (paired Student's *t*-test with alpha=0.05; Table 2).

Inhibition experiments are necessary in the biosensor analyses of sera, as no adequate difference in direct binding in PBS/AE was observed between TB-positive and TB-negative sera to provide improved diagnosis over that obtained by ELISA. This was thought to be due to serum components binding non-specifically to the liposomes. By incubating the serum in mycolic acid liposomes, the highly specific antibodies would be complexed and prevented from binding to the immobilized mycolic acid liposomes on the cuvette surface. We attempted to apply the same principle to the IgG samples. The purified IgG in PBS was mixed with either phosphatidylcholine or mycolic acid liposomes to afford a final concentration of 0.08 mg/ml of IgG in PBS/AE and the response was measured over 10 min as previously done for serum. The degree of binding of IgG to the immobilized mycolic acid liposomes after mycolic acid or phosphatidylcholine incubation was compared to determine whether the IgG could be inhibited.

The inhibition signals of IgG from patient 260 and the IgG negative control were measured on different channels of the IAsys biosensor. A significant difference in the ability of IgG 260 and IgG negative to be inhibited by mycolic acid liposomes was determined with Student's *t*-test using an alpha value of 0.05 (Table 2). These results correlate with previously obtained data using whole serum and confirm that it is the IgG fraction of serum that is inhibited from binding by preincubation with the mycolic acid antigen.

Table 2
Binding of IgG antibody purified from TB^+ and TB^- serum to mycolic acid liposomes

Biosensor signal	Patient (±SEM)	
	TB^+ (no. 260)	TB^- control
Serum binding (arc seconds)	462±35	248±24
IgG binding (arc seconds)	70±10	31±1
IgG binding inhibited with mycolic acid (%)	54±3.4	24±6

SEM, standard error of the mean; $n>4$.

4. Discussion

In Africa, TB is often the first manifestation of HIV infection; it is the leading cause of death among HIV-infected patients. Corbett et al. (2006) stated that every opportunity should be taken to screen HIV-infected patients for active TB in order to prevent rapid death when both diseases manifest themselves in an individual, and to provide safe antiretroviral (ARV) treatment. The shorter the time from sampling to the diagnostic result, the more lives will be saved. Serodiagnosis with mycolic acids as antigen provides such an opportunity (Verschoor and Onyebujoh, 1999).

Pan et al. (1999) indicated that the anti-cord factor antibodies (IgG) in TB patients specifically recognized the mycolic acid structure, especially methoxy mycolic acid methyl esters. Mycolic acid is presented by antigen-presenting cells (APC) through a mechanism that does not involve major histocompatibility complex (MHC)-class I or MHC-class II molecules (Moody et al., 1999). The anti-mycolic acid immune response could therefore be independent of the participation of $CD8^+$ or $CD4^+$ T cells that respond to antigen presented on MHC I and MHC II surface proteins, respectively. Other than the MHC-presented protein antigens, mycolic acid is presented on CD1, with the ability to induce proliferation of T cell lines, with or without the CD4 or CD8 molecules (Beckman et al., 1994; Goodrum et al., 2001). The production of antibodies to protein antigens generally depends on the help of $CD4^+$ T cells. It is known that infection with HIV results in depletion of $CD4^+$ T cells and inhibition of the function of the remaining T cells (Price et al., 2001). Thus, Hendrickson et al. (2000) showed a decreased antibody specificity and sensitivity to a mycobacterial 30-kDa protein antigen with ELISA when screening patients in a population that had a high prevalence of HIV. Ratanasuwan et al. (1997) showed that when a lipoarabinomannan (LAM) was used in serological tests on HIV-negative and TB-positive patients, it showed sensitivities varying from 21% to 89%, but only 7% to 40% in HIV-positive patients. Antunes et al. (2002) described the MycoDot serological assay for tuberculosis, which is based on the detection of specific IgG antibodies against the LAM antigen, fixed onto a solid support consisting of a plastic comb designed to fit into the wells of a microtitre 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. Daniel

et al. (1994) performed a field test in Mexico, and showed that 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 similar to the results of the Mexico test, the ELISA gave a sensitivity of 28% in 128 sera from HIV-positive donors. However, the immune response to mycolic acid could in principle proceed independently of the $CD4^+$/$CD8^+$ T cells. The human CD1 protein is known to mediate T cell responses by presenting at least three classes of mycobacterial lipids, i.e. free mycolates, glycosylated mycolates and diacylglycerol-based glyco-phospholipids. The alkyl chains of the mycolic acid antigen have 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 provide a possible explanation why the antibody response to mycolic acids is maintained in HIV-seropositive patients, despite a declining CD4 T lymphocyte count (Schleicher et al., 2002). Simonney et al. (2007) also suggested that the CD1-restricted lipid antigen presentation pathway is the likely mechanism accounting for the perseverance of high circulating antibody responses to PGL-Tb1 antigen in HIV-infected patients with TB. Simonney et al. (2007) showed that about half of HIV-positive individuals produce specific anti-glycolipid antibody several months before a diagnosis of TB disease can be made.

Here, a significant increase in sensitivity and specificity was shown for anti-mycolic acid antibody detection in patient serum with the inhibition assay on the biosensor, compared to that reported in our previous study using an ELISA assay (Schleicher et al., 2002). The false positive results observed amongst the TB^- HIV^+ population could show that the patients were true positive on the IAsys biosensor, since a sputum culture was used as the gold standard method for confirming their TB status. However, it is known that sputum culture of HIV-infected patients needs more incubation time than that of patients without HIV infection, which is consistent with the lower bacillary load seen in the sputum of HIV-infected patients (Brindle et al., 1993). The culture requires 10–100 viable *M. tuberculosis* per millilitre of sputum to give positive results (Colebunders and Bastian, 2000). It has also been shown that 15–20% of adults with pulmonary TB whose diagnosis was based on clinical, radiographic, and histopathological findings and response to anti-TB treatment have negative sputum cultures (Frieden et al., 2003).

The IAsys affinity biosensor was able to detect low affinity antibody binding to mycolic acids, in addition to high affinity antibody, which the conventional methods cannot generally achieve. In an ELISA, these antibodies would have been washed away before the final step and the patient would have tested false negative. The advantage of the biosensor lies in its real-time detection of antibody binding, without the need for prior washing away of the unbound antibody excess. In addition, the inhibition of binding as an endpoint eliminates much non-specific binding interference, which adds to the increased specificity of the biosensor assay. A disadvantage of the biosensor is that it is blind to the identity of the binding ligand from the serum sample. Here, the binding of IgG to mycolic acids was confirmed by showing that its binding inhibition could be reproduced with purified IgG from the same serum sample.

The few false negative results that still remain with the biosensor analysis are probably due to the inhibition of antibody activity by circulating mycolic acid antigen. Should this be the case, one can envisage that a duplicate test be run that is spiked with a stable source of anti-mycolic acid antibodies, such as monoclonal antibodies. A true negative will then return the spike signal, whereas a false negative will consume the signal. False positive results pose a more daunting technical challenge, but may be due to the cross-reactivity of antibodies to mycolic acids of non-tuberculous pathogenic mycobacteria, e.g. *M. avium*, which do occur at low frequency, especially in HIV-positive patients. More work is required to manage the specificity of the assay by, for instance, screening sera from patients that are TB negative, but test positive for *M. avium* disease. This work is currently underway.

Many serological assays have been developed for detection of specific antibody to lipid cell wall antigens in tuberculosis patients (Lyashchenko et al., 1998; Pan et al., 1999; Pottunarthy et al., 2000; Julian et al., 2002; Schleicher et al., 2002; Lopez-Marin et al., 2003; Fujita et al., 2005), but generally they do not meet the requirements on specificity and sensitivity (Attalah et al., 2005). The biosensor approach may improve that by means of its unique benefits reported here. However, the technique is technically quite difficult to perform in the laboratory and the technology is not yet suitable for large scale screening of patients.

Since only 61 patients were analyzed with the biosensor in this study, more patient and control sera will have to be analyzed to properly validate it as a reliable technique to determine anti-mycolic acid antibodies as surrogate markers for active tuberculosis. However, the

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

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Appendix B: Mycolic acids and cholesterol

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Cholesteroid nature of free mycolic acids from *M. tuberculosis*

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Abstract

Mycolic acids (MAs) are a major component of the cell walls of *Mycobacterium tuberculosis* and related organisms. These α-alkyl β-hydroxy long fatty acids have been the subject of numerous studies for their immunological properties. We previously reported that an interaction between cholesterol and mycolic acids could be responsible for the low accuracy in the serodiagnosis of TB when using free mycolic acid in an ELISA assay. The aim of this work was to investigate if this interaction could be due to a similarity in the structural properties between mycolic acids and cholesterol. The investigation revealed that patient sera cross-reacted with mycolic acids and cholesterol in an ELISA experiment suggesting that both molecules may present related functionality in a similar structural orientation. This relation was further supported by the interaction of mycolic acids with Amphotericin B (AmB), a known binding agent to ergosterol and cholesterol. Using a resonant mirror biosensor, we observed that AmB recognised both cholesterol and mycolic acids. In addition, a specific attraction was observed between mycolic acid and cholesterol by the accumulation of cholesterol from liposomes in suspension onto immobilized mycolic acids containing liposomes, detected with a biosensor technique. Combined, these results suggest that mycolic acids can assume a three-dimensional conformation similar to a sterol. This requires that mycolic acid exposes its hydroxyl group and assumes rigidity in its chain structure to generate a hydrophobic surface topology matching that of cholesterol. A particular folded conformation would be required for this, of which a few different types have already been proven to exist in monolayers of mycolic acids.

Keywords: Mycolic acids; Cholesterol; Lipid conformation; Amphotericin B; Resonant mirror biosensor

1. Introduction

Tuberculosis is one the most life-threatening infectious diseases. Its re-emergence has brought new interest in finding novel methods for its treatment and diagnosis, especially in developing countries where the population is most burdened by this pandemic and where the connection between TB and HIV/AIDS infection is particularly worrying. In fact, TB is the major cause of death in HIV/AIDS co-infected individuals (Uma Devi et al., 2003).

In the search for new surrogate markers for the diagnosis of tuberculosis, antibodies to cord factor (trehalose-6,6′-dimycolate, TDM) have attracted much attention. Although having a hydrophobic nature, TDM is a very immunogenic and biologically active substance present in the mycobacteria and a few related genera. TDM is a glycolipid consisting of trehalose, which is identical for all cord factors, and two of a set of mycolic acids (MAs) (**1**, Fig. 1), which differ within and among species and genera (Ryll et al., 2001). For example, *Mycobac-*

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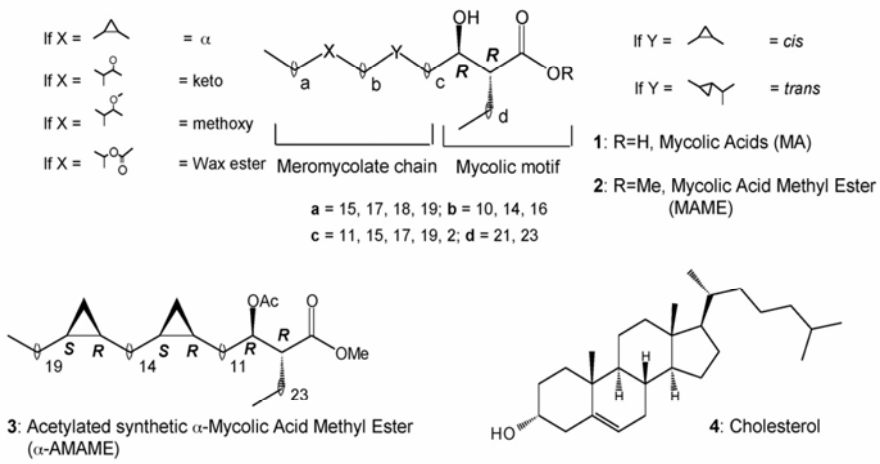


Fig. 1. Molecules under investigation for their similarity in function.

terium tuberculosis contains α-, methoxy- and keto-mycolic acids subclasses, whereas *Mycobacterium avium* contains wax ester—instead of methoxymycolic acids (Dobson et al., 1985). Fujiwara et al. (1999) identified the antigenic epitope in cord factor molecules to be the mycolic acids by proving that antibodies against TDM from TB patients could distinguish between mycolic acids subclasses.

We have reported the use of free mycolic acids as antigens for the serodiagnosis of tuberculosis; they have considerable potential because HIV–TB co-infected patients maintain high antibody levels to mycolic acids, despite the severity of the immune deficiency measured by the fall in CD4 T cell count (Schleicher et al., 2002). However, in an ELISA assay, the use of free mycolic acids was not adequate for serodiagnosis of tuberculosis (accuracy = 57%). An association between mycolic acids and cholesterol was hypothesised as a possible reason for this low accuracy (Schleicher et al., 2002). Cholesterol may be non-specifically attracted to mycolic acids by means of hydrophobic Van der Waals type of binding, or by a more specific interaction such as a hydrogen bond, arising from conformational features present in the two molecules.

Recently, free mycolic acids have been demonstrated to be able to adopt a folded conformation to give a hydrophobic surface. In particular, the "W" conformation has the alkyl chains folded to give four parallel arms, while in the "Z" conformation three folded arms provide the three-dimensional, curved hydrophobic surface (Villeneuve et al., 2007; Sekanka et al., 2007). The existence of these conformations has been suggested by analyses of Langmuir monolayers consisting of free mycolic acids over a range of temperatures (Villeneuve et al., 2005, 2007). In an extended form, the shape and structure of a mycolic acid appears very different to that of cholesterol and does not suggest particularly strong interactions between these molecules. However, the folded conformations of mycolic acids could be imagined to assume a shape similar to that of cholesterol.

The current study was designed to investigate the possibility that the low accuracy measured for a serodiagnostic ELISA assay based on the use of mycolic acid as antigen could be the result of the folding of free mycolic acids to resemble a "cholesteroid" shape. In order to investigate this, we have used an approach similar to that employed by Prendergast et al. (1998) to suggest molecular mimicry between microbial and self-structures in autoimmune diseases. The basic assumption of this approach is that the specific molecular recognition of two substances by an established binding agent indicates resemblance in the three-dimensional structure of the two compounds. Therefore we further investigated the interaction of mycolic acids and cholesterol with sera from human TB patients, using the ELISA technique described by Schleicher et al. (2002). Secondly, using a resonant mirror biosensor (Cush et al., 1993; Buckle et al., 1993; Athanassopoulou et al., 1999), we compared the interaction of these two lipids with Amphotericin B (AmB), a known cholesterol binding molecule (Baginski et al., 2002, 2005). Thirdly, the attraction between cholesterol and mycolic acids was studied, also using the resonant mirror biosensor.

2. Materials and methods

2.1. General procedures

^{1}H, ^{13}C and COSY nuclear magnetic resonance (NMR) were recorded on a Bruker advance DRX-500 spectrometer or Bruker AC-300 spectrometer with chemical shifts in ppm. Analytical thin layer chromatography (TLC) was performed on Alugram SIL G/UV, layer: 0.2 mm silica gel 60 with fluorescent indicator UV_{254} plates. The plates were viewed under ultraviolet light (254 and 366 nm). Flash column chromatography was performed using Merck silica gel. FT-IR was obtained with a

PerkinElmer BX-1 instrument of 2.00 cm^{-1} resolution fitted with a MIR source, an internal LiT aO_3 detector and FT-IR Spectrum Software version 5.1. Optical rotations were measured on a PerkinElmer model 341 polarimeter. For mycolic acids analyses HPLC was performed using a Merck Hitachi Chromatograph fitted with a Phenomenex Luna 5 μm C18 column and a Merck Hitachi L-4500 Diode Array detector. For AmB derivative purification a Waters high performance liquid chromatography (HPLC) system was used with a Waters 610 fluid unit, Waters 600 controller and a Waters 996 Photodiode Array detector. A Waters C18 reverse phase column (150 mm × 4.6 mm) was used for optimization of methods and a Phenomenex Luna C-18 reverse phase column (250 mm × 10 mm, 10 μm) was used to collect the product. Electrospray mass spectrometry performed on a Micromass Micro Triple quadrupole mass spectrometer in full scan mode, range 500–2000 amu, was used to characterise the product. Sample infusion was directly into the electron spray source by means of a Hamilton syringe pump at 10 μl/min.

2.2. Preparation of methyl mycolic acids isolated from M. tuberculosis (H37Rv)

A mixture of mycolic acids (**1**) was isolated from the cell wall of the virulent Erdman strain of *M. tuberculosis* as described by Goodrum et al. (2001). HPLC and NMR spectra were in agreement with the data reported in literatures (Steck et al., 1978; Watanabe et al., 2001).

To form the methyl esters, mycolic acids (**1**, 100 mg, ~0.1 mmol) were dissolved in a mixture of toluene:methanol (5:1, 18 ml), trimethylsilyldiazomethane (TDM, 2 M solution, 0.2 ml, 0.4 mmol) added, followed by a further 4 additions of TDM (0.1 ml, 0.2 mmol) every 45 min. The mixture was stirred for 72 h, and then quenched by evaporation. The residue was dissolved in dichloromethane (15 ml) and water (10 ml) was added. The two layers were separated and the water layer extracted with dichloromethane (2× 10 ml). The combined organic layers were dried and the solvent evaporated to give the desired compound (98 mg, ~97%). The NMR spectra of the compounds obtained corresponded to those reported in the literatures for methyl esters of mycolic acids (MAME, **2**) (Watanabe et al., 2001; Al Dulayymi et al., 2003, 2007; Koza and Baird, 2007).

The acetylated synthetic alpha-mycolic acid methyl ester (α-AMAME, **3**) was kindly provided by Dr. J. Al Dulayymi, University of Bangor, UK (Al Dulayymi et al., 2003, 2005). Cholesterol (**4**), isoniazid, AmB, $NaBH_4$, terephthalaldehyde and phosphatidylcholine were purchased from Fluka or Sigma–Aldrich. All organic solvents were purchased from Merck while the inorganic materials were from Sigma.

2.3. Synthesis of Amphotericin B derivative

2.3.1. Preparation of N′-(4-formylbenzylidene)isonicotinohydrazide (6)

Isoniazid (**5**, 0.5 g, 3.65 mmol) was added in portions over an hour to a solution of terephthalaldehyde (0.49 g, 3.65 mmol) in ethanol (50 ml) and stirred at room temperature (RT) overnight. The precipitate that formed was removed by filtration and the mother liquor was concentrated to give a crude product (0.15 g) that was purified by flash column chromatography on silica gel in ethyl acetate:methanol:ethanol containing 1% triethylamine (90:9:1, v/v) to give pure *N′*-(4-formylbenzylidene) isonicotinohydrazide (**6**, 0.12 g, 14% yield).

Molecular weight 253.2599 g mol^{-1}. R_f 0.57 (ethyl acetate:methanol:ethanol with 1% Et_3N (v/v) (90:9:1); mp 219–220 °C; IR ν_{max} 3465, 3197 (NH), 1698 (CHO) cm^{-1}. ^1H NMR (300 MHz, $(CD_3)_2$ SO) δ 12.25 (1H, s, NH), 10.06 (1H, s, 1a), 8.80 (2H, d, J = 5.4 Hz, 10a), 8.55 (1H, s, 6a), 8.01 (2H, d, J = 9 Hz, 3a), 7.97 (2H, d, J = 9 Hz, 4a), 7.84 (2H, d, J = 5.4 Hz, 9a); ^{13}C NMR (300 MHz, $(CD_3)_2$ SO) δ 193.1, 162.3, 150.7, 148, 140.7, 139.9, 137.4, 130.3, 128.1, 121.9 ppm; HR–MS (EI) calculated for $C_{14}H_{10}O_2N_3$ [M^+] 253.08513 g mol^{-1} found 253.08389 g mol^{-1}, *m/z* (EI) 122 (74), 106 (100), 79 (10), 78 (47), 51 (22).

2.3.2. Preparation of N-(4-((2-isonicotinoylhydrazono)methyl)benzyl)-AmB (7, AmB derivative)

In the next step, the aldehyde (**6**, 0.01 g, 0.04 mmol) and Amphotericin B (0.043 g, 0.05 mmol) were stirred at RT in 2 ml dimethylsulfoxide in the dark for an hour. The derivative that formed was reduced *in situ* with $NaBH_4$ (0.001 g, 0.04 mmol) overnight and purified by RP-HPLC (methanol:H_2O a gradient from 20% to 100% methanol). The column eluent was monitored at 300 and 407 nm and AmB derivative collected and concentrated.

Molecular weight 1159.35 g mol^{-1} (calculated), melting point not determined due to decomposition of compound at about 40 °C, $[\alpha]_D^{20}$ (*c* 2.0, DMF), IR ν_{max} 3391, (NH, OH's), 1564 (double bonds) cm^{-1}. ^1H NMR (500 MHz, $(CD_3)_2$ SO) δ 12.06 (1H, s, H–NH), 8.79 (2H, d, J = 3.3 Hz, H-10a), 8.46 (1H, s, H-6a), 7.83 (2H, d, J = 4.4 Hz, H-6a), 7.51 (2H, d, J = 7.9 Hz, H-4a), 7.7 (2H, d, J = 7.9 Hz, H-3a), 6.45–6.07 (m, 12H, olefinic), 5.95 (1H, dd, J = 13, 5.3 Hz, H-20), 5.43 (1H, dd, J = 11.3, 12 Hz, H-33), 5.21 (1H, m, H-37), 4.48 (1H, broad-s, H-1′), 4.4 (1H, m, H-19), 4.24 (1H, m, H-11), 4.23 (1H, t, H-17), 4.06 (1H, m, H-3), 3.99 (1H, dt, H-15), 3.89 and 3.67 (2H, s, H-1a), 3.73 (1H, d, H-2′), 3.54–3.46 (HOD plus 3H), 3.2 (1H, m, H-5′), 3.09 (2H, m, H-4′, H-35), 2.82 (1H, m, H-3′), 2.28 (1H, s, H-34), 2.16 (3H, m, H-2, H-18), 1.88–1.05 (14H, m, CH_2, CH), 1.87 (1H, t, J = 6.7 Hz, H-16), 1.16 (3H, d, J = 5.5 Hz, CH_3), 1.11 (3H, d, J = 5.6 Hz, CH_3), 1.04 (3H, d, J = 5.6 Hz, CH_3), 0.91 (3H, d, J = 6.5 Hz, CH_3), ^{13}C NMR (300 MHz, $(CD_3)_2$ SO) δ 174.4, 170.6, 161.54, 150.3, 149.1, 143.6, 140.51, 133.9, 136.8, 133.7–128.5, 131.9, 127.2, 121.5, 97.2, 77.1, 73.8, 74.5, 74.4, 69.8, 73.6, 69.4, 68.8, 66.2, 65.4, 65.2, 63, 57.1, 44.7, 49.5–44.3, 42.3, 42, 35.1, 29, 18.5, 12.1 ppm.

2.4. ELISA

The human sera used in the ELISA experiments were from two sources: the first was from a pulmonary TB positive collection that was made in 1994 by the MRC Clinical and Biomedical TB Research Unit at King George V Hospital, Dur-

ban, KwaZulu-Natal and donated by Dr. P.B. Fourie. The second source was from a collection made in the year 2000 from patients for another study by Schleicher et al. (2002). From the latter, five serum samples were selected from patients who were determined to be negative for both tuberculosis and HIV (TB^-). These TB negative patients were hospitalised for various reasons other than TB or AIDS.

Mycolic acids (MA, **1**), acetylated synthetic α-mycolic acid methyl ester (α-AMAME, **3**) and cholesterol were used at final concentrations of 60 μg/ml. To prepare the coating solutions, the antigens were heated in PBS buffer for 20 min at 85 °C. The hot solutions were sonicated and kept at 85 °C during loading into the ELISA plates at 3 μg antigen per well. The assay with sera was done according to Schleicher et al. (2002). The results obtained were analysed for statistical differences using the Student's *t*-test.

2.5. Preparation of liposomes

Empty liposomes were prepared consisting of only phosphatidyl choline (PC), whereas the other liposomes consisted of PC in some ratio to the lipid under investigation. Mycolic acids (MA, **1**) liposomes and other chemically prepared mycolic acid liposomes contained MA or MAME (**2**) or α-AMAME (**3**) (7.8 mol%; mycolic acid: PC = 1 mg: 9 mg), whereas the cholesterol (Chol, **4**) liposomes contained Chol (50 mol%, Chol:PC = 3 mg:6 mg). The appropriate lipids were initially dissolved in chloroform in an amber glass vial and vortexed to ensure mixing. The samples were then dried at 80 °C under a stream of N_2 and sonicated in 2 ml saline for 5 min at room temperature. Sonication was done using a Branson sonifier (model B30, Branson Sonicpower Co. USA) with 30% duty cycles and output of 5. Subsequently the liposomes were divided into 200 μl aliquots, freeze-dried and stored at −70 °C until required for use. Before use, the liposomes were reconstituted with 2 ml of phosphate buffered saline (PBS/AE) pH 7.4 containing EDTA (1 mM), sodium azide (0.025%, m/v), heated at 80 °C for 15 min and then sonified as above. The final liposome concentration came to 500 μg/ml. The liposome suspensions were analysed for their mycolic acid content by HPLC according to Goodrum et al. (2001). Recovery of mycolic acids ranged between 70% and 110% of the expected values.

2.6. Measurements of interaction between mycolic acids, cholesterol and Amphotericin B derivatives on an IAsys affinity biosensor

The binding interactions among Amphotericin B, cholesterol, mycolic acids and acetylated synthetic α-mycolic acid methyl ester were measured by means of an IAsys resonant mirror biosensor (IAsys Affinity Sensors, Bar Hill, Cambridge, UK) according to the activity sequence outlined in Fig. 2. IAsys software was used to set the device at a data-sampling interval of 0.4 s, temperature of 25 °C and stirring rate of 75% for all experiments on the biosensor. The cells of the cuvette were rinsed with ethanol (95%) 3 times prior to use, followed by extensive washing with PBS/AE. A 60-μl volume of PBS/AE was

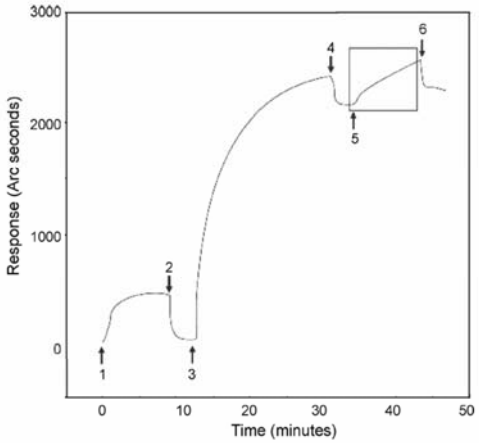
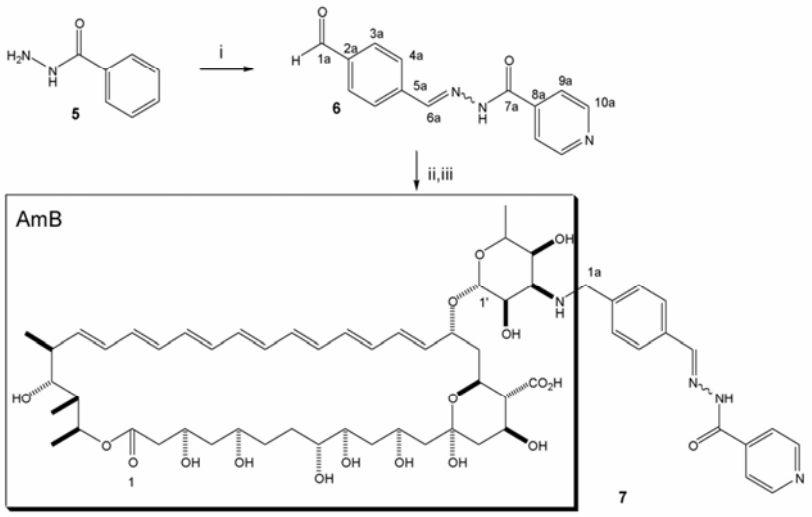


Fig. 2. Biosensor activity sequence to obtain sensorgrams of the binding of cholesterol from cholesterol-containing liposomes onto various immobilized lipid-containing liposomes. 1: activation of surface with CPC, 2: PBS/AE wash step, 3: addition of liposomes for coating, 4: PBS/AE wash step, 5: addition of the test solution, 6: PBS/AE wash step. The cholesterol binding sensorgram appears in the rectangle.

pipetted into each cell of the cuvette to obtain a stable baseline for 1 min. The PBS/AE was subsequently aspirated and the surface activated with 50 μl of cetyl-pyridinium chloride (CPC, 0.02 mg/ml in PBS/AE) for 10 min. This was followed by washing 5 times with 60 μl PBS/AE and then substituting with 25 μl PBS/AE for a new baseline before immobilization of mycolic acids (or cholesterol, or a synthetic α-AMAME, or PC only) containing liposomes to the surface for 20 min. The immobilized liposomes were then finally washed 5 times with 60 μl PBS/AE. The cuvette cell content was substituted with 25 μl of PBS/AE and allowed to equilibrate for 5–10 min to achieve a stable baseline. An addition of either 25 μl liposomes under investigation or 25 μl of a solution of AmB (1×10^{-4} M) or the AmB derivative (**7**, Scheme 1, 1×10^{-4} M) in PBS/AE was made. Direct interaction between the immobilized cholesterol/mycolic acids and the dissolved Amphotericin B compounds or lipids contained in the suspended liposomes was monitored for 5–10 min, after which the cuvette was washed 3 times with 60 μl PBS/AE. Finally, regeneration of the cuvette was effected by 5 times washing with 50 μl 95% ethanol for 1 min, followed by 7 times washing with 70 μl PBS/AE for 1 min. The surface was then finally treated for 5 times with 50 μl potassium hydroxide (12.5 M) for 2 min, followed by 7 times washing with 70 μl PBS/AE for 1 min. The results obtained were analysed for statistical differences using the Student's *t*-test.

2.7. Measurements of direct interaction between mycolic acids and cholesterol

After immobilization of either the mixture of natural mycolic acids (**1**), their methyl esters (**2**), or protected acetylated synthetic α-mycolic acid methyl ester (**3**) containing liposomes using the same method as described above and illustrated in



Scheme 1. Preparation of an AmB derivative: (i) terephthalaldehyde, (ii) AmB and (iii) $NaBH_4$.

Fig. 2, 25 μl cholesterol-containing liposomes were added. Direct interaction between the immobilized mycolic acids and cholesterol was monitored for 10 min, after which the cuvette was washed 3 times with 60 μl PBS/AE and regenerated as before.

3. Results and discussion

3.1. Interactions of patient sera to mycolic acids and cholesterol

It is known that all humans have anti-cholesterol antibodies (ACHA), which have been proven to be very specific for their interaction with the sterol. ACHA recognise selectively 3β-hydroxy-sterols in a stereospecific manner, but they cannot distinguish between enantiomers (Geva et al., 2001; Bíró et al., 2007). Concurrently, tuberculosis patients have been shown to produce antibodies against mycolic acids (Schleicher et al., 2002). Pan et al. (1999) reported that even small changes in the structure of the mycolic acids present in TDM are important for their antigenicity. In particular, among the three subclasses present in *M. tuberculosis*, methoxymycolic acids were shown to be the most antigenic. For this reason, we expected TB negative patient sera to react to cholesterol, but not to mycolic acids.

TB positive and TB negative sera were tested in ELISA for antibody binding to natural mycolic acid and cholesterol. To verify the specificity of the interaction between sera and mycolic acids, a synthetic α-mycolic acid (**3**), with the carboxylic acid protected as methyl ester and the hydroxyl group as acetyl ester, was used as negative control. This compound was chosen because it lacks a hydrogen donor at either the α-carboxylic acid, the β-hydroxyl group or in the meromycolate chain, which diminishes the possibility of hydrogen bonds with antibodies without alleviating the hydrophobic nature of the molecule that can be expected to be the major force of non-specific binding from serum components. Moreover, the protection of the polar groups in the mycolic motif also discourages any folded arrangement with the merochain (Villeneuve et al., 2005, 2007). Therefore, this change in the three-dimensional structure may be hypothesised to preclude specific interactions with antibodies against mycolic acids or cholesterol.

Coating of the wells with the lipid antigens was confirmed under the microscope as visible fatty deposits adsorbed on the polystyrene. Eleven TB positive patient sera were randomly chosen from the 1994 collection and five TB and HIV negative patients were randomly selected from the 2000 collection. For the TB negative controls, it was deemed important to exclude HIV positive individuals, for whom false negative TB diagnosis is known to occur at high frequency with the currently available TB diagnostics (Uma Devi et al., 2003). The sera were diluted 1:20, which was found to be the highest dilution where significant binding to the antigens on the plate could still be observed with most sera.

As expected there was a tendency for TB positive patient antibodies to bind more strongly to mycolic acid than those of the TB negative patients (average absorbance value 0.76 compared to 0.37 respectively, Fig. 3, $P<0.1$). The selectivity of binding with MAs was confirmed by the negligible antibody activity of the same patients to the synthetic alpha-mycolic acid in both groups of sera (Fig. 3, $P<0.0001$). Both TB positive and TB negative sera also recognised cholesterol as an antigen. In either TB positive or TB negative patients, the difference in ELISA signal between mycolic acid and cholesterol was not significant (Fig. 3, $P>0.25$). Although TB negative patients should not present antibodies against *M. tuberculosis* mycolic acids, the results obtained show that their sera recognise in a similar fash-

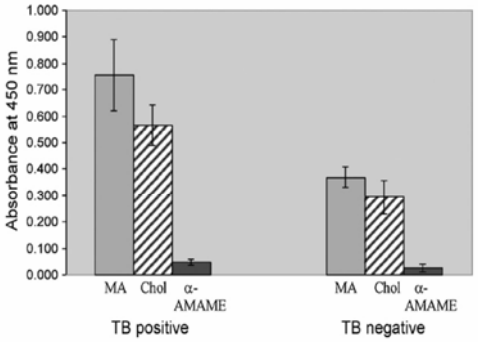


Fig. 3. Normalised ELISA results of TB positive ($n = 11$) and TB negative sera ($n = 5$) against natural mycolic acids (1) (MA, grey), cholesterol (4) (Chol, hatched) and an acetylated, synthetic α-mycolic acid methyl ester (3) (α-AMAME, black). The error bars indicate the standard error of the mean.

ion either cholesterol or mycolic acids. Such congruence of the titres of both antibody specificities could suggest cross-reactivity of recognition of cholesterol and mycolic acid by antibodies.

3.2. AmB cross-reactivity to mycolic acids and cholesterol

To further probe the possibility of resemblance in the nature of mycolic acid and cholesterol to bind to a ligand, an alternative experiment was designed using Amphotericin B (AmB) as a cholesterol binding agent. AmB is a macrolide antibiotic that exerts its antifungal activity by binding to ergosterol and cholesterol (Baginski et al., 2002). The major interaction between the macrolide and the sterol is based on the formation of a hydrogen bond between the amine or hydroxyl groups of the mycosamine moiety of AmB and the hydroxyl group of ergosterol (Herve et al., 1989; Matsumori et al., 2005). Other sterols, in particular cholesterol, are also recognised and bound by AmB, albeit with one order of magnitude weaker affinity. This is due to the more rigid hydrophobic sterol surface of ergosterol (which has two extra double bonds and a methyl group compared to that of cholesterol), making the Van der Waals interaction with the hydrophobic part of AmB stronger (Readio and Bittman, 1982; Baginski et al., 2002). The difference in binding affinity of AmB to ergosterol and cholesterol proves the specificity of the interaction between AmB and its sterol ligands. According to the approach of Prendergast et al. (1998), the specific molecular recognition of two substances by an established binding agent of one of them indicates a resemblance in the three-dimensional structure of the two compounds. For this reason, AmB was selected to assess its binding activity to cholesterol and to mycolic acid using resonant mirror biosensor technology. This technique was preferred to ELISA because it does not need a fluorescent tag on the binding agent that may affect the structure and interaction properties of AmB (Cush et al., 1993). Moreover using this technique it is possible to use liposomes which better mimic the biological environment within which these lipids are presented (MacKenzie et al., 1997).

In order to immobilize the liposomes onto the surface of the biosensor, a new procedure was used. This is based on the activation of the cuvette cell surface with cetyl-pyridinium chloride, a

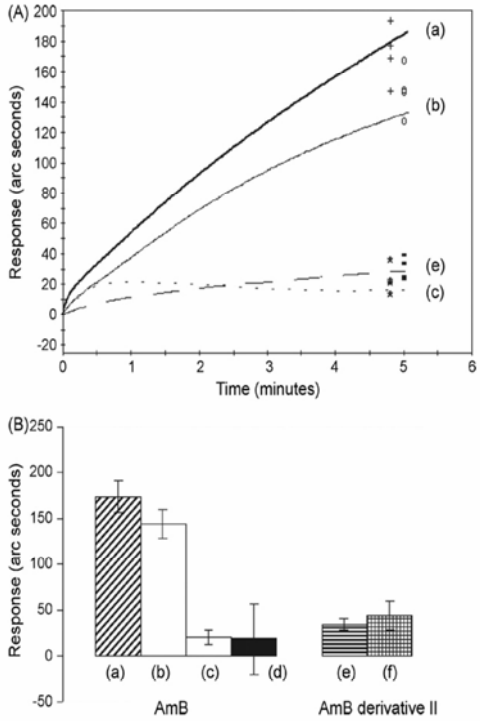


Fig. 4. Resonant mirror biosensor binding curves (A) and binding capacity (B) of AmB or AmB derivative (7) on immobilized lipid antigens. Binding of AmB on (a) mycolic acids (1) (thick line, +, hatched bar); (b) cholesterol (thin line, o, white bar); (c) synthetic protected α-MA (3) (dotted line, *, grey bar) or (d) PC liposomes (black bar). Binding of AmB derivative (7) on immobilized (e) cholesterol liposomes (dashed line, -, horizontal stripes bar) or (f) mycolic acid (1) containing liposomes (grid bar); $n = 5$ for each set. Immobilization of lipid ligate containing liposomes was monitored to achieve binding of at least 2000 arc seconds before contacting with AmB or AmB derivative (7) solutions.

frequently employed cationic, amphipathic compound that gives glazed and metal surfaces a hydrophobic character. Hydrophobic lipid antigen-containing liposomes can then adhere to the surface (see Fig. 2). Following the coating of the cuvette cells with either cholesterol-containing or empty liposomes, consisting only of phosphatidylcholine, the biosensor registered a direct accumulation of AmB on cholesterol liposomes, while it did not show any binding to empty liposomes (Fig. 4). Remarkably, a binding sensorgram comparable to that of immobilized cholesterol was obtained when AmB was allowed to interact with immobilized mycolic acids.

The binding between the AmB macrolide and cholesterol could be abrogated by covalent modification of the single amino group on the AmB macrolide by linking it to isoniazid to give Amphotericin B-derivative (**7**). The mycolic acid binding was similarly abrogated by the covalent modification of the AmB macrolide amino group. This modification to the amino group of the AmB molecule destroyed the latter's ability to bind to both cholesterol and the mycolic acid, showing that the attraction of AmB for both these lipids is determined by the fine structure of the ligand–receptor pair and possibly that this requires a specific hydrogen bond between the binding partners. Such an analysis would be in agreement with the literature, confirming the impor-

tance of the hydrogen bond for a stable interaction (Baginski et al., 2002). Matsumori et al. (2005) demonstrated that AmB's binding to sterols could be hindered when its amino-sugar moiety was forced in a conformation that prevented hydrogen bonding with its ligate. This suggests that the interaction between AmB and mycolic acids could also be based on the formation of a hydrogen bond with the amino-sugar moiety of AmB. It is noteworthy that AmB did not bind to the synthetic protected alpha-mycolic acid (**3**) immobilized on the sensor surface (Fig. 4), thus supporting the specificity of this interaction in terms of the requirement for both Van der Waal's and hydrogen bonding in the recognition event. These results imply that mycolic acid and cholesterol share structural features that are similarly recognised by AmB and that may provide the basis for the cross-reactivity observed with the TB patient antibodies.

3.3. Interaction between mycolic acids and cholesterol

In order to determine whether the presumed structural relatedness between mycolic acid and cholesterol would also lead to their interaction directly, the interaction between mycolic acids and cholesterol liposomes was analysed using the biosensor technique.

Following coating of the cuvette surface with either mycolic acid (**1**), mycolic acid methyl ester (**2**) or synthetic acetylated α-mycolic acid methyl ester (**3**), the cuvettes were exposed to cholesterol-containing test liposomes and the sensorgrams for the binding of cholesterol onto the different surfaces were recorded (Fig. 5). A mixture of methyl esters of natural mycolic acids (MAME), still containing hydrogen binding groups (β-hydroxyl and the oxygenated groups in the meromycolate chain), was also used as a negative control for this test. The experiments described earlier compared the interactions of mycolic acids and cholesterol with binding agents known to be able to differentiate between small conformational changes. The present experiment measured the attraction between two lipids and therefore it was important to verify that this is not just due to Van der Waals interactions between two hydrophobic molecules but also to a more specific conformational match between cholesterol and

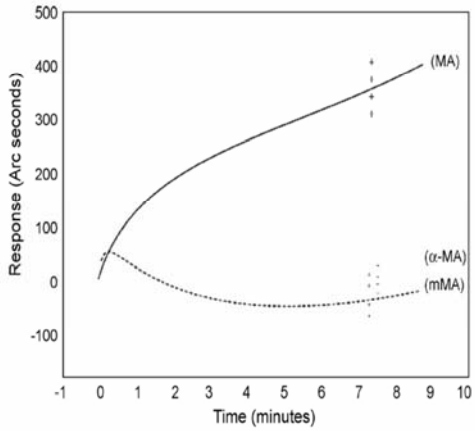


Fig. 5. Resonant mirror biosensor binding curves for the attraction of cholesterol to immobilized mycolic acids (1) (MA, thick line/+), their methyl esters (2) (mMA, dashed line/*) or synthetic protected alpha-mycolic acid (3) (α-MA, thin line/-). Each line represents a typical curve of five repeats with the end points of each indicated after 7.5 min of contact.

mycolic acid. The formation of a hydrogen bond between the carboxylic and the β-hydroxyl of mycolic acid is particularly favoured for the natural erythro configuration of 2*R*,3*R* 2-alkyl-3-hydroxy acids and has been shown to have a stabilising effect on the alignment of the alkyl chains, affecting the physical properties of these acids (Durand et al., 1979a,b). Therefore, the use of methyl esters of mycolic acids as a negative control is particularly important, as this modification will destroy the specific hydrogen bond in the mycolic motif that is expected to destabilise the natural conformations of the alkyl chains to assume a sterol fold, similar to that assumed by the steroid-precursor, the linear 2,3-oxidosqualene, before its conversion into lanosterol in cholesterol biosynthesis (Van Tamelen, 1982).

Fig. 5 shows that the mycolic acid liposomes-coated cuvette surface accumulated cholesterol from the solution while the methyl esters of mycolic acid (**2**) and the synthetic, acetylated alpha-mycolic acid methyl ester (**3**) were unable to do so (no significant difference between binding of cholesterol to (**2**) and (**3**), $P > 0.1$, but highly significant difference of cholesterol bind-

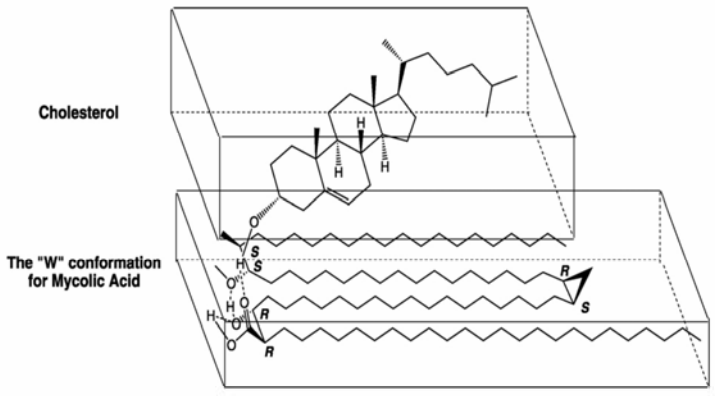


Fig. 6. A pictorial representation of a hypothesised mechanism of interaction between a "W"-folded methoxy MA (Villeneuve et al., 2005, 2007) and cholesterol. The absolute stereochemistry of the cyclopropane and the methoxy group are not completely clarified. Here for simplicity the S,R stereochemistry has been used for the cyclopropyl group and the SS for the methoxy-group. RR is known to be the absolute configuration of the mycolic motif. The "W" fold is drawn here in a flat plane, whereas in practice the fold shown in earlier models had a cylindrical three-dimensional structure (Villeneuve et al., 2005, 2007).

ing to mycolic acid or either the methyl esters of mycolic acid (**2**) and the synthetic, acetylated alpha-mycolic acid methyl ester (**3**), $P < 0.001$). These results demonstrate a pronounced attraction between cholesterol and mycolic acid that is determined by the presence of a free carboxylic acid group and the degree of structural fit by a particular conformation that free mycolic acids assume, which can be eliminated by formation of a simple methyl ester of the acid. In Fig. 6 a graphical representation is attempted of a mycolic acid, folded to assume a cholesteroid nature. A more accurate representation can be expected from virtual models based on Langmuir–Blodgett analysis of synthetic subclasses of mycolic acids interacting with cholesterol. The current advances in the stereo-controlled chemical synthesis of mycolic acids may make this possible in the near future.

4. Conclusion

The following observations support the hypothesis of a similarity in nature between the mycolic acids of *M. tuberculosis* and cholesterol that can affect the accuracy of serodiagnostic assays based on antibodies to mycolic acids as surrogate markers of active TB: (i) both mycolic acid and cholesterol are recognised by AmB and human TB sera, (ii) both human TB sera and AmB are able to distinguish between natural occurring mycolic acid (**1**) and a closely related chemically synthetic structure, i.e. acetylated alpha-mycolic acid methyl ester (**3**), (iii) TB negative patient sera containing antibodies to cholesterol (Bíró et al., 2007) bind to mycolic acid comparatively well, but not to the acetylated alpha-mycolic acid methyl ester (**3**) and (iv) the association of AmB to both cholesterol and mycolic acid could be disrupted by covalent modification of the AmB that prevented the formation of hydrogen bonds but not Van der Waals association. Likewise, modification of mycolic acid, aiming to disfavour its arranging into the "W" or any other folded conformation and preclude hydrogen bonding with ligate, abrogated its interaction with patient sera, AmB and cholesterol. The specific attraction between mycolic acid and cholesterol in liposomes probably depends on a particular conformation that free MAs assume in the phospholipid bilayer of liposomes, and this will probably also apply to biological membranes. The existence of a condensed conformation for MAs has already been proposed by several groups, based on Langmuir studies. Here we present the first evidence that these folded conformations could possibly exist in biological conditions to determine the antigenicity of mycolic acids.

The implications of such a discovery and of the relatedness between cholesterol and mycolic acids may be significant for several reasons.

First it could confirm the explanation of the poor results obtained with ELISA testing the usefulness of antibodies to mycolic acids as surrogate markers for tuberculosis (Schleicher et al., 2002).

Second, it impacts on the observation that mycolic acids, intraperitoneally or intratracheally administered as liposomes in mice, resulted in a pathogen-associated type of innate immune response that mainly affects macrophages, converting them into cholesterol-rich foam cells (Korf et al., 2005). Moreover, when administered intratracheally, mycolic acid could also prevent experimentally induced asthma in mice (Korf et al., 2006). The ability of mycolic acid to attract cholesterol to the macrophage may be critical for these phenomena.

Third, cholesterol has been shown to play a role in the entry and survival of *M. tuberculosis* in the host macrophage (Gatfield and Pieters, 2000; de Chastellier and Thilo, 2006). The cholesteroid nature of at least some mycolic acids may imply their active participation in this manifestation of virulence.

The different subclasses of mycolic acids from *M. tuberculosis* are currently under investigation to learn if the cholesteroid nature of mycolic acid is due only to the mycolic motif or to the other functional groups in the meromycolate chain. Finally, an improved understanding of the nature of the mycolate coat of *M. tuberculosis* could lead to the development of improved diagnostics and treatments of tuberculosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemphyslip.2008.01.004.

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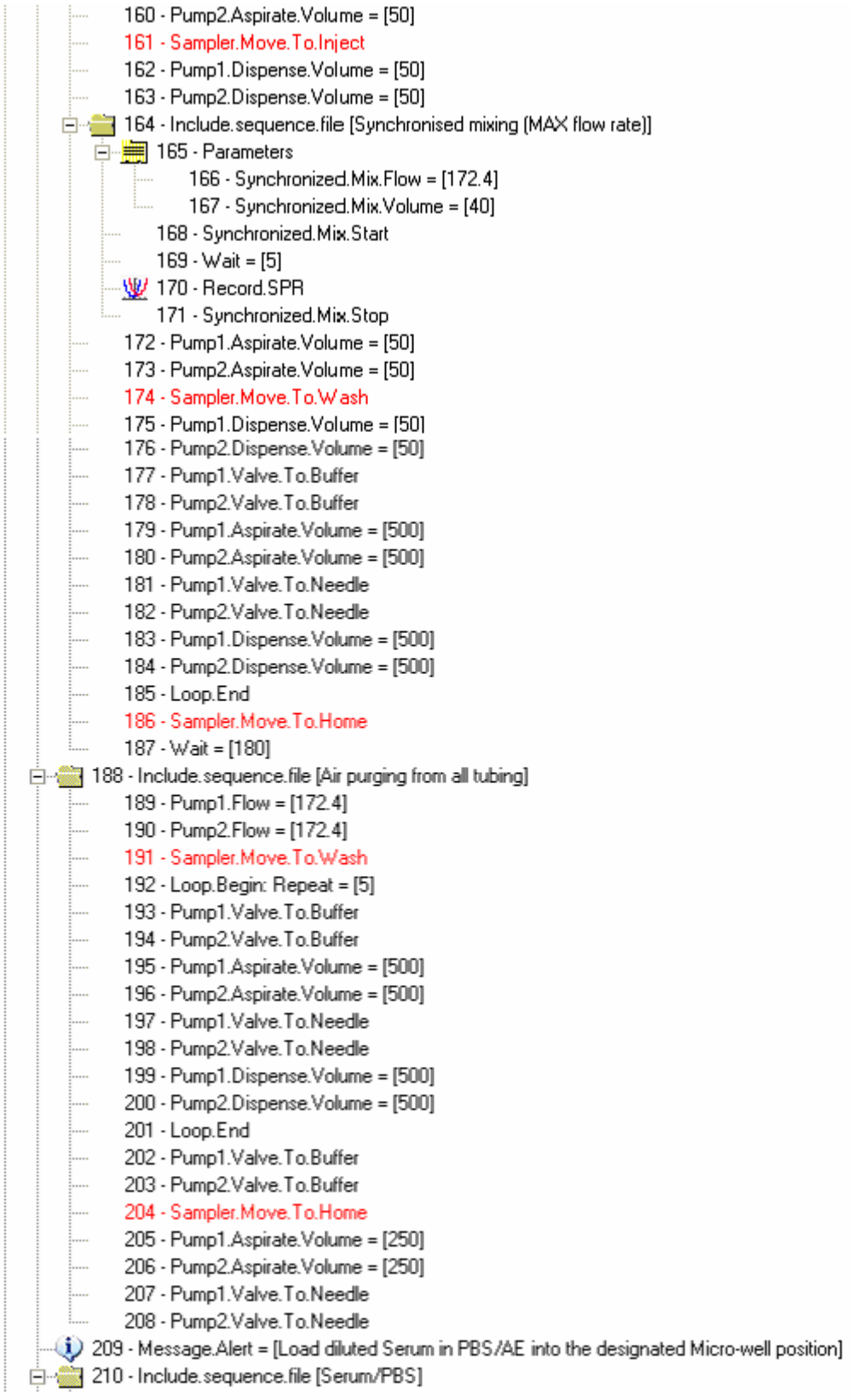
Appendix C: The optimal MARTI-test sequence

- 1 - Synchronized.Mix.Flow = [172.4]
- 2 - Measurement.Interval = [1]
- 3 - Measurement.Start.Both Channels
- 4 - Include.sequence.file [Full NEW sequence (without 'airlock' sequences)]
  - 5 - Pump1.Flow = [172.4]
  - 6 - Pump2.Flow = [172.4]
  - 7 - Include.sequence.file [BASELINE SETTING (leaving 50 uL in cuvette)]
    - 8 - Update/Add.Event.Item [Baseline setting BEGIN]
    - 9 - Sampler.Move.To.Stock[2]
    - 10 - Pump1.Aspirate.Volume = [50]
    - 11 - Pump2.Aspirate.Volume = [50]
    - 12 - Sampler.Move.To.Inject
    - 13 - Pump1.Dispense.Volume = [50]
    - 14 - Pump2.Dispense.Volume = [50]
    - 15 - Sampler.Move.To.Home
    - 16 - Record.SPR
    - 17 - Wait.Baseline (s)
    - 18 - Wait = [180]
    - 19 - Sampler.Move.To.Inject
    - 20 - Pump1.Aspirate.Volume = [50]
    - 21 - Pump2.Aspirate.Volume = [50]
    - 22 - Sampler.Move.To.Wash
    - 23 - Record.SPR
    - 24 - Update/Add.Event.Item [Baseline setting END]
    - 25 - Pump1.Dispense.Volume = [50]
    - 26 - Pump2.Dispense.Volume = [50]
    - 27 - Sampler.Move.To.Home
  - 28 - Include.sequence.file [Air purging from all tubing]
    - 29 - Pump1.Flow = [172.4]
    - 30 - Pump2.Flow = [172.4]
    - 31 - Sampler.Move.To.Wash
    - 32 - Loop.Begin: Repeat = [5]
    - 33 - Pump1.Valve.To.Buffer
    - 34 - Pump2.Valve.To.Buffer
    - 35 - Pump1.Aspirate.Volume = [500]
    - 36 - Pump2.Aspirate.Volume = [500]
    - 37 - Pump1.Valve.To.Needle
    - 38 - Pump2.Valve.To.Needle
    - 39 - Pump1.Dispense.Volume = [500]
    - 40 - Pump2.Dispense.Volume = [500]
    - 41 - Loop.End
    - 42 - Pump1.Valve.To.Buffer
    - 43 - Pump2.Valve.To.Buffer
    - 44 - Sampler.Move.To.Home
    - 45 - Pump1.Aspirate.Volume = [250]
    - 46 - Pump2.Aspirate.Volume = [250]
    - 47 - Pump1.Valve.To.Needle
    - 48 - Pump2.Valve.To.Needle
  - 49 - Message.Alert = [Load liposomes into the designated Micro-well plate position]
  - 50 - Include.sequence.file [Liposomes immobilisation]
    - 51 - Update/Add.Event.Item [Liposome immobilisation BEGIN]
    - 52 - Sampler.Move.To.Sample[137]

- 53 - Pump1.Aspirate.Volume = [50]
- 54 - Pump2.Aspirate.Volume = [50]
- 55 - Sampler.Move.To.Inject
- 56 - Pump1.Dispense.Volume = [50]
- 57 - Pump2.Dispense.Volume = [50]
- 58 - Include.sequence.file [Synchronised mixing (70% flow rate)]
  - 59 - Parameters
    - 60 - Synchronized.Mix.Flow = [172.4]
    - 61 - Synchronized.Mix.Volume = [20]
  - 62 - Synchronized.Mix.Start
  - 63 - Wait = [1200]
  - 64 - Record.SPR
  - 65 - Synchronized.Mix.Stop
- 66 - Pump1.Aspirate.Volume = [50]
- 67 - Pump2.Aspirate.Volume = [50]
- 68 - Sampler.Move.To.Wash
- 69 - Pump1.Dispense.Volume = [50]
- 70 - Pump2.Dispense.Volume = [50]
- 71 - Record.SPR
- 72 - Message.Alert = [Change to Non-Degassed Buffer]
- 73 - Include.sequence.file [PBS/AE wash sequence]
  - 74 - Update/Add.Event.Item [PBS/AE wash]
  - 75 - Record.SPR
  - 76 - Loop.Begin: Repeat = [5]
  - 77 - Sampler.Move.To.Stock[2]
  - 78 - Pump1.Valve.To.Needle
  - 79 - Pump2.Valve.To.Needle
  - 80 - Pump1.Aspirate.Volume = [50]
  - 81 - Pump2.Aspirate.Volume = [50]
  - 82 - Sampler.Move.To.Inject
  - 83 - Pump1.Dispense.Volume = [50]
  - 84 - Pump2.Dispense.Volume = [50]
  - 85 - Include.sequence.file [Synchronised mixing (70% flow rate)]
    - 86 - Parameters
      - 87 - Synchronized.Mix.Flow = [172.4]
      - 88 - Synchronized.Mix.Volume = [20]
    - 89 - Synchronized.Mix.Start
    - 90 - Wait = [5]
    - 91 - Synchronized.Mix.Stop
  - 92 - Pump1.Aspirate.Volume = [50]
  - 93 - Pump2.Aspirate.Volume = [50]
  - 94 - Sampler.Move.To.Wash
  - 95 - Pump1.Dispense.Volume = [50]
  - 96 - Pump2.Dispense.Volume = [50]
  - 97 - Pump1.Valve.To.Buffer
  - 98 - Pump2.Valve.To.Buffer
  - 99 - Pump1.Aspirate.Volume = [500]
  - 100 - Pump2.Aspirate.Volume = [500]
  - 101 - Pump1.Valve.To.Needle
  - 102 - Pump2.Valve.To.Needle
  - 103 - Pump1.Dispense.Volume = [500]
  - 104 - Pump2.Dispense.Volume = [500]
  - 105 - Loop.End

- 106 - Record.SPR
- 107 - Sampler.Move.To.Home
- 108 - Include.sequence.file [Air purging from all tubing]
  - 109 - Pump1.Flow = [172.4]
  - 110 - Pump2.Flow = [172.4]
  - 111 - Sampler.Move.To.Wash
  - 112 - Loop.Begin: Repeat = [5]
  - 113 - Pump1.Valve.To.Buffer
  - 114 - Pump2.Valve.To.Buffer
  - 115 - Pump1.Aspirate.Volume = [500]
  - 116 - Pump2.Aspirate.Volume = [500]
  - 117 - Pump1.Valve.To.Needle
  - 118 - Pump2.Valve.To.Needle
  - 119 - Pump1.Dispense.Volume = [500]
  - 120 - Pump2.Dispense.Volume = [500]
  - 121 - Loop.End
  - 122 - Pump1.Valve.To.Buffer
  - 123 - Pump2.Valve.To.Buffer
  - 124 - Sampler.Move.To.Home
  - 125 - Pump1.Aspirate.Volume = [250]
  - 126 - Pump2.Aspirate.Volume = [250]
  - 127 - Pump1.Valve.To.Needle
  - 128 - Pump2.Valve.To.Needle
- 129 - Message.Alert = [Load saponin into the designated Micro-well plate position]
- 130 - Include.sequence.file [Saponin blocking (0.5 mg/mL)]
  - 131 - Update/Add.Event.Item [Saponin blocking (0.5 mg/mL) BEGIN]
  - 132 - Sampler.Move.To.Sample[139]
  - 133 - Pump1.Aspirate.Volume = [50]
  - 134 - Pump2.Aspirate.Volume = [50]
  - 135 - Sampler.Move.To.Inject
  - 136 - Pump1.Dispense.Volume = [50]
  - 137 - Pump2.Dispense.Volume = [50]
  - 138 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
    - 139 - Parameters
      - 140 - Synchronized.Mix.Flow = [50.0]
      - 141 - Synchronized.Mix.Volume = [20]
    - 142 - Synchronized.Mix.Start
    - 143 - Wait = [300]
    - 144 - Record.SPR
    - 145 - Synchronized.Mix.Stop
  - 146 - Pump1.Aspirate.Volume = [50]
  - 147 - Pump2.Aspirate.Volume = [50]
  - 148 - Pump1.Valve.To.Needle
  - 149 - Pump2.Valve.To.Needle
- 150 - Sampler.Move.To.Wash
- 151 - Pump1.Dispense.Volume = [50]
- 152 - Pump2.Dispense.Volume = [50]
- 153 - Include.sequence.file [PBS/AE wash sequence]
  - 154 - Update/Add.Event.Item [PBS/AE wash]
  - 155 - Loop.Begin: Repeat = [5]
  - 156 - Sampler.Move.To.Stock[2]
  - 157 - Pump1.Valve.To.Needle
  - 158 - Pump2.Valve.To.Needle
  - 159 - Pump1.Aspirate.Volume = [50]


160 - Pump2.Aspirate.Volume = [50]
161 - Sampler.Move.To.Inject
162 - Pump1.Dispense.Volume = [50]
163 - Pump2.Dispense.Volume = [50]
164 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
165 - Parameters
166 - Synchronized.Mix.Flow = [172.4]
167 - Synchronized.Mix.Volume = [40]
168 - Synchronized.Mix.Start
169 - Wait = [5]
170 - Record.SPR
171 - Synchronized.Mix.Stop
172 - Pump1.Aspirate.Volume = [50]
173 - Pump2.Aspirate.Volume = [50]
174 - Sampler.Move.To.Wash
175 - Pump1.Dispense.Volume = [50]
176 - Pump2.Dispense.Volume = [50]
177 - Pump1.Valve.To.Buffer
178 - Pump2.Valve.To.Buffer
179 - Pump1.Aspirate.Volume = [500]
180 - Pump2.Aspirate.Volume = [500]
181 - Pump1.Valve.To.Needle
182 - Pump2.Valve.To.Needle
183 - Pump1.Dispense.Volume = [500]
184 - Pump2.Dispense.Volume = [500]
185 - Loop.End
186 - Sampler.Move.To.Home
187 - Wait = [180]
188 - Include.sequence.file [Air purging from all tubing]
189 - Pump1.Flow = [172.4]
190 - Pump2.Flow = [172.4]
191 - Sampler.Move.To.Wash
192 - Loop.Begin: Repeat = [5]
193 - Pump1.Valve.To.Buffer
194 - Pump2.Valve.To.Buffer
195 - Pump1.Aspirate.Volume = [500]
196 - Pump2.Aspirate.Volume = [500]
197 - Pump1.Valve.To.Needle
198 - Pump2.Valve.To.Needle
199 - Pump1.Dispense.Volume = [500]
200 - Pump2.Dispense.Volume = [500]
201 - Loop.End
202 - Pump1.Valve.To.Buffer
203 - Pump2.Valve.To.Buffer
204 - Sampler.Move.To.Home
205 - Pump1.Aspirate.Volume = [250]
206 - Pump2.Aspirate.Volume = [250]
207 - Pump1.Valve.To.Needle
208 - Pump2.Valve.To.Needle
209 - Message.Alert = [Load diluted Serum in PBS/AE into the designated Micro-well position]
210 - Include.sequence.file [Serum/PBS]

- 211 - Update/Add.Event.Item [Diluted serum blocking START]
- 212 - Sampler.Move.To.Sample[141]
- 213 - Pump1.Aspirate.Volume = [35]
- 214 - Pump2.Aspirate.Volume = [35]
- 215 - Sampler.Move.To.Inject
- 216 - Pump1.Dispense.Volume = [35]
- 217 - Pump2.Dispense.Volume = [35]
- 218 - Include.sequence.file [Synchronised mixing (70% flow rate)]
  - 219 - Parameters
    - 220 - Synchronized.Mix.Flow = [172.4]
    - 221 - Synchronized.Mix.Volume = [20]
  - 222 - Synchronized.Mix.Start
  - 223 - Wait = [480]
  - 224 - Synchronized.Mix.Stop
- 225 - Record.SPR
- 226 - Message.Alert = [Load Serum diluted in Liposomes into the designated Micro-well position]
- 227 - Include.sequence.file [Serum/liposomes]
  - 228 - Update/Add.Event.Item [Inhibition study START]
  - 229 - Sampler.Move.To.Sample[143]
  - 230 - Pump1.Aspirate.Volume = [35]
  - 231 - Pump2.Aspirate.Volume = [35]
  - 232 - Sampler.Move.To.Inject
  - 233 - Pump1.Dispense.Volume = [35]
  - 234 - Pump2.Dispense.Volume = [35]
  - 235 - Include.sequence.file [Synchronised mixing (70% flow rate)]
    - 236 - Parameters
      - 237 - Sampler.Move.To.Sample[227.3]
      - 238 - Synchronized.Mix.Volume = [20]
    - 239 - Synchronized.Mix.Start
    - 240 - Wait = [480]
    - 241 - Record.SPR
    - 242 - Synchronized.Mix.Stop
    - 243 - Pump1.Aspirate.Volume = [70]
    - 244 - Pump2.Aspirate.Volume = [70]
    - 245 - Sampler.Move.To.Wash
    - 246 - Pump1.Dispense.Volume = [70]
    - 247 - Pump2.Dispense.Volume = [70]
    - 248 - Loop.Begin: Repeat = [3]
    - 249 - Pump1.Valve.To.Buffer
    - 250 - Pump2.Valve.To.Buffer
    - 251 - Pump1.Aspirate.Volume = [500]
    - 252 - Pump2.Aspirate.Volume = [500]
    - 253 - Pump1.Valve.To.Needle
    - 254 - Pump2.Valve.To.Needle
    - 255 - Pump1.Dispense.Volume = [500]
    - 256 - Pump2.Dispense.Volume = [500]
    - 257 - Loop.End
  - 258 - Sampler.Move.To.Home
  - 259 - Update/Add.Event.Item [Inhibition study END]
- 260 - Include.sequence.file [PBS/AE wash sequence]
  - 261 - Update/Add.Event.Item [PBS/AE wash]
  - 262 - Loop.Begin: Repeat = [5]
  - 263 - Sampler.Move.To.Stock[2]

- 264 - Pump1.Valve.To.Needle
- 265 - Pump2.Valve.To.Needle
- 266 - Pump1.Aspirate.Volume = [50]
- 267 - Pump2.Aspirate.Volume = [50]
- 268 - Sampler.Move.To.Inject
- 269 - Pump1.Dispense.Volume = [50]
- 270 - Pump2.Dispense.Volume = [50]
- 271 - Include.sequence.file [Synchronised mixing (70% flow rate)]
  - 272 - Parameters
    - 273 - Synchronized.Mix.Flow = [172.4]
    - 274 - Synchronized.Mix.Volume = [40]
  - 275 - Synchronized.Mix.Start
  - 276 - Wait = [5]
  - 277 - Record.SPR
  - 278 - Synchronized.Mix.Stop
- 279 - Pump1.Aspirate.Volume = [50]
- 280 - Pump2.Aspirate.Volume = [50]
- 281 - Sampler.Move.To.Wash
- 282 - Pump1.Dispense.Volume = [50]
- 283 - Pump2.Dispense.Volume = [50]
- 284 - Pump1.Valve.To.Buffer
- 285 - Pump2.Valve.To.Buffer
- 286 - Pump1.Aspirate.Volume = [500]
- 287 - Pump2.Aspirate.Volume = [500]
- 288 - Pump1.Valve.To.Needle
- 289 - Pump2.Valve.To.Needle
- 290 - Pump1.Dispense.Volume = [500]
- 291 - Pump2.Dispense.Volume = [500]
- 292 - Loop.End
- 293 - Sampler.Move.To.Home
- 294 - Wait = [120]
- 295 - Include.sequence.file [Air purging from all tubing]
  - 296 - Include.sequence.file [Air purging from all tubing]
    - 297 - Pump1.Flow = [172.4]
    - 298 - Pump2.Flow = [172.4]
    - 299 - Sampler.Move.To.Wash
    - 300 - Loop.Begin: Repeat = [5]
    - 301 - Pump1.Valve.To.Buffer
    - 302 - Pump2.Valve.To.Buffer
    - 303 - Pump1.Aspirate.Volume = [500]
    - 304 - Pump2.Aspirate.Volume = [500]
    - 305 - Pump1.Valve.To.Needle
    - 306 - Pump2.Valve.To.Needle
    - 307 - Pump1.Dispense.Volume = [500]
    - 308 - Pump2.Dispense.Volume = [500]
    - 309 - Loop.End
    - 310 - Pump1.Valve.To.Buffer
    - 311 - Pump2.Valve.To.Buffer
    - 312 - Sampler.Move.To.Home
    - 313 - Pump1.Aspirate.Volume = [250]
    - 314 - Pump2.Aspirate.Volume = [250]
    - 315 - Pump1.Valve.To.Needle

- 316 - Pump2.Valve.To.Needle
- 317 - Message.Alert = [Put Isopropanol + 50 mM NaOH (2/3, v:v) in Stock 1]
- 318 - Include.sequence.file [NaOH and Isopropanol wash]
  - 319 - Update/Add.Event.Item [NaOH wash]
  - 320 - Loop.Begin: Repeat = [5]
  - 321 - Sampler.Move.To.Stock[1]
  - 322 - Pump1.Aspirate.Volume = [100]
  - 323 - Pump2.Aspirate.Volume = [100]
  - 324 - Sampler.Move.To.Inject
  - 325 - Pump1.Dispense.Volume = [100]
  - 326 - Pump2.Dispense.Volume = [100]
  - 327 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
    - 328 - Parameters
      - 329 - Synchronized.Mix.Flow = [172.4]
      - 330 - Synchronized.Mix.Volume = [40]
    - 331 - Synchronized.Mix.Start
    - 332 - Wait = [20]
    - 333 - Synchronized.Mix.Stop
  - 334 - Pump1.Aspirate.Volume = [100]
  - 335 - Pump2.Aspirate.Volume = [100]
  - 336 - Sampler.Move.To.Wash
  - 337 - Pump1.Dispense.Volume = [100]
  - 338 - Pump2.Dispense.Volume = [100]
  - 339 - Loop.End
- 340 - Include.sequence.file [PBSAE wash sequence]
  - 341 - Include.sequence.file [PBS/AE wash sequence]
    - 342 - Update/Add.Event.Item [PBS/AE wash]
    - 343 - Loop.Begin: Repeat = [5]
    - 344 - Sampler.Move.To.Stock[2]
    - 345 - Pump1.Valve.To.Needle
    - 346 - Pump2.Valve.To.Needle
    - 347 - Pump1.Aspirate.Volume = [100]
    - 348 - Pump2.Aspirate.Volume = [100]
    - 349 - Sampler.Move.To.Inject
    - 350 - Pump1.Dispense.Volume = [100]
    - 351 - Pump2.Dispense.Volume = [100]
    - 352 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
      - 353 - Parameters
        - 354 - Synchronized.Mix.Flow = [172.4]
        - 355 - Synchronized.Mix.Volume = [40]
      - 356 - Synchronized.Mix.Start
      - 357 - Wait = [5]
      - 358 - Record.SPR
      - 359 - Synchronized.Mix.Stop
    - 360 - Pump1.Aspirate.Volume = [100]
    - 361 - Pump2.Aspirate.Volume = [100]
    - 362 - Sampler.Move.To.Wash
    - 363 - Pump1.Dispense.Volume = [100]
    - 364 - Pump2.Dispense.Volume = [100]
    - 365 - Pump1.Valve.To.Buffer
    - 366 - Pump2.Valve.To.Buffer
    - 367 - Pump1.Aspirate.Volume = [500]
    - 368 - Pump2.Aspirate.Volume = [500]

- 369 - Pump1.Valve.To.Needle
- 370 - Pump2.Valve.To.Needle
- 371 - Pump1.Dispense.Volume = [500]
- 372 - Pump2.Dispense.Volume = [500]
- 373 - Loop.End
- 374 - Sampler.Move.To.Home
- 375 - Wait = [60]
- 376 - Message.Alert = [Put 96 % Ethanol in Stock 1]
- 377 - Include.sequence.file [EtOH wash sequence]
  - 378 - Include.sequence.file [EtOH wash]
    - 379 - Update/Add.Event.Item [EtOH wash]
    - 380 - Loop.Begin: Repeat = [5]
    - 381 - Sampler.Move.To.Stock[1]
    - 382 - Pump1.Aspirate.Volume = [100]
    - 383 - Pump2.Aspirate.Volume = [100]
    - 384 - Sampler.Move.To.Inject
    - 385 - Pump1.Dispense.Volume = [100]
    - 386 - Pump2.Dispense.Volume = [100]
    - 387 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
      - 388 - Parameters
        - 389 - Synchronized.Mix.Flow = [172.4]
        - 390 - Synchronized.Mix.Volume = [40]
      - 391 - Synchronized.Mix.Start
      - 392 - Wait = [20]
      - 393 - Synchronized.Mix.Stop
    - 394 - Pump1.Aspirate.Volume = [100]
    - 395 - Pump2.Aspirate.Volume = [100]
    - 396 - Sampler.Move.To.Wash
    - 397 - Pump1.Dispense.Volume = [100]
    - 398 - Pump2.Dispense.Volume = [100]
    - 399 - Loop.End
  - 400 - Include.sequence.file [PBSAE wash sequence]
    - 401 - Include.sequence.file [PBS/AE wash sequence]
      - 402 - Update/Add.Event.Item [PBS/AE wash]
      - 403 - Loop.Begin: Repeat = [5]
      - 404 - Sampler.Move.To.Stock[2]
      - 405 - Pump1.Valve.To.Needle
      - 406 - Pump2.Valve.To.Needle
      - 407 - Pump1.Aspirate.Volume = [100]
      - 408 - Pump2.Aspirate.Volume = [100]
      - 409 - Sampler.Move.To.Inject
      - 410 - Pump1.Dispense.Volume = [100]
      - 411 - Pump2.Dispense.Volume = [100]
      - 412 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
        - 413 - Parameters
          - 414 - Synchronized.Mix.Flow = [172.4]
          - 415 - Synchronized.Mix.Volume = [40]
        - 416 - Synchronized.Mix.Start
        - 417 - Wait = [5]
        - 418 - Record.SPR
        - 419 - Synchronized.Mix.Stop
      - 420 - Pump1.Aspirate.Volume = [100]

421 - Pump2.Aspirate.Volume = [100]
422 - Sampler.Move.To.Wash
423 - Pump1.Dispense.Volume = [100]
424 - Pump2.Dispense.Volume = [100]
425 - Pump1.Valve.To.Buffer
426 - Pump2.Valve.To.Buffer
427 - Pump1.Aspirate.Volume = [500]
428 - Pump2.Aspirate.Volume = [500]
429 - Pump1.Valve.To.Needle
430 - Pump2.Valve.To.Needle
431 - Pump1.Dispense.Volume = [500]
432 - Pump2.Dispense.Volume = [500]
433 - Loop.End
434 - Sampler.Move.To.Home
435 - Wait = [180]
436 - Measurement.End

Appendix D: SPR dips preparation and regeneration sequence

- 1 - Include.sequence.file [SPR dip calibration]
  - 2 - Message.Alert = [SPR dip calibration will now commence]
  - 3 - Include.sequence.file [Air purging from all tubing]
    - 4 - Pump1.Flow = [172.4]
    - 5 - Pump2.Flow = [172.4]
    - 6 - Sampler.Move.To.Wash
    - 7 - Loop.Begin: Repeat = [2]
    - 8 - Pump1.Valve.To.Buffer
    - 9 - Pump2.Valve.To.Buffer
    - 10 - Pump1.Aspirate.Volume = [500]
    - 11 - Pump2.Aspirate.Volume = [500]
    - 12 - Pump1.Valve.To.Needle
    - 13 - Pump2.Valve.To.Needle
    - 14 - Pump1.Dispense.Volume = [500]
    - 15 - Pump2.Dispense.Volume = [500]
    - 16 - Loop.End
    - 17 - Pump1.Valve.To.Buffer
    - 18 - Pump2.Valve.To.Buffer
    - 19 - Sampler.Move.To.Home
    - 20 - Pump1.Aspirate.Volume = [250]
    - 21 - Pump2.Aspirate.Volume = [250]
    - 22 - Pump1.Valve.To.Needle
    - 23 - Pump2.Valve.To.Needle
  - 24 - Update/Add.Event.Item [SPR dip calibration START]
  - 25 - Pump1.Valve.To.Needle
  - 26 - Pump2.Valve.To.Needle
  - 27 - Sampler.Move.To.Wash
  - 28 - Pump1.Dispense.Volume = [250]
  - 29 - Pump2.Dispense.Volume = [250]
  - 30 - Sampler.Move.To.Stock[1]
  - 31 - Pump1.Aspirate.Volume = [100]
  - 32 - Pump2.Aspirate.Volume = [100]
  - 33 - Sampler.Move.To.Inject
  - 34 - Pump1.Dispense.Volume = [100]
  - 35 - Pump2.Dispense.Volume = [100]
  - 36 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
    - 37 - Parameters
      - 38 - Synchronized.Mix.Flow = [172.4]
      - 39 - Synchronized.Mix.Volume = [50]
    - 40 - Synchronized.Mix.Start
    - 41 - Wait = [30]
    - 42 - Record.SPR
    - 43 - Synchronized.Mix.Stop
  - 44 - Pump1.Aspirate.Volume = [50]
  - 45 - Pump2.Aspirate.Volume = [50]
  - 46 - Sampler.Move.To.Wash
  - 47 - Pump1.Dispense.Volume = [50]
  - 48 - Pump2.Dispense.Volume = [50]
  - 49 - Include.sequence.file [Air purging from all tubing]
    - 50 - Pump1.Flow = [172.4]
    - 51 - Pump2.Flow = [172.4]
    - 52 - Sampler.Move.To.Wash
    - 53 - Loop.Begin: Repeat = [3]

- 54 - Pump1.Valve.To.Buffer
- 55 - Pump2.Valve.To.Buffer
- 56 - Pump1.Aspirate.Volume = [500]
- 57 - Pump2.Aspirate.Volume = [500]
- 58 - Pump1.Valve.To.Needle
- 59 - Pump2.Valve.To.Needle
- 60 - Pump1.Dispense.Volume = [500]
- 61 - Pump2.Dispense.Volume = [500]
- 62 - Loop.End
- 63 - Pump1.Valve.To.Buffer
- 64 - Pump2.Valve.To.Buffer
- 65 - Sampler.Move.To.Home
- 66 - Pump1.Aspirate.Volume = [250]
- 67 - Pump2.Aspirate.Volume = [250]
- 68 - Pump1.Valve.To.Needle
- 69 - Pump2.Valve.To.Needle
- 70 - Loop.Begin: Repeat = [4]
- 71 - Sampler.Move.To.Stock[1]
- 72 - Pump1.Aspirate.Volume = [50]
- 73 - Pump2.Aspirate.Volume = [50]
- 74 - Sampler.Move.To.Inject
- 75 - Pump1.Dispense.Volume = [50]
- 76 - Pump2.Dispense.Volume = [50]
- 77 - Include.sequence.file [Synchronised mixing (MAX flow
  - 78 - Parameters
    - 79 - Synchronized.Mix.Flow = [172.4]
    - 80 - Synchronized.Mix.Volume = [50]
  - 81 - Synchronized.Mix.Start
  - 82 - Wait = [30]
  - 83 - Record.SPR
  - 84 - Synchronized.Mix.Stop
- 85 - Pump1.Aspirate.Volume = [50]
- 86 - Pump2.Aspirate.Volume = [50]
- 87 - Sampler.Move.To.Wash
- 88 - Pump1.Dispense.Volume = [50]
- 89 - Pump2.Dispense.Volume = [50]
- 90 - Include.sequence.file [Air purging from all tubing]
  - 91 - Pump1.Flow = [172.4]
  - 92 - Pump2.Flow = [172.4]
  - 93 - Sampler.Move.To.Wash
  - 94 - Loop.Begin: Repeat = [1]
  - 95 - Pump1.Valve.To.Buffer
  - 96 - Pump2.Valve.To.Buffer
  - 97 - Pump1.Aspirate.Volume = [500]
  - 98 - Pump2.Aspirate.Volume = [500]
  - 99 - Pump1.Valve.To.Needle
  - 100 - Pump2.Valve.To.Needle
  - 101 - Pump1.Dispense.Volume = [500]
  - 102 - Pump2.Dispense.Volume = [500]
  - 103 - Loop.End
  - 104 - Pump1.Valve.To.Buffer
  - 105 - Pump2.Valve.To.Buffer

- 106 - Sampler.Move.To.Home
- 107 - Pump1.Aspirate.Volume = [250]
- 108 - Pump2.Aspirate.Volume = [250]
- 109 - Pump1.Valve.To.Needle
- 110 - Pump2.Valve.To.Needle
- 111 - Loop.End
- 112 - Include.sequence.file [PBS/AE wash sequence]
  - 113 - Loop.Begin: Repeat = [6]
  - 114 - Sampler.Move.To.Stock[2]
  - 115 - Pump1.Valve.To.Needle
  - 116 - Pump2.Valve.To.Needle
  - 117 - Pump1.Aspirate.Volume = [50]
  - 118 - Pump2.Aspirate.Volume = [50]
  - 119 - Sampler.Move.To.Inject
  - 120 - Pump1.Dispense.Volume = [50]
  - 121 - Pump2.Dispense.Volume = [50]
  - 122 - Include.sequence.file [Synchronised mixing (MAX flow rate)]
    - 123 - Parameters
      - 124 - Synchronized.Mix.Flow = [172.4]
      - 125 - Synchronized.Mix.Volume = [50]
    - 126 - Synchronized.Mix.Start
    - 127 - Wait = [10]
    - 128 - Record.SPR
    - 129 - Synchronized.Mix.Stop
  - 130 - Pump1.Aspirate.Volume = [50]
  - 131 - Pump2.Aspirate.Volume = [50]
  - 132 - Sampler.Move.To.Wash
  - 133 - Pump1.Dispense.Volume = [50]
  - 134 - Pump2.Dispense.Volume = [50]
  - 135 - Loop.Begin: Repeat = [1]
  - 136 - Pump1.Valve.To.Buffer
  - 137 - Pump2.Valve.To.Buffer
  - 138 - Pump1.Aspirate.Volume = [500]
  - 139 - Pump2.Aspirate.Volume = [500]
  - 140 - Pump1.Valve.To.Needle
  - 141 - Pump2.Valve.To.Needle
  - 142 - Pump1.Dispense.Volume = [500]
  - 143 - Pump2.Dispense.Volume = [500]
  - 144 - Loop.End
  - 145 - Loop.End
- 146 - Pump1.Valve.To.Buffer
- 147 - Pump2.Valve.To.Buffer
- 148 - Pump1.Valve.To.Needle
- 149 - Pump2.Valve.To.Needle
- 150 - Update/Add.Event.Item [SPR dip calibration END]
- 151 - Sampler.Move.To.Home
- 152 - Record.SPR
- 153 - Update/Add.Event.Item [50 uL now in cuvette]
- 154 - Include.sequence.file [Air purging from all tubing]
  - 155 - Pump1.Flow = [227.3]
  - 156 - Pump2.Flow = [227.3]
  - 157 - Sampler.Move.To.Wash
  - 158 - Loop.Begin: Repeat = [2]
  - 159 - Pump1.Valve.To.Buffer

160 - Pump2.Valve.To.Buffer
161 - Pump1.Aspirate.Volume = [500]
162 - Pump2.Aspirate.Volume = [500]
163 - Pump1.Valve.To.Needle
164 - Pump2.Valve.To.Needle
165 - Pump1.Dispense.Volume = [500]
166 - Pump2.Dispense.Volume = [500]
167 - Loop.End
168 - Pump1.Valve.To.Buffer
169 - Pump2.Valve.To.Buffer
170 - Sampler.Move.To.Home
171 - Pump1.Aspirate.Volume = [250]
172 - Pump2.Aspirate.Volume = [250]
173 - Pump1.Valve.To.Needle
174 - Pump2.Valve.To.Needle

Appendix E: Sequence for cleaning the needles and cuvette

- 1 - Loop.Begin: Repeat = [4]
- 2 - Sampler.Move.To.Wash
- 3 - Include.sequence.file [Safety lines]
  - 4 - Pump1.Flow = [227.3]
  - 5 - Pump2.Flow = [227.3]
  - 6 - Pump1.Valve.To.Buffer
  - 7 - Pump2.Valve.To.Buffer
  - 8 - Synchronized.Mix.Stop
- 9 - Wash.Speed = [150]
- 10 - Drain.Speed = [200]
- 11 - Loop.Begin: Repeat = [2]
- 12 - Wash.Start
- 13 - Drain.Start
- 14 - Pump1.Aspirate.Volume = [250]
- 15 - Pump2.Aspirate.Volume = [250]
- 16 - Pump1.Valve.To.Needle
- 17 - Pump2.Valve.To.Needle
- 18 - Pump1.Dispense.Volume = [500]
- 19 - Pump2.Dispense.Volume = [500]
- 20 - Wait = [2]
- 21 - Wash.Stop
- 22 - Pump1.Valve.To.Buffer
- 23 - Pump2.Valve.To.Buffer
- 24 - Synchronized.Mix.Stop
- 25 - Drain.Stop
- 26 - Loop.End
- 27 - Pump1.Dispense.Volume = [50]
- 28 - Pump2.Dispense.Volume = [50]
- 29 - Sampler.Move.To.Home
- 30 - Pump1.Flow = [16.7]
- 31 - Pump2.Flow = [16.7]
- 32 - Pump1.Valve.To.Needle
- 33 - Pump2.Valve.To.Needle
- 34 - Synchronized.Mix.Stop
- 35 - Loop.End

Appendix F: ESPRIT Biosensor signal percentage inhibition of patient serum antibody binding to MA using different dilutions of serum and inhibitory liposomes

Serum dilutions		% inhibition of serum antibody binding to MA			
High diluted in PBS/AE	Low diluted in MA or PC Liposomes	P135 (TB^+HIV^+)	P129 (TB^+HIV^-)	P94 (TB^-HIV^-)	P96 (TB^+HIV^-)
1/4000	1/250	-10.43*	17.29	-	-
	1/500	-21.51*	25.09	-0.23*	29.53
	1/1000	-45.85*	24.73	-	-
	1/2000	-23.32*	2.00*	-	-
1/2000	1/250	-	16.58*	-	-
	1/500	-	19.22*	7.61*	30.41
	1/1000	-	41.67	-	-5.12*

MA: mycolic acid, PC: phosphatidylcholine, TB: tuberculosis, HIV: human immunodeficiency virus, +: positive, -: negative. Results represent the average of triplicate values and *: no significant difference.