

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dated and has largely been replaced by surface plasmon resonance (SPR) based devices (Cush *et al.*, 1993).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-pipetting 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 anti-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 brought 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 Kretschmann'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.

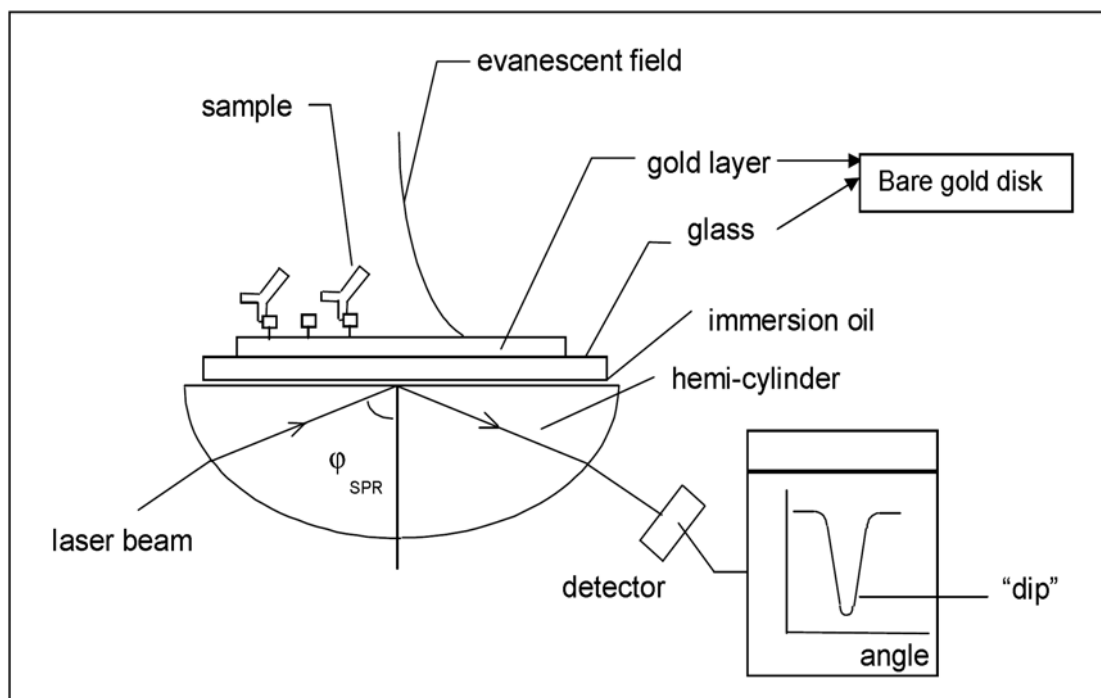


Figure 3.1: The Kretschmann configuration of a surface plasmon resonance biosensor (Eco Chemie B.V., Autolab ESPRIT manual).

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 flexibility 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).

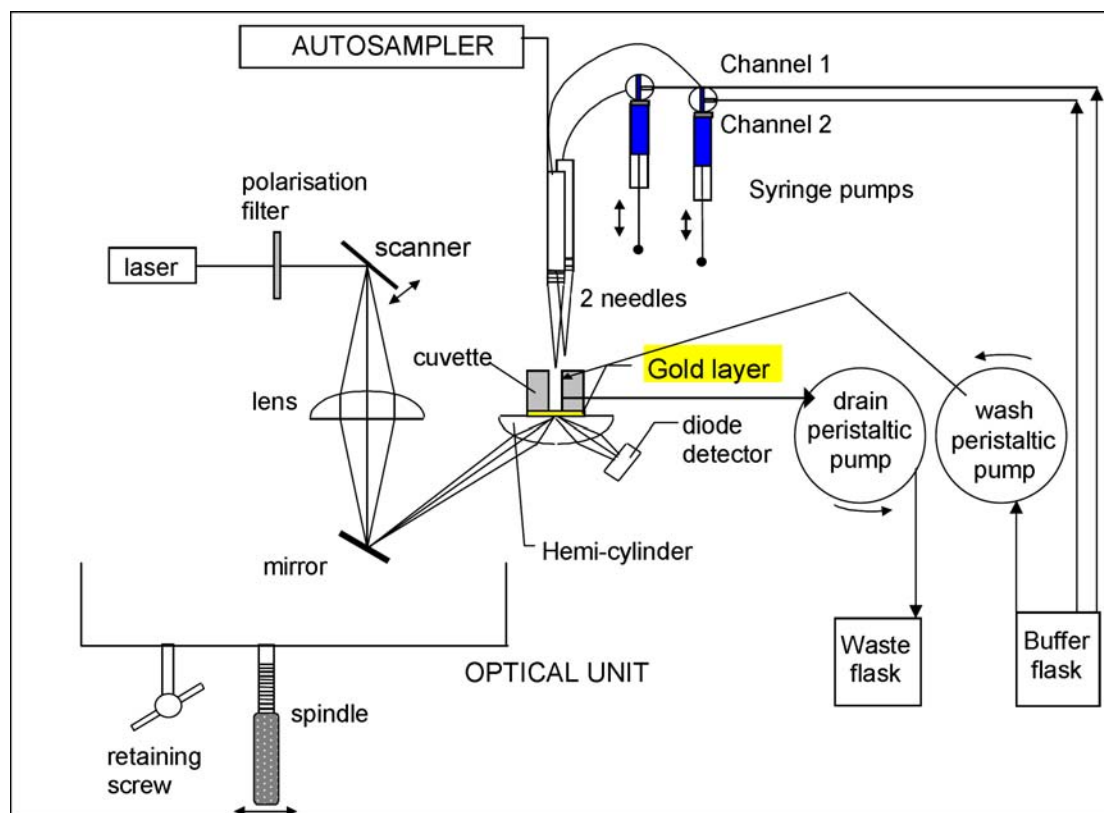


Figure 3.2: Schematic picture of the ESPRIT configuration (Eco Chemie B.V., ESPRIT biosensor manual).

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).

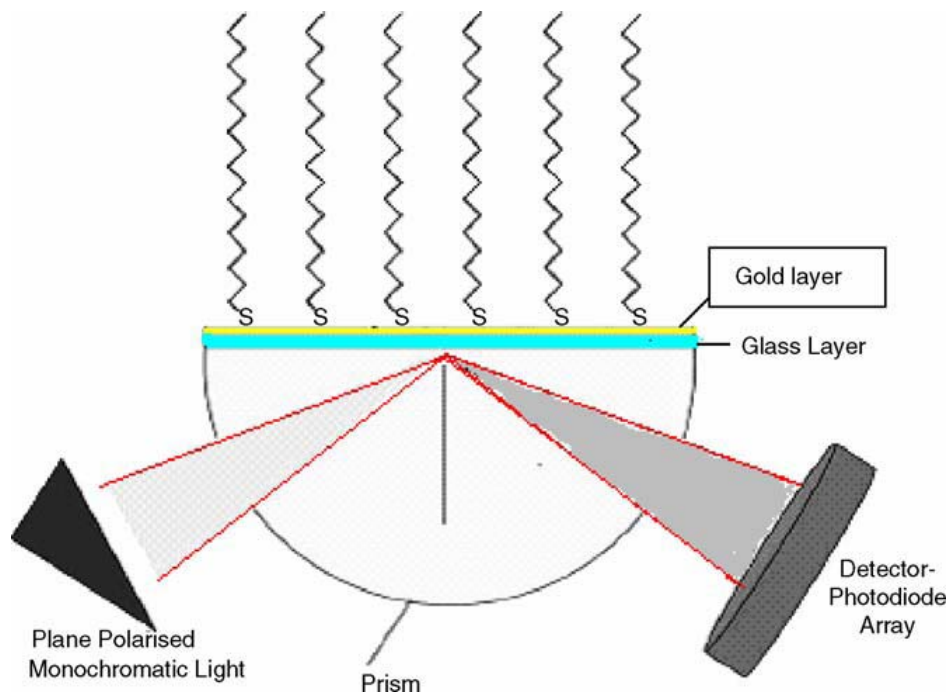


Figure 3.3: A schematic presentation of a hydrophobic SPR surface where a gold disk is coated with ODT (Arya *et al.*, 2006).

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 ferrocyanide/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™ 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 °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 Stellenbosch 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 $-70\text{ }^{\circ}\text{C}$ 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 $0.2\text{ }\mu\text{m}$ 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 ferrocyanide/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 μ l PBS/AE, followed by addition of 50 μ l MA liposomes on the disc for 20 minutes. The immobilized liposomes were then finally washed five times with 100 μ l PBS/AE, substituted with 50 μ l 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 μ l PBS/AE, the content of each cell substituted with 50 μ l 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 μ l) 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 μ l) 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 μ l mixture of isopropanol and 50 mM NaOH (2:3,

v/v) for 2 minutes and finally washed with 100 μ l of 99% absolute ethanol. The surface was washed 5 times with 100 μ l 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 underivatized 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 underivatized 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 μ l ethanol (96%) using the automatic dispenser with simultaneous draining, followed by brief (\sim 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.

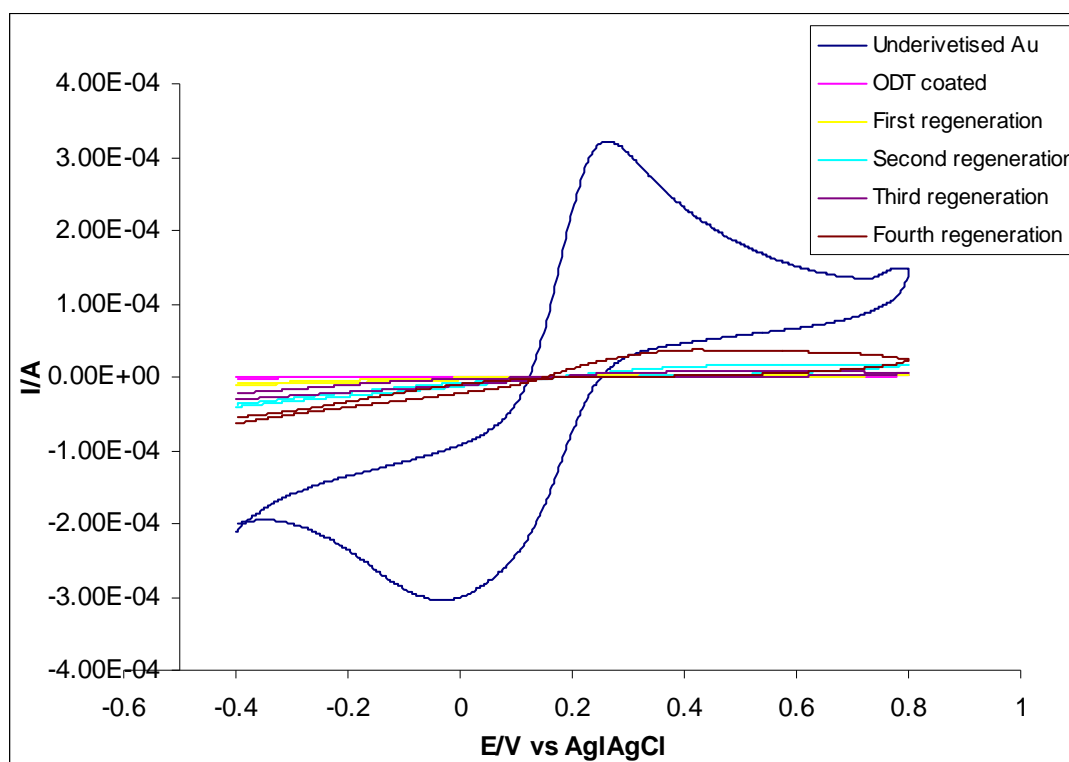


Figure 3.4: Testing of the octadecanethiol coated ESPRIT biosensor gold surface against sequential times of regeneration with a mixture of isopropanol and 50 mM NaOH (2/3, v/v) using cyclic voltammetry.

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).

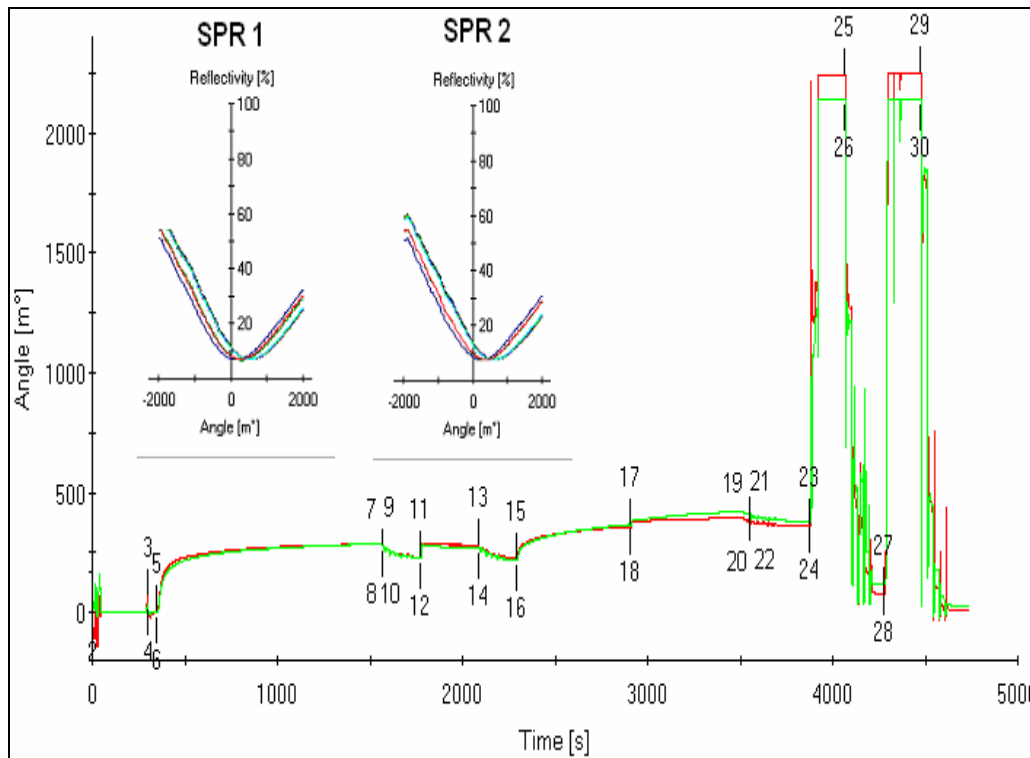


Figure 3.5: A representative ESPRIT sensorgram showing the full sequence of events to measure the inhibition of binding of human TB pos patient serum (HIV⁺TB⁺) antibodies to immobilized MA-liposomes on the ESPRIT biosensor. The event markers on the graphs are represented as follows: 3 – 4 baseline setting, 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 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.

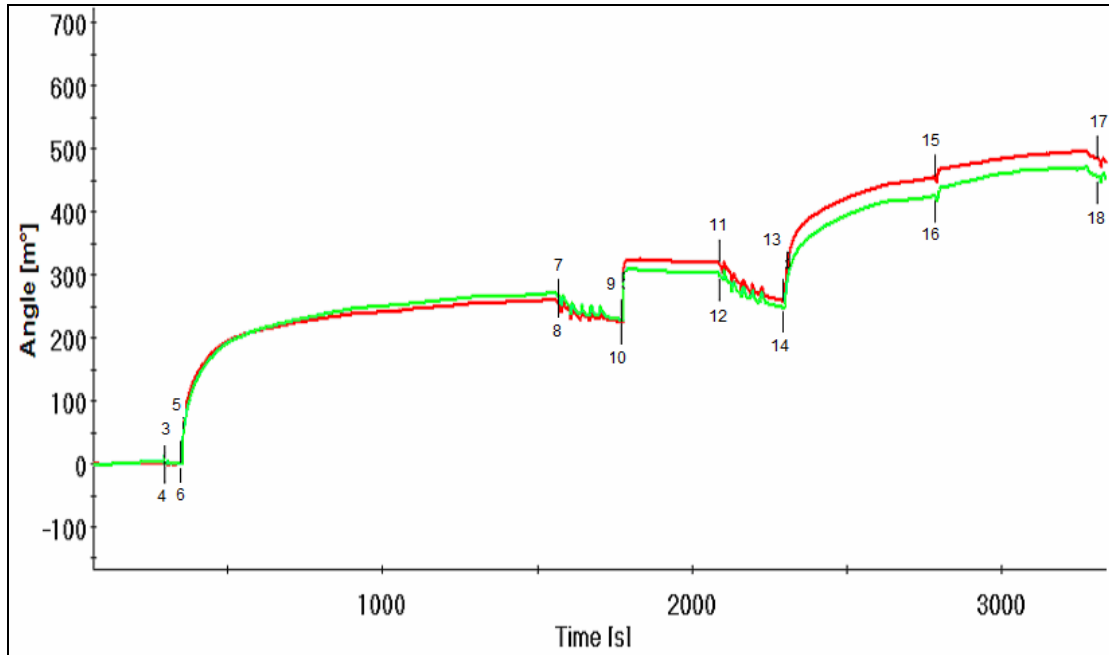


Figure 3.6: A representative ESPRIT sensorgram showing the full sequence of events to measure the inhibition of binding of human TB neg patient serum (HIV-TB) antibodies to immobilized MA-liposomes on the ESPRIT biosensor. The event markers on the graphs are represented as follows: 3 – 4 baseline setting, 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 IAys 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.

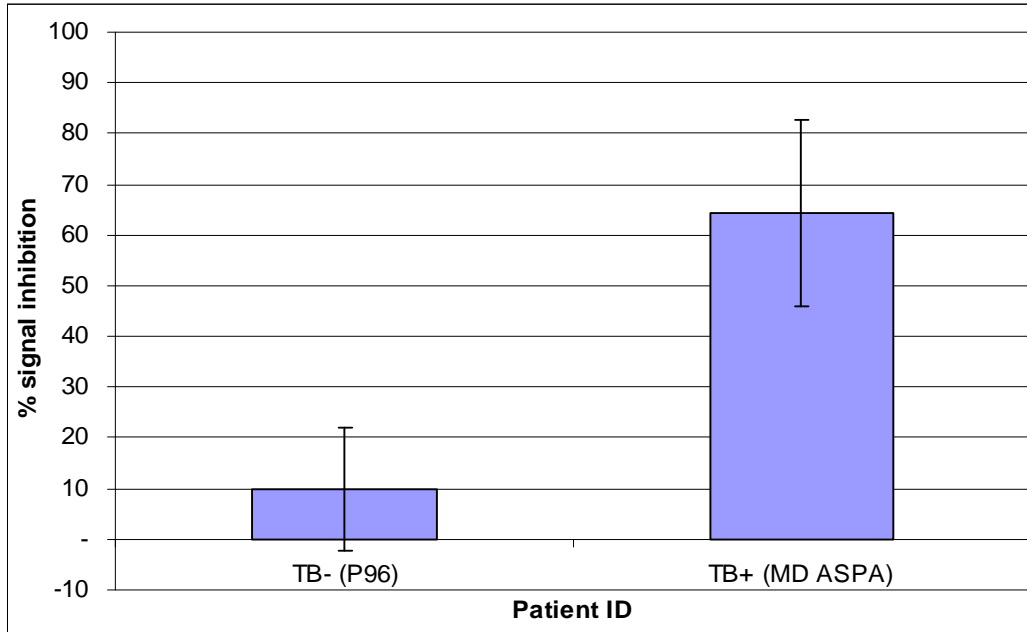


Figure 3.7: Inhibition of human serum antibody with mycolic acids in a TB negative (P96) and a TB positive (MD ASPA) patient serum on the ESPRIT biosensor, $p < 0.05$ ($n = 10$).

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.

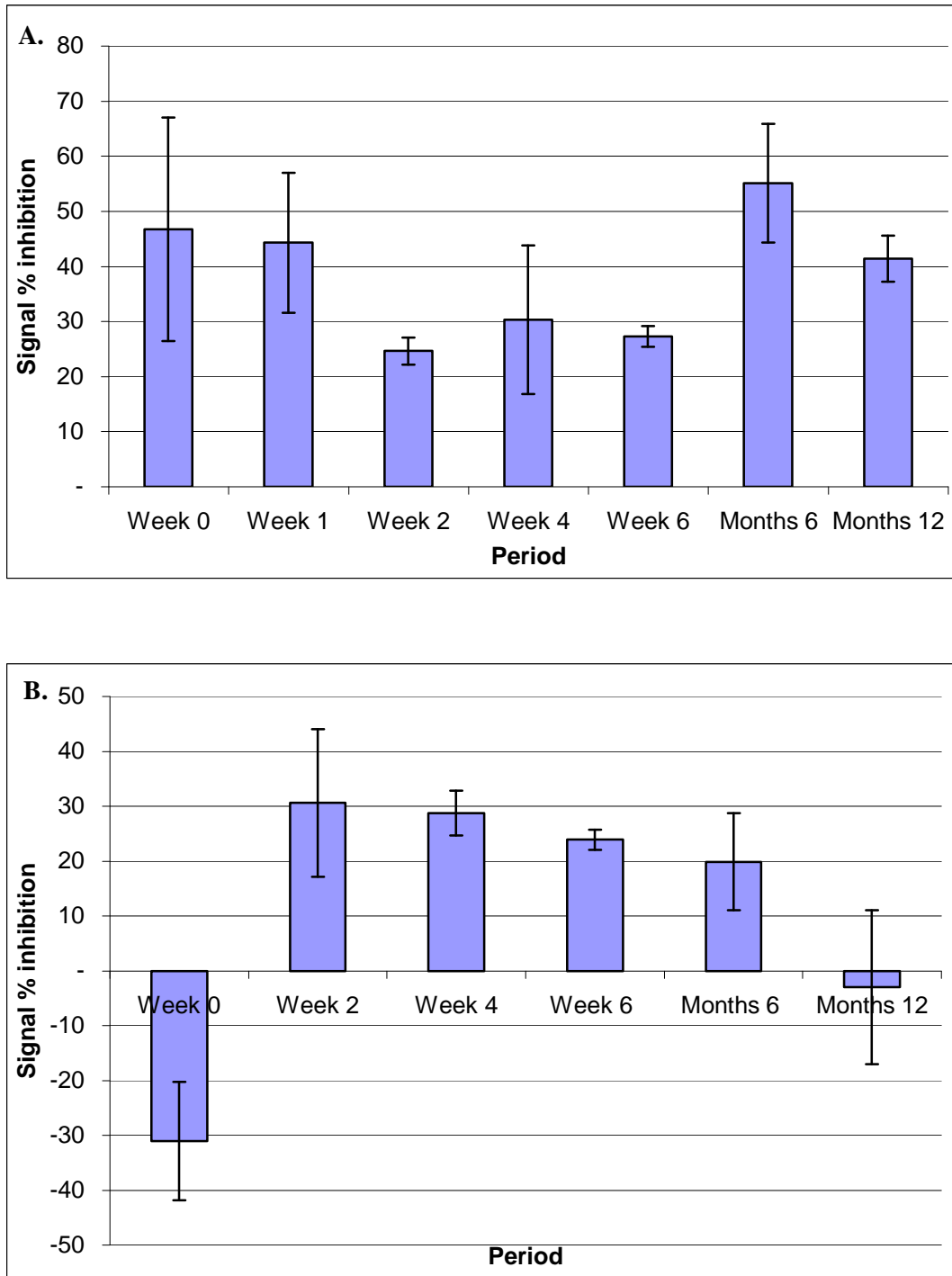


Figure 3.8: ESPRIT- MARTI test results of inhibition of human serum antibody binding to mycolic acids in TB patients (A = P5121 and B = P3897) before and during anti-TB chemotherapy on the ESPRIT biosensor ($n \geq 3$, except for P5121 in week 2 and 6, $n = 2$).

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.

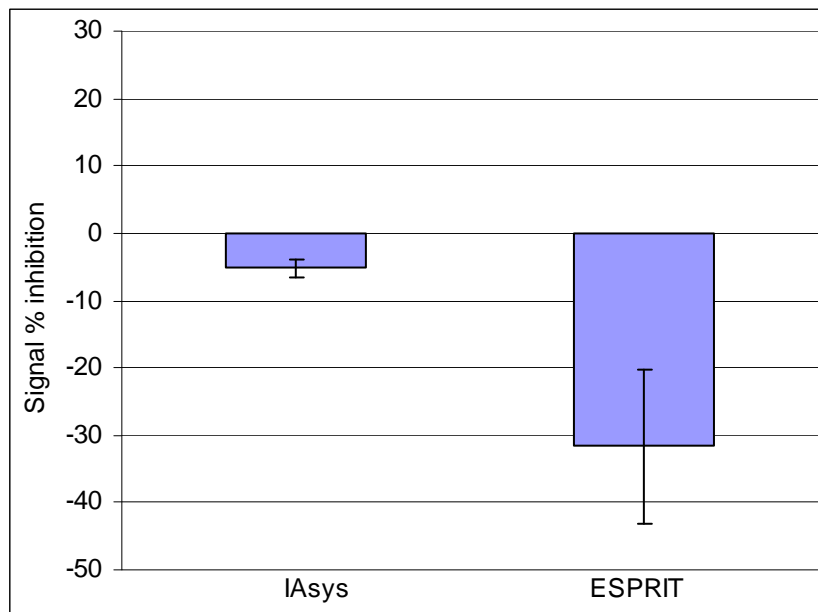


Figure 3.9: Comparison between IAsys and ESPRIT biosensor to determine the source of the false negative MARTI-test outcome of patient P3897 (week 0) on ESPRIT ($n = 3$).

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).

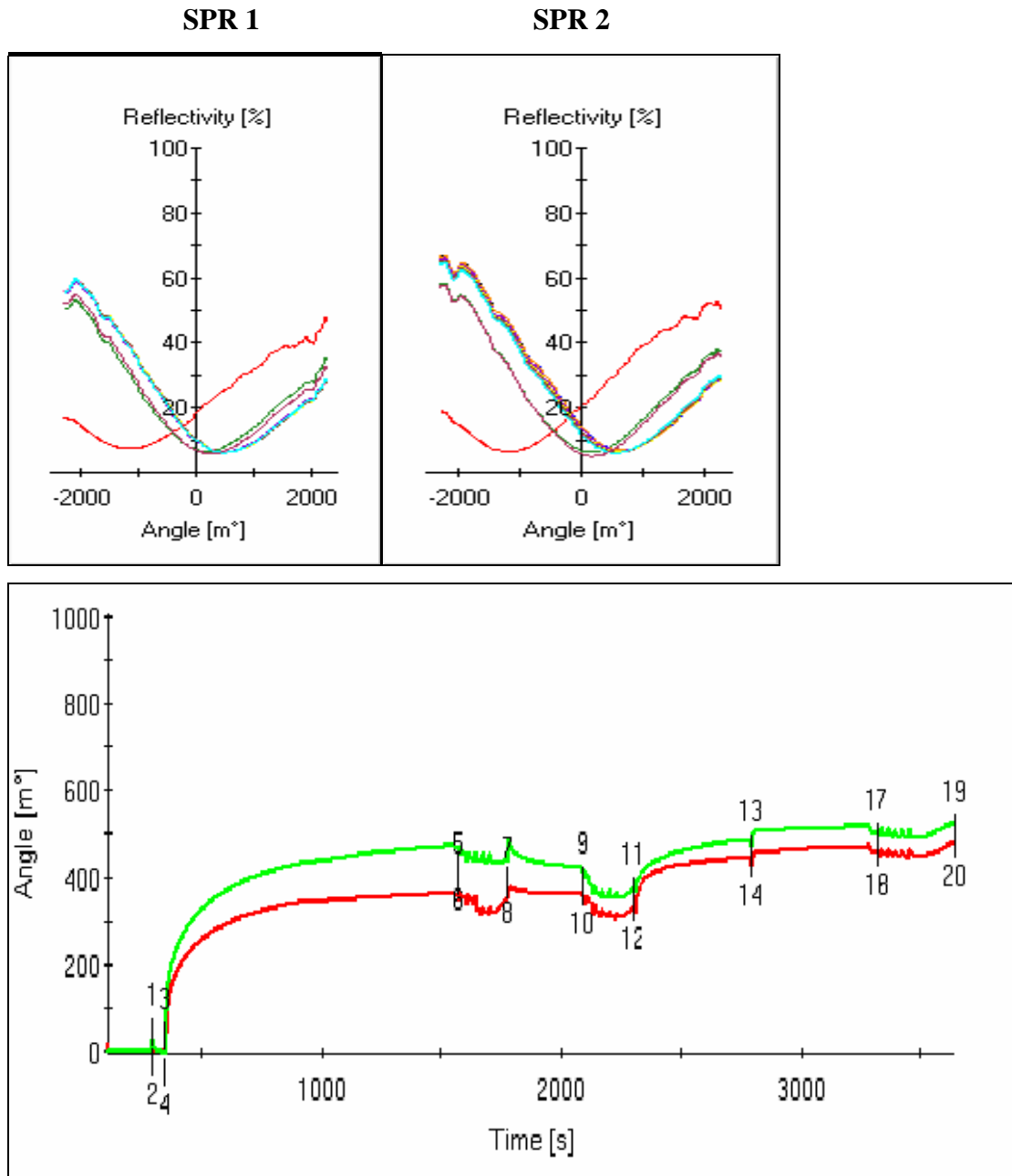


Figure 3.10: A representative ESPRIT sensorgram showing the full sequence of events to measure the inhibition of binding of human TB neg patient serum (HIV⁻TB⁻) antibodies to immobilized MA-liposomes on the ESPRIT biosensor before service. The unsymmetrical SPR dips (1 and 2) indicate the accumulation 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.

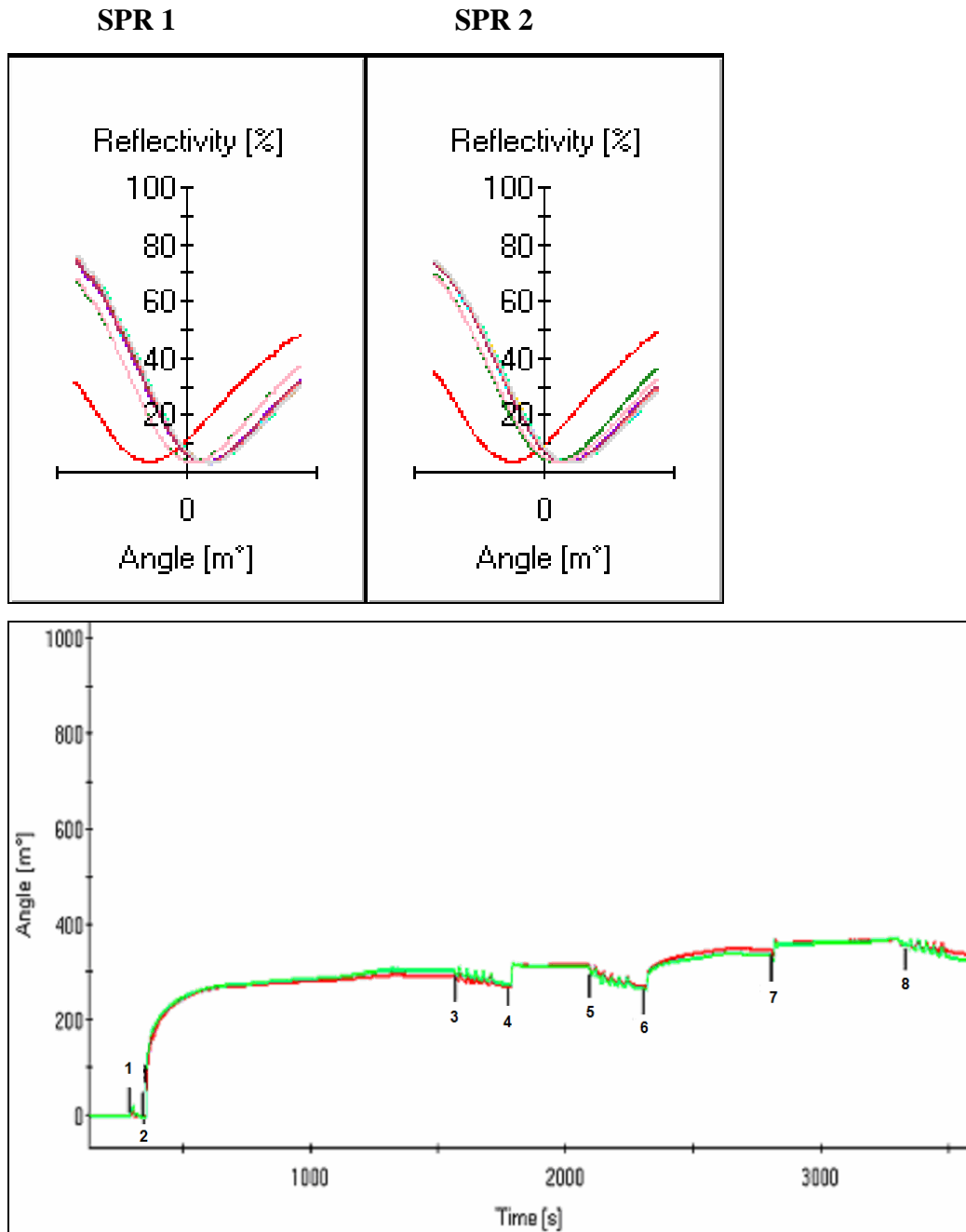


Figure 3.11: A representative ESPRIT sensorgram showing the full sequence of events to measure the inhibition of binding of human TB neg patient serum (HIV⁻TB⁻) antibodies to immobilized MA-liposomes on the ESPRIT after service. The symmetrical SPR dips (1 and 2) indicate free from dust optics. The event 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).

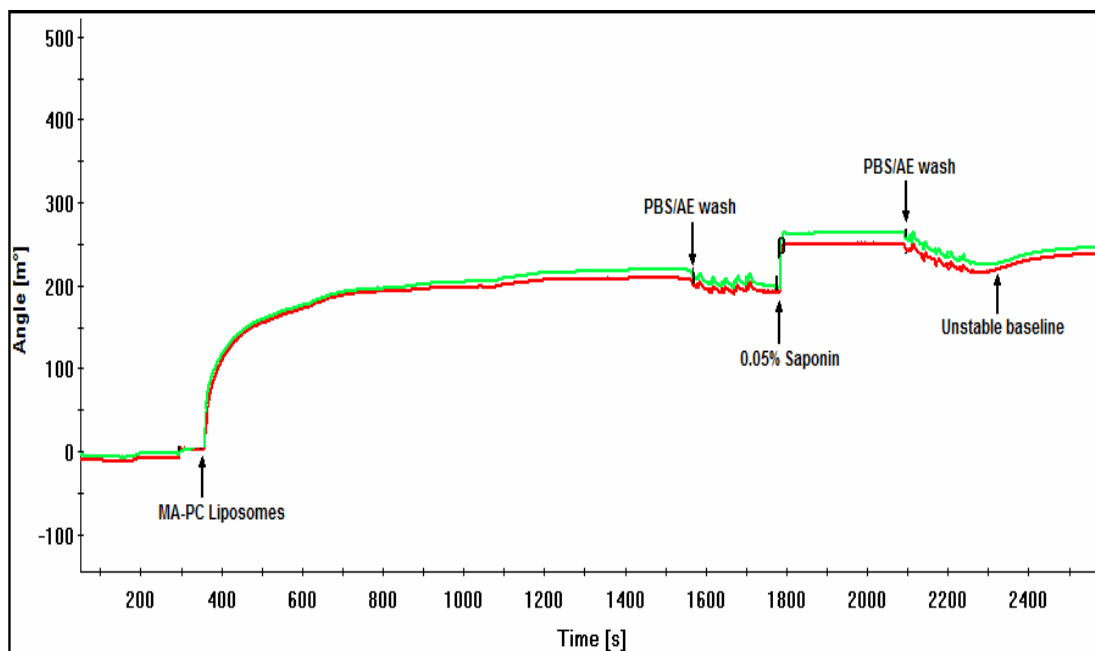


Figure 3.12: Effect of saponin (0.05%) on mycolic acid liposomes immobilized on the ESPRIT gold surface coated with octadecanethiol.

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 underivatized 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.