Appendix

Articles that this thesis contributed towards

1. Appendix A (p130-155):

   Lemmer, Y., Thanyani, S.T., Vrey, P.J., Driver, C.H.S., Venter, L., van Wyngaardt, S., ten Bokum, A.M.C., Ozoemena, K.I., Pilcher, L.A., Fernig, D.G., Stoltz, A.C., Swai, H.S., Verschoor, J.A., *Detection of Antimycolic Acid Antibodies by Liposomal Biosensors.* Methods Enzymol, 2009. **464**: p. 80-102.

2. Appendix B (p156-165):

   Semete, B., Booysen, L., Lemmer, Y., Kalombo, L., Katata, L., Verschoor, J., Swai, H.S., *In vivo evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems.* Nanomedicine, 2010. **6**(5): p. 662-671.

3. Appendix C (p166-174):
   Beukes, M., Lemmer, Y., Deysel, M., Al Dulayymi, J.R., Baird, M.,Koza, G., Iglesias, M.M., Rowles, R.R., Theunissen, C., Grooten, J., Toschi, G., Roberts, V.V., Pilcher, L., Van Wyngaardt, S., Mathebula, N., Balogun, M., Stoltz, A.C., Verschoor, J.A., *Structure-function relationships of the antigenicity of mycolic acids in tuberculosis patients.* Chem Phys Lipids, 2010. **doi:10.1016/j.chemphyslip.2010.09.006**.

CHAPTER FIVE

Detection of Antimycolic Acid Antibodies by Liposomal Biosensors

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Contents

Abstract

Antibodies to mycolic acid (MA) antigens can be detected as surrogate markers of active tuberculosis (TB) with evanescent field biosensors where the lipid antigens are encapsulated in liposomes. Standard immunoassay such as ELISA, where the lipid antigen is not encapsulated, but directly adsorbed to the well-bottoms of microtiter plates, does not yield the required sensitivity and

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specificity for accurate diagnosis of TB. One reason for this is the cross-reactivity of natural anticholesterol antibodies with MAs. MAs are the major cell wall lipids of mycobacteria. Mycobacterial MA has immunomodulatory properties and elicits specific antibodies in TB patients. Liposomes were optimized for their use as carriers both for the presentation of immobilized purified mycobacterial MA on sensor surfaces, and as a soluble inhibitor of antibody binding in inhibition assays. By using an inhibition assay in the biosensor, the interference by anticholesterol antibodies is reduced. Here, we describe the MA carrying capacity of liposomes with and without cholesterol as a stabilizing agent, optimized concentration and size of liposomes for use in the biosensor assay, comparison of the methods for wave-guide and surface plasmon resonance biosensors and how the cholesteroid nature of MA can be demonstrated by the biosensor when Amphotericin B is allowed to bind to MA in liposomes.

1. Introduction

As a result of the development of drug-resistant strains of *Mycobacterium tuberculosis* and the breakdown of the immune system of its host by HIV, tuberculosis (TB) is no longer a "controlled" disease and has become a major health problem in both developed and developing countries (Houghton *et al.*, 2002). Diagnosis of TB is no longer 100% reliable due to AIDS, and was never adequate for determining extrapulmonary and child TB. HIV coinfection and drug resistance appears to shorten the lifetime of a TB patient considerably, such that it becomes a matter of life and death to be able to diagnose TB within 24 h of sampling. The decision toward treatment cannot be taken lightly, as the treatment regime has to be maintained for at least 6 months to clear all the latent TB from the body. The current drive toward new tools for TB diagnosis arose from these facts, and that the mycobacterial pathogen isolated from sputum samples is slow growing, thereby requiring several weeks to become visible during *in vitro* growth (Reischl, 1996; Samanich *et al.*, 2000). Although DNA-amplifying technology has reduced the period from sampling to TB diagnosis to within days, it still uses mainly samples obtained from the lungs. Therefore, fast, affordable, and reliable diagnosis of TB has become a high priority in public health (Chan *et al.*, 2000), and is currently actively pursued in several laboratories. Mycolic acids (MAs) appeared to be promising antigens for the design of fast immunodiagnostics for TB (Verschoor and Onyebujoh, 1999).

The major cell wall lipids of mycobacteria are the MAs, which are long chain (C60–C90) α-branched, β-hydroxy fatty acids. Mycobacterial MAs are immunogenic. MA was the first nonprotein antigen shown to stimulate the proliferation of human CD4 , CD8 , and $CD4^+$ T-lymphocytes upon CD1 presentation (Beckman *et al.*, 1994; Goodrum *et al.*, 2001). In addition, anti-MA antibodies could be detected in the serum of patients with active

pulmonary TB (Pan *et al.*, 1999) using the standard ELISA procedure, indicating that free MAs are present in the circulation during active TB disease (Beatty *et al.*, 2000). The prevalence of anti-MA antibodies appeared to be independent of the degree to which a person was suffering from AIDS (Schleicher *et al.*, 2002). Whereas ELISA did not detect anti-MA antibodies well enough to be considered as a basis for a diagnostic test of active TB, biosensor analysis did (Thanyani *et al.*, 2008). It could achieve the required specificity and sensitivity to be regarded seriously as a solution to the current dilemma of standard TB diagnosis taking several weeks after sampling to produce a result. Even the highly sensitive PCR detection of mycobacterial DNA in patient sputum samples still takes a few days to deliver a diagnostic outcome.

MAs are soluble in extremely nonpolar organic solvents such as chloroform, dichloromethane, and hexane. Alternatively, they can be "solubilized" in boiling water or aqueous buffers. For the detection of anti-MA antibodies by means of ELISA, MA was either coated from hexane solutions (Pan *et al.*, 1999) or from boiling phosphate-buffered saline (PBS) (Schleicher *et al.*, 2002). This presentation of MA is clearly not physiological. Presentation of MA in liposomal environments more closely resembles the way in which MA is encountered in the body of the patient. Indeed, when encapsulated into liposomes and injected into mice, MA was shown to behave as a typical pathogen-associated molecular pattern (Korf *et al.*, 2005). It could also act as an immunomodulatory compound that suppresses experimental asthma through Treg cell intervention (Korf *et al.*, 2006). It was only logical to derive that diagnosis of TB by detection of surrogate marker antibodies against MA should perform better when MA antigen was presented in liposomes. This was demonstrated by Thanyani *et al.* (2008) by making use of biosensors. Here, we describe how liposomes are optimized for their use as carriers of MA for immobilization as antigens on sensor surfaces, and as soluble inhibitors of antibody binding in inhibition assays in a TB diagnostic assay. It is dubbed the MARTI-test, as an acronym of Mycolic acid Antibody Real-Time Inhibition. Whereas the original MARTI-test is described for a wave-guide biosensor (Thanyani *et al.*, 2008), we show here how the method is adjusted to also suit the more popularly used surface plasmon resonance (SPR) biosensors. Finally, we demonstrate the cholesteroid nature of MA with the biosensor by measuring the binding of Amphotericin B to MA in liposomes.

2. Experimental

2.1. Purification of mycobacterial MA

MAs are isolated and purified from *M. tuberculosis* H37Rv ATTC 27294 purchased from the American Type Culture Collection (ATTC, Baltimore, MD, USA) as described by Goodrum *et al.* (2001). The MA is dissolved in

chloroform (HPLC grade; Merck; Darmstadt, Germany) and aliquotted into glass vials. The chloroform is evaporated under nitrogen gas and the dried MA is stored at 4 °C until use. The purified MAs are checked for LPS contamination using the kinetic-QCL Limulus amoebocyte test kit (Sigma, St. Louis, MO). In the experiments shown in this chapter, no LPS is detected.

2.2. Fluorescent labeling of MA

MA is fluorescently labeled by derivatization with 5-bromomethyl fluorescein (5-BMF; Molecular Probes, Leiden, The Netherlands), as described by Korf *et al.* (2005). Quality control is performed by TLC on a silica gel GHL thin layer plate. Chromatography is performed in two dimensions, with chloroform:methanol:water as the mobile phase in the first dimension, and 100% methanol (Merck) as the mobile phase in the second dimension. Iodine vapor is used to visualize the MA. The absence of free 5-BMF, not associated with the MA spot, indicates that the label is covalently bound. Fluorescently labeled 5-BMF–MA is incorporated into liposomes for assessment by the biosensor or flow cytometry.

2.3. Determination of MA carrying capacity of liposomes

MAs are dissolved in chloroform and 100 μg quantities are aliquoted into amber vials and stored at 4 °C. Liposomes are prepared according to the method described by Bangham (1983). This involves the deposition of a thin lipid film from an organic solvent medium on the wall of a container, followed by agitation with an aqueous medium. In short, phosphatidylcholine (PC from egg yolk, 99% pure; Sigma), cholesterol (Sigma), and dried MA are all dissolved separately in chloroform. PC (9 mg) with or without 4.5 mg cholesterol and varying amounts of MA are mixed together in a glass vial. The chloroform is evaporated under nitrogen gas in a chemical hood. PBS (1 ml) is added and the mixture is heated to 80 °C until the lipid film dissociates from the wall of the vial. The hot mixture is then vortexed and sonicated for 5 min using a Branson sonifier for 50 pulses at a 20% output level.

MA-carrier liposomes are extracted using a triphase partition method, modified from Dennison and Lovrein (1997). Briefly, a sample (50–200 μl) of MA-carrier liposome solution is diluted to 600 μl with H_2O and extracted three times with equal volumes of *tert*-butanol and chloroform. The chloroform phases are collected and the chloroform is evaporated under nitrogen gas. MA remaining in the vials due to saturation of the liposomes is collected by chloroform rinses. HPLC of the extracted MA is carried out as described (Butler and Kilburn, 1990), after derivatization with *p*-bromophenacyl bromide. A high molecular weight internal standard

(Ribi ImmunoChem Research Company, Hamilton, MT, USA) is added to each sample to allow quantification of the extracted MA.

Liposomes are made with egg PC, with or without the addition of cholesterol. The maximum amount of MA that can be incorporated reproducibly is about 2 mg/ml for liposomes consisting of phosphatidyl choline and cholesterol, but only about 500 μg MA/ml into liposomes consisting of phosphatidyl choline only (Fig. 5.1). MA not incorporated into the liposomes can in most cases be quantitatively recovered from the wall of the vessel in which the liposomes are prepared (shaded part of the bars). At very high concentrations the recovery is not quantitative, as the MA tends to form clumps which float in the liposome suspension. The MA liposomes

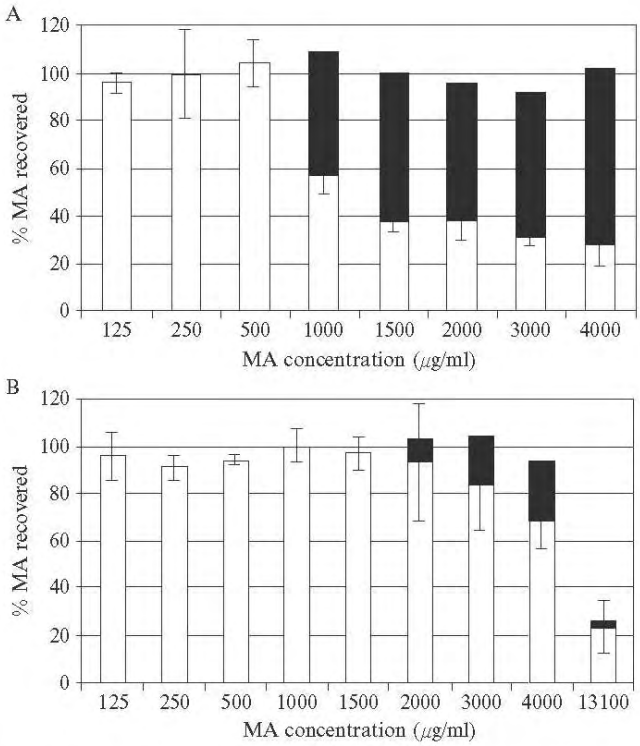

Figure 5.1 Titration of the maximum amount of MA that can be incorporated in liposomes consisting of egg phosphatidylcholine, with or without cholesterol: (A) liposomes consisting of egg phosphatidylcholine (99% pure) only; (B) liposomes consisting of egg phosphatidylcholine (99% pure) and cholesterol. The shaded part of the bars represent the percentage of MA recovered from the wall of the vessel in which the liposomes were prepared. Each bar represents the mean of three samples and the S.D.

can be lyophilized and reconstituted without significant loss of MA content. Heating to 80 °C in the reconstitution process is an important step to ensure full recovery of MA content.

2.4. MA liposome size is influenced by cholesterol and MA content, but not by pH

MA-containing liposomes are prepared with equal amounts of 5-BMF labeled and unlabeled MAs and varying amounts of cholesterol according to Table 5.1.

A liposome stock suspension is obtained by adding PBS (1 ml) to the dried lipid components, dissolving it on a heat block for 60 min after vortexing and sonicating the heated (80 °C) mixture with a Branson Sonifier B30 Cell Disrupter at 20% duty cycle and output control of 2 for 5 min.

Both the size distribution (forward scatter, FS) and the fluorescence (FL-1) of the liposomes are measured on a flow cytometer (a Beckman Coulter Epics Ultra instrument is used to obtain the results in Fig. 5.2). For the analysis, 10 times dilutions of the stock liposomes are prepared using PBS in plastic analyzing tubes covered with foil to prevent the photobleaching of the fluorescent marker. The tubes are kept in a water bath at 37 °C during the experiments. One gate is set on the analyzer to exclude background signals and debris events that have FS values below 10. A constant number of 50,000 events are counted per measurement. Flowset (3.6 μm) fluorospheres measured with each experiment is used to correlate the relative size of the liposomes to that of the beads when varying amounts of cholesterol are added to a constant amount of MA and PC.

The size distribution of the liposomes changes in accordance with a change in the cholesterol concentration (Fig. 5.2A). Two different liposome states can be identified. At the two highest concentrations of cholesterol, the liposomes are 2 log units bigger than the liposomes that contain none or the lowest concentrations of cholesterol. The liposomes obtained with the addition of 11.25 μl of cholesterol stock give an intermittent size of liposomes in between that of the two states. With an increase in the cholesterol concentration and liposome size, the liposomes also show an analogous increase of 2 log units in the amount of fluorescence per liposome that is

Table 5.1 Liposome compositions with various concentrations of cholesterol

Cholesterol (μl from 100 mg/ml stock)	0	5.63	11.25	22.5	45
PC (μl from 100 mg/ml stock)	90	90	90	90	90
5-BMF–MA (μg)	125	125	125	125	125
Unlabeled MA (μg)	125	125	125	125	125

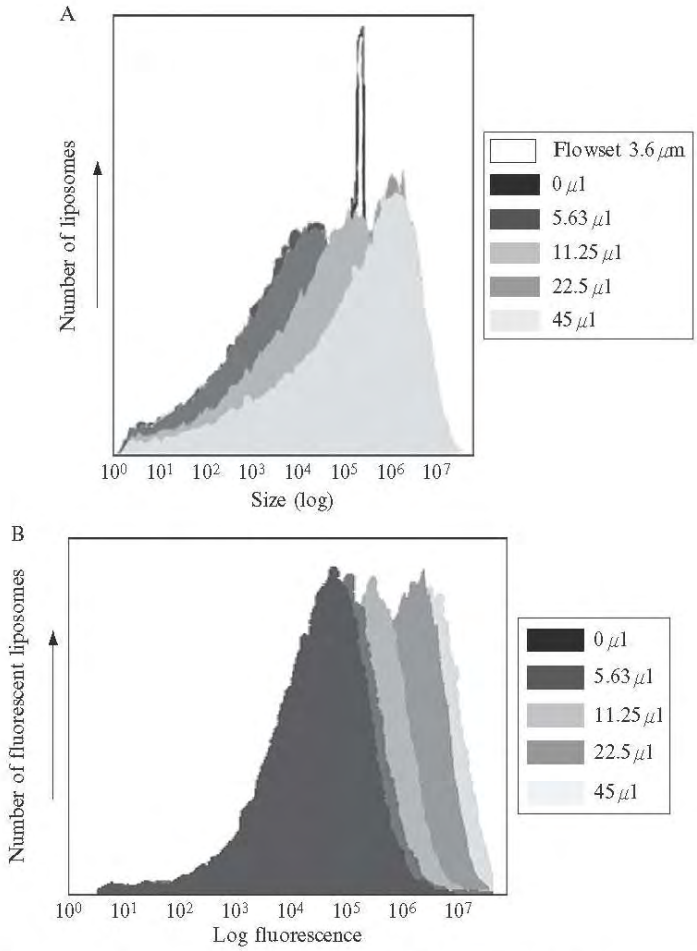

Figure 5.2 Size (A) and fluorescence (B) of MA-containing liposomes containing various amounts of cholesterol according to Table 5.1 as determined with flow fluorometry. Flowset beads (3.6 μm) were used as a marker for the size distribution of the liposomes.

emitted (Fig. 5.2B), indicating that the change from smaller to larger liposomes is not due to swelling with water/PBS, but to an accumulation of more MA-containing material into the bigger liposomes. The results imply that cholesterol content determines two states of liposomes, where higher concentrations of cholesterol induce the disordered state of the bilayer in liposomes that increase their size 100-fold. All cholesterol-containing liposomes used in the biosensor analysis were in the disordered state at the highest concentration of cholesterol shown here.

In Fig. 5.2, the biggest sized liposomes measured around 30 μm and were composed of PC (9 parts), cholesterol (4.5 parts), and MA (0.25 parts). When the phosphatidyl choline is combined with either the MA or the cholesterol, significant changes in the sizes of the liposomes occur. With cholesterol alone the size sharply increases, while with MA alone the size sharply decreases (Fig. 5.3). This effect is not significantly influenced in the pH range between 4 and 10 of the PBS to which the liposomes are exposed, probably due to the unchanged surface charge of phosphatidyl choline liposomes over these pH values. The anionic phosphatidyl residues ($pK_a \leq 3.5$) only become neutralized below pH 4, while the cationic state of choline ($pK_a = 13.9$) is maintained over the full pH range measured (Tatulian, 1993). It is therefore expected that the stability of the liposomes described here for use in the biosensor will be quite tolerant to a broad pH window extending to both sides of the typical pH of 7.4 of PBS.

2.5. MA liposome immobilization on IAsys biosensor cuvettes

A number of application notes are available for the adsorption of liposomes to hydrophobic sensor surfaces. These do not stand up as rigorous and reliable for the immobilization of MA antigen-containing liposomes aiming at antibody detection. This motivated the use of nonderivatized sensor cuvettes where the glass-like hafnium oxide surface can be made hydrophobic by treatment with the cationic detergent, cetylpyridinium chloride (CPC). CPC is well known for its use as an antiseptic agent. Its property

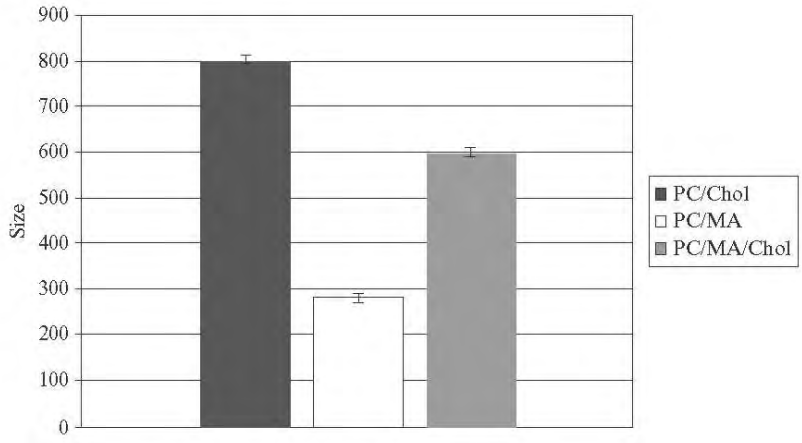

Figure 5.3 Relative sizes of PC/cholesterol and PC/mycolic acid liposomes and a mixture of the two liposomes. All liposome types contained 9 parts of phosphatidyl choline and either 4.5 parts of cholesterol, or 0.25 parts of MA, or both.

of binding to glass when applied at aqueous dilutions below the critical micellar concentration (i.e., <0.1 mg/ml) is long established (Westwell and Anacker, 1959), leaving a "greasy" surface (Hartley and Runnicles, 1938).

PBS/AE buffer consists of 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, and 1.05 g Na_2HPO_4 per liter of double distilled, deionized water containing 1 m*M* EDTA and 0.025% (m/v) sodium azide and is adjusted to pH 7.4. CPC (0.02 mg/ml) and saponin (1 mg/ml) are prepared in PBS/AE. The IAsys resonant mirror biosensor system and twin-cell nonderivatized cuvettes are from Affinity Sensors (Farfield Scientific, Crewe, UK). The sensor is set for a data sampling interval of 0.4 s, temperature of 25 °C, and stirring rate of 75% for all experiments. The cells are rinsed three times prior to use with 96% ethanol (Saarchem, South Africa), followed by extensive washing with PBS/AE. A 60 μl volume of PBS/AE is pipetted into each cell of the cuvette to obtain a stable baseline for 1 min. Cuvettes are washed with PBS/AE until a stable baseline could be maintained for at least 5 min. The cells are aspirated and 50 μl of a 20 μg/ml CPC in PBS/AE solution is added. After 10 min the cells are washed five times with 60 μl PBS/AE. PBS/AE (25 μl) is added until a stable baseline is achieved. Twenty-five microliters of the desired liposome solution are added and the binding response is monitored for 10 min. The MA-containing liposome concentration consisting of 9:3:1 (m/m) phosphatidyl choline:cholesterol:MA is titrated between 0.02 and 10 mg/ml of total lipid concentration. The MA-containing liposome concentration with the optimum binding capacity is found to be 500 μg total lipid/ml (Fig. 5.4). This concentration is used in all subsequent experiments.

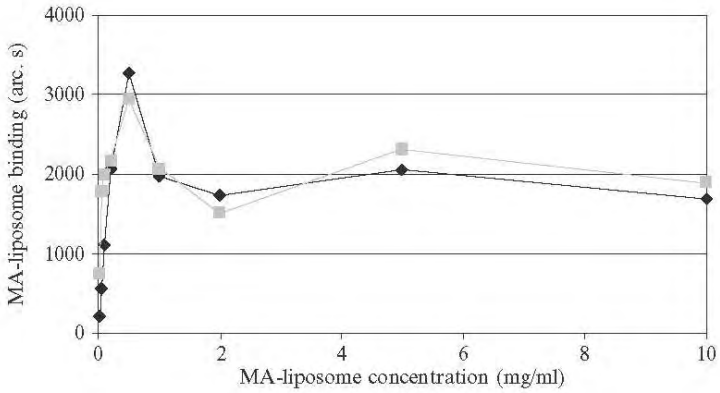

Figure 5.4 Titration of the optimal concentration of MA/cholesterol-containing liposomes for immobilization on a nonderivatized IAsys biosensor cuvette.

After liposome binding, the cells are washed five times with 60 μl PBS/AE and immediately after that, treated five times with 60 μl 1 mg/ml saponin in PBS/AE. Due to response differences among saponin batches, it is necessary to titrate the amount of saponin required each time a new batch of saponin is used. The cells are incubated with saponin for at least 10 min and until a stable baseline is achieved before a final five times wash with 60 μl PBS/AE. Antibody interaction analysis could be continued from this point as described by Thanyani *et al.* (2008). The cells are 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 are performed using patient's serum that is 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 is added in each cell, to compare the responses of the two cells over 10 min. A preincubation of 1/500 dilutions of serum with solutions of liposomes-containing MAs and empty liposomes (PC alone) is allowed for 20 min. These are then added (10 μl) for binding inhibition studies in the two cuvette cells, one with MAs liposomes and the other with empty liposomes as a control, and allowed to bind for 10 min. Finally, dissociation of antibodies is effected with three times PBS/AE washing and measurement of the response for 5 min.

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

A typical profile of a positive and negative TB test using this method, but applied in the ESPRIT biosensor, is shown in Fig. 5.11. The outcome of the test in the IAsys biosensor scored an overall specificity of 48.4% (15/31) and sensitivity of 86.7% (26/30) with 61 patient sera analyzed (Thanyani *et al.*, 2008). When adjusted for the inadequate performance of the reference culture test in HIV-infected patients, the MARTI-test scored a specificity of 76.9%.

A disadvantage of the IAsys biosensor in this application is the difficulty of aligning the relative basic signal strength of the two cells in a cuvette, which must be identical during the first serum exposure before a patient diagnosis can be derived from the difference of the binding signal during the second liposome-preincubated serum exposure. We experienced an average success rate of around 30% to obtain comparable signals during the first serum exposure. For this reason and due to the fact that the IAsys cuvettes were relatively expensive, we transferred the technology from the wave-guide IAsys biosensor to the more generally used SPR biosensors.

2.6. Technology transfer from the IAsys waveguide to the ESPRIT SPR biosensor

An ESPRIT SPR biosensor (Eco Chemie B.V., Utrecht, The Netherlands) is used in this study to detect antibodies to MA in human patient sera. 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) to indicate the binding of ligands, in this case anti-MA antibodies. Both IAsys and ESPRIT biosensors use a cuvette system. The light is totally internally reflected from the sensing surface by means of a prism in both biosensors. SPR signals are related to the refractive index close to the sensor surface, and therefore report the amount of macromolecules bound to the sensor surface. An SPR immunosensor is composed of several important components such as a light source, detector, prism with transducer surface and flow system (Shankaran *et al.*, 2007). The transducer surface is usually a gold film (50–100 nm, on which biomolecules, such as antibody or antigen, are immobilized) on a glass slide optically coupled to the glass prism through refractive index matching oil. The resonance conditions are influenced by the biomolecules interacting with their immobilized ligands 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.

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 use of self-assembled mono- and multilayers (SAMs) is increasing rapidly in various fields of research, and this applies especially to the construction of biosensors (Zhang *et al.*, 2000, 2008). The uncomplicated procedure for SAM formation and compatibility with metal substrates such as gold for electrochemical measurements enable special benefits for biosensor applications. The term self-assembly involves the spontaneous arrangement of atoms and molecules into an ordered stable form or even aggregate of functional entities (Tecilla *et al.*, 1990). For example, the highly ordered and dense nature of the long-chain alkanethiols of SAMs mimic the cellular microenvironment of lipid bilayer structures, thereby providing novel substrates for immobilized biomolecules (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).

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). Here, the preparation of octadecanethiol (ODT) in absolute ethanol to form a SAM is described. It is characterized by cyclic voltammetry (CV) and applied for the measurement of binding, or inhibition of binding of patient serum antibodies to MAs that are immobilized in liposomes onto the alkanethiol coated ESPRIT biosensor surface. The low solubility of ODT in ethanol is preferred to form the 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. Here, a full coverage of the underivatized Au surface is observed when 10 m*M* of ODT is used. This is confirmed by a strongly hindered redox reaction when the surface is characterized with a CV instrument. CV experiments are carried out using an Autolab potentiostat PGSTAT 30 from Eco Chemie (Utrecht, The Netherlands) driven by the General Purpose Electrochemical Systems (GPES) data processing software version 4.9.

Sodium dodecylsulphate (SDS) and absolute ethanol (analytical grade) were obtained from Merck (Gauteng, SA). ODT, 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, MO). Acetic acid (analytical grade), sodium hydrogen carbonate ($NaHCO_3$), isopropanol (chemically pure), and sodium hydroxide (NaOH) were obtained from Saarchem (Gauteng, SA). ODT (10 m*M*) is dissolved in absolute ethanol using a water bath sonifier (Ultrasonic Cleaner, Optima Scientific CC, Model: DC150H) for 30 min. Sodium hydrogen carbonate (0.2 *M*), SDS (0.5%, w/v), sodium hydroxide (50 m*M*), 1 m*M* ferrocyanide/ferricyanide, 1 *M* KCl, and urea (6 *M*) are prepared with sterile double distilled water. The coating of the gold disk cannot be monitored in real time, since the ODT is dissolved in absolute ethanol that generates too large refractive index jumps in the sensor signals when alternated with PBS/AE. The underivatized gold disk is incubated for 16 h at room temperature in 10 m*M* ODT. The ODT-coated gold disk is immersed in a solution of 1 m*M* ferrocyanide/ferricyanide (used as a redox probe) containing 1 *M* KCl and scanned at a rate of 25 and 50 mV/s at a potential window of −0.1 to 0.5 V (vs. Ag/AgCl, saturated KCl). The results in Fig. 5.5 show that there is a significant drop in the current response of the ODT-coated disk toward the redox probe in comparison to the uncoated gold disk, indicating the formation of a stable SAM of ODT by S–Au bonds on the surface of the gold disk. The SAM can be maintained after the exposure of the coated surface to several regeneration cycles of absolute ethanol and a mixture of 50 m*M* NaOH with isopropanol (2:3, v/v).

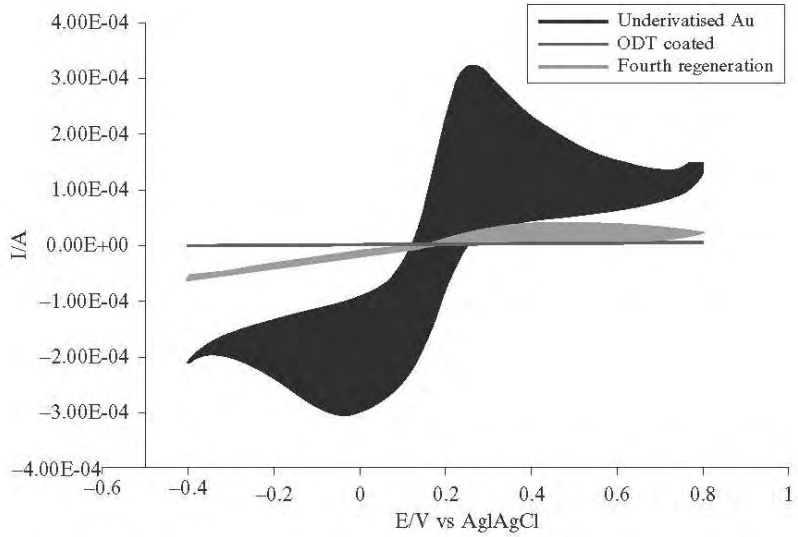

Figure 5.5 Testing of the octadecanethiol-coated ESPRIT biosensor gold surface against sequential times of regeneration with a mixture of isopropanol and 50 m*M* NaOH (2:3, v/v) using cyclic voltammetry. The voltammograms for the different surfaces are shaded to better identify the one from the other.

Kim *et al.* (2001) reported that partial ODT 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 ODT in ethanol is exposed to oxygen and oxidized to disulfide, the oxidized disulfide can be precipitated onto the monolayer. Here, the solution of ODT in absolute ethanol is covered with parafilm to avoid oxygen exposure.

The ODT-coated disk is subsequently inserted into the biosensor on a droplet of special refractive index oil, after wiping the glass bottom surface with lens tissue. The PBS/AE is filtered through a 0.2 μm particle retention membrane and degassed with helium for 30 min before use. Degassing is required whenever a hydrophobic surface is created that is exposed to air before liposome addition to prevent the formation of a "dissolved" air layer onto the hydrophobic surface that prevents SPR to occur. The cuvette is flushed with 500 μl ethanol (96%) using the automatic dispenser with simultaneous draining, followed by brief (~60 s) flow-washing with PBS/AE. An automated software program sequence can be created to control the addition of all the samples and liquids into the cuvette. Quality of the surfaces are monitored by determining the SPR dips after cleaning the Au ODT-coated surface with 96% ethanol and a mixture of isopropanol and 50 m*M* sodium hydroxide (2:3, v/v). The samples are transferred from a

384 multiwell plate (Bibby Sterilin Ltd, Stone, UK) to the cuvette surface by an autopipettor. First, the baseline of the ESPRIT biosensor is set with 10 μl PBS/AE, followed by addition of 50 μl MA liposomes on the disk for 20 min. The immobilized liposomes are then finally washed five times with 100 μl PBS/AE, to prepare for blocking the surface with saponin.

2.6.1. Use of degassed buffers after liposome coating of the sensor surface

A recent study (Eastoe and Ellis, 2007) showed that exposure of lipids to degassed buffers resulted in a detergent effect that destabilized the lipids. The removal of hydrophobic gas by pumping created a limited ability of the degassed water or aqueous buffer to dissolve lipids. To determine whether this will also be the case here where buffers are degassed by bubbling through helium gas, we exposed a liposome-coated surface repeatedly to either degassed or nondegassed buffer. The liposomes-containing MAs are immobilized on ODT-coated gold sensor disks for 20 min. The liposomes are washed five times with degassed or nondegassed PBS/AE, and left for 5 min with mixing to achieve a baseline. This procedure is repeated three times. Figure 5.6A demonstrates how the baseline is affected during movement of degassed PBS/AE over the liposome coat; compared to when buffer is used that is not degassed (Fig. 5.6B). A stable baseline is obtained only when a nondegassed PBS/AE is used. The rest of the procedure in the MARTI-assay that follows after liposome coating is subsequently done with buffer that is not degassed, taking special care that air bubbles do not develop in the fluid lines that could affect the working of the pumps. In several attempts where we test samples with continued use of degassed buffer after liposome coating, we obtain nonreliable results, with the liposome layer often coming apart at the final step of antibody incubation.

2.6.2. Optimization of saponin concentration

Different concentrations of saponin (%, m/v) prepared in PBS/AE (0.1%, 0.05%, 0.025%, 0.0125%, and 0.00625%) are tested to block the hydrophobic sites of the MA liposome layer.

The stock saponin concentration is 0.1% and the subsequent dilutions are prepared from this stock solution. From the results obtained (Fig. 5.7A), there is a tendency of an increase in saponin accumulation onto MA liposomes immobilized on an ODT-coated gold surface, as the saponin concentration is increased from 0.00625% to 0.05%. At a saponin concentration of 0.05% there is an amount of net saponin accumulation after PBS/AE buffer wash (Fig. 5.7B). An unstable baseline is also obtained when 0.05% saponin is used. A saponin concentration of 0.0125% is chosen as optimal, because it gives a stable baseline and acceptable variation after PBS/AE wash (Fig. 5.7B) as compared to 0.00625% and 0.025%. The differences in optimal saponin concentration used on the IAsys (0.03%) and current ESPRIT biosensors

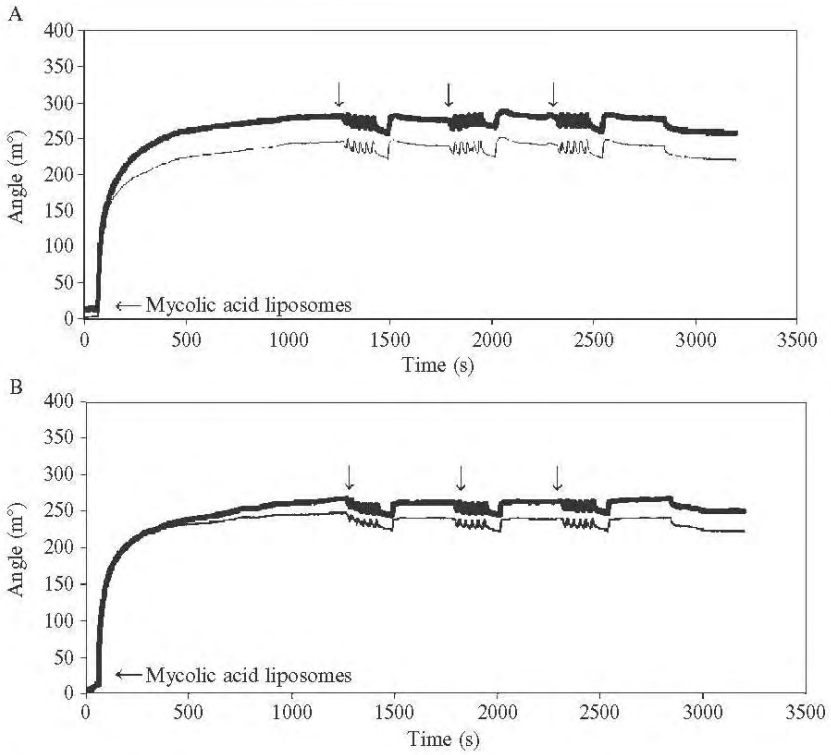

Figure 5.6 Effect of degassed (A) and nondegassed (B) buffer on immobilized mycolic acids liposomes in the ESPRIT biosensor. The arrows indicate where washing cycles with PBS/AE were introduced before allowing a baseline to be reached with mixing before substitution of cell content.

(0.0125%) could be due to different batches of saponin, or that the CPC and ODT activation before immobilization of the MA produces different surface properties.

2.6.3. Optimization of first serum exposure dilution in PBS/AE

After optimization of saponin concentration, the next step is to determine which concentration of serum is optimal for the MARTI-assay in the first exposure to antigen. Chung *et al.* (2005) indicated that serum should be diluted to minimize the nonspecific binding to the biosensor surface. Serum is a complicated protein mixture for direct application to a biosensor surface. The introduction of a first serum exposure at high dilution was previously done on the IAsys biosensor to provide a practical working dilution that did not fully saturate the antigen coat, but was still concentrated enough to give

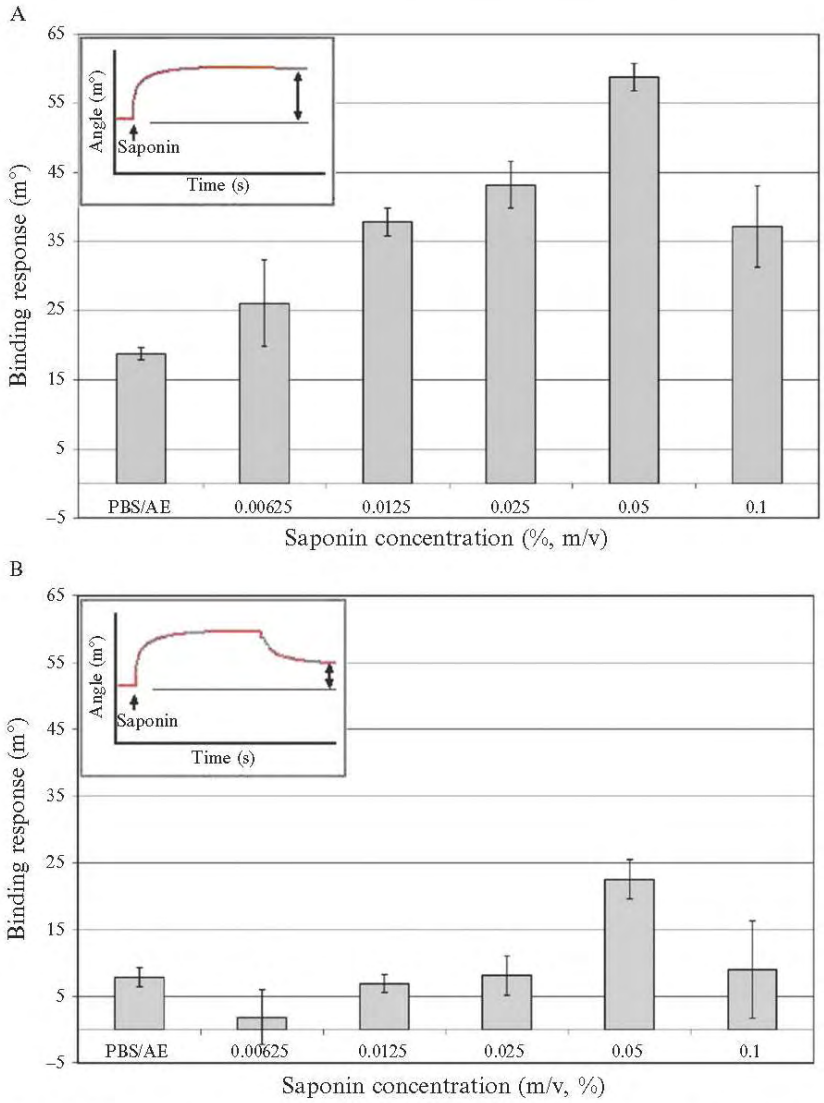

Figure 5.7 Optimization of saponin concentration to avoid nonspecific binding on immobilized mycolic acids on the Au surface coated with octadecanethiol. Accumulation of saponin is performed for 5 min (A) and washed with PBS/AE (B). The error bars indicate the standard error of the mean (S.E.M.) and $n = 3$.

a measurable signal to probe the comparability of the binding signals from the cuvette. This simultaneously blocked off the major nonspecific binding areas and hugely increased the accuracy of the MARTI-test.

Serum samples collected for another study (Schleicher *et al.*, 2002) were used that were obtained from 61 adult 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 TB. The TB-negative patients that were used as controls had medical conditions other than TB and were recruited from the general medical wards.

The liposomes are immobilized as described earlier, the surface blocked with 0.0125% saponin, after which 50 μl of PBS/AE is added and left for 5 min to affect a stable baseline. This is 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 preincubated in MAs-containing or empty liposomes is kept constant at 1/250 in all the experiments. The results show that the chosen serum dilution range of 1/4000–1/500 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. 5.8 gave a positive linear correlation with a coefficient (r^2) of 0.9749 between the serum concentrations and their signal binding response over the range measured, which is a requirement for a successful MARTI-assay.

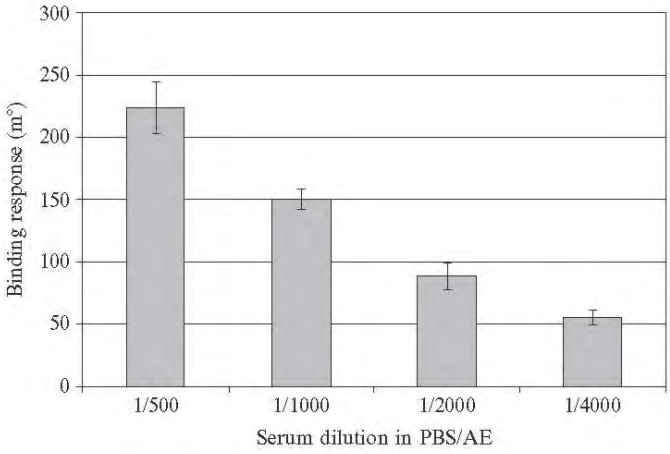

Figure 5.8 Optimization of the dilution of serum (P135) for the first exposure to antigen in the MARTI-assay, after 0.0125% saponin blocking of the mycolic acid liposome coat of the ESPRIT biosensor. The error bars indicate the standard deviation. Correlation coefficient (r^2) = 0.9749, $n \geq 3$.

The 1/4000 and 1/2000 dilutions are adequate for the first serum exposure, leaving enough room for a positive binding event at the second serum exposure.

2.6.4. Second serum exposure with liposome preincubation

P129 (TB-positive) serum was used to optimize the dilution of the second exposure to preincubated serum in MA-containing, or empty liposomes for inhibition studies, following on a first serum exposure to immobilized antigen at a dilution of either 1/4000 or 1/2000. The first exposure should avoid the saturation of antigen with antibody before the addition of preincubated serum. Different dilutions (1/250, 1/500, 1/1000, and 1/2000) of preincubated serum in MA-containing and empty liposomes are applied by 35 μl addition to either 1/4000 or 1/2000 of first serum exposure in PBS/AE, after 10 min of incubation. This is followed by washing away of the unbound antibody with five times 100 μl PBS/AE.

The TB-positive patient P129 serum showed a significant decrease of signal when the serum was preincubated in MA-containing liposomes, compared to empty liposomes over a range of 1/250, 1/500, or 1/1000 dilution (Fig. 5.9) after a first serum exposure at 1/4000. There was no inhibition of antibody by MA preincubation observed when 1/2000 dilution of serum was used and binding response signals were also too low.

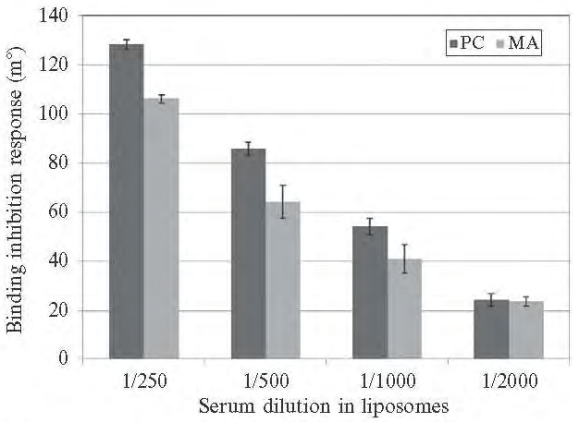

Figure 5.9 MARTI antibody-binding inhibition response of preincubated TB-positive P129 serum dilutions inhibited with mycolic acids (MAs)-containing and empty (PC) liposomes after first serum exposure of 1/4000 on immobilized MAs. The error bars indicate the standard deviation. P129 showed significant 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, $n = 3$.

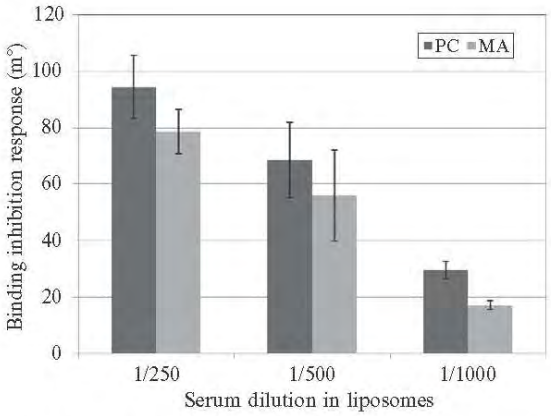

Figure 5.10 MARTI-binding inhibition response of various dilutions of preincubated TB-positive patient serum (P129) with mycolic acids (MA)-containing and empty (PC) liposomes after first exposure serum dilution of 1/2000 to surface immobilized MAs. The error bars indicate the standard deviation. No statistical difference (at 95% confidence limit) is obtained at 1/250 and 1/500 dilutions between MA- and PC-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) $n = 3$.

This shows that the lower limit of serum concentration was reached at 1/2000 dilution to measure the inhibition of anti-MA antibody binding.

The results in Fig. 5.10 indicate that inhibition values of 17%, 19%, and 41% were obtained at 1/250, 1/500, and 1/1000 dilutions of serum in liposome solution, respectively, after first serum exposure at 1/2000 dilution. 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 preincubated serum dilution at 1/1000 dilution (numerical difference: 12.50 millidegrees). However, when looking at the numerical signal difference between MA- 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 (numerical difference: 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 serum exposure at 1/4000 dilution, while only the 1/1000 dilution of inhibited serum produced a 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 restrict the 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 better inhibition response was obtained at the

preferred serum dilutions of 1/4000 and 1/500 for first and second serum exposures, respectively, compared to the result obtained with first exposure at 1/2000. 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.

2.6.5. Regeneration of the ODT-coated gold disks

After dissociation of the unbound serum antibodies to MAs, the surface is regenerated with 100 μl mixture of isopropanol and 50 m*M* NaOH (2:3, v/v) for 2 min and finally washed with 100 μl of 99% (absolute) ethanol. The surface is washed five 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.

2.6.6. Cleaning of cuvette and needles

A flow wash sequence is used to clean the needles after analyzing approximately 30 sample runs. Sequential washes with 0.5% (w/v) SDS, 6 *M* urea, 1% (v/v) acetic acid, 0.2 *M* sodium hydrogen carbonate ($NaHCO_2$), and ddd H_2O are done in order to maintain the quality of the SPR signals during repeated measurements.

2.6.7. The optimized MARTI-assay

With the lesson learnt of avoiding degassed buffers after liposome coating and the conditions optimized 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 is 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 is preincubated with antigen in order to effect an inhibition of binding signal, as graphically demonstrated in Fig. 5.11.

The SPR dips (Fig. 5.11 inserts) between 0% and 10% reflectivity that are associated with the binding profiles proved that the sensor surfaces remained intact and fully activated during the run of the experiments. Using this optimized protocol, four serum samples were selected from the Schleicher *et al.* (2002) collection and assessed for the presence of anti-MA antibodies. In Table 5.2, the MARTI-assay results are presented and compared with that obtained on ELISA by Schleicher *et al.* (2002).

From Table 5.2, P129 and P96 tested false negative on ELISA and true positive on ESPRIT biosensor, while P94 tested equivocally on ELISA and true negative on ESPRIT biosensor, as it was previously shown on the IAsys biosensor (Thanyani *et al.*, 2008). 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 applied to the

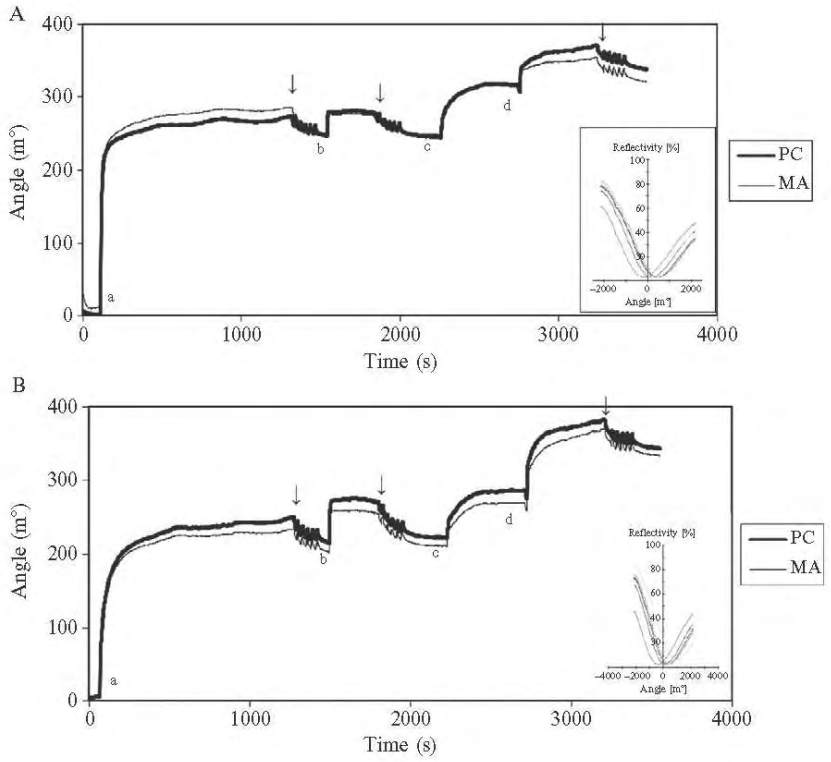

Figure 5.11 Typical sensorgrams summarizing the process of measuring serum antibody (A = TB-positive P129 and B = TB-negative P94) binding, or inhibition of binding by mycolic acid-containing and empty liposomes, on an ESPRIT biosensor with ODT-coated gold surface and immobilized mycolic acid liposomes. 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 empty (thick line) or mycolic acid-containing (thin line) liposomes at lesser dilution (d). The arrows indicate washing with PBS/AE.

Table 5.2 MARTI (ESPRIT biosensor) and ELISA analysis compared for their ability to detect antibody to MA in three selected human sera

Patient no.	TB/HIV status	ELISA assay[a]	MARTI-assay[b]
P129	TB^+HIV^-	1.59	25.09
P96	TB^+HIV^-	1.05	29.53
P94	TB^+HIV^-	1.69	−0.23

[a] Signal to background value of absorbance at 450 nm. Values higher than 2 are taken as positive.
[b] Percentage inhibition of antibody binding to MA liposomes. Values higher than 20% are taken as positive.

MARTI-test has a weakness in that the channels often do not give matching results, while the cuvettes are 10 times more expensive than the gold disks 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 pipettor 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 h of receipt of the serum. This is the first time that such reliability has been achieved. However, more sera need to be analyzed to confirm the reproducibility of the assay among the HIV-positive population, to prove the value of the MARTI-test against the many studies reported of low sensitivity and specificity with HIV-positive samples using standard techniques of TB diagnosis (Antunes *et al.*, 2002; Hendrickson *et al.*, 2000; Schleicher *et al.*, 2002).

2.7. The cholesteroid nature of MA demonstrated on the ESPRIT biosensor

In our previous studies, we have provided evidence for a structural relationship and attraction between free MAs and cholesterol (Benadie *et al.*, 2008). This was supported by demonstrating the interaction between MA and Amphotericin B—an antifungal macrolide agent known to bind to cholesterol (Baginski *et al.*, 2002)—on the IAsys biosensor system. The same principle was confirmed with the ESPRIT biosensor as demonstrated below, in an attempt to determine what the effect of labeling of MA would be on its manifestation of cholesteroid nature.

For the preparation of the different liposomes, phosphatidyl choline stock solution (90 μl, 100 mg/ml chloroform) is added to an amber glass vial containing either MA (1 mg) or an equimolar amount of 5-BMF labeled MA (1.35 mg). For the preparation of cholesterol-containing liposomes, phosphatidyl choline stock solution (60 μl, 100 mg/ml chloroform) is added to a cholesterol solution (30 μl, 100 mg/ml chloroform). The samples are mixed well until dissolved, then dried under a stream of N_2 gas at 85 °C. Saline (2 ml) is then added and the sample is heated on a heat block for 20 min at 85 °C. The sample is then vortexed for 1 min, sonicated with a Virsonic probe sonicator until a clear solution forms to indicate vesicle formation, aliquoted at 0.2 ml per vial, lyophilized and stored at −70 °C until use. Before use, lyophilized liposomes are reconstituted with buffer (2 ml). The liposomes are placed on a heat block for 30 min at 85 °C, vortexed for 2 min and sonified. The final liposome concentration comes to 500 μg lipid/ml.

The binding interaction between Amphotericin B and either cholesterol-, MA-, or 5-BMF labeled MA-containing immobilized liposomes are tested here. An ODT-coated gold disk is mounted in the ESPRIT instrument and the individual liposomes immobilized as described before. The instrument is operated and reagents used at RT. The liposomes are washed five times with nondegassed PBS/AE, and left for 5 min with mixing to obtain a baseline. Amphotericin B (1×10^{-4} M) is added to the liposome layer and the direct interaction is recorded for 10 min after which the disk is washed five times with nondegassed PBS/AE, and left for 5 min. The results (Fig. 5.12) confirm the ability of the ESPRIT biosensor to demonstrate that Amphotericin B recognizes both cholesterol and MA, as was previously shown with the IAsys biosensor. In addition, it shows for the first time the intolerance of the system for fettering with the structure of MA by adding a bulky label on its carboxylic acid group.

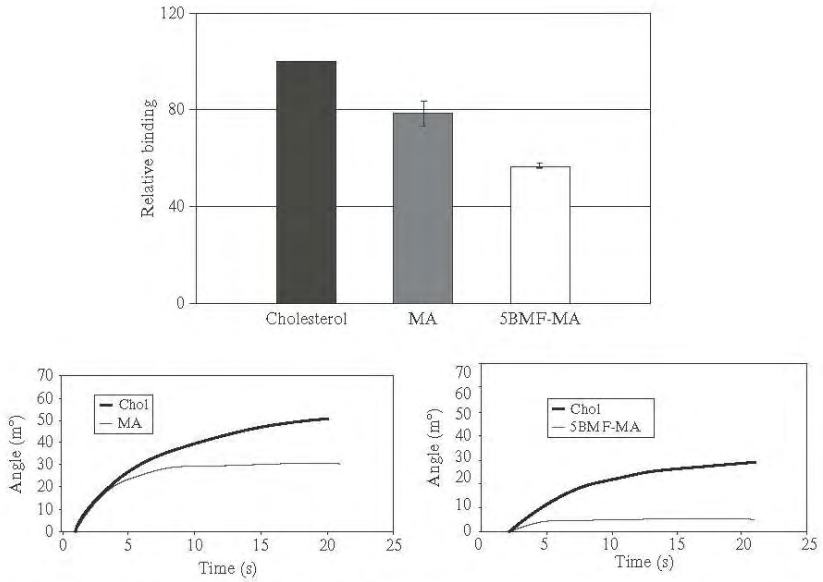

Figure 5.12 Normalized AmB-binding capacity on immobilized lipid antigens cholesterol, MA, and 5BMF–MA and binding curves of MA versus cholesterol and 5BMF–MA versus cholesterol. The error bars indicate the standard deviation, $n = 3$ for each set.

3. Conclusion

Antibodies to MA in serum as surrogate markers of active TB can be detected by making use of liposomes as MA antigen carriers in a wave-guide evanescent field biosensor (Thanyani *et al.*, 2008). However, SPR biosensors are more generally in use, more amenable to high-throughput screening, have lower running costs, and provide more comparable binding signals in twin-cell cuvettes. The more preferred technique of SPR biosensor technology, using standard methods to form a hydrophobic surface onto the gold disks on which the MA-containing liposomes can be immobilized, is reported here. This demonstrates the use of liposomes in biosensors to detect large biomolecules such as antibodies. By using Amphotericin B as a small ligand to bind cholesterol and MA in a wave-guide biosensor the cross-reactivity of antibodies to MAs in ELISA can be explained (Benadie *et al.*, 2008). This can also be demonstrated using an SPR biosensor. The biosensor antibody detection approach (MAR.TI—TB serodiagnostic test) remains unaffected by the antibody cross-reactivity between MA and cholesterol.

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