CHAPTER 4

The optimal MARTI-assay with ESPRIT biosensor

4.1 Introduction

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

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

4.2 Aim

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

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

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

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

4.3 Materials and Methods

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

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

4.3.2 Optimization of saponin concentration

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

4.3.3 Optimization of first serum exposure dilution in PBS/AE

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

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

4.3.4 Optimization of second serum exposure dilution in liposomes

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

4.3.5 Regeneration of the ODT coated gold discs

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

4.4 Results and Discussion

4.4.1 Effect of degassed buffer on immobilized MA liposomes

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

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

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

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

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

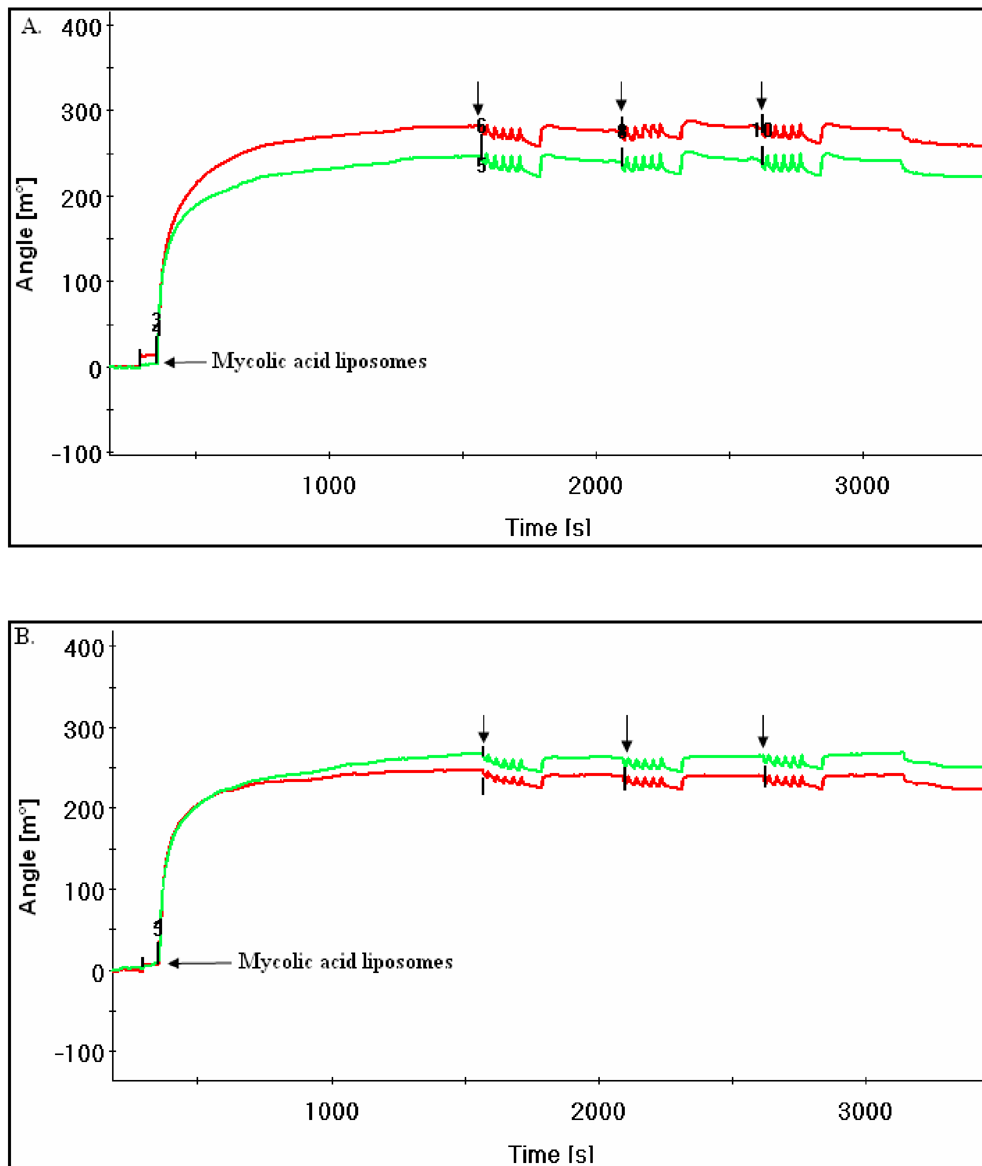

Figure 4.1: Effect of degassed (A) and non-degassed (B) buffer on immobilized mycolic acids liposomes in the ESPRIT biosensor. The arrows indicate where washing cycles with PBS/AE were introduced before allowing a baseline to be reached with mixing before substitution of cell content.

4.4.2 Optimization of saponin concentration

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

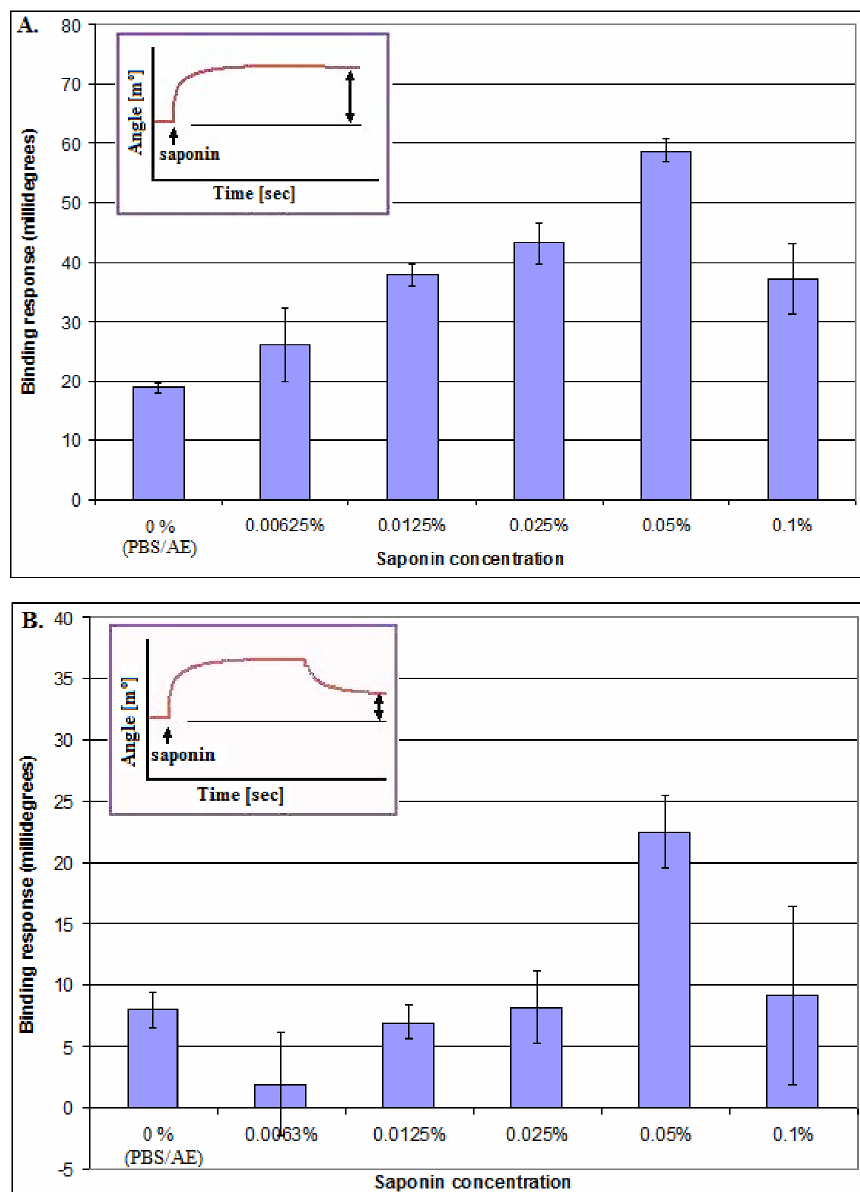

Figure 4.2: Optimization of saponin concentration to avoid non-specific binding on immobilized mycolic acids on the Au surface coated with octadecanethiol. Accumulation of saponin was performed for 5 minutes (A) and washed with PBS/AE (B). The error bars indicate the standard error of the mean (SEM)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

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

4.4.3 The optimized MARTI-assay

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

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

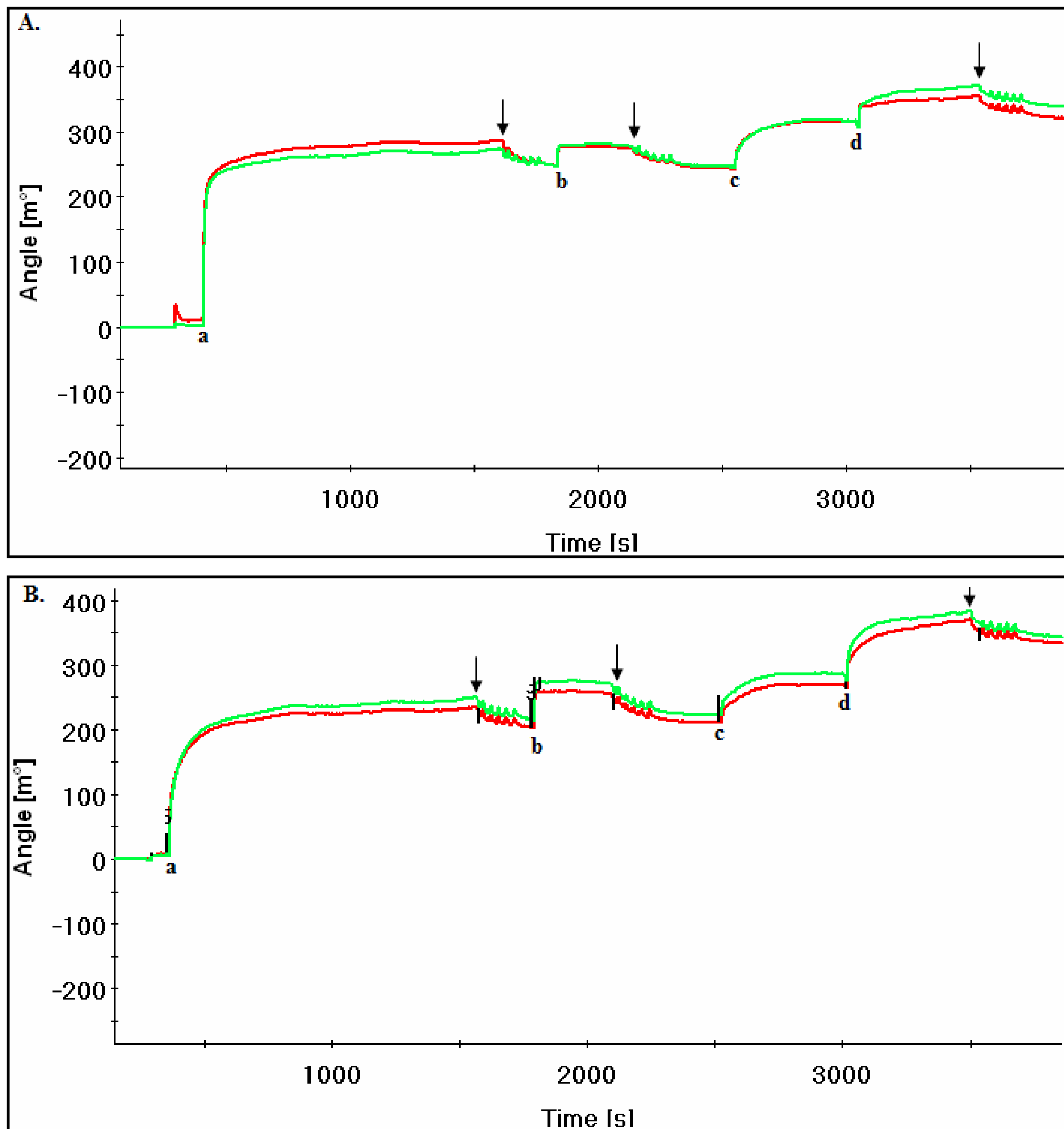

Figure 4.3: Typical sensorgrams summarizing the process of measuring serum antibody (A = TB positive P129 and B = TB negative P94) binding or inhibition of binding by mycolic acid containing and empty liposomes, on an ESPRIT biosensor with ODT coated gold surface and immobilized mycolic acid liposomes.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

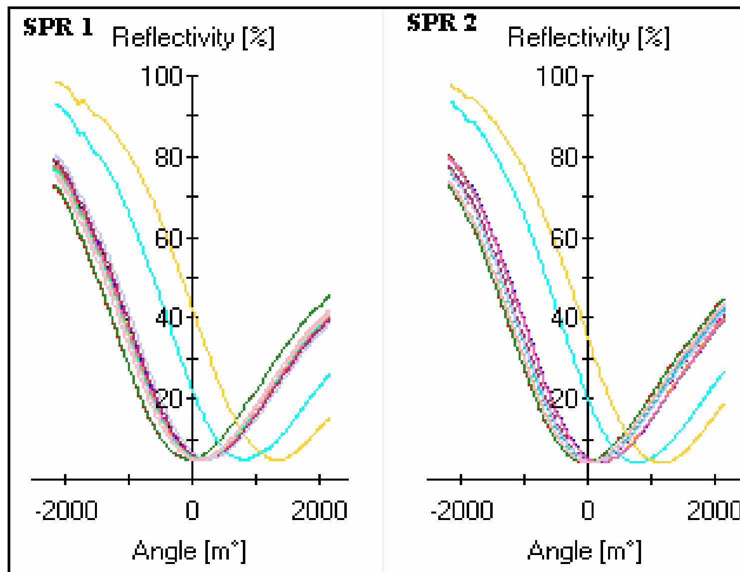

Figure 4.4: SPR dips reflecting the reliability of binding profiles during the experimental data acquisition period of the optimized MARTI-assay.

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

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

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

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

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

4.4.3.1 First serum exposure

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

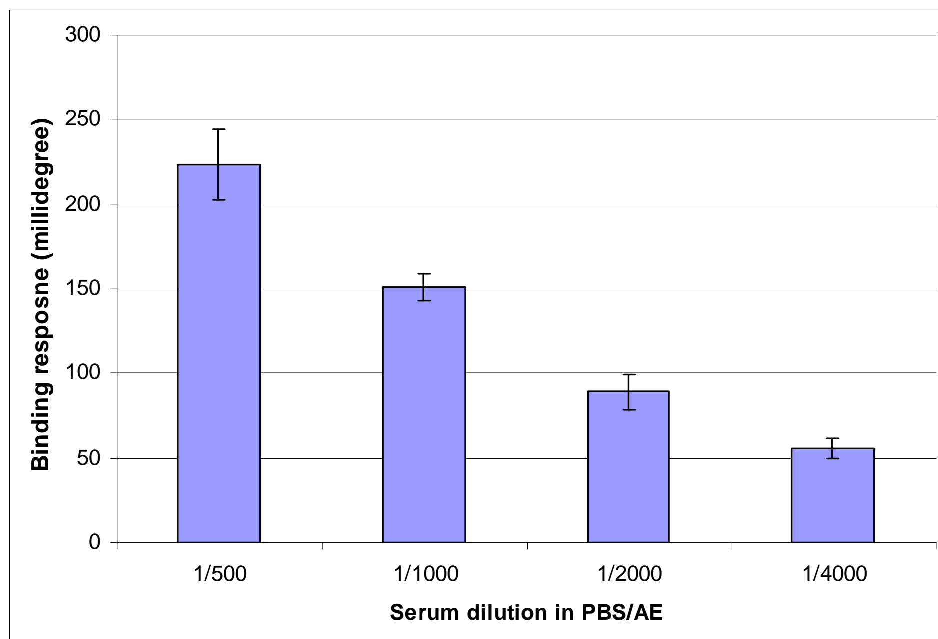

Figure 4.5: Optimization of the dilution of serum (P135) for the first exposure to antigen in the MARTI-assay, after 0.0125% saponin blocking of the mycolic acid liposome coat of the ESPRIT biosensor. The error bars indicate the standard deviation. Correlation co-efficient (r^2) = 0.9749, $n \geq 3$.

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

4.4.3.2 Second serum exposure with liposome pre-incubation

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

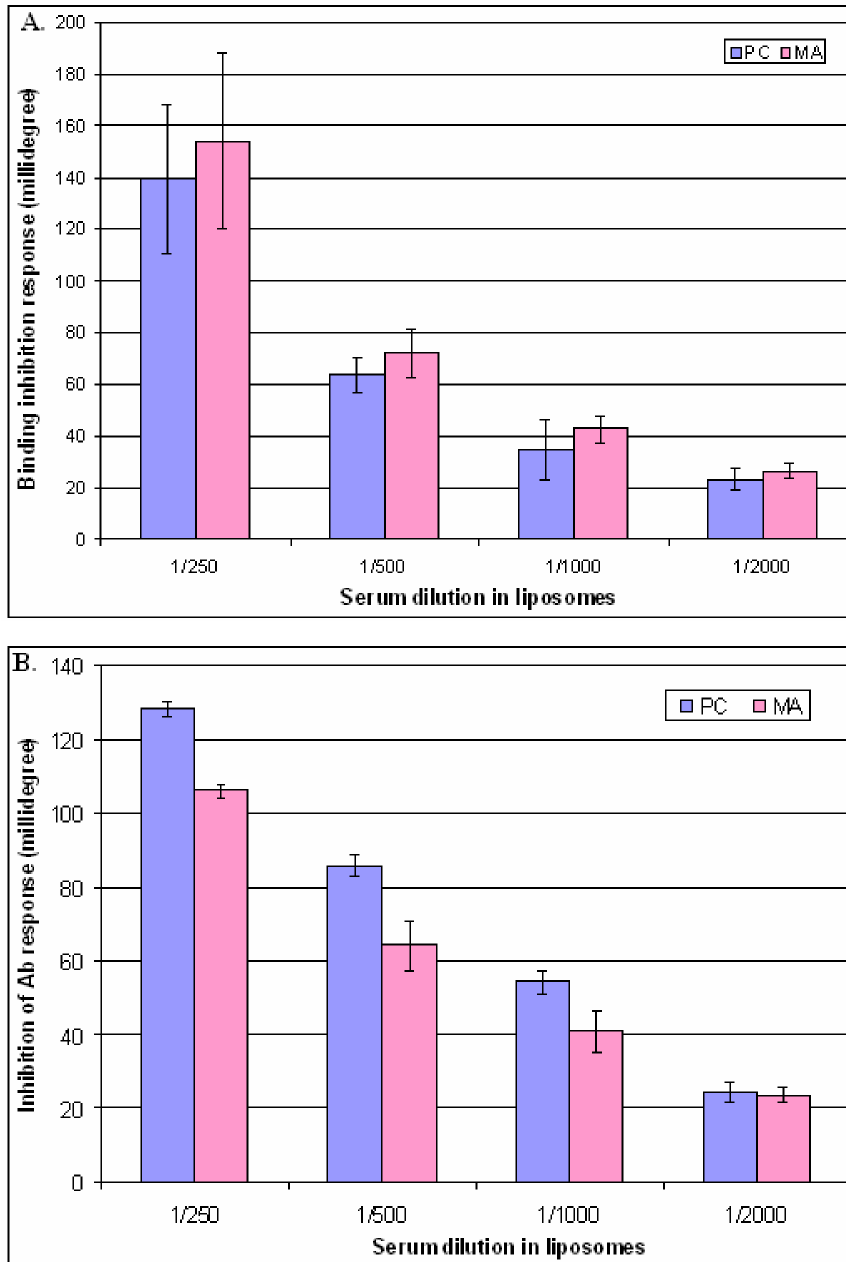

Figure 4.6: MARTI-antibody binding inhibition response of pre-incubated serum dilutions inhibited with mycolic acids (MA) and phosphatidylcholine (PC) after first serum exposure of 1/4000 on immobilized mycolic acids liposomes. A = results with TB false neg. P135 serum. B = results with TB pos. P129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

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

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

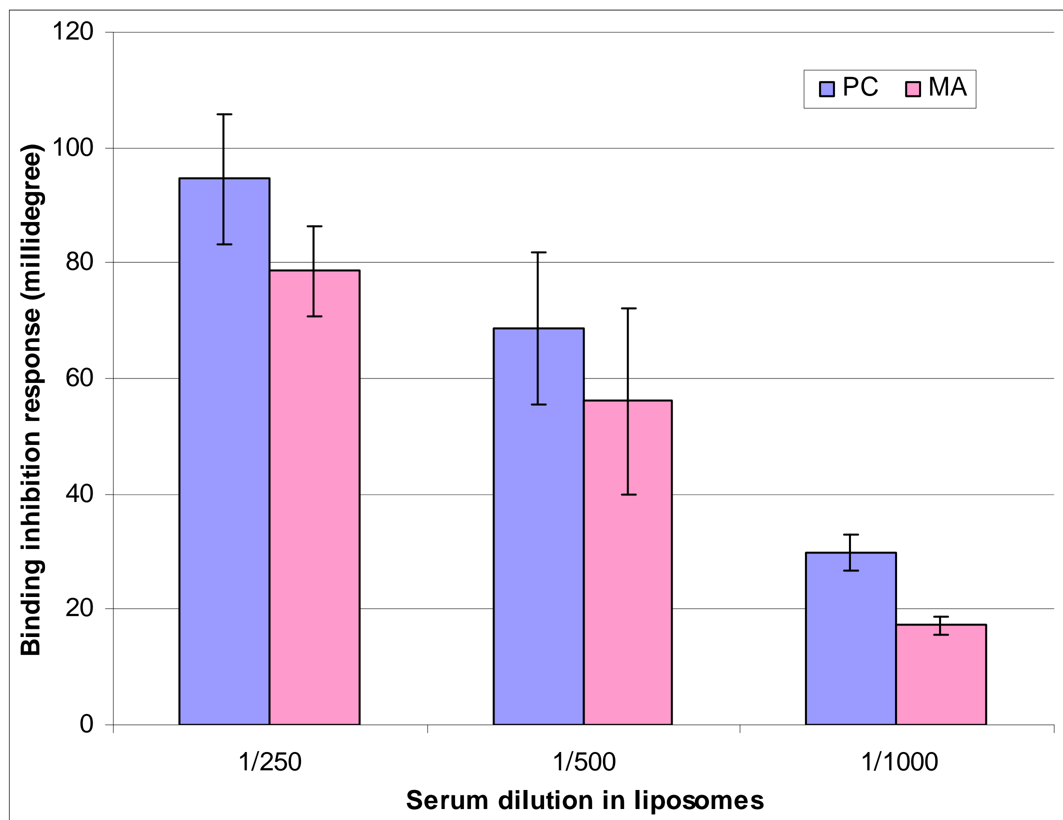

Figure 4.7: MARTI-binding inhibition response of various dilutions of pre-incubated TB positive patient serum (P129) with mycolic acids (MA) and phosphatidylcholine (PC) after first exposure serum dilution of 1/2000 to surface immobilized mycolic acids liposomes. The error bars indicate the standard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

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

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

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

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

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

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

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

CHAPTER 5

Concluding Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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