CHAPTER 1

General Introduction

1.1 Tuberculosis

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

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

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

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

1.2 History of tuberculosis diagnosis

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

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

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

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

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

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

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

1.2.1.1 Tuberculin skin test

The tuberculin skin test which is based on the intradermal injection of mycobacterial purified protein derivative (PPD), a crude mixture of *M. tuberculosis* proteins widely shared among *M. tuberculosis*, *M. bovis* BCG, and most environmental mycobacteria. It is the most generally used method for identifying TB infection. The technique is based on the injection of 0.1 ml of a solution of tuberculin, a purified protein derivative (PPD), intradermally into the volar or dorsal surface of the forearm. If positive, this produces a discrete, pale elevation of the skin, 6 mm to 10 mm in diameter, 48 to 72 hours after injection (Charnace and Delacourt, 2001). The reading is based on a measurement of swelling. The specificity is low as purified protein derivative contains many antigens widely shared among mycobacteria. Some persons may react to the tuberculin skin test though they are not infected with *M. tuberculosis* (Doherty *et al*., 2002; Anderson *et al*., 2000). Several studies have demonstrated that PPD cannot reliably distinguish between previous *Mycobacterium bovis* BCG vaccination, exposure to environmental mycobacteria, or infection with *M. tuberculosis* (Charnace and Delacourt, 2001; Chan *et al*., 2000; Ewer *et al*., 2003). It is currently estimated that almost one third of people positive to tuberculin skin test (TST) do not actually have TB infection. The sensitivity of the skin test is estimated to be around 70% of alternatively confirmed active TB cases. The sensitivity decreases to as low as 30% in immunocompromised people (Palomino *et al*., 2007). The TST is useful for proving infection, but not necessarily the disease. A positive test only suggests prior exposure to the antigen, not active infection. If the patient is in an immunosupressed state, a negative test does not rule out TB infection (Hornum *et al*., 2008). The TST can give false positive results leading to inappropriate initiation of chemotherapy, which can be a waste of health care resources (Kunst, 2006) and a discomfort to the patient.

1.2.1.2 Interferon gamma assay

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

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

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

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

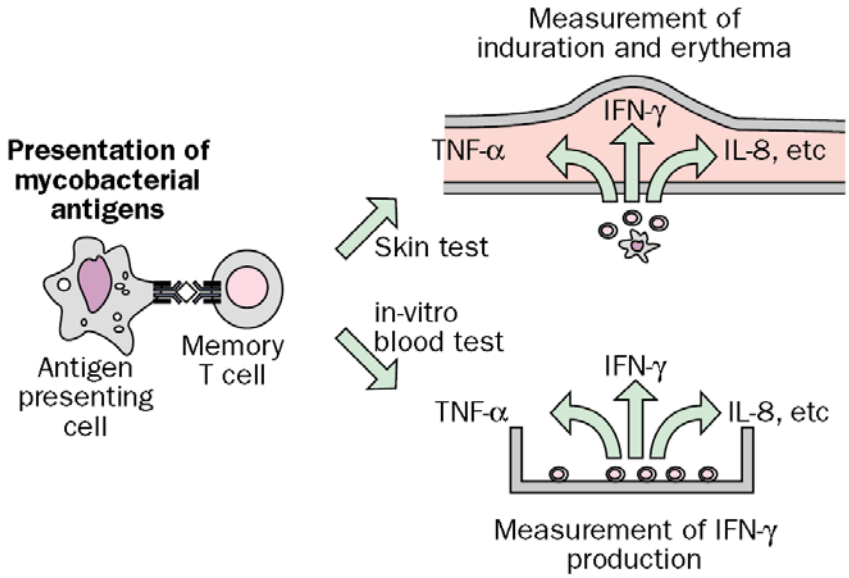


Figure 1.1: *In vivo* and *in vitro* diagnostic tests for tuberculosis. Both *in vivo* (skin test) and *in vitro* (blood test) depend on the elaboration of inflammatory cytokines by T cells previously sensitised to mycobacterial antigens (Anderson *et al*., 2000).

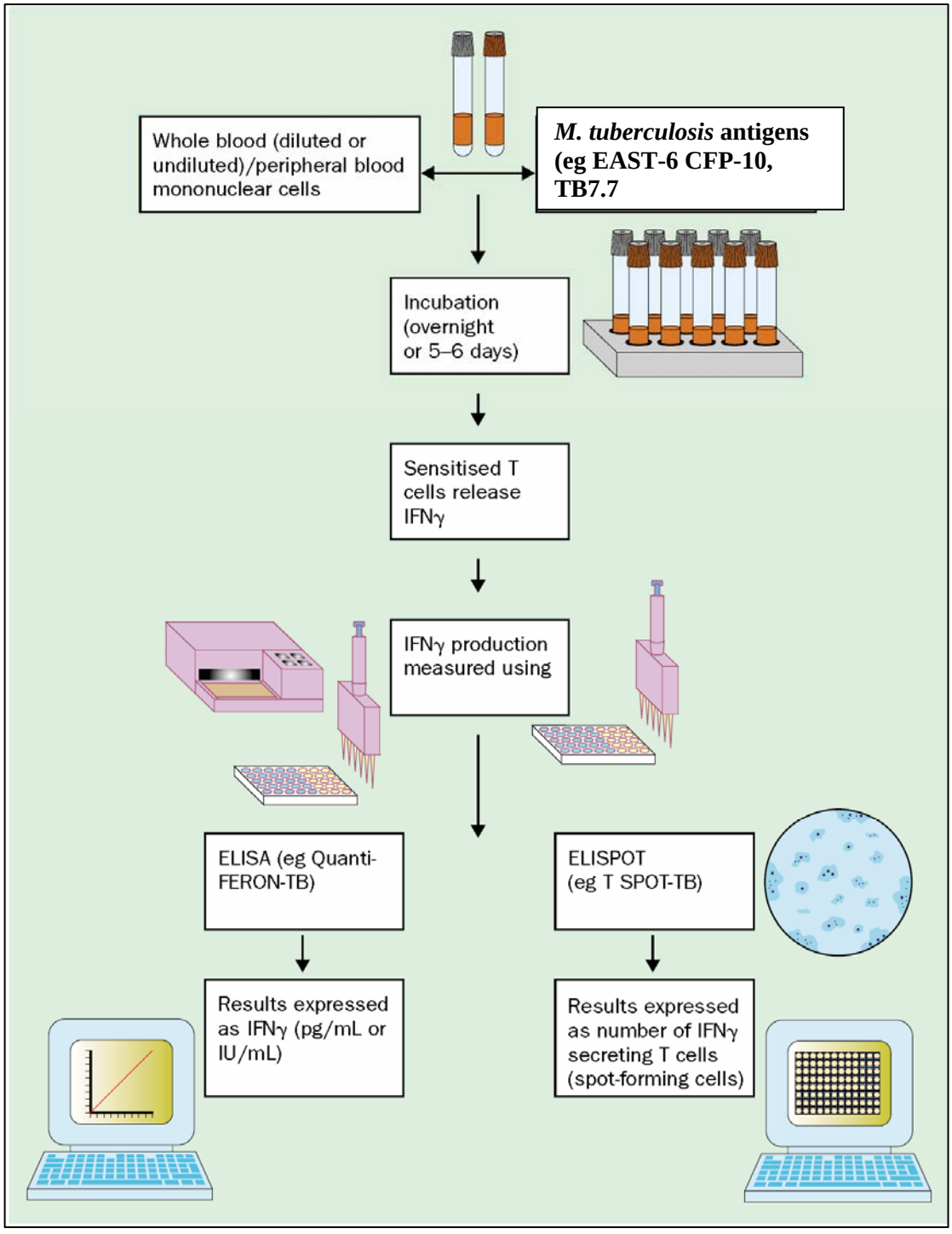


Figure 1.2: An overview of the interferon γ assay technology. The test uses the protein antigens early secretory antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP10) and a peptide from *M. tuberculosis* specific antigen (TB7.7) that are major targets for INF-γ-secreting T lymphocytes in *M.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

1.2.2 Tests for active TB diagnosis

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

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

1.2.2.1 Direct microscopy

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

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

The direct microscopy of sputum for AFB is reliable for pulmonary tuberculosis, but is not very sensitive. It may give false negative results and requires a high degree of bacillary load - of up to 10 000 bacilli/ml of sputum (Mitarai *et al*., 2001). Direct microscopy is not valid for diagnosing extrapulmonary tuberculosis or child tuberculosis (Charnace and Delacourt, 2001; Tiwari *et al*., 2007). Unfortunately, this technique can't distinguish among the various possible mycobacterial species. It is therefore standard protocol that the result of microscopy of the smear be confirmed by culture.

1.2.2.2 Chest X-rays

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

1.2.2.3 Culture based method

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

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

1.2.2.4 Fast techniques

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

1.2.2.4.1 Polymerase chain reaction assays for TB

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

1.2.2.4.2 FASTPlaque TB test

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

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

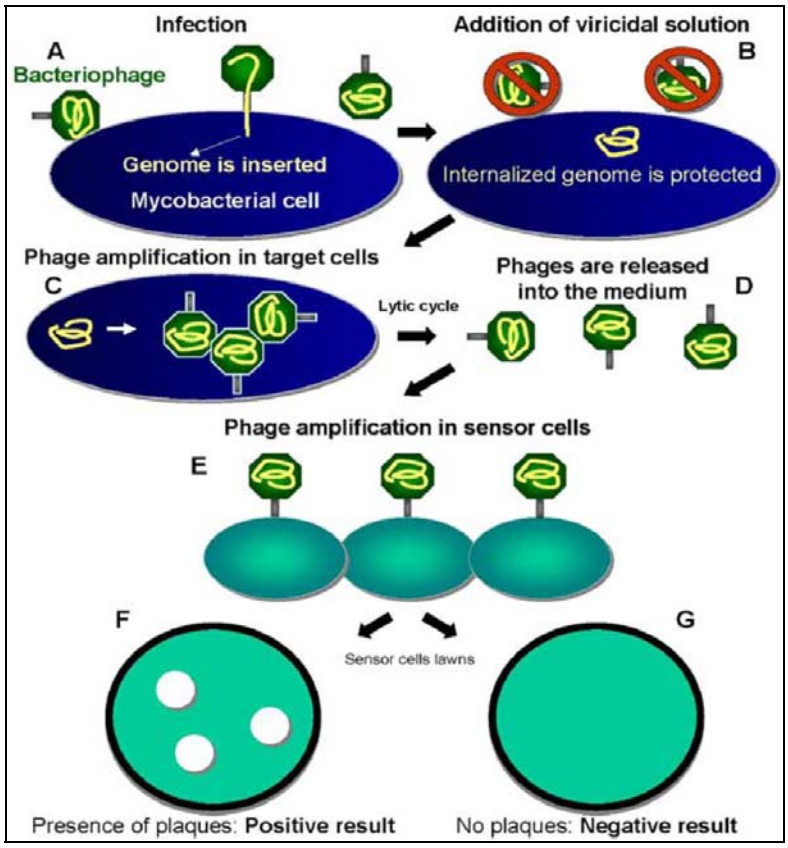


Figure 1.3: An overview of the phage amplification assay (Hazbon, 2004).

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

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

1.3 Mycobacterial antigens for serodiagnosis of TB

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

The cell wall of mycobacteria has several unique features, which distinguishes it from all other prokaryotes, thereby qualifying as an ideal target for diagnosis of infection (Khasnobis *et al*., 2002; Chatterjee *et al*., 1997). It consists of a plasma membrane surrounded by a lipid and carbohydrate rich shell, which in turn is encircled by a capsule of polysaccharides, proteins and lipids. The insoluble matrix is composed of covalently attached macromolecules, i.e. peptidoglycan, arabinogalactan and mycolic acid (Fig. 1.4). Despite its low sensitivity and specificity, a large number of native and recombinant antigens of *M. tuberculosis* such as purified protein derivative, acylated trehalose family and 38 kDa respectively have been assessed, showing substantial progress for the serodiagnosis of TB (Antunes *et al*., 2002; Thanyani, 2003; Verma and Jain, 2007; Palomino *et al*., 2007).

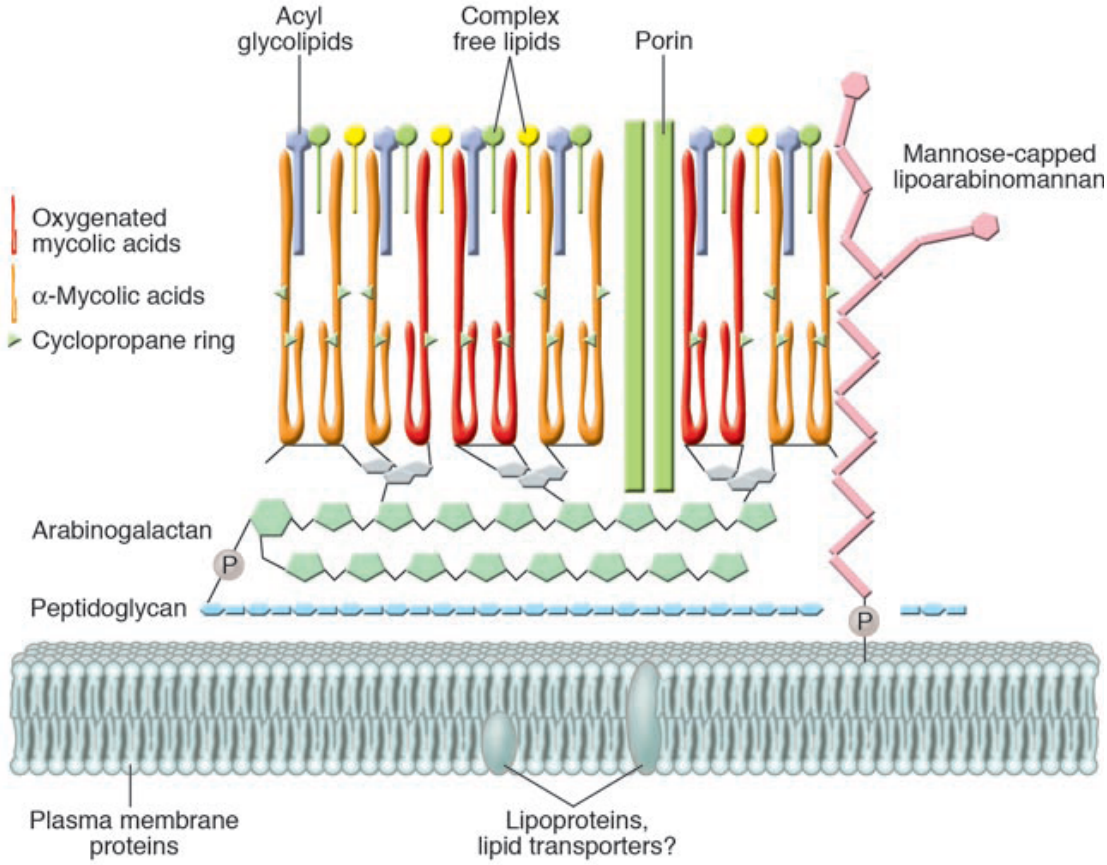


Figure 1.4: Schematic representation of *Mycobacterium tuberculosis* cell envelope (Riley, 2006).

1.3.1 38 kDa antigen

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

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

1.3.2 Lipoarabinomannan antigen

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

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

1.3.3 Acylated trehalose antigen

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

1.3.4 Mycolic acid antigen

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

Mycolic acids are unique 60-90 carbon length branched α-alkyl, β-hydroxy fatty acids, which form an outer waxy lipid layer around the mycobacteria (Dobson *et al.*, 1985). Three families of mycolic acids are known; α-mycolic acids without any

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

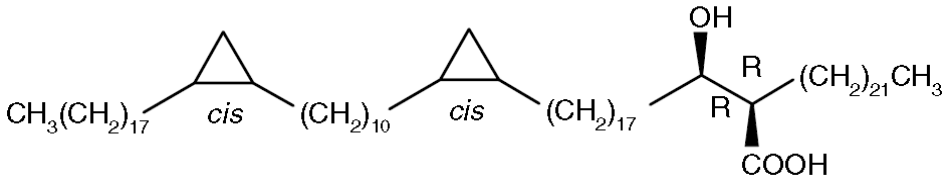
Mycolic acid is presented by antigen-presenting cells (APC) through a mechanism that does not involve MHC-class I or MHC-class II molecules. Mycolic acid is a CD1 restricted antigen with the ability to induce proliferation of specialized T-cells of low abundance in the blood (Beckman *et al*., 1994). The human CD1 protein is known to mediate T-cell responses by presenting at least the three classes of mycobacterial lipids, i.e. free mycolates, glycosylated mycolates and diacylglycerol-based glyco-phospholipids such lipoarabinomannan (Beckman *et al*., 1994; Moody *et al*., 1997). The alkyl chains of the mycolic acid antigen have been proposed to bind directly within the hydrophobic groove of CD1 resulting in presentation of the hydrophilic caps to the T-cell's antigen receptor (Porcelli *et al*., 1996; Moody *et al*., 1999). The CD1-restricted lipid antigen presentation pathway could probably be the reason why the antibody response to mycobacterial lipid antigens is preserved in HIV-seropositive patients, despite a declining CD4 T-lymphocyte count (Schleicher *et al*., 2002; Simonney *et al*., 2007).

Schleicher *et al*. (2002) showed with ELISA that there is a significantly higher anti-mycolic acid antibody level in TB positive than in TB negative patients. They investigated the diagnostic potential of an ELISA, based on the detection of antibodies to *M. tuberculosis* mycolic acids in sera of HIV seropositive and HIV seronegative tuberculosis patients, in a population with a high prevalence of TB. Although they did observe a higher signal of antibody to mycolic acids in TB positive patients than in TB negative patients, they also found quite a number of false positive and false negative results. From their studies, they then concluded that the ELISA has poor

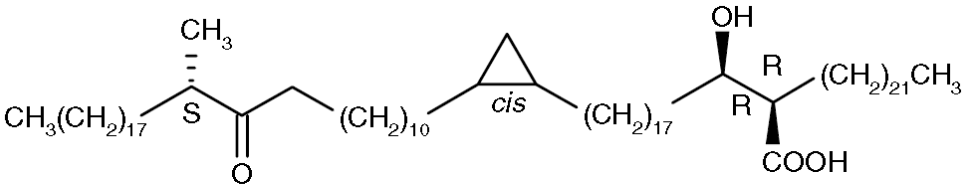
sensitivity and specificity to detect anti-mycolic acid antibody and is therefore not suitable as a reliable serodiagnostic assay for the diagnosis of pulmonary TB.

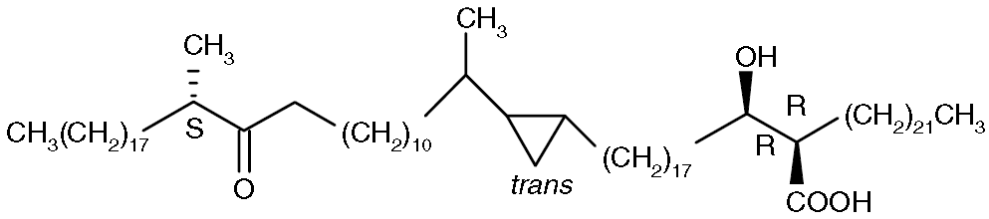
Our previous study on an IAsys biosensor showed its potential to detect antibodies to mycolic acids in active TB patient sera (Thanyani, 2003; Siko, 2002). The current study will focus on the validation of the MARTI (Mycolic acid Antibody Real Time Inhibition)-assay on IAsys biosensor and its further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

(a) Alpha Mycolic acids

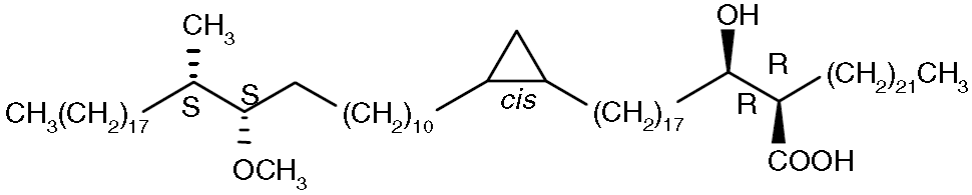


(b) Keto Mycolic acids





(c) Methoxy Mycolic acids



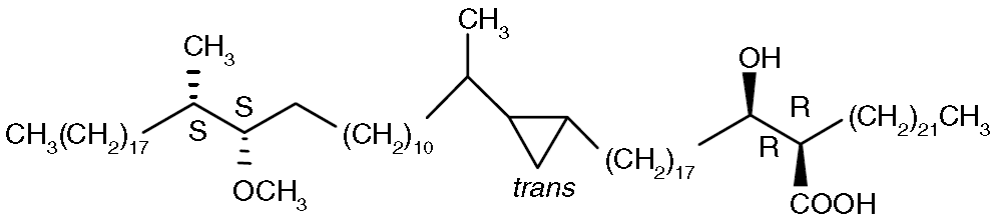


Figure 1.5: Structures of mycolic acids from *M. tuberculosis* (Khasnobis *et al*., 2002).

1.4 Modern alternative tests for serodiagnosis of TB

In spite of new technologies such as PCR, no reliable and affordable tests have been generally accepted in the market for the diagnosis of TB (Ahmad *et al*., 1998). Our preliminary study on IAsys affinity biosensor showed the detection of anti-mycolic acids antibody in human TB patient sera (Thanyani, 2003). The introduction of optical biosensors in 1990, based on the phenomenon of surface plasmon resonance (SPR), has revolutionized the measurement of binding interactions in biochemistry (Malmqvist and Karlsson, 1997; Marcheini *et al*., 2007). Most optical biosensors rely upon a phenomenon called the evanescent field to monitor changes in refractive index occurring within a few hundred nanometers of the sensor surface. Such changes are generated as a result of the binding of a molecule to a surface immobilized receptor (or the subsequent dissociation of this complex). Real-time monitoring of these effects allows binding constants to be derived (Cush *et al*., 1993). Optical biosensors can be used to provide qualitative information, such as whether two molecules interact, and quantitative information, such as kinetic and equilibrium constants for complex formation for a wide range of biological systems (Fig. 1.6). Different chemicals can be used to regenerate the surface for re-use when molecules are immobilized on the surface.

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

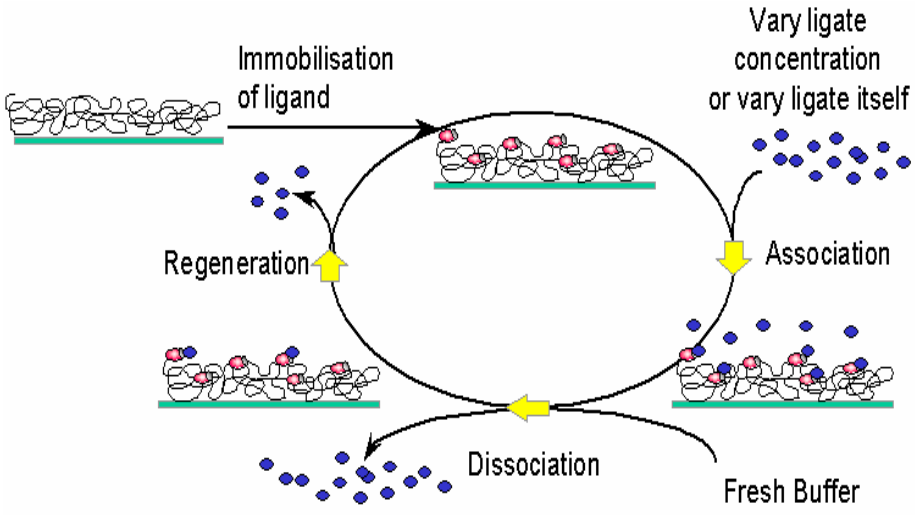


Figure 1.6: An experimental cycle of a sensor surface after regeneration (IAsys Manual).

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

Biosensor technology enables researchers to detect molecules with low affinity in a biological medium. This new technology makes it possible to visualize on a computer screen the progress of binding of biomolecules as a function of time, in terms of changes in mass accumulation occurring on a sensor surface. Biosensor instruments make it possible to determine how fast and how strongly molecules interact and what the binding stochiometry is (Van Regenmortel, 1999). They provide rich information on the specificity, affinity, and kinetics of biomolecular interactions and the concentration levels of an analyte of interest from a complex sample (Shankaran *et al*., 2007). The independence from labeling requirements and low sample

consumption have made optical biosensors an essential component of both academic and commercial laboratories (Myszka, 1999). The biosensor technology offers sensitive detection of surface adsorption, but all adsorbed molecules are detected, thereby putting very high demands on the measures to avoid unwanted interactions (Malmqvist, 1999).

Conventional methods for the detection and identification of bacteria mainly rely on specific microbiological and biochemical identification, while biosensors methods can be fast, sensitive, relatively affordable and able to generate both qualitative and quantitative information on the number and the nature of the microorganisms tested (Leonard *et al*., 2002). While conventional methods of pathogen detection require time-consuming steps to arrive at a useable measurement (Jongerius-Gortemaker *et al*., 2002; He and Zhang, 2002), biosensor technology can significantly reduce the time as well as detect trace amounts of pathogens with fewer false positives. However conventional methods are being used despite their long turnover times because of their high selectivity and sensitivity. Biosensors have the potential to shorten the time span between sample uptake and results, but their future lies in reaching selectivities and sensitivities comparable to established methods, but at a fraction of the cost (Lazcka *et al*., 2007).

Biosensors have many applications, especially in health and medical fields (Frostell-Karlsson *et al*., 2000; Rogers, 2000). They have become increasingly popular for determining the affinity and kinetics of interactions of biological macromolecules (Schuck, 1996; Myszka *et al*., 1999; Markgren *et al*., 2000). Most of the commercially available biosensor systems are applied in the clinical and pharmaceutical markets (Rodriguez-Mozaz *et al*., 2004). The optical biosensors that measure refractive index changes caused by bound macromolecules permit one to monitor the time dependence of the binding of label-free macromolecules to receptors immobilized on a surface (Malmqvist, 1999; Van Regenmortel, 1999). They are used to study binding in a number of different applications, e.g., antigen-antibody interactions, protein-protein interactions, protein-DNA interactions, and in interaction of HIV-1 protease with inhibitors (Schuck, 1996; Markgren *et al*., 2000; Scheller *et al*., 2001). Additional uses include epitope mapping, ligand fishing and small molecule screening (Muller *et al*., 1998; Myszka, 1999).

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

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

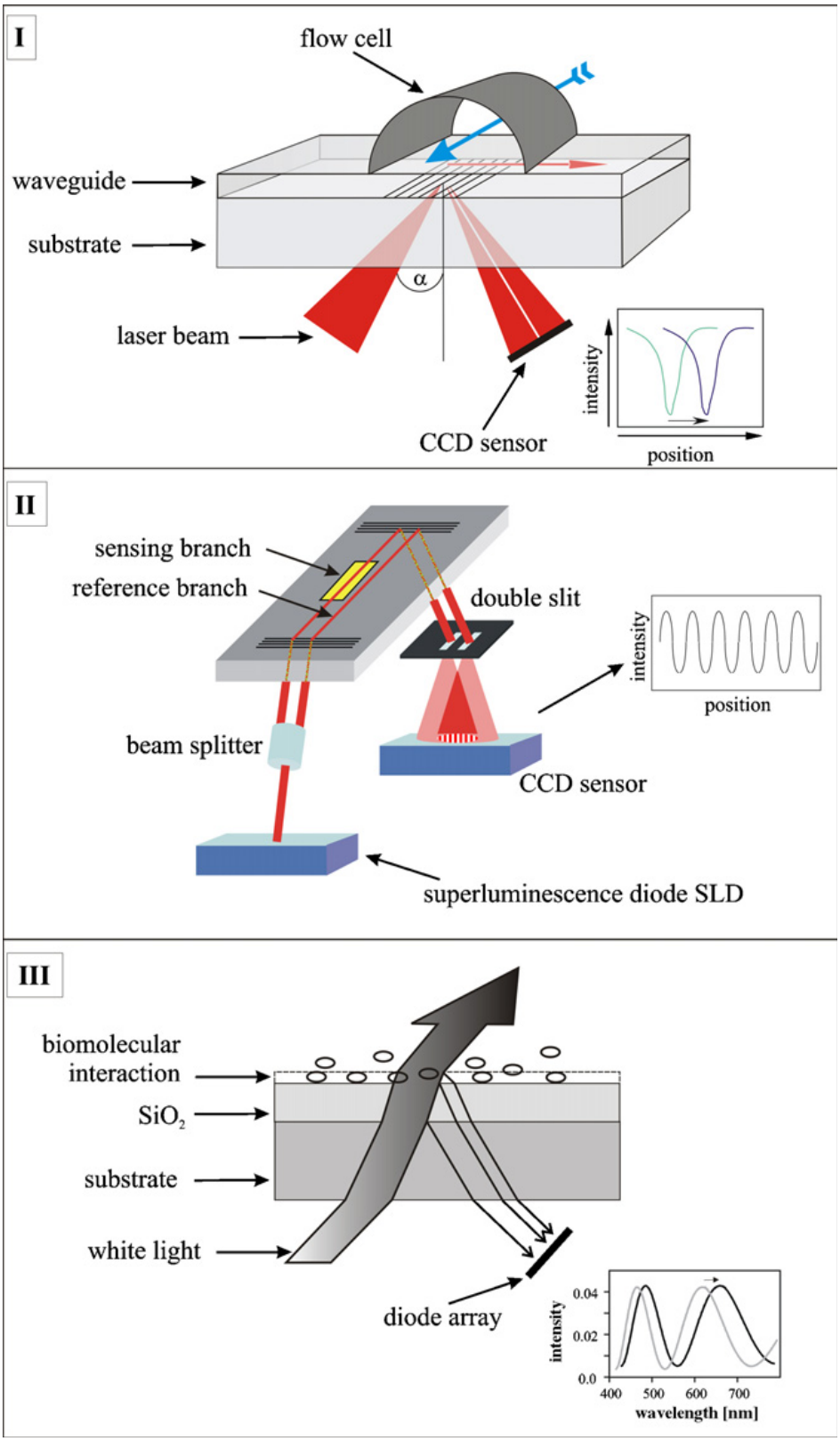


Figure 1.7: Configurations of the three used optical label free devices to diagnose TB: grating coupler (I), interferometric biosensor (II) and the RIfs system (III), (Nagel *et al.*, 2007).

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

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

1.4.1 IAsys biosensor

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

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

The resonant mirror is a simple structure of two dielectric layers of glass. The device consists of a high refractive index waveguide separated from a high refractive index

prism block by an intervening, low refractive index coupling layer (Fig. 1.8) (Cush *et al*., 1993). Changes in refractive index due to the interaction of ligand-analyte at the surface of the device (the biological layer) changes the angle at which light can be made to propagate in the waveguide. At the resonance angle, light of a high intensity passes from the prism, through the coupling layer, to propagate in the waveguide as a surface evanescent wave. The light returns through the coupling layer, emerging to strike the detector, which is then monitored in real-time as the binding of molecules occurs (Cush *et al.,* 1993; Schuck, 1996).

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

The IAsys CMD cuvette has been used in a very wide range of interaction analyses including those between proteins, nucleic acids and carbohydrates. It is hydrophilic and charged with derivatizable carboxylate groups that allow the unique feature of efficient electrostatic binding prior to covalent immobilization (Morgan *et al*., 1998). Planar surfaces provide enhanced sensitivity for exploring and comparing biomolecular interactions using alternative immobilization chemistry. It allows ligate interaction to take place close to the biosensor surface where the evanescent field is most intense. Both amino and carboxylate surfaces can be useful for the analysis of high molecular weight ligates or particulates which may be unable to enter the CMD matrix. The biotinylated planar surface is ideal for rapid, convenient and well controlled capture of biotinylated ligands including proteins, lipids, nucleic acids and glycoproteins with streptavidin linking the ligands to the surface. The hydrophobic surface enables hydrophobic binding of biomolecules, such as lipid monolayers and proteins (Altin *et al*., 2001). The non-derivatized surface offers an alternative to the hydrophobic cuvette for simple immobilization of lipids and carbohydrates. In our previous studies, we showed how the IAsys technology could be applied in the detection of anti-mycolic acid antibodies as surrogate markers for active TB on a non-derivatized cuvette coated with mycolic acid liposomes (Thanyani, 2003). McConkey *et al*. (2002) reported that the sensitivity of serologic tests for TB depended on the

origin of the sample and the clinical spectrum of the disease groups prevalent in that area. Therefore, each new serodiagnostic test should be validated with cases and control specimens from the countries/regions in which it will be used. In the current study, an IAsys biosensor was used to validate the mycolic acid antibody real-time inhibition (MARTI)-assay for its application to detect anti-mycolic acid antibodies in human serum samples from patients suffering from active tuberculosis due to infection with *M. tuberculosis*.

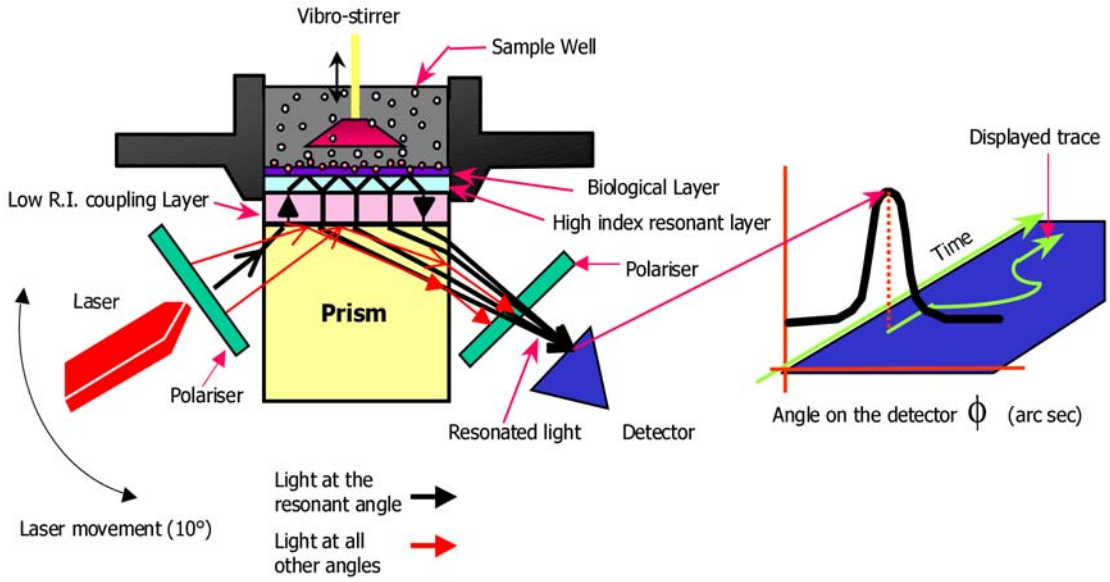


Figure 1.8: Cross section of the IAsys Affinity Biosensor cuvette and how the resonant mirror works (IAsys technical manual).

1.4.2 ESPRIT biosensor

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

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

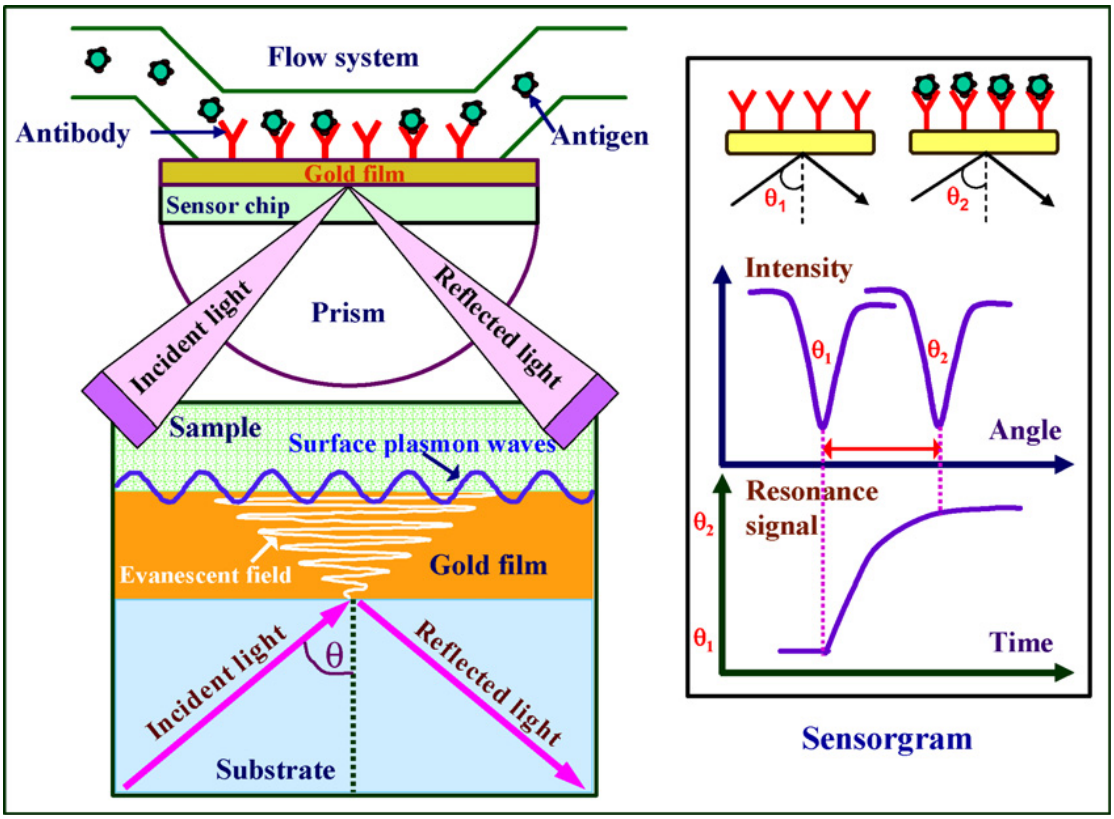


Figure 1.9: Schematic view of the surface plasmon resonance immunoassay technique (Shankaran *et al*., 2007).

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

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

1.5 Application of biosensors as immunosensors

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

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

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

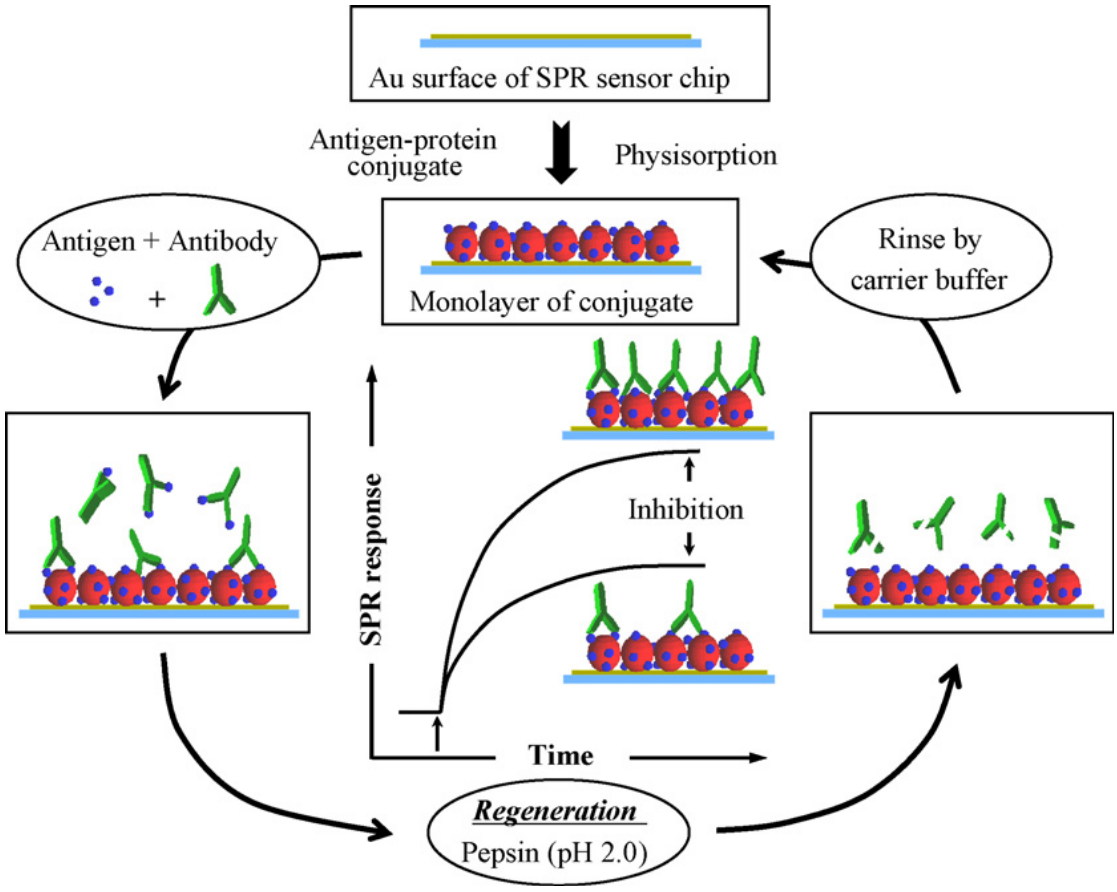


Figure 1.10: Schematic view of the indirect competitive inhibition immunoassay (Shankaran *et al*., 2007).

1.6 Advantages of biosensors in immunoassays

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

1.7 Hypothesis

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

1.8 Aims

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

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

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

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