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The development of a novel sensor for the detection of TB

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The Development of a Novel Sensor for the Detection of TB

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Contents

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Abstract

Tuberculosis (TB) is an immense global problem with 8.8 million new cases, leading to an estimated 1.45 million deaths from the disease in 2010 alone. Although current serological tests have been deemed inconsistent and imprecise by the World Health Organisation, they have stated that a point of care serological test for the detection of TB would be of extreme value. This work sought to provide such a device.

It was known that ELISA assays using trehalose di-mycolates, trehalose mono-mycolates and mycolic acids as antigens could be used to detect the presence of anti-TB antibodies in serum samples. It was hoped that by binding these antigens to a gold surface, the performance of the assays could be improved. Using information gained from ELISA assays, work was undertaken to develop a novel, reliable, easy to use point of care sensor for the detection of TB.

It was decided to use gold nanoparticles as part of the sensor, and thiol modified antigens, as well as non-thiol modified antigens (*via* a linker compound) were successfully bound to their surfaces. It was discovered that addition of a sodium chloride solution to the antigen coated gold nanoparticles led to their aggregation, in turn, changing the colour of the solution from red to blue. It was also discovered that this process could be inhibited by the addition of a TB positive serum sample to the coated gold nanoparticles prior to adding the sodium chloride solution.

These observations were used to develop a novel assay for the detection of TB, which could be performed in as little as 15 minutes. Large numbers of serum samples from high burden TB populations were analysed by the developed assay, and although a number of false positive samples were detected, largely from patients with a record of infection with malaria, almost all of the positive samples could be detected with multiple antigens. The assay developed was then incorporated into a device which is fully portable and easy to use, and can be read by eye, by observing the colour of the coated gold nanoparticle solution, and potentially, by measuring its absorbance at two different wavelengths.

Chapter 1

Introduction

1.1 Tuberculosis

Tuberculosis (TB) is a contagious disease caused by *Mycobacterium tuberculosis*.[1] Once an individual has been infected with the mycobacterium, they remain infected for years, possibly for life,[2] and can develop clinical disease at any point in time.[3] TB has an ancient history both in humans and other mammals, with the earliest detection of *M. tuberculosis* being in the remains of bison dating from as far back as 15,000 BC.[4] Recent discoveries have also shown that the skeletal remains of prehistoric humans confirm the presence of TB in humans dating back to around 7000 BC.[5]

TB started to become recognised as a public health problem during the Industrial Revolution. Cities were formed rapidly during this period, and living conditions in these cities were very poor, as was the healthcare service, thus leading to the swift spreading of TB,[6] and during the 18th and 19th centuries TB was the cause of 25% of adult deaths in major European cities.[7] The death rate from tuberculosis began to decline at the start of the 20th century and this has been credited to an improvement in living conditions, better healthcare service and also to an increase in the segregation of people, which in turn leads to a decrease in exposure to the disease.[3] Medical developments during the 1940s, in developed countries, helped to further combat TB, with the production and use of streptomycin being an important breakthrough.[8] However, despite these medical advances, the number of people infected with TB began to rise again during the latter part of the 20th century (from approximately 1980) even among developed countries such as the United States of America and China.[9] This new increase in the annual incidence rate of tuberculosis is attributable to a decline in living conditions, but is mostly due to the spreading of Human Immunodeficiency Virus (HIV) in Africa.[10,11,12]

Figure 1.1.1 shows the estimated number of new cases of TB per 100,000 population in 2010, while Figure 1.1.2 shows the estimated number of TB patients co-infected with HIV. It can be seen from these Figures that TB is widespread, especially throughout the continents of Africa and Asia. It can also be observed that the regions with the highest incidence rate of new cases of TB, such as South Africa, tend to also be the regions with the highest rate of co-infection with HIV, hence co-infection with both of these diseases is a major problem in these areas.

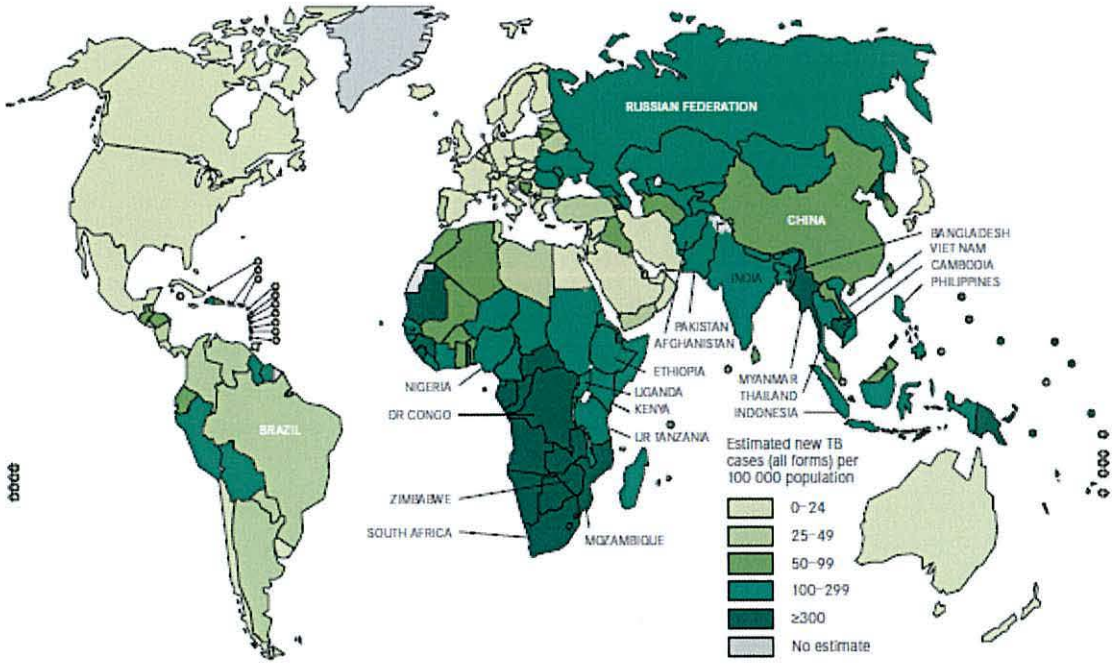

Figure 1.1.1: Map showing the estimated number of new TB cases in 2010.[16]

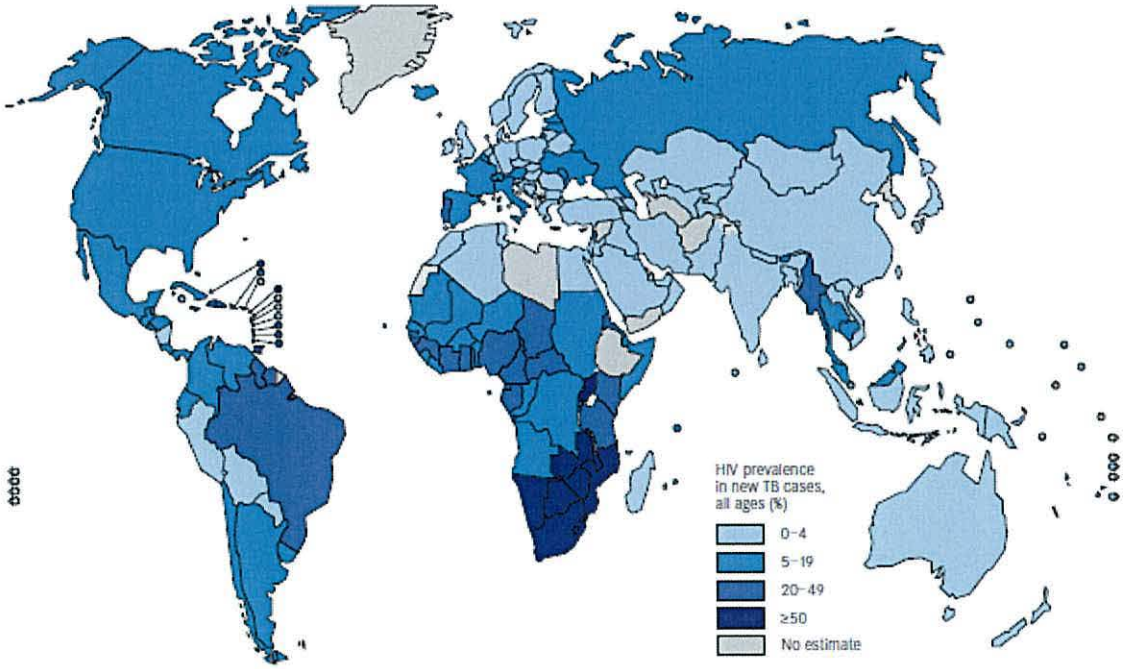

Figure 1.1.2: Map showing the estimated number of TB patients co-infected with HIV in 2010.[16]

TB and HIV work in conjunction with each other and form a lethal combination, each increasing the progression rate of the other disease. It has been stated by the World Health Organisation (WHO) that HIV is the most influential cause of increasing the risk of developing active TB, with a person suffering from HIV ten times as likely to develop active TB as a person who is not HIV infected.[13] It has been established that a person suffering from HIV while also being co-infected with *M. tuberculosis* is about 20-40 times more likely to die from TB than a person who is infected with *M. tuberculosis* but is HIV-negative.[14,15,16] This leads to a need for a rapid and accurate method to detect TB, that works with HIV infection, which would be available at a low cost and could be used in developing counties, such as in Africa where HIV and TB are a large problem.[16]

In addition to an increase in the number of people infected with TB since 1980, there has also been a large increase in the number of drug-resistant tuberculosis cases. This drug resistance occurs as a result of the improper use of antibiotics in the chemotherapy of drug-susceptible TB, which includes improper administration of the antibiotics, or due to the patient failing to complete the whole course of treatment.[17]

As a result of the above factors, tuberculosis remains an immense global problem. From a recent WHO report on Global Tuberculosis Control, it is estimated that in 2010 alone there were 8.8 million new cases of TB, with an estimated 1.45 million deaths.[16] Although these figures continue the trend of a decrease in the incidence rate of TB per year, which has been declining since 2006,[16] it still remains a major problem and an estimated budget of approximately $4.4 billion ($4 billion of which is used for diagnosis and treatment) was funded to attempt to address and control the TB problem in 2012, which is an increase from the budget of $3.5 billion for 2006.[10,16]

1.2 *Mycobacterium tuberculosis*

TB is mainly caused in humans by *M. tuberculosis* which can be seen in Figure 1.2.1 below.[1]

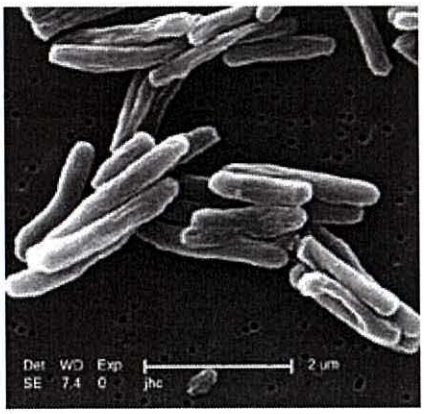

Figure 1.2.1 Scanning Electron Micrograph of M. tuberculosis.[18] Reprinted by permission.

M. tuberculosis is classified as an 'acid-fast' gram positive bacterium. It is gram positive due to the lack of an outer cell membrane, and is 'acid-fast' because it does not retain methyl violet stain well.[18] The bacterium does however stain bright red in Ziehl-Neelsen stain, which is a test for acid-fast bacteria.[19] Since its complete genome sequence was solved in 1998, much more is now known and understood about the bacterium.[20] *M. tuberculosis* has a complex envelope around its outside, which is of unusually low permeability to hydrophilic molecules, and which contributes to its resistance to host defence mechanisms.[21,22] Because of these unusual characteristics, knowledge of the cell envelope is crucial. A simplified representation of the cell envelope of *M. tuberculosis* can be seen in Figure 1.2.2.

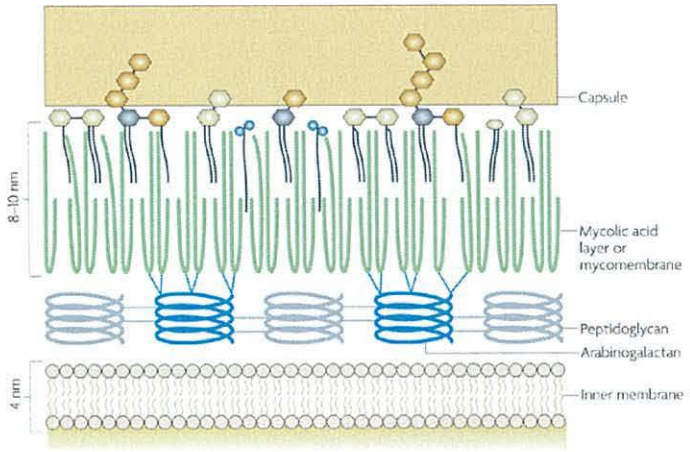

Figure 1.2.2 A simplified representation of the cell envelope of M. tuberculosis.[23] Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, copyright 2007.

The cell envelope is made up of three structural components, the plasma (or inner) membrane, the cell wall and the 'capsule', which mainly contains proteins and polysaccharides as well as a smaller amount of lipids which are not present in most other types of bacteria.[24,25] In these mycobacteria, it has been discovered that the cell wall is made up of three different types of macromolecules: peptidoglycan, arabinogalatan and mycolic acids, all of which are covalently connected as shown in Figure 1.2.2.[21,23] Mycolic acids are the main constituents of the cell wall, but in addition to this, there are also a large number of other lipids present, which make up to 40% of the dry weight of the cell envelope. A lot of research has been carried out on these lipids, in particular to try and determine their structure, biosynthesis and to understand the capability of the mycobacteria to cause disease.[26,27,28] One of the most interesting and potentially useful glycolipids found in *M. tuberculosis* is 'cord factor', also known as trehalose dimycolate (TDM), which is a trehalose which has been esterified at both primary alcohol positions with mycolic acids.

1.3 Mycolic Acids

Mycolic acids are long chain α-alkyl, β-hydroxylated fatty acids, and were first isolated from *M. tuberculosis* by Anderson *et al.* in 1938.[29] At that time, Anderson defined the 'mycolic acid' as the "ether-soluble, unsaponifable, hydroxy acids of the human *tubercle bacillus*".[29,30] Advances in analytic techniques have shown that what was once considered to be 'mycolic acid', a single component of *M. tuberculosis*, actually consists of a family of over 500 different mycolic acids, which have closely related chemical structures.[31] Mycolic acids are now known to be characteristic of all mycobacteria; however, the mycolic acids present in the cell envelope of different mycobacteria vary in both the number of carbon atoms and also the functional groups present in the molecules.[32] Figure 1.3.1 below shows the general structure of a mycolic acid:

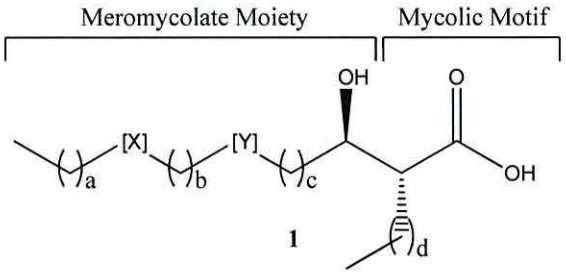

Figure 1.3.1 General Structure of Mycolic Acids.

There are two main moieties in each mycolic acid: the main branch, which is called the meromycolate moiety, and the mycolic motif. In the meromycolate moiety, the position

labelled [X] is called the distal position and for mycolic acids from *M. tuberculosis*, can contain double bonds, cyclopropane rings, methoxy or carbonyl functional groups, while the position labelled [Y] is called the proximal position and can be either a double bond or a cyclopropane ring.[33,34]

Due to the possible variations in the functional groups that can be present in mycolic acids, Watanabe *et al.* proposed a broad classification method.[35] This method splits the mycolic acids into three types: Type 1, Type 2 and Type 3. Type 1 mycolic acids have a cyclopropane ring in the proximal position (which can be either *cis* or *trans*); Type 2 mycolic acids have a *trans* double bond in the proximal position, while a Type 3 mycolic acid has a *cis* double bond in the proximal position.[35] These three different types can be seen in Figure 1.3.2 below.

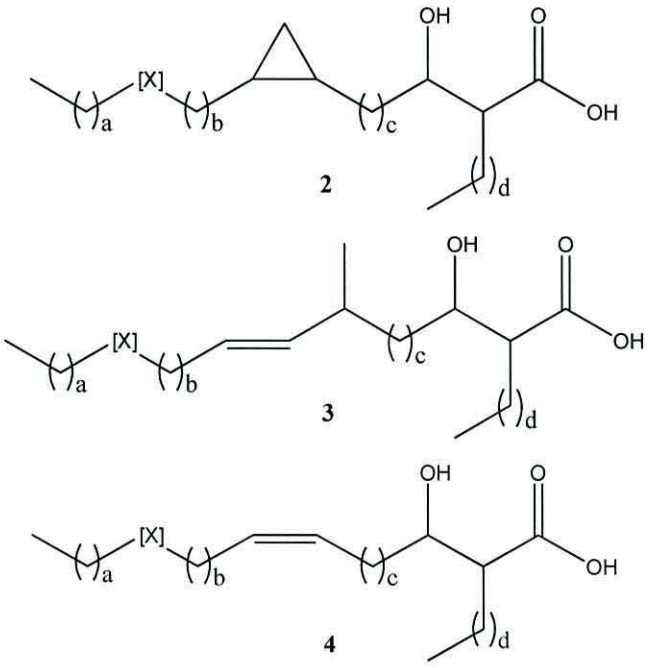

Figure 1.3.2. (top) Type 1 Mycolic Acid (cis version is shown); (middle) Type 2 Mycolic Acid and (bottom) Type 3 Mycolic Acid.

Although the cyclopropyl groups and the double bonds can be in either the *cis* or *trans* configuration, it has been discovered that whenever they are in the *trans* configuration, they always contain a methyl group on the adjacent distal carbon. When they are in a *cis* configuration this is not the case, with no methyl group being seen on the adjacent carbon atom.[31,32] (See Figure 1.3.3 - Figure 1.3.7 on the following page for examples.)

The identification of different mycolic acids has been possible recently due to advances in analytic techniques. Techniques such as 2D-TLC, HPLC, GC-MS and LC-MS have been

widely used to determine the structures of some of the mycolic acids present in *M. tuberculosis*.[36,37,38,39,40,41] It has been discovered that three main types are present in the bacterium: (i) cyclopropyl mycolic acids, which are called α, and contain two cyclopropane rings (usually in the *cis* configuration) (Figure 1.3.3), (ii) keto-mycolic acids (Figure 1.3.4 and Figure 1.3.5), and, (iii) methoxy-mycolic acids (Figure 1.3.6 and Figure 1.3.7).[42,43,44,45]

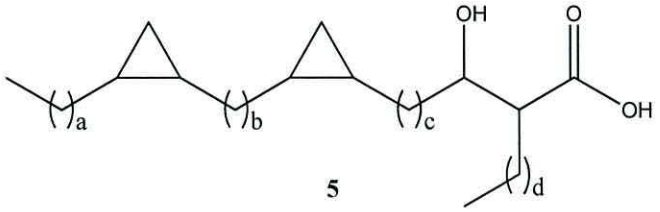

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Figure 1.3.3 α-mycolic acid.

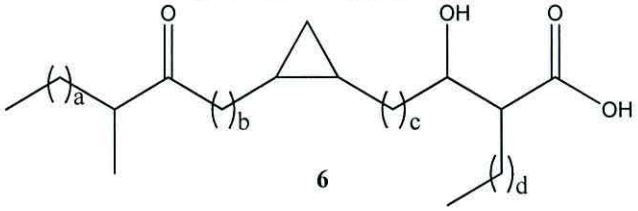

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Figure 1.3.4 Keto-mycolic acid with a cyclopropane ring in the cis-configuration.

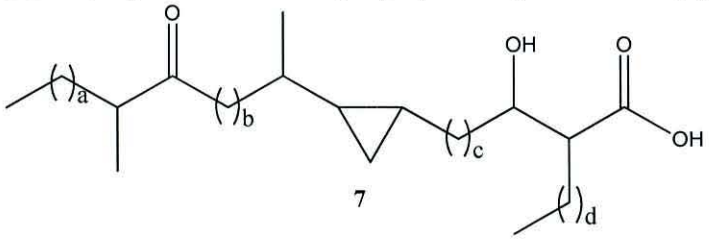

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Figure 1.3.5 Keto-mycolic acid with a cyclopropane ring in the trans-configuration.

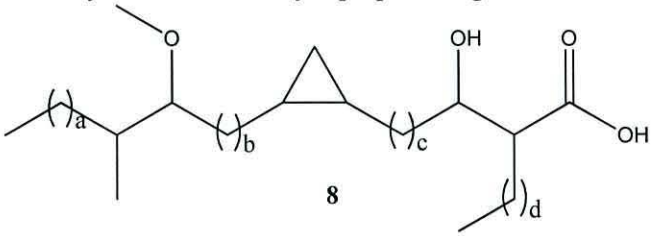

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Figure 1.3.6 Methoxy-mycolic acid with a cyclopropane ring in the cis-configuration.

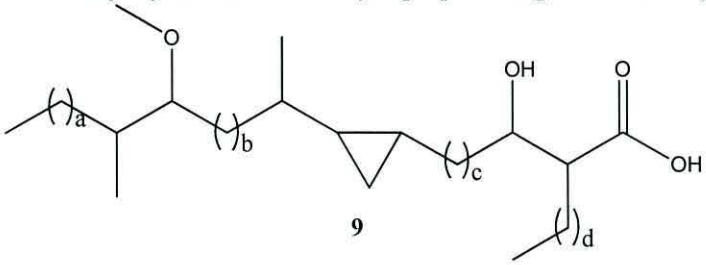

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Figure 1.3.7 Methoxy-mycolic acid with a cyclopropane ring in the trans-configuration.

There are also other minor types of mycolic acids present in *M. tuberculosis*, such as keto, methoxy or α-mycolic acids, which have a double bond instead of a cyclopropyl ring in the proximal position.[46] It has been established however that α-mycolic acids are the most common type in *M. tuberculosis*.[47] Even though the formation of a cyclopropane ring in the proximal position seems to be energetically unfavourable, it does occur readily in slow growing mycobacteria such as *M. tuberculosis*.[46] This is believed to occur because the cyclopropyl mycolic acids are much less susceptible to oxidative environments, and much less likely to undergo oxidation than their double bond counterparts.[48] It is also proposed that these cyclopropyl mycolic acids significantly contribute to the structural integrity of the cell wall complex in *M. tuberculosis*. This theory was proposed by Hasegawa *et al.*, and they also proposed that the mycolic acids can exist in either a folded or an unfolded state, depending on the external pressure.[49]

Mycolic acids also have various other roles in mycobacteria. For example, they are the reason that *M. tuberculosis* and other mycobacteria have such a low permeability to hydrophilic molecules.[21,22,50] It has been discovered, however, that changing the functional groups in the mycolic acids, and also changing the chain lengths changes the permeability.[51,52] Mycolic acids are also crucial for the virulence of mycobacteria.[51,52] Furthermore, the mycolic acids permit mycobacteria, such as *M. tuberculosis*, to grow inside macrophages, preventing them being 'attacked' by the host's immune system.[53]

A single enantiomer of an α-mycolic acid from *M. tuberculosis* was first synthesised by Al Dulayymi *et al.* in 2003.[54] Since then, the same group have successfully synthesised single enantiomers of various other α-,[55] methoxy[56] and keto[57,58] mycolic acids, including an example of a series of three stereoisomers of the major methoxy mycolic acid present in *M. tuberculosis*.[59] In addition to the synthesis of mycolic acids commonly found in *M. tuberculosis*, the first epoxy-mycolic acid, commonly found in *Mycobacterium fortuitum*, was also successfully synthesised.[60]

Synthesis of these compounds has for the first time allowed their biological activity to be studied. Work has been done to try and treat diseases such as asthma, where mycolic acids have been found to block immune responses which cause the symptoms of disease.[61] The synthesis of a large range of these mycolic acids has allowed study of single analogues, some of which have shown the ability to block the immune response which causes the symptoms of asthma.[62]

Mycolic acids are also known to be antigenic to anti-TB antibodies,[63,64] therefore the synthesis of individual mycolic acids will allow the antigenic properties of each individually synthesised mycolic acid, and any combination thereof, to be investigated.

1.4 Cord Factors

Cord factor, also known as trehalose dimycolate (TDM), is a trehalose which is esterified at both primary alcohol positions with mycolic acids.[65] Figure 1.4.1 below shows an example of a cord factor.

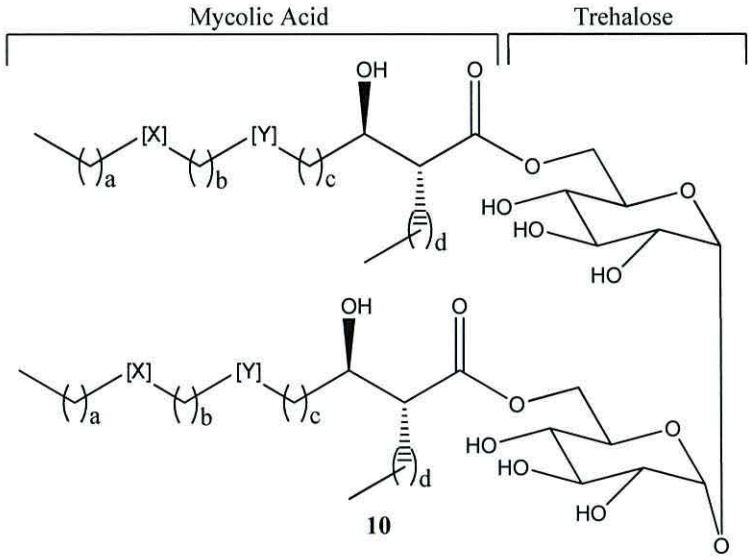


Figure 1.4.1 Cord Factor.

Cord factors were studied as early as 1884, when Robert Koch noticed that certain strains of *tubercle bacilli* formed long strands or 'cords'.[66] However, real interest in cord factors began after Middlebrook *et al.* discovered that the formation of 'serpentine cords' was characteristic of virulent strains of *tubercle bacilli.*[67] 'Cord factor' got its name in 1950 from H. Bloch, who isolated the glycolipid from the 'sticky' substance on the surface of *tubercle bacilli* cells.[68] The first time cord factor was purified was by Noll *et al.* in 1956, who proposed it to be a 6,6'-dimycolate of α,α-D-trehalose.[69] At that time, the structure of mycolic acids in mycobacteria was not known, thus the structure by Noll *et al.* only contained an approximate structure for cord factor.[70] Further work on the structure of mycolic acids in mycobacteria, in particular by Kato and Asselineau led to the confirmation of the structure of cord factor.[71]

After the discovery of cord factor, its toxic properties were the main reason for its study. It was first reported in the 1960s that cord factor causes a stimulation in the activity of

nicotinamide adenine dinuclease (NAD+), which is similar to the effect that tuberculosis causes.[72,73,74] Interest in cord factors further intensified after the discovery by Bekierkunst that by injecting 10 to 20 μg of cord factor directly into the blood of mice, granulomas were formed in their lungs, which led to local immunity for the mice against airborne infection by *M. tuberculosis*.[75]

This glycolipid also possesses numerous other desirable biological functions which are being investigated, and could be of extreme importance, such as being anti-tumour.[76,77] Cord factor is also a powerful adjuvant, which enhances the immune system of the host to generic antigens.[78,79] However, possibly the most important reason for current research into cord factors and mycolic acids is as possible antigens. This is because antibody responses to mycolic acids and cord factors are conserved in patients who have been tested HIV-positive.[63] This is in contrast to antibodies to proteins, which are depleted in HIV/AIDS co-infected patients.[80] This is an important attribute as some of the methods currently being used to detect TB or latent TB, such as the Tuberculin Skin Test (see section 1.5.3), are ineffective when the patient is co-infected with HIV.

As well as mycolic acids, Al Dulayymi *et al.* also reported the first synthetic cord factor in 2009, with the method used by the group being modifiable to produce cord factor or trehalose monomycolates (TMM) from any mycolic acid synthesised.[81] As with mycolic acids, the synthesis of single isomers of TDM and TMM compounds allows the antigenic properties of each of these, and any combination thereof, to be investigated.

1.5 Detection of Tuberculosis

Currently, there are five main methods approved by the WHO for detecting active TB or latent TB; namely bacterial culture;[82,83] smear microscopy;[83] skin test;[84] interferon-γ release assay;[84] and nucleic acid amplification test.[85]

1.5.1 Bacterial Culture

Bacterial culture is considered the 'gold standard' for detecting active TB and is the only method that can definitively diagnose a patient as being TB positive.[86] This method involves growing *M. tuberculosis* cultures from sputum on media. Traditionally, diagnosis was done using Lowenstein-Jensen media or Middlebrook media for culturing *M. tuberculosis*; however development in the area has led to quicker culture growth using BACTEC medium, without losing accuracy of results. Even though this method can identify whether a patient is infected with TB, it can take 3-8 weeks for the cultures to

grow.[82,87] There are three main reasons why cultivating the mycobacteria should be undertaken. Firstly, this method is much more sensitive than smear microscopy and has a limit of detection of approximately 10 bacilli/ml of sample.[88] Culturing the mycobacteria allows testing for drug susceptibility,[83] and growth of the mycobacteria is required for specific species classification.[83]

Even though bacterial culture is considered the 'gold standard' for detection of active TB, and has a specificity[I,89] of approximately 98 %, it is reported to only have a sensitivity[I,89] of 80-85 %.[90,91] In real terms, this means that in 15-20% of the cases where a person does have active TB, this test diagnoses them as TB negative.

1.5.2 Sputum Smear Microscopy

Sputum smear microscopy is a rapid and inexpensive test for the detection of acid-fast bacteria (AFB) and is considered the most vital method for diagnosing pulmonary TB in low-income and middle-income countries.[92,93] Commonly, samples are stained with Ziehl-Neelsen stain and the amount of AFB present in the sample is quantified, however, in higher income regions aruamine-rhodamine staining is more common as it increases sensitivity.[93] The staining process itself can be performed in less than 1 hour, leading to results being available much quicker than for bacterial culture.[13] One of the main drawbacks of this method is that it detects all AFBs and not specifically *M. tuberculosis*, so a positive result indicates the occurrence of mycobacteria in the sample, but not necessarily *M. tuberculosis*.[83] Another disadvantage of this method is that for extra-pulmonary tuberculosis, samples need to be collected from different areas of the body, such as obtaining cerebrospinal fluid (CSF); however the sensitivity of this test, for this type of TB, can vary between 10 % and 90 %.[94]

Although sputum smear microscopy is a much quicker method than bacterial culture, another drawback is that its limit of detection is between 5,000 and 10,000 bacilli/ml of sample, compared to approximately 10 bacilli/ml for bacterial culture.[83] Reports claim a high specificity of 91 – 100 % but sensitivities ranging from 50 – 80 %.[83,93]

In select settings, however, higher and lower sensitivity values have been reported. In setting, such as where the prevalence of TB is low, but that of chronic coughing is high, values of as low as 22 % have been reported.[95] However, in select settings, such as when

[I] Sensitivity is the proportion of people/samples who have the disease who test positive for it, while specificity is the proportion of people/samples who do not have the disease who test negative for it.

each smear sample analysed were from patients who were culture positive and were confirmed to have pulmonary TB, sensitivities of greater than 80 % have been stated.[96] It has also been reported than HIV co-infection reduces the sensitivity of this test.[97,98]

1.5.3 The Tuberculin Skin Test

There are currently two types of Tuberculin Skin Test (TST) that are used for the detection of *M. tuberculosis* infection, the Mantoux test and the Heaf test. The Mantoux test is the most widely used, especially after the Heaf test was discontinued in countries such as the UK in 2005.[83,99] This method involves the injection of tuberculin purified protein derivative (tuberculin PPD), isolated by protein purification from tubercle bacilli, into the patient's skin and reading the 'lesion' in 2 to 3 days.[83]

Unlike bacterial culture, this method is used to detect latent TB (which is defined as a condition where a person is infected with *Mycobacterium tuberculosis*, but do not show any TB symptoms[100]); however it cannot distinguish between latent tuberculosis infection and active tuberculosis disease.[101] A major drawback of this method is that it can give both false positive and false negative results. False positive results can be obtained for patients who are infected with mycobacteria other than *M. tuberculosis*, patients who have been vaccinated with the Bacillus Calmette-Guérin (BCG) vaccination and also from repeat testing with this method, while false negative results can be obtained for patients with compromised immune systems, such as those infected with HIV.[82,102,103,104,105,106]

Another drawback of this method is that the results largely depend on the interpretation by the person observing the lesion, due to it relying on the lesion size. This can lead to discrepancies from person to person measuring, which can lead to misdiagnosis of the patient.[102,103] These combined effects lead to reported sensitivities of between 57 % and 100 %,[107,108] with specificities of 93 – 100 % in non-BCG vaccinated countries,[108,109] but specificities ranging from 35 – 79 % in BCG vaccinated countries.[110,111] These sensitivity and specificity values are approximate and carry an error due to there being no 'gold standard' for the detection of latent tuberculosis.[101] As opposed to active tuberculosis, where bacterial culture is used as the gold standard, and positive and negative samples are compared against the results of this assay, there is no 'reference assay' for detecting latent TB, therefore no assay to which the results can be compared to determine sensitivity and specificity accurately.[101] This leads to sensitivity and specificity values generally being quoted as a range, with a set confidence interval.[101]

1.5.4 Interferon-γ Release Assays

Interferon-γ release assays (IGRAs) are a relatively new method used for the detection of *M. tuberculosis* infection, however they are quickly becoming more popular, with the QuantiFERON-TB Gold and T-SPOT.TB test kits both being commercially available.[101] This method works based on the fact that interferon-γ will be secreted from white blood cells in response to them coming into contact with TB antigens, ESAT-6 and CFP-10, if the patient is infected with *M. tuberculosis*. The interferon-γ can then be quantified by ELISPOT or ELISA.[112,113] As with the tuberculin skin test, IGRAs detect both latent and active TB and cannot distinguish the two.[101,113]

Although this method has some advantages over the tuberculin skin tests, it also has some drawbacks. The main benefits are that results can be obtained within 24 hours; no false positive results occur if the patient has been previously vaccinated with the BCG vaccination, and the method does not depend on the reader's interpretation, which can lead to variability with the skin test method.[84,114,115]

The disadvantages of this method are, firstly, serum samples must be properly stored and processed within 12 hours of collection, or the accuracy of the test diminishes. Another drawback is that although the method is reported to be able to detect latent and active TB,[101,113] there is some uncertainty as to the performance of the test in detecting active TB, as it has also been reported that it is not yet certain if it can detect TB once it has become active, as it is reported that active TB is known to suppress γ-interferon response.[115] Also, there is only a limited amount of clinical and laboratory experience with this assay, which leads to the method not being recommended for children under the age of eighteen or pregnant women due to the lack of information and insufficient data. Further studies are required in order to determine whether it can be recommended for these people.[84,114,115]

The QuantiFERON-TB Gold assay has reported sensitivities of 55 – 93 %,[108,116] and specificities of 89 – 100 %,[117] while the T-SPOT.TB assay has reported sensitivities of 80 – 100 %,[118,119] and specificities of 85 – 100 %.[108,111] As with the tuberculin skin test, these values for sensitivity and specificity are only approximate and do contain errors, due to there being no 'gold standard' for the detection of latent tuberculosis.[101]

One key drawback for this method, as well as the TST, is that approximately 2 billion people are infected with latent TB.[16] It is therefore impractical to treat all of the patients who appear positive by these tests for TB.

1.5.5 Nucleic Acid Amplification Test

Nucleic acid amplification tests (NAATs) are a rapid test for pulmonary tuberculosis, with one of the most common tests being the Amplified *Mycobacterium tuberculosis* Direct Test (MTD).[85] Briefly, NAATs work by releasing nucleic acids from mycobacteria through sonication before heat denaturing is carried out to disturb the ribosomal RNA's (in the case of MTD) secondary structure. The ribosomal RNA is then amplified and a single-stranded DNA sequence, modified to contain a chemiluminescence marker, is added which selectively binds to *M. tuberculosis* complex-specific sequences.[120] One of the main advantages of this method is that the assay can be run in approximately 2.5 to 3.5 hours once the process has commenced, which is comparable in time to sputum smear microscopy.[120,121] Another advantage of NAATs compared to sputum smear microscopy is that they have a detection limit of less than 100 bacilli/ml of sample which equates to 1 – 10 bacilli per reaction.[122] However, these low limits of detection require that great care must be taken in order to minimise contamination.[123]

This method does however also have some drawbacks. Firstly, it detects the presence of *Mycobacterium tuberculosis* complex, but cannot distinguish between the different mycobacteria and it is stated that bacterial culture must be performed alongside the MTD test.[120] There is also limited knowledge available about this assay when samples other than sputum are used; therefore samples containing blood may cause false-positive readings.[120] Another drawback is that the precision of the assay is much worse for sputum smear-negative patients compared to sputum smear-positive patients.[124] NAATs have consistently been reported with high specificities of greater than 95 %,[122] however the sensitivity of the test has been reported to vary between 63 – 99 %,[85,125,126,127] with the value of 63 % being reported for smear-negative patients and 99 % for smear positive patients.[85]

1.5.6 ELISA

Enzyme Linked Immunosorbent Assay (ELISA) is a system to detect the presence of antibodies in a sample, and has been used in order to try and detect TB antibodies.[64,128]

Briefly, an ELISA assay to detect antibodies is carried out as follows:

1. A known amount of antigen is coated onto the surface of an ELISA plate.
2. Any free non-specific binding sites are blocked.
3. The sample containing the primary antibody is added to the plate.
4. Any excess antibodies are washed away in a washing step, leaving only the antibodies that have bound to the antigen on the plate.
5. A secondary antibody, labelled with an enzyme, is added to the well, and binds to the primary antibody.
6. Any excess unbound secondary antibody is washed away from the plate in another washing step.
7. A chemical is added which is converted by the enzyme on the secondary antibody into a detectable form.
8. The signal (absorbance or fluorescence *etc.*) is measured after a given time, and the magnitude of the signal is used to infer the relative amount of antibodies in the sample.[129,130]

The ELISA assay can selectively measure the levels of specific antibodies by varying the antigens and secondary antibody used. It can be used for the detection of IgA,[131] IgD,[132] IgE,[133] IgG[134] and IgM[135] antibodies.

Commercial Use

The ELISA method is currently commercially used in order to detect diseases such as Lyme disease.[136] Although not recommended by the WHO for the detection of TB,[137] a recent report has stated that in India, which is the country with the largest burden of TB, with approximately 2 – 2.5 million cases of TB in 2010,[16] around 1.5 million ELISA assays are carried out each year to diagnose the disease.[138] This leads to an estimated spending of approximately US $15 million each year in India on these tests alone.[139] As well as being used in India, these tests are also extensively used in many countries, including China, Indonesia, Pakistan and Uganda.[139]

There are many kits available, such as Qualisa TB IgG which claims a sensitivity of 100 % and a specificity of 99.2 %,[140] and Serocheck-MTB which claims sensitivity and specificity values of 100 %.[141] Indeed most of these tests report sensitivity and specificity values of greater than 95 % on their packages without any published data to support those claims.[137] It has however been estimated in a recent study that the true values for the ELISA tests being used in the countries mentioned above are 59 % for sensitivity and 87 % for specificity.[138]

These values for sensitivity and specificity lead to a danger of misdiagnosis of TB, which is detrimental to both the patients and to the general public, with each failure to diagnose a patient with TB resulting in added spreading of the disease.[139] The routine use of these tests in the mentioned countries also inhibits validated tests from being introduced and being routinely used.[139]

Along with other investigations into serological tests available for the detection of TB, these findings led to the WHO releasing a statement declaring:

"Commercial serological tests provide inconsistent and imprecise findings resulting in highly variable values for sensitivity and specificity. There is no evidence that existing commercial serological assays improve patient-important outcomes, and high proportions of false-positive and false-negative results adversely impact patient safety. Overall data quality was graded as very low and it is strongly recommended that these tests not be used for the diagnosis of pulmonary and extra-pulmonary TB."[137]

Even though this statement was made by the WHO, they acknowledge the fact that a point-of-care serological test would be extremely valuable for the rapid detection of TB, in particular where access to on-site laboratories is not available.[137] It has also been reported in a recent review that significant research into TB biomarkers is urgently required in order to produce accurate, portable and consistent serological tests to replace the unsuccessful and inconsistent assays that are currently used.[139]

Research using Lipid Antigens for the Detection of Anti-TB Antibodies

Research has been undertaken by Schleicher *et al.,* using ELISA to try and detect anti-mycolic acid antibodies from *M. tuberculosis* in serum samples from patients infected with TB and HIV, and from patients infected with TB alone.[63] This group used natural mycolic acids, isolated from *M. tuberculosis*, as antigens for their assay and they discovered that

the antibody levels were significantly higher for TB positive sera than for TB negative sera and that antibody levels remained largely unchanged between HIV-positive and HIV-negative samples, suggesting that antibody responses to mycolic acids are also conserved in patients who have been tested HIV-positive.[63] In spite of the fact that a higher signal was observed for TB positive sera than for TB negative sera, a large number of false positive and false negative responses were observed with a sensitivity of 51 % and specificity of 63 % being quoted for the assay.[63]

Continuation of this work was done by Beukes *et al.*, where they compared the antibody responses to natural mycolic acid extracted from *M. tuberculosis* with synthetic mycolic acids produced by Al Dulayymi *et al.*, as well as investigating their corresponding methyl esters.[142] It has been discovered that antibody recognition is much higher for free mycolic acids than their corresponding methyl esters suggesting that the carboxylic acid section of the mycolic acids either has a large contribution to the binding of the mycolic acids to the antibodies, or that they stabilise a particular antigen conformation.[142] It was also discovered that oxygenated mycolic acids are more antigenic than α- mycolic acids but none of the synthetically produced mycolic acids could notably and reproducibly distinguish TB positive sera from TB negative sera.[142] However, the authors believe that methoxy mycolic acids in particular, could be used to improve the sensitivity of a serological test for the diagnosis of TB when detecting anti-lipid antibodies.[142] There are no quoted values for sensitivity and specificity for this research because pooled sera was used.[142]

In addition to the research done on free mycolic acids as antigens, Pan *et al.* have undertaken similar investigations with cord factor.[47] Here, the antigenic properties of natural cord factor, isolated from *M. tuberculosis* and *M. avium* as well as separated sub-classes of cord factor, were analysed. Firstly, it was discovered that cord factor isolated from *M. tuberculosis* detected many more antibodies at the end of the ELISA assays than cord factor isolated from *M. avium*.[47] This suggests that it may be possible to detect antibodies to specific mycobacterial diseases by varying the cord factor used for their detection. It was also discovered that the sub-class of mycolic acid present in the cord factor is recognised by the antibodies, with the largest amount of binding occurring to cord factors containing methoxy mycolic acids whereas binding to cord factors containing α- and keto mycolic acids was found to be relatively low.[47] For this study, sera were not pooled; however sensitivity and specificity values for the ELISA assays with various cord

factors as antigens were not quoted, with only the average optical density value for positive and negative sera quoted.[47]

1.5.7 Other Methods

In addition to the methods mentioned above, there are some other methods for detecting TB that have been developed, but are not being currently widely used. Three of note are the IAsys (interaction assay system) affinity biosensor developed by Thanyani *et al.*,[64] the optoelectronic biosensor developed by Sliva *et al.*,[143] and an impedance based sensor developed by Ozoemena *et al.*[144]

The IAsys Biosensor

An IAsys is a method for looking at the real time interactions between molecules on the surface of a cuvette, by means of an optical reading. A change in refractive index is measured, which is directly related to the change in mass on the surface.[145,146,147] The method developed by Thanyani *et al.* works by preparing liposomes containing mycolic acids, then immobilising them on the surface of a cuvette before adding the serum sample under investigation. The refractive index is then measured at different intervals, and the amount of binding is inferred from the change in the measurement.[64]

The main advantage of this method is that real time data is available which directly relates to the amount of antibodies binding to antigens. This method however does have some drawbacks. Firstly, there is no information on the type of molecule binding to the mycolic acid containing liposomes, although the group has inferred from protein-A based affinity chromatography that IgG antibodies do bind and have an effect on the refractive index. From the results published, a total of 61 serum samples were analysed from patients who were infected with TB alone, HIV alone, co-infected with both or were uninfected, and 16 false positive and 4 false negative results were obtained, giving sensitivity and specificity values of 87 % and 48 % respectively. It must also be noted that the method requires multiple washing steps which becomes time consuming and limits the number of samples that can be analysed in a day.[64]

An Optoelectronic Biosensor

The second method is the optoelectronic biosensor developed by Sliva *et al.*[143] In brief, oligonucleotides modified to contain a terminal thiol group are mixed with gold nanoparticles, and binding occurs between them through a gold-sulfur (Au-S) bond. The resulting coated gold nanoparticles are then mixed with the DNA sample under

investigation (which has been amplified by PCR), denatured by heat then re-cooled before $MgCl_2$ is added. Measurements are taken 15 minutes after addition of the $MgCl_2$. The sample acts like a 'colour filter', and red (523 nm) and green (628 nm) light is passed through the sample solution. The ratio of transmittance between that for the red and green light is then taken, and normalised to give a final responsitivity reading.[143]

This method has shown promise in distinguishing between positive and negative samples, however it is still in its early stages and only a small number of samples have been analysed so far. The device produced is also reported to be cost effective and is portable, which is very important if the method for TB diagnosis is to be used in remote areas. The major drawback of this method is that DNA samples, which need to be amplified by PCR, are used for the diagnosis. This increases the amount of time required for a successful diagnosis and also the amount of equipment needed in order to be able to make the diagnosis. The fact that a PCR amplification step is required prevents this method from being completely portable, even though the unit for analysis itself is, and therefore doesn't answer the problem of being able to give a 'point of care' diagnosis.[143]

An Impedance Based Sensor

The final method discussed in this section is the impedance based biosensor developed by Ozoemena *et al.*[144] This method involves binding mycolic acids to a gold electrode, *via* a linker compound, and measuring the difference in impedance when analysing TB positive and TB negative serum samples.

Using this method, a distinction was reported between one TB positive and one TB negative serum sample, and it was also reported that this difference in signal was much greater when using the impedance based method, as compared to when the same samples were analysed by ELISA.[144] The authors also comment that the impedance based sensor can detect serum samples of low concentrations, *i.e.* at a dilution of 1 in 2,000, and comment that this method could be developed into a very sensitive sensor for the detection of active TB.[144]

Further work has been carried out by the group to further characterise the gold electrode surface and look at the structure of the mycolic acid layer formed on these electrodes,[148] however to date, no further analysis of serum samples by this method have been reported.

1.5.8 Summary of Tests Available for TB Detection

The following table gives a summary of the tests currently commercially available for the diagnosis of TB.

Table 1.5.1 Summary of the current test being used for the detection of TB.

Test	Comments	Sensitivity (%)	Specificity (%)
Bacterial Culture	• Considered the 'gold standard' for detecting TB[86] • 3-8 weeks are required for a result[82,87] • Can distinguish drug resistant strains from non-drug resistant strains[83]	80-85	98
Sputum Smear Microscopy	• Quick screening method for mycobacterial disease[92,93] • Results available much quicker than for bacterial culture with the staining process being performed in less than 1 hour[13] • Cannot distinguish tuberculosis from other mycobacterial diseases[83]	50-80	91-100
Tuberculin Skin Test	• Detects both latent and active TB and cannot distinguish them[101] • Results are obtained in 2 to 3 days[83] • False positive samples include patient vaccinated with the BCG vaccination[82,102,103,104,105,106] • False negative samples include patients co-infected with HIV[82,102,103,104,105,106]	57-100*	35-79* [II] 93-100* [III]
Interferon γ-Release Assay	• Detects both latent and active TB and cannot distinguish them[101,113] • Results obtained in 24 hours[84,114,115] • No false positive samples due to BCG vaccination[84,114,115] • Limited amount of clinical and laboratory experience with the assay[84,114,115]	55-93* [IV] 80-100* [V]	89-100* [IV] 85-100* [V]
Nucleic Acid Amplification Test	• Detects the presence of *Mycobacterium tuberculosis* complex, but cannot distinguish the individual mycobacteria[120] • Results obtained in 2.5 to 3.5 hours[120,121] • Low limits of detection[122] • Limited data when not using sputum[120]	95	63-99
ELISA	• Detects antibodies produced against *M. tuberculosis*[64,128] • WHO has strongly recommended that these assays are not to be used [137]	59**	87**

* Estimated sensitivity or specificity for either active or latent TB.

** Estimated true sensitivity or specificity for the ELISA assays that are currently commercially available in countries such as India, China, Indonesia, Pakistan and Uganda.

All of these techniques are used in conjunction with monitoring the patient's clinical symptoms in order to fully diagnose the patient. Although there are a number of methods

[II] BCG vaccinated countries.
[III] Non-BCG vaccinated countries.
[IV] QuantiFERON-TB Gold
[V] T-SPOT.TB

available for the detection of TB, it can be seen from the above summary that there is a need for a fast, efficient and cost effective sensor for the detection of TB, which can be easily operated and is fully portable, for use in developing countries such as in Africa.

1.6 The Use of Coated Gold Nanoparticles as a Potential Sensor

1.6.1 The Gold-Sulfur (Au-S) Bond

The formation of gold-sulfur (Au-S) bonds has been known for a number of years, with the first self-assembled monolayer (SAM) on a gold surface being reported by Nuzzo *et al.* in 1983, where monolayers of dialkyl disulfides were produced on a gold surface.[149] However, in spite of much research, the nature of this bond is still largely unknown, in both 2D (planar gold) and 3D (gold nanoparticle) systems, and is still a topic of interest for researchers.[150]

Several groups have studied the gold - alkane thiol bond, mainly using X-ray photoelectron spectroscopy (XPS), however, the results obtained were mostly inconclusive.[151,152,153,154,155,156,157] Early work concluded that the sulfur bound in the form of a thiolate.[153,158,159] However, more recently, studies have analysed the oxidation state of the gold, and their results suggest otherwise.[150] Research was done by Bourg *et al.*, where the binding energy for the Au 4f and S 2p electrons were measured by XPS for planar gold SAMs, gold nanoparticle SAMs, and for monolayers formed on Au (I) complexes. The results suggest that for both the 2D and 3D SAMs, the gold only exhibits one oxidation state, which is the Au (0) state, which corresponds to a peak at 83.9 eV, and in addition, there are no peaks reported which correspond to Au (I). For the sulfur, the binding energy for the S $2p_{3/2}$ was 1.6 - 2.2 eV less than the theoretical value for elemental sulfur (S_8) which may suggest that the sulfur carries a charge of approximately -0.2 *e*.[150] It is also recognized that the Au–S bonds are virtually the same for SAMs on both planar gold and on colloidal gold.[150]

It has also been discovered that the Au-S bonding tends to be the same regardless of whether the starting material is a thiol or disulfide with both producing the same species on the gold surface.[151]

A point of contention has been whether or not the carbon-sulfur (C-S) bond cleaves upon an organothiol binding to gold, with early work suggesting that a cleavage of this bond did occur.[158] This however has been disproved with the immobilisation of biological

molecules, particularly oligonucleotides modified with thiol groups, onto gold plates and gold nanoparticles.[160,161,162,163,164,165] However it is not only oligonucleotides that have been bound to gold by Au-S bonds, simple thiols,[150] disulfides[166] and thioethers[167] have also been successfully bound.

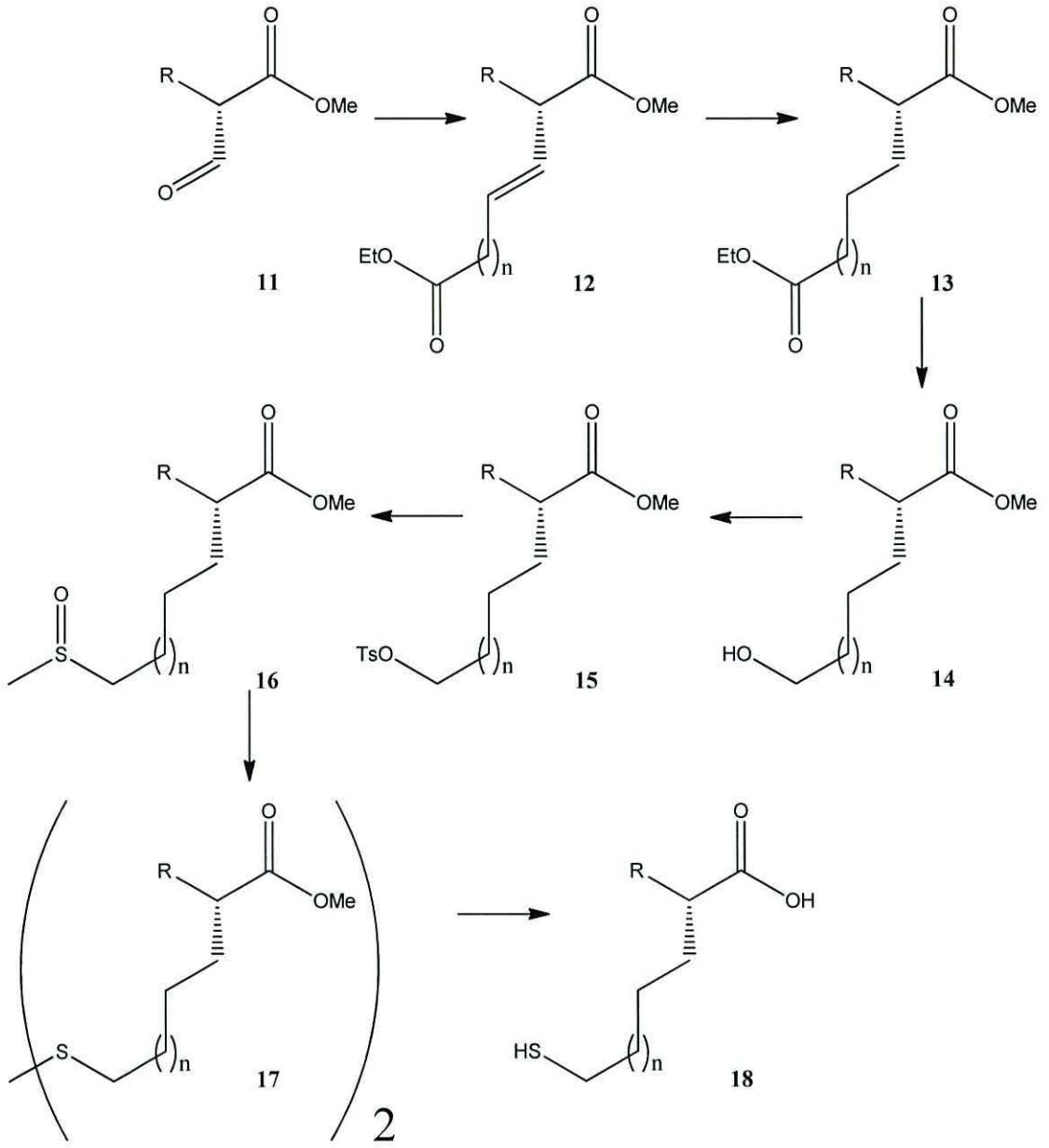
An important fact to note is that loss of bioactivity often transpires when biological molecules are adsorbed straight onto a naked bulk metal surface due to denaturation, but adsorption onto a nanoparticle surface generally preserves bioactivity due to the biocompatibility of nanoparticles.[168]

Finally, it has been discovered that the formation of SAMs on larger nanoparticles are more ordered than for smaller nanoparticles,[158] which could be important when attempting to develop a sensor that provides consistent results.

The synthesis of Thiol Modified Mycolic Acids

In order to bind mycolic acids to gold surfaces *via* an Au-S bond, work was undertaken in both Bangor and Pretoria on the synthesis of thiol modified mycolic acids.[169] Two different approaches were taken to incorporate a thiol functional group into the mycolic acid molecules: the first approach involved modification of the alkyl chain to contain a thiol group; while the second approach involved modification of the acid functional group.[169]

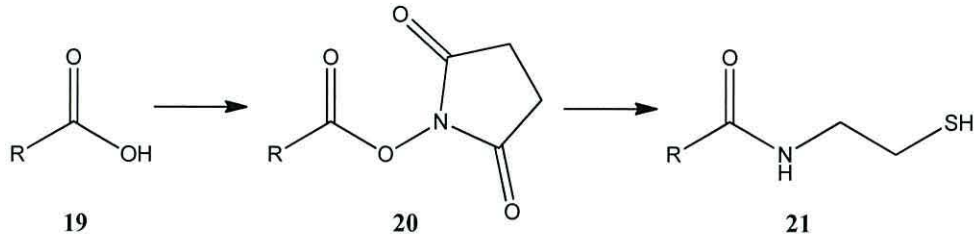
In brief, the synthesis of the α-chain moiety of the mycolic acid was modified to contain a thiol group as follows:



Scheme 1.6.1 Synthesis of thiol modified mycolic acids with a thiol group on the α-chain (18).

Firstly, aldehyde (**11**) was coupled with a sulfone containing ester functionality to produce the alkene (**12**), which was then hydrogenated to form compound **13**. This introduced the correct chain length to the α-chain, while also containing an ethyl ester functional group that could be selectively modified. This ester was then selectively reduced to the corresponding alcohol (**14**) which was then activated by conversion into a tosylate (**15**). This tosylate was then treated with potassium thioacetate to produce the thioacetate (**16**), which was then hydrolysed to give the disulfide (**17**). This disulfide could then be treated with dithiothreitol to cleave the disulfide bond and yield the thiol modified mycolic acid (**18**).[169]

The second approach, to modify the acid group of the mycolic acids, were carried out as follows:



Scheme 1.6.2 Synthesis of thiol modified mycolic acids with a thiol group on the acid group (21).

Using this approach, the mycolic acids (**19**) were firstly activated by reaction with *N*-hydroxysuccinimide to give the ester (**20**). This ester was then reacted with 2-mercaptoethylamine hydrochloride to yield the mycolic acid containing a thiolated linker on the acid group.[169]

Both of these approaches were successful in forming thiol modified mycolic acids, therefore making binding mycolic acids directly to gold surfaces, *via* an Au-S bond, possible.

1.6.2 Gold Nanoparticles

Gold nanoparticles, which are also referred to as colloidal gold, are thought to have been around since about the 5th century B.C., where they were mainly used to give glass and ceramics a ruby colour.[170] Colloidal gold, or 'soluble or drinkable gold' as it was referred to, was known during the Middle Ages and was used to cure diseases, such as epilepsy as well as being a diagnostic tool for syphilis.[170] The first known synthesis of colloidal gold was performed by Faraday in 1857, where chlorate anions ($AuCl_4^-$) were reduced by phosphorus, using carbon disulfide as a solvent.[171]

Interest in colloidal gold intensified towards the middle of the 20th century, during which time various different methods for its synthesis were developed. The earliest and simplest method was developed by Turkevitch *et al.* in 1951.[172,173] This method is used to produce spherical gold nanoparticles with a diameter of approximately 20 nm, by adding sodium citrate to a solution of chlorauric acid at 95 °C, with vigorous stirring.[172] This was later modified by Frens in 1973 to produce spherical gold nanoparticles of various diameters by changing the ratios of chlorauric acid and sodium citrate used.[174]

Towards the end of the 20th century, a method for synthesising gold nanoparticles in organic solvents was developed by Brust *et al.*[175] This involves the addition of chlorauric acid in water to tetraoctylammonium bromide (TOAB) in toluene and stirring the mixture until the chlorauric acid is transferred into the organic layer. A solution of aqueous sodium borohydride is then added to reduce the Au^{3+} to Au^{0}, after which a thioalkane (dodecanethiol) is added in order to prevent aggregation of the gold nanoparticles that were being formed. This method generally produces gold nanoparticles of up to approximately 5 nm diameter, and has allowed new research in this field as it was the first method to produce air stable and thermally stable gold nanoparticles with such small diameters, while still allowing control of the particle sizes.[175]

In 2009, Perrault *et al.* synthesised monodispersed gold nanoparticles of controlled sizes and uniform shape of up to 200 nm in diameter.[176] The method used was an adaptation of a method used by Brown *et al.*[177] where gold nanoparticles were grown onto gold nanoparticle seeds. Perrault *et al.* created the seeds by using the citrate method developed by Turkevitch *et al.*, then adding a solution of aqueous gold chloride with hydroquinone (which has a weak reduction potential of -0.669 V) to the seeds. The method works based on the fact that reducing gold chloride in the presence of metal clusters has a different standard potential (+1.002 V) than the standard potential required to reduce isolated gold chloride (-1.5 V).[178] The difference in potentials is large enough so that reduction of isolated gold chloride is not spontaneous with hydroquinone while this reduction does occur at the surfaces of gold clusters.[176] This method allows the formation of gold nanoparticles with specific diameters, which can be controlled by the amount of seeds used.[176]

Metal nanoparticles, and specifically gold nanoparticles, have been studied for many reasons, including being used to immobilise antibodies to improve their stability,[179] as a sensor for H_2O_2,[180] and in conjunction with semiconductors as a DNA sensor.[143,181]

However, one of the main reasons for studies involving gold nanoparticles is because they exhibit unique optical properties,[182,183] and display different chemical and electronic properties compared to bulk materials.[168] Additional interest has developed in the gold nanoparticle field due to aqueous gold nanoparticles appearing red in colour; this is due to the surface plasmon band, which is a broad absorption band at approximately 520 nm.[170] It has been shown that atoms on the nanoparticle surface have an incomplete valence,

i.e. they contain a free (6s) electron,[184] which can move between the vacant orbitals of the surface atoms upon exposure to electromagnetic radiation of the correct wavelength causing oscillations of the 'free electron gas' density.[170,184,185]

This surface plasmon band is missing for bulk gold and for nanoparticles of less than 2 nm diameter.[170] The maximum wavelength of this absorption band varies with nanoparticle diameter, with smaller diameter nanoparticles exhibiting a surface plasmon resonance band at a lower wavelength than for nanoparticles with a larger diameter.[170] Another important factor is the charge on the gold nanoparticle core. The addition of electronic charge causes the position of the surface plasmon resonance band to move to a lower wavelength while removal of electronic charge shifts the band to a higher wavelength.[182,186,187,188]

Recent research has used gold nanoparticles as a means for detecting diseases by binding oligonucleotides to the particles. A DNA sample (often amplified by PCR) is then added to the modified nanoparticles, and binding of complementary DNA sequences occurs between the bound oligonucleotide and the DNA under investigation.[143,189,190,191,192,193] The gold nanoparticles appear red in aqueous solution due to a surface plasmon resonance band absorption at approximately 520 nm, however, upon addition of salt, the solution becomes blue due to aggregation of the gold nanoparticles leading to a red shift (a shift to higher wavelength) of the surface plasmon resonance band.[194,195] It has been observed that aggregation can be stopped following salt addition if complementary binding has occurred. This is due to an increase in steric hindrance from the complementary DNA, which prevents aggregation from occurring.[196] This means that it is possible to distinguish between positive and negative samples by looking at the solution's absorbance at different wavelengths.[143]

1.7 Concluding Remarks and Aims for Sensor Development

TB is currently an immense global problem with approximately 8.8 million new cases per year. The current methods for the detection of TB have many limitations (Table 1.5.1), and all serological tests for its detection have been deemed to be unreliable by the World Health Organisation. They have however acknowledged that a point-of-care serological test would be extremely valuable for the rapid detection of TB, in particular in regions where access to on-site laboratories is not available.

Research has shown that mycolic acids and cord factors are antigenic to anti-TB antibodies. However, to date, complex mixtures of natural compounds have been the most

widely investigated. In the instance where synthetic analogues have been analysed, pooled sera has been used, thus no sensitivity and specificity values were quoted, leading to no indication of whether a sensor detecting these antibodies would have good sensitivity and specificity. It may however be possible, that by investigating individual TB positive and TB negative serum samples against individual isomers of synthetic mycolic acids and cord factors, or any combination thereof, a method with sufficient sensitivity and specificity can be developed to differentiate TB positive serum samples from TB negative serum samples.

Baird's research group at Bangor University has reported the synthesis of a range of mycolic acids in a series of papers starting in 2005. Just before the project began, the first syntheses of trehalose di- and mono-mycolates were completed. A number of the synthetic mycolic acids had been examined as antigens by Verschoor's group at the University of Pretoria, South Africa, using a rather complex design of sensor to optimise TB positive / TB negative differences. In order to simplify the sensor design and improve sensitivity and specificity, work began in both Bangor and Pretoria on the synthesis of thiolated mycolic acids for binding to gold. In South Africa, the aim was to use an electrochemical approach based on impedance to achieve this. The priority in Bangor was to develop a method based on mycolic acid coated gold nanoparticles, although initial studies had also been carried out using an electrochemical approach. This project was one of several being completed at the same time to identify the best combination of synthetic mycolic acids or related trehalose esters to distinguish TB positive and TB negative serum samples and to apply these in a novel design of sensor.

The central aim of this project was to develop a biosensor by forming a self assembled monolayer (SAM) of mycolic acids on gold nanoparticles (AuNPs) *via* Au-S bonds. Access to synthetic mycolic acids modified to contain a thiol functional group would make it possible to bind mycolic acids to gold nanoparticles without the loss of bioactivity.[168] Various configurations of sensor would be examined.

The sensor developed needs to be rapid, portable, reliable and easy to use in remote regions where access to on-site laboratories is not possible.

1.8 References

1 V. Kumar, A. K. Abbas, N. Fausto and R. N. Mitchell, *Robbins Basic Pathology (8th ed.)*, Saunders/Elsevier, 2008, 516-522.

2 J. B. Bass, L. S. Farer, P. C. Hopewell, R. F. Jacobs and D. E. Sneider, **Diagnostic standards and classification of tuberculosis,** *Am. Rev. Repir. Dis.*, 1990, **142,** 725-735.

3 J. D. H. Porter and K. P. W. J. McAdam, *Tuberculosis: Back to the Future.*, John Wiley & Sons Ltd., 13-31.

4 B. Rothschild, L. Martin, G. Lev, H. Bercovier, G. Bar-Gal, C. Greenblatt, H. Donoghue, M. Spigelman and D. Brittain, ***Mycobacterium tuberculosis* complex DNA from an extinct bison dated 17,000 years before the present,** *Clin. Infect. Dis.*, 2001, **33**, 305-311.

5 I. Hershkovitz, H. D. Donoghue, D. E. Minnikin, G. S. Besra, O. Y-C. Lee, A. M. Gernaey, E. Galili, V. Eshed, C. L. Greenblatt, E. Lemma, G. K. Bar-Gal and M. Spigelman, **Detection and molecular characterisation of 9,000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in the Eastern Mediterranean,** *PLoS ONE,* 2008, **3**, 3426.

6 W. Stead, **The origin and erratic spread of tuberculosis: How the past explains the present and is the key to the future,** *Clin. Chest Med.*, 1997, **18**, 65-77.

7 K. A. Sepkowitz, J. Raffalli, L. Riley, T. E. Kiehn and D. Armstrong, **Tuberculosis in the AIDS era,** *Clin. Microbiol. Rev.*, 1995, **8**, 180-199.

8 H. H. Fox, **The chemical approach to the control of tuberculosis,** *Science,* 1952, **162**, 129-134.

9 C. Dye, **Global epidemiology of tuberculosis,** *The Lancet,* 2006, **367**, 938-940.

10 WHO, *Global Tuberculosis Control: Surveillance, Planning, Financing. Geneva: World Health Organisation,* 2006, 242.

11 C. Dye, C. J. Watt, D. M. Bleed, S. M. Hossein and M. C. Raviglione, **Evolution of tuberculosis control and prospects for reducing tuberculosis incidence, prevalence, and deaths globally,** *JAMA,* 2005, **293**, 2767-2775.

12 E. L. Corbett, C. J. Watt, N. Walker, D. Maher, B. G. Williams, M. C. Raviglionea and C. Dye, **The growing burden of tuberculosis – Global trends and interactions with the HIV epidemic,** *Arch. Intern. Med.*, 2003, **163**, 677-686.

13 A. D. Harries, D. Maher and S. Graham, *TB/HIV: A clinical manual (2nd ed.),* World Health Organisation, Geneva, 2004.

14 L. Kremer and G. S. Besra, **Re-emergence of tuberculosis: Strategies and treatments,** *Expert Opinion on Investigational Drugs,* 2002, **11**, 153-157.

15 J. D. H. Porter and K. P. W. J. McAdam, *Tuberculosis: Back to the Future*, John Wiley & Sons Ltd., 1994, 35-49.

16 WHO Report 2011, *Global Tuberculosis Control,* 2011.

17 *The WHO/IUATLD Global Project on Anti-Tuberculosis Drug Resistance Surveillance; Anti-Tuberculosis Drug Resistance in the World (4th global report),* 2007.

18 B. A. Forbes, D. F. Sahm and A. S. Weiddfeld, *Bailey & Scott's Diagnostic Microbiology (12th ed.),* 2007.

19 T. Flowers, **Quarantining the noncompliant TB patient: Catching the "red snapper",** *Journal of health and hospital law: a publication of the American Academy of Hospital Attorneys of the American Hospital Association,* 1995, **28,** 95-105.

20 S. T. Cole, R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gorodon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S.

Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rurrer, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead and B. G. Barrell, **Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence,** *Nature,* 1998, **393,** 537-544.

21 M. Daffé and P. Draper, **The envelope layers of mycobacteria with reference to their pathogenicity,** *Adv. Microb. Phys.,* 1998, **39,** 131-203

22 V. Jarlier and H. Nikaido, **Permeability barrier to hydrophobic solutes in *Mycobacteria chelonei,*** *J. Bacteriol.,* 1990, **172,** 1418-1423.

23 A. M. Abdallah, N. C. G. van Pittius, P. A. D. Champion, J. Cox, J. Luirink, C. M. J. E. Vandenbroucke-Grauls, B. J. Appelmelk and W. Bitter, **Type VII secretion – Mycobacteria show the way,** *Nat. Rev. Microbiol.,* 2007, **5,** 883-891.

24 A. Ortalo-Magné, M. A. Dupont, A. Lemassu, A. B. Andersen, P. Gounon and M. Daffé, **Molecular composition of the outermost capsular material of the tubercle bacillus,** *Microbiol.,* 1995, **141,** 1609-1620.

25 O. Neyrolles and C. Guilhot, **Recent advances in deciphering the contribution of *Mycobacterium tuberculosis* lipids to pathogenesis,** *Tuberculosis,* 2011, **91,** 187-195.

26 C. Asselineau and G. Laneelee, **Mycobaterial lipids: A historical perspective,** *Frontiers in Bioscience,* 1998, **3,** 164-174.

27 P. C. Karkousis, W. R. Bishai and S. E. Dorman, ***Mycobacterium tuberculosis* cell envelope lipids and the host immune response,** *Cell. Microbiol.,* 2004, **6,** 105-116.

28 P. E. Kolattukundy, N. D. Fernandes, A. K. Azad, A. M. Fitzmaurice and T. D. Sirakova, **Biochemistry and molecular genetics of the cell-wall lipid biosynthesis in mycobacteria,** *Molec. Microbiol.,* 1997, **24,** 263-270.

29 R. J. Anderson, **Structural peculiarities of acid-fast bacterial lipids,** *Chem. Rev.*, 1941, **29**, 225-202.

30 F. H. Stodola, A. Lesuk and R. J. Anderson, **The chemistry of the lipids of tubercle bacilli: LIV. The isolation and properties of mycolic acids,** *J. Biol. Chem.,* 1938, **126**, 505-513.

31 C. E. Barry, R. E. Lee, M. Khisimusi, A. E. Sampson, B.G. Schroeder, R. A. Slayden and Y. Yuan, **Mycolic acids: Structure, biosynthesis and physiological functions,** *Progress in lipid research*, 1998. **37**, 143-179.

32 P. J. Brennan and H. Nikaido, **The envelope of mycobacteria,** *Annu. Rev. Biochem.,* 1995, **64**, 29-63.

33 D. E. Minnikin, *The biology of mycobacteria; C. Ratledge, J. Stanford, Eds.; Academic Press, Inc.: San Diego,* 1982, 95-184.

34 M. Watanabe, Y. Aoyagi, H. Mitome, T. Fujita, H. Naoki, M. Ridell and D. E. Minnikin, **Location of functional groups in mycobacterial meromycolate chains; The recognition of new structural principles in mycolic acids,** *Microbiol.,* 2002, **148,** 1881-1902.

35 M. Watanabe, Y. Aoyagi, M. Ridell and D. E. Minnikin, **Separation and characterization of individual mycolic acids in representative mycobacteria,** *Microbiology,* 2001, **147**, 1825-1837.

36 W. R. Butler, D. G. Ahearn and J. O. Kilburn, **High–performance liquid chromatography of mycolic acids as a tool in the identification of *Corynebacterium, Nocardia, Rhodococcus,* and *Mycobacterium* species,** *J. Clin. Microbiol.,* 1986, **23,** 182-185.

37 D. E. Minnikin, I. G. Hutchinson, A. B. Caldicott and M. Goodfellow, **Thin layer chromatography of methanolysates of mycolic acid-containing bacteria,** *J. Chromatog. A.,* 1980, **188,** 221-233.

38 T. Kusaka and Y. Mori, **Pyrolysis gas chromatography-mass spectrometry of mycobacterial mycolic acid methyl esters and its application to the identification of *Mycobacterium lerpae*,** *J. Gen. Microbiol.,* 1986, **132,** 3403-3406.

39 G. O. Guerrant, M. A. Lambert and C. W. Moss, **Analysis of short-chain acids from anaerobic bacteria by high-performance liquid chromatography,** *J. Clin. Microbiol.,* 1982, **16,** 355-360.

40 S. E. Glickman, J. O. Kilburn, W. R. Butler and L. S. Ramos, **Rapid identification of mycolic acid patterns of mycobacteria by high-performance liquid chromatography using pattern recognition software and a Mycobacterium library,** *J. Clin. Microbiol.,* 1994, **32,** 740-745.

41 P. L. Valero-Giullén, F. Pacheco and F. Martín-Luengo, **Fatty acid composition and mycolic acid pattern of some chromogenic mycobacteria,** *J. Appl. Microbiol.,* 1985, **59,** 113-126.

42 D. E. Minnikin and N. Polgar, **Structural studies on the mycolic acids,** *Chem. Commun. (London),* 1967, **7,** 312-314.

43 D. E. Minnikin and N. Polgar, **The methoxymycolic and ketomycolic acids from human tubercle bacilli,** *Chem. Commun. (London),* 1967, **22,** 1172-1174.

44 D. E. Minnikin and N. Polgar, **Stereochemical studies on the mycolic acids,** *Chem. Commun. (London),* 1966, **18,** 648-649.

45 D. E. Minnikin and N. Polgar, **Studies on the mycolic acids from human tubercle bacilli,** *Tetrahedron Let.,* 1966, **7,** 2643-2647.

46 K. M. George, Y. Yuan, D. R. Serman, C. E. Barry and J. Liu, **The biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis* – Identification and functional analysis of CMAS-2,** *J. Biol. Chem.,* 1995, **270,** 27292-27298.

47 J. Pan, N. Fujiwara, S. Oka, R. Maekura, T. Ogura and I. Yano, **Anti-cord factor (trehalose 6,6'-dimycolate) IgG antibody in tuberculosis patients recognizes mycolic acid subclasses,** *Microbiol. Immunol.,*1999, **43,** 863-869.

48 D. W. Grogan and J. E. J. Cronan, **Characterization of *Escherichia coli* mutants completely defective in synthesis of cyclopropane fatty acids,** *J. Bacteriol.,* 1986, **166,** 872-877.

49 T. Hasegawa, S. Amino, S. Kitamura, M. Matsumoto, S. I. Katada and J. Nishijo, **Study of the molecular conformation of α- and keto-mycolic acid monolayers by the Langmuir-Blodgett technique and fourier transfer infrared reflection-absorption spectroscopy,** *Langmuir,* 2003, **19,** 105-109.

50 L. Wang, R. A. Slayden, C. E. Barry and J. Liu, **Cell wall structure of a mutant of *Mycobacterium smegmatis* defective in the biosynthesis of mycolic acids,** *J. Biol. Chem.,* 2000, **275,** 7224-7229.

51 Y. Yuan and C. E. Barry, **A common mechanism for the biosynthesis of methoxy and cyclopropyl mycolic acids in *Mycobacterium tuberculosis*,** *Proc. Natl. Acad. Sci. USA,* 1996, **93,** 12828-12833.

52 Y. Yuan, Y. Zhu, D. D. Crane and C. E. Barry, **The effect of oxygenated mycolic acid composition on cell wall function and macrophage growth in *Mycobacterium tuberculosis*,** *Molec. Microb.,* 1998, **29,** 1449-1458.

53 C. Asselineau and J. Asselineau, **Stereochemistry of the corynomycolic acids,** *Bull. Soc. Chim. France,* 1966, **6,** 1992-1999.

54 J. R. Al Dulayymi, M. S. Baird and R. Roberts, **The synthesis of a single enantiomer of a major α-mycolic acid of *Mycobacterium tuberculosis*,** *Chem. Commun.*, 2003, **2,** 228-229.

55 M. Muzael, G. Koza, J. R. Al Dulayymi and M. S. Baird, **The synthesis of a major α-mycolic acid of *Mycobacterium smegmatis*,** *Chem. Phys. Lipids,* 2010, **163,** 678-684.

56 R. Rowles, **The synthesis of mycolic acids,** *PhD thesis, Bangor University,* 2010.

57 G. Koza, C. Theunissen, J. R. Al Dulayymi and M. S. Baird, **The synthesis of single enantiomers of mycobacterial ketomycolic acids containing *cis*-cyclopropanes,** *Tetrahedron,* 2009, **65,** 10214-10229.

58 G. Koza and M. S. Baird, **The first synthesis of single enantiomers of ketomycolic acids,** *Tetrahedron letters,* 2007, **48,** 2165-2169.

59 J. R. Al Dulayymi, M. S. Baird, E. Roberts, M. Deysel and J. Verschoor, **The first syntheses of single enantiomers of the major methoxymycolic acid of *Mycobacterium tuberculosis*,** *Tetrahedron,* 2007, **63,** 2571-2592.

60 D. Z. Al Kremawi, J. R. Al Dulayymi and M. S. Baird, **The first synthesis of epoxy-mycolic acids,** *Tetrahedron letters,* 2010, **51,** 1698-1701.

61 J. E. Korf, G. Pynaert, K. Tournoy, T. Boonefaes, A. Van Oosterhout, D. Ginneberg, A. Haegeman, J. A. Verschoor, P. De Beatselier and J. Grooten, **Macrophage reprogramming by mycolic acid promotes a tolerogenic reponse in experimental asthma,** *Am. J. Respir. Crit. Care Med.,* 2007, **174,** 152-160.

62 S. V. Beken, J. R. Al Dulayymi, T. Naessens, C. Koza, M. Maza-Iglesias, R. Rowles, C. Theunissen, J. De Medts, E. Lanckacker, M. S. Baird and J. Grooten, **Molecular structure of the *Mycobacterium tuberculosis* virulence factor, mycolic acid, determines the elicited inflammatory pattern,** *Eur. J. Immunol.*, 2011, **41,** 450-460.

63 G. K. Schleicher, C. Feldman, Y. Vermaak and J. A. Verschoor, **Prevalence of anti-mycolic acid antibodies in patients with pulmonary tuberculosis co-infected with HIV,** *Clin. Chem. Lab. Med.,* 2002, **40,** 882-887.

64 S. T. Thanyani, V. Roberts, D. G. R. Siko, P. Vrey and J. A. Verschoor, **A novel application of affinity biosensor technology to detect antibodies to mycolic acid in tuberculosis patients,** *J. Immun. Methods,* 2008, **332,** 61-72.

65 H. Noll, H. Bloch, J. Asselineau and E. Lederer, **The chemical structure of the cord factor of *Mycobacterium tuberculosis,*** *Biochem. Biophys. Acta.,* 1956, **20,** 299-309.

66 R. Koch and K. Mitt, *Gesundheitsamt,* 1884, **2,** 1-88.

67 G. Middlebrook, R. J. Dubos and C. J. Pierce, **Virulence and morphological characteristics of mammalian tubercle bacilli,** *J. Exp. Med.,* 1947, **86,** 175-184.

68 H. Bloch, **Studies on the virulence of tubercle bacilli: Isolation and biological properties of a constituent of virulent organisms,** *J. Exp. Med.,* 1950, **91,** 197-218.

69 G. Middlebrook, C. M. Coleman and W. B. Schaefer, **Sulfolipid from virulent tubercle bacilli,** *Proc. Nat. Acad. Sci.,* 1959, **45,** 1801-1804.

70 E. Lederer, **Cord factor and related trehalose esters,** *Chem. Phys. Lipids,* 1976, **16,** 91-106.

71 M. Kato and J. Asselineau, **Relationship between chemical structure and biochemical activity of 6,6' dimycolate (cord factor) of *Mycobacterium tuberculosis*,** *Jpn. J. Med. Sci. Biol.,* 1975, **28,** 94-97.

72 M. Artman, A. Bekierkunst and I. Goldenberg, **Tissue metabolism in infection: Biochemical changes in mice treated with cord factor,** *Arch. Biochem. Biophys.,* 1964, **105,** 80-85.

73 R. Shankaran and T. A. Venkitasubramanian, **Effect of cord factor on carbohydrate metabolism in mice,** *Amer. Rev. Resp. Dis.,* 1970, **101,** 401-407.

74 H. S. R. Murthy, K. S. Kumar and T. A. Venkitasubramanian, **NAD- and NADP- specific dehydrogenases in cord-factor-treated mice,** *Biochim. Biophys. Acta.,* 1967, **146,** 584-586.

75 A. Beierkunst, I. S. Levij, E. Yarkoni, E. Vilkas, A. Adam and E. Lederer, **Granuloma formation induced in mice by chemically defined mycobacterial fractions,** *J. Bacteriol.,* 1969, **100,** 95-102.

76 A. Beierkunst, I. S. Levij, E. Yarkoni, E. Vilkas and E. Lederer, **Suppression of urethan-induced lung adenomas in mice treated with trehalose-6,6'-dimycolate (cord factor) and living BCG,** *Science,* 1971, **174,** 1240-1242.

77 R. Ryll, Y. Kumazawa and I. Yano, **Immunological properties of trehalose dimycolate (cord factor) and other mycolic acid-containing glycolipids – A review,** *Microbiol. Immunol.,* 2001, **45,** 801-811.

78 E. Ribi, E. L. Granger, K. C. Milner, K. Yamamoto, S.M. Strain and R. Parker, **Induction of resistance to tuberculosis in mice with defined components of mycobacteria and with some unrelated materials,** *Immunol.,* 1982, **46,** 297-305.

79 R. Saito, A. Tanka, K. Sugiyama, I. Azuma and Y. Yamamura, **Adjuvant effect of cord factor, a mycobacterial lipid,** *Infect. Immun.,* 1976, **13,** 776-781.

80 J. Ivanyi, **Serodiagnosis of tuberculosis: Due to shift track,** *Tuberculosis,* 2012, **92,** 31-37.

81 J. R. Al Dulayymi, M. S. Baird, M. Maza-Iglesias, S. V. Beken and J. Grooten, **The first unique synthetic mycobaterial cord factors,** *Tetrahedron letters,* 2009, **50,** 3702-3705.

82 A. K. Bello and C. H. Njoku, **Tuberculosis: Current trends in diagnosis and treatment,** *Niger. J. Clin. Pract.,* 2005, **8,** 118-124.

83 N. E. Dunlap, J. Bass, P. Fujiwara, P. Hopewell, C. R. Horsburgh, M. Salfinger and P. M. Simone, **Diagnostic standards and classification of Tuberculosis in adults and children,** *Am. J. Respir. Crit. Care Med.,* 2000, **4,** 1376-1395.

84 S. Pottumarthy, A. J. Morris, A. C. Harrison and V. C. Wells, **Evaluation of the tuberculin gamma interferon assay: Potential to replace the Mantoux skin test,** *J. Clin. Microbiol.,* 1999, **37,** 3229-3232.

85 R. L. Guerra, N. M. Hooper, J. F. Baker, R. Alborz, D. T. Armstrong, G. Maltas, J. A. Kiehlbauch and S. E. Dorman, **Use of the Amplified *Mycobacterium tuberculosis* Direct Test in a public health laboratory,** *Chest,* 2007, **132,** 946-951.

86 F. Vlaspolder, P. Singer and C. Roggeveen, **Diagnostic value of an amplification method (Gen-Probe) compared with that of culture for diagnosis of tuberculosis,** *J. Clin. Microbiol.,* 1995, **33,** 2699-2703.

87 P. Anargyos, D. S. J. Astill and I. S. L. Lim, **Comparison of improved BACTEC and Lawenstein-Jensen media for culture of mycobacteria from clinical specimen,** *J. Clin. Microbiol.,* 1990, **28,** 1288-1291.

88 H. J. Yeagar, J. Lacy, L. Smiths and C. LeMaistre, **Quantitative studies of mycobactrial populations in sputum and saliva,** *Am. Rev. Respir. Dis.,* 1967, **95,** 998-1004.

89 D. G. Altman and J. M. Bland, **Diagnostic tests 1: Sensitivity and specificity,** *BMJ,* 1994, **308,** 1152.

90 M. A. Morgan, C. D. Horstmeier, D. R. DeYoung and G. D. Roberts, **Comparison of a radiometric method (BACTEC) and conventional culture media for recovery of mycobacteria from smear-negative specimens,** *J. Clin. Microbiol.,* 1983, **18,** 384-388.

91 S. Ichiyama, K. Shimokataa and J. Takeuchi, **Comparative study of a biphasic culture system (Roche MB check system) with a conventional egg medium for recovery of mycobacteria,** *Tuberc. Lung Dis.,* 1993, **74,** 338-341.

92 C. Dye, S. Scheele, P. Dolin, V. Pathania and M. C. Raviglione, **Consensus statement. Global burden of tuberculosis: Estimated incidence, prevalence, and mortality by country,** *JAMA,* 1999, **282,** 677-686.

93 K. R. Steingart, V. Ng, M. Henry, P. C. Hopewell, A. Ramsay, J. Cunningham, R. Urbanczik, M. D. Perkins, M. A. Aziz and M. Pai, **Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: A systematic review,** *Lancet Ingect. Dis.,* 2006, **6,** 664-674.

94 M. P. Golden and H. R. Vikram, **Extrapulomonary tuberculosis: An overview,** *Am. Fam. Physician.,* 2005, **72,** 1761-1786.

95 R. Urbanczik, **Present position of microscopy and culture in diagnostic Mycobacteriology,** *Zbl. Bakt. Hyg [A],* 1985, **260,** 81-87.

96 M. A. Behr, S. A. Warren, H. Salmon, P. C. Hopewell, A. Ponce de Leon, C. L. Daley and P. M. Small, **Transmission of *Mycobacterium tuberculosis* from patients smear-negative for acid-fast bacilli,** *Lancet,* 1999, **353,** 444-449.

97 A. M. Elliott, K. Namaabo, B. W. Allen, N. Luo, R. J. Hayes, J. O. Pobee and K. P. McAdam, **Negative sputum smear results in HIV-positive patients with pulmonary tuberculosis in Lusaka, Zambia,** *Tuber. Lung Dis.,* 1993, **74,** 191-194.

98 J. L. Johnson, M. J. Vjecha, A. Okwera, E. Hatanga, F. Byekwaso, K. Wolski, T. Aisu, C. C. Whalen, R. Huebner, R. D. Mugerwa and J. J. Ellner, **Impact of human immunodeficiency virus type-1 infection on the initial bacteriologic and radiographic manifestations of pulmonary tuberculosis in Uganda,** *Int. J. Tuberc. Lung Dis.,* 1998, **2,** 397-404.

99 L. Materson, I. Srouki, R. Kent and A. P. Bath, **Nasal tuberculosis – An update of current clinical and laboratory investigation,** *J. Laryngol. Otol.,* 2011, **125,** 210-213.

100 F. R. Hauk, B. H. Neese, A. S. Panchal and W. El-Amin, **Identification and management of latent tuberculosis infection,** *Am. Fam. Physician.,* 2009, **79,** 879-886.

101 M. Pai, A. Zerling and D. Menzies, **Systematic review: T-Cell-based assays for the diagnosis of latent tuberculosis infection: An update,** *Ann. Intern. Med.,* 2008, **149,** 177-184.

102 S. D. Chaparas, H. M. Vandiviere, I. Melvin, G. Koch and C. Becker, **Variability with the Mantoux procedure,** *Am. Rev. Respir. Dis.,* 1985, **132,** 175-177.

103 L. B. Reichman, **A scandalous incompetence...continued,** *Chest,* 1998, **113,** 1153-1154.

104 L. Wang, M. O. Turner, R. K. Elwood, M. Schulzer and J. M. Fitzgerald, **A meta-analysis of the effect of Bacille Calmatte Guerin vaccination on tuberculin skin test measurement,** *Thorax,* 2002, **57,** 804-809.

105 R. Menzies and B. Vissandjee, **Effect of bacilli Calmette-Guerin vaccination on tuberculin reactivity,** *Am. Rev. Respir. Dis.,* 1992, **145,** 621-625.

106 D. Menzies, **Interpretation of repeated tuberculin tests – Boosting, conversion and reversion,** *Am. J. Respir. Crit. Care. Med.,* 1999, **159,** 15-21.

107 Y. Kobashi, K. Mouri, S. Yagi, Y. Obase, M. Fukuda, N. Miyashita and M. Oka, **Usefulness of the QuantiFERON TB-2G test for the differential diagnosis of pulmonary tuberculosis,** *Intern. Med.,* 2008, **47,** 237-243.

108 A. K. Detjen, Y. Keil, S. Roll, B. Hauer, H. Mauch, U. Wahn and K. Magdorf, **Interferon-gamma release assays improve the diagnosis of tuberculosis and nontuberculous mycobacterial disease in children in a country with low incidence of tuberculosis,** *Clin. Infect. Dis.,* 2007, **45,** 322-328.

109 I. Brock, K. Weldingh, E. M. Leyten, S. M. Arend, P. Ravn and P. Andersen, **Specific T-cell epitopes for immunoassay-based diagnosis of *Mycobacterium tuberculosis* infection,** *J. Clin. Microbiol.,* 2004, **42,** 2379-2387.

110 T. Mori, M. Sakatani, F. Yamagishi, Y. Kawabe, K. Nagao, E. Shigeto, N. Harada, S. Mitarai, M. Okada, K. Suzuki, Y. Inoue, K. Tsuyuguchi, Y. Sasaki, G. H. Mazurek and I. Tsuyuguchi, **Specific detection of tuberculosis infection: An interferon-gamma-based assay using new antigens,** *Am. J. Respir. Crit. Care. Med.,* 2004, **170,** 59-64.

111 J. Y. Lee, H. J. Choi, I. N. Park, S. B. Hong, Y. M. Oh, C. M. Lim, S. D. Lee, Y. Koh, W. S. Kim, D. S. Kim, W. D. Kim and T. S. Shim, **Comparison of two commercial interferon-gamma assays for diagnosing *Mycobacterium tuberculosis* infection,** *Eur. Respir. J.,* 2006, **28,** 24-30.

112 R. L. V. Skjot, T. Oettinger, I. Rosenkrands, P. Ravn, I. Rock, S. Jacobsen and P. Andersen, **Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens,** *Infect. Immun.,* 2000, **68,** 214-220.

113 K. Dheda, R. van Zyl Smit, M. Badri and M. Pai, **T-cell interferon-[gamma] release assays for the rapid immunodiagnosis of tuberculosis: Clinical utility in high-burden vs. low-burden settings,** *Curr. Opin. Pulm. Med.,* 2009, **15,** 188-200.

114 Y. A. Kang, H. W. Lee, H. I. Yoon, B. Cho and S. K. Han, **Discrepancy between the tuberculin skin test and the whole-blood interferon γ assay for the diagnosis of latent tuberculosis infection in an intermediate tuberculosis-burden country,** *JAMA,* 2005, **293,** 2756-2761.

115 G. H. Mauzurek, J. Jereb, P. LoBue, M. F. Iademarco, B. Metchock and A. Vernon, **Guidlines for using the QuantiFERON-TB Gold Test for detecting *Mycobacterium tuberculosis* infection, United States,** *CDC MMWR,* 2005, **54,** 49-55.

116 G. Ferrara, M. Losi, M. Meacci, B. Meccugni, R. Pirlo, P. Roversi, B. M. Bergamini, R. D'Amico, P. Marchegiano, F. Rumpianesi, L. M. Fabbri and L. Richeldi, **Routine hospital use of a new commercial whole blood interferon-gamma assay for the diagnosis of tuberculosis infection,** *Am. J. Respir. Crit. Care. Med.,* 2005, **172,** 631-635.

117 I. Brock, M. E. Munk, A. Kok-Jensen and P. Andersen, **Performance of the whole blood IFN-gamma test for tuberculosis diagnosis based on PPD or the specific antigens ESAT-6 and CFP-10,** *Int. J. Tuberc. Lung Dis.,* 2001, **5,** 462-467.

118 A. Soysal, T, Torun, S. Efe, H. Gencer, K. Tahaoglu and M. Bakir, **Evaluation of cut-off values of interferon-gamma-based assays in the diagnosis of *M. tuberculosis* infection,** *Int. J. Tuberc., Lung Dis.,* 2008, **12,** 50-56.

119 C. Jafari, M. Ernst, B. Kalsdorf, U. Greinert, R. Diel, D. Kirsten, K. Marienfield, A. Lalvani and C. Lange, **Rapid diagnosis of smear-negative tuberculosis by bronchoalveolar lavage enzyme-linked immunospot,** *Am. J. Respir. Crit. Care Med.,* 2006, **174,** 1048-1054.

120 Gen-Probe, **Amplified *Mycobacterium tuberculosis* Direct Test for *in vitro* diagnostic use,** available at http://www.gen-probe.com/pdfs/pi/IN0014.pdf.

121 American Thoracic Society – Conference Report, **Rapid diagnostics test for tuberculosis: what is appropriate use?** *Am. J. Respir. Crit. Care Med.,* 1997, **155,** 1804-1814.

122 A. Sloutsky, L. L. Han and B. G. Werner, **New method for the detection of *Mycobacterium tuberculosis* direct test inhibitors in clinical specimens,** *Diag. Microbiol. Infect. Dis.,* 2004, **50,** 109-111.

123 E. A. Mothershed and A. M. Whitney, **Nucleic acid-based methods for the detection of bacterial pathogens: Present and future considerations for the clinical laboratory,** *Clin. Chim. Acta,* 2006, **363,** 206-220.

124 J. Dinnes, J. Deeks, H. Kunst, A. Gibson, E. Cummins, N. Waugh, F. Drobniewski and A. Lalvani, **A systematic review of rapid diagnostic tests for the detection of tuberculosis infection,** *Health Tech. Assess.,* 2007, **11,** 1-178.

125 P. Chedore and F. B. Jamieson, **Routine use of the Gen-Probe MTD2 amplification test for detection of *Mycobacterium tuberculosis* in clinical specimens in a large public health mycobacteriology laboratory,** *Diagn. Microbiol. Infect. Dis.,* 1999, **35,** 185-191.

126 C. E. O'Sullivan, D. R. Miller, P. S. Schneider and G. D. Roberts, **Evaluation of Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test by using respiratory and nonrespiratory specimens in a tertiary care center laboratory,** *J. Clin. Microbiol.,* 2002, **40,** 1723-1727.

127 M. B. Smith, J. S. Bergmann, M. Onoroto, G. Mathews and G. L. Woods, **Evaluation of Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test by using respiratory and nonrespiratory specimens in a tertiary care center laboratory,** *Arch. Pathol. Lab. Med.,* 1999, **123,** 1101-1103.

128 N. Somonney, H. Labrousse, T. Ternynch and P. H. Lagrange, **Recycling of ELISA plates for the serological diagnosis of tuberculosis using a *Mycobacterium tuberculosis*-specific glycolipid antigen,** *J. Immunol. Methods.,* 1996, **199,** 101-105.

129 E. Nassau, E. R. Parsons and G. D. Johnson, **The detection of antibodies to *Mycobacterium tuberculosis* by microplate enzyme-linked immunosorbent assay,** *Tubercle,* 1976, **57,** 67-70.

130 R. M. Lequin, **Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA),** *Clin. Chem.,* 2005, **51,** 2415-2418.

131 K. Csorba, S. Schmidt, F. Florea, N. Ishii, T. Hashimoto, M. Hertl, S. Karpati, L. Bruckner-Tuderman, W. Nishie and C. Sitaru, **Development of an ELISA for sensitive and specific detection of IgA autoantibodies against BP180 in pemphigoid diseases,** *Orpha. J. Rare Dis.,* 2011, **6,** 31-40.

132 D. E. Mosedale, M. S. Sandhu, J. Luan, M. Goodall and D. J. Grainger, **A new sensitive and specific enzyme-linked immunosorbent assay for IgD,** *J. Immunol. Methods,* 2006, **313,** 74-80.

133 T. Okayama, Y. Matsuno, N. Yasuda, T. Tsukui, Y. Suzuta, M. Koyanaai, M. Sakaguchi, Y. Ishii, T. Olivry and K. Masuda, **Establishment of a quantitative ELISA for the measurement of allergen-specific IgE in dogs using anti-IgE antibody cross-reactive to mouse and dog IgE,** *Vet. Immunol. Immunopath.,* 2011, **139,** 99-106.

134 B. Wang, L. J. Hak, M. V. Relling, C. H. Pui, M. H. Woo and M. C. Storm, **ELISA to evaluate plasma anti-asparaginase IgG concentrations in patients with acute lymphoblastic leukemia,** *J. Immunol. Methods,* 2000, **239,** 75-83.

135 M. K. Sharief and E. J. Thompson, **A sensitive ELISA system for the rapid detection of virus specific IgM antibodies in the cerebrospinal fluid,** *J. Immunol. Methods,* 1990, **130,** 19-24.

136 PHO Technical Report: Update on Lyme Disease Prevention and Control February 2012.

137 WHO, **Commercial serodiagnostic tests for diagnosis of tuberculosis: Policy statement,** 2011.

138 D. W. Dowdy, K. R. Steingart and M. Pai, **Serological testing versus other strategies for diagnosis of active tuberculosis in India: A cost-effectiveness analysis,** *PLoS Med.,* 2011, **8,** e1001074.

139 J. Grenier, L. Pinto, D. Nair, K. Steingart, D. Dowdy, A. Ramsay and M. Pai, **Widespread use of serological tests for tuberculosis: data from 22 high-burden countries,** *Eur. Respir. J.,* 2011, **39,** 502-505.

140 Qualisa TB IgG, **Enzyme linked immunosorbent assay (ELISA) for the detection of IgG antibodies to *Mycobacterium tuberculosis* in human serum, plasma and other body fluids.**

141 Serocheck-MTB, **Rapid test for detection of antibodies to *Mycobacterium tuberculosis*.**

142 M. Beukes, Y. Lemmer, M. Deysel, J. R. Al Dulayymi, M. S. Baird, G. Koza, M. M. Iglesias, R. R. Rowles, C. Theunissen, J. Grooten, G. Toschi, V. V. Roberts, L. Pilcher, S. Van Wyngaardt, N. Mathebula, M. Balogun, A. C. Stoltz and J. A. Verschoor, **Structure-function relationships of the antigenicity of mycolic acids in tuberculosis patients,** *Chem. Phys. Lip.*, 2010, **163,** 800-808.

143 L. B. Silva, B. Veigas, G. Dorian, P. Costa, J Inacio, R. Martins, E. Fortunato and P. V. Baptista, **Portable optoelectronic biosensing platform for identification of mycobacteria from the *Mycobacterium tuberculosis* complex,** *Biosens. Bioelectron.*, 2011, **26,** 2012-2017.

144 N. S. Mathebulla, J. Pillay, G. Toschi, J. A. Verschoor and K. I. Ozoemena, **Recognition of anti-mycolic acid antibody at self-assembled mycolic acid antigens on a gold electrode: A potential impedimetric immunosensing platform for active tuberculosis,** *Chem. Commun. (Camb.),* 2009, **23,** 3345-3347.

145 R. Karlsson, A. Michaelsson and L. Mattsson, **Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system,** *J. Immunol. Methods,* 1991, **145,** 229-240.

146 P. E. Buckle, R. J. Davies, T. Kinning, D. Yeung, P. R. Edwards and D. Pollard-Knight, **The resonant mirror: A novel optical sensor for direct sensing of biomolecular interactions part II: Applications,** *Biosens. Bioelectron.*, 1993, **8,** 355-368.

147 R. Cush, J. M. Cronin, W. J. Stewart, C. H. Maule, J. Molloy and N. J. Goddard, **The resonant mirror: A novel optical biosensor for direct sensing of biomolecular interactions Part I: Principle of operation and associated instrumentation,** *Biosens. Bioelectron.*, 1993, **8,** 347-354.

148 K. I. Ozoemena, N. S. Mathebula, J. Pillay, G. Toschi and J. A. Verschoor, **Electron transfer dynamics across self-assembled *N*-(2-mercaptoethyl) octadecanamide/mycolic acid layers: Impedimetric insights into the structural integrity and interaction with anti-mycolic acid antibodies,** *Phys. Chem. Chem. Phys.*, 2010, **12,** 345-357.

149 R. G. Nuzzo and G. L. Allara, **Adsorption of bifunctional organic disulfides on gold surfaces,** *J. Am. Chem. Soc.*, 1983, **105,** 4481-4483.

150 M. Bourg, A. Badia and R. B. Lennox, **Gold-sulfur bonding in 2D and 3D self-assembled monolayers: XPS characterization,** *J. Phys. Chem. B,* 2000, **104,** 6562-6567.

151 C. D. Bain, H. A. Biebuyck and G. M. Whitesides, **Comparison of self-assembled monolayers on gold: Coadsorption of thiols and disulphides,** *Langmuir,* 1989, **5,** 723-727.

152 D. E. Weisshaar, M. M. Walczak and M. D. Porter, **Electrochemically induced transformations of monolayers formed by self-assembly of mercaptoethanol at gold,** *Langmuir,* 1993, **9,** 323-329.

153 G. D. Castner, K. Hinds and D. W. Grainger, **X-ray photoelectron spectroscopy sulfur 2p study of organic thiol and disulfide binding interactions with gold surfaces,** *Langmuir,* 1996, **12,** 5083-5086.

154 H. Riley, N. Price, T. L. Smith and S. Yang, **Photo-oxidation and photo-reduction in alkylthiol monolayers self-assembled on gold,** *J. Chem. Soc., Faraday Trans.*, 1996, **92,** 3629-3634.

155 M. M. Walczak, C. A. Alves, B. D. Lamp and M. D. Porter, **Electrochemical and X-ray photoelectron spectroscopic evidence for differences in the binding sites of alkanethiolate monolayers chemisorbed at gold,** *J. Electroanal. Chem.,* 1995, **238,** 308-312.

156 C. Zubragel, C. Deuper, F. Schneider, M. Neumann, M. Grunze, A. Schertel and C. Woll, **The presence of two different sulfur species in self-assembled films of *n*-alkanethiols on Au and Ag surfaces,** *Chem. Phys. Lett.,* 1995, **238,** 308-312.

157 M. J. Hostetler, J. E. Wingate, C. Zhong, J. E. Harris, R. W. Vachet, M. R. Clark, J. D. Londono, S. J. Green, J. J. Stokes, G. D. Wignall, G. L. Glish, M. D. Porter, N. D. Evans and R. W. Murray, **Alkanethiolate gold cluster molecules with core diameters from 1.5 to 5.2 nm: Core and monolayer properties as a function of core size,** *Langmuir,* 1998, **14,** 17-30.

158 H. Sellers, A. Ulman, Y. Shindman and J. E. Eilers, **Structure and binding of alkanethiolates on gold and silver surfaces: Implications for self-assembled monolayers,** *J. Am. Chem. Soc.,* 1993, **115,** 9389-9401.

159 C. Zhong, J. Zak and M. D. Porter, **Voltammetric reductive desorption characteristics of alkanethiolate monolayers at single crystal Au(111) and (110) electrode surfaces,** *J. Electroanal. Chem.,* 1997, **42,** 9-13.

160 H. Cai, C. Xu, P. He and Y. Fang, **Colloid Au-enhanced DNA immobilization for the electrochemical detection of sequence-specific DNA,** *J. Electroanal. Chem.,* 2001, **510,** 78-85.

161 A. P. Alivisatos, K. P. Johnsson, X. Peng, T. E. Wilson, C. J. Loweth, M. P. Bruchez and P. G. Schultz, **Organization of 'nanocrystal molecules' using DNA,** *Nature,* **382,** 609-611.

162 R. Elghanian, J. J. Stornhoff, R. C. Mucic, C. A. Mirkin and R. L. Letsinger, **Selective colorimetric detection of polynucleotides based on the distance-dependant optical properties of gold nanoparticles,** *Science,* 1997, **277,** 1078-1081.

163 J. J. Storhoff, R. C. Mucic and C. A. Mirkin, **Strategies for organizing nanoparticles into aggregate structures and functional materials,** *J. Clust. Sci.,* 1997, **8,** 179-216.

164 C. A. Mirkin, R. L. Letsinger, R. C. Mucic and J. J. Storhoff, **A DNA-based method for rationally assembling nanoparticles into macroscopic materials,** *Nature,* 1996, **382,** 607-609.

165 J. J. Stornhoff and C. A. Mirkin, **Programmed materials synthesis with DNA,** *Chem. Rev.,* 1999, **99,** 1849-1862.

166 L. A. Porter, D. Ji, S. L. Westcott, M. Graupe, R. S. Czernuszewicz, N. J. Halas and T. R. Lee, **Gold and silver nanoparticles functionalized by the adsorption of dialkyl disulfides,** *Langmuir,* 1998, **14,** 7378-7386.

167 X.-M. Li, M. R. De Jong, K. Inoue, S. Shinkai, J. Huskens and D. N. Reinhoudt, **Formations of gold colloids using thioether derivatives as stabilizing ligands,** *J. Mater. Chem.,* 2001, **11,** 1919-1923.

168 X. Luo, A. Morrin, A. J. Killard and M. R. Smyth, **Application of nanoparticles in electrochemical sensors and biosensors,** *Electroanal.,* 2006, **4,** 319-326.

169 M. O. Balogun, E. Huws, M. M. Sirhan, A. D. Saleh, J. R. Al Dulayymi, L. Pilcher, J. A. Verschoor and M. S. Baird, **Thiol modified mycolic acids,** *Chem. Phys. Lipids,* 2013 (In Press).

170 M.-C. Daniel and D. Astruc, **Gold nanoparticles: Assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology,** *Chem. Rev.,* 2004, **104,** 293-346.

171 M. Faraday, **The Bakerian lecture: Experimental relations of gold (and other metals) to light,** *Philos. Trans.*, 1857, **147,** 145-181.
172 J. Turkevitch, P. C. Stevenson and J. Hillier, **A study of the nucleation and growth process in the synthesis of colloidal gold,** *Discuss. Faraday Soc.*, 1951, **11,** 55-75.
173 J. Kimling, M. Maier, B. Okenve, V. Kotaidis, H. Ballot and A. Plech, **Turkevich method for gold nanoparticle synthesis revisited,** *J. Phys. Chem. B,* 2006, **110,** 15700-15707.
174 G. Frens, **Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions,** *Nature: Phys. Sci.*, 1973, **241,** 20-22.
175 M. Brust, M. Walker, D. Bethell, D. J. Schiffrin and R. Whyman, **Synthesis of thiol-derivatised gold nanoparticles in a two-phase liquid-liquid system,** *J. Chem. Soc., Chem. Commun.*, 1994, **7,** 801-802.
176 P. D. Perrault and W. C. W. Chan, **Synthesis and surface modification of highly monodispersed, spherical gold nanoparticles of 50 – 200 nm,** *J. Am. Chem. Soc.*, 2009, **131,** 17042-17043.
177 K. D. Brown, D. G. Walter and M. J. Natan, **Seeding of colloidal Au nanoparticle solutions. 2. Improved control of particle size and shape,** *Chem. Matter.*, 2000, **12,** 306-313.
178 E. Gachard, H. Remita, J. Khatouri, B. Keita, L. Nadko and J. Belloni, **Radiation-induced and chemical formation of gold clusters,** *New J. Chem.*, 1998, **22,** 1257-1265.
179 Y. Zhuo, R. Yuan, Y. Chai, D. Tang, Y. Zhang, N. Wang, X. Li and Q. Zhu, **A reagentless amperometric immunosensor based on gold nanoparticles/thionine/Nafion-membrane-modified gold electrode for determination of α-1-fetoprotein,** *Electrochem. Commun.*, 2005, **7,** 355-360.
180 P. A. Fiorito, V. R. Goncales, E. A. Ponzio and S. I. Córdoba de Torresi, **Synthesis, characterization and immobilization of Prussian blue nanoparticles. A potential tool for biosensing devices,** *Chem. Commun.*, 2005, **3,** 366-368.
181 H. Cai, N. Zhu, Y. Jiang, P. He and Y. Fang, **Cu@Au alloy nanoparticle as oligonucleotide labels for electrochemical stripping detection of DNA hybridization,** *Biochem. Bioelecton.*, 2003, **18,** 1311-1319.
182 S. Link and M. A. El-Sayed, **Size and temperature dependence of the plasmon absorption of colloidal gold nanoparticles,** *J. Phys. Chem. B,* 1999, **103,** 4212-4217.
183 K. L. Kelly, E. Coronado, L. L. Zhao and G. C. Schatz, **The optical properties of metal nanoparticles: The influence of size, shape, and dielectric environment,** *J. Phys. Chem. B,* 2003, **107,** 668-677.
184 H. E. Toma, V. M. Zamarion, S. H. Toma and K. Araki, **The coordination chemistry at gold nanoparticles,** *J. Braz. Chem. Soc.*, **21,** 1158-1176.
185 J. W. M. Chon, C. Bullen, P. Zijlstra and M. Gu, **Spectral encoding on gold nanorods doped in silica sol-gel matrix and its application to high-density optical data storage,** *Adv. Funct. Matter.*, 2007, **17,** 875-880.
186 T, Ung, L. M. Liz-Marzan and P. Mulvaney, **Gold nanoparticle thin films,** *Colloids Suf. A: Physicochem. Eng. Asp.*, 2002, **202,** 119-126.
187 N. L. Swanson and B. D. Billard, **Optimization of extinction from surface plasmon resonances of gold nanoparticles,** *Nanotechnology,* 2003, **14,** 353-357.
188 B. Yan, Y. Yang and Y. Wang, **Comments on "Simulation of the optical absorption spectra of gold nanorods as a function of their aspect ratio and the effect of the medium dielectric constant",** *J. Phys. Chem.*, 2003, **107,** 9159.
189 T.-L. Chang, C.-Y. Tsai, C.-C. Sun, R. Uppala, C.-C. Chen, C.-H. Lin and P.-H. Chen, **Electrical detection of DNA using gold and magnetic nanoparticles and bio bar-code DNA between nanogap electrodes,** *Microelectron. Eng.*, 2006, **83,** 1630-1633.

190 S.-H. Chen, V. C. H. Wu, Y.-C. Chuang and C.-S. Lin, **Using oligonucleotide-functionalized Au nanoparticles to rapidly detect foodborne pathogens on a piezoelectric biosensor,** *J. Microbiol. Methods,* 2008, **73,** 7-17.

191 Y.-T. Chen, C.-L. Hsu and S.-Y. Hou, **Detection of single-nucleotide polymorphisms using gold nanoparticles and single-strand-specific nucleases,** *Anal. Biochem.,* 2008, **375,** 299-305.

192 E. Liandris, M. Gazouli, M. Andreadou, M. Čomor, N. Abazovic, L. A. Sechi and J. Ikonomopoulos, **Direct detection of unamplified DNA from pathogenic mycobateria using DNA derivatized gold nanoparticles,** *J. Microbiol. Methods,* 2009, **78,** 260-264.

193 Z.-S. Wu, J.-H. Jiang, L. Fu, G.-L. Shen and R.-Q. Yu, **Optical detection of DNA hybridization based on fluorescence quenching of tagged oligonuvleotide probes by gold nanoparticles,** *Anal. Biochem.,* 2006, **353,** 22-29.

194 L. Brus, **Noble metal nanocrystals: Plasmon transfer photochemistry and single-raman spectroscopy,** *Acc. Chem. Res.,* 2008, **41,** 1742-1749.

195 A. N. Shipway, M. Lahav, R. Gabai and I. Willner, **Investigations into the electrostatically induced aggregation of Au nanoparticles,** *Langmuir,* 2000, **16,** 8789-8795.

196 P. Costa, A. Amaro, A. Botelho, J. Inácio and P. V. Baptista, **Gold nanoprobe assay for the identification of mycobacteria of the *Mycobacterium tuberculosis* complex,** *Clin. Microbiol. Infect.,* 2010, **16,** 1464-1469.

Chapter 2

Materials and Methods

2.1 General Considerations

All chemicals used were purchased from commercial suppliers. All reagents and solvents used were of analytical grade unless otherwise stated. Pipette tips and Eppendorf tubes were sterilised prior to use. Assays were carried out on 96-well, flat-bottomed polystyrene micro-plates, purchased from Fishcher Scientific (product code – FB56416), which were untreated prior to use to allow for medium binding and were purchased sterile and used immediately upon opening. Gold nanoparticle solutions were obtained from Aldrich or BBInternational, with all solutions containing 0.01 % Au and a mean gold nanoparticle diameter of 20 nm. Gold nanoparticle solutions that were coated with antigens were stored at 4 °C until used. Human serum samples were obtained from the World Health Organisation (WHO) and from the School of Environment, Natural Resources and Geography, Bangor University. All of the serum samples used had been irradiated prior to use. Washing of the 96-well micro-plates was carried out using a Labtech LT-3500 Plate Washer and absorbance measurements were obtained on a Labtech LT-4000 Plate Reader. Centrifugation was carried out in an Eppendorf Centrifuge 5415D. Cyclic voltammetry was carried out using Autolab PGSTAT30 and analysed using GPES software. UV-visible measurements were carried out using a JASCO V-550 UV/VIS Spectrophotometer. The chemical structures, as well as the origins, of all of the antigens used can be found in the Appendix, Section 9.1.1.

2.2 Solutions

20x Phosphate buffered saline (PBS) stock solution: 20x PBS stock solution was prepared by dissolving NaCl (160 g), KCl (4 g), KH_2PO_4 (4 g) and Na_2HPO_4 (23 g) in ultra-pure double distilled de-ionised water (ddd H_2O) (900 ml). The solution was made up to a final volume of 1 L using dddH_2O and filtered through a 0.22 µl membrane filter.

0.5 % Casein/PBS buffer: 20x PBS stock solution (50 ml) was added to ddd H_2O (700 ml) in a litre flask. Casein (5 g, from bovine milk, carbohydrate and fatty acid free) was added to this solution and dissolved by stirring at 37 °C for 2 hours. The solution was stored overnight at 4 °C, and the pH adjusted to 7.4 on the following day using 1 M NaOH before being made up to a final volume of 1 L using ddd H_2O.

Secondary antibody conjugate: See Section 2.2.1. All solutions were prepared 5 minutes prior to use.

0.1 M Citrate buffer: Citric acid (0.1 M, 450 ml) was added to a tri-sodium citrate solution (0.1 M, 450 ml) until the pH was 4.5. The volume was then made up to 1 L using ddd H_2O.

o-Phenylenediamine dihydrochloride (OPD) substrate: OPD (10 mg) and crushed pellets of H_2O_2 urea adducts (35 % H_2O_2, 8 mg) was added to 0.1 M citrate buffer (10 ml). The substrate was prepared 5 minutes prior to use.

1 M Phosphate buffer (pH 7.4): K_2HPO_4 (1 M, 80.2 ml) and KH_2PO_4 (1 M, 19.8 ml) were mixed and the pH was checked to be 7.4.

10 mM Phosphate buffer (pH 7.4): 1 M Phosphate buffer (pH 7.4, 1 ml) was made to a total volume of 100 ml using dddH_2O.

2.2.1 Secondary Antibody Conjugates

Anti-human IgG (whole molecule) secondary antibody conjugate: A 1 in 1000 dilution of anti-human IgG (whole molecule) secondary antibody conjugate was prepared by dissolving Anti-human IgG (whole molecule)-peroxidase antibody produced in goat (Sigma) (10 µl) in 0.5 % Casein/PBS buffer (9.99 ml).

Anti-human IgG (Fc specific) secondary antibody conjugate: A 1 in 1000 dilution of anti-human IgG (Fc specific) secondary antibody conjugate was prepared by dissolving Anti-human IgG (Fc specific)-peroxidase antibody produced in goat (Sigma) (10 µl) in 0.5 % Casein/PBS buffer (19.99 ml).

Anti-human IgG (γ-chain specific) secondary antibody conjugate: A 1 in 333 dilution of anti-human IgG (γ-chain specific) secondary antibody conjugate was prepared by dissolving Anti-human IgG (γ-chain specific)-peroxidase antibody produced in goat (Sigma) (30 µl) in 0.5 % Casein/PBS buffer (9.97 ml).

Anti-human IgA (α-chain specific) secondary antibody conjugate: A 1 in 1667 dilution of anti-human IgA (α-chain specific) secondary antibody conjugate was prepared by dissolving Anti-human IgA (α-chain specific)-peroxidase antibody produced in goat (Sigma) (10 µl) in 0.5 % Casein/PBS buffer (16.66 ml).

Anti-human IgM (µ-chain specific) secondary antibody conjugate: A 1 in 333 dilution of anti-human IgM (µ-chain specific) secondary antibody conjugate was prepared by

dissolving Anti-human IgM (μ-chain specific)-peroxidase antibody produced in goat (Sigma) (30 μl) in 0.5 % Casein/PBS buffer (9.97 ml).

Anti-human IgG1 (γ-chain) secondary antibody conjugate: A 1 in 1000 dilution of anti-human IgG1 (γ-chain) secondary antibody conjugate was prepared by dissolving Mouse monoclonal [4E3] secondary antibody to human IgG1 – gamma chain (HRP) conjugate (Abcam) (10 μl) in 0.5 % Casein/PBS buffer (9.99 ml).

2.3 Cyclic Voltammetry[1]

A saturated calomel electrode was used as a reference electrode and a platinum electrode as the counter electrode. The working electrode was the gold-coated slides coated with antigen. Prior to use, the cell was cleaned using a 50:50 mixture of concentrated H_2SO_4:HNO_3 followed by rinsing in $dddH_2O$ (nominal resistivity > 18 MΩ cm at 25 °C), cleaning in a steam bath, and drying in the oven. A seal was made between the working electrode and the electrolyte solution with an o-ring defining a geometric area of 0.6 cm^2. The electrolyte solution in the cell was 0.1 M NaOH unless otherwise stated, and all electrolyte solutions were de-gassed prior to use. Prior to forming a layer of modified antigens on the gold-coated slides, the slides were treated to produce a flat surface with strong Au (111) character.[2] The slides were flame annealed using a Bunsen burner until they achieved red heat three times with air cooling between each annealing stage. After the final annealing stage, the slides were allowed to cool in air for a short time before being quenched in $dddH_2O$.

The gold-coated slides were modified with antigen by submerging the slides in a solution of the antigen under investigation dissolved in a suitable solvent, and left for 72 hours. After formation of the modified antigen layer on the gold-coated slides, they were washed numerous times with $dddH_2O$ prior to use.

The potential difference between the electrodes was swept from 0 V to -1.3 V and back to 0 V, and this was repeated twice, at a scan rate of 50 mV/s.

2.4 Coating of the Gold Nanoparticles

Coating with Thiol Modified Antigen

To a glass bottle, 10 mM phosphate buffer (pH 7.4, 4 ml), gold nanoparticles (0.01 % Au, 4 ml) and thiol modified antigen (5 μM solution in hexane, 1 ml) were added and left on a shaker for 16 hours.

The volumes of the above solutions can be varied, provided that the ratio is kept as 4:4:1.

Coating with Non-Thiol Modified Antigen

To a glass bottle, 10 mM phosphate buffer (pH 7.4, 4 ml), gold nanoparticles (0.01 % Au, 4 ml) and thio-stearic acid (5 μM solution in hexane, 1 ml) were added and left on a shaker for 24 hours. After this time, antigen (5 μM solution in hexane, 1 ml) was added and the solution was left on the shaker for a further 16 hours.

In later experiments, it was discovered that octadecanethiol (ODT) could perform the same function as thio-stearic acid, so this procedure could be modified to contain ODT as a direct replacement for thio-stearic acid.

2.5 The Rate of Binding of Antibodies to the Coated Gold Nanoparticles

The development of the assay described in this section is described in Chapter 3.

900 μl from the aqueous layer of the solutions described in Section 2.4 was transferred to a cuvette and serum (100 μl) was added. The absorbance of the solution was then measured from 200 – 800 nm continuously over 30 minutes (1 scan per 100 s).

In instances where the assay was performed on 96-well plates, 180 μl from the aqueous layer of the solutions described in Section 2.4 was transferred to a 96-well micro-plate and serum (20 μl) was added. The absorbance of the solution was then measured at 540 nm at 2 minute intervals for a period of 30 minutes.

2.6 Gold Nanoparticle Assay with Induction of Colour Change

The development of the assays described in this section are discussed in detail in Chapters 4 and 5, and the following procedures were used to analyse serum samples with these assays unless otherwise stated.

2.6.1 Protocol when the Assay was Performed in Cuvettes

a. With Centrifugation of the Gold Nanoparticles Prior to Use

1 ml aliquots of the aqueous layer from the solutions described in Section 2.4 were put in Eppendorf tubes and centrifuged for 12 minutes. In instances where gold nanoparticles coated with thiol modified antigens were used, centrifugation was performed at 12,000 RCF, however in instances where gold nanoparticles coated with non-thiol modified antigens were used, centrifugation was performed at 7,000 RCF. The supernatant was then removed, and the gold nanoparticle 'pellet' was re-suspended in 10 mM phosphate buffer (pH 7.4, 1 ml). 900 µl of this solution was then transferred to a cuvette. Serum was diluted to a concentration of 1:2,500 in 10 mM phosphate buffer (pH 7.4) and was then added to the cuvette (100 µl). The absorbance of the solution was then measured from 200 – 800 nm continuously over 30 minutes (1 scan per 100 s). A saturated aqueous solution of NaCl (100 µl) was then added to the cuvette and the absorbance of the solution was again measured from 200 – 800 nm continuously over 15 minutes (1 scan per 100 s).

b. Without Centrifugation of the Gold Nanoparticles Prior to Use

The same method as described above (Section 2.6.1.a) was used, with the exception that 900 µl from the aqueous layer of the solutions described in Section 2.4 was directly transferred to a cuvette without prior centrifugation.

2.6.2 Protocol when the Assay was Performed on 96-Well Plates

a. With Centrifugation of the Gold Nanoparticles Prior to Use

1 ml aliquots of the aqueous layer from the solutions described in Section 2.4 were put in Eppendorf tubes and centrifuged for 12 minutes. In instances where gold nanoparticles coated with thiol modified antigens were used, centrifugation was performed at 12,000 RCF, however in instances where gold nanoparticles coated with non-thiol modified antigens were used, centrifugation was performed at 7,000 RCF. The supernatant was then removed, and the gold nanoparticle 'pellet' was re-suspended in 10 mM phosphate buffer (pH 7.4, 1 ml). This solution was then transferred to a 96-well

micro-plate (180µl/well). Serum was diluted to a concentration of 1:2,500 in 10 mM phosphate buffer (pH 7.4) and was then added to the plate (20 µl/well) and left for 30 minutes before the absorbance of the solution was measured at 540 nm and 630 nm. A saturated aqueous solution of NaCl (20 µl/well) was then added to the plate and left for a further 15 minutes before the absorbance of the solution was again measured at 540 nm and 630 nm. Four replicates of each sample was measured unless otherwise stated.

b. Without Centrifugation of the Gold Nanoparticles Prior to Use

The same method as described above (Section 2.6.2.a) was used, with the exception that 180 µl from the aqueous layer of the solutions described in Section 2.4 was directly transferred to a well on a 96-well micro-plate without prior centrifugation (this was repeated for each well which was going to be used).

2.6.3 The Removal of IgM from the Serum Samples Prior to Use in the Assay

The same procedures as described in Sections 2.6.1 and 2.6.2 are used, with the following amendment:

Prior to addition of the serum samples to the coated gold nanoparticles, serum was diluted to a concentration of 1:2,500 in 10 mM phosphate buffer (pH 7.4) which was modified to contain dithiothreitol (DTT) (20 µM). This solution was then left for 30 minutes before being used for the assay required.

In instances where the serum concentration was changed from 1:2,500, the concentration of DTT in the buffer was also amended in order to keep the ratio of serum to DTT the same.

2.7 ELISA Protocol for Human Samples[3]

ELISAs were carried out on 96-well micro-plates and each sample was run four times unless otherwise stated. Antigens were dissolved in hexane to give an antigen solution of concentration 60 µg/ml. This solution was then added to the micro-plates (50 µl/well), and the solvent was left to evaporate at room temperature for 2 hours before the plates were stored at 4 °C overnight in a plastic bag. Control wells were coated with hexane (50 µl/well) only. Blocking was done by adding 0.5 % casein/PBS buffer (400 µl/well), and incubating at 25 °C for 2 hours. The buffer was then removed and any excess buffer was flicked out onto paper towels until the plates were dry. Serum was diluted to a concentration of 1:20 in 0.5 % casein/PBS buffer and was added to the plates (50 µl/well)

and incubated at 25 °C for a further 1 hour. The plates were then washed with 0.5 % casein/PBS buffer (400 µl/well) three times using an automatic washer, and any excess buffer was flicked out onto a paper towel until dry. Secondary antibody conjugate (50 µl/well) (see Section 2.2.1 for different secondary antibody conjugates) was then added to the plates, and incubated at 25 °C for 30 minutes. The plates were again washed three times with 0.5 % casein/PBS buffer (400 µl/well) using an automatic washer, and any excess buffer was again flicked out onto a paper towel until the plates were dry. OPD substrate (50 µl/well) was then added, and incubated for a further 30 minutes at 25 °C. The colour reaction was terminated by adding H_2SO_4 (2.5 M, 50 µl/well), and the absorbance was read at 492 nm.

2.8 References

1 C. D. Gwenin, M. Kalaji, P. A. Williams and R. M. Jones, **The orientationally controlled assembly of genetically modified enzymes in an amperometric biosensor,** *Bios. Bioelectron.,* 2007, **22,** 2869-2875.

2 W. Haiss, D. Lackey, J. K. Sass and K. H. Besocke, **Atomic resolution scanning tunnelling microscopy images of Au(111) surfaces in air and polar organic solvents,** *J. Chem. Phys.,* 1991, **95,** 2193-2196.

3 S. T. Thanyani, V. Roberts, D. G. R. Siko, P. Vrey and J. A. Verschoor, **A novel application of affinity biosensor technology to detect antibodies to mycolic acid in tuberculosis patients,** *J. Immunol. Methods,* 2008, **332,** 61-72.

Chapter 3

Towards the Development of a Sensor for the Detection of TB

3.1 Introduction

In order to work towards the development of a novel, rapid and easy to use point of care sensor for the detection of TB, a number of different approaches were investigated within the group. One part of the project focused on using ELISA assays as a method for detecting anti-TB antibodies using mycolic acids, TMMs and TDMs as antigens, to distinguish between TB positive (TB+) and TB negative (TB-) serum samples. This work was carried out to give an insight into which mycolic acids, TMMs and TDMs are most potent as antigens towards anti-TB antibodies, thus giving an indication as to which would be most suitable for use in a sensor for the detection of TB. The other part of the project focused on working towards the development of a sensor for the detection of TB, based on the antigens used in the ELISA and the detection of anti-TB antibodies that bound to them. This thesis will focus on the work carried out towards the development of a sensor for the detection of TB based on gold nanoparticles.

It was decided to use gold nanoparticles (AuNPs) as part of the sensor because they exhibit unique chemical and optical properties and display different chemical and electronic properties compared to bulk gold.[1,2,3] Also, crucially for the development of a sensor based on antibody/antigen interactions, molecules bound to gold nanoparticles do not lose their bioactivity.[3] Incorporating a sulfur atom into the antigen would allow binding of the antigen to the gold surface with controlled orientation due to the antigen binding to the gold surface through a gold-sulfur bond (see Section 1.6). By modifying the location of the sulfur atom within the antigen molecule, the orientation of the antigen on the surface could be controlled, allowing investigations into the effect of the antigen's orientation on the binding.

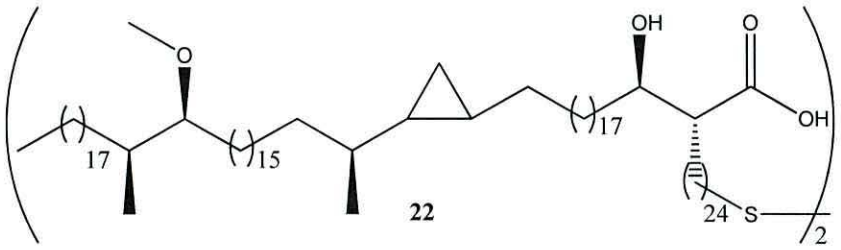
For these studies, serum samples were obtained from multiple sources. Human serum samples were obtained from the WHO, and were divided into three groups: group 1 consisted of 50 samples which were un-blinded prior to performing any experiments on them; group 2 consisted of 50 samples which were tested blind and were un-blinded at a later point in the project; while the remaining 249 serum samples were also tested blind before being un-blinded, essentially at the end of the project. All of the serum samples obtained from the WHO came from TB endemic populations and they were all designated as either TB positive or TB negative (by bacterial culture, smear microscopy and clinical observation) by the WHO. A full list of these samples, as well as some of the key

information supplied, can be found in the Appendix, Section 9.1.2. The serum samples were obtained from patients from ten different countries, and in this thesis are identified by the first two letters in the serum code, and are as follows; BA – Bangladesh, BR – Brazil, CA – Canada, CO – Colombia, GA – Gambia, KE – Kenya, PE – Peru, SA – South Africa, SP – Spain and VI – Vietnam.

Serum samples from a non-endemic, UK, population were obtained from Dr. Prysor Williams, School of Environment, Natural Resources and Geography, Bangor University, and formal ethical approval was granted for the use of all of the serum samples prior to their use in this project.

3.2 Confirming that the Thiol Modified Antigens Bound to Gold Surfaces

After the first thiol modified mycolic acid[1], **22**, illustrated in Figure 3.2.1, was produced within the research group, the first step in developing a sensor, of the nature described above, was to determine whether such modified mycolic acids would bind to gold surfaces and onto gold nanoparticle surfaces.



22

Figure 3.2.1 Structure of the thiol modified mycolic acid, 22.

Three different techniques were used to try and confirm this: stripping voltammetry; transmission electron microscopy (TEM); and particle size and zeta (ζ)-potential analysis. Gold surfaces and gold nanoparticle surfaces were coated with the thiol modified mycolic acid (**22**) according to the procedures described in Sections 2.3 and 2.4.

3.2.1 Stripping Voltammetry

In order to determine whether the thiol modified mycolic acid, **22**, bound to gold surfaces, stripping voltammetry was performed. This is a technique that can be used to remove substrates from an electrode surface.[4] Reduction of the gold-sulfur bond produces a

[1] In some instances a thiol group has been incorporated into the antigen molecule, however in some instances, such as compound **22**, a disulfide molecule was synthesised. Both of these types of molecules are referred to as 'thiol modified' in this thesis due to there being no difference between the two types of molecules when they are bound to gold surfaces and to gold nanoparticles (see Section 1.6.1).

current, which is observed as a current peak in the current-potential graph, and is related to the electron transfer from the electrode to the chemisorbed substrate.[5]

A self-assembled monolayer (SAM) of the thiol modified mycolic acid, **22**, and subsequent stripping voltammetry of the resulting modified gold-coated slides were performed according to the procedure described in Section 2.3, and the following voltammogram was obtained:

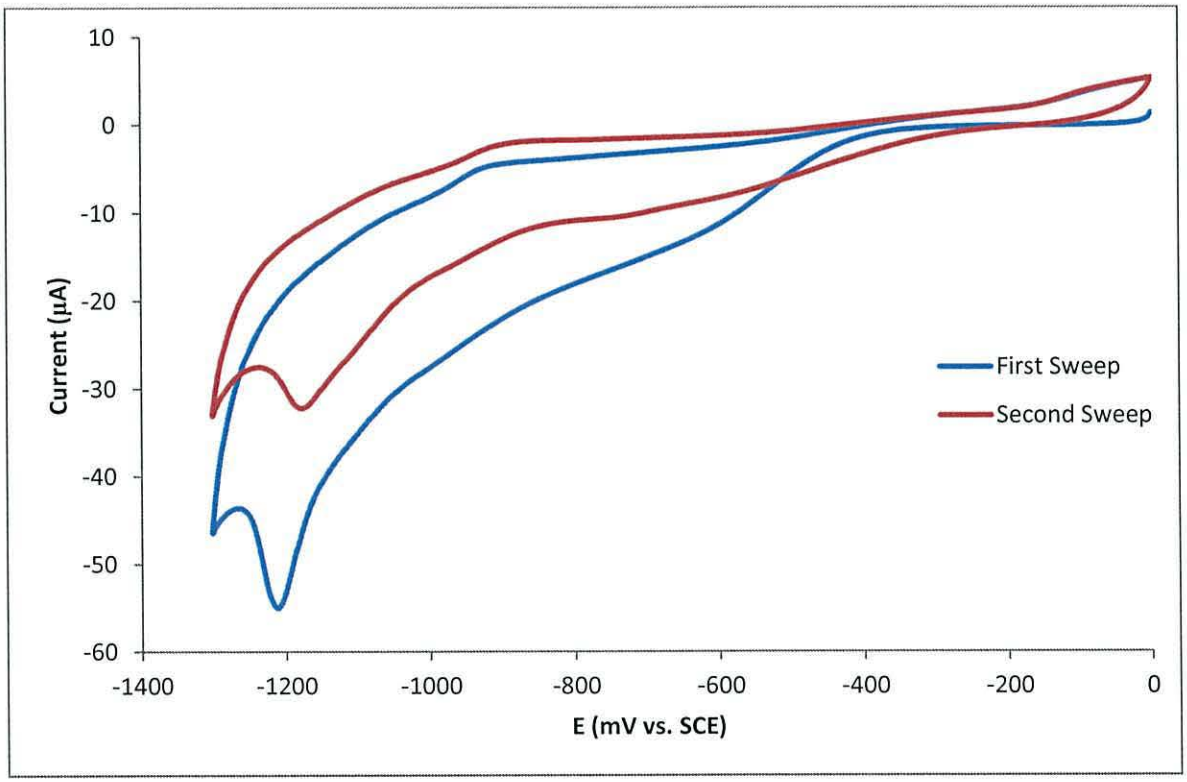


Figure 3.2.2 Voltammogram of a gold (111) electrode modified with a thiol modified mycolic acid, 22, recorded at a sweep rate of 50 mV/s in 0.1 M NaOH.

The reduction peak at -1211 mV is characteristic of thiol desorption.[5] The small oxidation peak at -885 mV corresponds to re-adsorption of some of the thiolated species onto the gold surface and this can be confirmed by the reduction peak at -1178 mV in the second sweep. This peak again corresponds to thiol desorption from the gold surface, however this peak is smaller than the thiol desorption peak from the first sweep, suggesting that only a few of the thiolated species which were desorbed during the first sweep were re-adsorbed onto the gold surface. The presence of these reduction peaks at -1211 and -1178 mV confirmed that the thiol modified mycolic acid, **22**, bound to the gold surface.

3.2.2 Transmission Electron Microscopy

Gold nanoparticles which had been coated with **22**, as well as gold nanoparticles which had been un-treated were analysed by Transmission Electron Microscopy (TEM), in order to see whether the mycolic acid coating could be detected. The micrographs were run by Dr Chris von Ruhland, School of Medicine, Cardiff University. The following Figure shows the TEM images obtained for gold nanoparticles which had not been coated with antigen and for gold nanoparticles which were coated with **22**:

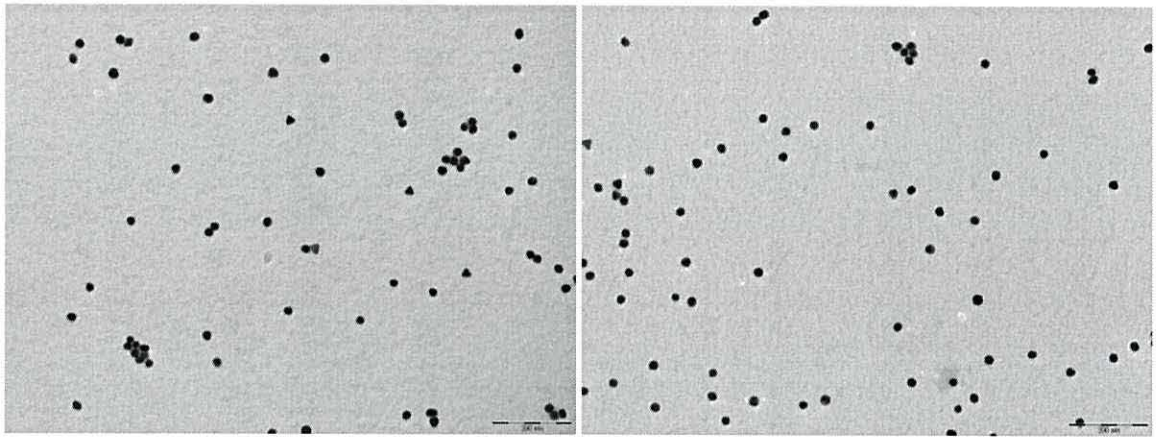


Figure 3.2.3 TEM images of gold nanoparticles which were not coated with antigen (left) and gold nanoparticles which were coated with 22 (right).

It can be seen from these images that there is no observable difference between gold nanoparticles which were un-coated, and those which were coated with **22**. This may be due to this method not being able to detect the presence of small organic molecules on the surface of larger metal particles. An important observation however is that there is no evidence that any aggregation of the gold nanoparticles had occurred after coating them with **22**, suggesting that the gold nanoparticle solution[II] remains stable after the coating process was performed.

Since using TEM did not confirm the presence of the modified antigen (**22**) on the gold nanoparticle surface, other techniques were used in order to try and confirm their presence.

3.2.3 Particle Size and Zeta (ζ)-Potential Analysis

Particle size and zeta potential measurements were performed by Mr. Mark Bumiller, HORIBA Scientific, using a HORIBA SZ-100Z system (Kyoto, Japan). Particle size analysis measurements were made at a 90 degree scattering angle and standard Dynals

[II] The terms 'gold nanoparticle solution' and 'gold nanoparticle suspension' are both commonly used in the literature, therefore the term 'solution' will be used throughout this thesis for consistency.

analysis algorithm at 25 °C. Results are reported as the z-average from the intensity distribution. None of the samples required dilution or other sample preparation steps. The system was first verified using both a 100 nm PSL standard (Duke Scientific cat no. 3100A) and a 10 nm colloidal gold reference material from NIST (NIST reference material 8011). Zeta potential measurements were made on the same system using disposable measurement cells with carbon coated electrodes in order to minimize sample interaction at the electrode surface. The samples were also analysed without dilution for the zeta potential analysis.

The particle size analysis showed a small increase in the average particle size from 23.9 nm for the un-treated gold nanoparticles to 24.0 nm for the mycolic acid coated gold nanoparticles, however, the zeta potential analysis showed a decrease in the magnitude of the zeta potential of the particles from -17.6 mV for the un-treated gold nanoparticles to -11.3 mV for the mycolic acid coated gold nanoparticles.

Zeta potential is a measure of the electric potential across the mobile part of the double layer, which is responsible for electrokinetic phenomena.[6] Un-treated gold nanoparticles are coated with charged citrate ions whereas the mycolic acid coated gold nanoparticles are expected to be coated with a lipid layer containing less charge. The change in the coating layer from a charged surface to a more un-charged surface should lead to a reduction in the magnitude of the zeta potential.

The results obtained suggest that this is the case, with a decrease in the magnitude of the zeta potential observed for the mycolic acid coated gold nanoparticles, suggesting a reduction in the surface charge of the coated gold nanoparticles, in turn suggesting that the mycolic acids have successfully been bound to the surfaces of the gold nanoparticles.

3.3 Initial Experiments

Having successfully bound thiol modified mycolic acids to gold nanoparticle surfaces, work was undertaken to develop a sensor for the detection of TB using these coated gold nanoparticles. The objectives of the project were to develop a sensor for the detection of TB that would be rapid and easy to use, and would be based on the interaction of antigens on the surfaces of the modified gold nanoparticles with anti-TB antibodies in serum

samples.[III] This antigen-antibody interaction was investigated in detail by ELISA,[7] and it was believed that the patterns observed during those studies would hold true when the antigens investigated were bound onto gold nanoparticle surfaces.

For these studies it was decided to purchase gold nanoparticles from an external supplier (Aldrich for initial experiments and BBInternational for future experiments) in order to ensure gold nanoparticles of consistent sizes were used for the experiments, which would lead to more consistent and re-producible results in the assay developed. It was decided to use gold nanoparticles of 20 nm in diameter because they were readily available, and have a characteristic absorbance peak at approximately 521 nm,[8] which would allow analysis of the gold nanoparticles using UV-visible spectroscopy.

Initial experiments focussed on developing conditions for the coated gold nanoparticles, under which they would remain stable in solution. Also, any chemicals or reagents used in the developed assay, could not have absorbance peaks in the region of 500 - 600 nm, as this would interfere with the absorbance peak of the gold nanoparticles themselves.

For the ELISA assays performed,[7] 0.5 % casein/PBS buffer had been used in the assay and for serum dilution. However, it was believed that this buffer would not be compatible with the coated gold nanoparticles as its salt content would cause them to aggregate.[9] It was decided to confirm this by re-suspending gold nanoparticles in 0.5 % casein/PBS buffer. This was performed by centrifuging gold nanoparticles at 12,000 RCF for 12 minutes before removing the supernatant and re-suspending them in 0.5 % casein/PBS buffer and analysing the solution using UV-visible spectroscopy. Un-treated gold nanoparticles (*i.e.* gold nanoparticles purchased from Aldrich) were also analysed for comparison, and the results of this experiment can be seen in Figure 3.3.1:

[III] Evidence that it was antibodies that were binding to the coated gold nanoparticles is supplied in Section 6.2.

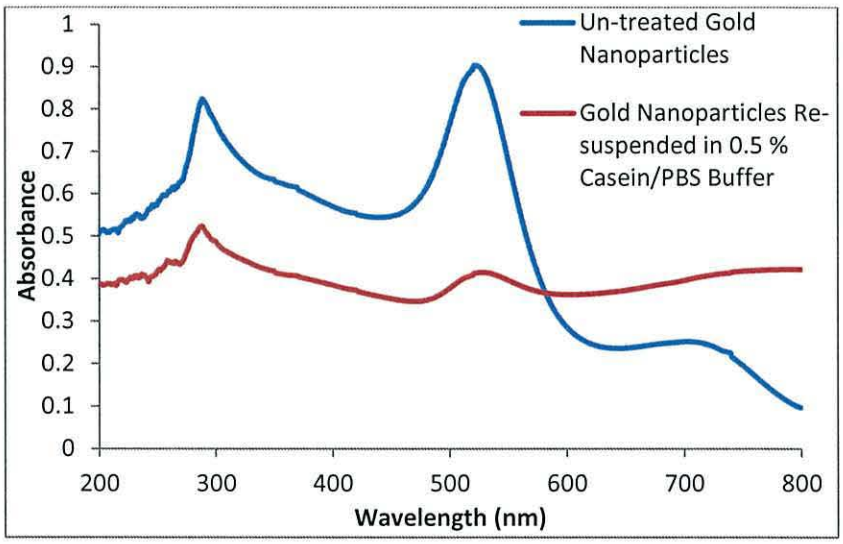


Figure 3.3.1 UV-visible spectrum of un-treated gold nanoparticles and gold nanoparticles re-suspended in 0.5 % casein/PBS buffer.

The spectrum depicted in Figure 3.3.1 shows a change in the maximum absorbance peak when re-suspending the gold nanoparticles in 0.5 % casein/PBS buffer. It can be observed that there is a significant reduction in the absorbance peak at 523 nm, as well as an increase in the peak at above 700 nm, suggesting aggregation of the gold nanoparticles when they are re-suspended in 0.5 % casein/PBS buffer.[9] Due to aggregation of the nanoparticles when using this buffer, another buffer would be needed, therefore a phosphate buffer was tested. The same experiment was repeated, but, instead of re-suspending the gold nanoparticles in 0.5 % casein/PBS buffer, they were this time re-suspended in 10 mM phosphate buffer (pH 7.4). The results are illustrated in Figure 3.3.2:

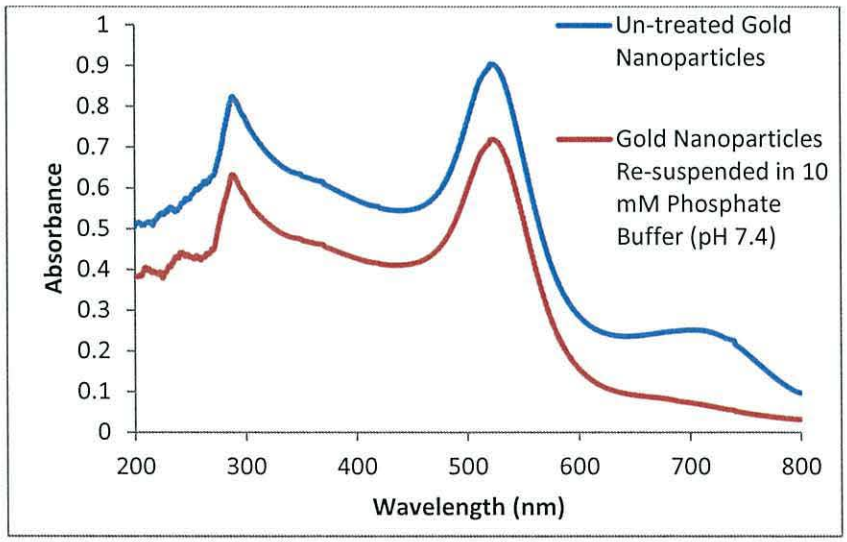


Figure 3.3.2 UV-visible spectrum of un-treated gold nanoparticles and gold nanoparticles re-suspended in 10 mM phosphate buffer (pH 7.4).

It can be seen from this spectrum that there is no shift in this case in the maximum absorbance peak when re-suspending the gold nanoparticles in 10 mM phosphate buffer (pH 7.4). The reduction in the absorbance value can be attributed to a loss of some of the nanoparticles during the centrifugation and re-suspension process. These results suggest that there is no aggregation in the gold nanoparticles when they are re-suspended in 10 mM phosphate buffer (pH 7.4), which makes this a suitable buffer to use in an assay or device based on gold nanoparticles.

It can also be observed that when re-suspending the gold nanoparticles in 10 mM phosphate buffer (pH 7.4), the small peak at approximately 700 nm is lost. This could be due to some of the gold nanoparticles purchased from Aldrich forming small amounts of aggregates in solution, leading to this small peak. The centrifugation and re-suspension process removed these aggregates from the solution, leading to the loss of this peak in the above spectrum. Using this buffer would also be advantageous when using serum samples due to its pH being very similar to that of human serum which is usually in the range of 7.35-7.45.[10]

In order to check that this trend was also observed when using coated gold nanoparticles, gold nanoparticles were coated with the thiol modified mycolic acid, **22**, and re-suspended in the same buffers as used above. Upon re-suspension, the same pattern was observed, with aggregation occurring in 0.5 % Casein/PBS buffer, while no aggregation was observed when using 10 mM phosphate buffer (pH 7.4) (see Figure 3.3.3). The absorbance values observed for the different solutions vary due to differing nanoparticle concentrations being present at various stages of the coating process and some being lost during the centrifugation and re-suspension process.

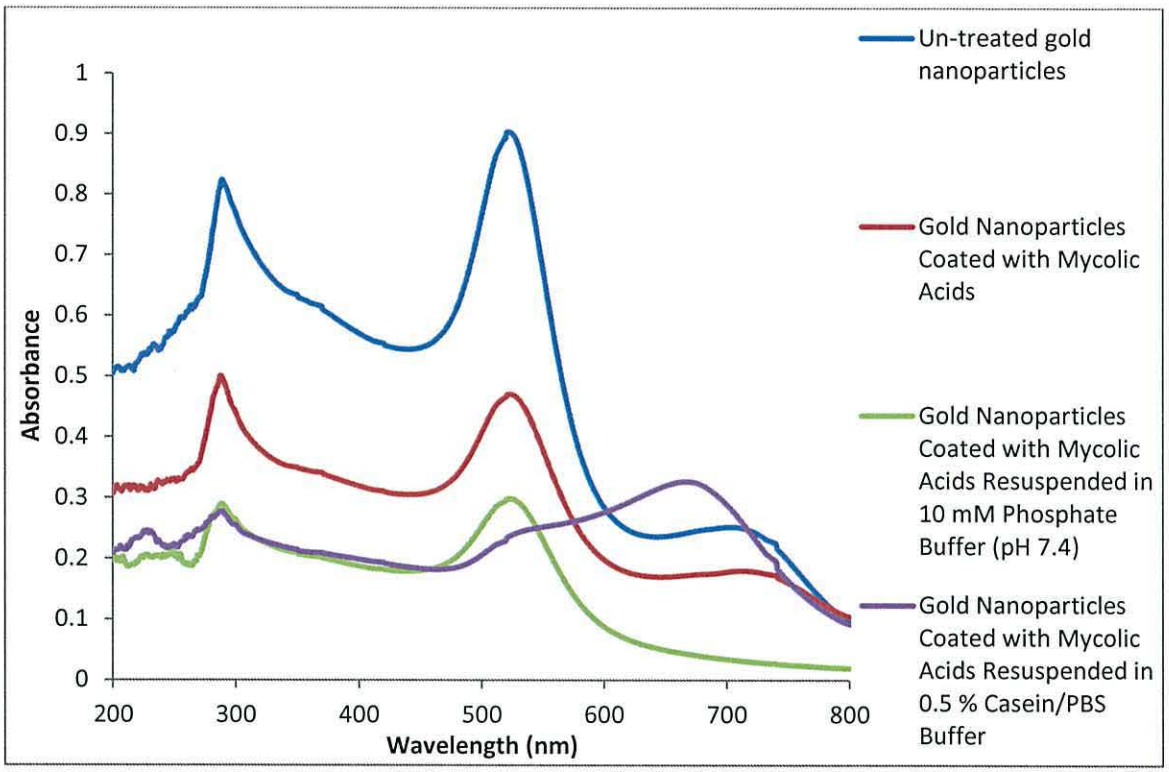


Figure 3.3.3 Comparison of the UV-visible spectra for un-treated gold nanoparticles, gold nanoparticles coated in mycolic acid (22), gold nanoparticles coated in mycolic acid and re-suspended 10 mM phosphate buffer (pH 7.4) and gold nanoparticles coated in mycolic acid re-suspended in 0.5 % casein/PBS buffer.

It can be observed from Figure 3.3.3 that coating the gold nanoparticles with the modified mycolic acid does not cause a significant change in the wavelength at which the maximum absorbance occurs. It can again be observed, that when re-suspending the coated gold nanoparticles in 10 mM phosphate buffer (pH 7.4), the small peak at approximately 700 nm is lost. This again suggests that the centrifugation and re-suspension process removes these aggregates from the solution.

These experiments suggested that 10 mM phosphate buffer (pH 7.4) would be suitable for use in an assay or sensor based on coated gold nanoparticles, and that the centrifugation and re-suspension process may remove the presence of any aggregate that is present in the solution either from the supplier or formed as a result of the coating process.

All of the other materials that would be used in the assay or sensor were also examined to ensure that they would not give peaks that interfere with those observed for the coated gold nanoparticles. UV-visible spectra for these materials can be seen in the Appendix, Section 9.2.1.

The spectrum for the thiol modified mycolic acid showed no significant peaks, while those for both TB+ and a TB- serum samples showed large peaks in the region of 280 nm, with

no peaks in the region above 500 nm, where the coated gold nanoparticles absorb. Therefore when performing experiments on coated gold nanoparticles using these materials, any change in the spectrum above 500 nm can be attributed to a change in the coated nanoparticle peak.

3.4 Initial Experiments to Detect Anti-TB Antibodies Using Coated Nanoparticles

Having successfully coated gold nanoparticles with a thiol modified mycolic acid, and identifying a suitable buffer in which those coated gold nanoparticles would not aggregate, work was then carried out towards developing an assay and/or sensor for the detection of TB.

The first approach taken was to look at the interaction of antibodies in the serum samples with the antigens coated onto the surface of the gold nanoparticles. This was done by looking at the change in the absorbance peak for the coated gold nanoparticles with time, following the addition of a serum sample.

Interactions of species in solutions with those coated onto gold nanoparticles have been investigated before. Previously, antibodies have been bound to gold nanoparticles which were then subjected to samples containing antigens specific to those antibodies.[11] In this study, gold nanoparticles were coated with antibodies before samples of various protein antigens were added, and a change in absorbance at a wavelength above the surface plasmon resonance (SPR) peak was recorded. Recording the data at a wavelength higher than the SPR was believed to give a greater sensitivity than recording the data at the SPR wavelength. The change in absorbance observed is credited to a change in the refractive index of the individual particles following antigen binding providing a further layer of coating to the gold nanoparticles.[11] This principle has also been further investigated in order to look at real-time biomolecular interactions on gold nanoparticle surfaces.[12]

It was believed that interactions of antibodies with antigens coated onto the surface of gold nanoparticles could be investigated in a similar way. It was believed that upon binding of anti-TB antibodies to the mycolic acids coated onto the surfaces of gold nanoparticles, the extra layer of coating on the gold nanoparticles would lead to a change in the refractive index of the individual particles, in turn, leading to a change in the SPR peak. By

observing the UV-visible spectrum with time, it was expected that information about the rate of binding of the antibodies to the mycolic acids could also be inferred.

3.4.1 Initial Experiments

Initially, serum (100 µl) diluted to a concentration of 1 in 5 in 10 mM phosphate buffer (pH 7.4) was added to the coated gold nanoparticles (900 µl) and the absorbance of the solution was then measured from 200 – 800 nm continuously over 30 minutes (1 scan per 100 s). One TB+ serum sample, KE 3, and one TB- serum sample, BA 13, were initially used in order to see if any difference would be observed in the SPR peak of the coated gold nanoparticles. These serum samples were part of a set provided by the WHO defined as either TB+ to smear and culture and to clinical observation, or negative to the same tests. The particular samples were chosen due to the TB+ serum sample giving consistently high absorbance values with all of the antigens investigated by ELISA,[7] and the TB- serum sample giving consistently low absorbance values with most of the antigens investigated.[7] The UV-visible spectra obtained from these initial experiments can be seen in Figure 3.4.1. Only the region from 450 to 650 nm is shown in order to clearly see any changes in the SPR peak. There were no significant changes observed in the spectra at wavelengths below 450 nm and above 650 nm.

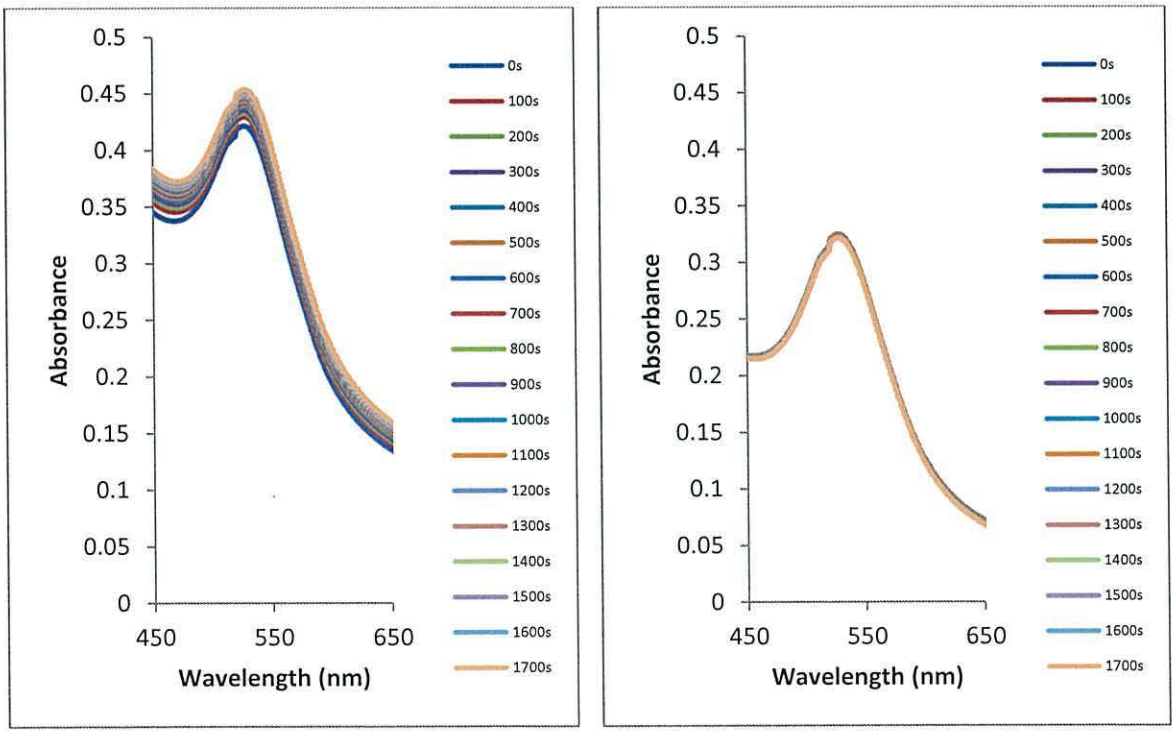


Figure 3.4.1 (left) UV-visible spectrum for the mycolic acid coated gold nanoparticles at 100 s intervals after the addition of the TB positive serum sample, KE 3. (right) UV-visible spectrum for the mycolic acid coated gold nanoparticles at 100 s intervals after the addition of the TB negative serum sample, BA 13.

A clear difference can be seen between the TB+ and TB- serum samples in Figure 3.4.1. For the TB+ sample, KE 3, a clear increase in absorbance can be observed with time, while for the TB- sample, BA 13, very little change in the absorbance is observed with time. This suggests that for the TB+ serum sample, after addition of the serum, anti-TB antibodies bind to the mycolic acid coated onto the gold nanoparticles, increasing the layer of the coating around the gold nanoparticles, in turn, changing the refractive index of the molecules, leading to an increase in the absorbance of the solution. For the negative serum sample, after addition of the serum, no antibodies bound to the mycolic acids coated onto the gold nanoparticles, in turn leading to no change in the refractive index of the solution with time leading to no change in the absorbance of the solution.

This difference is highlighted in Figure 3.4.2, which shows the change in absorbance at 540 nm with time for both of the above solutions. The SPR peak occurred at 527 nm, therefore a wavelength slightly higher was selected in order to improve the sensitivity of the test.[11]

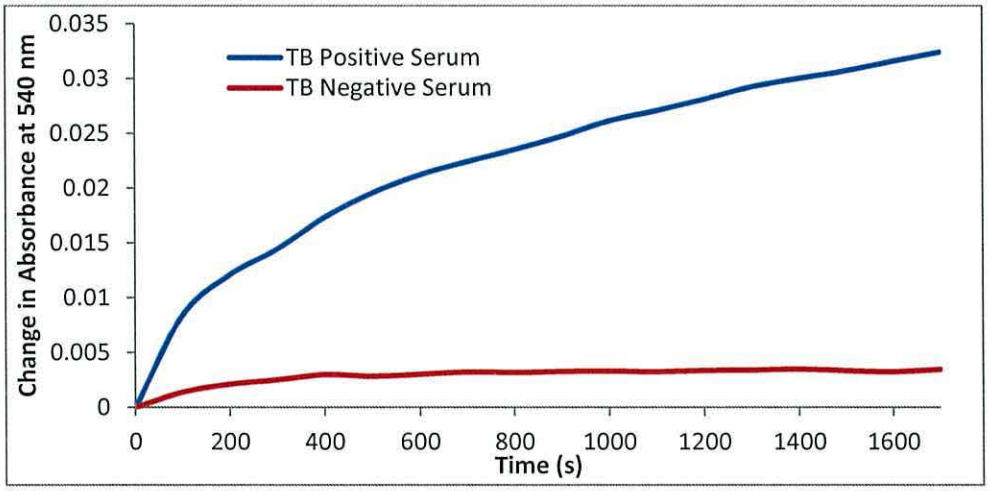


Figure 3.4.2 Graph showing the change in absorbance at 540 nm with time for the coated gold nanoparticle solution after addition of the TB positive serum sample, KE 3 and the TB negative serum sample, BA 13.

It can be seen from this Figure that for the TB+ serum sample, an increase in absorbance at 540 nm is observed with time, while for the TB- serum sample, very little increase in absorbance is observed with time. The graph is also consistent with what would be expected for antibodies binding to antigens on the surface of a gold nanoparticle. The gradients of the graphs for both samples are greatest immediately after addition of the serum sample to the coated gold nanoparticle solution, suggesting that binding of the antibodies to the mycolic acids occurs quickest at this time. As time progresses, the

gradients of the graphs decrease as fewer antibodies remain un-bound in solution and the amount of antigens without any antibodies bound to them also decrease.

These Figures strongly suggest that the change in absorbance observed is directly influenced by antibodies in the serum samples binding to mycolic acids, themselves bound on the gold nanoparticle surfaces, and that looking at this change in absorbance gives information about the amount of antibodies that bind to the antigens, as well as the rate of binding of these antibodies to the antigens.

Being able to investigate this interaction using mycolic acids bound onto gold nanoparticles is advantageous over using ELISA for a number of reasons. Firstly, the interaction can be monitored in real-time giving more information about the interaction; secondly, no washing steps, secondary antibody conjugates or additional colour reagents are required for this assay; and finally, it is a much quicker assay with results being obtained within 30 minutes.

3.4.2 Varying the Serum Concentration

One of the drawbacks of this assay is that it required 20 µl of serum sample per test, compared to only 2.5 µl per test for ELISA. Due to only a limited amount of serum being available, it was decided to to check whether this amount could be reduced. The above experiments were repeated at serum concentrations ranging from 1 in 5 to 1 in 640 in 10 mM phosphate buffer (pH 7.4). In order to preserve sera, and since this experiment was looking at the trend observed when using serum of different concentrations, different TB+ and TB- serum samples were used. Sample BA 3 was used as the TB+ sample while CO 18 was used as the TB- sample. These serum samples were selected because a large amount of each was still available at this point in the project and they had given high and low absorbance values respectively with a number of antigens by ELISA.[7] The results obtained from these experiments can be seen in Figure 3.4.3:

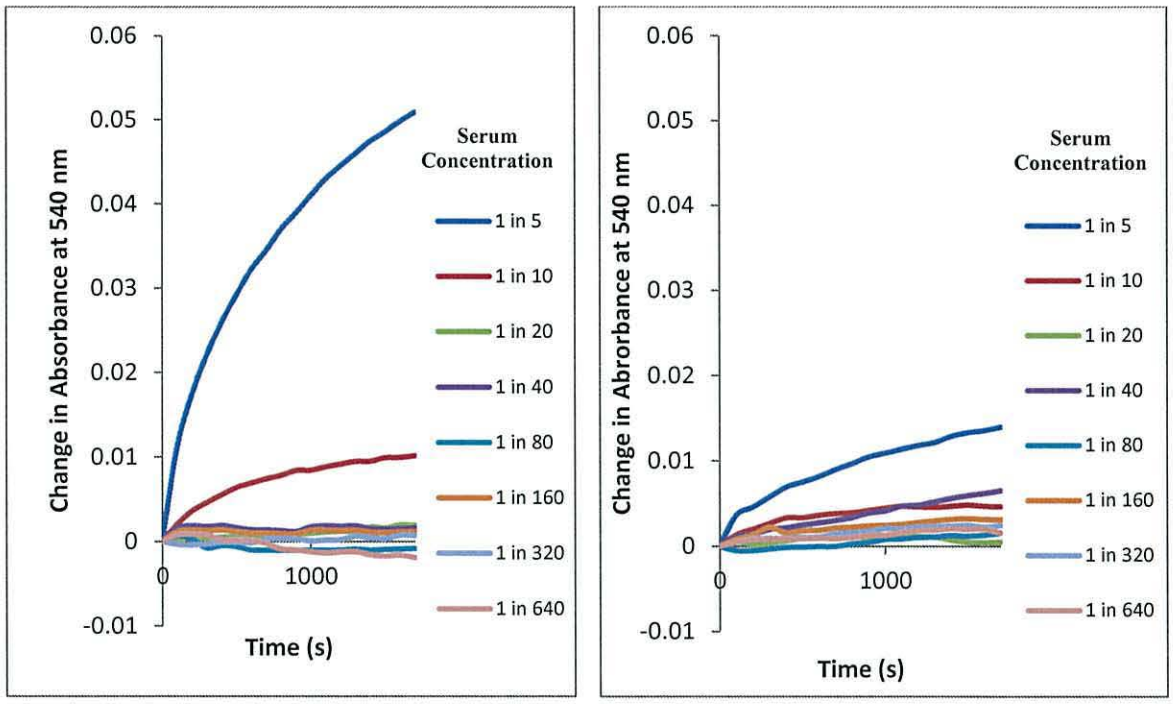


Figure 3.4.3 (left) Graph showing the change in absorbance at 540 nm for the TB positive serum sample, BA 3, at various concentrations with time. (right) Graph showing the change in absorbance at 540 nm for the TB negative serum sample, CO 18, at various concentrations with time.

A similar trend can be observed for both serum samples. The largest change in absorbance is observed at a 1 in 5 serum concentration and the change in absorbance is significantly reduced as the serum concentration is reduced. This would suggest that a serum concentration of 1 in 5 is needed for this assay. The trend observed in these results suggests that neat serum would give an even greater change in absorbance, in turn resulting in a better distinction between TB+ and TB- serum samples. However, as 100 μl of serum would be needed per sample to perform the assay, and due to the limited availability of most of the serum samples, it was decided, at this point in the project, to continue using serum at a concentration of 1 in 5 for this assay.

3.4.3 Testing a Larger Number of Serum Samples

Having observed that this method can distinguish a TB+ serum sample from a TB- serum sample, and also that a serum concentration of 1 in 5 is required to see a significant change in the absorbance at 540 nm with time, it was decided to analyse more serum samples using this method. Five different TB+ serum samples and five different TB- serum samples were analysed, and the change in absorbance at 540 nm with time, following the addition of the serum sample to the gold nanoparticles coated with **22** was recorded, Figure 3.4.4. The serum samples chosen for this experiment were chosen at random, with one of the TB- serum samples being from an individual in the UK. This sample was

chosen in order to compare the results from a TB- serum sample from a low burden TB country with the TB- serum samples obtained from the WHO, from high burden TB endemic populations: the UK serum sample was expected to give a small change in absorbance as it was not expected to contain many anti-TB antibodies.

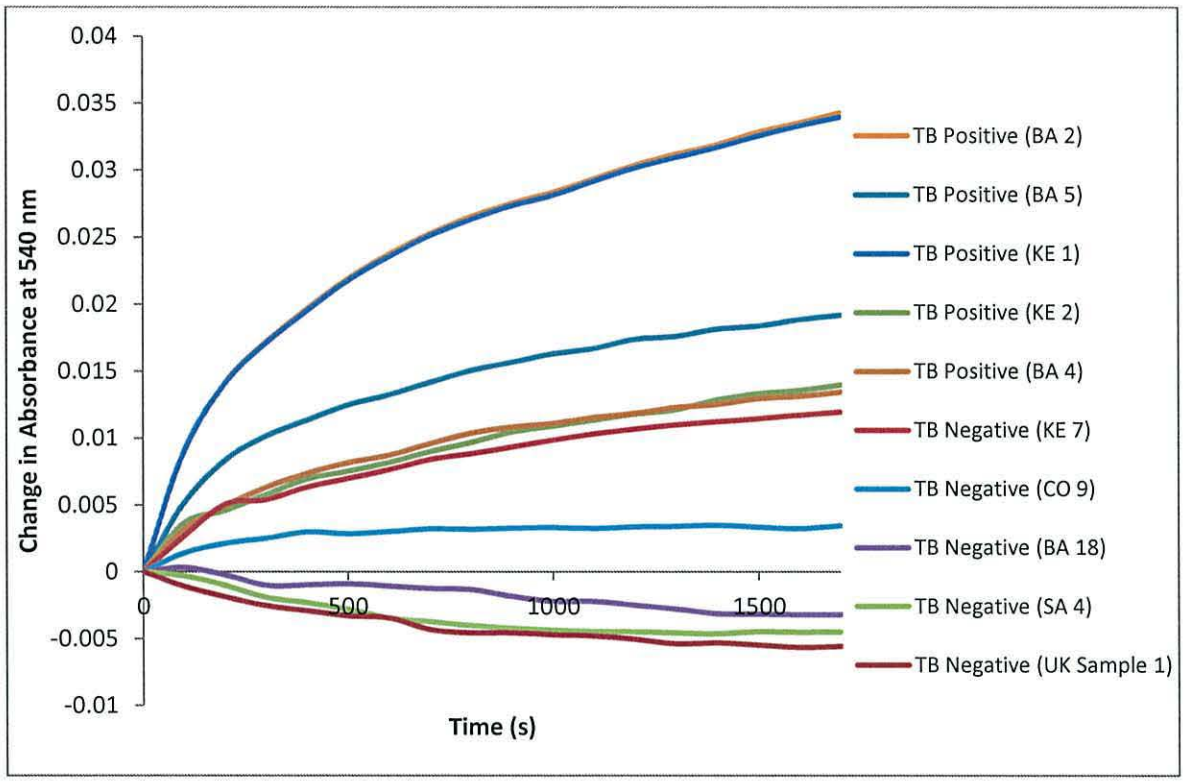


Figure 3.4.4 Graph showing the change in absorbance at 540 nm for multiple different serum samples with time, using mycolic acid coated gold nanoparticles for the assay.

It can be seen from this graph that there is an increase in absorbance at 540 nm with time after addition of all of the TB+ serum samples, while for the TB- serum samples, generally, there is only a small change in absorbance with time. Even though, in some instances, the difference between the TB+ and TB- samples is only very small (for example samples BA 4 and KE 7), a distinction is observed between the TB+ and TB- serum samples, with all of the coated gold nanoparticle solutions tested with TB+ samples having a greater change in absorbance than those tested with TB- serum samples.

3.4.4 Using More Concentrated Serum

In order to try and overcome this small difference between some of the TB+ and TB- serum samples, it was decided to use more concentrated serum samples in the assay. Previous results (see Figure 3.4.3) suggested that using a more concentrated serum sample would result in a larger change in absorbance, therefore possibly giving more of a

distinction between TB+ and TB- serum samples. The assay was therefore run with neat serum in order to see if a bigger change in absorbance was observed with time after addition of the serum samples, and whether or not a good distinction would be observed between TB+ and TB- serum samples. As previously mentioned, the drawback of performing this test with neat serum was that 100 µl of serum was required per test, and not enough serum was available to perform this test with many of the serum samples or to carry out replicates. One TB+ and one TB- serum samples were therefore examined. The UV-visible spectra obtained from these experiments at 100 s intervals over a period of 30 minutes can be seen in Figure 3.4.5. Again, only the region from 450 nm to 650 nm is shown in order to clearly see any changes in the SPR peak in this region.

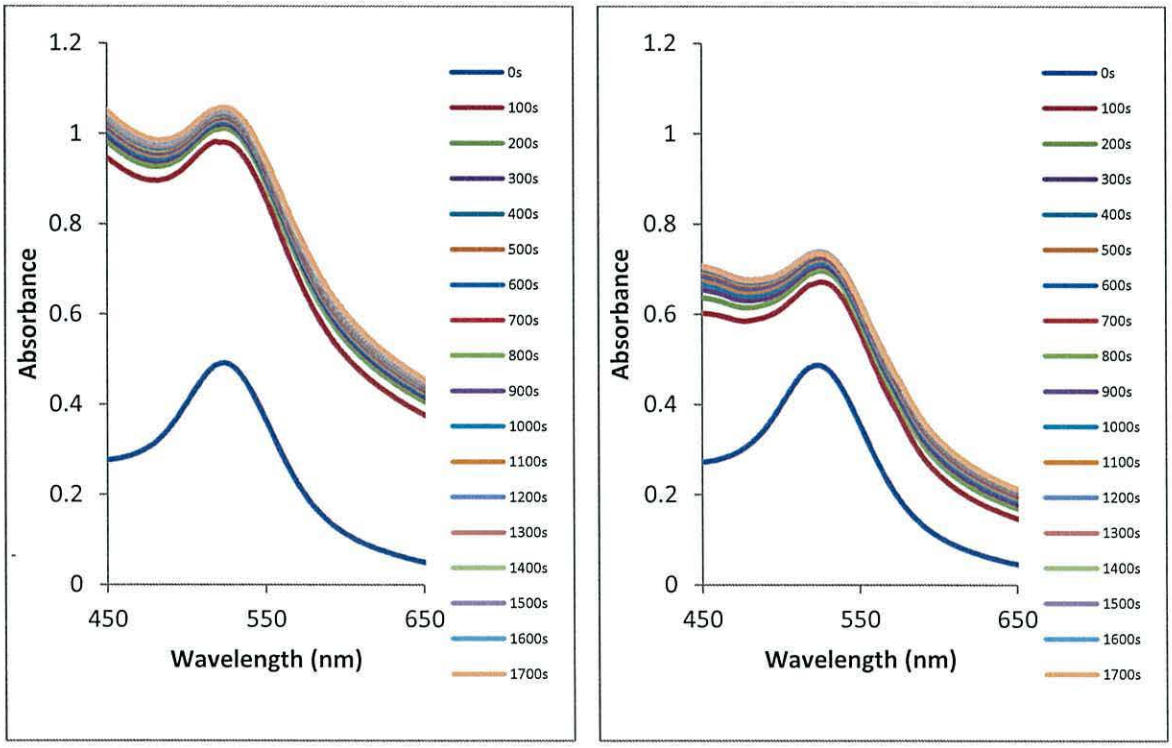


Figure 3.4.5 (left) UV-visible spectrum for mycolic acid coated gold nanoparticles coated at 100 s intervals after addition of the TB positive serum sample, GA 1. (right) UV-visible spectrum for mycolic acid coated gold nanoparticles coated at 100 s intervals after addition of the TB negative serum sample, BA 13.

The first observation from these graphs is that the change in absorbance observed when using neat serum samples is much greater than that when using more dilute serum. This would be expected due to a higher concentration of antibodies (and other species) being present in the samples leading to more interactions with the antigens on the surface of the gold nanoparticles. This in turn leads to a bigger change in the refractive index of the individual particles, in turn leading to a larger change in the absorbance observed.

It can also be seen that, although a change in the SPR peak is observed with both the TB+ and TB- serum samples with time, the increase in absorbance is somewhat higher for the TB+ serum sample than for the TB- serum sample. This suggests that a larger difference between TB+ and TB- serum samples may be obtained by using neat serum.

In order to check whether this pattern would hold true for multiple serum samples, a new approach for the assay was required due to the assay requiring a large amount of each serum samples (as compared to ELISA), and only a limited amount being available. It was therefore decided to perform the assay on 96-well plates, which required smaller volumes of coated gold nanoparticles and serum per experiment.

3.4.5 Performing the Assay on 96-Well Plates

Reducing the amount of gold nanoparticles and antigen required per test reduced the cost of the assay, while reducing the amount of serum required made it possible to analyse more serum samples by this method. The only drawback of performing the assay on 96-well plates was that the amount of data obtained during the assay was reduced, as it was only possible to read the plates at a certain pre-defined wavelength throughout the whole assay, and not obtain the full spectrum.

In order to make sure that the assay could be successfully performed on 96-well plates, one TB+ serum sample (KE 1) and one TB- serum sample (CO 9) were analysed using neat serum and also at a dilution of 1 in 5 in 10 mM phosphate buffer (pH 7.4), and four replicates of each experiment were performed in order to test the reproducibility of the assay. The absorbencies of the solutions were measured at 540 nm at 2 minute intervals following the addition of the serum sample to the coated gold nanoparticle solution, over a period of 30 minutes, and the results obtained from these experiments can be seen in Figure 3.4.6.

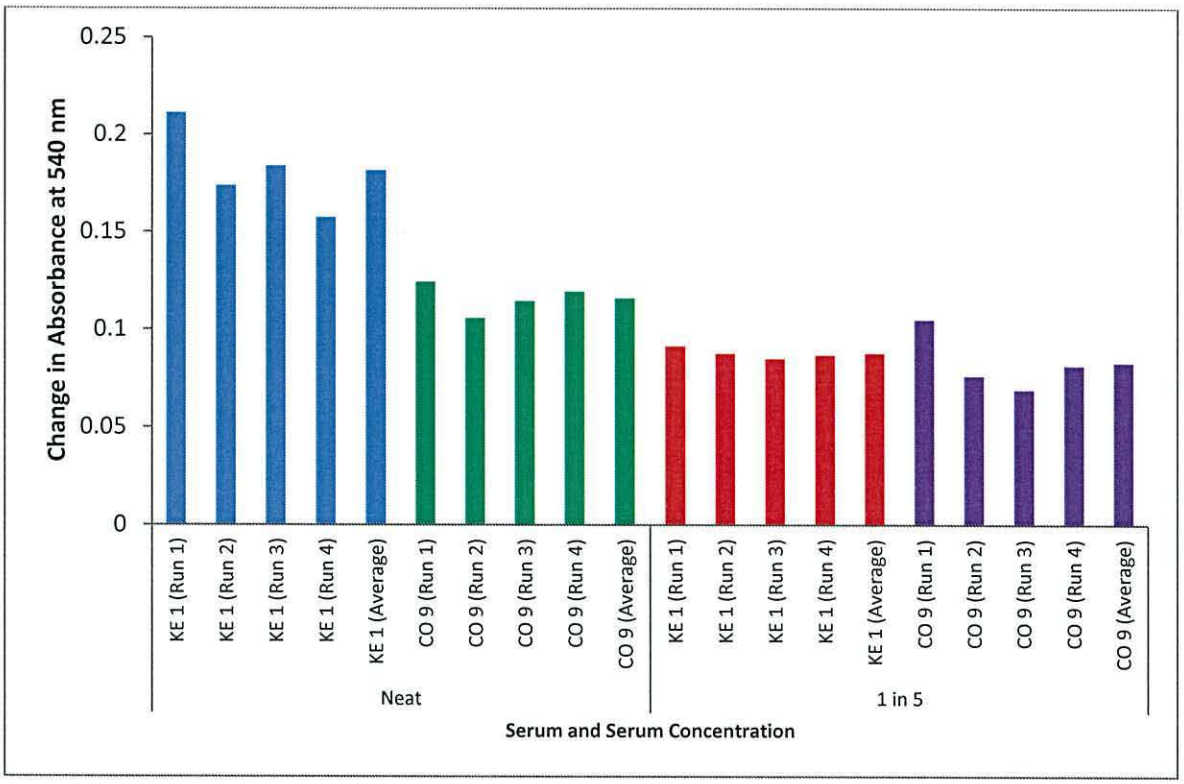


Figure 3.4.6 Graph showing the change in absorbance at 540 nm for the mycolic acid coated gold nanoparticle solutions over 30 minutes following addition of the serum sample.

It can be observed from this graph that there is a greater change in absorbance for both TB+ and TB- serum samples when using neat serum (compared to when using serum at a concentration of 1 in 5), and also, that there is a larger difference in the change in absorbance between the TB+ and TB- serum samples when using neat serum (compared to when using serum at a 1 in 5 concentration). This again suggests that using neat serum for this assay would lead to a better distinction between TB+ and TB- serum samples. Another observation is that the changes in absorbance for the four replicates with each serum sample are very similar, thus confirming the reproducibility of the assay, which is crucial when developing a sensor for the detection of TB.

The average change in absorbance with time for each serum sample can be seen in the following Figure. Figure 3.4.7 (left) shows the average change in absorbance at 540 nm for the coated gold nanoparticle solution after addition of serum diluted to a concentration of 1 in 5 with time, while Figure 3.4.7 (right) shows the change in absorbance observed after addition of neat serum samples.

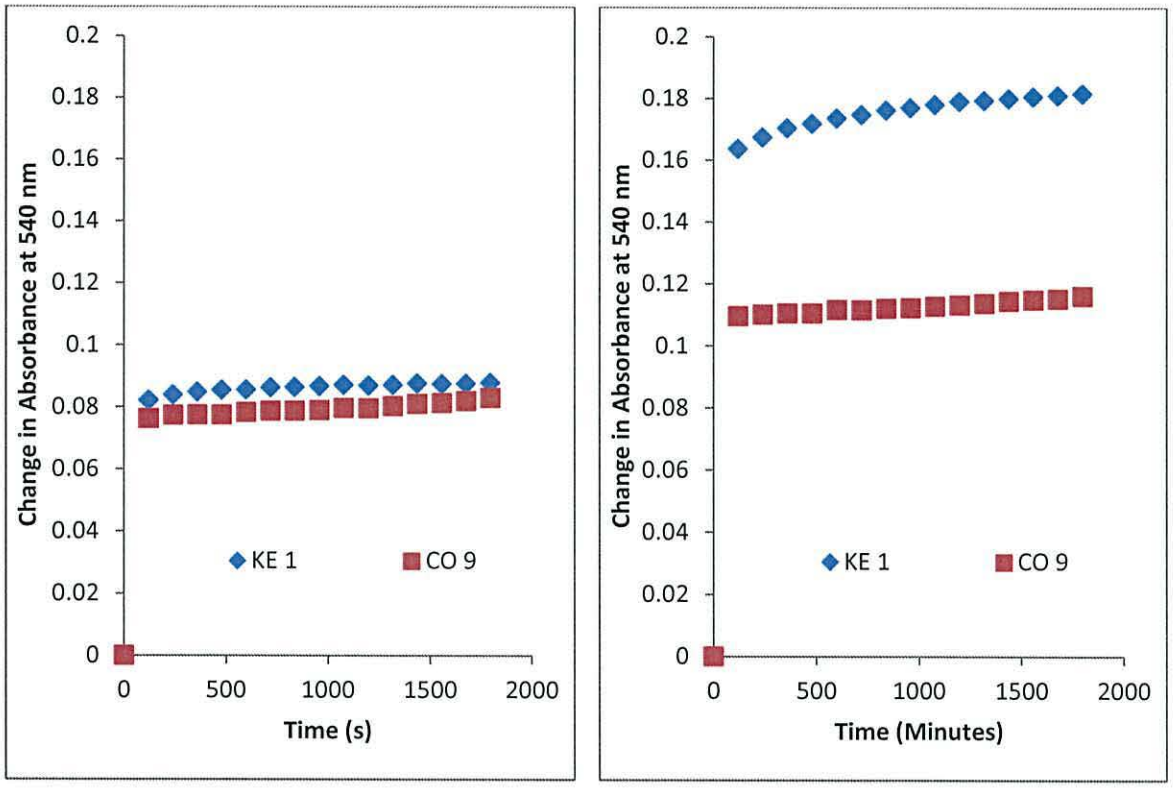


Figure 3.4.7 (left) Graph showing the average change in absorbance at 540 nm with time for the mycolic acid coated gold nanoparticles following addition of the serum samples at a concentration of 1 in 5. (right) Graph showing the average change in absorbance at 540 nm with time for the mycolic acid coated gold nanoparticles following addition of neat serum samples.

It can again be observed from these Figures that there is a larger difference between the TB+ and TB- serum samples when using neat serum for this assay. This suggested that all assays of this nature should be performed with neat serum samples in order to get the best distinction between TB+ and TB- serum samples.

3.4.6 Analysis of a Larger Set of Serum Samples

Having established that the assay can be performed on 96-well plates, and that using neat serum samples gave a greater change in absorbance with time, as well as a better distinction between TB+ and TB- serum samples, it was decided to test a larger set of samples.

Due to neat serum being required for this assay, and due to only a limited amount of some of the samples being available at this point in the project, serum from all three groups (see Section 9.1.2) was used for these experiments, and some of the serum samples were therefore tested blind due to those samples not being un-blinded at this point in the project. The Figures in this section were produced after un-blinding of the serum samples, and all samples that were run blind are designated by an asterisk in the Figures. As some of the

samples were tested blind, the serum samples selected for these experiments were chosen based on the ELISA results for those samples.[7] Ten serum samples known or believed to be TB+ and ten known or believed to be TB- were analysed, and these results can be seen in Figure 3.4.8. Two replicates of each sample were run (designated as Run 1 and Run 2 in Figure 3.4.8), and the average change in absorbance for these two replicates is also shown.

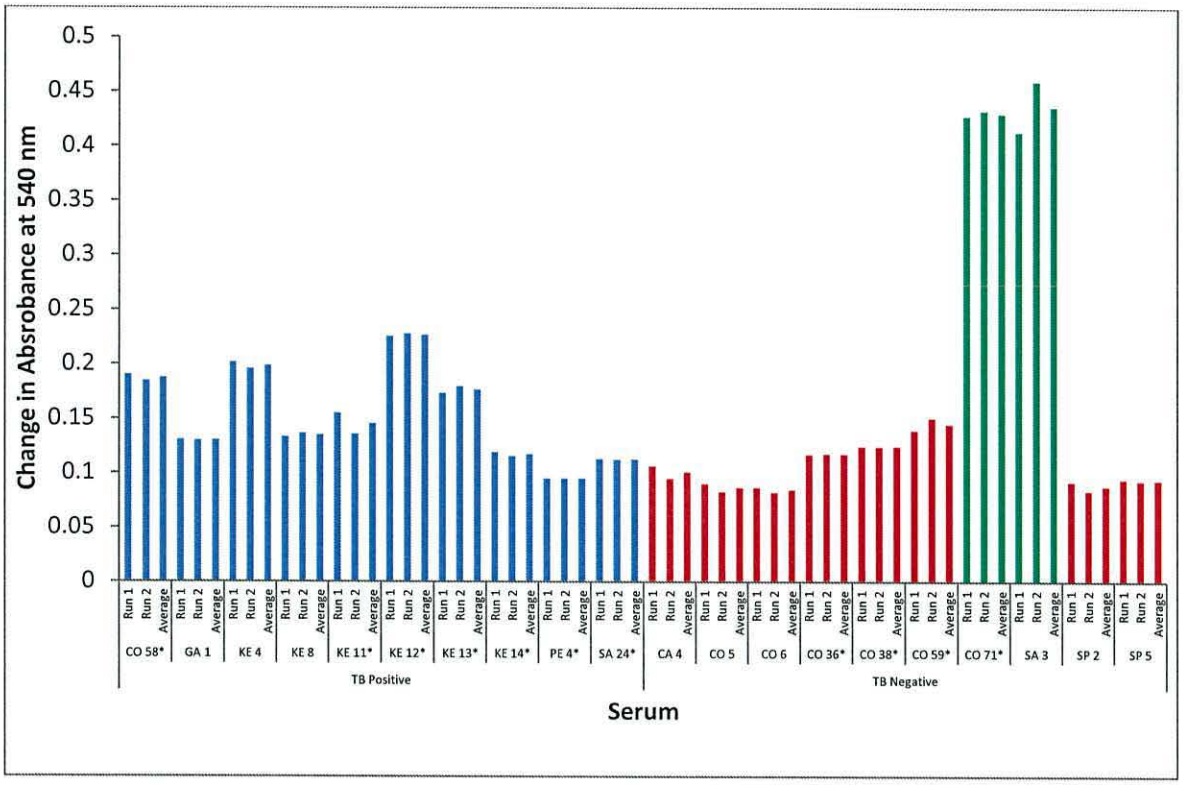


Figure 3.4.8 Graph showing the change in absorbance at 540 nm for the mycolic acid coated gold nanoparticle solutions over 30 minutes following addition of various serum samples.

The first observation from the graph is that the replicates of each sample are very consistent, which is important when developing a sensor for the detection of TB. Secondly, excluding the samples marked in green in the above graph, there is generally, a bigger change in the absorbance at 540 nm when using TB+ serum samples as compared to using TB- serum samples; this difference is, however, only small in some cases.

The samples shown above in green were clear outliers in the set of TB- serum samples, therefore it was decided to investigate these samples further. Upon inspection of these samples, it was found that they appeared 'cloudy' or opaque, whereas the other serum samples used in the experiment were transparent. This suggested that the change in absorbance with these two samples was not due to any changes in the SPR peak of the

coated gold nanoparticles, but to the addition of an opaque solution increasing the absorbance.

In order to test this hypothesis, other serum samples which appeared 'cloudy'/opaque were selected and analysed by this method, and these results can be seen in Figure 3.4.9 below:

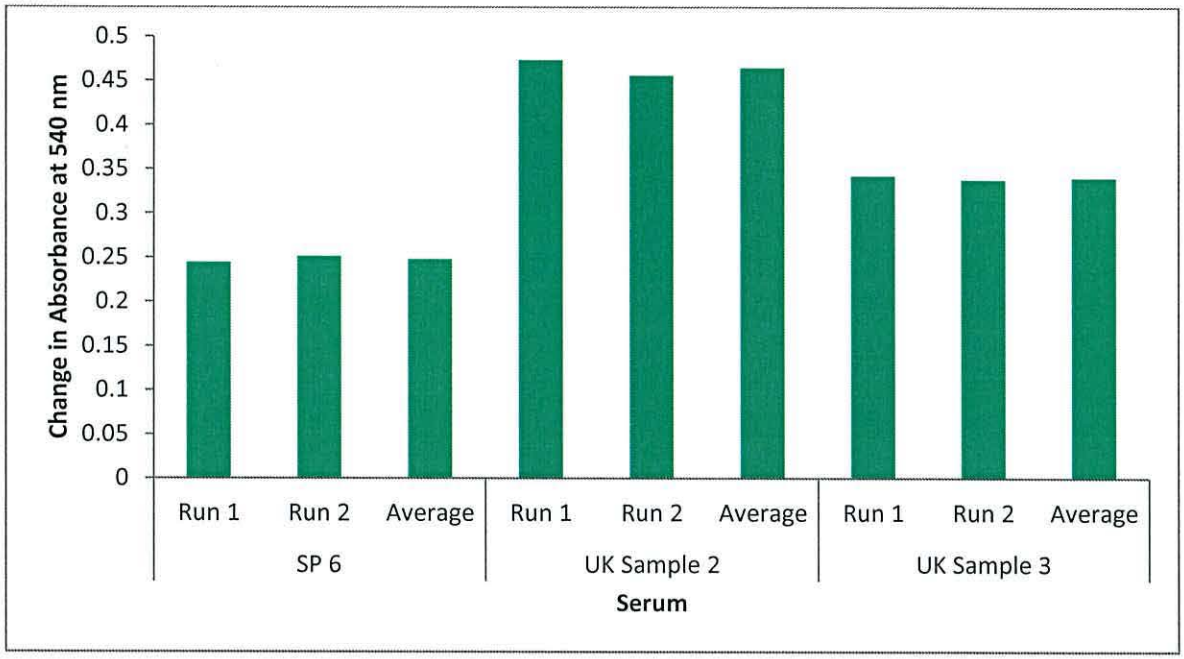


Figure 3.4.9 Graph showing the change in absorbance at 540 nm for the mycolic acid coated gold nanoparticle solutions over 30 minutes following addition of various 'cloudy'/opaque serum samples.

As can be seen from this graph there is a large change in absorbance over time with all of these samples, further confirming the hypothesis that the change in the absorbance for these samples is likely to be due to them being opaque, and not to a change in the SPR peak of the coated gold nanoparticles.

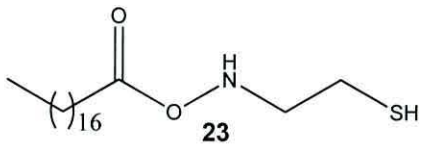
These observations therefore suggest that although this method shows some distinction between TB+ and TB- serum samples (see Figure 3.4.8), a drawback is that the method only works if the samples used are transparent.

3.4.7 Using Non-Thiol Modified Antigens

Later in the project, a method of binding non-thiol modified antigens onto gold nanoparticles was discovered (see Chapter 5). The experiments mentioned in this section were performed after this discovery.

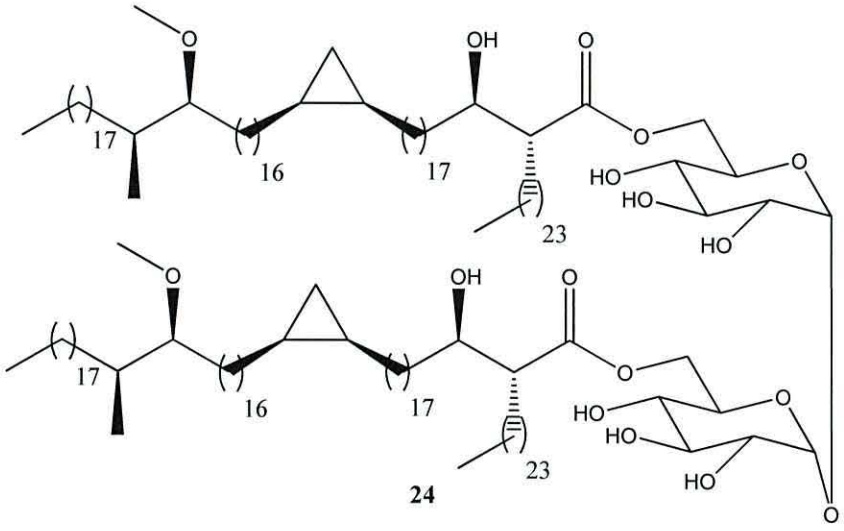
Running the Assay in Cuvettes

In order to establish whether the assay would work when using gold nanoparticles coated with non-thiol modified antigens, one TB+ serum sample (GA 1) and one TB- serum sample (BA 13) were analysed using gold nanoparticles coated with thio-stearic acid (**23**) and then coated with a methoxy TDM (**24**), the structures of which can be seen in Figure 3.4.10 and Figure 3.4.11, (the coating method can be seen in Section 2.4) and the UV-visible spectrum was measured over 30 minutes after addition of the serum sample to the coated gold nanoparticles.



23

Figure 3.4.10 Structure of thio-stearic acid (23).



24

Figure 3.4.11 Structure of the methoxy TDM (24).

Neat serum was again used for these experiments because using this concentration had previously given the best distinction between TB+ and TB- samples using this assay (see Section 3.4.4). The UV-visible spectra obtained from these experiments can be seen in the Appendix, Section 9.2.2. A graph showing the change in absorbance at 540 nm after serum addition, for both the TB+ and TB- serum samples, is shown in Figure 3.4.12.

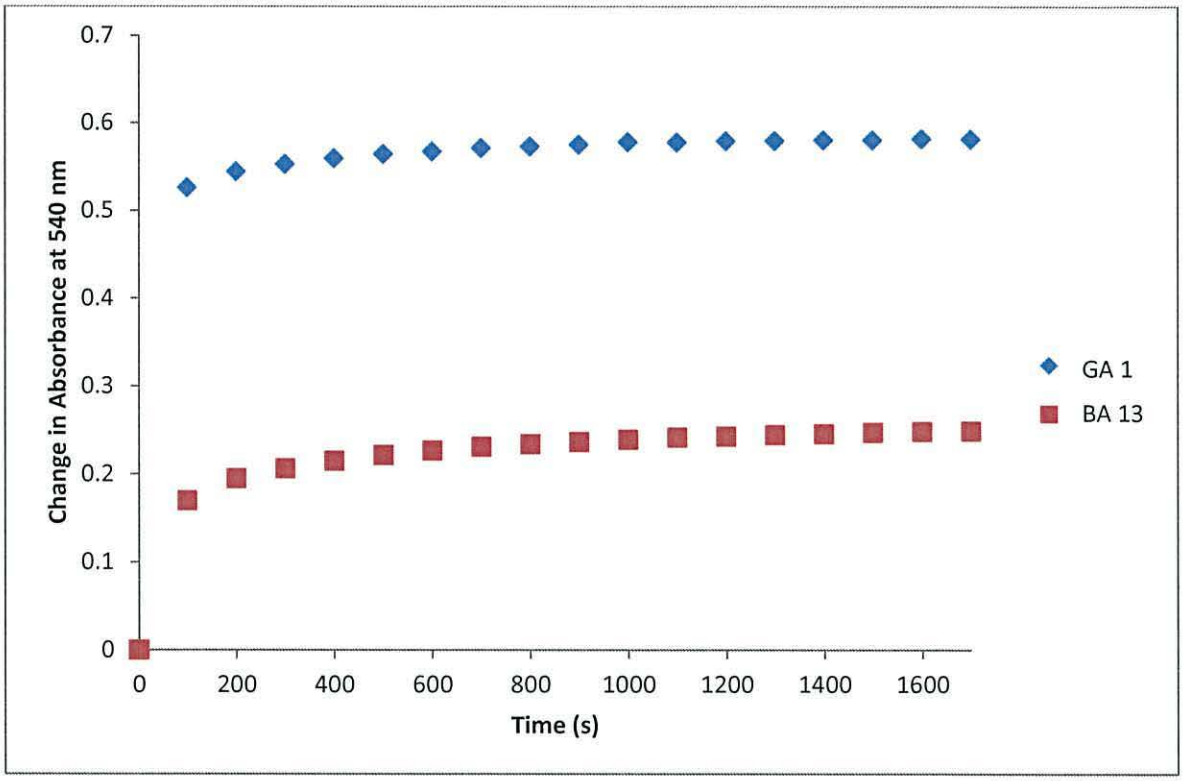


Figure 3.4.12 Graph showing the change in absorbance at 540 nm with time for the gold nanoparticles coated with thio-stearic acid followed by 24 after addition of a TB positive (GA 1) and a TB negative (BA 13) serum sample.

As when using gold nanoparticles coated directly with thiolated mycolic acid (see Section 3.4.4), a change in absorbance at 540 nm was observed when analysing both the TB+ and TB- samples. However, the change in absorbance was significantly larger when analysing the TB+ sample than when analysing the TB- sample. This would again be expected as there are more interactions with the antigen on the gold nanoparticle surface when analysing a TB+ sample, in turn leading to a larger change in absorbance at 540 nm.

These results show, that similar to those obtained when using gold nanoparticles coated directly with mycolic acid, a distinction between the TB+ and TB- serum samples can be obtained. For these experiments, no thiol modified antigen was required, which is a major advantage when synthesising antigens for use in a sensor for the detection of TB.

Running the Assay on a 96-well Plate

Having shown that using gold nanoparticles coated with non-thiol modified antigens can distinguish a TB+ serum sample from a TB- serum sample, it was decided to analyse a larger set of samples. The same serum samples as those used with gold nanoparticles coated directly with mycolic acid were used (see Figure 3.4.8), and the experiments were again performed on 96-well plates in order to reduce the amount of serum sample required

per test. Again, two replicates of each experiment were performed (designated as Run 1 and Run 2 in Figure 3.4.13) and the average change in absorbance for these two replicates is also shown.

Again, these experiments were carried out before the un-blinding of some of the serum samples and all samples which were tested blind are again designated by an asterisk in the graph.

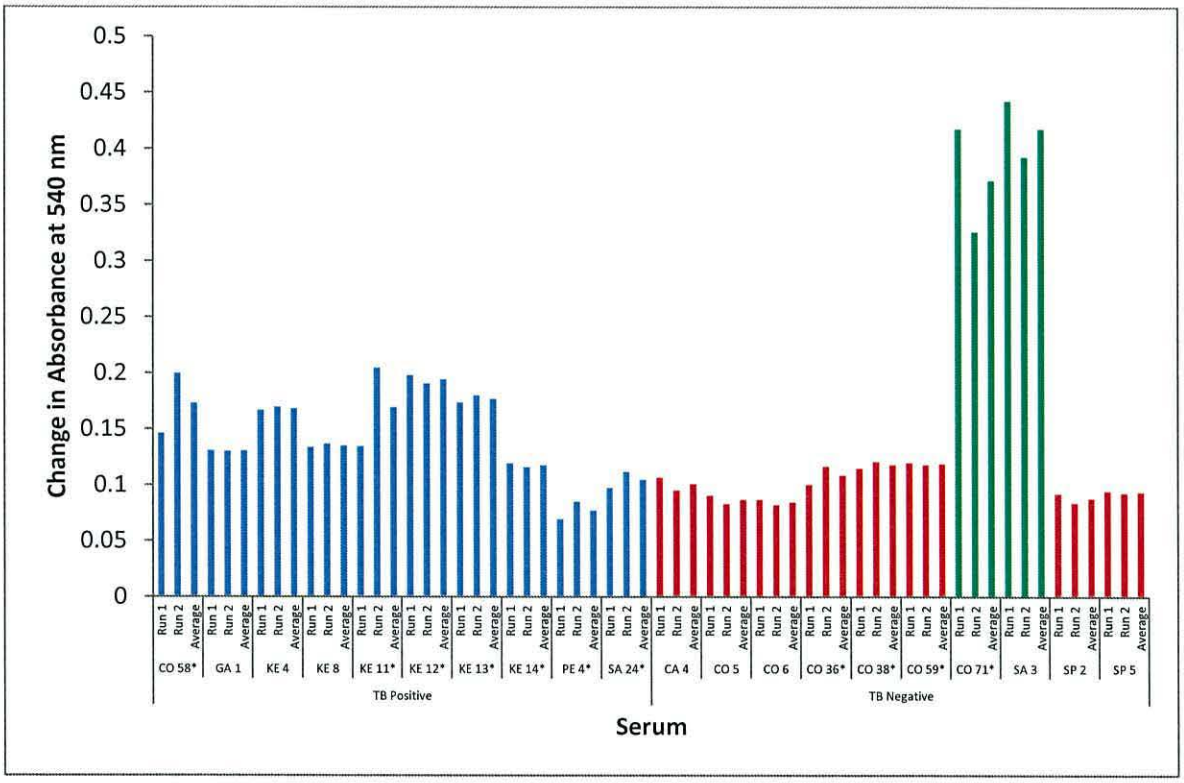


Figure 3.4.13 Graph showing the change in absorbance at 540 nm for the solutions of gold nanoparticles coated in thio-stearic acid followed by 24, over 30 minutes, following addition of various serum samples.

From this graph, it can be seen that, with the exception of the two samples labelled in green, a slightly bigger change in absorbance is again generally observed with the TB+ serum samples than with the TB- serum samples. However, similar to the results obtained when using gold nanoparticles coated directly with mycolic acid (see Figure 3.4.8), in some instances, these differences are only small.

The two samples labelled in green, CO 71 and SA 3, again show a very large change in absorbance in these experiments, again suggesting that only transparent serum samples can be used in this assay.

3.5 Concluding Remarks

Thiol modified mycolic acids have been successfully immobilised onto a gold surface and onto the surface of gold nanoparticles. An assay has been developed and optimised to detect the change in the SPR peak of the coated gold nanoparticles with time following addition of serum samples to the coated gold nanoparticles. This change in the SPR peak is caused by a change in the refractive index of the individual particles due to biomolecular interactions between the antigens coated onto the surface of the gold nanoparticles, and substances (believed to be antibodies) in the serum samples.

Even though in some cases the differences in the change in absorbance between TB positive and TB negative serum samples are small, the assay developed does show a slight distinction between TB positive and TB negative serum samples, and this distinction has been shown to be greatest when using un-diluted (neat) serum samples.

The assay can also be performed with non-thiol modified antigens, with similar results to those using a thiol modified mycolic acid being obtained. Even though the assay showed some distinction between the TB positive and TB negative serum samples, a problem was observed with samples which appeared 'cloudy' or opaque. In these cases, an increase in absorbance of the coated gold nanoparticle solution was observed after serum addition, which was un-related to biomolecular interactions, and caused purely due to the addition of an opaque solution. This suggested that this assay was only suitable for use on serum samples which appeared transparent.

3.6 References

1 S. Link and M. A. El-Sayed, **Size and temperature dependence of the plasmon absorption of colloidal gold nanoparticles,** *J. Phys. Chem. B,* 1999, **103,** 4212-4217.

2 K. L. Kelly, E. Coronado, L. L. Zhao and G. C. Schatz, **The Optical Properties of Metal Nanoparticles: The influence of size, shape, and dielectric environment,** *J. Phys. Chem. B,* 2003, **107,** 668-677.

3 X. Luo, A. Morrin, A. J. Killard and M. R. Smyth, **Application of nanoparticles in electrochemical sensors and biosensors,** *Electroanal.,* 2006, **4,** 319-326.

4 M. M. Walczak, D. D. Popenoe, R. S. Deinhammer, B. D. Lamp, C. Chung and M. D. Porter, **Reductive desorption of alkanethiolate monolayers at gold: A measure of surface coverage,** *Langmuir,* 1991, **7,** 2687-2693.

5 A. S. Viana, M. Kalaji, and L. M. Abrantes, **Self-assembled monolayers of vitamin B12 disulphide derivatives on gold,** *Electrochim. Acta,* 2002, **47,** 1587-1594.

6 IUPAC, **Compendium of chemical terminology, 2nd *ed.* (the "gold book"),** compiled by D. McNaught and A. Wilkinson, *Blackwell Scientific Publications, Oxford* (1997).

7 Confidential unpublished work.

8 M.-C. Daniel and D. Astruc, **Gold nanoparticles: Assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis and nanotechnology,** *Chem. Rev.,* 2004, **104,** 293-346.

9 S. K. Ghosh and T. Pal, **Interparticle coupling effect on the surface plasmon resonance of gold nanoparticles: From theory to applications,** *Chem. Rev.,* 2007, **107,** 4797-4862.

10 D. Roy, J. Quiles, D. C. Gaze, P. Collinson, J. C. Kaski and G. F. Baxter, **Role of reactive oxygen species on the formation of novel diagnostic marker ischaemia modified albumin,** *Heart,* 2006, **92,** 113-114.

11 P. Englebienne, **Use of colloidal gold surface plasmon resonance peak shift to infer affinity constants from the interactions between protein antigens and antibodies specific for single or multiple epitopes,** *Analyst,* 1998, **123,** 1599-1603.

12 N. Nath and A. Chilkoti, **A colorimetric gold nanoparticle sensor to interrogate biomolecular interactions in real-time on a surface,** *Anal. Chem.,* 2002, **74,** 504-509.

Chapter 4

The Development of a Colour-Based Assay for the Detection of TB

4.1 Introduction

Due to the limitations of the assay developed in the previous chapter, it was decided to try and develop another assay for the detection of TB, which would not be hindered by these limitations (see Section 3.5). With the aim of the project being to develop a rapid, portable, reliable and easy to use sensor for the detection of TB, a colour sensor (or assay) was targeted. Reports in the literature had shown that gold nanoparticles appear red in aqueous solution however upon addition of salt; the solution becomes blue due to aggregation of the gold nanoparticles leading to a red shift (a shift to higher wavelength) of the surface plasmon resonance band.[1] It has also been shown that aggregation can be prohibited following salt addition if complementary binding has occurred to modified oligonucleotides bound to the surface of gold nanoparticles.[2]

This chapter investigates the use of these principles to develop a colour sensor for the detection of TB. It was shown in the previous chapter that addition of serum samples to mycolic acid coated gold nanoparticles led to biomolecular interactions (believed to be antibody-antigen interactions) at the coated gold nanoparticle surface (see Section 3.4). It was also shown that a larger change in absorbance at 540 nm was generally observed when analysing TB positive serum samples than when analysing TB negative serum samples, suggesting more interactions at the coated gold nanoparticle surface when analysing TB positive serum samples (see Section 3.4).

It was believed that complementary binding to the lipid antigens coated onto the surface of the gold nanoparticles, when analysing TB positive serum samples, would prevent aggregation upon salt addition, leading to a red colour being observed for the coated gold nanoparticle solution. In instances where no complimentary binding occurs (when analysing TB negative serum samples), the coated gold nanoparticles would aggregate leading to a change in the colour of the solution from red to blue.

4.2 Initial Experiments

4.2.1 Inducing a Colour Change in the Coated Gold Nanoparticles

The first step in developing a sensor of this nature was to see if aggregation could be induced in the gold nanoparticles and in the mycolic acid coated gold nanoparticles. Firstly, it was decided to test if gold nanoparticles aggregated upon salt addition. Figure 4.2.1 (left) shows the UV-visible spectrum for un-coated gold nanoparticles (diluted

to a concentration of 1 in 2 in 10 mM phosphate buffer (pH 7.4), and Figure 4.2.1 (right) shows the UV-visible spectrum for the un-coated gold nanoparticles over 15 minutes after addition of a 1 M NaCl solution (100 µl in 900 µl of un-coated gold nanoparticles).

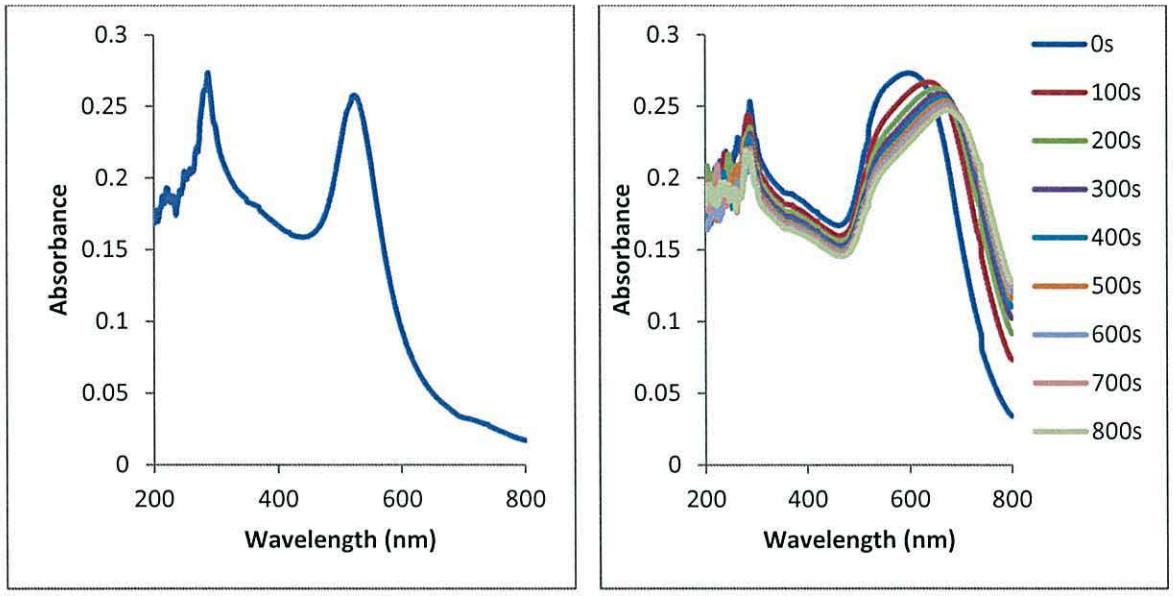


Figure 4.2.1 (left) UV-visible spectrum for un-coated gold nanoparticles. (right) UV-visible spectrum for un-coated gold nanoparticles over 15 minutes, after addition of a 1 M NaCl solution.

It can be seen from Figure 4.2.1 that aggregation does occur upon NaCl addition, with a change in wavelength at which the maximum absorbance is observed from 525 to 675 nm, corresponding to a change in the colour of the solution from red to blue. This colour change can be seen in Figure 4.2.2, which shows that there is a clear distinction in colour between the non-aggregated gold nanoparticles (left) and the gold nanoparticles which have aggregated after being subjected to the 1 M NaCl solution (right):

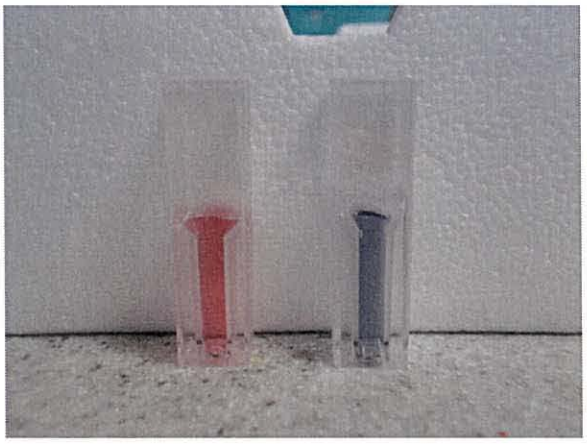


Figure 4.2.2 Cuvettes showing non-aggregated gold nanoparticles (left) and gold nanoparticles which have aggregated after being subjected to the 1 M NaCl solution (right).

Having seen that un-coated gold nanoparticles aggregate upon salt addition, the next step was to check whether this would still occur when using gold nanoparticles coated with mycolic acids. Figure 4.2.3 (left) shows the UV-visible spectrum for the mycolic acid (**22**) coated gold nanoparticles over 15 minutes after the addition of a 1 M NaCl solution and Figure 4.2.3 (right) shows the UV-visible spectrum over 15 minutes after the addition of a saturated aqueous solution of NaCl. In each instance, 100 μl of NaCl solution was added to 900 μl of coated gold nanoparticle solution.

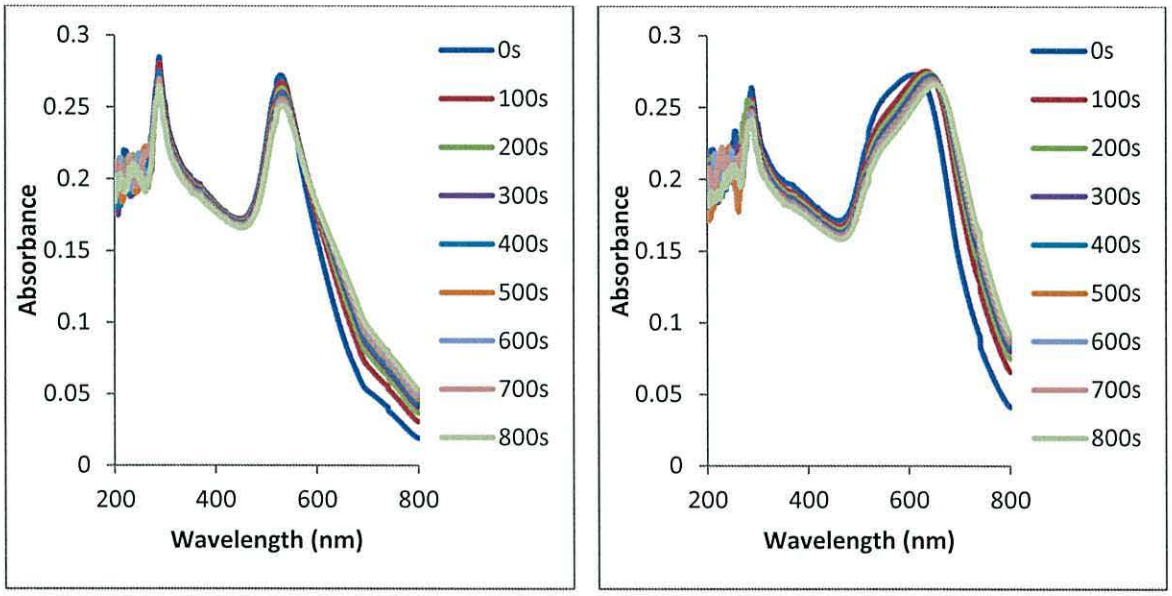


Figure 4.2.3 (left) UV-visible spectrum for gold nanoparticles coated with 22 over 15 minutes, after addition of a 1 M NaCl solution. (right) UV-visible spectrum for gold nanoparticles coated with 22 over 15 minutes, after addition of a saturated aqueous solution of NaCl.

It can be seen from these spectra that firstly, coated gold nanoparticles can aggregate upon salt addition. This can be observed by the change in the wavelength at which the maximum absorbance occurs after the addition of a saturated aqueous solution of NaCl (Figure 4.2.3 (right)), from 525 to 651 nm. It can also be observed that a higher concentration of NaCl was required to cause aggregation of the coated gold nanoparticles compared to the un-coated gold nanoparticles. Un-coated gold nanoparticles aggregated when subjected to a 1 M NaCl solution, however, only partial aggregation occurred with gold nanoparticles coated with **22**. This decrease in aggregation is likely to be due to mycolic acids binding to the gold nanoparticle surfaces, increasing steric hindrance between the particles and making it harder for them to aggregate. These results suggested that in order to give the coated gold nanoparticles the best chance of aggregating, a saturated aqueous solution of NaCl should be used.

4.2.2 Towards the Development of a Colour Sensor

Using the information gained above, it was decided to modify the previous assay (see Section 3.4), so that after a serum sample was incubated with the coated gold nanoparticles for 30 minutes, a saturated aqueous solution of NaCl was added, and a change in the UV-visible spectrum was recorded over 15 minutes. The full protocol for these experiments can be seen in Section 2.6.1.

Initially, a serum concentration of 1 in 5 was used for this experiment because this concentration had previously worked with the previous assay (see Section 3.4), and not enough serum was available to use it un-diluted. Initially, one TB+ (KE 1) and one TB- serum sample (KE 7) were used. It was expected that complimentary binding of antibodies to the mycolic acids coated onto the gold nanoparticle surfaces would occur with the TB+ sample, leading to no aggregation upon addition of the saturated aqueous solution of NaCl, while for the TB- sample, no complimentary binding was expected, leading to aggregation of the mycolic acid coated gold nanoparticles upon the addition of the salt solution.

No aggregation occurred when using the TB+ serum sample, however, there was no aggregation when using the TB- serum sample either (see Appendix, Section 9.3.1).

The assay therefore needed to be modified further. It was observed that at a 1 in 5 serum dilution no aggregation occurred (see Appendix, Section 9.3.1), but with no serum (effectively a 1 in infinity dilution), aggregation did occur (see Figure 4.2.3 (right)). It was therefore decided to dilute the serum samples used, and see at what concentration aggregation would start to occur for a TB- sample. This value would then be taken as a starting point, and a TB+ sample at the same concentration would also be run to see whether any aggregation occurred for it as well.

The TB- serum sample, KE 7, was analysed at the following concentrations: 1 in 10, 1 in 20, 1 in 40, 1 in 80, 1 in 160, 1 in 320, 1 in 2,500, 1 in 5,000 and 1 in 10,000. It was found that although there was slight aggregation of the coated gold nanoparticles when using a 1 in 2,500 serum concentration, more aggregation was observed when using a 1 in 5,000 serum concentration (Figure 4.2.4 (left)). It was therefore decided to test a TB+ sample (KE 1) at this concentration to see if any aggregation would occur. No significant aggregation was observed for this serum sample (see Figure 4.2.4 (right)) indicating that at this concentration, a difference could be observed between the TB+ and the TB- serum samples.

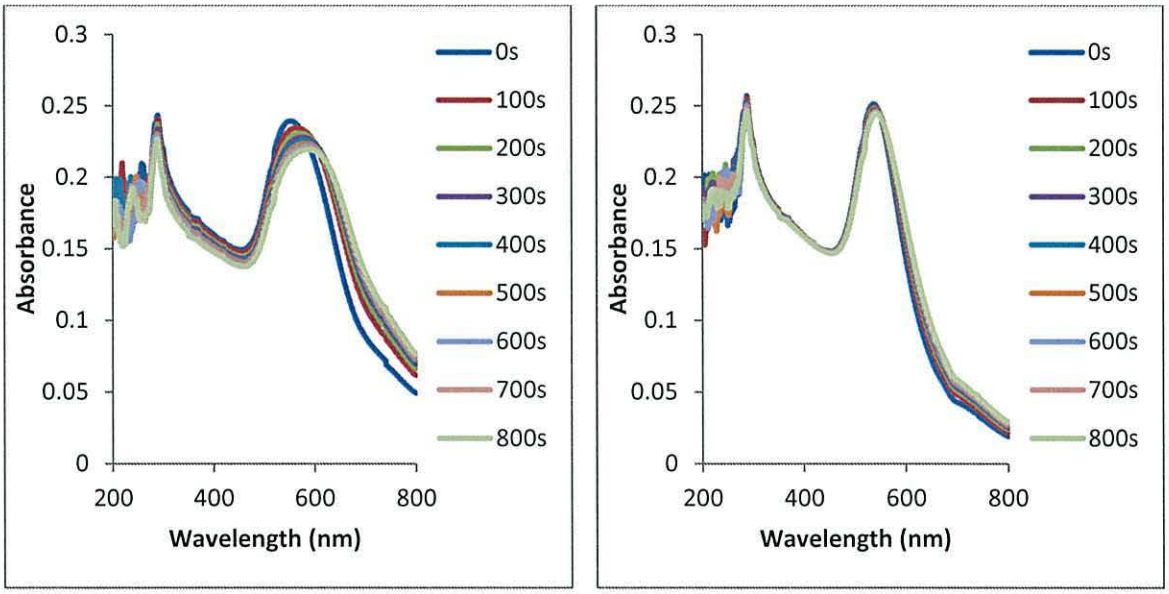


Figure 4.2.4 (left) UV-visible spectrum of 22 coated gold nanoparticles with KE 7 (a TB negative serum) (diluted to a concentration of 1 in 5,000) for 15 minutes after addition of saturated aqueous NaCl. (right) UV-visible spectrum of 22 coated gold nanoparticles with KE 1 (a TB positive serum) (diluted to a concentration of 1 in 5,000) for 15 minutes after addition of saturated aqueous NaCl.

It was decided from the results obtained to use a 1 in 5,000 serum dilution from this point forward unless otherwise stated (this concentration was changed to 1 in 2,500 when gold nanoparticles from BBInternational were used later in the project see Section 5.6). After being able to distinguish a TB+ serum sample from a TB- serum sample using this method, the next step was to see if the results could be reproduced. This was done using the same serum samples, and the experiment was repeated three times with each sample (the results can be seen in the Appendix, Section 9.3.1). The same pattern was observed with all of the replicates, with very little aggregation observed when using the TB+ sample, while aggregation was observed when using the TB- sample.

4.2.3 Testing a Larger Number of Serum Samples

In order to determine whether or not the assay worked for other serum samples, two more TB+, BA 1 and BA 2, and two more TB- samples, BA 6 and CO 11, were analysed, using gold nanoparticles coated with **22**, and the results of these experiments can be seen in the Appendix, Section 9.3.2. In essence, it could be seen from these spectra that the assay does work when analysing different serum samples, because these serum samples also showed a clear distinction between the TB+ and TB- sample.

Having seen a distinction between TB+ and TB- serum samples, more samples were analysed by this method. It was decided to run the seven remaining TB+ samples from the first group of serum samples, as well as ten TB- samples in order to further evaluate the

assay. At this point, each sample took 45 minutes to run, therefore, it was decided to only measure the UV-visible spectrum 15 minutes after the addition of a saturated solution of NaCl to the coated gold nanoparticle and serum solution instead of over 15 minutes after its addition.

In order to better visualise the data obtained, the results from these experiments are presented as a ratio of their absorbance at 540 nm to their absorbance at 630 nm. The absorbance value at 540 nm represents an absorbance value for the SPR peak without any aggregation of the gold nanoparticles, while the absorbance at 630 nm represents an absorbance value for the SPR peak of aggregated gold nanoparticles. This value therefore gives a representation of how much aggregation occurred in the coated gold nanoparticles. A large value represents little to no aggregation in the coated gold nanoparticles, while a small value represents an increase in the absorbance at 630 nm which relates to aggregation of the coated gold nanoparticles. Therefore, when analysing a TB+ serum sample, a large value would be expected, and when analysing a TB- serum sample, a small value would be expected.

Table 4.2.1 shows the results obtained from these experiments and Figure 4.2.5 shows a graphical representation of the data.

Table 4.2.1 Table showing the ratio of absorbance at 540 nm : 630 nm for multiple serum samples using gold nanoparticles coated with the thiol modified mycolic acid, 22.

TB Positive Serum Samples		**TB Negative Serum Samples**	
Serum Code	**Ratio of Absorbance at 540 nm : 630 nm**	**Serum Code**	**Ratio of Absorbance at 540 nm : 630 nm**
BA 4	0.907	SA 1	1.254
BA 5	1.167	SA 4	0.872
BA 11	1.181	BA 10	0.873
KE 2	1.974	BA 11	1.181
KE 3	1.788	CO 5	1.178
KE 4	1.034	CO 9	1.031
KE 5	2.065	CO 11	1.059
		CO 17	1.855
		CO 18	1.004
		PE 2	1.373

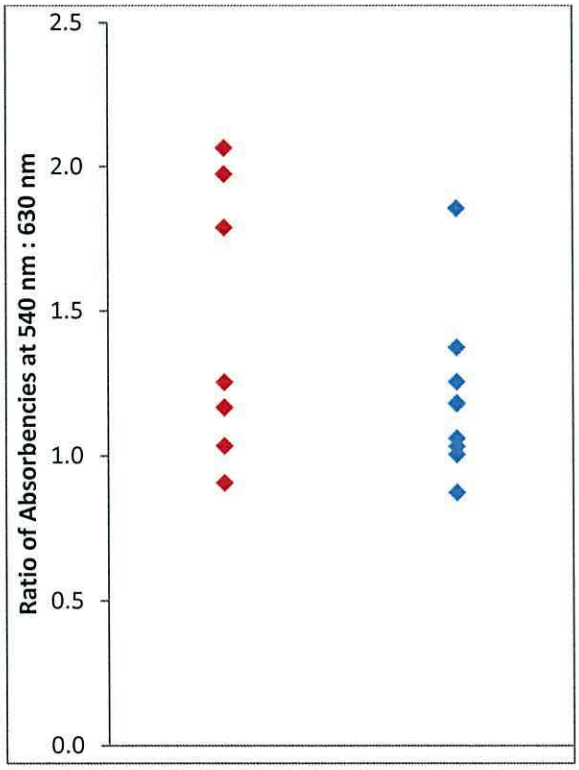


Figure 4.2.5 Graph showing the ratio of absorbencies at 540 nm : 630 nm for 7 TB positive serum samples (red) and 10 TB negative serum samples (blue).

It can be seen from this data that even though there is a distinction between some of the TB+ and some of the TB- serum samples, there is no clear overall distinction between the two sets of samples. Three of the seven TB+ samples have a ratio of higher than 1.5, while nine of the ten TB- samples have a ratio below this value. This does suggest a slight distinction between the TB+ and TB- serum samples, however four of the seven TB+ samples gave a ratio value indicative of a TB- sample and one of the ten TB- samples gave a ratio value indicative of a TB+ sample.

This suggested that the assay required further optimisation, and possibly that the antigen that was currently coated onto the gold nanoparticles was not the best one to distinguish TB+ serum samples from TB- serum samples. This had also been found for the ELISA results for human serum samples which suggested that mycolic acids do not distinguish TB+ serum samples from TB- serum samples as well as TDMs and TMMs.[3] Another drawback of this assay was the time required to analyse serum samples. Even though a single serum sample could be analysed much quicker than one serum sample could be analysed by ELISA, with each sample only taking 45 minutes to analyse, only one serum sample could be analysed at a time with the current setup, without any replicates.

Therefore, work was also undertaken to develop a new format for this assay which would allow more samples to be run simultaneously.

Due to potentially being able to analyse more serum samples and/or more variables of the assay if a different platform was developed, this was investigated first.

4.3 Performing the Assay on 96-Well Plates

Instead of performing the assay in cuvettes, it was decided to try to perform it on 96-well plates. This had the advantage of being able to analyse more samples at a time, with replicates, while also requiring less gold nanoparticles and antigen per serum sample analysed, therefore reducing the cost of the assay. The only drawback of performing the assay on 96-well plates was that the amount of data obtained would be reduced, with absorbencies at certain wavelengths only available at the end of the assay, and not the full spectrum over a period of time as was available when performing the assay in cuvettes.

The full protocol for performing these experiments can be seen in Section 2.6.2, and any amendments to this procedure are discussed where appropriate in this Chapter. For the initial experiments, the absorbance at 570 and 630 nm was read at the end of the assay instead of at 540 and 630 nm, due to no filter for reading the 96-well plates at 540 nm being available at this point in the project.

4.3.1 Initial Experiments Using Gold Nanoparticles Coated with 22

In order to make sure that the method worked on 96-well plates, it was decided to analyse multiple serum samples with gold nanoparticles coated with the thiol modified mycolic acid, **22**. It was decided to analyse all 50 samples from the first group of serum samples (see Appendix 9.1.2) as well as 25 TB- serum samples from the UK. A full list of the serum samples used for these experiments can be seen in the Appendix, Section 9.3.3.

The following Figure shows the absorbance values obtained at 630 nm at the end of the assay for each serum sample by category. The individual value obtained with each serum sample can be seen in the Appendix, Section 9.3.3. The absorbance value at 630 nm is quoted because this gives a representation of how much aggregation has occurred for the coated gold nanoparticles at the end of the assay (see Section 4.2.3). Therefore, for a TB+ serum sample, a low absorbance value would be expected at 630 nm at the end of the assay due to little or no aggregation of the coated gold nanoparticles, while for TB- serum

samples, a higher absorbance value at 630 nm would be expected due to aggregation of the coated gold nanoparticles.

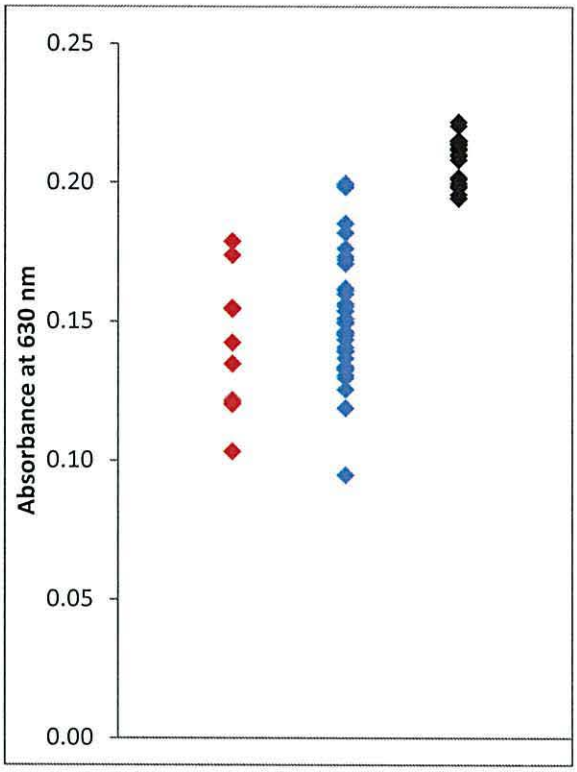


Figure 4.3.1 Graph of the absorbencies at 630 nm of different serum samples by category (red – 10 TB positive samples obtained from the WHO, blue – 40 TB negative samples obtained from the WHO, black – 25 TB negative samples from the UK).

It can be seen that the TB+ serum samples generally gave lower absorbencies at 630 nm at the end of the assay than TB- serum samples, with the TB+ samples giving an average absorbance value of 0.14 while the TB- samples gave an average absorbance value of 0.15. The TB- serum samples from the UK (which have an average absorbance value of 0.21) gave a higher absorbance than the TB- samples obtained from the WHO, meaning more aggregation occurred for those samples. All of the serum samples obtained from the WHO came from patients who were suspected of having TB, and who had TB like symptoms, while the pateients from which the samples were obtained were also generally more likely to be exposted to TB than the individuals from which the UK samples were obtained. This could lead to these TB- serum samples obtained from the WHO containing more antibodies that can bind to the mycolic acid antigen coated onto the gold nanoparticle surface, in turn leading to less aggregation in the coated gold nanoparticles analysed with those samples, compared to those analysed with TB- serum samples from the UK.

One of the problems in analysing the results obtained from the assay by only looking at the absorbance at the end of the assay at 630 nm is that slight natural variation in the absorbance value of the coated gold nanoparticles at this wavelength would not be accounted for, and could influence the results obtained. Therefore, as was done earlier (see Page 82), it was decided to take the ratio of two different absorbencies at the end of the assay, as this was expected to eliminate this problem. Any natural variation in absorbance at 630 nm, should also be reflected by a natural variation in absorbance at 540 or 570 nm, and taking a ratio of the absorbance at 540 nm : 630 nm (or 570 nm : 630 nm for the initial experiments) should eliminate it. Another possible source of variation in absorbance was during the centrifugation and re-suspension process in coating and preparing the gold nanoparticles for use in the assay (see Section 2.6). Slightly varying gold nanoparticle concentrations could be obtained from day to day due to possible loss of the nanoparticles during this process, which would lead to a different 'base' absorbance each day. Again, taking the ratio of absorbencies at 540 nm : 630 nm (or 570 nm to 630 nm) would help negate this problem because a decrease in absorbance at 630 nm due to a decrease in the concentration of the coated gold nanoparticles would also be reflected by a decrease in the absorbance at 540 nm (or 570 nm).

Figure 4.3.2 shows a graphical representation of the data already presented in Figure 4.3.1 now as a ratio of absorbencies at 570 nm : 630 nm. When analysing the data by looking at the ratio of absorbencies at 570 nm : 630 nm (or 540 nm : 630 nm) a larger value represents little to no aggregation, while smaller values represent more aggregation (due to an increase in the absorbance at 630 nm). Therefore, when analysing TB+ serum samples, large values would be expected, while smaller values would be expected when analysing TB- serum samples. The values obtained with each individual serum sample can be observed in the Appendix, Section 9.3.3.

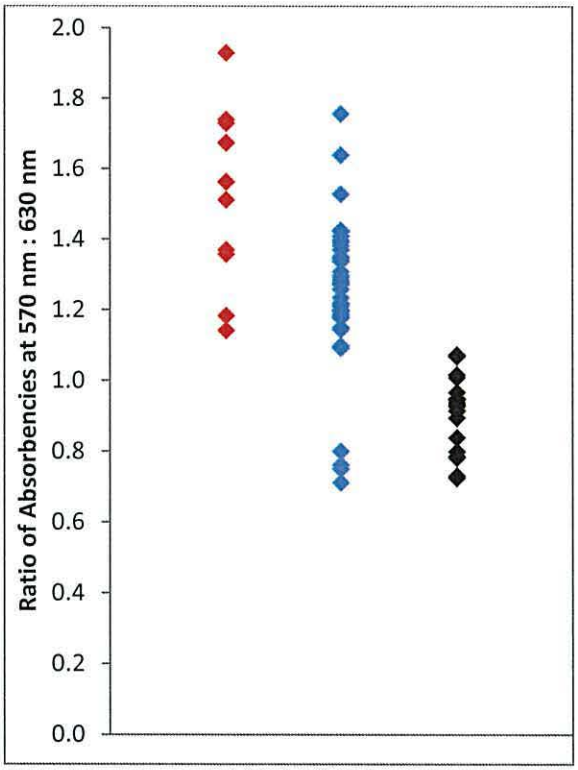


Figure 4.3.2 Graph showing the ratio of absorbencies at 570 nm : 630 nm for different serum samples by category (red – 10 TB positive samples obtained from the WHO, blue – 40 TB negative samples obtained from the WHO, black – 25 TB negative samples from the UK).

Although some overlap was observed between the TB+ and the TB- serum samples obtained from the WHO, this graph does again show some distinction between the TB+ and the TB- serum samples, with six of the ten TB+ samples having a ratio value of greater than 1.5, while only three of the sixty five TB- samples analysed gave a ratio value of greater than 1.5.

4.3.2 Comparison of Results with ELISA Results

In order to determine whether or not the antigen used was a good antigen for differentiating TB+ serum samples from TB- serum samples, the corresponding non-thiolated mycolic acid, **25**, was analysed with the same serum samples by ELISA. These samples were run by Dr Alison Jones, using anti-human IgG (whole molecule) secondary antibody conjugate, and the results can be seen in Figure 4.3.3. A full list of the values obtained with each individual serum sample can be seen in the Appendix, Section 9.3.3.

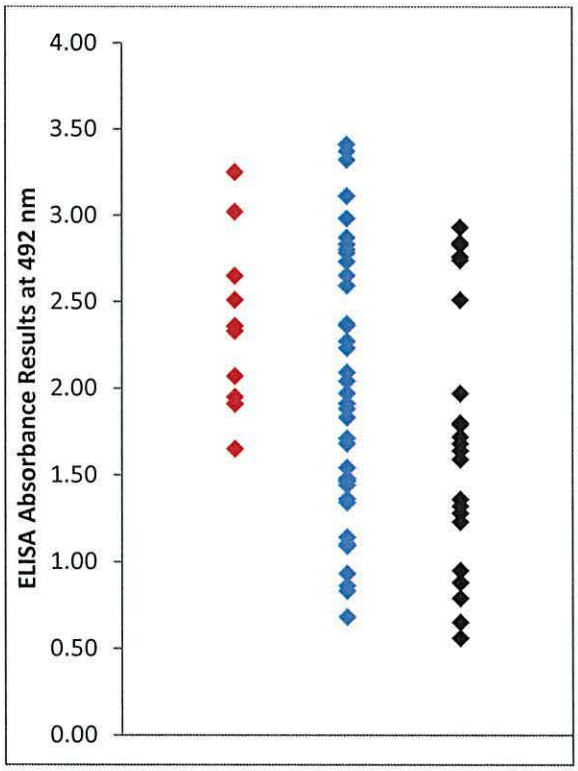


Figure 4.3.3 Graph showing the ELISA results obtained when using 25 as the antigen for different serum samples by category (red – 10 TB positive samples obtained from the WHO, blue – 40 TB negative samples obtained from the WHO, black – 25 TB negative samples from the UK).

By looking at Figure 4.3.3, it can be seen that there is some distinction between the TB+ serum samples and the TB- serum samples, however, as with the results obtained when using the gold nanoparticle based assay, a lot of overlap in the data is observed between the TB+ samples and the TB- samples obtained from the WHO. These results suggest that the methoxy mycolic acid used was not a great antigen for distinguishing TB+ serum samples from TB- serum samples.

A promising observation for the development of the gold nanoparticle assay was that the data for the TB- serum samples from the UK seemed much more closely packed when using that assay than the data obtained using the ELISA assay.

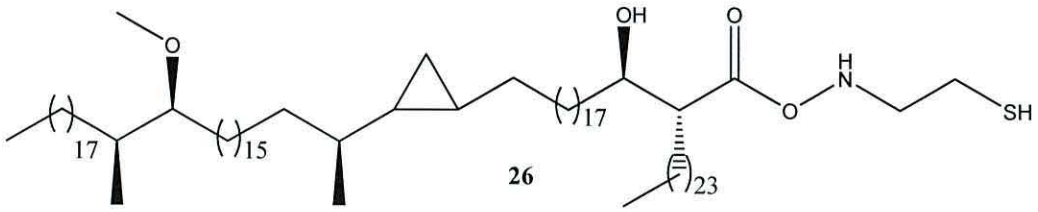
4.4 Analysis of Serum Samples with Other Antigens

Having observed some distinction between TB+ and TB- serum samples when using gold nanoparticles coated with **22**, it was decided to analyse serum samples with different thiolated antigens. At this point in the project, three other thiolated antigens were available for use: a thiolated methoxy mycolic acid with the thiol functional group on the acid group of the molecule (**26**) (the structure of which can be seen in Figure 4.4.1 on the next page);

a thiolated natural mycolic acid mixture (with the thiol group on the acid group of the molecules); and thio-stearic acid (**23**).

Analysis of Serum Samples with Gold Nanoparticles Coated with a Methoxy Mycolic Acid with the Thiol Functional Group on the Acid End of the Molecule

Firstly, it was decided to investigate the effect of coating the gold nanoparticles with a methoxy mycolic acid with the thiol functional group on the acid group of the molecule, the structure of which can be seen in Figure 4.4.1, on the assay. If this compound could distinguish TB+ serum samples from TB- serum samples as well as, or better than, **22**, which contains the thiol functional group on the alkyl chain of the molecule, this would be advantageous. The syntheses of mycolic acids with a thiol functional group on the acid group of the molecule are much quicker than the synthesis of mycolic acids with the thiol functional group on the alkyl chain of the molecule. Also, any mycolic acid can be modified to contain a thiol functional group on its acid group in two synthetic steps.[4]



26

Figure 4.4.1 Structure of the thiol modified mycolic acid, 26.

Because gold nanoparticles coated with **26** were going to be compared to gold nanoparticles coated with **22**, a smaller set of twenty two serum samples was analysed, including nine TB+ samples, five TB- samples obtained from the WHO and eight TB- samples from the UK. These samples were selected because they gave a good spread of data (*i.e.* values far apart) within their respective categories when using gold nanoparticles coated with **22** for the assay, so they would therefore give a good overview of the serum set. Some serum samples, for example the TB+ sample obtained from the WHO which gave the highest response previously (KE 1), was not used because only a small amount of the sample remained, therefore it was decided to preserve these serum samples in case they would be more valuable for future experiments.

In order to show a clear comparison of the results, Figure 4.3.2 was simplified to contain the data obtained with **22**, for only the 22 serum samples used in this experiment (Figure 4.4.2 (left)), and the results obtained when using gold nanoparticles coated with **26** for the

assay is shown in Figure 4.4.2 (right). The values obtained with each individual serum sample can be seen in the Appendix, Section 9.3.4.

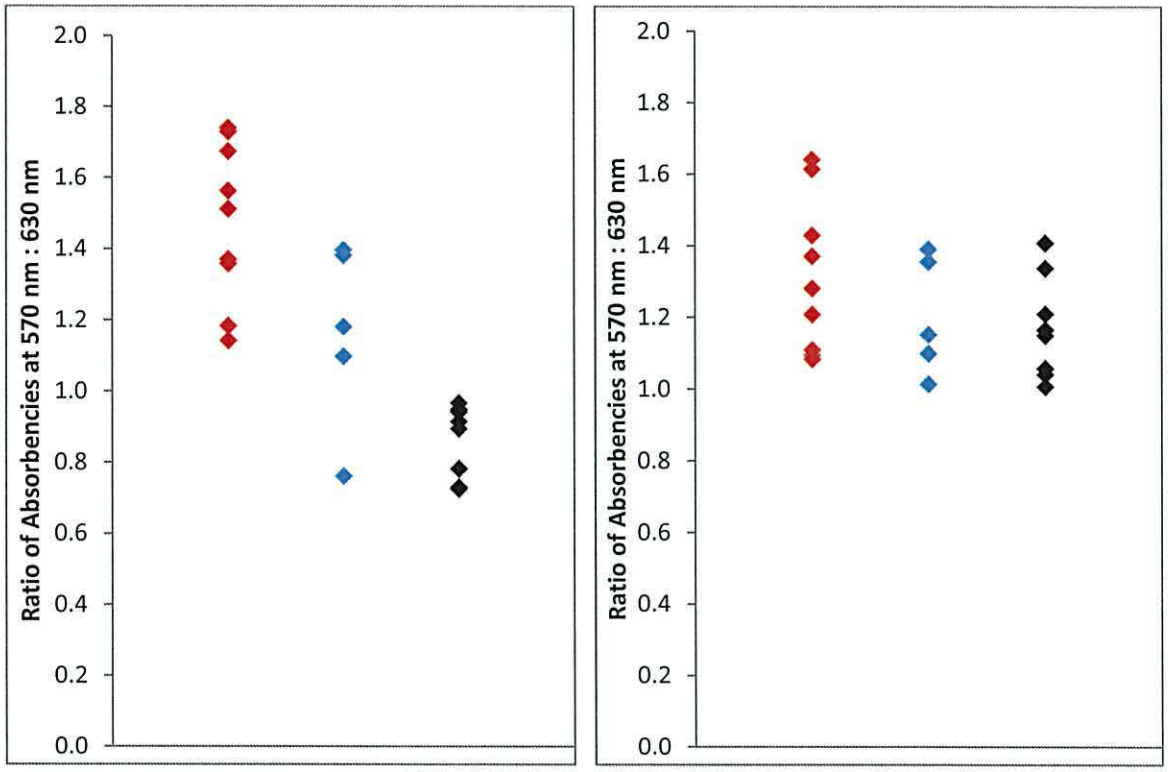


Figure 4.4.2 Graph showing the ratio of absorbencies at 570 nm : 630 nm for different serum samples by category when using gold nanoparticles coated with 22 (left) and 26 (right) for the assay (red – 9 TB positive samples obtained from the WHO, blue – 5 TB negative samples obtained from the WHO, black – 8 TB negative samples from the UK).

It can be seen that only a slight distinction is observed between the TB+ and TB- serum samples when using gold nanoparticles coated with **26** in the assay compared to when **22** was used. When using **22**, an average value of 1.47 was obtained with the TB+ samples, and 1.16 with the TB- samples while values of 1.31 and 1.16 respectively were obtained when using **26**. A more significant difference was observed between using gold nanoparticles coated with **22** and **26** when analysing the TB- samples from the UK, with the values obtained for these samples being much higher when using gold nanoparticles coated with **26** (an average value of 1.17) than they were when using gold nanoparticles coated with **22** (an average value of 0.86).

These results suggest that the orientation of the antigen on the gold nanoparticle surfaces has a significant effect on the results obtained in the assay. It seems that by placing the thiol group on the alkyl chain of the mycolic acid, selective binding occurs between the antibodies in the serum and the mycolic acid leading to a significant difference being

observed in the values obtained at the end of the assay for TB+ and TB- serum samples. However, placing the thiol group on the acid group of the molecule significantly reduces the distinction observed. When using gold nanoparticles coated with this antigen, all of the values obtained for the TB+ serum samples were above 1, suggesting that only partial aggregation of the gold nanoparticles has occurred. However, the values obtained for all of the TB- serum samples, in Figure 4.4.2 (right) are also above 1, again suggesting that aggregation of the coated gold nanoparticles is inhibited. These results therefore suggest that when using a mycolic acid with the thiol group on the acid side of the molecule, more non-selective binding occurs between the mycolic acid and the antibodies in the serum, thus leading to a poorer distinction between the TB+ and TB- serum samples when compared to gold nanoparticles coated with **22**, having a thiol group on the alkyl chain.

Analysis of Serum Samples with Gold Nanoparticles Coated with a Natural Mycolic Acid Mixture with the Thiol Functional Group on the Acid End of the Molecule

Having attempted to perform the assay with gold nanoparticles coated with a methoxy mycolic acid containing a thiol group on the acid group of the molecule, **26**, it was decided to perform the assay with a natural mycolic acid mixture containing a thiol group on the acid group of the molecules. Even though the results obtained with **26** were worse than when using **22**, another antigen with a thiol on the acid group of the molecule was used in order to make sure that this trend was observed again. The same serum samples were used as for the previous experiments in this Section, and the results obtained are shown in Figure 4.4.3. The values obtained with each individual serum sample can be seen in the Appendix, Section 9.3.4:

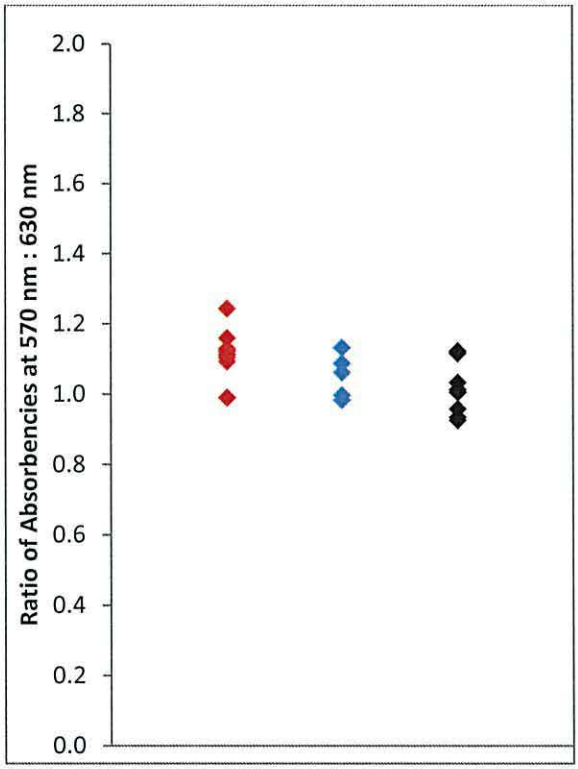


Figure 4.4.3 Graph showing the ratio of absorbencies at 570 nm : 630 nm for different serum samples by category when using gold nanoparticles coated with a natural mycolic acid mixture with a thiol group on the acid group of the molecules for the assay (red – 9 TB positive samples obtained from the WHO, blue – 5 TB negative samples obtained from the WHO, black – 8 TB negative samples from the UK).

Again, from this Figure, it can be seen that no real distinction is observed between the TB+ and TB- serum samples, with the TB+ samples giving an average value of 1.12 and the TB- samples giving an average value of 1.03. These results further suggest that mycolic acid antigens bound onto gold nanoparticles *via* a thiol group on the acid group of the molecules do not give as good a distinction between TB+ and TB- serum samples as antigens that are bound to gold nanoparticles *via* a thiol group on their alkyl chains, when using this assay.

Analysis of Serum Samples with Gold Nanoparticles Coated with Thio-stearic acid with the Thiol Functional Group on the Acid End of the Molecule

The final thiol modified antigen that was used at this point was thio-stearic acid (**23**). This compound was not expected to distinguish TB+ samples from TB- samples because it is not known to be antigenic to antibodies against TB, therefore it acted as a control in order to make sure that it was the mycolic acids coated onto the gold nanoparticle surface that were responsible for the distinction observed previously, between TB+ and TB- serum samples.

The same serum samples as were used for the previous experiments in this Section were used in the assay with gold nanoparticles coated with thio-stearic acid, and the results obtained are shown in Figure 4.4.4. The values obtained with each individual serum sample can be seen in the Appendix, Section 9.3.4:

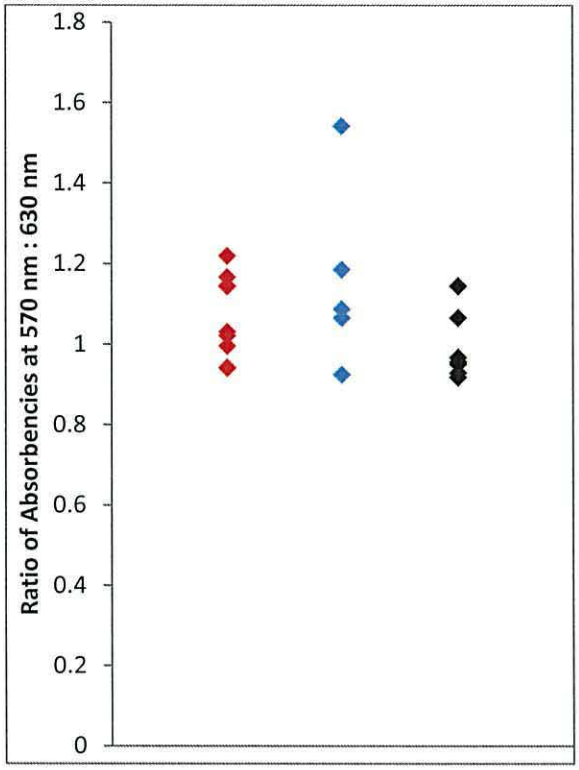


Figure 4.4.4 Graph showing the ratio of absorbencies at 570 nm : 630 nm for different serum samples by category when using gold nanoparticles coated with thio-stearic acid for the assay (red – 9 TB positive samples obtained from the WHO, blue – 5 TB negative samples obtained from the WHO, black – 8 TB negative samples from the UK).

It can be seen that there is no distinction between the TB+ (which gave an average absorbance value of 1.07) and the TB- serum samples (which gave an average value of 1.05) when using gold nanoparticles coated with thio-stearic acid. With the exception of one serum sample, SA 1, all of the ratio values obtained at the end of the assay were relatively low, suggesting aggregation of the coated gold nanoparticles had occurred after addition of NaCl, in turn suggesting no selective binding had occurred to the antigen coated onto the gold nanoparticle surfaces, and no distinction was observed between the TB+ and TB- serum samples. These results therefore suggest that the distinction between TB+ and TB- serum samples previously observed when coating gold nanoparticles with thiol modified mycolic acids (see Figure 4.3.2 and Figure 4.4.2) was due to the presence of the mycolic acid bound onto the surface of gold nanoparticles.

4.5 Further Optimisation of the Assay

It was decided at this stage to further optimise the assay. Since the assay did not involve many steps, only a few variables needed to be investigated.

The optimum serum concentration required had been investigated earlier (see Section 4.2.2), while it had also been determined that a saturated aqueous solution of NaCl should be used due to lower concentrations not being able to aggregate coated gold nanoparticles, and to give coated gold nanoparticles subjected to TB- serum samples the best chance to aggregate (see Section 4.2.1). The variables left to investigate were the amount of thiol modified antigen that was used to coat the gold nanoparticles, the amount of time the serum was left with the coated gold nanoparticles before addition of the NaCl solution and the amount of time allowed between addition of the NaCl solution and the measurement of the solution's absorbance. These were therefore investigated in turn.

4.5.1 Changing the Amount of Antigen

The first variable that was investigated was the amount of antigen used to coat the gold nanoparticles. For the previous experiments, a 5 μM solution of **22** had been used, therefore it was decided to vary the concentration of this solution, and look at any changes observed at the end of the assay. Thiol modified antigen solutions of the following concentrations were prepared, and used to coat gold nanoparticles: 0.31 μM, 1.25 μM, 20 μM, 80 μM.

The same serum samples were used as for the experiments in Section 4.4. The data obtained previously when gold nanoparticles were coated with a 5 μM solution of **22** as well as the results obtained from these experiments are shown in Figure 4.5.1 while a summary of the results are shown in Table 4.5.1. The values obtained with each individual serum sample can be seen in the Appendix, Section 9.3.5:

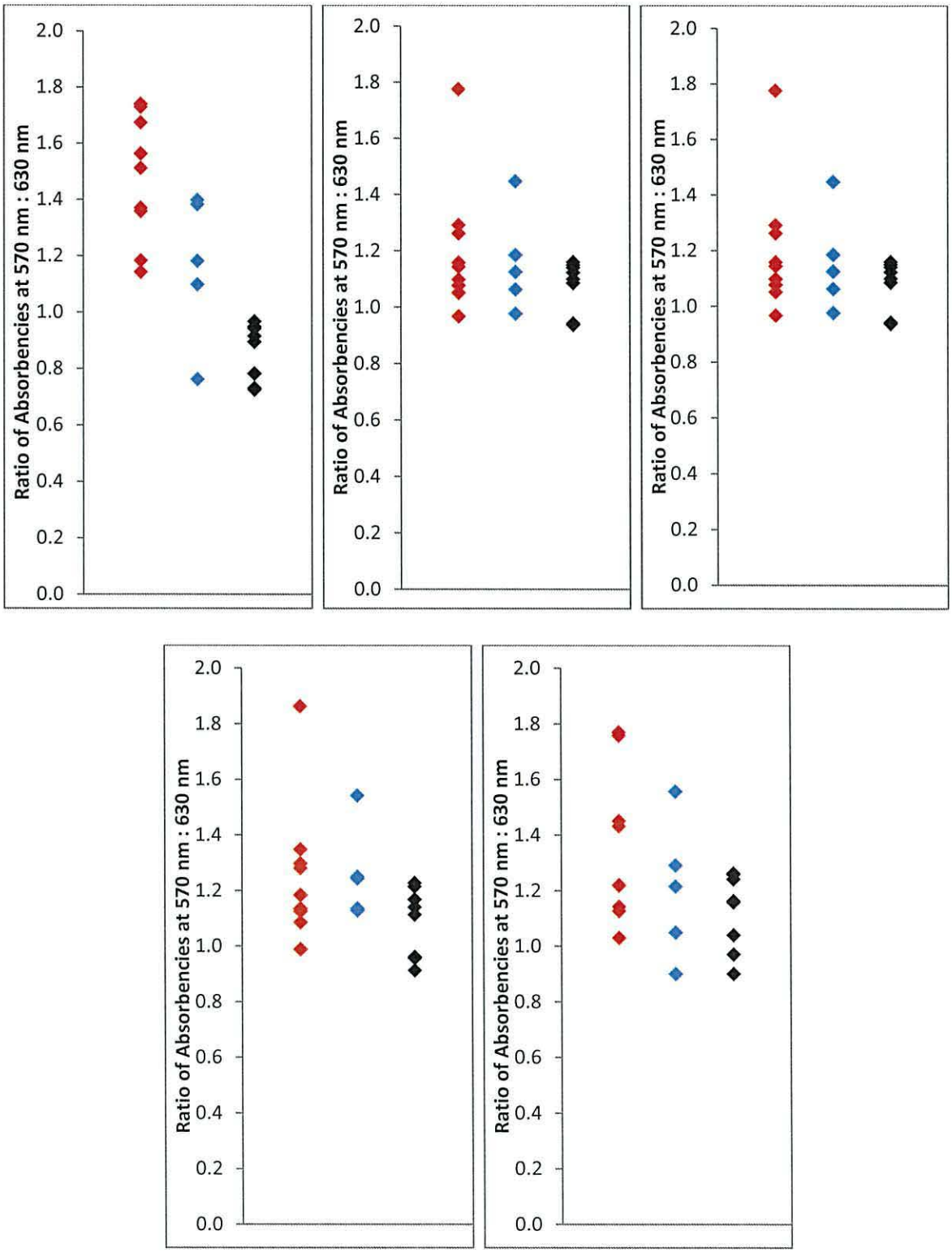


Figure 4.5.1 Graph showing the ratio of absorbencies at 570 nm : 630 nm for different serum samples by category when using gold nanoparticles coated with a 5 μM (top left), 0.31 μM (top middle), 1.25 μM (top right), 20 μM (bottom left) and 80 μM (bottom right)solution of 22 for the assay (red – 9 TB positive samples obtained from the WHO, blue – 5 TB negative samples obtained from the WHO, black – 8 TB negative samples from the UK).

Table 4.5.1 Table showing the average ratio of absorbencies at 570 nm : 630 nm for the different serum classes.

	Concentration of the Solution of 22				
Serum Category	0.31 μM	1.25 μM	5 μM	20 μM	80 μM
TB+	1.19	1.22	1.47	1.24	1.34
TB-	1.16	1.18	1.16	1.26	1.20
UK-	1.08	1.05	0.86	1.09	1.12

It can be seen, that generally changing the amount of antigen used to coat the gold nanoparticles does not have a large effect on the results obtained. The results did however suggest that the best distinction between the TB+ and TB- samples was observed when coating the nanoparticles with a 5 μM solution of antigen, suggesting that there was no advantage to using more thiol modified antigen to coat the nanoparticles. It was therefore decided to continue coating the gold nanoparticles with a 5 μM solution of antigen.

4.5.2 Varying the Times for Each Stage of the Assay

The final variables investigated were the amount of time the serum was left with the coated gold nanoparticles before addition of the NaCl solution and the time between addition of the NaCl solution and the measurement of the solution's absorbance. Prior to performing these experiments, serum samples were left with the coated gold nanoparticles for 30 minutes before saturated aqueous NaCl was added, and in instances where the assay was performed on 96-well plates, the absorbance of the solution was measured 15 minutes after addition of the NaCl solution.

When developing an assay or sensor for the detection of TB, rapid analysis of the serum samples is an important factor. It would therefore be advantageous to try and make the procedure as quick as possible without compromising the quality of the results obtained. An experiment was therefore designed to look at the effect of changing the time the serum was left with the coated gold nanoparticles before addition of the NaCl solution. One TB+, KE 3, and one TB- serum sample, KE 7, were used for these experiments, and the gold nanoparticles were again coated with **22**. The serum was left with the coated gold nanoparticles for various times before saturated aqueous NaCl was added, and an UV-visible spectrum was taken 15 minutes after its addition. A typical UV-visible spectrum obtained when analysing the TB+ and TB- samples can be seen in Figure 4.5.2 (red and blue respectively). As there was no significant change in the spectra obtained at

various times, only spectra obtained after 5 minutes are shown here; all others can be seen in the Appendix, Section 9.3.6.

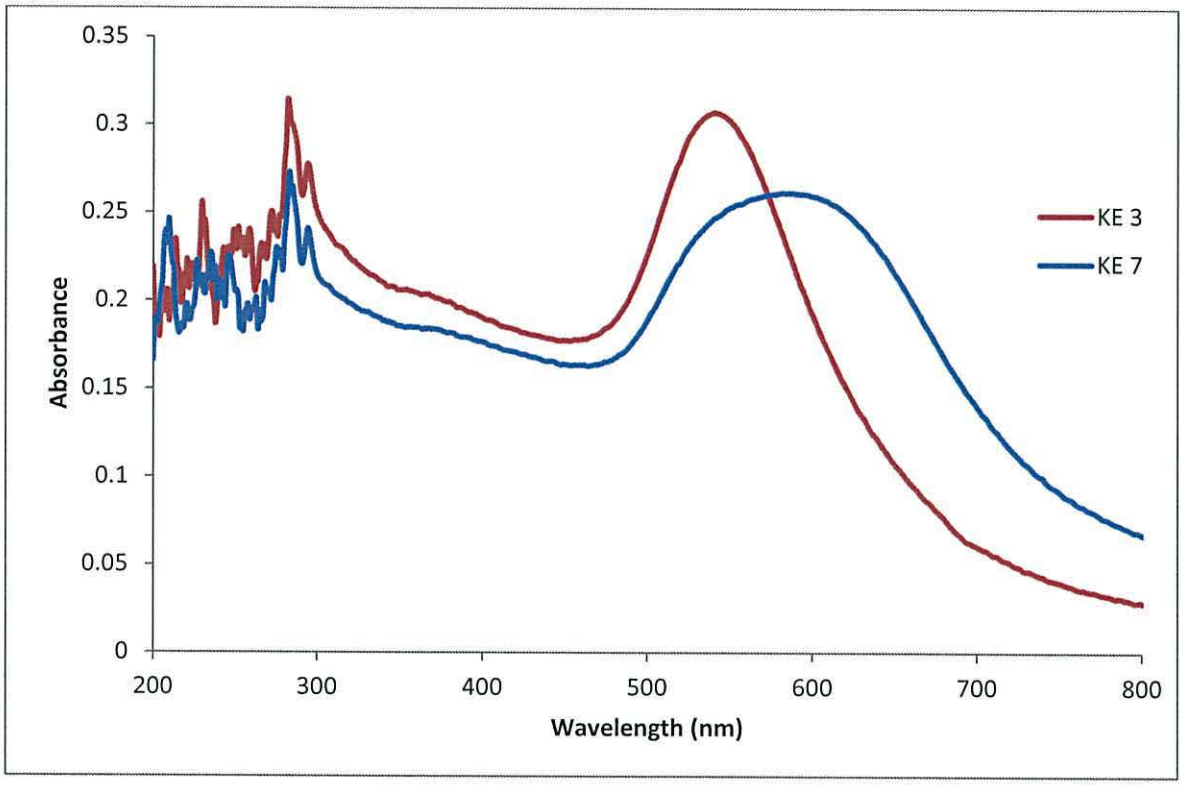


Figure 4.5.2 UV-visible spectrum after leaving the TB positive serum sample KE 3 (red) and the TB negative serum samples KE 7 (blue) with gold nanoparticles coated with 22 for 5 minutes (spectrum taken 15 minutes after NaCl addition).

The UV-visible spectra obtained, when analysing each serum sample in turn, have the same profile regardless of how long the serum sample is left with the coated gold nanoparticles before the NaCl solution is added, suggesting that it may be possible to reduce the time of this step to as little as 5 minutes instead of the 30 minutes that had been used up to this point. For practical reasons, it was decided to shorten this time to only 15 minutes when performing the assay in 96-well plates, and in order to keep the assay consistent, the same time was used when performing this assay in cuvettes.[I]

This experiment was repeated with a further two TB+ and two TB- serum samples in order to make sure that this trend was observed, and this was found to be the case with the same

[I] An exception to this change in timings was performed when performing initial experiments in cuvettes with gold nanoparticles coated with a linker compound and non-thiol modified antigen later in the project (see Section 5.3). For those experiments, the time of this step was reverted to 30 minutes in order to gain a fair comparison with the results obtained when using gold nanoparticles coated with **22**.

pattern being observed each time. The only slight difference was that for one of the TB- samples, there was slightly less aggregation when leaving the serum with the coated gold nanoparticles for 45 and 60 minutes than there was when they were left for 30 minutes. A possible reason for this may be that leaving the serum with the coated gold nanoparticles for too long can cause non-selective binding to occur to the antigens. The results from these experiments again suggested that it may be possible to reduce the amount of time for this step to as low as 5 minutes, confirming the results and observations stated earlier in this section. These spectra can be seen in the Appendix, Section 9.3.6.

Varying the Time for the Second Part of the Assay

After analysing the first step of the experiment, the time allowed for the second part of the experiment was also investigated. Serum samples were left with gold nanoparticles coated with **22** for a constant time before saturated aqueous NaCl was added. An UV-visible spectrum was then taken every 100s. These experiments were again initially performed with one TB+ (KE 1) and one TB- (KE 7) serum sample and showed no significant difference in the UV-visible spectrum with time when analysing the TB+ serum sample, see Appendix, Section 9.3.7. However, for the TB- serum sample analysed, there was a change in the UV-visible spectrum for the first few minutes after addition of the NaCl solution, however, there is no significant changes in the spectrum after approximately 500 s. These results therefore suggest that it is possible to reduce the time for this step of the assay to 10 minutes instead of the 15 minutes that was previously used. Further confirmation of these results can be found in the Appendix, Figure 9.3.10 - Figure 9.3.12. In these experiments, an UV-visible spectrum was recorded every 100 s for 15 minutes following the addition of the saturated aqueous solution of NaCl and a similar pattern was observed with no great change in the UV-visible spectrum being observed after approximately 500 s.

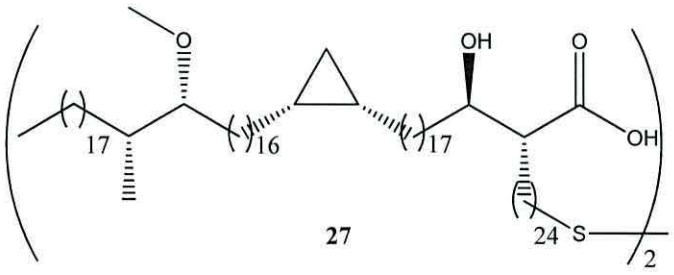
It was therefore decided to measure the absorbance of the solutions 10 minutes after addition of the NaCl solution when performing future experiments.[II]

[II] An exception to this change in timings was again performed when performing initial experiments in cuvettes with gold nanoparticles coated with a linker compound and non-thiol modified antigen later in the project, (see Section 5.3) as well was the experiments performed after re-optimisation of the assay with gold nanoparticles purchased from BBInternational (see Section 5.6). For those experiments, the time of this step was reverted to 15 minutes in order to gain a fair comparison with the results obtained when using gold nanoparticles coated with **22**.

Reducing the amount of time required for this step, as well as shortening the time required for the first step of the assay, reduced the overall time required to analyse a serum sample by this assay from 45 minutes to 25 minutes. This could be further reduced in a sensor for the detection of TB to approximately 15 minutes, which would potentially make this test a very rapid method for the detection of TB.

4.6 Analysis of Serum Samples with a Different Thiol Modified Mycolic Acid

After further optimising the assay, it was decided to analyse serum samples with gold nanoparticles coated with a different thiol modified mycolic acid, **27**, the structure of which can be seen in Figure 4.6.1.



27

Figure 4.6.1 Structure of 27.

The same serum samples as had been used for the experiments in Sections 4.4 were used and the assay was again performed on 96-well plates. The results obtained from these experiments can be seen in Figure 4.6.2 and the values obtained with each individual serum sample can be seen in the Appendix, Section 9.3.8.

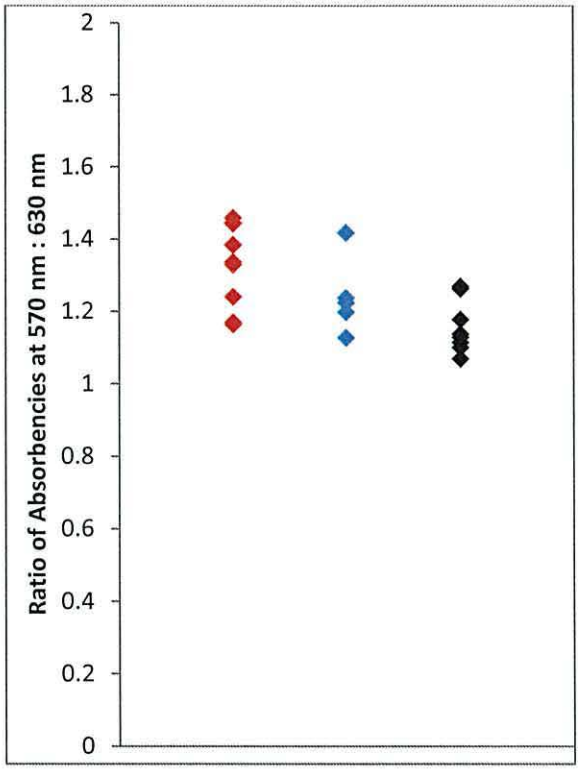


Figure 4.6.2 Graph showing the ratio of absorbencies at 570 nm : 630 nm for different serum samples by category when using gold nanoparticles coated with 27 for the assay (red – 9 TB positive samples obtained from the WHO, blue – 5 TB negative samples obtained from the WHO, black – 8 TB negative samples from the UK).

It can be seen from this Figure that generally, a higher ratio value is observed at the end of the assay when analysing TB+ serum samples (which gave an average value of 1.33) than when analysing TB- samples (which gave an average value of 1.19), and therefore a distinction is observed. When comparing this data to the data obtained when using gold nanoparticles coated with **22** (Figure 4.4.2), a similar pattern is observed with both antigens, however the 'spread' of the data seemed to be smaller when using gold nanoparticles coated with **27**. The likely reason for this is that the gold nanoparticles used with both antigens came from different batches, although from the same supplier (Aldrich). The highest absorbance at 570 nm observed when using gold nanoparticles coated with **27** was 0.19 (see Section 9.3.8), while the highest absorbance at 570 nm when using gold nanoparticles coated with **22** was 0.22 (see Appendix, Section 9.3.3). Due to the possibility of different batches of gold nanoparticles (of slightly different concentrations) giving rise to slightly different results, a method of interpreting the data was required, which would take this into account and negate any possible effect that it may have. Such a method to interpret the data was devised later in the project, and is explained in detail in Section 5.4.

Measuring the Absorbance at 540 nm and 630 nm

Prior to performing these experiments, a new filter was obtained for the plate reader which allowed for the reading of the absorbance of the solutions at 540 nm. It was believed that a better distinction could be observed between TB+ and TB- serum samples if the absorbance at 540 nm was measured instead of at 570 nm because the absorbance reading at 540 nm would give a truer reflection of the amount of non-aggregated coated gold nanoparticles present in the solution. Figure 3.3.3 shows the UV-visible spectrum for the coated gold nanoparticles, and it can be seen that the SPR peak of the coated gold nanoparticles is much closer to 540 nm than to 570 nm, therefore measuring the absorbance at 540 nm would give a truer reflection of the height of this peak, in turn giving a truer reflection of the amount of gold nanoparticles not aggregating after addition of the NaCl solution.

The results obtained when measuring the ratio of absorbencies at 540 nm : 630 nm for the experiments in this Section are shown in Figure 4.6.3, and the values obtained with each individual serum sample can be seen in the Appendix, Section 9.3.8.

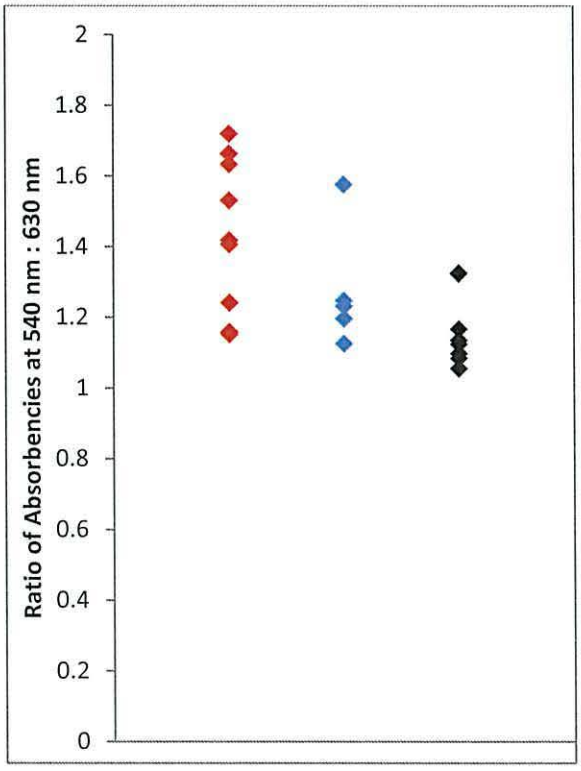


Figure 4.6.3 Graph showing the ratio of absorbencies at 540 nm : 630 nm for different serum samples by category when using gold nanoparticles coated with 27 for the assay (red – 9 TB positive samples obtained from the WHO, blue – 5 TB negative samples obtained from the WHO, black – 8 TB negative samples from the UK).

By comparing Figure 4.6.3 to Figure 4.6.2, it can be seen that there is a bigger 'range' in the ratio values when using 540 nm instead of 570 nm. It can also be observed that the serum samples which gave positive results (*i.e.* a high ratio value) gave a higher value when analysing the ratio of absorbencies at 540 nm : 630 nm, while there is only little to no change with serum samples which gave a negative result (*i.e.* a low ratio value). When analysing the ratio of absorbencies at 540 nm : 630 nm, the TB+ samples gave an average value of 1.44 while the TB- samples gave an average value of 1.21, compared to the values of 1.33 and 1.19 respectively that were obtained when analysing the ratio of absorbencies at 570 nm : 630 nm.

These results suggested that measuring the absorbance of the solutions at the end of the assay at 540 and 630 nm gave a more meaningful value than measuring the absorbance at 570 and 630 nm. In addition, they suggest that a better distinction could be observed between serum samples which give a positive result and serum samples that give negative results when measuring the absorbance of the solutions at 540 and 630 nm. It was therefore decided, for future experiments, to measure the absorbance of the solutions at 540 and 630 nm at the end of the assay, when performing the gold nanoparticle assay on 96-well plates.

4.7 Concluding Remarks

A colour change was successfully induced in gold nanoparticles coated with a thiol modified mycolic acid antigen, due to the aggregation of these coated gold nanoparticles when they are subjected to a saturated aqueous solution of NaCl. This aggregation process was found to be inhibited by the addition of a TB positive serum sample to the coated gold nanoparticles prior to adding NaCl to the solution. In instances where a TB negative serum sample was added to the coated gold nanoparticles, aggregation could still occur, thus leading to a means of differentiating TB positive serum samples from TB negative serum samples by measuring the amount of aggregation observed in the coated gold nanoparticles, and also by observing the colour of the coated gold nanoparticle solution at the end of the assay.

Fifty serum samples obtained from the WHO were analysed with the assay developed using gold nanoparticles coated with **22**, and even though there was some overlap in the data obtained for the TB positive and TB negative samples, a distinction between both was observed. Upon analysing the same serum samples with a non-thiolated version of the

same mycolic acid antigen by ELISA, it was found that the antigen being used was not a great antigen for distinguishing TB positive serum samples from TB negative samples.

Investigations suggested that the position of the sulfur atom within the mycolic acid antigen was important. A better distinction was observed between the TB positive and TB negative serum samples when the sulfur atom was placed on the alkyl chain of the mycolic acid compared to when it was placed on the acid group of the molecule. It was also confirmed that the mycolic acid was required to give a distinction between the TB positive and TB negative serum samples, and not any long chain fatty acid, with thio-stearic acid **(23)** showing no distinction between TB positive and TB negative serum samples.

Further optimisation showed that the assay could be performed in as little as 15 minutes without compromising the results obtained. This could potentially lead to the development of a sensor for the detection of TB, with results being available in 15 minutes, which is much quicker than any of the methods currently available for the detection of TB (see Table 1.5.1), giving a colour response that could potentially be read by eye, and/or by measuring the absorbance of the solution at two different wavelengths.

4.8 References

1 A. N. Shipway, M. Lahav, R. Gabai and I. Willner, **Investigations into the electrostatically induced aggregation of Au nanoparticles,** *Langmuir,* 2000, **16,** 8789-8795.

2 P. Costa, A. Amaro, A. Botelho, J. Inácio and P. V. Baptista, **Gold nanoprobe assay for the identification of mycobacteria of the *Mycobacterium tuberculosis* complex,** *Clin. Microbiol. Infect.,* 2010, **16,** 1464-1469.

3 Confidential unpublished work.

4 M. O. Balogun, E. Huws, M. M. Sirhan, A. D. Saleh, J. R. Al Dulayymi, L. Pilcher, J. A. Verschoor and M. S. Baird, **Thiol modified mycolic acids,** *Chem. Phys. Lipids,* 2013 (In Press).

Chapter 5
Implementing Non-Thiol Modified Antigens

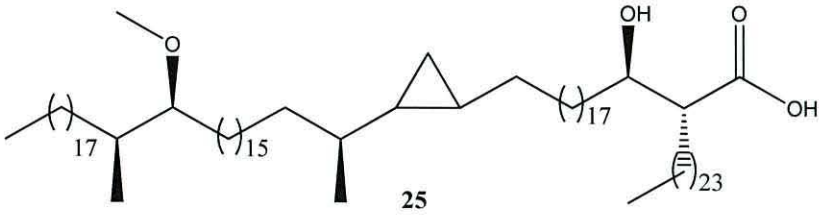
5.1 *Introduction*

Having successfully developed an assay that showed a distinction between TB positive and TB negative serum samples (see Chapter 4), it was decided to try to incorporate non-thiol antigens into this gold nanoparticle assay. One of the problems with the assay described above was that thiol modified antigens were required in order to bind the antigen to the gold nanoparticle surfaces, by a gold-sulfur bond, and that the process of incorporating this thiol group into the antigen molecule makes the synthesis more time consuming.[1] Developing a method which could incorporate non-thiol modified antigens would therefore be advantageous, as methods for the synthesis of mycolic acids, TMMs and TDMs are known. This would also allow all of the currently synthesised non-thiol modified antigens to be analysed using this gold nanoparticle based aggregation assay.

5.2 *Initial Experiments*

5.2.1 Direct Interaction of the Antigen with the Gold Nanoparticles

In order to make sure that non-thiol modified antigens did not interact directly with gold nanoparticles, it was decided to try to coat gold nanoparticles directly with them. Gold nanoparticles were coated using the protocol in Section 2.4, however instead of using a thiol modified antigen for the coating process a 5 μM solution of a natural TDM mixture (isolated from *M. tuberculosis*), a natural mycolic acid mixture (isolated from *M. tuberculosis*) and **25** (the non-thiolated version of **22**), the structure of which can be seen in Figure 5.2.1, were used. These three different 'coated' gold nanoparticle solutions were then analysed with the same serum samples as were used for the experiments in Sections 4.4 and 4.5, performing the assay in 96-well plates according to the protocol found in Section 2.6.2.



25

Figure 5.2.1 Structure of compound 25.

The results obtained from these experiments can be seen in Figure 5.2.2, and the values obtained with each individual serum sample with each individual antigen can be seen in the Appendix, Section 9.4.1. The results obtained when analysing the serum samples with gold nanoparticles coated with **22** are also provided in that section for comparison.[I]

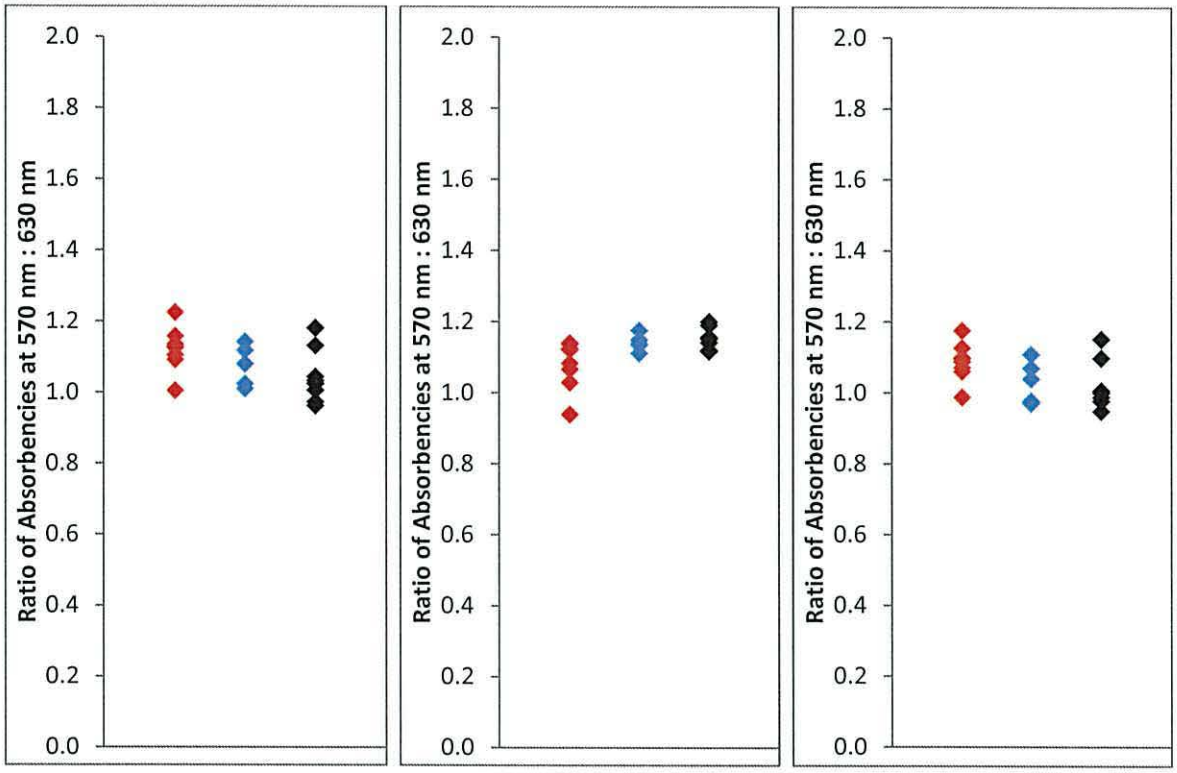


Figure 5.2.2 Graph showing the ratio of absorbencies at 570 nm : 630 nm for different serum samples by category when using gold nanoparticles coated with 5 μM solutions of natural TDM mixture (left), natural mycolic acid mixture (middle) and 25 (right) (red – 9 TB positive samples obtained from the WHO, blue – 5 TB negative samples obtained from the WHO, black – 8 TB negative samples from the UK).

It can be seen from these figures that no distinction was observed between the TB+ and TB- serum samples, and that all of the values obtained at the end of the assay were relatively low, suggesting essentially complete aggregation of the gold nanoparticles after addition of the NaCl solution. This suggested that none of the antigens had coated onto the gold nanoparticles, which was expected because none of them contained a thiol functional group which would have provided them with the ability to bind to the gold nanoparticle surfaces by forming a gold-sulfur bond.

These experiments suggested that non-thiol modified antigens could not be directly bound to gold nanoparticles, therefore another approach was attempted.

[I] Even though previous results had suggested that measuring the absorbance of the solution at 540 nm and 630 nm at the end of the assay gave a more meaningful ratio value, it was decided to measure the absorbance of the solutions at the end of these experiments at 570 nm and 630 nm in order to be able to directly compare the results obtained with the results obtained when using gold nanoparticles coated with **22**.

5.3 *Incorporating a Linker Compound*

Another approach to incorporate non-thiol modified antigens into the assay was to bind a linker compound to the gold nanoparticles, which in turn would interact with the non-thiol modified antigen, leading to a coating of the non-thiol modified antigen around the gold nanoparticles.

Firstly, gold nanoparticles were coated with a 5 μM solution of thio-stearic acid (**23**), which would bind to the gold nanoparticles through the thiol group on the acid group of the molecule (forming a gold-sulfur bond), leading to a long alkyl chain being 'exposed' around the gold nanoparticles. It was expected that this layer around the gold nanoparticles would be lipophilic in nature; therefore it would interact with other lipophilic substances such as mycolic acids, TMMs and TDMs. This in turn would lead to a 'coating' of the lipophilic substance (mycolic acid, TMM or TDM) around the gold nanoparticles.

Gold nanoparticles were coated with thio-stearic acid (**23**) followed by a natural TDM mixture (isolated from *M. tuberculosis*) according to the protocol for coating with non-thiol modified antigens in Section 2.4. This antigen was chosen as a starting point because it had given a good distinction between TB+ and TB- serum samples by ELISA[2] and it was readily available commercially.

5.3.1 Characterisation of Non-Thiol Antigen Binding to Gold Nanoparticles

The first requirement was to characterise whether the natural TDM bound to the coated gold nanoparticles. As there was no thiol group on the TDM molecules, and due to the use of a linker compound, electrochemical stripping could not be used to try and characterise whether or not binding had occurred. Electrochemical stripping analysis would look at molecules directly bound to the gold surface, and therefore would not determine whether the TDM had bound to the linker compound.[3]

Transmission Electron Microscopy (TEM)

One method that was used to determine whether the natural TDM mixture had bound to the linker compound on the coated gold nanoparticles was TEM. As with the gold nanoparticles coated with **22**, these coated nanoparticles were analysed by TEM following the same procedure as previously discussed (see Section 3.2.2). The following TEM image was obtained for the gold nanoparticles coated with thio-stearic acid followed by a natural

TDM mixture; the image observed for gold nanoparticles not coated with antigen is also shown for comparison.

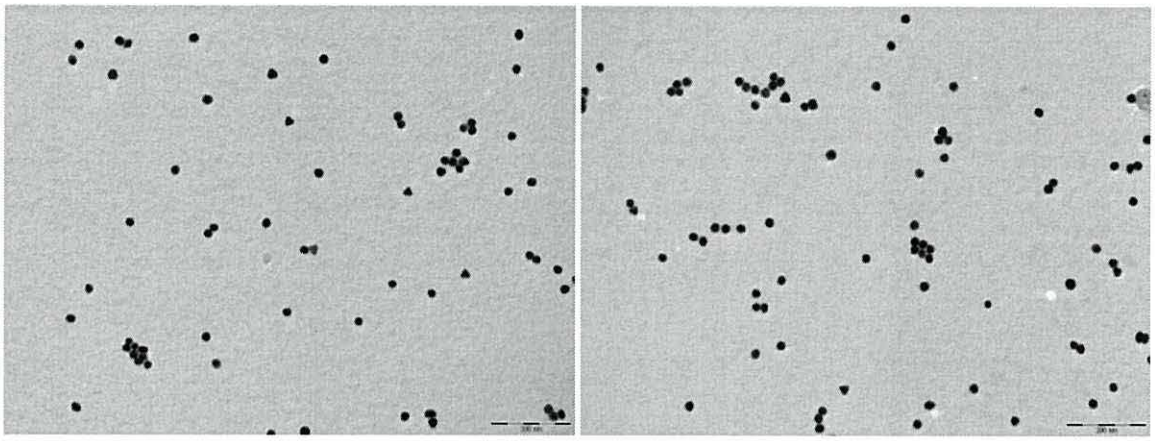


Figure 5.3.1 TEM images of gold nanoparticles which were not coated with antigen (left) and gold nanoparticles which were coated with thio-stearic acid followed by a natural TDM mixture isolated from M. tuberculosis (right).

As was observed for gold nanoparticles coated with **22** by TEM (see Section 3.2.2), no significant difference was observed between the gold nanoparticles which were not coated with antigen and those which were coated with thio-stearic acid followed by a natural TDM mixture. This again may be due to this method not being able to detect the presence of small organic molecules on the surface of larger metal particles.[4]

Since using TEM did not confirm the presence of the TDM mixture on the gold nanoparticles, another technique was used in order to try and confirm their presence.

Particle Size and Zeta (ζ)-Potential Analysis

The next methods that were used to attempt to identify whether the natural TDM mixture had bound to the gold nanoparticles were particle size and zeta potential calculations. These analyses were carried out following the same procedure as was previously discussed (see Section 3.2.3).

Particle size analysis showed an increase in particle diameter from 23.9 nm for the un-treated coated gold nanoparticles to 35.3 nm for the TDM coated gold nanoparticles. This increase in particle diameter strongly suggests that the TDM had successfully been coated onto the gold nanoparticle surfaces.

This was further confirmed by the zeta potential analysis. This showed a decrease in the magnitude of the zeta potential of the particles from -17.6 mV for the un-treated gold nanoparticles to -13.2 mV for the TDM coated gold nanoparticles. This again suggests that

a less charged species had been coated onto the surface of the gold nanoparticles, in turn suggesting that the TDM had successfully been bound to the surfaces of the gold nanoparticles.

Confirming that the TDM Coated Gold Nanoparticles Could Aggregate Upon NaCl Addition

After confirming that the natural TDM mixture had coated onto the gold nanoparticles, it was decided to check whether or not the TDM coated gold nanoparticles aggregated upon the addition of NaCl. 100 µl of a saturated aqueous solution of NaCl was added to 900 µl of the coated gold nanoparticles, and the UV-visible spectra observed before and 15 minutes after addition of the NaCl solution is shown in Figure 5.3.2. An image of a cuvette containing natural TDM coated gold nanoparticles and natural TDM coated gold nanoparticles 15 minutes after addition of saturated aqueous NaCl is shown in Figure 5.3.3.

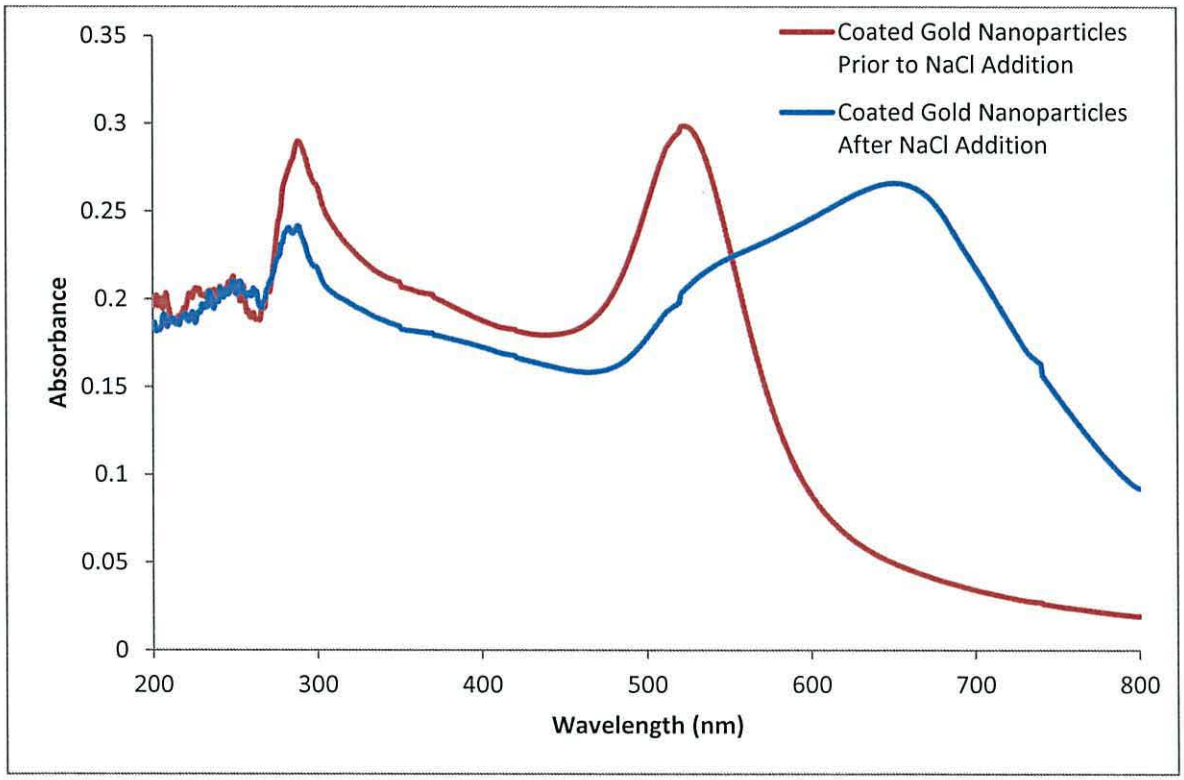


Figure 5.3.2 UV-visible spectrum of gold nanoparticles coated with a natural TDM mixture (using thio-stearic acid as a linker) (red) and the coated gold nanoparticles 15 minutes after addition of saturated aqueous NaCl (blue).

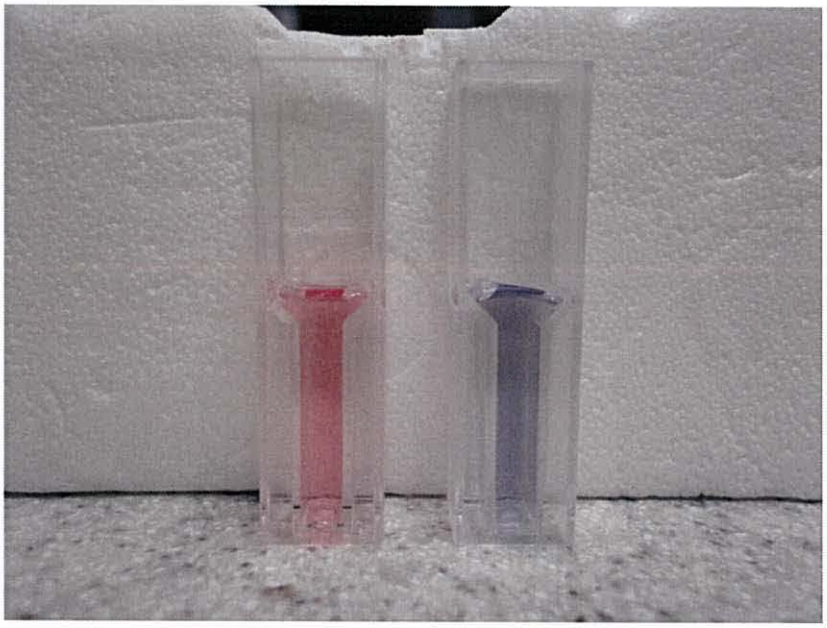


Figure 5.3.3 Cuvettes showing gold nanoparticles coated with natural TDM mixture (using thio-stearic acid as a linker) (left) and gold nanoparticles coated with natural TDM mixture (using thio-stearic acid as a linker) 15 minutes after addition of saturated aqueous NaCl (right).

As was observed when performing this test on gold nanoparticles coated with **22** (see Section 4.2.1), aggregation of the coated gold nanoparticles occurred after addition of the NaCl solution, with a change in the wavelength at which the maximum absorbance occurs from 524 to 651 nm, and a change in the colour of the solution from red to blue was observed.

The results of the experiments discussed in this section show that gold nanoparticles can be coated with non-thiol modified antigens *via* a thio-stearic acid linker compound, and aggregation of these coated gold nanoparticles can be achieved by addition of a saturated aqueous solution of NaCl. This suggested that these coated nanoparticles would also be suitable for use in the assays developed in Chapters 3 and 4, allowing a large range of TDM, TMM and mycolic acid antigens which had already been synthesised in Bangor to be used in these assays, without requiring any modifications to the antigens.

5.3.2 Using Gold Nanoparticles Coated with a Natural TDM Mixture

Having shown that gold nanoparticles coated with a non-thiol modified antigen (using thio-stearic acid (**23**) as a linker compound) could be used in the assay, it was decided to try and use them in the assay to see whether any distinction would be observed between TB+ and TB- serum samples. It was initially decided to perform the assay on 96-well plates, with all of the serum samples from the first group of samples obtained from the WHO (see Appendix, Section 9.1.2). This was done to get a broad overview of whether or not the

assay would work with these natural TDM coated gold nanoparticles, and if the assay did work, whether or not there would be a better or worse distinction between the TB+ and TB- serum samples when they were analysed in this way, compared to using gold nanoparticles coated directly with antigen *i.e.* with **22** or **27**.

It was again expected that complementary binding of antibodies to the antigen (the natural TDM mixture) coated to the gold nanoparticles would occur for the TB+ serum samples, leading to no aggregation upon addition of the NaCl solution, while for the TB- serum samples, no complimentary binding was expected, leading to aggregation of the TDM coated gold nanoparticles upon addition of the NaCl solution.

The results obtained from these experiments can be seen in Figure 5.3.4, and the values obtained with each individual serum sample can be seen in the Appendix, Section 9.4.2.[II]

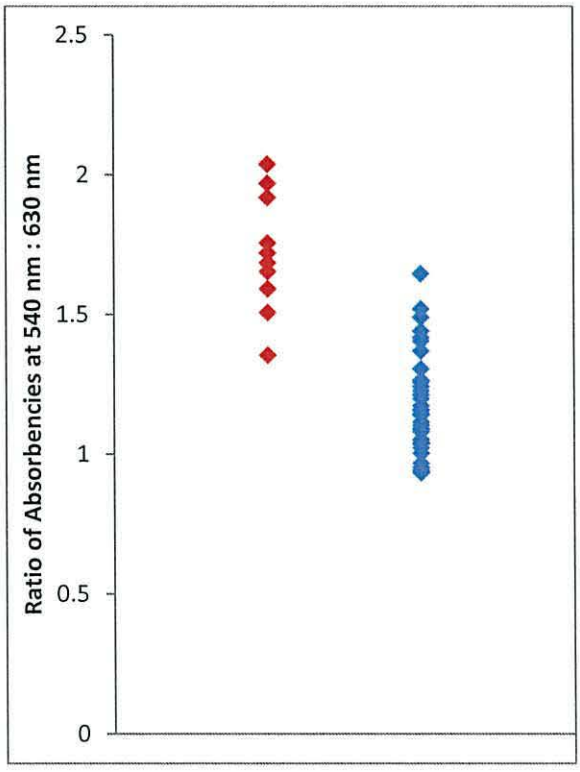


Figure 5.3.4 Graph showing the ratio of absorbencies at 540 nm : 630 nm for 50 different serum samples by category when using gold nanoparticles coated with a natural TDM mixture for the assay (red – 10 TB positive samples obtained from the WHO, blue – 40 TB negative samples obtained from the WHO).

[II] A point to note is that when centrifuging these coated gold nanoparticles prior to using them in the assay, some aggregation occurred when centrifuging at 12,000 RCF. When the speed of the centrifuge was reduced to 7,000 RCF, no aggregation was observed. It was therefore decided to centrifuge all gold nanoparticles coated with non-thiol modified antigen at 7,000 RCF.

The results obtained were very encouraging. Firstly, it was observed that the assay did work with the gold nanoparticles coated with the natural TDM mixture, and secondly, a distinction was observed between the TB+ and TB- serum samples. This confirmed that non-thiol modified antigens could be used in the assay, allowing a large range of TDM, TMM and mycolic acid antigens which had already been synthesised to be used in this assay. This Figure also suggests a better distinction between the TB+ and TB- serum samples when using gold nanoparticles coated with the natural TDM mixture than when using gold nanoparticles coated with **22** (see Figure 4.3.2). When using gold nanoparticles coated with the natural TDM mixture, nine of the ten TB+ serum samples gave a ratio value of higher than 1.5, while only two of the forty TB- serum sample analysed gave a value of greater than 1.5. This led to a sensitivity of 90 % and a specificity of 95 % for this assay using gold nanoparticles coated with a natural TDM mixture (using this method of interpreting the data).

In order to confirm whether the observation made in Section 4.6, which suggested that reading the absorbance of the solution at the end of the assay at 540 and 630 nm gave a truer reflection of the aggregation occurring and also a better distinction between TB+ serum samples and TB- serum samples, the solutions for the above experiments were also measured at 570 nm. The values obtained when calculating the ratio of absorbencies at 570 nm : 630 nm are shown in Figure 5.3.5 below, and a full list of the values obtained with each individual serum sample can be seen in the Appendix, Section 9.4.2.

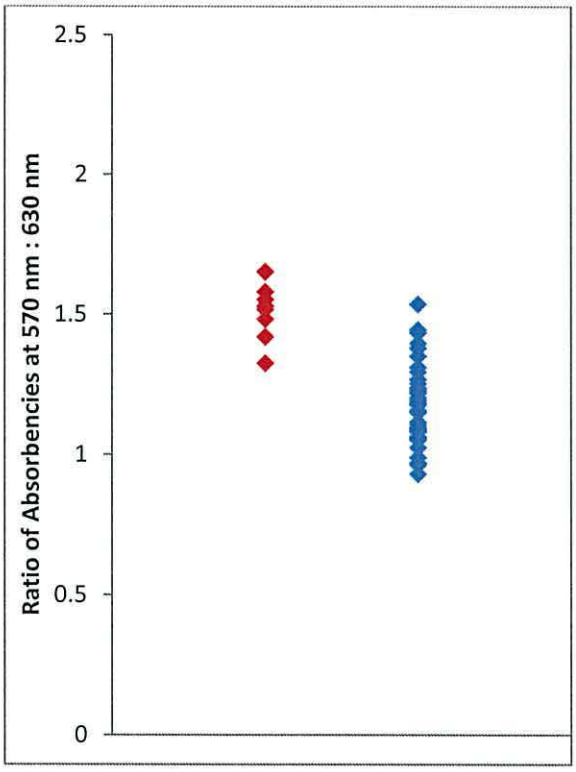


Figure 5.3.5 Graph showing the ratio of absorbencies at 570 nm : 630 nm for 50 different serum samples by category when using gold nanoparticles coated with a natural TDM mixture for the assay (red – 10 TB positive samples obtained from the WHO, blue – 40 TB negative samples obtained from the WHO).

Similar to the observations made in Section 4.6, there is much less 'range' in the data when observed at 570 and 630 nm (as compared to 540 and 630 nm), and there is also more 'overlap' in the values obtained with the TB+ and TB- serum samples at these wavelengths. When analysing the data at 570 and 630 nm, average values of 1.52 and 1.17 were obtained with the TB+ and TB- samples respectively, however an increase in the difference between TB+ and TB- samples was observed when reading the solutions at 540 and 630 nm, with average values of 1.72 and 1.16 respectively obtained. These results again suggested that the absorbance of the solutions at the end of the assay should be read to 540 and 630 nm.

5.4 Percentage Aggregation as a Method of Quantifying the Results

Having developed a method for incorporating non-thiol modified antigens into the assay, and having established that the absorbance of the solutions should be read at 540 and 630 nm to give the best reflection of the amount of aggregation occurring, and to give the best distinction between TB+ and TB- serum samples, it was decided to develop a method to more accurately quantify the results obtained.

It was suggested from the results obtained in Section 4.6 that different batches of gold nanoparticles could have slightly different concentrations which could influence the ratio value obtained at the end of the assay. In order to overcome this problem, it was decided to analyse the data obtained from the assay as a 'percentage aggregation'.

Standards were to be placed on each 96-well plate on which the assay would be performed. One standard would represent 0 % aggregation, and this standard would only contain gold nanoparticles coated with the antigen being investigated, and another standard would represent 100 % aggregation, which would contain gold nanoparticles coated with the antigen being investigated in the assay and a saturated aqueous solution of NaCl. The first standard represented 0 % aggregation because it would not be subjected to NaCl, therefore no aggregates should be present in this solution. The second standard represented 100 % aggregation because it would be subjected to the NaCl solution, therefore it would fully aggregate.

The value obtained at the end of the assay for the coated gold nanoparticles, which had been subjected to the serum sample under investigation, would then be compared against these two standards and converted into a percentage aggregation. The use of these two standards on each 96-well plate negated the effect of any problems that may arise from using gold nanoparticles from different batches, while also giving a quick method to compare data obtained when analysing serum samples with gold nanoparticles coated with various different antigens.

Percentage aggregation was calculated as follows:

$$A = \frac{Absorbance\ at\ 540\ nm}{Absorbance\ at\ 630\ nm}$$

$$A_0 = \frac{Absorbance\ at\ 540\ nm\ when\ 0\ \%\ aggregation}{Absorbance\ at\ 630\ nm\ when\ 0\ \%\ aggregation}$$

$$A_{100} = \frac{Absorbance\ at\ 540\ nm\ when\ 100\ \%\ aggregation}{Absorbance\ at\ 630\ nm\ when\ 100\ \%\ aggregation}$$

$$Percentage\ Aggregation = \frac{A_0 - A}{A_0 - A_{100}} \times 100$$

A_0 is obtained from the first standard, while A_{100} is obtained from the second standard.

Due to slight differences in the volumes of the standards and the volumes of the solutions containing the serum samples at the end of the assay, and due to some slight natural variations in gold nanoparticle concentrations from one well to another, values of slightly lower than 0 % and slightly higher than 100 % can be obtained using this approach. All values presented in Figures that are lower than 0 % are adjusted to 0 % and all values greater than 100 % are adjusted to 100 % for this thesis.

Using this method of calculation, a gold nanoparticle solution analysed with a TB+ serum sample was expected to give a high value for 'A' in turn giving a low percentage aggregation value, while a gold nanoparticle solution analysed with a TB- serum sample was expected to give a low value for 'A' in turn giving a high percentage aggregation value.[III]

Later in the project, it was discovered that addition of a serum sample to coated gold nanoparticles caused a slight change in the absorbance values at 540 and 630 nm (without the addition of any NaCl), and these slight changes were not related to aggregation, but to the addition of the serum sample to the coated gold nanoparticles (believed to be by the same phenomena as discussed in Chapter 3). As a result of this observation, another standard for 0 % aggregation, containing coated gold nanoparticles and serum, was added to each 96-well plate on which the assay was performed, and two values for percentage aggregation were then calculated and quoted (see Section 5.7 onwards). 'Method 1' for percentage aggregation used the standard containing coated gold nanoparticles only as the standard for 0 % aggregation, while 'Method 2' for percentage aggregation used the standard containing coated gold nanoparticles and a serum sample for 0 % aggregation.

In order to quantify how well the method performed with various antigens, sensitivity and specificity values are quoted for each antigen. For each antigen investigated, a 'cut-off' percentage aggregation value was taken and all of the samples which gave a percentage aggregation value equal to or above this value were designated as TB-, while all of the samples which gave a lower percentage aggregation value were designated as TB+. This 'cut-off' absorbance value is specified with each antigen used.

In order to help determine an appropriate value for this cut-off absorbance, receiver operating characteristics (ROC) analysis was performed on the data obtained from the

[III] Note: Values and graphs using this method therefore are in reverse to values and graphs shown earlier where the ratio of absorbance at 540 nm : 630 nm was used.

assays. The ROC curves that were produced were used to aid in deciding on a 'cut-off' percentage aggregation value and the area under the curve (AUC) was also recorded. The AUC corresponds to the probability that the test will rank a randomly chosen positive sample higher than a randomly chosen negative sample (provided that a high value is indicative of a positive sample), and is commonly used to represent the expected performance of a given test.[5] An AUC value of 0.5 therefore represents a test that cannot distinguish positive samples from negative samples, and increasing this value towards one increases the probability that the test can distinguish positive samples from negative samples.

Percentage Aggregation Values for the Gold Nanoparticle Assay Using Gold Nanoparticles Coated with a Natural TDM Mixture

Having developed a method to quantify the results obtained from the assay as a percentage aggregation, these values were calculated for the experiments described in Section 5.3.2 (this could be performed because standards had been run on those plates), and the values obtained can be seen in the Appendix, Section 9.4.2.

By representing the data as a percentage aggregation, a truer reflection of the aggregation which had occurred in the solutions could be identified. Generally, the percentage aggregation value obtained very closely reflects the ratio of absorbencies at 540 nm : 630 nm, however there are some slight discrepancies, for example, when looking at serum samples KE 5 and BA 14. Serum sample KE 5 gave the higher ratio value and would therefore be expected to give the lowest percentage aggregation value (as a more positive serum sample would be expected to give a higher ratio value, and a lower percentage aggregation value). The percentage aggregation value for this sample, however, was slightly higher than that for BA 14. The reason for this slight discrepancy is that the serum samples were run on different 96-well plates and different lots of gold nanoparticles were used on each plate. By using the percentage aggregation calculation, this difference in lots is accounted for, thus giving a truer reflection of the amount of aggregation that has occurred.

Using the values for percentage aggregation, eight of the ten TB+ serum samples gave a value of lower than 50 %, while thirty five of the forty TB- serum samples gave a value that was higher than 50 %. This led to a sensitivity of 80 % and a specificity of 88 % for this assay using gold nanoparticles coated with a natural TDM mixture. These values are

slightly lower than the sensitivity and specificity values calculated in Section 5.3.2, however the values quoted here were expected to be a fairer representation of the solutions at the end of the assay. A probable reason for this slight discrepancy between using ratios and percentage aggregation for the analysis of the data was that eight of the ten TB+ serum samples were analysed on the same 96-well plate, therefore the gold nanoparticles used for the assay on that plate may have been of a slightly different concentration to the gold nanoparticles used on the other plates, leading to a slight difference in the values obtained. Analysing using the percentage aggregation method, however, negated the effect of the serum samples being run on different plates and gave a truer reflection of the aggregation which had occurred in the solutions during the assay.

It was therefore decided to analyse all of the data obtained when using this assay with the percentage aggregation analysis for all future experiments (where the assay was performed on 96-well plates).

5.5 Analysis of Serum Samples with Various Antigens

Having obtained a distinction between TB+ and TB- serum samples when running the assay using gold nanoparticles coated with thio-stearic acid followed by a natural TDM mixture, it was decided to see if a distinction would also be observed when using gold nanoparticles coated with synthetic antigens, *via* a thio-stearic acid (**23**) linker compound. It was again decided to use the 50 serum samples from the first group of samples obtained from the WHO, using gold nanoparticles coated with a methoxy TDM (**24**), methoxy TMM (**28**) and methoxy mycolic acid (**25**) (the structures of which can be seen in the Appendix, Section 9.1.1) for these experiments. It was also decided to analyse the serum samples with gold nanoparticles coated with thio-stearic acid only to make sure that any distinction observed between the TB+ and TB- serum samples was due to the presence of the TDM, TMM or mycolic acid antigens, and not due to any non-specific binding to thio-stearic acid. The percentage aggregation values obtained for these experiments can be seen in the Appendix, Section 9.4.3, and a graphical representation of the data can be seen in Figure 5.5.1. The values obtained when using gold nanoparticles coated with a natural TDM mixture are also included for comparison.

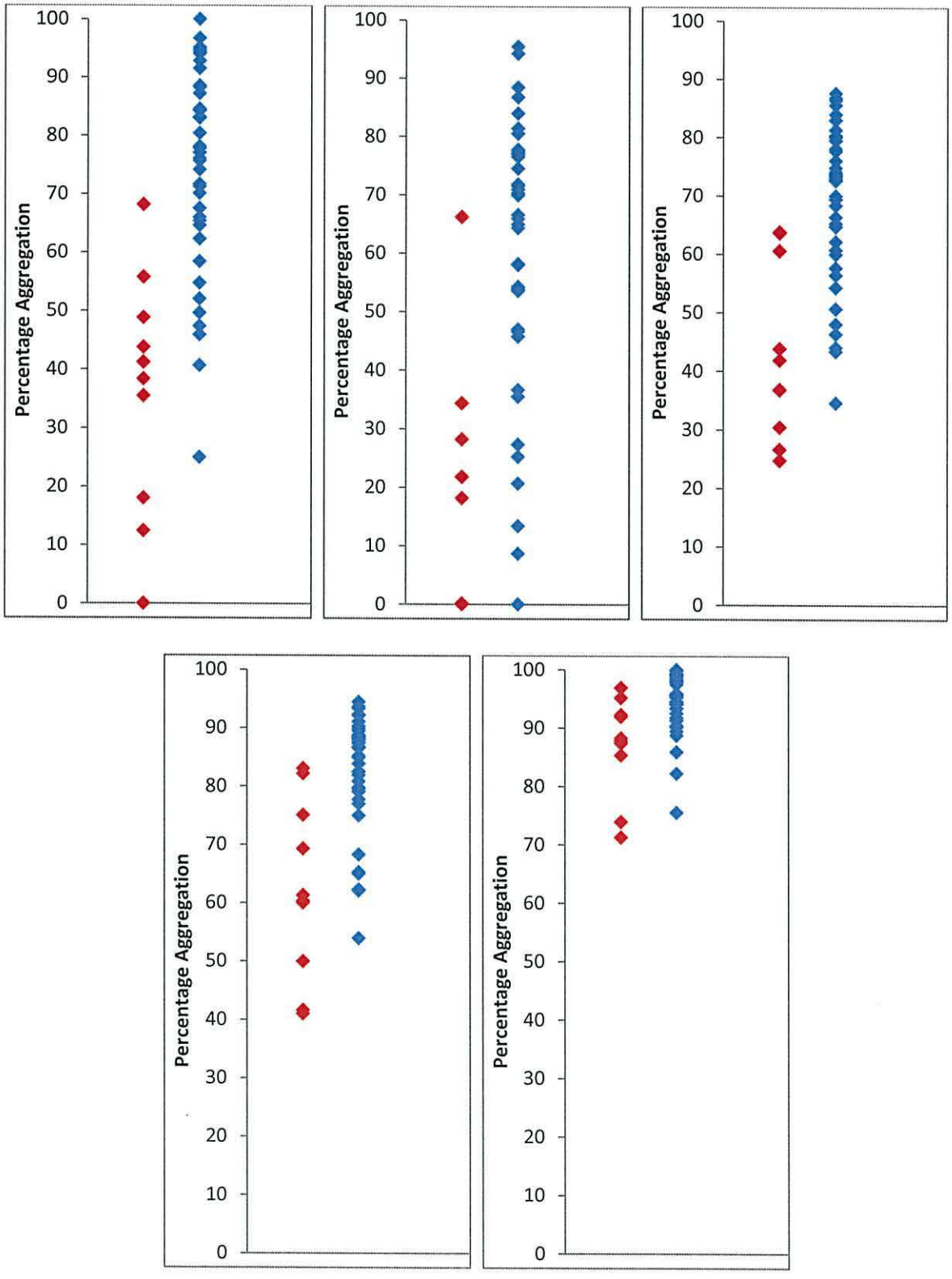


Figure 5.5.1 Graph showing the percentage aggregation for 50 different serum samples by category when using gold nanoparticles coated with a natural TDM mixture (top left), 24 (top middle), 28 (top right), 25 (bottom left) and thio-stearic acid (bottom right) for the assay (red – 10 TB positive samples obtained from the WHO, blue – 40 TB negative samples obtained from the WHO).

The data obtained from these experiments were very encouraging. Firstly, when using gold nanoparticles coated with stearic acid only, all of the percentage aggregation values were high (greater than 70 %) suggesting that no binding had occurred to the stearic acid coated onto the gold nanoparticles. This in turn suggested that the low percentage aggregation values observed with some of the other antigens was due to specific binding to the antigen coated onto the gold nanoparticles, prohibiting aggregation from occurring after addition of the NaCl solution at the end of the assay.

Another encouraging observation was that a distinction could be observed between the TB+ serum samples and the TB- serum samples when using gold nanoparticles coated with synthetic antigens. By taking a 'cut-off value' of 50 % aggregation, and designating all serum samples which gave a value below this cut-off as TB+ and all serum samples which gave a value above this cut-off as TB-, the following sensitivity and specificity values were obtained:

Table 5.5.1 Table showing the sensitivity and specificity values obtained when analysing the first set of serum samples with gold nanoparticles coated with various antigens.

Antigen	Positive Samples Correctly Detected	Negative Samples Correctly Detected	Sensitivity (%)	Specificity (%)
Natural TDM	8	35	80	88
24	9	29	90	73
28	7	35	70	88
25	3	40	30	100
Thio-stearic acid	0	40	0	100

The values obtained for sensitivity and specificity were relatively high when using **24** and **28** as the antigens, and were comparable to the values obtained when analysing serum samples with those antigens by ELISA (a sensitivity and specificity of 90 % and 68 % respectively with **24** and 90 % and 65 % respectively with **28**.[2] This was encouraging because the gold nanoparticle assay developed could be performed in as short a time as 15 minutes, without the need of any washing steps, making this platform suitable to use in a device for the detection of TB.

The results obtained also seem to give an indication of the amount and strength of binding to the antigen coated onto the gold nanoparticles. For example, when analysing serum sample BA 5 with the synthetic antigens, a percentage aggregation value of 0.16 was obtained when using gold nanoparticles coated with **24** (a methoxy TDM), 30.43 with **28**

(a methoxy TMM) and 49.98 with **25** (a methoxy mycolic acid). These values suggested that either more binding occurs to the TDM than the TMM and the mycolic acid and/or that the binding that occurred to the TDM was stronger than the binding to the TMM and the mycolic acid. They also suggest that the least and/or weakest binding occurred to the mycolic acid. These results are consistent with the observations made when analysing serum samples by ELISA.[2]

5.6 Further Optimisation of the Assay

5.6.1 Purchasing Gold Nanoparticles from a Different Supplier

Having been able to bind synthetic antigens onto the surfaces of gold nanoparticles, and observing a distinction between TB+ and TB- serum samples when using gold nanoparticle coated with these synthetic antigens, it was now decided to examine gold nanoparticles from a different source, which were more cost effective than those previously used (from Aldrich).

Given the results above, it was possible for all of the synthetic TDM, TMM and mycolic acid antigens to be analysed using this assay, thus large quantities of gold nanoparticles would therefore be required to perform these analyses. Due to financial constraints, the gold nanoparticles purchased from Aldrich were too expensive to purchase in large quantities, therefore a large quantity of gold nanoparticles was purchased from BBInternational. The specifications for these gold nanoparticles reported that, similar to those purchased from Aldrich, they had a mean diameter of 20 nm, and a 0.01 % Au concentration.

In order to confirm these gold nanoparticles were in fact of the same concentration as those purchased from Aldrich, they were coated with **22**, and an UV-visible spectrum was taken after they had been centrifuged (at 12,000 RPM for 12 minutes) and re-suspended in 10 mM phosphate buffer (pH 7.4). The spectrum obtained can be seen in Figure 5.6.1; the spectrum obtained when using gold nanoparticles from Aldrich is also shown for comparison.

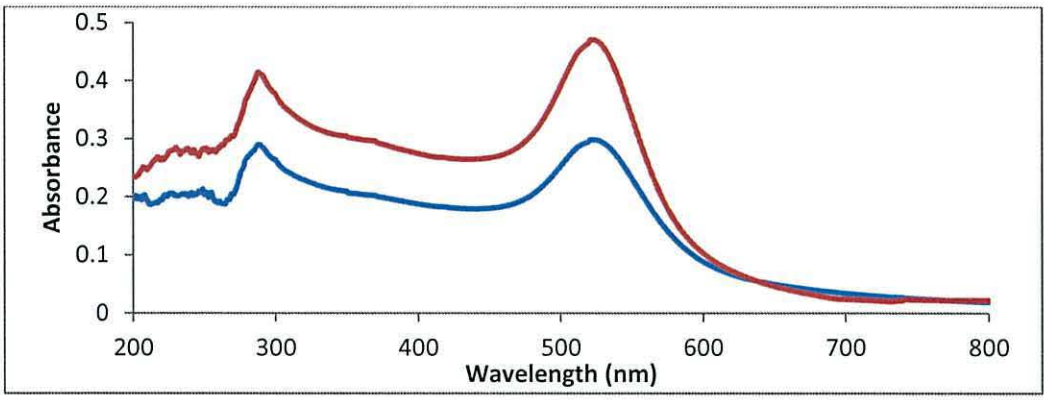


Figure 5.6.1 Comparison of the UV-visible spectra obtained with gold nanoparticles coated with 22 after re-suspension in 10 mM phosphate buffer (pH 7.4) (blue – gold nanoparticles from Aldrich, red – gold nanoparticles from BBInternational).

It can be seen from this Figure that there is a significant difference in absorbance at the SPR peak with both of these solutions. The absorbance for the gold nanoparticles purchased from BBInternational (coated with **22**) was over 1.5 times greater than that for those purchased from Aldrich (coated with **22**). This suggested a significant difference in concentrations between both solutions.

It was shown from previous experiments (see Section 4.2.2) that the ratio of gold nanoparticles to serum sample was important for the performance of the assay, therefore in light of the gold nanoparticles purchased from BBInternational being more concentrated than those purchased from Aldrich, re-optimisation of the assay was required. Another possible method of overcoming this difference would have been to re-suspend the coated gold nanoparticles in more buffer than was used when using gold nanoparticles purchased from Aldrich. This approach was not taken because omitting the centrifugation and re-suspension step prior to performing the assay was a target (see Section 5.6.2). It was also believed that using gold nanoparticles of a higher concentration (therefore having a higher absorbance) may lead to a clearer difference in colour between red (non-aggregated gold nanoparticles) and blue (aggregated gold nanoparticles) at the end of the assay.

5.6.2 Re-Optimisation of the Assay

Unless otherwise stated, all of the experiments for this re-optimisation process were performed on 96-well plates to allow more serum samples to be analysed in a shorter time. The protocol described in Section 2.6 was used for all of these experiments, but changing only one variable at a time. All assays from this point forward were performed with gold nanoparticles purchased from BBInternational.

Removal of the Centrifugation and Re-Suspension Steps Prior to Performing the Assay

It was decided to try to remove the centrifugation and re-suspension process prior to performing the assay to be able to analyse more serum samples in a shorter time. It was believed that the accuracy of the assay could also be increased due to there being less chance of slight variations in the concentration of the coated gold nanoparticles from well to well or from plate to plate. The only downside to not performing the centrifugation and re-suspension prior to using the coated gold nanoparticles in the assay was that any aggregated gold nanoparticles would not be removed from the solutions prior to the assay being performed (see Figure 3.3.2).

An experiment was performed to determine whether or not performing the centrifugation and re-suspension steps were necessary for the assay. Gold nanoparticles were coated with thio-stearic acid (**23**) followed by a natural TDM mixture isolated from *M. tuberculosis* (according to the procedure found in Section 2.4), and half were centrifuged and re-suspended in 10 mM phosphate buffer (pH 7.4), while the other half was not treated further prior to use. Eight different serum samples were analysed with both 'sets' of coated gold nanoparticles, and the percentage aggregation values obtained at the end of these experiments can be seen in Table 5.6.1:

Table 5.6.1 Table showing the percentage aggregation values obtained when analysing various serum samples with gold nanoparticles coated with a natural TDM mixture with and without centrifugation of the coated gold nanoparticles prior to their use.

Serum Code	TB Status	Without Centrifugation	With Centrifugation
BA 4	Positive	101.06	42.59
BA 5	Positive	82.86	14.18
KE 1	Positive	51.94	10.40
KE 2	Positive	46.30	6.44
BA 10	Negative	101.84	70.80
CO 13	Negative	101.91	60.29
UK Sample 7	Negative	99.58	72.42
UK Sample 25	Negative	95.52	74.29

It can be seen that, although a distinction is observed between the TB+ and TB- serum samples both with and without centrifugation, there was a greater distinction with centrifuged coated gold nanoparticles. Also, when the assay was performed without centrifugation higher percentage aggregation values were obtained. This suggested that the

serum that was being used for these experiments was too dilute, and more concentrated serum samples were required for use in the assay when using gold nanoparticles from BBInternational. The results also suggested that some of the coated gold nanoparticles were being lost in the centrifugation process leading to lower percentage aggregation values being observed (due to a higher coated gold nanoparticles to serum sample ratio).

It was therefore decided to change the amount of 10 mM phosphate buffer (pH 7.4) in which the centrifuged coated gold nanoparticles were re-suspended, in order to keep the concentration the same for both centrifuged and non-centrifuged coated gold nanoparticles. An experiment was therefore designed to determine how much 10 mM phosphate buffer (pH 7.4) should be used for the re-suspension of the coated gold nanoparticles after they had been centrifuged. An UV-visible spectrum was measured for 1 ml of non-centrifuged gold nanoparticles coated with a natural TDM mixture, and for centrifuged gold nanoparticles coated with a natural TDM mixture (1 ml prior to being centrifuged) re-suspended in various amounts of 10 mM phosphate buffer (pH 7.4). The spectra obtained from these experiments can be seen in Figure 5.6.2.

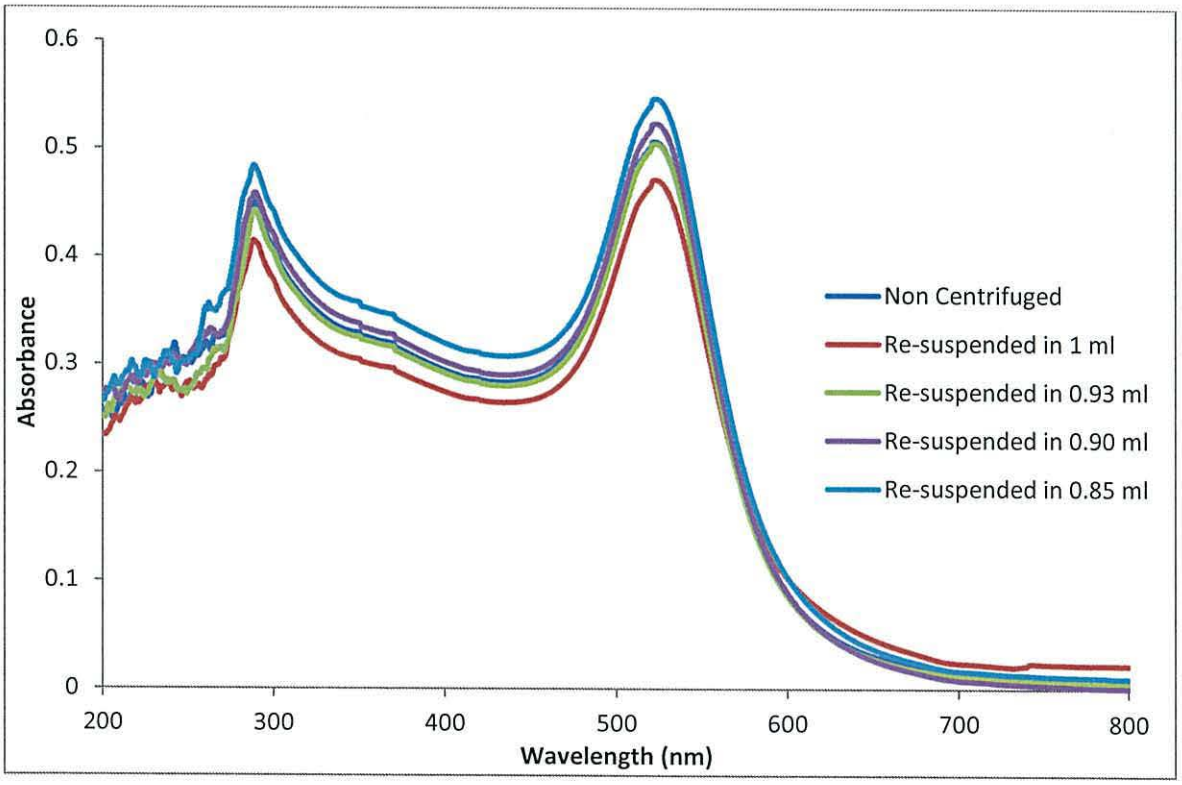


Figure 5.6.2 UV-visible spectra for gold nanoparticles coated with a natural TDM mixture without centrifugation, and after re-suspension in various amounts of 10 mM phosphate buffer (pH 7.4) after being centrifuged.

From this Figure, it was determined that after centrifugation of the coated gold nanoparticles, re-suspension in 0.93 ml of 10 mM phosphate buffer (pH 7.4) gave the same absorbance as the non-centrifuged coated gold nanoparticles, suggesting that the concentrations in both of these solutions were identical. It was therefore decided to re-suspend centrifuged gold nanoparticles (1 ml prior to being centrifuged) in 0.93 ml of 10 mM phosphate buffer (pH 7.4).

Varying the Serum Concentration

Since the values obtained in Table 5.6.1 suggested that the serum was too dilute when using gold nanoparticles purchased from BBInternational, an experiment was designed in order to determine which serum concentration was most suitable to use with these gold nanoparticles. Gold nanoparticles were again coated with a natural TDM mixture and were not centrifuged prior to use. Four serum samples (two TB+ samples (KE 1 and KE 2) and two TB- samples (BA 10 and CO 13)) were used at serum concentrations of 1 in 500, 1 in 1,000, 1 in 1,500, 1 in 2,000, 1 in 2,500, 1 in 3,000, 1 in 3,500 and 1 in 4,000. The percentage aggregation values obtained at the end of these experiments can be seen in Table 5.6.2 below:

Table 5.6.2 Table showing the percentage aggregation values obtained when analysing various serum samples of various concentrations using gold nanoparticles coated with a natural TDM mixture.

	Serum Code			
Serum Concentration	**KE 1**	**KE 2**	**BA 10**	**CO 13**
1 in 500	-11.58	-7.08	-23.21	-51.66
1 in 1,000	-10.20	-4.17	-22.65	-31.56
1 in 1,500	-6.19	-1.55	-8.13	11.96
1 in 2,000	0.22	23.48	32.92	78.27
1 in 2,500	17.81	26.89	84.48	98.07
1 in 3,000	26.58	54.53	101.06	102.45
1 in 3,500	47.52	73.48	103.79	104.38
1 in 4,000	73.92	87.37	103.77	105.45

It can be seen that at serum concentrations of 1 in 1,500 or greater, all of the samples gave a low percentage aggregation value, suggesting that these concentrations are too high. At a serum concentration of 1 in 2,000, the TB- serum sample CO 13 gave a high percentage aggregation value, however serum sample BA 10 again gave a low value suggesting that this concentration is also too high.

At a serum concentration of 1 in 2,500 both TB+ samples gave low percentage aggregation values, while both TB- samples gave high percentage aggregation values. Therefore at this concentration, a good distinction was observed between the TB+ and TB- samples.

As the serum concentration was diluted further, the percentage aggregation values obtained with the TB+ samples increased. At a 1 in 3,000 serum concentration, one of the TB+ samples gave a percentage aggregation value of higher than 50 %, while at a serum concentration of 1 in 4,000, both TB+ samples gave a percentage aggregation value of higher than 50 %.

These results suggested that the best distinction between the TB+ and TB- serum samples was observed at a serum concentration of 1 in 2,500, therefore this concentration was used for the experiments from this point forward.

Varying the NaCl Concentration

Previous experiments had suggested that a saturated aqueous solution of NaCl should be used for the assay (see Section 4.2.1), however these experiments had been performed using gold nanoparticles directly coated with a thiol modified mycolic acid. An experiment was therefore designed to make sure that this was still the case when using gold nanoparticles coated with non-thiol modified antigens (using thio-stearic acid (**23**) as a linker compound). Gold nanoparticles were again coated with a natural TDM mixture, and the same four serum samples as were used in the previous experiment were used. 100 µl of NaCl solutions of various concentrations were added at the end of the assay. The percentage aggregation values obtained at the end of these experiments can be seen in Table 5.6.3:

Table 5.6.3 Table showing the percentage aggregation values obtained when analysing various serum samples using gold nanoparticles coated with a natural TDM mixture in the gold nanoparticle assay and using NaCl solutions of various concentrations.

	Serum Code			
NaCl Concentration	**KE 1**	**KE 2**	**BA 10**	**CO 13**
1 M	16.66	23.45	52.70	81.34
2 M	14.00	20.52	65.95	91.43
3 M	14.05	20.57	77.40	95.48
4 M	19.51	20.83	82.13	97.14
Saturated	17.81	26.89	84.48	98.07

It can be seen that when analysing the TB+ serum samples (KE 1 and KE 2), the concentration of the NaCl solution added does not have a great effect on the results obtained. However, with the TB- serum samples, increasing the NaCl concentration greatly increases the aggregation that occurs. This leads to the best distinction between the TB+ and TB- serum samples occurring when using a saturated aqueous solution of NaCl at the end of the assay.

Therefore it was decided to continue using a NaCl solution of this concentration in the assay.

Varying the Amount of Antigen Used for Coating the Gold Nanoparticles

Previous experiments had suggested that a 5 μM solution of antigen in hexane should be used for the assay, however these experiments had again been performed using gold nanoparticles directly coated with a thiol modified mycolic acid. It was therefore decided to confirm that this concentration of antigen was the most suitable to use when coating gold nanoparticles with non-thiol modified antigens (using a thio-stearic acid (**23**) linker compound). Gold nanoparticles were firstly coated with a 5 μM solution of thio-stearic acid (**23**) followed by a solution of the natural TDM mixture at concentrations ranging from 0.13 μM to 50 μM. These coated nanoparticles were then analysed with eight different serum samples, and the percentage aggregation values obtained at the end of the assay is shown in Table 5.6.4 below:

Table 5.6.4 Table showing the percentage aggregation values obtained when analysing various serum samples using gold nanoparticles coated with various amounts of a natural TDM mixture.

		Concentration of Natural TDM Solution Used for the Coating Process (μM)							
Serum Code	**TB Status**	**0.13**	**0.25**	**1.25**	**2.5**	**5**	**10**	**25**	**50**
BA 4	Positive	89.22	87.20	92.40	91.75	49.65	90.41	46.35	23.51
BA 5	Positive	92.58	93.09	96.85	96.05	40.29	72.71	38.00	17.08
KE 1	Positive	31.90	31.00	37.41	27.13	26.12	36.08	22.47	14.87
KE 2	Positive	75.21	72.55	74.81	74.48	34.92	53.50	29.89	16.75
BA 10	Negative	99.92	99.42	100.46	99.66	83.14	100.09	95.12	77.53
CO 13	Negative	102.72	101.40	101.99	102.53	89.80	98.63	98.19	88.60
UK Sample 7	Negative	104.87	102.71	102.17	103.94	97.17	104.45	102.10	98.03
UK Sample 25	Negative	105.06	102.93	105.00	105.30	95.69	102.68	99.88	96.85

The best distinction between TB+ and TB- serum samples was observed when coating the gold nanoparticles with a 50 μM solution of the natural TDM mixture, however, a good

distinction is also observed when coating the gold nanoparticles with a 5 μM solution. The results obtained when using a 2.5 μM or weaker solution of the natural TDM mixture suggest that a lot of aggregation occurred, in turn suggesting little binding to the antigens coated onto the gold nanoparticle surface, suggesting that more antigen was required to coat the gold nanoparticles for use in this assay.

The results obtained when coating the gold nanoparticles with a 5 μM solution and a 25 μM solution were very similar, suggesting that the results obtained with gold nanoparticles coated with a 10 μM solution were an anomaly. Due to there being no significant improvement in distinction between TB+ and TB- serum samples unless a 50 μM solution of antigen was used, it was decided that since the amount of antigen available was limited, a 5 μM solution of antigen would still be used to coat the nanoparticles from this point forward.

Coating the Gold Nanoparticles with Antigen Dissolved in Chloroform

Since all of the TDMs, TMMs and mycolic acids that have been synthesised may not be soluble in hexane, it was decided to see if the gold nanoparticles could be coated with antigens dissolved in other solvents. Because the natural TDM mixture was soluble in both hexane and chloroform, it was again used as the antigen for this experiment. Gold nanoparticles were coated with a 5 μM solution of the natural TDM mixture in chloroform, and these were then analysed with eight different serum samples. The percentage aggregation values obtained are shown in Table 5.6.5, and values obtained when using gold nanoparticles coated with the natural TDM mixture dissolved in hexane are also included for comparison.

Table 5.6.5 Table showing the percentage aggregation values obtained when analysing various serum samples using gold nanoparticles coated with a natural TDM mixture (dissolved in hexane or chloroform).

Serum Code	TB Status	Antigen Dissolved in Hexane	Antigen Dissolved in Chloroform
BA 4	Positive	49.65	50.23
BA 5	Positive	40.29	49.39
KE 1	Positive	26.12	17.32
KE 2	Positive	34.92	30.63
BA 10	Negative	83.14	96.54
CO 13	Negative	89.80	98.82
UK Sample 7	Negative	97.17	105.17
UK Sample 25	Negative	95.69	102.64

The percentage aggregation values obtained when using the two different sets of coated gold nanoparticles were very similar, and both sets were able to distinguish the TB+ serum samples from the TB- serum samples equally well.

The results suggested that the solvent in which the antigen is dissolved for the coating process does not have a significant effect on the results obtained. It was therefore decided to continue coating gold nanoparticles with antigen dissolved in hexane, however, in instances where the antigen was not soluble in hexane, a different solvent (*e.g.* chloroform) could be used, and if this is the case, it will be specified.

Using Various Linker Compounds

The final experiments in this re-optimisation involved varying the linker compound. Up to this point, thio-stearic acid (**23**) had been used, however it was also decided to try to use thio-TBSA (**29**),[IV,6,7,8,9,10] the structure of which can be seen in Figure 5.6.3, and octadecanethiol.

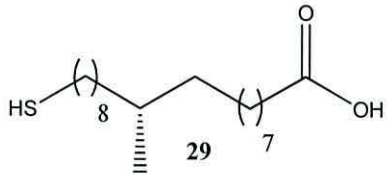


Figure 5.6.3 Structure of thio-TBSA (29)

Gold nanoparticles were therefore coated with the linker compound to be investigated (a 5 μM solution in hexane), followed by the natural TDM mixture. These coated nanoparticles were then used for the assay and analysed with eight different serum samples. The percentage aggregation values obtained are shown in Table 5.6.6:

[IV] Tuberculostearic acid (TBSA) was first isolated in 1927 from the phosphatide fraction of *M. tuberculosis*, and is also present in *M. bovis*, *M. leprae* and *M. phlei*, but it is not present in *M. avium*.

Table 5.6.6 Table showing the percentage aggregation values obtained when analysing various serum samples using gold nanoparticles coated with various linker compounds followed by a natural TDM mixture.

		Linker Compound Used		
Serum Code	**TB Status**	**Thio-stearic acid**	**Thio-TBSA**	**Octadecanethiol**
BA 4	Positive	49.65	72.16	59.92
BA 5	Positive	40.29	66.77	38.33
KE 1	Positive	26.12	65.21	29.22
KE 2	Positive	34.92	66.26	28.97
BA 10	Negative	83.14	85.64	79.92
CO 13	Negative	89.80	90.02	92.63
UK Sample 7	Negative	97.17	91.26	90.69
UK Sample 25	Negative	95.69	94.98	88.40

It can be seen that the percentage aggregation values obtained when using thio-stearic acid (**23**) and octadecanethiol were very similar. The values obtained when using thio-TBSA (**29**), however, were different. The values for the TB+ serum samples were higher with this linker compound, suggesting that less binding had occurred to the antigen coating the gold nanoparticles, or possibly that less antigen had bound in the first place, in turn reducing the amount of antibodies that could bind. Also, the distinction between the TB+ and TB- samples was worse when using this linker compound compared to when thio-stearic acid (**23**) or octadecanethiol were used.

These results suggested that either thio-stearic acid (**23**) or octadecanethiol should be used as the linker compound when coating gold nanoparticles with non-thiol modified antigen. It was therefore decided to keep using thio-stearic acid (unless otherwise stated) to be consistent with the previous experiments.

5.6.3 Confirming the Re-Optimised Conditions

Having re-optimised the assay to use gold nanoparticles purchased from BBInternational, it was decided to make sure that these conditions gave a good distinction between TB+ and TB- serum samples. This was done by performing the assay in cuvettes using gold nanoparticles coated with thio-stearic acid (**23**) followed by a natural TDM mixture, and using the protocol described in Section 2.6.1. For these experiments, the coated gold nanoparticles were not centrifuged prior to use. Five TB+ serum samples and five TB- serum samples were analysed, and the UV-visible spectra of the solutions obtained after the addition of the NaCl solution can be seen in the Appendix, Section 9.4.4.

These results confirmed that the assay worked with gold nanoparticles purchased from BBInternational, and that a distinction could be observed between TB+ and TB- serum samples, with essentially no aggregation observed when analysing all of the TB+ samples, while aggregation of the coated gold nanoparticles was observed when analysing all of the TB- samples.

5.7 Analysis of the First Set of Serum Samples

Having established that the assay, using gold nanoparticles from BBInternational, could distinguish TB+ serum samples from TB- serum samples when coated with a natural TDM mixture, using thio-stearic acid as a linker compound, it was decided to analyse a larger number of serum samples with a range of different antigens.

During initial experiments for this Section, it was discovered that a second standard for the calculation of percentage aggregation should be added to each 96-well plate (as mentioned in Section 5.4), therefore two values for percentage aggregation are quoted with each serum sample from this point forward. Unless otherwise stated, 'Method 2' for percentage aggregation is used for all of the analysis as this method is believed to be the more accurate representation of percentage aggregation since it takes into account any change in absorbance at 540 or 630 nm due to serum addition to the coated gold nanoparticles.

'Cut-off' values, in order to calculate the sensitivity and specificity values for the assay when using different antigens, were determined from the ROC analysis of the results, with a value below the 'cut-off' value being designated as TB+ (due to having low percentage aggregation), while a value equal to or greater than the 'cut-off' value being designated as TB- (due to having high percentage aggregation).

In addition to percentage aggregation values determined from measured absorbance values, predictions were made by eye by two individuals, independently, on whether a serum sample was TB positive or TB negative, by observing the colour of the wells. These predictions will be termed 'eye tests' in the following discussion. Solutions which appeared red in colour, giving a prediction of a TB positive serum sample are designated as a '+', those that appeared blue in colour, giving a prediction of a TB negative serum sample are designated as a '-', while solutions which did not appear either clearly red or clearly blue, leading to an unsure prediction are designated as a '?'.

5.7.1 Using a Natural TDM Mixture as the Antigen

The first antigen used in this assay was the natural TDM mixture isolated from *M. tuberculosis*, in order to obtain data for all the fifty serum samples from group 1 with this antigen after re-optimising the assay conditions. It was believed from previous experiments (see Section 5.6.2) that centrifugation of the coated gold nanoparticles was not required prior to their use in the assay for the assay to work, however, it had still not been determined whether the assay would perform better or worse without this centrifugation step. Therefore, it was decided to use gold nanoparticles coated with a natural TDM mixture with centrifugation prior to their use in the assay (labelled 'centrifuged' in the following tables), and without centrifugation prior to their use in the assay (labelled 'non-centrifuged' in the following tables).

The percentage aggregation values obtained at the end of the assay, as well as the eye test results are shown in Table 5.7.1, while Figure 5.7.1 shows a graphical representation of the data obtained (using 'method 2' for percentage aggregation).

Table 5.7.1 Table showing the percentage aggregation values obtained and eye test results when analysing 50 different serum samples using gold nanoparticles coated with a natural TDM mixture.

		Non-Centrifuged				Centrifuged			
		Percentage Aggregation		Eye Test		Percentage Aggregation		Eye Test	
Serum Code	TB Status	Method 1	Method 2	1	2	Method 1	Method 2	1	2
BA 1	Positive	96.48	94.62	-	-	85.12	76.78	-	-
BA 2	Positive	69.91	53.96	+	+	47.16	17.53	+	+
BA 3	Positive	72.75	58.30	+	?	54.36	28.77	+	+
BA 4	Positive	52.16	26.80	+	+	37.82	2.96	+	+
BA 5	Positive	71.03	55.67	?	?	56.22	31.68	+	+
KE 1	Positive	37.63	4.56	+	+	30.72	-8.12	+	+
KE 2	Positive	61.65	38.67	+	+	49.26	20.80	+	+
KE 3	Positive	59.94	35.93	+	+	45.23	14.50	+	+
KE 4	Positive	63.66	44.40	+	+	47.27	17.71	+	+
KE 5	Positive	69.97	54.05	+	+	56.18	31.61	?	+
BA 6	Negative	75.05	61.83	?	+	61.29	39.58	?	?
BA 7	Negative	59.17	37.53	+	+	52.42	25.74	+	+
BA 8	Negative	89.74	84.29	-	-	79.45	67.93	?	-
SA 1	Negative	64.85	43.79	+	+	60.79	38.79	+	+
SA 2	Negative	58.01	32.85	+	+	49.15	20.62	+	+
SA 3	Negative	80.45	68.73	?	-	62.62	41.65	?	+
SA 4	Negative	79.97	67.96	?	-	68.25	50.44	?	?
SA 5	Negative	77.04	63.27	?	-	68.01	50.06	?	?
BA 9	Negative	72.22	57.49	+	+	58.41	35.09	+	+

BA 10	Negative	99.40	99.03	-	-	91.08	87.31	-	-
BA 11	Negative	91.74	87.35	-	-	73.29	58.32	?	?
BA 12	Negative	49.70	18.85	+	+	31.72	2.89	+	+
BA 13	Negative	87.49	80.87	?	-	72.41	56.95	?	?
BA 14	Negative	89.08	83.29	?	-	72.15	56.53	?	?
BA 15	Negative	72.63	58.12	+	?	61.11	39.31	+	+
BA 16	Negative	91.02	85.51	?	-	70.18	57.59	?	?
BA 17	Negative	95.70	93.06	?	-	81.52	73.72	?	-
BA 18	Negative	83.28	73.03	-	-	62.08	46.07	?	+
CO 1	Negative	90.63	85.01	-	-	84.04	75.09	?	-
CO 2	Negative	93.36	89.38	-	-	83.44	74.15	?	-
CO 3	Negative	88.16	81.06	-	-	70.66	54.20	?	?
CO 4	Negative	93.71	89.94	-	-	77.77	65.30	?	-
CO 5	Negative	95.72	93.15	-	-	93.36	89.63	-	-
CO 6	Negative	71.68	54.71	?	?	60.89	38.95	?	+
CO 7	Negative	83.20	73.14	-	-	69.53	52.44	?	?
CO 8	Negative	85.89	77.43	-	-	72.27	56.72	?	?
CO 9	Negative	75.51	60.83	?	-	60.97	39.07	?	?
CO 10	Negative	75.90	61.46	?	?	59.36	36.56	?	?
CO 11	Negative	69.57	51.34	?	?	49.12	20.58	+	?
CO 12	Negative	81.56	70.51	?	-	65.76	46.56	?	-
CO 13	Negative	93.95	90.75	-	-	88.14	81.49	?	-
CO 14	Negative	85.43	76.49	?	-	64.44	49.43	?	?
CO 15	Negative	71.37	53.81	+	?	48.07	26.15	+	+
CO 16	Negative	94.95	91.85	-	-	80.02	71.59	?	?
CO 17	Negative	98.10	96.93	-	-	81.99	74.39	-	-
CO 18	Negative	96.51	94.37	-	-	85.40	79.24	-	-
KE 6	Negative	52.84	24.57	+	+	36.59	1.01	+	+
KE 7	Negative	92.24	88.12	-	-	74.75	60.60	?	-
PE 1	Negative	88.28	82.06	?	-	73.08	57.99	?	?
PE 2	Negative	65.56	47.30	+	+	51.48	24.27	+	+

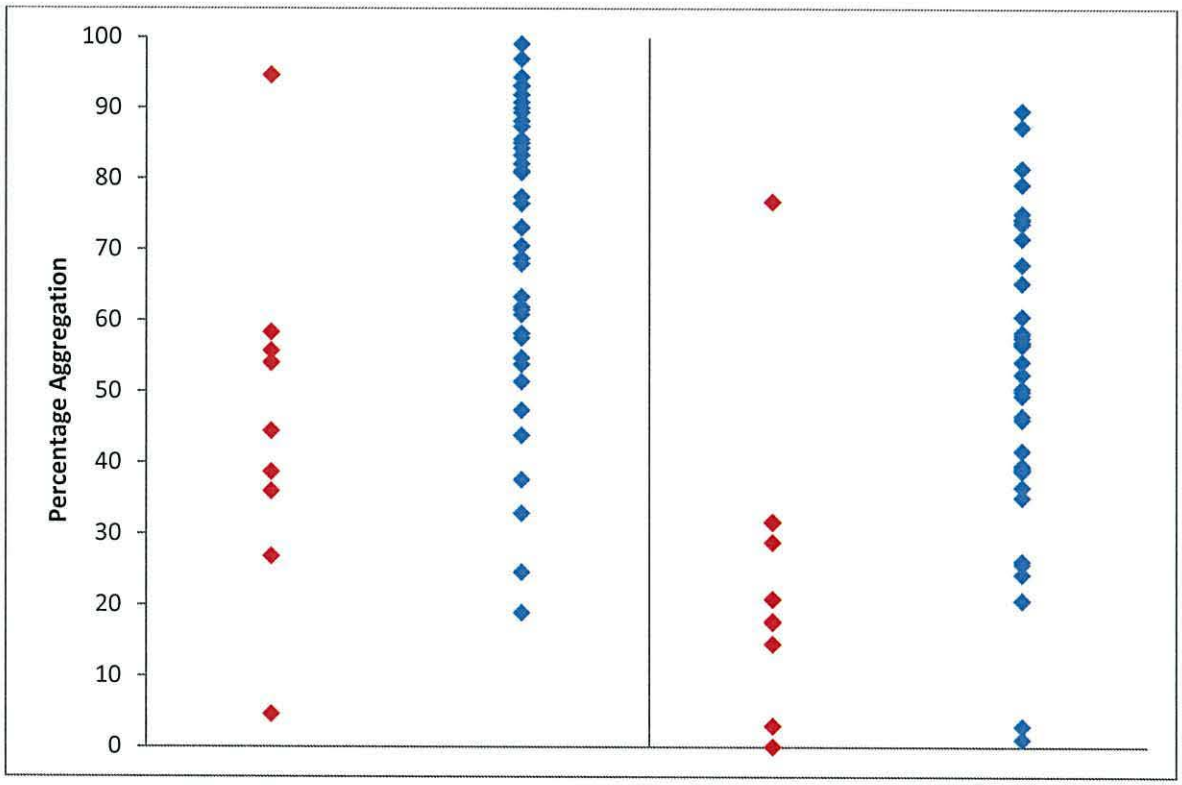


Figure 5.7.1 Graph showing the percentage aggregation values (method 2) for 50 different serum samples by category when using gold nanoparticles coated with a natural TDM mixture for the assay (left – non-centrifuged, right – centrifuged)(red – 10 TB positive samples obtained from the WHO, blue – 40 TB negative samples obtained from the WHO).

It can be seen that a distinction is observed between the TB+ and the TB- serum samples with and without centrifugation. Another observation is that there seems to be a difference in the results obtained with the two different sets of coated gold nanoparticles. When using coated gold nanoparticles that were centrifuged prior to their use in the assay, the percentage aggregation values for the TB+ samples are generally very low, and lower than the percentage aggregation values for the same samples when the coated gold nanoparticles were not centrifuged prior to their use. However, the values obtained for the TB- samples are also lower when using coated gold nanoparticles that were centrifuged prior to their use. This therefore has no effect on the overall classification of the serum samples, but may therefore suggest that a different 'cut-off' value should be taken for both of these sets of data, *i.e.* centrifuged and non-centrifuged, in order to obtain the best distinction between the TB+ and the TB- samples.

Although different values are obtained when using 'method 1' and 'method 2' for calculating the percentage aggregation, the patterns of the values are almost identical (*i.e.* the highest values with 'method 1' are the highest values with 'method 2', and the

lowest values with 'method 1' are the lowest values with 'method 2'). This suggests that using only one of these methods is sufficient when evaluating the assay.

When using coated gold nanoparticles that were not centrifuged prior to their use, an AUC value of 0.780, and sensitivity and specificity values of 90 % and 73 % respectively were obtained (with a cut-off value of 60 %). However, when using gold nanoparticles that had been centrifuged prior to use, the AUC value was raised to 0.827, and sensitivity and specificity values of 90 % and 83 % respectively were obtained (with a cut-off value of 32 %). Both of these sets of values are comparable to the results previously obtained with this antigen when using gold nanoparticles purchased from Aldrich (see Section 5.5), confirming earlier observations, that the re-optimised conditions and use of gold nanoparticles from BBInternational were suitable for use in the assay.

The above observations suggest that the gold nanoparticles coated with the natural TDM mixture should be centrifuged prior to their use in the assay to give the best distinction between TB+ and TB- serum samples by percentage aggregation. However, when performing eye tests on the solutions at the end of the assay, many more '?' designations were made when using the coated gold nanoparticles which had been centrifuged prior to their use, as compared to when using those which had not been centrifuged. This suggested that if a colour sensor designed to be read by eye were developed, coated gold nanoparticles which had not been centrifuged should be used, however, if a colour sensor is designed to be read by a machine at two different wavelengths, coated gold nanoparticles which had been centrifuged should be used.

Due to these findings, it was decided to analyse all of the serum samples with both 'centrifuged' and 'non-centrifuged' coated gold nanoparticles unless otherwise stated.

5.7.2 Analysis of the Serum Samples Using Various Synthetic Antigens

Having performed the assay with gold nanoparticles coated with a natural TDM mixture, and found that a distinction was observed between the TB+ and TB- serum samples analysed, it was decided to analyse the same serum samples with a range of different synthetic antigens. A wide range of new antigens had been synthesised by this point in the project, thus analysis of the serum samples with all of these antigens was possible.

Each antigen in turn was coated onto gold nanoparticles (using thio-stearic acid as a linker compound where needed) and analysed with the 50 serum samples from group 1.

A summary of the data obtained from these experiments can be seen in Table 5.7.2, and the values obtained when using the natural TDM mixture are included for comparison. The individual percentage aggregation values obtained at the end of the assay, as well as the eye test results can be seen in the Appendix, Section 9.4.4. In Table 5.7.2, two sets of values are quoted for some antigens:[V] The top set of values represent the values obtained when using coated gold nanoparticles which had not been centrifuged prior to their use (non-centrifuged coated gold nanoparticles); while the bottom set of values represent those obtained with coated gold nanoparticles which had been centrifuged prior to their use (centrifuged coated gold nanoparticles). Again, all of the analysis has been performed on the values obtained for percentage aggregation calculated using 'method 2'. The structures of all of the antigens used can be seen in the Appendix, Section 9.1.1.

[V] Some antigens were not performed 'centrifuged' because they either showed a poor distinction between the TB positive and TB negative serum samples when they were analysed 'non-centrifuged', or a high cut-off percentage aggregation value was required in order to obtain a good sensitivity and specificity value with that antigen, thus would not be suitable for use in a colour sensor.

Table 5.7.2 Table showing a summary of the results obtained when analysing the first group of 50 serum samples from the WHO using gold nanoparticles coated with various antigens.

Antigen	Antigen Type	Cut-Off Percentage Aggregation	Sensitivity (%)	Specificity (%)	Average Percentage Aggregation for TB Positive Serum Samples	Average Percentage Aggregation for TB Negative Serum Samples	AUC
Natural TDM Mixture		60.00	90	73	46.70	70.78	0.780
		32.00	90	83	23.42	50.60	0.827
24	*cis*[VI]-Methoxy TDM	59.35	90	68	44.36	67.77	0.777
		39.50	90	78	29.09	50.52	0.797
30	*cis*-Methoxy TDM	71.50	100	75	18.88	79.91	0.900
		55.00	100	80	22.28	69.82	0.912
28	*cis*-Methoxy TMM	66.90	90	75	47.93	75.80	0.780
		56.25	90	73	37.82	63.53	0.780
31	*trans*-Methoxy TMM	71.80	90	63	52.68	71.69	0.737
		43.60	90	70	30.59	55.04	0.790
32	*cis*-Methoxy Mycolic Acid	60.00	100	80	21.01	79.26	0.915
		57.70	90	83	40.39	76.63	0.922
22	Thiol Modified *trans*-Methoxy Mycolic Acid	55.40	60	53	41.58	52.49	0.540
27	Thiol Modified *cis*-Methoxy Mycolic Acid	71.00	90	70	54.55	75.81	0.777
		52.50	90	68	36.36	60.59	0.807
33	Thiol Modified *cis*-Methoxy Mycolic Acid	70.90	80	70	41.12	71.19	0.757
34	*cis*-α-TDM	77.25	80	68	59.81	80.04	0.732
		45.20	90	68	31.07	52.66	0.762
35	*cis*-α-TMM	31.00	100	70	-16.92	46.09	0.840
		22.00	100	73	2.77	47.59	0.872
36	*cis*-α-Mycolic Acid	91.70	100	73	36.48	91.21	0.860
37	Thiol Modified *cis*-α-Mycolic Acid	64.00	80	63	40.53	64.21	0.715
38	*cis*-Keto TDM	40.50	100	83	19.94	65.20	0.892
		43.50	100	80	10.13	55.10	0.905
39	*trans*-Keto TDM	74.00	90	68	53.66	76.96	0.787
		64.60	90	65	44.14	67.96	0.765
40	*cis*-Keto Mycolic Acid	82.00	100	75	45.62	88.55	0.875
41	*cis*-Epoxy Mycolic Acid	92.50	100	75	46.62	92.38	0.855

[VI] The pre-fix *cis* and *trans*, refer to the stereochemistry of the cyclopropane ring in the proximal position of the meromycolate moiety.

The above table shows that in general, there were no significant differences in the results obtained at the end of the assay when it was carried out using centrifuged or non-centrifuged coated gold nanoparticles. In five of the eleven cases where the assay was performed with both centrifuged and non-centrifuged coated gold nanoparticles, the best combined sensitivity and specificity values were obtained with the non-centrifuged coated gold nanoparticles, while in the other six cases, the best values were obtained with centrifuged coated gold nanoparticles. However, when looking at the eye test results (see Section 9.4.4), a much higher number of samples are designated as '?' when using centrifuged coated gold nanoparticles for the assay, suggesting that it was harder to make a distinction between the TB+ and TB- serum samples by eye when using these.

With the aim of the project being to develop a rapid, easy to use, point-of-care sensor for the detection of TB, a colour sensor, which could be read by eye, would be desirable. For this reason, and due to the values obtained when using centrifuged and non-centrifuged coated gold nanoparticles being very similar, it was decided to only use the results obtained with non-centrifuged gold nanoparticles for the analysis of these serum samples.

From the data obtained, it can be seen that the thiol modified antigens (**22**, **27** and **37**) in general give a reasonably poor distinction between the TB+ and TB- samples. The best distinction between the TB+ and TB- serum, using a thiol modified mycolic acid, was observed with **27**, which gave sensitivity and specificity values of 90 % and 70 % respectively. The worst distinction was observed with **22** which gave values of 60 % and 53 % respectively. Due to the poor performance of these antigens in these experiments it was decided that future experiments should be concentrated on non-thiol modified antigens. Therefore, **22**, **33** and **37** were not used in future experiments. It was decided to continue using **27** because it was the best performing thiol modified antigen, and it was believed that using a thiol modified antigen with a large set of serum samples might reveal some distinction in those serum samples which may have otherwise been overlooked.

Another observation was that using gold nanoparticles coated with free mycolic acids generally gave a good distinction between the TB+ and TB- serum samples. With the free mycolic acids (**32**, **36**, **40** and **41**), very high sensitivity and specificity values could be obtained. The lowest values were obtained when using **36** as the antigen which gave values of 100 % and 73 % respectively, while the highest values were obtained when using **32** which gave values of 100 % and 80 % respectively.

Although good sensitivity and specificity values were obtained when using these antigens, a very high cut-off value for the percentage aggregation, with the exception of when using **32**, was required in order to obtain these values. Cut-off values of 91.70 %, 82.00 % and 92.50 % were required for **36**, **40** and **41** respectively. This suggested that even though a distinction could be observed when calculating percentage aggregation, this was not possible by observing the colour of the solutions by eye, as some of the solutions which were analysed with TB+ serum samples appeared blue at the end of the assay. When observing the results of the eye tests (see Section 9.4.4), this was confirmed to be the case. When using **36** as the antigen, only three of the ten TB+ serum samples were correctly identified by both persons performing the eye tests, while five of the ten TB+ serum samples were correctly identified with **40**, and four of the ten when using **41**. This is in contrast to when **32** was used as the antigen where all ten of the TB+ serum samples were correctly identified by both persons performing the eye test.

These observations suggested that free mycolic acids may distinguish TB+ serum samples from TB- serum samples, however, with the exception of **32**, none of those analysed would be suitable for use in a device which would be read by eye. However, in light of the high sensitivity and specificity values obtained with these antigens, it was decided to continue using these free mycolic acids as antigens for the next set of experiments.

When using gold nanoparticles coated with TDMs and TMMs for the assay, varying results were obtained. The best distinction between the TB+ and TB- serum samples was observed when using **38** and **30** as the antigens, which gave sensitivity and specificity values of 100 % and 83 %, and 100 % and 75 % respectively. These results were very encouraging because it was with these antigens that the best distinction between the TB+ and TB- serum samples had also been observed by ELISA.[2] This was encouraging because this implied that the results obtained by this assay were related to those obtained by ELISA, which could suggest that it was antibodies that were being detected by this assay.

Crucially, with these antigens, intermediate percentage aggregation cut-off values were required to obtain these sensitivity and specificity values. In contrast to when the free mycolic acids **36**, **40** and **41** were used as the antigens, when **38** was used as the antigen, all ten TB+ serum samples were correctly identified by the eye tests (one of the persons performing the tests designated one of the samples as '?' while the other designated the

sample as '+'), and when using **30** as the antigen, nine of the ten TB+ samples were correctly identified by the eye tests, while the other sample was designated as '?' by both persons. These results were very encouraging for developing a colour sensor, which could be read by eye (or by machine) for the detection of TB.

An interesting observation could be made when looking at antigens of the same class (*e.g.* methoxy TDM), but which contained different stereochemistries. For example, **24** and **30** are both methoxy TDMs, however **30** performs much better in this assay than **24**. This strongly suggests that the stereochemistry of the antigen used to coat the gold nanoparticles for this assay has a crucial role in the amount of binding occurring to it.

Another interesting observation could be made when comparing how well TDMs and TMMs with the same mycolic acid moiety performed. Compounds **24** and **28** both contain the same methoxy mycolic acid, however the TMM, **28**, performed better than its TDM counterpart when analysing these serum samples, with sensitivity and specificity values of 90 % and 75 % respectively being obtained, as compared to values of 90 % and 68 % respectively obtained when using **24** as the antigen. The same pattern was again observed when comparing the results obtained when using **34** (α-TDM) and **35** (α-TMM), which contain the same α-mycolic acid. Again, the TMM out-performed its TDM counterpart, with sensitivity and specificity values of 100 % and 70 % respectively being obtained with **35**, while the values reduced to 80 % and 68 % when using **34**.

In light of these results, it was believed that the TMM counterparts to **30** and **38** would give even better distinctions between TB+ and TB- serum samples. However, when attempting to coat gold nanoparticles with the corresponding TMM to **38**, aggregation of the coated gold nanoparticles occurred. This was re-attempted multiple times with the same result. Upon further inspection, it was discovered that the TMM appeared orange/red in colour while the TDM appeared white/colourless. This suggested that this compound may have contained an impurity that led to aggregation of the coated gold nanoparticles during the coating process. It was therefore not possible to carry out the analysis of the serum samples using this assay with this antigen. Also by the end of this project, the TMM counterpart to **30** had not been synthesised, thus could also not be used in this project.

Another important observation from these results was that many of the results obtained with synthetic antigens were better, or gave more distinction between the TB+ and TB- serum samples, than those obtained when using the natural TDM mixture. This was very

encouraging as it suggested that synthetic antigens would potentially be better to use in a sensor for the detection of TB than naturally occurring antigens.

5.7.3 Analysis of 50 Blind Serum Samples

Since a good distinction between TB+ and TB- serum samples had been obtained by this assay with some of the antigens analysed, it was decided to move on to look at a larger set of serum samples. It was decided to test fifty serum samples (from group 2) in a blind experiment (a full list of the serum samples can be found in Table 9.1.2). This ensured no bias in the assay due to knowing the TB status of the serum samples beforehand, while also giving a good indication of how effective the assay performed with various antigens.

It was decided to use all of the antigens which had been used in the previous experiments (see Section 5.7.2), with the exception of the poorest performing thiol modified antigens (**22**, **37** and **33**). Performing the assay with gold nanoparticles coated with these various antigens was done to gain a good overview of how well the assay performed, while also allowing analysis of a larger set of data, which would hopefully give further information as to which antigens would be best used in a sensor for the detection of TB.

As these serum samples were tested blind, predictions were made on whether they were TB+ or TB- by using the optimal percentage aggregation cut-off values for the un-blinded serum samples (from group 1). A summary of these predictions can be seen in Table 5.7.3, and a full list of the individual percentage aggregation values obtained, as well as the eye test results can be seen in the Appendix, Section 9.4.6. As was seen in Table 5.7.2, two sets of values are quoted with some antigens and again the top set of values represent those obtained when using non-centrifuged coated gold nanoparticles, while the bottom set represent data obtained when using centrifuged coated gold nanoparticles.

Table 5.7.3 Table showing a summary of the predictions made with the second group of 50 serum samples from the WHO, run blind, and then un-blinded, using gold nanoparticles coated with various antigens.

Antigen	Antigen Type	Cut-Off Percentage Aggregation (Based on Initial Set of 50 Serum Samples)	Number of TB Positive Serum Samples Predicted	Number of TB Negative Serum Samples Predicted	Number of TB Positive Serum Samples Correctly Identified	Number of TB Negative Serum Samples Correctly Identified	Positive Serum Samples Correctly Identified (%)	Negative Serum Samples Correctly Identified (%)
Natural TDM Mixture		60.00	32	18	14	15	82	45
		32.00	29	21	14	17	82	52
24	*cis*-Methoxy TDM	59.35	34	16	15	14	88	42
		39.50	35	15	15	13	88	39
30	*cis*-Methoxy TDM	71.50	32	18	17	18	100	55
		55.00	30	20	17	20	100	61
28	*cis*-Methoxy TMM	66.90	30	20	14	17	82	52
		56.25	34	16	15	14	88	42
31	*trans*-Methoxy TMM	71.80	39	11	17	11	100	33
		43.60	40	10	16	9	94	27
32	*cis*-Methoxy Mycolic Acid	60.00	23	27	14	24	82	73
		57.70	28	22	16	21	94	64
27	*cis*-Thiol Modified Methoxy Mycolic Acid	71.00	30	20	14	17	82	52
		52.50	22	28	14	25	82	76
34	*cis*-α-TDM	77.25	37	13	16	12	94	36
		45.20	33	17	15	15	88	45
35	*cis*-α-TMM	31.00	25	25	14	22	82	67
		22.00	20	30	12	25	71	76
36	*cis*-α-Mycolic Acid	91.70	26	24	14	21	82	64
38	*cis*-Keto TDM	40.50	23	27	16	26	94	79
		43.50	32	18	17	18	100	55
39	*trans*-Keto TDM	74.00	39	11	16	10	94	30
		64.60	42	8	17	8	100	24
40	*cis*-Keto Mycolic Acid	82.00	30	20	17	20	100	61
41	*cis*-Epoxy Mycolic Acid	92.50	32	18	16	17	94	52

The results of this blind test were encouraging. Upon un-blinding the TB status of the serum samples, 17 TB+ and 33 TB- samples were identified. Although a significant number of false positive results were obtained with some antigens, the majority of the TB+ samples were correctly identified with most of the antigens. The best results were obtained with **30**, **40** and **38**, with all of the TB+ samples and 61 % of the TB- samples being correctly identified with **30** and **40**, and 94 and 79 % of the TB+ and TB- samples respectively correctly identified with **38**.

Due to the cut-off percentage aggregation values being based on only a small number of samples, it is possible that these values are not optimal for giving the best distinction between TB+ and TB- samples. Therefore, after un-blinding the TB status of the serum samples, further analysis was performed, once the results and their interpretation had been formally recorded.

A summary of the data obtained from this analysis can be seen in Table 5.7.4, while that performed with the data obtained with all 100 serum samples can be seen in Table 5.7.5. Again, two sets of values are quoted with some antigens and again the top set of values represent those obtained when using non-centrifuged coated gold nanoparticles, while the bottom set represent data obtained when using centrifuged coated gold nanoparticles.

Table 5.7.4 Table showing a summary of the results obtained when analysing the second group of 50 serum samples from the WHO, after their TB status was un-blinded, using gold nanoparticles coated with various antigens using the gold nanoparticle assay.

Antigen	Antigen Type	Cut-Off Percentage Aggregation	Sensitivity (%)	Specificity (%)	Average Percentage Aggregation for TB Positive Serum Samples	Average Percentage Aggregation for TB Negative Serum Samples	AUC
Natural TDM Mixture		39.20	82	79	20.62	56.10	0.807
		27.25	82	76	7.31	36.03	0.845
24	*cis*-Methoxy TDM	38.70	82	82	17.95	53.46	0.832
		23.50	82	82	14.48	37.20	0.859
30	*cis*-Methoxy TDM	60.00	100	64	17.32	69.55	0.879
		46.70	100	70	22.51	60.39	0.884
28	*cis*-Methoxy TMM	39.50	82	82	23.15	63.70	0.841
		29.70	82	82	9.57	46.28	0.865
31	*trans*-Methoxy TMM	56.85	88	67	21.09	58.01	0.831
		22.22	82	61	2.70	29.37	0.806
32	*cis*-Methoxy Mycolic Acid	73.55	100	64	44.30	74.84	0.800
		43.00	94	85	25.30	61.43	0.861
27	*cis*-Thiol Modified Methoxy Mycolic Acid	51.55	82	82	33.07	64.45	0.822
		47.40	82	85	38.93	57.96	0.863
34	*cis*-α-TDM	50.70	82	76	25.81	63.52	0.809
		18.90	82	88	9.41	45.47	0.854
35	*cis*-α-TMM	39.90	94	61	11.90	51.78	0.759
		35.80	94	58	14.26	43.30	0.758
36	*cis*-α-Mycolic Acid	96.20	100	58	59.03	85.50	0.784
38	*cis*-Keto TDM	45.85	100	67	21.77	60.85	0.879
		19.00	100	79	3.31	43.71	0.884
39	*trans*-Keto TDM	53.80	82	70	27.32	59.37	0.791
		19.60	82	88	12.52	44.67	0.834
40	*cis*-Keto Mycolic Acid	81.40	100	61	54.71	78.86	0.772
41	*cis*-Epoxy Mycolic Acid	79.70	84	70	58.29	80.66	0.729

From this table, it can be seen that the results obtained when analysing these serum samples generally agree with those for the first set of serum samples, suggesting that there was no bias in the results obtained from the first set even though the TB status of those samples were known prior to their analysis by this assay (see Section 5.7.2).

As with the results for the first 50 samples, the results obtained when using centrifuged coated gold nanoparticles and when using non-centrifuged gold nanoparticles are very similar with most antigens. Again, however, when observing the eye test results (see Section 9.4.6) many more '?' designations were made, and more false positive samples were predicted, when using centrifuged gold nanoparticles. This would again suggest that even though in some instances, centrifuged coated gold nanoparticles may give a slightly better distinction between TB+ and TB- serum samples, using percentage aggregation, the results obtained when using non-centrifuged gold nanoparticles would be more applicable for the development of a colour sensor which could be read both by eye, and by measuring the absorbance of the solutions at two different wavelengths.

It can also be observed that the thiol modified mycolic acid, **27**, performed slightly better with this second set of serum samples, which suggested that it may be suitable for use in the development of a sensor for the detection of TB, and that a larger number of serum samples need to be analysed with this type of antigen before ruling them out completely.

The free mycolic acids (**32**, **36**, **40** and **41**) again performed well in the assay, giving reasonably high sensitivity and specificity values with the second set of serum samples. However, with the exception of **32**, the same problem, as with the first set of samples, was observed in that a very high percentage aggregation cut-off value was required in order to obtain these high sensitivity and specificity values. These results again suggested that, even though a distinction could be observed between the TB+ and TB- serum samples with free mycolic acids, if a sensor designed to be read by eye were to be developed, these antigens would not be as useful due to only a small number of the samples being correctly identified as TB+ by the eye tests (7 of 17 were correctly identified when using gold nanoparticles coated with **40** and **36** and only 6 of 17 when using **41**).

Again, it was also observed that the antigens which gave the best distinction between the TB+ and TB- samples with the first set of serum samples, **38** and **30**, were also those which gave the best distinction here. This again suggests that there was no bias in the results obtained when analysing the first set of serum samples.

A slight discrepancy, however, was observed between the two sets of data when comparing how well TDMs and TMMs with the same mycolic acid moiety performed. With the first set of 50 serum samples, the TMMs out-performed their TDM counterpart in both instances (see Section 5.7.2). However, when analysing the second set of serum samples,

24 and **28** both performed equally well giving sensitivity and specificity values of 82 %, while **34** (α-TDM) slightly out-performed **35** (α-TMM) giving sensitivity and specificity values of 82 and 76 % compared to the values of 94 and 60 % obtained when using **35**.

Despite this discrepancy, in general the results obtained when analysing the second set of serum samples did agree with those obtained with the first set of serum samples, suggesting that there was no bias in the results obtained from the first set of serum samples.

It was therefore decided to combine the data obtained with all 100 serum samples tested with each antigen, in order to determine which antigens were best at distinguishing TB+ from TB- serum samples. This was performed to give an insight into which antigens should be coated onto gold nanoparticles for use in the assay when performing a blind test of 249 serum samples later in the project (see Section 5.7.5).

A summary of the results obtained with all 100 serum samples can be seen in Table 5.7.5.

Table 5.7.5 Table showing a summary of the results obtained when analysing the 100 serum samples from the WHO, after their TB status was un-blinded, using gold nanoparticles coated with various antigens using the gold nanoparticle assay.

Antigen	Antigen Type	Cut-Off Percentage Aggregation	Sensitivity (%)	Specificity (%)	Average Percentage Aggregation for TB Positive Serum Samples	Average Percentage Aggregation for TB Negative Serum Samples	AUC
Natural TDM Mixture		58.31	85	64	30.27	64.14	0.813
		34.80	89	69	13.28	44.01	0.846
24	*cis*-Methoxy TDM	55.60	82	66	22.73	61.30	0.815
		43.66	96	53	19.89	44.50	0.840
30	*cis*-Methoxy TDM	71.35	100	66	17.90	75.23	0.894
		54.15	100	71	22.43	65.56	0.898
28	*cis*-Methoxy TMM	66.90	85	64	32.33	70.33	0.825
		57.40	96	56	20.04	55.73	0.841
31	*trans*-Methoxy TMM	64.15	82	62	32.79	65.50	0.800
		38.00	82	62	13.03	43.43	0.798
32	*cis*-Methoxy Mycolic Acid	73.55	100	69	35.50	77.26	0.856
		72.45	96	69	30.89	69.76	0.896
27	*cis*-Thiol Modified Methoxy Mycolic Acid	66.65	78	71	41.02	71.13	0.815
		48.35	82	75	37.98	59.40	0.834
34	*cis*-α-TDM	69.85	74	66	38.40	72.57	0.788
		45.75	96	58	17.43	49.41	0.829
35	*cis*-α-TMM	39.90	96	66	1.22	48.67	0.787
		35.80	96	64	10.01	45.65	0.804
36	*cis*-α-Mycolic Acid	95.60	100	64	50.86	88.63	0.821
38	*cis*-Keto TDM	45.90	100	74	20.10	63.24	0.885
		20.00	93	81	5.84	49.95	0.886
39	*trans*-Keto TDM	59.10	74	74	37.08	69.01	0.801
		46.30	78	67	24.23	57.43	0.809
40	*cis*-Keto Mycolic Acid	81.65	100	69	51.34	84.17	0.835
41	*cis*-Epoxy Mycolic Acid	92.05	96	66	53.97	87.08	0.790

From this data, it can be seen that **38**, **32** and **30** seem to be the best antigens at distinguishing TB+ serum samples from TB- serum samples by this assay, while also having the potential to be used in a colour sensor (to be read by eye) for the detection of TB. With each of these three antigens, a sensitivity of 100 % was achieved, with

specificity values of 74 % for **38**, 69 % for **32** and 66 % for **30**. These values for sensitivity and specificity were much higher than those observed when using gold nanoparticles coated with the natural TDM mixture for the assay, suggesting that the synthetic antigens may be best to use in a sensor for the detection of TB.

A graphical representation of the data obtained with these three antigens can be seen in Figure 5.7.2:

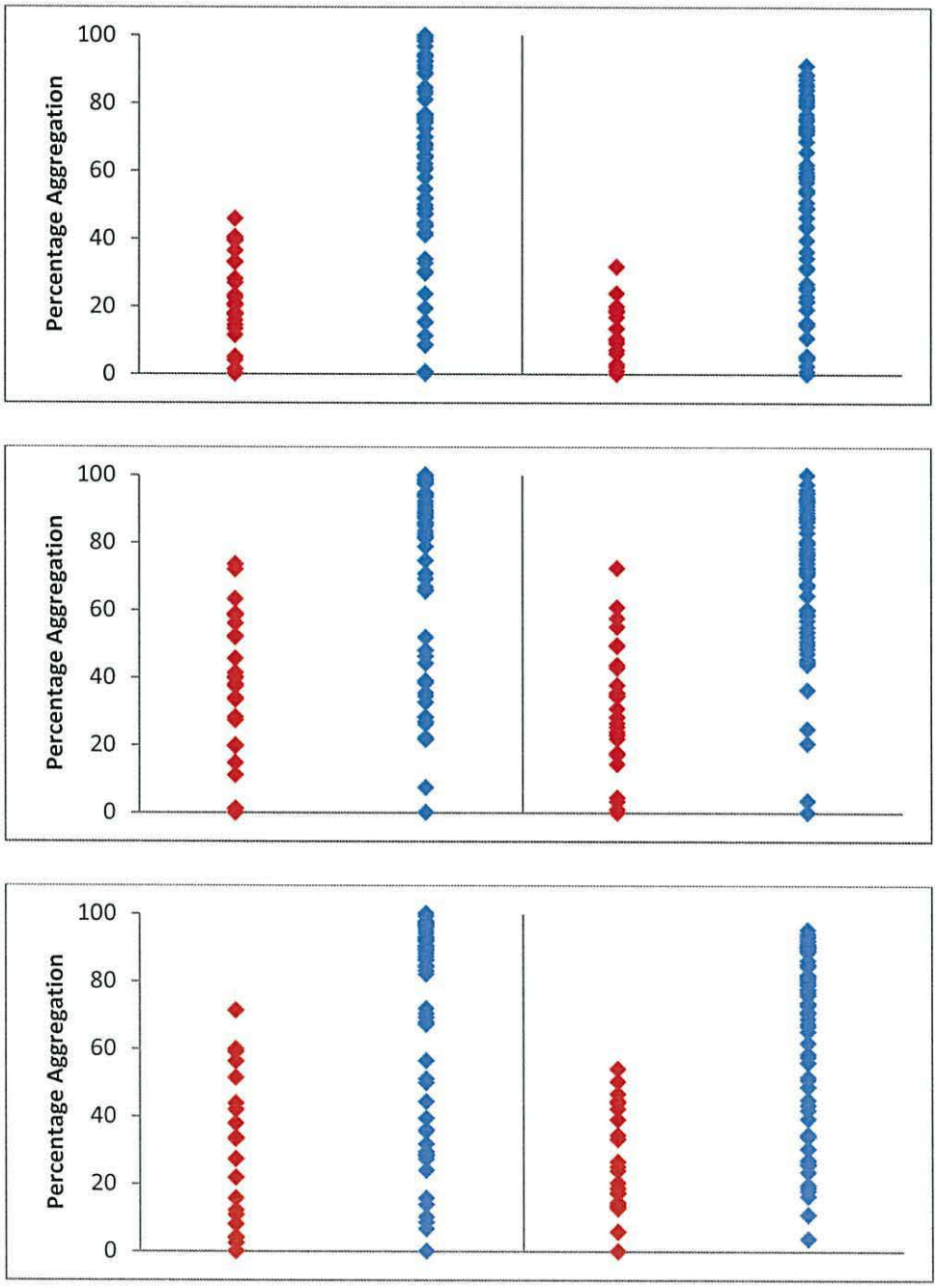


Figure 5.7.2 Graph showing the percentage aggregation values (method 2) for 100 different serum samples by category when using gold nanoparticles coated with 38 (top), 32 (middle) and 30 (bottom) for the assay (left – non-centrifuged, right – centrifuged)(red – 27 TB positive samples obtained from the WHO, blue – 73 TB negative samples obtained from the WHO).

As the aim of the project was to develop a rapid, portable, reliable and easy to use sensor, which could be used in remote regions where access to on-site laboratories is not possible, it was decided to try and further evaluate the assay and analyse as many of the antigens as possible with a large set of blind serum samples in order to gain a greater overview of which antigens would be best to use in a sensor for the detection of TB. Due to time and financial constraints, all of the antigens could not be used for these experiments, therefore the antigens to be used were chosen based on which had previously best distinguished TB+ serum samples from TB- serum samples.

The first three antigens, **38**, **32** and **30**, were chosen because a good distinction between TB+ and TB- serum samples was previously observed when using these antigens in the gold nanoparticle based assay. When selecting the remaining antigens it was decided to analyse as broad a range as possible, and therefore to use one antigen from each 'class' of antigens. It was also decided to try and investigate TDMs and TMMs with the same mycolic acid moiety in order to check which would perform best, while also trying to investigate the effect that different stereochemistry would have on the results obtained.

With these criteria, **34** and **35** were selected for use as they were the only α-TDM and α-TMM antigens which had been analysed with the previous 100 serum samples. Using these antigens would also give an indication as to whether TDMs or TMMs with the same mycolic acid moiety could distinguish TB+ serum samples from TB- serum samples better than the other. In order to investigate this further, a methoxy TDM (**24**) and TMM (**28**) were also chosen. Using **24** along with **30** also allowed the investigation of antigens only differing in their stereochemistry.

Although **27** had shown a poorer distinction between the TB+ and TB- serum samples already tested, compared to some antigens, giving a sensitivity and specificity of 78 % and 71 % respectively, it was decided that this antigen should be used in these experiments in order to test a thiol modified antigen with a large set of serum samples, and this was the best antigen of this type available (see Section 5.7.2).

Even though the other free mycolic acids (**36**, **40** and **41**) showed a distinction between the TB+ and TB- samples with the gold nanoparticle based assay, when analysing by percentage aggregation values, these antigens were not used with the blind set of serum samples because the eye test results when analysing the serum samples with these antigens showed many TB+ samples being misclassified as TB- (see Section 5.7.2 and 5.7.3). Free

mycolic acids had also given a poor distinction between the TB+ and TB- serum samples by ELISA.

The natural TDM mixture could not be used because it was no longer supplied by Aldrich, while the keto TMM counterpart to **38** could not be used because it could not be coated onto gold nanoparticles (see Section 5.7.2).

5.7.4 Analysis of TB Negative Serum Samples from the UK

Prior to performing a blind test on an additional 249 serum samples (from the third group of serum samples, see Section 9.1.2), it was decided to make completely sure that serum samples from healthy patients gave high percentage aggregation values by this assay. This was done by analysing serum samples obtained from persons in the UK who had no TB like symptoms, and were thought to be healthy. Twenty five serum samples were analysed with gold nanoparticles coated with the various antigens that were to be used with the 249 blind serum samples from TB indigenous populations.

A summary of the percentage aggregation values as well as a summary of the eye test results when performing the assay with these serum samples can be seen in Table 5.7.6, and a full list of the percentage aggregation values and eye test results can be seen in the Appendix, Section 9.4.7. It was decided to perform these experiments using non-centrifuged coated gold nanoparticles only, because this method has shown the most promise for being developed into a sensor for the detection of TB which could be read both by eye, and by measuring the absorbance of the solution at the end of the assay at two different wavelengths (see Section 5.7.2 and 5.7.3).

Because no TB+ serum samples were present in this set of samples, sensitivity and specificity values are not quoted for this set of data. For the eye test results, in instances where both persons designated the colour of the solution to be indicative of a TB+ serum sample, the sample is designated as '+'. When both persons designate the serum sample as '?', or when different designations were given to the solutions at the end of the assay by both persons performing the eye test, the sample is designated as '?'. When both persons designate the colour of the solution to be indicative of a TB- serum sample, the sample is designated as a '-'.

The serum samples were also tested in the assay with gold nanoparticles coated with the three different free mycolic acids (**36**, **40** and **41**) for comparison, and the results obtained from these experiments are also included in Table 5.7.6 for completeness.

Table 5.7.6 Table showing a summary of the results obtained when analysing the 25 TB negative serum samples from the UK, using gold nanoparticles coated with various antigens using the gold nanoparticle assay.

Antigen	Antigen Type[VII]	Average Percentage Aggregation	Eye Test Results (Number of Serum Samples Designated as the Following)		
			+	?	-
24	Methoxy TDM	71.43	0	11	14
30	Methoxy TDM	94.57	0	0	25
28	Methoxy TMM	90.18	0	1	24
32	Methoxy Mycolic Acid	85.45	0	2	23
27	Thiol Modified Methoxy Mycolic Acid	76.23	1	3	21
34	α-TDM	85.11	0	5	20
35	α-TMM	97.65	0	0	25
38	Keto TDM	91.45	0	0	25
36	α-Mycolic Acid	103.16	0	0	25
40	Keto Mycolic Acid	97.72	0	0	25
41	Epoxy Mycolic Acid	99.23	0	0	25

It can be seen from this table that high percentage aggregation values are observed with all of the antigens, suggesting that no, or very little, binding of antibodies occurred to the antigens coated onto the gold nanoparticle surfaces. It can also be seen, that very promising eye test results were obtained with these serum samples. With the exception of one sample analysed with gold nanoparticles coated with **27**, no samples were designated as TB+ by both of the persons performing the eye tests. When using gold nanoparticles coated with the three best antigens for distinguishing TB+ serum samples from TB- serum samples by this assay (**38**, **32** and **30**), all 25 samples were identified as TB- by the eye tests by both persons performing the test (as well as by the percentage aggregation values (see the Appendix, Section 9.4.7)) when using **38** and **30** as the antigens, and 23 of the 25 serum samples were correctly identified when using **32**.

These results were very encouraging because all of the serum samples tested, from healthy patients, from a non-endemic TB population, gave high percentage aggregation values, and

[VII] All of the antigens used with this assay from this point foreward contained a *cis* cyclopropane ring in the proximal position of the meromycolate moiety, therefore the prefix, *cis* or *trans*, is no longer stated.

were identified as negative by the eye tests, at the end of the assay, with the majority of the antigens. These results therefore further support previous observations suggesting that any serum samples tested by this assay that results in a red/pink coloured solution and also has a low percentage aggregation value at the end of the assay is from an infected/unhealthy person.

5.7.5 Analysis of 249 Blind Serum Samples

Having confirmed that serum samples obtained from healthy patients did give high percentage aggregation values at the end of the assay (see Section 5.7.4), and that TB+ serum samples could be distinguished from TB- serum samples with a reasonable degree of accuracy with some antigens (see Section 5.7.3), it was decided to further evaluate the assay by blind testing 249 serum samples obtained from the WHO. These samples were tested with gold nanoparticles coated with the antigens chosen in Section 5.7.3, and were performed with centrifuged and non-centrifuged coated gold nanoparticles.

As these serum samples were tested blind, predictions were again made on whether they were TB+ or TB- by using the optimal percentage aggregation cut-off values for the un-blinded serum samples (from groups 1 and 2). A summary of these predictions can be seen in Table 5.7.7, and a full list of the individual percentage aggregation values obtained, as well as the eye test results for these 249 blind samples can be seen in the Appendix, Section 9.4.8. Two sets of values are again quoted with each antigen and again the top set of values represent the values obtained when using non-centrifuged coated gold nanoparticles, while the bottom set of values represent data obtained when using centrifuged coated gold nanoparticles.

In addition to the percentage aggregation values and eye test results obtained when performing the assay on these samples, results of statistical analysis, which were performed by Dr. James Gibbons, are also included. The values obtained for the statistical analysis (labelled as statistics), were obtained by a statistical combination of the results obtained when analysing the previous 100 serum samples with the four antigens which gave the best distinction between TB+ and TB- serum samples, and for the purposes of this analysis, these statistical outputs are treated as a separate 'antigen'. For the reasons previously discussed (see Section 5.7.2 and 5.7.3), the statistical analysis was performed on coated gold nanoparticles which had not been centrifuged prior to their use in the assay.

Table 5.7.7 Table showing a summary of the predictions made with the third group of 249 serum samples from the WHO, run blind, and then un-blinded, using gold nanoparticles coated with various antigens.

Antigen	Antigen Type	Cut-Off Percentage Aggregation (Based on Initial Set of 100 Serum Samples)	Number of TB Positive Serum Samples Predicted	Number of TB Negative Serum Samples Predicted	Number of TB Positive Serum Samples Correctly Identified	Number of TB Negative Serum Samples Correctly Identified	Positive Serum Samples Correctly Identified (%)	Negative Serum Samples Correctly Identified (%)
24	Methoxy TDM	55.60	175	74	55	57	77	33
		43.66	202	47	66	38	88	22
30	Methoxy TDM	71.35	179	70	70	65	93	37
		54.15	239	10	75	10	100	6
28	Methoxy TMM	66.90	185	64	63	52	84	30
		57.40	230	19	74	18	99	10
32	Methoxy Mycolic Acid	73.55	187	62	60	47	80	27
		72.45	204	45	68	38	91	22
27	Thiol Modified Methoxy Mycolic Acid	66.65	193	56	65	46	87	26
		48.35	219	30	69	24	92	14
34	α-TDM	69.85	225	24	73	22	97	13
		45.75	192	57	67	49	89	28
35	α-TMM	39.90	156	93	51	69	68	40
		35.80	197	52	69	46	92	26
38	Keto TDM	45.90	159	90	67	82	89	47
		20.00	93	156	38	119	51	68
Statistics		0.50	143	106	62	93	83	53

The first observation made was that a significant number of false positive results were obtained in this set of data with all of the antigens used, with on average, only 30 % of the TB- samples being correctly identified. However, it was again noted that the majority of the TB+ samples were correctly identified with most antigens, with on average 86 % of the TB+ samples being correctly identified.

Again, antigens **38** and **30** were the best at predicting TB+ and TB- serum samples with 89 and 47 % of the TB+ and TB- samples respectively being correctly identified with **38**, and 93 and 37 % respectively being correctly identified with **30**. It could also be noted that the

statistical analysis gave a better prediction of TB+ and TB- samples than most individual antigens.

Although the majority of the TB+ serum samples were correctly identified with most of the antigens, the high number of false positive results was a concern, therefore further analysis was performed, after un-blinding of the TB status of the serum samples investigated, once the results and their interpretation had been formally recorded. For these analyses, the data obtained from these blind experiments were combined with the previous results obtained with the 100 serum samples already tested, and this analysis can be seen in Table 5.7.8. Two sets of values are again quoted with each antigen, and the top set of values again represent the values obtained when using non-centrifuged coated gold nanoparticles, while the bottom set of values represent data obtained when using centrifuged coated gold nanoparticles.

In addition to the percentage aggregation values and eye test results obtained when performing the assay on these samples, statistical analysis results, which were performed by Dr. James Gibbons, are again included, and for the purposes of this analysis, these statistical outputs are again treated as a separate 'antigen'.

Table 5.7.8 Table showing a summary of the results obtained when analysing the 349 serum samples obtained from the WHO, using gold nanoparticles coated with various antigens using the gold nanoparticle assay.

Antigen	Antigen Type	Cut-Off Percentage Aggregation	Sensitivity (%)	Specificity (%)	Average Percentage Aggregation for TB Positive Serum Samples	Average Percentage Aggregation for TB Negative Serum Samples	AUC
24	Methoxy TDM	48.50	71	50	30.98	48.19	0.640
		29.10	71	51	19.10	30.23	0.634
30	Methoxy TDM	64.00	92	55	33.01	61.46	0.757
		24.00	78	53	14.65	34.42	0.697
28	Methoxy TMM	52.80	78	54	26.25	52.98	0.701
		29.30	71	53	19.70	32.00	0.621
32	Methoxy Mycolic Acid	80.37	90	36	35.78	53.75	0.637
		46.40	71	60	35.23	53.00	0.684
27	Thiol Modified Methoxy Mycolic Acid	66.63	84	41	36.05	54.54	0.676
		38.95	80	38	20.37	30.02	0.586
34	α-TDM	30.70	59	64	27.62	45.76	0.665
		26.25	71	58	18.09	35.28	0.693
35	α-TMM	41.60	78	47	21.11	38.10	0.629
		21.90	81	51	5.74	29.42	0.738
38	Keto TDM	40.50	89	62	24.63	51.51	0.749
		33.60	90	51	16.90	37.51	0.746
Statistics		0.44	90	61	0.733	0.349	0.801

From this table, the first observation was that all of the antigens analysed by this assay showed some distinction between the TB+ and TB- serum samples. It can also be seen that the results obtained when using centrifuged coated gold nanoparticles, and those obtained when using non-centrifuged coated gold nanoparticles were again generally very similar. However, with the two antigens that show the best distinction between the TB+ and TB- serum samples, *i.e.* **38** and **30**, using non-centrifuged coated gold nanoparticles gave a better distinction. When using **38**, sensitivity and specificity values of 89 and 62 % were obtained when using non-centrifuged coated gold nanoparticles, however, when centrifuged coated gold nanoparticles were used, the specificity dropped to 51 %. A similar trend was also observed when using **30** as the antigen for the assay, with sensitivity and specificity values of 92 and 55 % being obtained with the non-centrifuged coated gold nanoparticles, however these values again dropped to 78 and 53 % when centrifuging the

coated gold nanoparticles prior to their use in the assay. For these reasons, and for the reasons discussed in the previous sections (see Section 5.7.2 and 5.7.3), all further analysis of this data set was performed on non-centrifuged coated gold nanoparticles only.

An encouraging observation made from this data was that two out of the three antigens which could best distinguish TB+ serum samples from TB- serum samples when analysing the 100 previously tested samples (see Section 5.7.3), remained the antigens which gave the best distinction after testing the 249 blind serum samples *i.e.* **38** and **30**.

An important observation was that the statistical analysis gave a better distinction between the TB+ and the TB- serum samples than any individual antigen. The sensitivity and specificity values of 90 and 61 % respectively were reasonably high, and the AUC value of 0.801 (stating that when using these statistics, there is an 80.1 % probability that a TB+ serum sample will give a higher value than a TB- serum sample) was higher than any of the AUC values obtained with the individual antigens. This suggested than when developing a sensor for the detection of TB, a better distinction between TB+ and TB- samples may be obtained by using a combination of antigens. This again confirms earlier observations and suggests that any device developed for the detection of TB based on this assay, should include multiple antigens.

It can also be observed from this table that many of the patterns that were observed when analysing the results obtained with the first 100 serum samples tested (see Sections 5.7.2 and 5.7.3) were still true when analysing the results obtained with all 349 serum samples. Firstly, when looking at the two antigens which were of the same class (methoxy TDM), but which had different stereochemistries, **30** and **24**, **30** still performed much better in this assay than **24**. This strongly suggests that the stereochemistry of the antigen used to coat the gold nanoparticles for this assay has a crucial role in the amount of binding occurring to it. A range of antigens from the same class, differing only in their stereochemistry should be investigated in order to investigate this further.

Previous analysis of the results obtained after testing the first 100 serum samples (see Section 5.7.3) were inconclusive as to whether TDMs or TMMs coated onto gold nanoparticles gave the best distinction between TB+ and TB- serum samples by this assay, and the results obtained after testing the 349 serum samples remained inconclusive. When comparing **24** and **28**, the methoxy TMM, **28**, gave the better distinction between the TB+ and TB- serum samples, giving sensitivity and specificity values of 78 and 54 %

respectively compared to the values of 71 and 50 %, which were obtained with **24**. However, when comparing **34** and **35**, the performance of both antigens were similar. These results suggest that in some instances TDMs perform better than their TMM counterpart, however in other instances, TMMs perform better than their TDM counterpart. Further analysis investigating a wider range of TDM and TMM antigens, containing the same mycolic acid moiety, needs to be carried out in order to gain a greater insight as to whether TDMs or TMMs give the best distinction between TB+ and TB- serum samples.

Analysis of Serum Samples from Patients Co-Infected with Malaria

Upon further analysing the results obtained with these serum samples, and the data provided by the WHO about the samples, it was discovered that many of the serum samples which were obtained from patients who were co-infected with malaria gave false positive values by this assay (see Section 9.1.2 for a full list of the serum samples and any co-infection they may have). The data provided indicated that 79 of the serum samples were from patients that were co-infected with malaria, or who had been infected with malaria within the year prior to the sample being taken. Of these 79 serum samples 61-68 of the samples generally gave a positive response in this assay, with most of the antigens (see Section 9.4.8).

Table 5.7.9 shows the sensitivity and specificity values obtained for the different antigens when analysing the results obtained with serum samples co-infected with malaria; serum samples not co-infected with malaria; serum samples co-infected with any other co-infection; and serum samples from patients who had no co-infection. The sensitivity and specificity values obtained for the antigens, when analysing all 349 serum samples, are also shown in Table 5.7.9 for comparison, and the cut-off values for all of these calculations have been kept the same as they were when analysing the full set of 349 serum samples. Only the data obtained when using non-centrifuged coated gold nanoparticles are included for the analysis.

Table 5.7.9 Table showing the sensitivity and specificity values obtained with non-centrifuged coated gold nanoparticles, by category of co-infection.

Antigen	Antigen Type	Co-Infection Status	Sensitivity (%)	Specificity (%)
24	Methoxy TDM	All 349 Serum Samples	71	50
		Co-Infected with Malaria	95	25
		Not Co-Infected with Malaria	66	59
		Non-Malaria Co-Infection	56	48
		No Co-Infection	69	66
30	Methoxy TDM	All 349 Serum Samples	92	55
		Co-Infected with Malaria	100	24
		Not Co-Infected with Malaria	91	65
		Non-Malaria Co-Infection	93	59
		No Co-Infection	90	69
28	Methoxy TMM	All 349 Serum Samples	78	54
		Co-Infected with Malaria	94	22
		Not Co-Infected with Malaria	74	65
		Non-Malaria Co-Infection	75	55
		No Co-Infection	74	71
32	Methoxy Mycolic Acid	All 349 Serum Samples	90	36
		Co-Infected with Malaria	100	18
		Not Co-Infected with Malaria	88	42
		Non-Malaria Co-Infection	69	38
		No Co-Infection	93	44
27	Thiol Modified Methoxy Mycolic Acid	All 349 Serum Samples	84	41
		Co-Infected with Malaria	100	18
		Not Co-Infected with Malaria	81	48
		Non-Malaria Co-Infection	75	40
		No Co-Infection	83	54
34	α-TDM	All 349 Serum Samples	59	64
		Co-Infected with Malaria	88	33
		Not Co-Infected with Malaria	54	74
		Non-Malaria Co-Infection	75	60
		No Co-Infection	49	83
35	α-TMM	All 349 Serum Samples	78	47
		Co-Infected with Malaria	88	24
		Not Co-Infected with Malaria	77	54
		Non-Malaria Co-Infection	56	47
		No Co-Infection	81	60

38	Keto TDM	All 349 Serum Samples	89	62
		Co-Infected with Malaria	100	29
		Not Co-Infected with Malaria	87	73
		Non-Malaria Co-Infection	88	73
		No Co-Infection	87	74
Statistics		All 349 Serum Samples	90	61
		Co-Infected with Malaria	100	29
		Not Co-Infected with Malaria	88	72
		Non-Malaria Co-Infection	88	69
		No Co-Infection	89	74

From this table, it can be seen that there are significant differences in the sensitivity and specificity values obtained when analysing serum samples from patients who were co-infected with malaria. With each antigen, the specificity value is significantly lower with the samples from patients who were co-infected with malaria, compared to the specificity values obtained when analysing samples with no malaria co-infection. For example, when using **30** as the antigen for the assay, when analysing serum samples from patients with no malaria co-infection, the assay had a sensitivity and specificity of 91 % and 65 % respectively, but when analysing serum samples from patients who had malaria co-infection these values changed to 100 % and 24 %. When using **38** as the antigen for the assay, a drop in specificity from 73 % to 29 % was observed, and for the statistical analysis, a drop in specificity from 72 % to 29 % was observed.

All of these values suggest that when malaria co-infection is present, many false positive results are observed.

Interestingly, when analysing serum samples from patients who had co-infections other than malaria (a full list of these co-infections can be seen in the Appendix, Section 9.1.2), the sensitivity and specificity values obtained were very similar to the values obtained when analysing the data for all 349 serum samples. For example, when using **30** as the antigen for the assay, when analysing the data obtained with all 349 serum samples, sensitivity and specificity values of 92 % and 55 % respectively were obtained, while sensitivity and specificity values of 93 % and 59 % were obtained when analysing serum samples from patients who were co-infected with a disease other than malaria. This suggests that there is no interference in the assay due to these other co-infections. In

addition to this, no interference occurs due to BCG vaccination (which does cause false positive results by tuberculin skin tests (see Table 1.5.1)), with the two best antigens for distinguishing TB+ and TB- samples, **38** and **30**, giving sensitivity and specificity values of 88 and 65 %, and 90 and 58 % respectively with serum samples from patients who had been vaccinated with the BCG vaccination, and values of 93 and 55 %, and 100 and 51 % respectively with serum samples from individuals who had not been vaccinated with the BCG vaccination. This suggests that this assay could be used in all countries, irrespective of whether or not the BCG vaccination is commonly used or not.

Finally, when analysing the results obtained with serum samples from patients who had no co-infection, generally, the sensitivity results obtained were very similar to those obtained when analysing the results from all 349 serum samples, however, generally the specificity values obtained were significantly higher. The most likely reason for this higher specificity is due to the 349 samples containing many false positive results from the serum samples from patients co-infected with malaria, reducing the overall specificity of that set.

In the literature, no obvious link between TB and malaria was found, however, it has been reported that a large increase in lipid levels occur in red blood cells of individuals infected with malaria.[11] It may therefore be possible that the false positive results observed due to malaria co-infection are due to weak interactions of cross-reacting anti-lipid antibodies to the antigens bound to the surfaces of the gold nanoparticles, preventing aggregation upon addition of the NaCl solution in the assay.

Further work should be undertaken in order to confirm whether malaria co-infection interferes with the gold nanoparticle based assay. If it does interfere, new antigens, or modifications to the assay to remove or take into account this interference, would be required.

Analysis of Serum Samples by Country

Having performed the assay on multiple serum samples, which had been obtained from patients from various countries, it was possible to analyse the data obtained in relation to which country the patient's serum sample was taken. The serum samples were obtained from patients from ten different countries, and are identified by the first two letters in the serum code, and are as follows; BA – Bangladesh, BR – Brazil, CA – Canada, CO – Colombia, GA – Gambia, KE – Kenya, PE – Peru, SA – South Africa, SP – Spain

and VI – Vietnam. Serum samples obtained from Brazil and Canada were excluded from these analyses due to no TB+ serum samples being obtained from those countries.

Each serum sample was grouped according to the country from which the patient's serum sample was taken, and receiver operating characteristics (ROC) analysis was performed on the data obtained from the assays. The ROC curves that were produced were then used to aid in deciding on a 'cut-off' percentage aggregation value, and the area under the curve (AUC) was also recorded. These AUC values were used to give an indication of how well that particular antigen could distinguish TB+ serum samples from TB- serum samples from a specific country.

Table 5.7.10 and Table 5.7.11 show the AUC values obtained, for serum samples obtained from various countries. In Table 5.7.10, the statistical analysis performed by Dr. James Gibbons (labelled as 'statistics') are included and are treated as an 'antigen', while in Table 5.7.11, these statistical analysis data are omitted. Cells highlighted in blue represent the highest AUC value for that particular country, suggesting that that antigen is the best antigen for distinguishing TB+ serum samples from TB- serum samples in that country. Cells highlighted in green represent the second highest AUC value, and cells highlighted in red represent the lowest AUC value, suggesting that that antigen is the worst antigen for distinguishing TB+ serum samples from TB- serum samples in that country. The final three rows of each table represent a count of the number of times each antigen gave: the highest AUC value for a specific country (labelled 'Best'); the second highest AUC value for a specific country (labelled 'Second Best'); and the lowest AUC value for a specific country (labelled 'Worst').

Table 5.7.10 Table showing the AUC values obtained when using each antigen in the gold nanoparticle assay, with serum samples from various countries. (Statistics are treated as an 'antigen'.)

	Antigen								
Country	**24**	**28**	**27**	**34**	**35**	**32**	**38**	**30**	**Statistics**
Gambia	0.934	0.804	0.884	0.817	0.578	0.716	0.743	0.878	0.751
South Africa	0.586	0.586	0.514	0.779	0.607	0.636	0.874	0.814	0.800
Spain	0.688	0.715	0.771	0.681	0.736	0.722	0.840	0.812	0.917
Bangladesh	0.762	0.813	0.817	0.828	0.670	0.799	0.817	0.832	0.963
Vietnam	0.586	0.686	0.586	0.571	0.686	0.571	0.724	0.686	0.614
Colombia	0.693	0.712	0.689	0.775	0.610	0.683	0.874	0.836	0.855
Kenya	0.700	0.800	0.800	0.800	0.600	0.567	0.700	0.567	0.933
Peru	0.714	0.750	0.893	0.786	0.786	0.857	0.857	0.893	0.857
Best	1	0	1	0	0	0	3	1	3
Second Best	0	2	2	1	1	1	1	3	2
Worst	1	0	0	2	3	2	0	1	0

Table 5.7.11 Table showing the AUC values obtained when using each antigen in the gold nanoparticle assay, with serum samples from various countries. (Statistics values are excluded.)

	Antigen							
Country	**24**	**28**	**27**	**34**	**35**	**32**	**38**	**30**
Gambia	0.934	0.804	0.884	0.817	0.578	0.716	0.743	0.878
South Africa	0.586	0.586	0.514	0.779	0.607	0.636	0.874	0.814
Spain	0.688	0.715	0.771	0.681	0.736	0.722	0.840	0.812
Bangladesh	0.762	0.813	0.817	0.828	0.670	0.799	0.817	0.832
Vietnam	0.586	0.686	0.586	0.571	0.686	0.571	0.724	0.686
Colombia	0.693	0.712	0.689	0.775	0.610	0.683	0.874	0.836
Kenya	0.700	0.800	0.800	0.800	0.600	0.567	0.700	0.567
Peru	0.714	0.750	0.893	0.786	0.786	0.857	0.857	0.893
Best	1	1	2	1	0	0	4	2
Second Best	1	1	1	1	1	1	2	4
Worst	1	0	1	2	3	2	0	1

These tables show that an antigen's performance with serum samples from different countries can vary significantly, with an antigen which can distinguish TB+ serum samples from TB- serum samples well in one country, not performing as well in another country. For example, when analysing the AUC values obtained when using **24** and **34** as the antigens, **24** has the highest AUC value (of the two) when analysing serum samples from patients from Gambia, Spain and Vietnam, while **34** had a higher AUC value than **24** when analysing serum samples from patients from all of the other countries.

If the statistical analysis is not taken into consideration, **38** had the highest AUC value for serum samples from patients from four different countries, **30** had the highest value for two countries (one shared), **27** had the highest value for two countries (both shared), **24** had the high value in one country while **28** and **34** also both had the highest AUC value in one country (shared). Having different antigens performing better than others with serum samples from patients from different countries suggests that having a device containing gold nanoparticles coated with multiple antigens may be of benefit.

It has been reported that different strains of *M. tuberculosis* contain different compositions of mycolic acid classes.[12] This might suggest that antibodies produced against these different strains of *M. tuberculosis* may have higher affinities to some mycolic acids, or sugar esters of mycolic acids, than to others. This could lead to some mycolic acids, or their sugar esters, being able to detect some strains of TB better than others, and may therefore account for this variation in results observed with serum samples from patients from different countries.

Having performed analysis to determine the AUC values of each antigen with serum samples from patients from various countries, it was decided to calculate the sensitivity and specificity values that could be obtained with the best performing antigens for each country. The cut-off percentage aggregation was selected with each antigen so that either 100 % sensitivity or 100 % specificity could be obtained (as specified in the corresponding table) for serum samples from patients from the country being investigated. These sensitivity and specificity values can be seen in Table 5.7.12 and Table 5.7.13. In order to show the most accurate analysis of the TDM, TMM and mycolic acid antigens, the sensitivity and specificity values obtained with the data from the statistical analysis are not included in these tables. In the event of more than one antigen sharing the highest or second highest AUC value with serum samples from a patient from a specific country, all antigens sharing that AUC value were analysed, and the antigen with the highest specificity value when the sensitivity values was at 100 % is quoted in these tables.

Table 5.7.12 Table showing the sensitivity and specificity values for the best and second best antigens using the gold nanoparticle assay with serum samples analysed from each country individually. (Cut-off percentage aggregation value was chosen in order to obtain 100 % sensitivity.)

Country	Best Antigen		Second Best Antigen	
	Sensitivity	Specificity	Sensitivity	Specificity
Gambia	24		27	
	100	69	100	50
South Africa	38		30	
	100	50	100	50
Spain	38		30	
	100	78	100	69
Bangladesh	30		34	
	100	62	100	8
Vietnam	38		28	
	100	0	100	0
Colombia	38		30	
	100	59	100	57
Kenya	27		28	
	100	50	100	50
Peru	27		30	
	100	50	100	50

Table 5.7.13 Table showing the sensitivity and specificity values for the best and second best antigens using the gold nanoparticle assay with serum samples analysed from each country individually. (Cut-off percentage aggregation value was chosen in order to obtain 100 % specificity.)

Country	Best Antigen		Second Best Antigen	
	Sensitivity	Specificity	Sensitivity	Specificity
Gambia	24		27	
	18	100	27	100
South Africa	38		30	
	36	100	7	100
Spain	38		30	
	0	100	0	100
Bangladesh	30		34	
	29	100	29	100
Vietnam	38		28	
	50	100	43	100
Colombia	38		30	
	11	100	0	100
Kenya	27		28	
	60	100	60	100
Peru	27		30	
	79	100	79	100

These tables show, that with the exception of Vietnam, by setting a cut-off percentage aggregation value to obtain a sensitivity of 100 %, a specificity of 50 % or greater could be obtained with at least two antigens for each country. Although only a limited number of serum samples from patients from some countries were present in the sample set, this data suggests that if a device for the detection of TB were developed, which incorporated gold nanoparticles coated with all six of the antigens present in Table 5.7.12 and Table 5.7.13, a sensitivity of 100 % and a specificity of at least 50 % could be obtained in most countries by varying which antigen(s) would be used for the analysis of the serum samples in different countries.

5.8 Comparison of Results with ELISA Results

In order to further evaluate the gold nanoparticle based assay, it was decided to compare the results obtained with those obtained by ELISA. The 349 serum samples obtained from the WHO were tested in ELISA assays with the antigens which had previously given the best distinction between TB+ and TB- serum samples from groups 1 and 2 by ELISA, and were run by Dr. Alison Jones (who led this aspect of the project), Mr. A. Saleh and myself.

The following analysis of the data obtained from these assays was performed after un-blinding the TB status of the serum samples, once the results and their interpretations had been formally recorded. A summary of the results can be seen in Table 5.8.1 and a full list of the individual absorbance values obtained at the end of the assay can be seen in the Appendix, Section 9.4.9. The structures of the antigens used can be seen in the Appendix, Section 9.1.2.

In addition to the absorbance values obtained when performing the assay on these serum samples, statistical analysis results, which were again performed by Dr. James Gibbons, are also included. The values obtained for the statistical analysis (which are again labelled as statistics), were obtained by a statistical combination of the results obtained when analysing the 100 serum samples from groups 1 and 2 with the four antigens which gave the best distinction between TB+ and TB- serum samples, and for the purposes of this analysis, these statistical outputs are again treated as a separate 'antigen'.

Table 5.8.1 Table showing a summary of the results obtained when analysing the 349 serum samples obtained from the WHO, using ELISA with various antigens.

Antigen	Antigen Type	Cut-Off Absorbance Value	Sensitivity (%)	Specificity (%)	Average Absorbance Value for TB Positive Serum Samples	Average Absorbance Value for TB Negative Serum Samples	AUC
Natural TDM Mixture		2.10	84	64	2.89	1.75	0.823
24	Methoxy TDM	0.85	88	60	2.60	1.05	0.843
30	Methoxy TDM	1.92	80	87	2.88	1.00	0.886
28	Methoxy TMM	2.07	65	88	2.36	0.98	0.825
34	α-TDM	1.05	95	62	3.08	1.25	0.871
38	Keto TDM	1.02	93	75	3.01	0.88	0.907
42	Keto Mycolic Acid[VIII]	1.02	59	50	1.40	1.18	0.573
Statistics		0.50	86	87	0.77	0.14	0.924

From this table, the first observation made was that all of the antigens analysed showed some distinction between the TB+ and TB- serum samples. Another encouraging observation made from this data was that the best two antigens at distinguishing the TB+ serum samples from the TB- serum samples with the gold nanoparticle based assay (**38** and **30**) were also the best two when analysing the serum samples by ELISA.

Another important observation was that the statistical analysis performed again gave a better distinction between the TB+ serum samples and the TB- serum samples than any of the individual antigens. The sensitivity and specificity values of 86 % and 87 % respectively were high, and the AUC value of 0.924 (stating that when using these

[VIII] This antigen contains a *trans* cyclopropane ring in the proximal position of the meromycolate moiety.

statistics, there is a 92.4 % probability that a TB+ serum sample will give a higher value than a TB- serum sample) was higher than any of the AUC values obtained with the individual antigens. This again suggested than when developing a sensor for the detection of TB, a better distinction between TB+ and TB- serum samples may be obtained by using a combination of antigens, rather than a single antigen, suggesting that any device developed for the detection of TB should include multiple antigens.

It can also be observed from this table that many of the patters which were observed when analysing the results obtained with the gold nanoparticle based assay were still true when analysing the serum samples by ELISA. Firstly, when looking at the two antigens which were of the same class (methoxy TDM), but which contained different stereochemistries, **24** and **30**, **30** again performed better than **24** when analysing the serum samples by ELISA. This again strongly suggests that the stereochemistry of the antigen used has a crucial role in the amount of binding occurring to it.

Previous analysis of the results obtained from the gold nanoparticle based assay was inconclusive as to whether TDMs or TMMs gave a better distinction between the TB+ and TB- serum samples. Only one such comparison could be made with the ELISA data, by comparing the results obtained with **24** and **28**. When comparing these antigens by ELISA, the methoxy TDM (**24**), gave a slightly better distinction between the TB+ and TB- serum samples, giving an AUC value of 0.843 compared to the value of 0.825 which was obtained with **28**. Further work with TDMs and TMMs from different classes with all 349 serum samples by ELISA and by the gold nanoparticle based assay is required to confirm this trend.

Analysis of Serum Samples by Country

Having performed the assay on multiple serum samples, which had been obtained from patients from various countries, it was again possible to analyse the data obtained in relation to which country the patient's serum sample was taken. As with the results obtained from the gold nanoparticle based assay, serum samples obtained from Brazil and Canada were excluded from these analyses due to no TB+ serum samples being obtained from those countries.

Again, each serum sample was grouped according to the country from which the patient's sample was taken, and receiver operating characteristics (ROC) analysis was performed on the data obtained from the assays. The ROC curves that were produced were then again

used to aid in deciding on a 'cut-off' absorbance value, and the area under the curve (AUC) was also recorded. As with the results obtained from the gold nanoparticle based assay, these AUC values obtained when using each antigen for the assay (with serum samples from each country) were used to give an indication of how well that particular antigen could distinguish TB+ serum samples from TB- serum samples from a specific country.

Table 5.8.2 and Table 5.8.3 show the AUC values obtained when using each antigen in turn for the ELISA, for serum samples obtained from various countries. In Table 5.8.2 the statistical analysis performed by Dr. James Gibbons (labelled as statistics) are included and are treated as an 'antigen', while in Table 5.8.3, these statistical analysis data are omitted. As with the analysis of the results obtained from the gold nanoparticle based assay, cells highlighted in blue represent the highest AUC value for that particular country, suggesting that that antigen is the best antigen for distinguishing TB+ serum samples from TB- serum samples in that country. Cells highlighted in green again represent the second highest AUC value, and cells highlighted in red represent the lowest AUC value, suggesting that that antigen is the worst antigen for distinguishing TB+ serum samples from TB- serum samples in that country. The final three rows of each table represent a count of the number of times each antigen gave; the highest AUC value for a specific country (labelled 'Best'); the second highest AUC value for a specific country (labelled 'Second Best'); and the lowest AUC value for a specific country (labelled 'Worst').

Table 5.8.2 Table showing the AUC values obtained when using each antigen in the ELISA, with serum samples from various countries. (Statistics are treated as an 'antigen'.)

	Antigen							
Country	**Natural TDM Mixture**	**24**	**30**	**28**	**34**	**38**	**42**	**Statistics**
Gambia	0.823	0.856	0.875	0.848	0.899	0.952	0.580	0.971
South Africa	0.871	0.889	0.868	0.743	0.943	0.939	0.607	0.946
Spain	0.868	0.847	0.858	0.743	0.840	0.910	0.736	0.944
Bangladesh	0.721	0.805	0.887	0.797	0.864	0.939	0.736	0.957
Vietnam	0.629	0.643	0.729	0.671	0.636	0.786	0.700	0.679
Colombia	0.906	0.921	0.929	0.854	0.925	0.899	0.512	0.944
Kenya	1.000	0.967	1.000	0.833	1.000	1.000	1.000	1.000
Peru	0.857	0.786	0.857	1.000	0.786	0.821	0.750	0.804
Best	1	0	1	1	1	2	1	6
Second Best	1	1	3	0	1	3	0	0
Worst	1	0	0	1	0	0	5	0

Table 5.8.3 Table showing the AUC values obtained when using each antigen in the ELISA, with serum samples from various countries. (Statistics values are excluded.)

	Antigen						
Country	**Natural TDM Mixture**	**24**	**30**	**28**	**34**	**38**	**42**
Gambia	0.823	0.856	0.875	0.848	0.899	0.952	0.580
South Africa	0.871	0.889	0.868	0.743	0.943	0.939	0.607
Spain	0.868	0.847	0.858	0.743	0.840	0.910	0.736
Bangladesh	0.721	0.805	0.887	0.797	0.864	0.939	0.736
Vietnam	0.629	0.643	0.729	0.671	0.636	0.786	0.700
Colombia	0.906	0.921	0.929	0.854	0.925	0.899	0.512
Kenya	1.000	0.967	1.000	0.833	1.000	1.000	1.000
Peru	0.857	0.786	0.857	1.000	0.786	0.821	0.750
Best	1	0	2	1	2	5	1
Second Best	2	1	3	0	2	1	0
Worst	2	0	0	1	0	0	5

As was observed with the analysis of the results obtained from the gold nanoparticle based assay, it can again be seen that an antigen's performance with serum samples from different countries can vary significantly. It can also be seen that an antigen which can distinguish TB+ samples from TB- samples accurately in one country, may not perform as well in another country. For example, when analysing the AUC values obtained when

using **28**, it gave the lowest AUC value for serum samples from patients from Kenya however it gave the highest AUC value for serum samples from patients from Peru.

If the statistical analysis is not taken into consideration, **38** had the highest AUC value for serum samples from patients from five different countries (one shared), **30** and **34** had the highest value for two countries (one shared), **28** had the highest value for one country, while the natural TDM mixture and **42** also had the highest AUC value in one country (shared). Having different antigens performing differently with serum samples from patients from different countries again suggests that having a device for the detection of TB containing multiple antigens may be of benefit.

Having performed analysis to determine the AUC values of each antigen with serum samples from patients from various countries, it was again decided to calculate the sensitivity and specificity values that could be obtained with the best performing antigens for each country. The cut-off absorbance value was again selected with each antigen so that either 100 % sensitivity or 100 % specificity could be obtained (as specified in the corresponding table) for serum samples from patients from the country being investigated. These sensitivity and specificity values can be seen in Table 5.8.4 and Table 5.8.5. In order to show the most accurate analysis of the TDM, TMM and mycolic acid antigens, the sensitivity and specificity values obtained with the data from the statistical analysis are again not included in these tables. In the event of more than one antigen sharing the highest or second highest AUC value with serum samples from a patient from a specific country, all antigens sharing that AUC value were analysed, and the antigen with the highest specificity value when the sensitivity values were at 100 % is again quoted in these tables.

Table 5.8.4 Table showing the sensitivity and specificity values for the best and second best antigens in the ELISA with serum samples analysed from each country individually. (Cut-off absorbance value was chosen in order to obtain 100 % sensitivity.)

Country	Best Antigen		Second Best Antigen	
	Sensitivity	Specificity	Sensitivity	Specificity
Gambia	**38**		**34**	
	100	77	100	60
South Africa	**34**		**38**	
	100	20	100	20
Spain	**38**		Natural TDM Mixture	
	100	67	100	53
Bangladesh	**38**		**30**	
	100	64	100	36
Vietnam	**38**		**30**	
	100	0	100	0
Colombia	**30**		**34**	
	100	64	100	68
Kenya	**38**		**30**	
	100	100	100	100
Peru	**28**		**30**	
	100	100	100	63

Table 5.8.5 Table showing the sensitivity and specificity values for the best and second best antigens in the ELISA with serum samples analysed from each country individually. (Cut-off absorbance value was chosen in order to obtain 100 % specificity.)

Country	Best Antigen		Second Best Antigen	
	Sensitivity	Specificity	Sensitivity	Specificity
Gambia	**38**		**34**	
	9	100	0	100
South Africa	**34**		**38**	
	93	100	86	100
Spain	**38**		Natural TDM Mixture	
	50	100	25	100
Bangladesh	**38**		**30**	
	76	100	52	100
Vietnam	**38**		**30**	
	64	100	50	100
Colombia	**30**		**34**	
	33	100	0	100
Kenya	**38**		**30**	
	100	100	100	100
Peru	**28**		**30**	
	100	100	79	100

Although only a limited number of serum samples from patients from some countries were present in the serum sample set, these tables show, that with the exception of South Africa and Vietnam, by setting a cut-off absorbance value to obtain a sensitivity of 100 %, a specificity of 64 % or greater could be obtained with at least one antigen for each country. This data again suggests that any developed device for the detection of TB which is based upon binding of antibodies to TDM, TMM or mycolic acid antigens should contain multiple antigens to obtain the best sensitivity and specificity values.

Summary

When comparing the results obtained from the developed assay based on coated gold nanoparticles and the results obtained from the ELISA assays, an encouraging observation can be made, in that the best antigens by ELISA *i.e.* **38** and **30**, were also the two best antigens by the developed assay based on coated gold nanoparticles.

One difference between the data sets is that there is no evidence of malaria co-infection having any effect on the ELISA results, though for many of the false positive samples using the coated gold nanoparticle based assay there is malaria co-infection. This suggests that further work is required with the gold nanoparticle based assay in order to determine whether this link still exists on larger sample sets, and if the link persists, methods of overcoming this should be investigated.

In general, it could be observed that sensitivity, specificity and AUC values are higher by ELISA. However, when analysing serum samples by country, comparable results are obtained with both assays, with a sensitivity value of 100 % and a specificity value of 50 % or greater being obtained with at least two antigens for each country (with the exception of Vietnam) with the developed assay based on coated gold nanoparticles, and a sensitivity of 100 % and a specificity of 64 % or greater being obtained with at least one antigen for each country (with the exception of Vietnam and South Africa) with ELISA.

5.9 Concluding Remarks

Non-thiol modified antigens have successfully been 'bound' to gold nanoparticles *via* a linker compound (initially thio-stearic acid). Aggregation could then be induced in these coated gold nanoparticles by addition of a saturated aqueous solution of NaCl. This 'binding' of non-thiol modified antigens onto gold nanoparticles allowed for a large range of antigens to be analysed by the assay developed.

Initial experiments using gold nanoparticles coated with a natural TDM mixture, isolated from *M. tuberculosis*, showed a good distinction between TB positive and TB negative serum samples. A method of calculating percentage aggregation was then developed in order to quantify the results obtained at the end of the assay. Synthetic antigens were then 'coated' onto gold nanoparticles and initial experiments using these coated gold nanoparticles again showed a good distinction between TB positive and TB negative serum samples.

The ability to use non-thiol modified antigens in the assay, and encouraging results for the initial experiments using these antigens, brought a need for a new source of gold nanoparticles in order to be able to analyse a large number of serum samples with a large range of antigens. Re-optimisation of the assay was required and performed with gold nanoparticles from a new source and, after this re-optimisation process was completed, a set of fifty serum samples were re-analysed using gold nanoparticles coated with a natural TDM mixture, isolated from *M. tuberculosis*, yielding comparable results to those obtained when using gold nanoparticles from the initial source.

Fifty serum samples whose TB status was known prior to performing the assay, and fifty serum samples whose TB status were unknown prior to performing the assay were then analysed with a range of different synthetic antigens. Upon un-blinding the unknown serum samples, sensitivity and specificity values of 100 % and 67 % respectively were obtained when using **38** as the antigen for the assay (higher values of 100 % and 79 % were obtained when using **38** as the antigen for the assay and centrifuging the coated gold nanoparticles prior to their use).

Upon obtaining these results, a blind test on 249 serum samples obtained from the WHO was performed. Upon un-blinding these serum samples, it was discovered that a problem occurred with serum samples from patients who were co-infected with malaria, however, no interference was observed in the assay due to other co-infections, or due to the patient being vaccinated with the BCG vaccination. Despite this problem with malaria co-infection, it was discovered that some antigens were better than others at distinguishing TB positive serum samples from TB negative serum samples from patients from different countries. Using gold nanoparticles coated with different antigens for serum samples from different countries led to, with the exception of Vietnam, a sensitivity of 100 % and a specificity of at least 50 % being obtained with at least two antigens for each country.

These sensitivity and specificity values obtained were very encouraging and suggested that the assay developed had the potential to be further developed into a device, which could be very useful as a quick screening test, which could be used in remote locations, without the need for an on-site laboratory, for the detection of TB.

5.10 References

1 M. O. Balogun, E. Huws, M. M. Sirhan, A. D. Saleh, J. R. Al Dulayymi, L. Pilcher, J. A. Verschoor and M. S. Baird, **Thiol modified mycolic acids,** *Chem. Phys. Lipids,* 2013 (In Press).

2 Confidential unpublished work.

3 C. A. Widrig, C. Chung and M. D. Porter, **The electrochemical desorption of n-alkanethiol monolayers from polycrystalline Au and Ag electrodes,** *J. Electroanal. Chem.,* 1991, **310,** 335-359.

4 X. Liu, H. Bai, L. Zhang and E. Wang, **Lipid-based strategies in inorganic nano-materials and biomineralization study,** *Adv. Planar Lipid Bilayers. Liposomes,* 2008, **7,** 203-220.

5 T. Fawcett, **An introduction to ROC analysis,** *Pat. Rec. Letters,* 2006, **27,** 861-874.

6 R. J. Anderson, **A study of the phosphatide fraction of tubercule bacilli,** *J. Biol. Chem.,* 1927, **74,** 537-551.

7 M. L. Burt and R. J. Anderson, **The chemistry of the lipoids of tubercle bacilli XXIV. Analysis of the acetone-soluble fat of the bovine tubercule bacillus,** *J. Biol. Chem.,* 1931, **94,** 451-463.

8 R. J. Anderson and N. Uyei, **The chemistry of the lipoids of tubercule bacilli XXVII. The composition of the phosphatide fraction of the bacillus leprae,** *J. Biol. Chem.,* 1932, **97,** 617-637.

9 M. C. Pangborn and R. J. Anderson, **The chemistry of the lipoids of tubercule bacilli XXV. The composition of the phosphatide fraction of the timothy bacillus,** *J. Biol. Chem.,* 1931, **94,** 465-472.

10 R. J. Anderson and E. Gilman Roberts, **The chemistry of the lipoids of tubercule bacilli XI. The phosphatide fraction of the avian tubercule bacilli,** *J. Biol. Chem.,* 1930, **85,** 519-528.

11 S. Déchamps, S. Shastri, K. Wengelnik and H. J. Vial, **Glycerophospholipid acquisition in *Plasmodium* – A puzzling assembly of biosynthetic pathways,** *Int. J. Parasitol.,* 2010, **40,** 1347-1365.

12 J. A. Verschoor, M. S. Baird and J. Grooten, **Towards understanding the functional diversity of cell wall mycolic acids of *Mycobacterium tuberculosis*,** *Prog. Lip. Res.,* 2012, **51,** 325-339.

Chapter 6

Classifying the Antibodies Binding to the Coated Gold Nanoparticles

6.1 Introduction

Having been able to distinguish TB positive serum samples from TB negative serum samples, using the assay developed, with a reasonable degree of accuracy, it was decided to try and characterise which class, or classes, of antibodies (if any) bound to the antigens on the gold nanoparticle surfaces.

Due to no secondary antibody conjugate being used in the assay developed based on coated gold nanoparticles, there was no indication of which class of antibodies was binding to the antigens coated onto the gold nanoparticle surfaces. Gaining knowledge of this could possibly allow the assay to be modified, and lead to an improvement in its sensitivity and specificity.

6.2 Characterising the Class of Antibodies Binding

In order to work towards characterising the class, or classes, of antibodies that bound to the antigens on the gold nanoparticle surfaces, it was decided to analyse a set of serum samples both by ELISA using various secondary antibody conjugates, and by the gold nanoparticle based assay. Analysis of the serum samples by ELISA allowed for calculation of the relative amount of each class of antibody binding to the antigen, due to the absorbance obtained at the end of the assay being directly related to the amount of antibodies that bound to the antigen under investigation.[1] The absorbance values obtained were then compared to the percentage aggregation values obtained with the same serum samples, from the developed assay based on coated gold nanoparticles, to check for a correlation between the data.

Due to only small quantities of some of the serum samples obtained from the WHO being left at this point in the project,[1] a set of twenty serum samples were chosen for these experiments consisting of three TB+ samples obtained from the WHO and seventeen TB- samples from UK individuals. The assay based on coated gold nanoparticles was performed using the protocol found in Section 2.6, using non-centrifuged coated gold nanoparticles. The ELISA assays were performed using the protocol found in Section 2.7 with the exception that serum was diluted to a concentration of 1 in 80 instead of 1 in 20 for these experiments. Dilute serum samples were used which were expected to lead to reasonably small absorbance values at the end of these assays, because at serum (and antibody) concentrations that were too high, the relationship between the absorbance at the

[1] These experiments were performed prior to the un-blinding of the 249 serum samples from group 3.

end of the assay and the amount of antibodies being analysed (and binding to the antigen) no longer held true.[1]

6.2.1 Introduction to Various Classes of Antibodies

There are five main classes of antibodies,[2] three of which are more prominent in serum samples, IgA, IgG and IgM, with IgG being the most abundant.[2] It is believed that approximately 13.5 mg/ml of IgG antibodies, 3.5 mg/ml of IgA antibodies and 1.5 mg/ml of IgM antibodies are present in human serum.[3] General structures for these different classes of antibodies are depicted below:

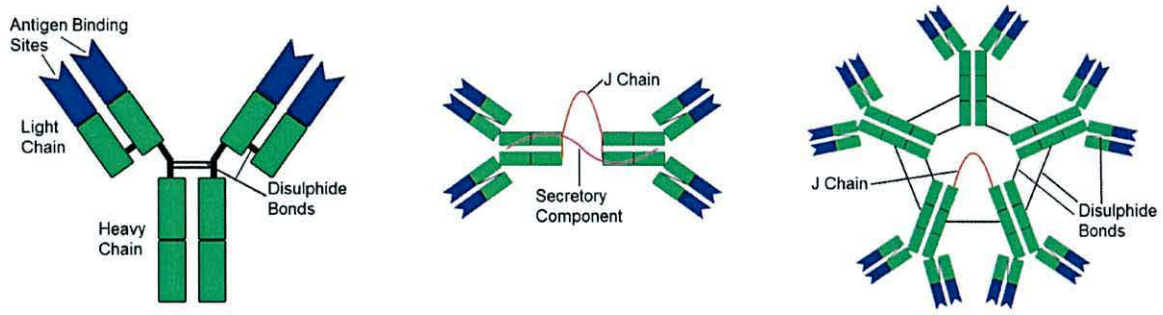


Figure 6.2.1 General structures for IgG antibodies (left), IgA antibodies (middle) and IgM antibodies (right).

These antibodies are produced as an immune response to invading pathogens (*e.g.* bacteria), and each class of antibodies contains a light chain and a heavy chain, with the heavy chain being specific to each class of antibody: IgG antibodies contain a γ-chain; IgA antibodies contain an α-chain; while IgM antibodies contain a μ-chain.[2] The light chains of the antibodies can exist in two form, λ and κ, and any given antibody can either contain λ chains or κ chains, but never one of each, and any of the two types of light chains may be present in any given antibody from any of the major classes of antibodies.[2] IgG antibodies exist as monomers, while IgA and IgM can both exist as polymers. Secreted IgA can exist as either monomers or dimers, while IgM forms pentamers in serum.[3]

IgM is always the first antibody produced against infection.[4] Due to IgM containing ten antigen-binding sites, it is said to be of high avidity, and it is very efficient at binding to micro-organisms.[5] IgG is then produced as a secondary response, and IgG antibodies are of extreme value due to the ability of their Fc region to bind to receptors on phagocytic cells,[5] allowing engulfment and destruction of the pathogen by phagocytic cells.[4] IgA is the foremost antibody present in mucosal secretions, where it is protected from digestion

by its secretory component. The main function of this class of antibody is to prevent entry of pathogens from outside to the host's system.[5]

IgG (whole molecule) secondary antibody conjugates can bind to the heavy or light chains of an IgG antibody. Even though the heavy chain is specific to the IgG isotype (γ-chain), the light chains are common for every class of antibody.[2] This would suggest that by using this secondary antibody conjugate, it may be possible to detect the presence of not only IgG, but also IgA and IgM in the serum samples.

It was therefore decided to use various secondary antibody conjugates in order to determine how much of each class of antibody, produced against TB, is present in the serum samples.

6.2.2 Initial Experiments

For these experiments, the methoxy TDM, **24**, was chosen as the antigen, and the following table states which secondary antibody conjugates were used in the ELISA assays (which were prepared using the procedures found in Section 2.7) to detect the following antibody classes:

Table 6.2.1 Table showing the secondary antibody conjugates used to detect various different antibody classes.

Class of Antibody Being Detected	Secondary Antibody Conjugate Used
IgG	Anti-human IgG (whole molecule) secondary antibody conjugate
IgG	Anti-human IgG (γ-chain specific) secondary antibody conjugate
IgM	Anti-human IgM (μ-chain specific) secondary antibody conjugate
IgA	Anti-human IgA (α-chain specific) secondary antibody conjugate
IgG1	Anti-human IgG1 (γ-chain specific) secondary antibody conjugate

These secondary antibody conjugates were chosen in order to give a good overview of the amount of the three main classes of antibodies present in serum samples (IgG, IgM and IgA) as well as the major sub-group of the IgG antibody class, IgG1.

The following Figure show correlations of the absorbance values obtained from the ELISA assays, and the percentage aggregation values obtained from the assay based on coated gold nanoparticles, when using each secondary antibody conjugate in turn for the ELISA assay. Individual percentage aggregation and absorbance values with each of the serum samples analysed can be found in Section 9.5.1.

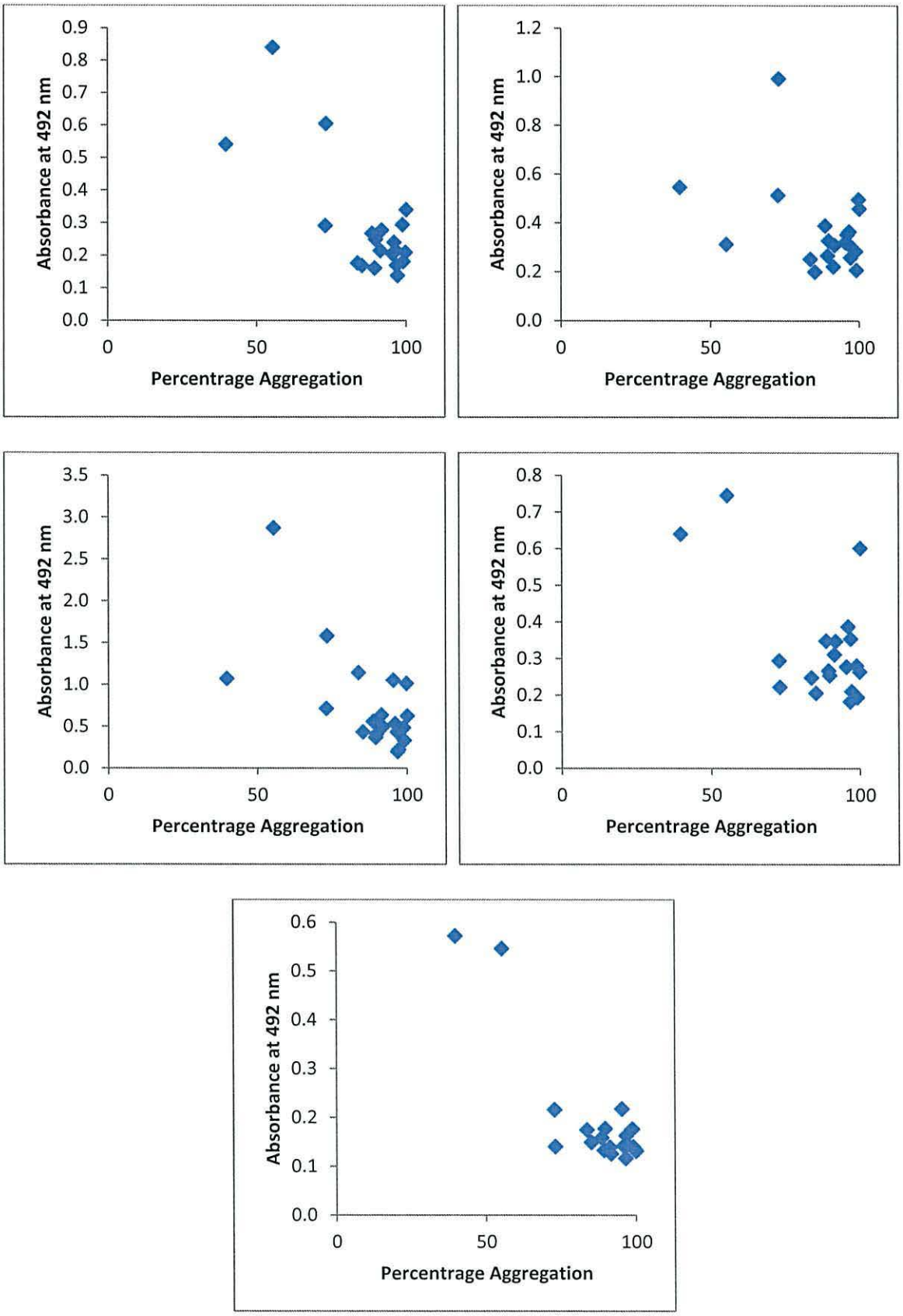


Figure 6.2.2 Correlation of the percentage aggregation values (from the coated gold nanoparticle assay) and the absorbance values at 492 nm (from the ELISA) when using Anti-human IgG (whole molecule) (top left), IgG (γ-chain specific) (top right), IgM (μ-chain specific) (middle left), IgA (α-chain specific) (middle right) and IgG1 (γ-chain specific) (bottom) secondary antibody conjugates.

These Figures do show some correlation between most of the antibodies being investigated and the results obtained from the assay using the coated gold nanoparticles, however, there is no evidence that it is only antibodies from one antibody class that is binding to the antigens coated onto the gold nanoparticles.

Looking at Figure 6.2.2 (top left), a good correlation between the amount of IgG antibodies binding to **24**, and the percentage aggregation values obtained at the end of the gold nanoparticle assay can be seen. Generally, all of the samples which gave high absorbance values at the end of the ELISA assays gave low percentage aggregation values at the end of the gold nanoparticle assay, while samples which gave low absorbance values at the end of the ELISA assays gave high percentage aggregation values at the end of the gold nanoparticle assay. This suggests that IgG antibodies do bind to the antigens on the coated gold nanoparticles.

This can be further inferred from the data in Figure 6.2.2 (bottom) which shows a good correlation between the percentage aggregation values and the amount of IgG1 antibody (the most abundant IgG sub-group) binding to **24** in the ELISA. This again suggests that IgG antibodies, specifically IgG1 antibodies in this instance, do bind to the antigens on the coated gold nanoparticles.

In addition to this correlation with IgG antibodies, there is also some correlation between the percentage aggregation values, and the absorbance values obtained when detecting the presence of IgM antibodies (see Figure 6.2.2 (middle left)). Again, generally the higher the absorbance value obtained with the ELISA assay, the lower the percentage aggregation value obtained from the gold nanoparticle assay. An exception is however seen with sample KE 1 (the serum sample which gave the lowest percentage aggregation value), which gave a reasonably low absorbance value at the end of the ELISA assay when detecting IgM antibodies. This suggests, that even though IgM antibodies may bind to the antigens on the coated gold nanoparticles, they are not the only class of antibody that are binding, and this low absorbance value with sample KE 1 suggests that IgM antibodies are not the most abundant antibodies binding to **24** on the gold nanoparticle surfaces.

When looking at Figure 6.2.2 (middle right), a reasonable correlation is also observed between the percentage aggregation values obtained and the absorbance values obtained when detecting IgA antibodies. An outlier is again observed in this set with UK Sample 54 giving a high percentage aggregation value at the end of the gold nanoparticle assay, but

also giving a reasonably high absorbance value at the end of the ELISA. These results suggest that IgA may also bind to **24** coated onto the gold nanoparticles, however the high absorbance value obtained with UK Sample 54 suggests that IgA antibodies are also not the most abundant antibodies binding to **24** on the gold nanoparticle surfaces.

It can therefore be inferred from this data, that it is likely that antibodies from each of the three classes investigated bound to the antigen coated onto the gold nanoparticle surface, with IgG, specifically IgG1, being the most abundant (due to the best correlation of the percentage aggregation values being with the absorbance values obtained when detecting the presence of IgG1 antibodies).

6.3 Towards the Removal of IgM Antibodies

As the results discussed in the previous section suggested that antibodies from the three different antibody classes investigated bind to the antigens coated onto the surfaces of the gold nanoparticles, it was decided to try and develop a method of removing the IgM antibodies from the serum samples prior to using the samples in the developed assay based on coated gold nanoparticles. Previous results from ELISA assays suggested that analysing IgG antibodies gave a better distinction between TB+ and TB- serum samples than when analysing IgA and IgM, with IgM giving the worst distinction.[1] The detection of IgM antibodies by ELISA also, generally, gave high absorbance values throughout, suggesting that there was a large amount of binding of this antibody class to TDMs, TMMs and mycolic acids.[1] It was therefore believed that removing IgM antibodies from the serum samples prior to using the serum samples in the assay based on coated gold nanoparticles would improve the distinction between TB+ and TB- serum samples, and in turn improve the sensitivity and specificity of the assay.

When searching the literature, a method of inactivating IgM antibodies in serum samples, while keeping IgG antibodies active was found.[6] It was stated that adding 2-mercaptoethanol or dithiothreitol (DTT) to serum samples containing both IgM and IgG antibodies inactivated the IgM antibodies by breaking the molecules' disulfide bonds, thus breaking the IgM polymer. IgM antibodies which have been de-polymerised do not agglutinate. This is in contrast to IgG antibodies which are resistant to 2-mercaptoethanol and DTT, and are not inactivated in their presence.[6]

6.3.1 Confirming the Removal of IgM antibodies by ELISA

Eight serum samples (four TB+ and four TB-) were analysed by ELISA using the standard protocol found in Section 2.7, and also with a modified method where the serum samples were diluted with 0.5 % casein/PBS buffer (pH 7.4) containing 20 μM DTT (and left to incubate for 1 hour prior to using them in the assay). In both instances, the serum samples were run and analysed with both anti-human IgG (γ-chain specific) secondary antibody conjugate and anti-human IgM (μ-chain specific) secondary antibody conjugate.

Using the IgG secondary antibody conjugate allowed for analysis of how much IgG antibodies remained in the serum samples after addition of DTT, while using the IgM secondary antibody conjugate allowed for analysis of how much IgM antibodies were removed from the serum samples after addition of DTT. **38** was used as the antigen for these experiments as it had previously shown a good distinction between TB+ and TB- serum samples. Of the four TB- serum samples chosen, two samples had previously given low absorbance values by ELISA with this antigen, while the other two had given reasonably high absorbance values. These samples were chosen to give a broader overview of whether using DTT would be beneficial in improving the distinction between TB+ and TB- serum samples. The results obtained are shown in Figure 6.3.1:

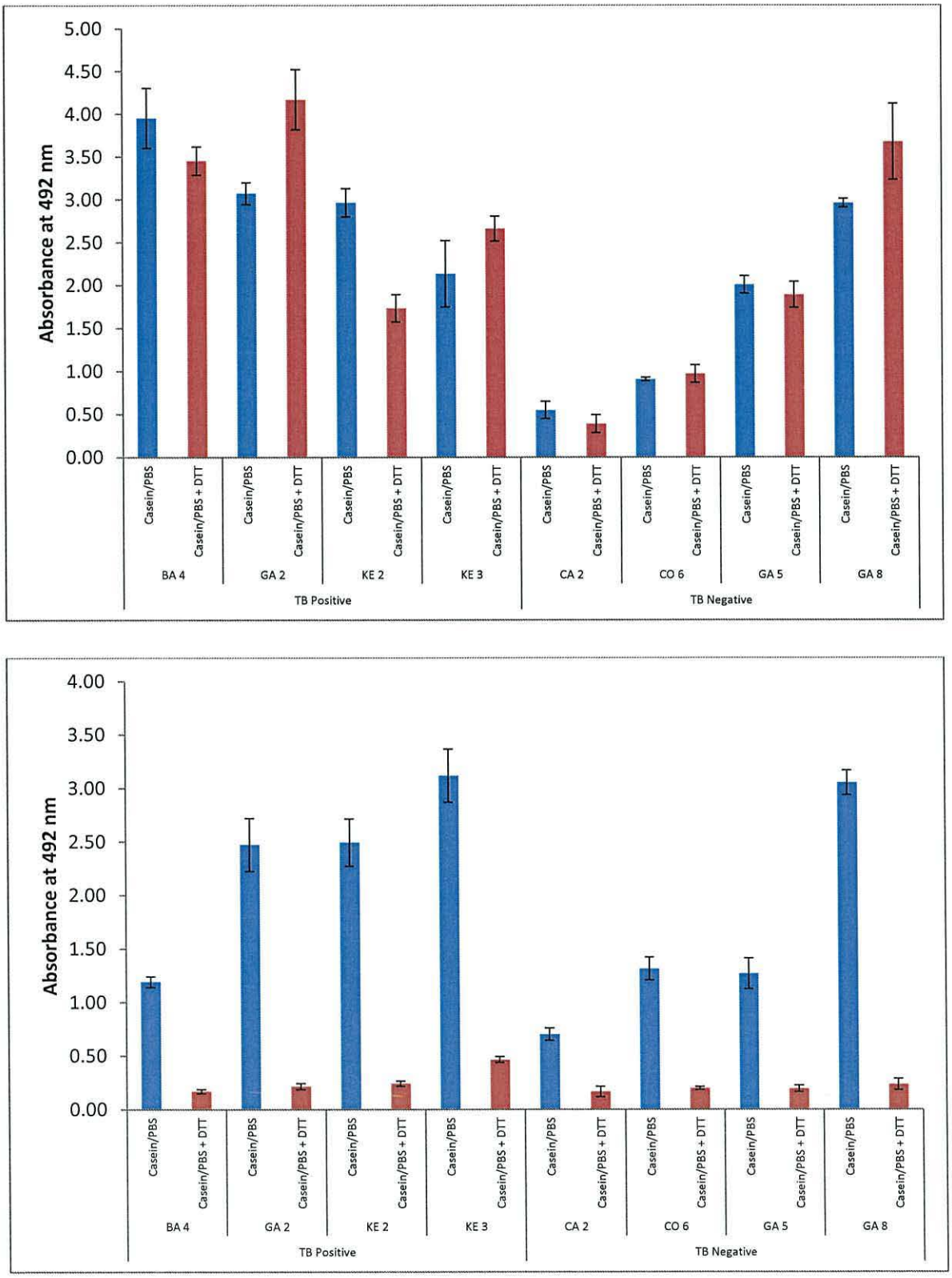


Figure 6.3.1 The absorbance at 492 nm of different serum samples, diluted with 0.5 % casein/PBS buffer (blue) and 0.5 % casein/PBS buffer containing 20 μM DTT (red) when using anti-human IgG (γ-chain specific) (top) and IgM (μ-chain specific) (bottom) secondary antibody conjugates for the ELISA.

The results obtained when using anti-human IgG (γ-chain specific) secondary antibody conjugate (Figure 6.3.1 (top)) show very little change in the absorbance when diluting the serum samples with the two different buffers suggesting that IgG antibodies remain largely un-affected by the addition of the DTT to the serum samples. However, when using anti-human IgM (μ-chain specific) secondary antibody conjugate for the assay, a significant difference in absorbance is observed between the two sets of results. When the serum samples were subjected to DTT prior to their use in the assay, a dramatic decrease in absorbance was observed at the end of the assay (as compared to the results obtained when DTT was not used), suggesting that after being subjected to DTT, the IgM antibodies in the serum samples were inactivated, and did not bind to **38**.

Incorporating the Inactivation of IgM Antibodies into the Gold Nanoparticle Assay

Having shown that IgM antibodies could be selectively inactivated in the presence of IgG antibodies, it was decided to incorporate this into the gold nanoparticle based assay. DTT was therefore added to the serum samples prior to their use in the assay (see Section 2.6.3 for the full procedure) in order to inactivate the IgM antibodies. In order to test whether this method worked, and to see whether it would give a better distinction between TB+ and TB- serum samples than was previously observed, gold nanoparticles coated with **24** and **30** in turn were tested with the 100 serum samples from the first two groups of serum samples (see Section 9.1.2). Table 6.3.1 below shows a summary of the results obtained from these experiments, and a full list of the percentage aggregation values obtained from these experiments can be seen in Section 9.5.2.

Table 6.3.1 Table showing a summary of the results obtained when inactivating IgM secondary antibodies prior to addition of the serum samples to the coated gold nanoparticles in the gold nanoparticle assay.

Antigen	With / Without DTT	Sensitivity (%)	Specificity (%)	AUC
24	Without DTT	82	66	0.815
	With DTT	82	58	0.747
30	Without DTT	100	66	0.894
	With DTT	85	52	0.729

It can be seen from the table that even though a distinction was observed between the TB+ and TB- serum samples when inactivating the IgM antibodies, unexpectedly, the distinction was not as good as when not inactivating them. With both antigens, a reduction in sensitivity and specificity values, as well as a reduction in the AUC value, was observed.

A possible reason for these reductions is that as DTT contains thiol functional groups, it may displace the linker compound and in turn the antigen from the gold nanoparticle surfaces. If this were to happen, a decrease in sensitivity and specificity would be expected because there would be less antigen present for the anti-TB antibodies to bind to, and anti-TB antibodies would not selectively bind to DTT. Due to this possibility, no further experiments were carried out using DTT.

6.4 Concluding Remarks

Experiments were performed to characterise the types of antibodies that bound to the coated gold nanoparticles. From the results obtained there was no evidence that it is only antibodies from one antibody class that were binding to the antigens coated onto the gold nanoparticles. However, the results did suggest that binding of IgG was most abundant.

ELISA results suggested that detecting IgG antibodies gave the best distinction between TB positive and TB negative serum samples while detecting IgM antibodies gave a poor distinction. A method was therefore sought which would remove or inactivate IgM antibodies in the presence of IgG antibodies, and this was achieved by adding a solution of DTT to the serum samples, and confirmed by ELISA. This process was then incorporated into the gold nanoparticle based assay; however, the sensitivity, specificity and AUC values obtained were lower than when not using DTT in the assay. A possible reason for this is that DTT may displace the thiol linker compounds, and in turn antigens, from the gold nanoparticle surfaces.

As the work reported in this Section involved initial steps towards characterising which class of antibodies bind to the antigens on the surfaces of the gold nanoparticles, and removing IgM antibodies from the serum samples, further work on this is needed. Firstly, investigations to look at the correlation between the results obtained by ELISA and those obtained with the gold nanoparticle based assay should be undertaken with a larger number of antigens and serum samples. Also, further investigations into methods of removing IgM antibodies in the presence of IgG antibodies, without using DTT, or any other thiol containing compound, in solution, are required.

6.5 References

1 Confidential unpublished work.

2 C. A. Janeway, P. Travers, M. Walport and M. Shlomchik, *Immunobiology: The immune system in health and disease (5th ed.),* Churchill Livingston, 2001, 92-122.

3 C. A. Janeway, P. Travers, M. Walport and M. Shlomchik, *Immunobiology: The immune system in health and disease (5th ed.),* Churchill Livingston, 2001, 123-154.

4 P. Parham, *The immune system,* Garland Publishing, 2000, 1-30.

5 J. H. L. Playfair, *Immunology at a glance (5th ed.),* Blackwell Scientific Publications, 1992.

6 G. C. Klein and K. A. Behan, **Determination of brucella immunoglobin G agglutinating antibody titre with dithiothreitol,** *J. Clin. Microbiol.,* 1981, **14,** 24-25.

Chapter 7

Towards the Development of a Portable, Easy to Use Device

7.1 *Introduction*

Having developed an assay, which can be read either by an instrument or by eye, which can distinguish TB positive serum samples from TB negative serum samples with a reasonable degree of accuracy (see Chapters 4 and 5), it was decided to try and incorporate this assay into a portable and easy to use device.

7.2 *The Developed Device*

A tube-like device was developed to incorporate the assay, and an example of one of the tubes developed can be seen in Figure 7.2.1 below:

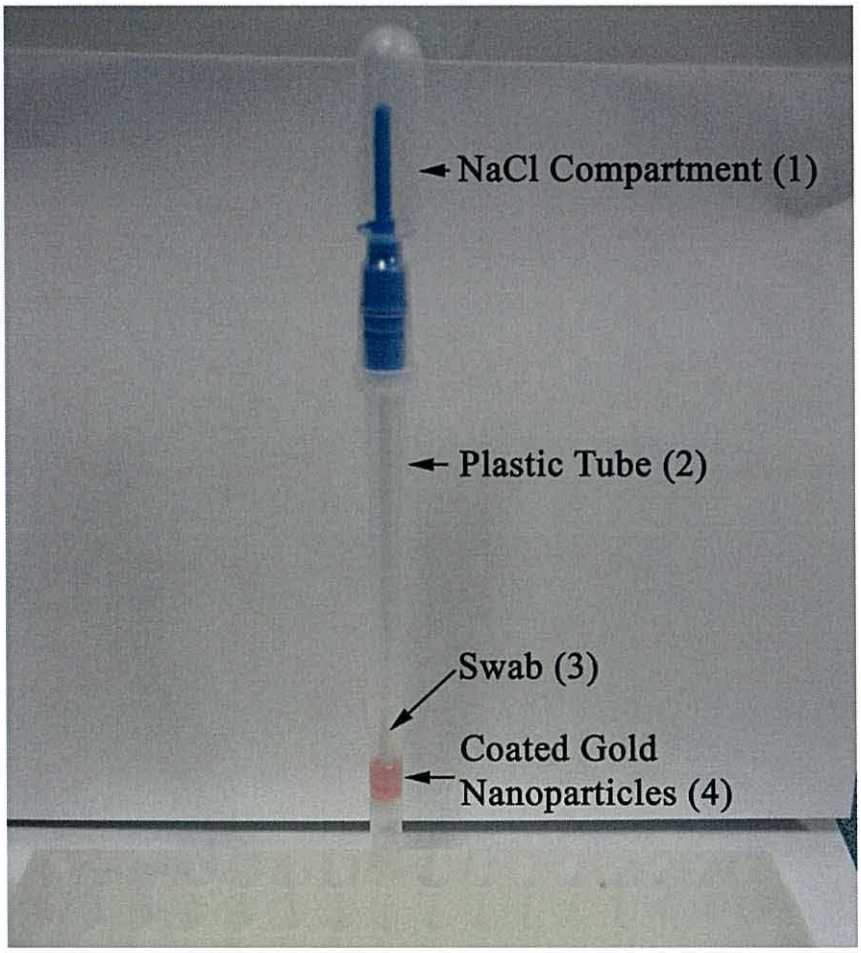


Figure 7.2.1 Image of the tube-like device developed.

Prior to performing the assay, a saturated aqueous solution of NaCl (70 µl in these examples) is placed in the NaCl compartment (1) and coated gold nanoparticles (450 µl in these examples) are placed in the bottom of the plastic tube (2). The NaCl compartment (1) also contains a breakable blue rod. Upon bending the blue rod (by hand), the NaCl solution held in this compartment flows down the plastic tube (2) and interacts with the

coated gold nanoparticles (4). The device also contains a swab (3) which can contain accurate amounts of liquids (in these examples 50 µl), and are used to transfer serum samples from outside the device to interact with the coated gold nanoparticles (4).

To perform the test, serum was diluted to a concentration of 1 in 2,500 in 10 mM phosphate buffer (pH 7.4) and 50 µl of this solution was transferred *via* the swab to interact with the coated gold nanoparticles. The tube was then shaken to allow interaction of the coated gold nanoparticles with the serum sample before being left for 10 minutes. The blue rod was then broken to allow the NaCl solution to flow down the plastic tube and interact with the coated gold nanoparticles which had been allowed to incubate with serum. The tube was again shaken before being left for a further 5 minutes. The colour of the gold nanoparticle solution was then observed.

7.3 Testing of Serum Samples

In order to make sure that the devices developed worked, and that TB+ serum samples could be distinguished from TB- serum samples in the devices, fourteen different serum samples were analysed. Seven TB+ serum samples (KE 8, PE 4, BA 20, GA 2, PE 3 and SA 6) and seven TB- serum samples (CA 3, CA 4, CA 5, SP 2, SP 5, SP 7 and GA 7) (chosen as strong TB+ and good TB- samples based on previous results on 96-well plates) were used (in order from left to right in Figure 7.3.1), and the gold nanoparticles were coated with **30**. The tubes at the end of these experiments can be seen in Figure 7.3.1:

Figure 7.3.1 Image of the tubes at the end of the experiments.

It can be seen from this image that a clear colour distinction is observed between the solutions obtained when testing TB+ serum samples and those observed when testing TB- serum samples. When testing the selected seven TB+ serum samples, a red (or pink) colour was observed for the coated gold nanoparticle solution at the end of the tests, while a blue colour was observed at the end of the tests when analysing the selected seven TB- serum samples.

Due to only a limited number of tubes being available for use, these serum samples were chosen, to test whether the tube-like devices worked as expected, because the TB+ samples gave low percentage aggregation values while the TB- samples gave high values, therefore a clear colour distinction could be observed. If serum samples which gave '?' designations by the previous eye tests performed (see Chapter 5) were used, the distinction between TB+ and TB- samples would not have been expected to be as clear.

In order to overcome this problem, as well as to overcome potential problems due to different individuals interpreting the colour of the solutions differently (and potentially some individuals *e.g.* colour blind individuals, not being able to see a visual difference between TB+ and TB- samples), a colour scale, an example of which can be seen in Figure 7.3.2, could be developed.

Figure 7.3.2 Colour Scale

The development of such a scale would allow the solutions obtained at the end of the assays to be directly compared against it, to give a value from 1 to 10. Solutions giving values of, for example, 1-4 could be designated as TB+, while values of, for example 7-10 being designated as TB- (values of 5-6 may require re-testing with the same and/or possibly other antigens, or further confirmation by another method). The development of such a system may overcome the problem of some of the serum samples previously leading to '?' designations being easier to interpret, while also overcoming the problem of different individuals interpreting the colour of the solutions differently because all of the solutions would be directly compared against the same colour scale.

Even though only a limited number of serum samples had been tested, these results suggest that the TB+ serum samples tested could be distinguished from the TB- serum samples tested when performing the developed assay in these devices, which are fully portable and

easy to use while giving a result in 15 minutes which can be read by eye or potentially by an instrument.

7.4 Concluding Remarks

A device has been produced to incorporate the developed assay based on coated gold nanoparticles, and initial results testing a limited number of serum samples using these devices were very encouraging, with a clear distinction being observed in the coated gold nanoparticle solutions which had been subjected to the selected TB positive and TB negative serum samples.

The limitation to performing the assay in these devices is that currently serum samples which gave '?' designations previously (see Chapter 5) would also be difficult to interpret in these devices, possibly leading to different interpretations of the solutions at the end of the assay by different individuals, as well as difficulty in interpreting the colour by some individuals *e.g.* those who are colour blind. These limitations may be overcome by developing a colour scale to which all of the solutions would be compared, significantly reducing discrepancies in interpretation of the solutions by different individuals.

Although further testing with a larger set of serum samples is required, these initial experiments suggest that the gold nanoparticle based assay developed could be successfully used in these devices to potentially produce a rapid, portable, reliable and easy to use sensor for the detection of TB, with results being available in 15 minutes. Moreover, these devices could be used in remote regions where access to on-site laboratories is not possible due to the results being readable by eye, as well as in laboratories where the results could also potentially be read by an instrument.

Chapter 8
General Conclusions

8.1 Conclusions

Anti-TB antibodies in both bovine and human serum samples were successfully detected using TDMs, TMMs and mycolic acids as antigens in ELISA, and the relative amount of antibodies in different serum samples could be determined due to the absorbance value observed at the end of the assay being directly related to the amount of antibodies in the serum sample. Analysing serum samples with various antigens suggested that TDMs and TMMs were more potent as antigens than mycolic acids for the detection of anti-TB antibodies. It was also shown that the best distinction between TB positive and TB negative serum samples was observed when detecting IgG antibodies.

Analysis of a large number of serum samples with various antigens by ELISA showed a good distinction between the TB positive and TB negative serum samples with some of the antigens tested, with the best distinction being observed with the keto TDM, **38**. The results from this analysis, showing how well the different antigens could distinguish TB positive serum samples from TB negative serum samples, gave an insight as to which antigens would be most suitable to use in a sensor for the detection of TB.[1]

The current project sought to take this work forward towards the development of a novel sensor for the detection of TB which would work in a few minutes and not require any laboratory facilities. It was decided to use gold nanoparticles as part of the detection element of the sensor. Thiol modified antigens, as well as non-thiol modified antigens, *via* a linker compound, were successfully bound to gold nanoparticles, and an assay was developed to detect changes in the SPR peak of these coated gold nanoparticles (as a result of biomolecular interactions at the coated gold nanoparticle surfaces). Although promising initial results were obtained, limitations were discovered in the assay when opaque serum samples were used, suggesting that a new approach was required.

This new approach again focussed on gold nanoparticles. It was discovered that gold nanoparticles coated with antigen aggregated when they are subjected to a saturated aqueous solution of NaCl, leading to a change in colour from red to blue. It was also discovered that this process could be inhibited by the addition of a TB positive serum sample to the coated gold nanoparticle solution prior to adding the NaCl solution. In instances where TB negative serum samples were added to the coated gold nanoparticle solution, aggregation could still occur upon addition of the NaCl solution, leading to a means of differentiating TB positive serum samples from TB negative serum samples by

the amount of aggregation observed in the coated gold nanoparticles, and in turn, the colour of the coated gold nanoparticle solution at the end of the assay. Though a similar salt induced aggregation method has been observed for gold nanoparticles bound to oligonucleotides, this lipid coated gold nanoparticle approach has been protected by a patent, 'Method for Determining the Presence or Absence of a Biomarker', WO/2012/153111.

An assay based on these principles was developed and optimised, and it was discovered that it could be performed in as little as 15 minutes without compromising the results obtained. This suggested that a sensor for the detection of TB, based on this assay, could also be developed, which could be performed in 15 minutes (which is quicker than any of the methods currently available for the detection of TB (see Table 1.5.1)) by giving a colour response that could be read by eye, and/or by measuring the absorbance of the solution at two different wavelengths.

Initially, in order to test how well the assay worked, 50 serum samples whose TB status were known, and 50 serum samples whose TB status were unknown were analysed by the developed assay using gold nanoparticles coated with a range of different TDM, TMM and mycolic acid antigens. Encouraging results were obtained from these experiments with sensitivity and specificity values of 100 % and 67 % respectively being obtained when using **38** as the antigen. In this case, the assignment was made based on percentage aggregation values obtained (calculated by measuring the absorbance of the solutions at 540 and 630 nm). When a visual assessment was made, carried out independently by two individuals, the results were less clear with some '?' designations being made.

Upon obtaining these encouraging results, it was decided to perform a blind test on a further 249 serum samples obtained from the WHO. In spite of discovering a problem with TB negative serum samples from patients who were co-infected with malaria often giving positive responses, encouraging results were again obtained, with no evidence of interference in the assay due to co-infection with any other disease studied, or due to the patient being vaccinated with the BCG vaccination. In addition, statistical analysis of the results obtained from these experiments showed that a combination of the results obtained with individual antigens gave a better distinction between the TB positive and TB negative serum samples than was observed with any single antigen alone. This suggested that a device developed for the detection of TB, based on interactions of antibodies to TDM,

TMM or mycolic acid antigens should contain multiple antigens. This is further supported by the findings that some antigens distinguished TB positive serum samples from TB negative serum samples from patients from some countries better than others. It was discovered that using gold nanoparticles coated with different antigens for serum samples from patients from different countries led to, with the exception of Vietnam, a sensitivity of 100 % and a specificity of at least 50 % being obtained with at least two antigens for each country. These values were comparable to those obtained by ELISA, where a sensitivity of 100 % and a specificity of 64 % or greater was obtained with at least one antigen for each country (with the exception of Vietnam and South Africa).

Having analysed a large set of serum samples with various antigens, attempts were made to classify the antibodies that bound to the antigens that were bound to the gold nanoparticles, due to the assay developed giving no indication of which antibodies bound, due to it not containing a secondary antibody conjugate. Although these results remained inconclusive, with no evidence that antibodies from only a single antibody class were binding to the antigens coated onto the gold nanoparticles, the results did suggest that the binding of IgG was most abundant.

Previous findings from ELISA assays had suggested that detecting IgG antibodies gave the best distinction between TB positive and TB negative serum samples, and that detecting IgM gave a less clear distinction. Attempts were therefore made to remove the IgM antibodies from the serum samples, prior to their use in the gold nanoparticle based assay as it was hoped that their removal might lead to an improvement in the sensitivity and specificity of the assay. The removal of IgM antibodies from serum samples using DTT was confirmed by ELISA analysis of the samples, however, their removal did not improve the distinction observed in the gold nanoparticle based assay (possibly due to the DTT displacing antigen on the gold nanoparticles' surfaces). If a method of removing the IgM antibodies, without the need for a compound containing a sulfur atom were discovered, however, it may still lead to an increase in sensitivity and specificity of the developed assay.

The removal of IgM antibodies however may not be necessary, or indeed desirable, if the assay developed were to be used as a quick screening test. The lack of secondary antibody conjugate allows detection of all of the antibody classes simultaneously, allowing a general overview of the level of antibodies (that can bind to the antigen on the gold nanoparticle

surfaces) to be observed. Therefore a quick method, which determines the general antibody level of the patient, and is able to detect all TB positive cases, while correctly identifying at least 50 % of TB negative cases, may be of great advantage as a screening test for TB, due to it being able to correctly rule out the presence of TB in at least 50 % of the cases. All those giving a positive result could then be further tested, reducing the number of patients required to be analysed by more time consuming or more expensive tests.

Finally, a device was produced to incorporate the developed assay, which is fully portable and easy to use, gives a result within 15 minutes, and which can be read either by eye or potentially by an instrument. Analysis of a limited number of serum samples using these devices was successful, with a clear visual distinction observed between the colour of the coated gold nanoparticle solution that had been subjected to the selected TB positive and TB negative serum samples. As the assay was the same assay as was perfomed in 96-well plates, serum samples which were identified as '?' would also be difficult to assign when the assay is performed in this format. A possible method of overcoming this problem could be to develop a colour scale, against which each solution would be compared and graded, leading to a reduction in individuals interpreting the colour of the solutions differently. The encouraging initial results suggest that the gold nanoparticle based assay developed could be used in these devices to produce a rapid, portable, reliable and easy to use sensor for the detection of TB, which could be used in remote regions where access to on-site laboratories is not possible due to the results being readable by eye, as well as in laboratories where the results could also potentially be read by an instrument.

8.2 Further Work

Having observed differences in sensitivity and specificity values with antigens that only differ in their stereochemistry, *i.e.* **24** and **30**, further analysis of antigens of this nature from each class should be performed with a large set of serum samples. Also, work discussed in this thesis proved inconclusive as to whether TDMs or TMMs containing the same mycolic acid moiety gave the best distinction between TB positive and TB negative serum samples. Further work, to analyse a large set of serum samples, should therefore be undertaken with a number of TDMs and TMMs with the same mycolic acid moiety, and also possibly the corresponding free mycolic acid, in order to determine which can best distinguish TB positive serum samples from TB negative serum samples. These analyses could possibly lead to the discovery, and/or towards the development of antigens which

can distinguish TB positive serum samples from TB negative serum samples with greater accuracy than the antigens discussed in this thesis, and in turn lead to a more accurate sensor for the detection of TB.

Another possible method of increasing the sensitivity and specificity of the assay developed, and in turn a sensor for the detection of TB, may be to remove IgM antibodies from the serum samples prior to their use in the assay. Ideally, any new method for the removal of IgM antibodies will not involve the use of a compound containing a sulfur atom, in order to ensure that no displacement of the antigen occurs at the gold nanoparticle surface, which may in turn lead to less specific binding of antibodies occurring to the coated layer on the gold nanoparticles.

Further work should also be carried out on serum samples from patients who are co-infected with other diseases, including other mycobacterial diseases and malaria. As previously reported, many false positive results were obtained when using the developed assay based on coated gold nanoparticles when using serum samples from patients who were co-infected with malaria. Further investigations should be made with these serum samples in order to try and identify an antigen that does not detect the malaria co-infection, or to attempt to identify what is interfering with the assay when this co-infection occurs, and attempt to remove it from serum samples prior to their use in the assay. Another approach could also be taken to try and identify an antigen that detects all of the malaria samples as positive. This approach could lead to the development of a combined screening method for either TB or malaria. Another common co-infection with TB that occurs in some regions is with HIV. Although reports claim that antibody responses to mycolic acids are conserved in patients who have tested HIV-positive,[2] this should be further investigated with TDMs and TMMs in order to ensure that similar observations are made.

In addition to analysing serum samples from patients who are co-infected with other diseases, further investigations with serum samples from patients from various countries may also be beneficial. A fairer set of serum samples, containing greater numbers of TB positive and TB negative serum samples from each country should be analysed with a number of different antigens. Further investigations may lead to a greater insight into which antigen(s) perform best with serum samples from patients from various countries, thus giving further insight into which antigens would be best to use in a sensor for the detection of TB.

Finally, there is a need to analyse a larger number of serum samples, including samples which gave false positive and false negative results, as well as those which gave '?' designations, with the developed device in order to ensure that the same distinction is still observed between the TB positive and TB negative serum samples as was observed on the 96-well plates. The development of a colour scale should also be performed in order to reduce the number of '?' designations made, and also help negate individuals interpreting the colour of the solutions differently, by comparing all of the solutions against a standard colour scale. Investigations into other platforms may also be required in order to check whether similar or better distinctions between TB positive and TB negative serum samples may be achieved, and in order to further maximise the assay's capabilities.

8.3 References

1 Confidential unpublished work.

2 G.K. Schleicher, C. Feldman, Y. Vermaak and J. A. Verschoor, **Prevalence of anti-mycolic acid antibodies in patients with pulmonary tuberculosis co-infected with HIV,** *Clin. Chem. Lab. Med.,* 2002, **40,** 882-887.

Chapter 9

Appendix