Novel optical sensing method for investigating microbial activity

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2005

Pariwono, A. M. (2005). Novel optical sensing method for investigating microbial activity. Master's thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/5577>

<https://doi.org/10.32657/10356/5577>

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NOVEL OPTICAL SENSING METHOD FOR INVESTIGATING MICROBIAL ACTIVITY

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A thesis submitted to the Nanyang Technological University
In fulfillment of the requirement for the degree of
Master of Engineering

2005

ABSTRACT

The increasing number of tuberculosis cases all around the world has become one of the recurring health concerns. Ninety-five percent of these cases occur in developing countries, where the detection methods are limited by the lack of trained manpower and cost. The most frequently used methods are the laboratory-based acid fast smearing and culturing techniques. Detection of acid fast bacilli (AFB) by conventional smearing techniques has a sensitivity of 50% compared to the latter method. Acid fast smearing's specificity is poor because it is unable to distinguish between *Mycobacterium tuberculosis* (*M. tuberculosis*) and other bacteria those are AFB. In contrast, culturing methods have very good specificity and sensitivity. However, it takes about 6 to 8 weeks for diagnosis. Therefore, in order to facilitate a faster detection of *M. tuberculosis*, a new optics-based biosensor method was developed.

The developed biosensor is designed to be portable, easy to use and affordable for applications in third world countries. It senses a specific metabolite produced by *M. tuberculosis*, named nicotinic acid (niacin). This particular substance was selected because the current niacin detection method in *M. tuberculosis* cultures has a specificity of 95% compared to other metabolic products detection.

Studies to investigate preferred optical setups and culture media were carried out. Four optical setups have been examined to measure different niacin concentrations. The configurations tested were as follows: optical probe, extrinsic optical sensor, single decladded fibre optic sensor and double decladded fibre optic sensor. From the experimental results, the most suitable setup was the extrinsic optical sensor. In this setup, light was shone from a light source, and attenuated by the sample (niacin

mixed with the chemical reagents) and finally captured by a detector. This optical setup has the best sensitivity and repeatability compared to the other three tested setups. Also, because of an indirect contact of the sensor to the sample, analysts have less exposure risk to the toxicity and pathogenicity introduced from the investigated sample.

Sample growth and niacin production on three different culture media were also investigated. The culture media tested were Lowenstein-Jensen egg media, Ogawa egg media and Middlebrook 7H9 broth. Experiment results have shown that Ogawa was able to facilitate specimens' growth and niacin production better than the other media.

With the selected optical setup and culture media, the novel optical sensor was developed. The sensor was built out of a photodiode, a sensing region where the sample was placed and an LED that has a maximum operating wavelength of 472 nm. The sensed sample was obtained by extracting niacin from patient specimens that has been culture on Ogawa media. The liquid extract was then reacted with chemical reagents and placed in the sensing region of the sensor. The sensor would then detect spectrum changes that occur, by detecting the absorbance level of the sample at a particular wavelength. When a high concentration of niacin was present, the absorbance level detected decreased. The decrease in absorbance level would then indicate the presence of *M. tuberculosis*.

Clinical trials have been conducted on 15 acid fast smeared sputum samples. The biosensor was able to detect *M. tuberculosis* in 5 to 7 days, which is around 10 times faster compared to the 6 to 8 weeks time frame needed by culturing. Testing cost is estimated to be 17 times cheaper than the current molecular methods. These results have demonstrated the capability of using this optical technique to perform rapid *M. tuberculosis* metabolite detection.

ACKNOWLEDGEMENTS

This Master's thesis owes its completion to several supportive and kind people. These people have rendered their precious and much appreciated help towards the completion of this report. The author would like to give them her most sincere thanks for making this research an enjoyable and enriching one.

The author wishes to thank her project supervisor, A/P Lim Chu Sing for his inputs, guidance, patience and encouragement throughout the project.

The author also wishes to thank A/P Chan Yew Weng, Senior Consultant Department of Anesthesia & Surgical Intensive Care at Singapore General Hospital, and Dr. Wang Su Xing, Chief Scientist from Singapore General Hospital Tuberculosis Laboratory, for their insights.

The author would also like to extend her grateful appreciation to doctors and clinicians in Rumah Sakit Tuberkulosa dan Paru-Paru (Indonesia), her colleagues, technicians from Biomedical Engineering Laboratory and Advance Materials Research Center for their support in assisting and assistance in some of the testing. It has been a fun and enjoyable experience working with all of them. Also, to her parents who supported her morally and given her much encouragement.

Finally, the author would like to thank the School of Mechanical and Production Engineering of Nanyang Technological University, Singapore for the generous resources and financial support provided during her first year of study.

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CHAPTER 1

INTRODUCTION

1.1. BACKGROUND

Based on the World Health Organization (WHO) 2003 statistics, the number of tuberculosis cases worldwide in the year 2002 is estimated to be 10.6 million, of which 1.8 million resulted in deaths [1]. Tuberculosis is caused by a particular bacterium, named as *Mycobacterium tuberculosis* (*M. tuberculosis*). This disease is also known to be the disease of the poor because it occurs mostly in areas with low sanitary level and in overcrowded populations [2, 3]. Hence, 95% of the cases occur in developing countries, and the remaining 5% in industrialized countries [4]. Sixty percent of the tuberculosis cases in the developing countries are found in Asia [5]. Tuberculosis often affects people with weak immune system. Hence, tuberculosis cases are expected to further increase at an alarming rate due to its close relation to Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS), which is spreading rapidly [6, 7]. In recent years, the bacterium has mutated to offer resistance to antibiotics, thus making it a major threat to healthcare in general.

Many methods have been used to detect the presence of *M. tuberculosis* in humans. Firstly, there are screening methods that include tuberculin skin test and X-rays. However, these tests are inaccurate and therefore require other tests to confirm the presence of *M. tuberculosis*. Secondly, there are the acid fast smearing and culturing tests that are most commonly practiced in developing countries [8]. Acid fast smearing results can be obtained within hours, but it has a low specificity and

cannot differentiate *M. tuberculosis* from other acid fast bacilli (AFB). Although culturing technique takes a long time (between 6 to 8 weeks), it gives the most specific results [9]. Thirdly, assays and molecular methods have been developed to provide faster detection with equal sensitivity. However, they tend to be prone to false positive results, which frequently occur because of cross contaminations [10]. Apart from requiring purified products, these new methods (assays and molecular methods) require trained personnel to process the samples.

Since most tuberculosis occurrences are in developing countries, its detection methods are limited by costs and laboratory facilities. The most affordable and accurate methods used in these countries are the **acid fast staining** and **culturing methods**. But due to the long detection period of culturing methods, the infection might become worse and the disease might spread more widely. It is therefore crucial to be able to detect the presence of *M. tuberculosis* early as possible, and yet, at an affordable cost. Therefore, this project set out to investigate several optic-based sensor strategies with the aim of developing a rapid sensor that is able to screen and detect the presence of *M. tuberculosis* in cultured patient samples.

Some metabolic products are specific only for *M. tuberculosis* and its presence can be analyzed by using colourimetric methods. When the substance of interest is present, with the help of certain chemical reagents, a specific colour emerges. The amount of metabolite will affect the colour density, and hence, when the amount of substance is small, the colour change is not obvious to human eyes. This situation will cause observers to misinterpret the results. Therefore, to overcome this situation, optical methods are investigated as part of this work.

Interaction of light with medium results in a modulation of intensity, wavelength, phase, or polarization [11]. In colourimetric methods, two factors are observed: intensity and wavelength shifts. Objects have different colours because they absorbed different wavelengths, and its intensity level is determined by the amount of the light being absorbed. The absorbed wavelength and intensity is specific for each medium. Optical methods detect either intensity of light or shift in wavelength,

or both. The advantage of optical methods is that they can be used to detect minute changes in an investigated sample, which may not be obvious to human eyes. Also, optical sensors can be designed to be portable and relatively inexpensive without being less sensitive.

Based on this principle, the sensor was developed. The sensitivity of the optical approach makes possible detection of the low levels of metabolite produced by *M. tuberculosis* in its early growth stage, allowing the sensor to yield rapid results.

1.2. OBJECTIVES

With the above considerations, this project has the following objectives in mind:

1. Research and develop a novel optic-based sensor to detect highly specific *M. tuberculosis* metabolites.
2. Optimize sensor parameters for rapid *M. tuberculosis* detection.
3. Derive algorithms specific to *M. tuberculosis* detection using the developed sensor.
4. Test the sensitivity of the sensor by conducting bench top and clinical trials.
5. Derive a usable algorithm for patient trials.
6. Compare existing identification methods to the developed sensor.
7. Establish a protocol for the application for the developed sensor.

1.3. SCOPE

In the first part of the project, the study focuses on investigating the most suitable optical setup to identify *M. tuberculosis* metabolic products. Bench top experiments were first conducted with a simulation of *M. tuberculosis* metabolic production. This was done to minimize exposure of the researcher to live bacteria. The most suitable configuration was then applied for the development of the new sensor.

In the second part of the project, the study focuses on testing the developed sensor for clinical trials, using samples from suspected tuberculosis patients. The experiment was conducted in Rumah Sakit Tuberkulosa dan Paru-Paru (RSTP) at Cisarua, Indonesia.

CHAPTER 2

LITERATURE REVIEW (PART 1)

MYCOBACTERIUM TUBERCULOSIS

Tuberculosis is a disease caused by *Mycobacterium tuberculosis* (Figure 2.1). Humans usually acquire infection by breathing in infectious droplets, which have been expelled from respiratory tract of infected persons. *M. tuberculosis* damages the lungs, resulting in necrotic cavities [12]. The bacterium may spread to any other organ system by entering the blood stream. It will then infect other parts of the body, including bones, joints, liver, gastro-intestinal tract and brain. The disease progresses slowly and chronically if untreated, which can result in death.

Figure 2.1. *Mycobacterium tuberculosis* [13]

People have been neglecting the danger of tuberculosis and its way of spreading because for years it seemed to be under control [5]. However, World Health Organization's (WHO) survey shows the contrary. In the year 2002, tuberculosis is the third leading cause of death in the world (1.8 million deaths), after Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Disease Syndrome

(AIDS) and heart disease [14, 15]. The total number of tuberculosis cases in 2002 (excluding the number of tuberculosis deaths) is estimated by WHO to be 8.8 million cases [1]. Of these, 95% occur in developing countries [5] (Figure 2.2), and 60% of these cases are in Asian countries [16].

1. Estimated TB incidence rates, 2002

Figure 2.2. Estimated Number of Tuberculosis Cases in 2002 [17]

Raviglione [5] has shown that the number of tuberculosis cases in United States has increased significantly from 1989 and is still increasing until today. One of the reasons identified for the increase is the HIV/AIDS epidemic [18]. HIV and AIDS patients are considered to have 113 and 170 times, respectively, more likely to develop infections after exposure to tuberculosis [19].

Emergence of multi drug-resistant *M. tuberculosis* strains (MDR-TB) [20, 21] have also contributed to the increase of tuberculosis cases. Two percent of all the tuberculosis cases are multi-drug resistant [22]. A multi-drug-resistant strain is defined as *M. tuberculosis* resistant to isoniazid and rifampin, with or without resistance to other drugs [23]. The emergence of drug resistance represents the

survival of random pre-existing mutations that is caused by monotherapy, erratic drug ingestion, omission of one or more drugs, suboptimal dosage, poor drug absorption or an insufficient number of active drugs in a regimen [24, 25].

Hospital employees have higher chances of being infected by *M. tuberculosis*, because of daily contact with the tuberculosis patients [26-28]. Prevention of the tuberculosis spreading has been done by introducing BCG vaccine, but its efficacy is still highly doubted by medical science [29-32].

For many years, people have been trying to reduce the number of tuberculosis cases, which could be done by observing characteristic of *M. tuberculosis* and by applying easier detection methods. Hence, this chapter will elaborate general characteristics of *M. tuberculosis* and detection methods currently adopted.

2.1. GENERAL CHARACTERISTICS

Mycobacterium tuberculosis has a shape of a slender rod. It sometimes forms filaments and tends to grow in clumps. The general characteristics of *M. tuberculosis* are summarized in Table 2.1. Its main characteristic is that it has a very thick cell wall, which is built from **mycolic acid**. Mycolic acid is a type of lipid found only in the cell walls of *Mycobacteria* and *Corynebacteria*, which enables them to be distinguished from other types of bacteria.

Table 2.1 General characteristics of *Mycobacterium tuberculosis* [33]

Items	Characters
Morphology	
<i>Shape</i>	Slender or slight curved rods
<i>Size</i>	1-4 um (length), 0.3-.6 um (breadth)
Culture morphology	
<i>On glycerol egg medium</i>	Rough, raised, thick, nodular or wrinkled surface, irregular thin margin
<i>On oleic acid albumin medium</i>	Flat, rough, corded, dry
<i>In liquid medium</i>	Growth at bottom of tube with a later pellicle growth on the surface
<i>In Tween-albumin liquid medium</i>	Readily disperse submerged growth

The cell-wall is composed of upper and lower segments [34]. The former consists of the peptidoglycan layer attached with the arabinogalactan and mycolic acid. This segment is known as the *cell wall core*. The latter is composed of cytoplasmic membrane, lipoarabinomannan, proteins, and phosphoinositol. This complicated cell-wall configuration protects the bacteria from outside threats [35]. A schematic of the wall composition is shown in Figure 2.3. This bacterium appears to be an obligate-aerobe type but it may have acquired the ability to come into an anaerobic survival form when oxygen becomes depleted [36].

Figure 2.3. *Mycobacterium tuberculosis* cell wall structure [37]

This waxy and water-resistant layer acts as a protective shield for the bacteria to survive in harsh environment (such as drying, general chemicals and drugs). On the other hand, its unique cell wall characteristic prevents fast nutrient penetration. This causes the bacteria to have a very slow growth rate [33].

Based on 16S ribosomal RNA analysis, this organism belongs to the branch of Actinomycetes, which traditionally joins the low gram-positive class, together with *Staphylococci* and *Streptococci*. The gram-positive classification is based on the evolutionary distance between the nearest ancestral units. However, Fu and Liu [38] showed the contrary. Based on the bacteria's cell wall characteristics, genomic G+C content and orthologous genes, they classified *M. tuberculosis* to be gram-negative

bacteria. Regardless of Fu and Liu's findings, scientists still classify *M. tuberculosis* as gram-positive bacteria.

2.2. GROWTH PHASES AND CONDITIONS

Similar to all kinds of bacteria, *M. tuberculosis* has a growth profile that depends on its surroundings. If the condition is suitable, but not optimum, bacteria may grow at a slower rate. Essential requirements for growth include: supply of suitable nutrients, source of energy, water, and appropriate temperature, pH and oxygen level. The optimum conditions for *M. tuberculosis* are tabulated in Table 2.2.

Table 2.2. Optimum growth conditions for *Mycobacterium tuberculosis* [33]

Item	Condition
<i>Optimum temperature</i>	37 °C
<i>Optimum pH</i>	6.4-7.0
<i>Generation time (in optimum condition on solid media)</i>	14-20 hrs

A **culture** is a liquid or solid medium that contains bacteria, which are growing and multiplying. **Incubation** is the process of maintaining the culture at a particular temperature so that the growth of the bacteria is optimum. The initial process of adding cells to the medium is **inoculation**. **Generation time** (time taken for one complete cell cycle) is measured during this period. For minimum generation time, optimum growth conditions are necessary.

The growth period of *M. tuberculosis* can be divided into four stages, i.e. lag, log, stationary and death phase (Figure 2.4). When *M. tuberculosis* is introduced into a new environment, e.g. a fresh medium, cell division may not begin immediately because they need to go through an adaptation phase or **lag phase**. During this time, they synthesize macromolecules required for multiplication, including enzymes, ribosomes and nucleic acid and they generate energy in the form of ATP.

Length of the lag phase depends on conditions of the original medium where the bacteria are and the latter medium where they are cultured. If bacteria are transferred from a rich nutrient medium to a medium that has a shortage in nutrient, the lag time tends to be longer. This is because the bacteria need to make enzymes to synthesize missing components in the new medium. In contrast, if cells are transferred to a medium similar in composition, the lag time will be shorter. It was observed by microbiologist that the lag phase of *M. tuberculosis* originated from sputum samples is around 4-5 days in solid media or 2 days in liquid media [39, 40].

Figure 2.4. Bacteria growth curve

(1) lag phase, (2) log phase, (3) stationary phase, (4) death phase

In the **log phase**, bacteria cells divide at a constant rate and their numbers increase by the same percentage during each time interval, which leads to what is called **exponential growth**, which can be expressed in the following equation [41]:

$$N = a \times 2^n \quad \dots(2.1)$$

N is the total number of cells, a is the number of organisms present in the original inoculums (cells on the culture), n is the number of generations (number of division) occurring during a given period of time. Plotting the number of organisms present as a function of times generates a curvilinear function, whereas plotting the logarithm of the number will result in a linear function.

Bacteria's activities shift as they enter a stage called late log phase, which marks the transition to **stationary phase**. This change occurs in response to multiple factors that are inevitable in a closed system, such as depletion of nutrients and build up of waste products. The total number of viable bacteria in the population remains relatively constant, but some cells are dying while others are multiplying. Cells that die release peptides and nucleic acids, which provides a source of nutrient and energy to fuel the growth of other cells. In this phase, bacteria become more resistant to harmful chemicals and radiations because of the changes in the composition of their cell walls and cytoplasmic membranes.

Death phase is a period when the total number of viable bacteria in the population decreases as cells die off at a constant rate. Like bacteria growth, death has an exponential profile but usually at a slower rate.

Growth on Solid Medium

Growth on solid medium is done as one way to investigate the bacteria present in a sample. This method is very accurate, because it shows the morphological shape of the bacteria analysed [42]. If a single bacterium is placed on the surface of the solid media, the cell will grow and multiply. The growth and multiplication will continue and the original cell eventually reaches such immense numbers that they form a compact heap of cells that is usually visible to the naked eyes. This mass of cells is called a **colony**. The size of the colony is limited by the exhaustion of the nutrients surrounding it. For this reason, crowded colonies are generally smaller than well-spaced ones (Figure 2.5).

Figure 2.5. Illustration of variation in colony sizes on solid media

There are two types of solid media used for culturing *M. tuberculosis*, namely agar and egg media. Agar based media are recommended for samples from non-sterile sites, such as urine samples. Agar media are frequently used to test the drug resistance of the culture because agar media is able to retain the exact concentration of the added drug. The types of culture media are listed in Table 2.3.

The most common egg media used for culturing *M. tuberculosis* are the Lowenstein-Jensen and Ogawa media. Ogawa media have similar composition as LJ media, only that they do not use asparagin [43]. Ogawa medium discovers drug resistant strains compared to LJ medium [44].

Growth in Liquid Medium

In liquid medium, or commonly known as **broth**, bacteria can move freely inside the nutrient. Thus, nutrients are able to diffuse into the bacteria cells much faster compared to solid medium and each cell is able to absorb the same amount of nutrient. As the number of cell increases, the medium becomes increasingly turbid.

M. tuberculosis can be cultured on liquid or solid media. Broth cultures that are widely used are Middlebrook liquid medium (7H9, 7H10, 7H11) [45] and Tween albumin liquid medium. In these broth media, *Mycobacteria* grows rapidly [46]

Table 2.3. *Mycobacteria* culture media [46]

Name	Type	Advantages	Limitations
Dubos	Agar-based Broth	- Specific for <i>M. tuberculosis</i>. - Rapid cultivation (colonies can be observed in 10-12 days). - Drugs may be added.	- Negative cultures do not rule out active infection by <i>Mycobacteria</i>
Middlebrook	Agar-based Broth	- Low concentration of malachite green to allow primary isolation. - Contains casein that stimulates the growth of <i>M. tuberculosis</i> - Susceptibility testing	- Negative cultures do not rule out active infection by <i>Mycobacteria</i>
American Trudeau Society (ATS)	Egg-based	- Allows early detection of <i>Mycobacteria</i> colonies	- Suited only for samples with low contamination level.
Lowenstein-Jensen (LJ)	Egg-based	- Able to differentiate <i>Mycobacteria</i> - Can be used for niacin testing	- Requires 5% of NaCl to decontamination. - Contaminating proteolytic organisms tend to liquefy the medium
Ogawa	Egg-based	- Able to differentiate <i>Mycobacteria</i> - Can be used for niacin testing - Cheaper compared to LJ	- Contaminating proteolytic organisms tend to liquefy the medium
Petragnani	Egg-based	- Contains large amount of malachite green to inhibit the growth of contaminating organism.	- Suited only for samples with low contamination level.

2.3. METABOLITE PRODUCTS

As *Mycobacterium tuberculosis* grows, it synthesizes certain metabolite products. A **metabolite** is a chemical substance that is produced or required by bacteria in a metabolic process. Some of these products are unique to a particular bacterium or it might be shared among other types of bacteria. Common metabolites produced by *M. tuberculosis* are elaborated as follows:

1. Carbon Dioxide (CO₂)

Growth of *M. tuberculosis* would result in an increase of carbon dioxide [47]. The production of CO₂ is a metabolic product of palmitic acid in *M. tuberculosis* [48]. Therefore, by investigating the increase of carbon dioxide, one can identify the bacteria presence. However, it should be noted that CO₂ is also a metabolic product of most other bacteria; and thus is not specific to *M. tuberculosis*.

2. Niacin

Niacin (nicotinic acid) is produced by *M. tuberculosis* for the synthesis of NAD and NADP [33, 49]. Niacin has a chemical structure of C₆H₅NO (Figure 2.6). Niacin plays a vital role in the oxidation-reduction reactions that occur during metabolic processes in all *Mycobacteria*. However, only *M. tuberculosis* and some isolates of *M. simiae* do not consume niacin further, and therefore accumulate more. Niacin production by *M. tuberculosis* and *M. simiae* has a sensitivity of 95% and 63%, respectively [50]. Niacin synthesis sequence is presented in Figure 2.6 [51].

Figure 2.6. *M. tuberculosis* niacin synthesis sequence [51]

3. Nitrate reduction

M. tuberculosis is one of the strongest nitrate reducers among other mycobacteria genus. It converts nitrate to nitrite using nitroreductase enzyme [52]. The chemical reaction takes place depending on the age of the colonies, temperature, and pH of the culture media.

4. Hydrogen Peroxide (H₂O₂)

M. tuberculosis, like all other bacteria, has an ability to convert flavoproteins into reduced-flavoprotein. When reduced-flavoproteins reacts with oxygen and oxides in the respiratory chain, two toxic compounds are formed, namely: hydrogen peroxide (H₂O₂) and super oxide radical (O₂⁻) [52].

There is no specific metabolite that is solely produced by *M. tuberculosis*. Mostly, some other bacteria produce these metabolic products as well. Ideally, a correct result will be obtained if the culture used for detection only contains *M. tuberculosis*.

2.4. EXISTING IDENTIFICATION METHODS

There are various ways to identify *M.tuberculosis* infections in a suspected tuberculosis patient. **Conventional methods** are simpler methods that have been used and proven for years to identify *M. tuberculosis*. **New detection method** involves the use of technology that is faster yet usually expensive and unproven. Types of identification methods used by health centres depend on several factors, such as cost of maintenance, number of trained practitioners.

2.4.1. CONVENTIONAL METHODS

Conventional methods have been used for many years. These methods are simple to conduct in health care centres with minimum facilities. Based on the usage frequency, a list of preference methods is described as follows:

2.4.1.1. Screening Methods

a. Symptoms

First indication of tuberculosis infection is by observing its symptoms: fever, drastic weight loss, chest pain, and prolonged chronic cough for more than 3 weeks. However, pulmonary cancer, which has similar indications, is often mistaken as tuberculosis.

b. Chest Radiograph

Chest radiograph is a radiology (x-ray) test that involves exposing the chest briefly to radiation to produce an image of the chest and the internal organs of the chest. A tuberculosis-infected patient is marked by cavitations, apical distribution, bilateral distribution, pulmonary fibrosis, shrinkage and calcification of the lungs [4]. This method is often used as screening method for mass tuberculosis detection in prison and immigrants [53-55].

c. Tuberculin Skin Test

Tuberculin skin test has been best used to identify tuberculosis in children. Tuberculin solution is injected into the volar or dorsal surface of the forearm. A pale elevation of the skin should appear. After 48 and 72 hr of injection, the area of indurations is measured at its largest transverse diameter and recorded in millimetres [56]. Infection by other mycobacteria and BCG vaccination may result in false positive results [54, 57, 58].

d. Microscopic

Results of these methods are immediate, but their specificity is very low. Microscopic methods observe specific characteristic of *M. tuberculosis* morphology for identification, which is frequently done by staining the bacteria cell wall. There are two methods frequently used: **acid fast** and **fluorescence** staining.

All bacteria that can sustain such staining method are called **Acid Fast Bacteria** (AFB). Bacteria are stained by carbol-fuchsin dye that is introduced by boiling (Ziehl-Neelsen staining) [59]. Only bacteria with thick cell walls could sustain the dye, and type one AFB is mycobacteria [37].

Acid fast smearing requires 5000-10000 AFB to be present per ml of sputum [50, 60], which is equal to a sensitivity of 45-75% compared to culture methods [45, 61]. A study was conducted to investigate if different amounts of sputum would affect the sensitivity of the results [61]. Experimental results showed that with a minimum sputum volume of 5 ml, the sensitivity increased to 92%.

This method has its greatest clinical impact on the first time diagnosis of tuberculosis for patients who have not been treated and who have not previously had culture-positive sputum. It also gives a semi-quantitative estimation of the number of bacteria being excreted, reflecting the patient's infectivity [56].

In countries with high AIDS and HIV prevalence, acid fast smearing has a poor prognosis. AIDS/HIV patients tend to excrete significantly fewer organisms per millilitre of sputum [57]. This leads to false count or non-detectable AFB. Negative sputum smears may also be due to deficiencies of collection, processing and interpretation of sputum smears or because of administrative errors, and these false-negative results should be remembered as possible confounding factor [59]. In children, smearing can only detect less than 20% of tuberculosis cases [56].

A method to reduce the possibility of negative sputum is by sputum induction. This procedure is used for patients who are unable to produce sputum spontaneously, such as children [10]. However, this method has caused the patients to feel unwell, causing vomiting and nausea.

Fluorescent testing has similar procedure with acid fast staining method but it uses auramine-O and rhodamine-B for the dye. Unlike Ziehl-Neelsen method, the stained bacteria appear yellow or orange. This colour will vary depending on the type of filter used. This method is 10-fold more sensitive than the acid fast staining method but it has less specificity [50].

2.4.1.2. Culturing Methods

Although longer time is required to obtain the results, culture methods are still the **gold standard** for identification. Their results are highly specific. Culture methods would normally take 6 to 8 weeks for a person to be certain that he/she is infected by *M. tuberculosis*. Additional tests to identify drug susceptibility of the bacteria requires another 2 to 6 weeks incubation [62]. Some biochemical methods have been introduced to differentiate *M. tuberculosis* from other mycobacterium. This has helped a faster detection by reducing the time to around 3-6 weeks. The most frequent biochemical tests conducted are niacin and nitrate reduction test.

a. Niacin Test

Cultures grown on egg medium yield the most consistent results for niacin test. Detection of niacin level has been widely used for *M. tuberculosis* identification because the results have 95% accuracy (Appendix 1) [50]. Other mycobacterium, i.e. *M. simiae*, may give a positive reaction but can be differentiated from *M. tuberculosis* based on morphological characteristics and temperature that is required for the bacteria to grow [63].

Niacin, which belongs to the aromatic amide group, can be detected by three methods, namely: colourimetry, microbiology and ultraviolet detections. Advantages and disadvantages of these methods are presented in Table 2.4. In **colourimetry** [64], niacin chemically reacted with a specific reagent. The chemical reaction involves an interaction between niacin, cyanogen halide and an aromatic amine; which is known as **Koenig reaction** (equation 2.7 and 2.8) [65]. Aromatic amines used for these purpose are p-aminoacetophenone, metol (p-methylaminophenol), aniline, β -naphthylamine, and p-phenylenediamine [66]. The chemical product (equation 2.8) appears yellow or red [67].

Table 2.4. Comparison of niacin detection methods

Method	Advantages	Disadvantages
Colorimetric	Rapid Inexpensive Simple	Toxic chemical involved
Microbiology	More sensitive compared to colorimetric method	Involves other bacteria Samples need to be pre-processed Longer detection time
Ultraviolet	Most sensitive	Expensive Trained operator required

The **microbiological** method is based on the growth of *Lactobacillus arabinosis* [68] and its ability to produce lactic acid. *L. arabinosis* requires niacin to grow. When a high niacin level is present in a culture media, there will be lots of bacteria present. This method is 100 times more sensitive compared to

colourimetry method. However, it requires 16 hours for determining niacin's presence [66]. **Ultraviolet light** is used to detect chemical bonding that is present in a sample. This method is very specific, but the sample needs to be pure in order to get good results.

b. Nitrate Reduction Test

Similar to niacin test, nitrate reduction test is a biochemical test that requires reagents for its identification. It has also been used to identify drug resistant strains [69]. HCl, sulfanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride) are added into the sample solution, positive results are indicated by colour changes from a colourless sample to be red or pink [33, 52, 70]. When nitrate reacts with sulfanilic acid, it forms a complex nitrite-sulfanilic acid. The nitrate-sulfanilic acid will then react with alpha-naphthylamin to form a red precipitate.

Angeby [69] developed a method based on the reduction of nitrate by *M. tuberculosis*. The test is able to give results in 7 days for majority of the isolates. Limitation of this method is that nitrite might be further reduced to nitric oxide, which cannot be detected by the reagents used. When nitrate reduction test is performed for the purpose of species identification, zinc powder was added to all negative tubes. Zinc reduces nitrate rapidly, and a true negative test will directly turn red, while there will be no colour change in a tube where reduction has passed beyond nitrite.

c. Carbon Dioxide Test

Dehydrogenase enzymes, which are present in the electron transport system (ETS) of bacteria, reduce tetrazolium salts. These enzymes convert each tetrazolium salts to insoluble formazans, which can then be extracted with an organic solvent and measured colourimetrically. The amount of formazan produced is used as to quantify the number of bacteria present in a sample [71, 72].

d. β -glucosidase

β -glucosidase is an enzyme used by *M. tuberculosis* to cleavage β -glucoside into glucose and galactose. β -glucosidase is only present if the media has β -glucoside.

The chemical reaction is as follows [52]:

2.4.2. NEW DETECTION METHODS**a. Carbon dioxide test**

Semi-automated liquid culture systems are able to monitor bacterial growth [47, 73]. The instrument is composed of broth culture vials that contain radiolabelled ^{14}C -palmitate as its sole carbon source. As bacteria grows, there will be an increase in CO_2 level [58, 74]. Since the CO_2 produced is radiolabelled, a fluorescence sensor detects it. This sensor has a detection limit of 4000 bacteria/ml of patient's sample [75]. Positive results can be detected in 2 weeks or less for the vast majority of sputum smear-positive samples and with in 3 weeks for smear-negative ones [74, 76-81]. The widespread adoption of semi-automated rapid liquid culture systems was frustrated by the demands on staff time and the difficulties associated with the disposal of radioisotopes.

New fully automated systems that rely on non-radiometric methods are currently being developed; such as MB/BacT, BACTEC 9000, and MGIT. Many studies have compared these different culture systems. These systems measure changes in gas pressure, carbon dioxide production or oxygen consumption either fluorimetrically or colourimetrically [74, 76-81].

These systems' specificity relies on the ability of the culture medium to suppress growth of contaminants, and only allowing mycobacteria to grow. Identification of species is not possible for all these systems [82], except BACTEC 460TB. This system is able to differentiate *M. tuberculosis* from other types of mycobacteria [39, 74].

b. Bronchoscopy

Flexible bronchoscopy is a rapid but invasive procedure. It confirms the tuberculosis diagnosis by direct visualization of the airway abnormalities. This test is normally conducted to children with an abnormal chest radiograph who are being assessed for possible TB infection. It can also be used to collect samples for microbiological investigation.

c. Serological Methods

Serological methods are methods that use antibody or enzyme for identification. When a foreign cell enters a human body system (antigen), the body will protect itself by binding the antigen to a substance (antibody) so that the foreign cell can be destroyed. This antigen-antibody has a specific binding between each other, which is described as a “key-lock” feature. The working principle of the immunoassays sensors is based on the properties of these specific bindings.

Serological methods are sometimes known as immunoassays. This method is associated with a particular polypeptide (protein). However, the presence of most mycobacterial antigens is non-specific because these proteins are shared among other species and genus [83]. When people have been in contact with *M. tuberculosis* but no infection occurs, there is a chance that the antibody has been activated, giving false positive results. To avoid these non-specific results, there have been efforts to purify or to semi purify antigens that might be unique to the *M. tuberculosis*.

Serological methods can be used with ELISA techniques. ELISA is used as an antigen filtrate for *M. tuberculosis*. It was discovered that tuberculin does not influence the IgM and IgA antibody levels, only IgG. Their sensitivities and specificities are 68% and 98%, respectively [83]. However, most studies performed in adults yield poor sensitivity (16-57%) but good specificity (62-100%) [56]. The specificity and sensitivity of these sensors might vary depending where they are being used. In developing countries, particularly in tropical areas, people are constantly exposed to various pathogens and therefore

develop cross-reacting antibodies. This causes poor specificity. Hence, the sensitivity and specificity of these methods need to be validated with the control of the country where the test will be applied [84].

There are a number of antigens being used for *M. tuberculosis* identification, e.g. 38 kDa antigen, 30 kDa antigen, and 19 kDa antigen. All these antigens have their own advantages and disadvantages [16]. 38 kDa antigen has been designated to be the most common used protein for this purpose but this antigen also contains non-specific epitopes found in other *Mycobacteria*.

d. Molecular Methods

There are two molecular methods frequently used: nucleic acid probes and PCR. Molecular methods are incredibly powerful and capable of detecting very low numbers of DNA target, thus very low level of contamination can lead to false positive [12, 85]. In theory a single organism can be detected in a clinical sample, but in practice the limit is usually around 10 bacteria [56]. Another limitation is that when exposed to new drugs, *M. tuberculosis* will mutate as its way for survival. Each mutation introduces a new DNA sequence. One example is the nucleotide change from G to A in an isoniazid drug resistant *M. tuberculosis* [86]. With this DNA sequence mutation, the probe has to be updated regularly.

Despite its limitations, molecular method has provided a sensitive and rapid means of detection. In fact, if the cost was low enough, they could replace sputum smear for the purpose of making diagnosis of pulmonary tuberculosis in previously untreated patients [87].

Nucleic Acid Probes

DNA fingerprints can provide valuable insights into the pathogenesis of tuberculosis and may help to identify *M. tuberculosis* strains with specific properties. DNA and RNA probes have proven to be very useful in detecting *M. tuberculosis* in a sample only if a large number of organisms are present (10^5

bacteria/ml sample). If the patient's specimen does not provide sufficient numbers of bacteria, the sample should be cultured first [83].

Nucleic acid probes are usually made of a short sequence of nucleotide bases that will bind or hybridise with highly specific regions of targeted nucleotide sequences. Ideally, the probe consists of 15-30 nucleotides. They can be either DNA or RNA, but DNA is more commonly used. The probe requires a specific target of nucleic acid and this target must be unique to the bacteria to give good identification results. Probes are labelled with isotopes such as ^{32}P , ^{35}S and ^{125}I , so that after hybridisation process, the system can detect bindings that are produced. Commonly used *Mycobacteria* probes are Gen-Probe system and SNAP. Gen-Probe uses labelled DNA to detect ribosomal RNA in *M. tuberculosis*, while SNAP utilizes alkaline-phosphates-labeled probe.

IS6110 has been chosen as the DNA sequence for *M. tuberculosis* detection. A single probe on its own cannot provide sufficient information to establish the identification [88, 89]. With addition of molecular epidemiological algorithms, it may be possible to have a more accurate DNA probe. Siddiqi et al [89] pointed out that the IS6110 DNA probe could not be used to identify drug-resistant strains. The processing time required for each sample is within 1 to 2 hours with accuracy estimated to be above 90% [58].

Polymerase Chain Reaction (PCR)

PCR relies on the specific double helix binding of DNA strands [50, 83]. Each strand has its own specific binding that can be used as an identification method. The process starts with extraction of DNA from *M. tuberculosis* cells using French pressure cell, sonicator, mini-bead beater or zirconium beads [90]. All these methods are found to be expensive. It also involves elaborate, messy and time-consuming work. The method of Kumar et al. [90] made use of beach sand to overcome the tedious cell extraction.

After DNA extraction, chemical bonds between the two strands are broken or denatured to produce single-stranded molecules. After bond separations, short-

synthetic pieces of DNA - called primers, will bind with the targeted DNA strand. Once the binding process is completed, the whole sequence will be repeated to obtain large number of DNA copies. Conventional bond-breakage method is done using labour-intensive chemicals that use lysomzyme and silica particles. Afgani and Stutmant [91] conducted a simple boiling experiment to break DNA bonds. They showed that this boiling method has better sensitivity compared to the chemical method.

In order to identify *M. tuberculosis*, PCR amplifies 16S rRNA [88]. IS16110 is frequently used as the target sequence [92], although drug resistance is not linked to this pattern [12]. Other target genes include MBP64, rpob and hsp65 [58]. In order to perform PCR on tuberculosis patient samples, they are required to be cultured for 1 or 2 days.

Limitation of this method is that gram-positive, gram-negative bacteria and several species of *Mycobacterium* (e.g. *M. avium*, *M. intracellulare* and *M. kansasii*) have the same primers as *M. tuberculosis*, which might give false-positive results. Cross contaminations affect accuracy of the results [10].

PCR has a sensitivity range of 79-97.8% and specificity range of 83-99.6%. Reports have shown that PCR and culture method do not have much difference in terms of specificity and sensitivity [93, 94]. Kraus et al. improve the sensitivity of the PCR method by combining it with fluorogenic probes [95]. Fluorogenic probes show graph profiles that are unique for each bacterial species within a sample. However, the profiles are too similar to be used to reliably distinguish the *Mycobacterial* species. Thus, by combining the fluorogenic probe and PCR, the sensitivity is improved.

e. High Performance Liquid Chromatography (HPLC)

HPLC detects molecule bonds that exist in a sample. A peak at a particular wavelength indicates the investigated chemical bonding. HPLC utilizes a liquid mobile phase to separate certain components out of a mixture. The sample is

dissolved in a specific solvent, and it is then forced to flow into a column under high pressure. In this column, the mixture is resolved into its components. For *M. tuberculosis* detection, HPLC detects mycolic acid, which is a chemical compound found in all mycobacteria cell walls. After the column is filled with a specific chemical, the sample will react and cause ion exchange. Leopold and Fischer used an octyl-sepharose column to identify lipoglycans of *M. tuberculosis* [96].

HPLC tests can be performed in a few hours. However, the disadvantage is that the organism must be in pure culture and it cannot distinguish *M. tuberculosis* from *M. bovis* [83].

f. Tuberculostearic Acid

Frequency-pulsed electron-captured gas-liquid chromatography has been used to detect femtomole quantities of TBSA in patient specimens. This method allows tuberculosis meningitis to be diagnosed within 3 hours. This method gives a specificity of 91% and sensitivity of 95% [83].

Most frequently used methods, elaborated above, are listed in Table 2.5 and Table 2.6. Table 2.5 tabulates the develop methods (and the inventor) used to detect tuberculosis in chronological order. Table 2.6 compares the sensitivity, specificity, cost required for testing one sample and the minimum number of *M. tuberculosis* cells per millilitre of patient specimens. Cost of testing is calculated based on labour and material cost. Information for tabulation came from several resources. Also, each method has its own advantages and disadvantages as tabulated in Table 2.7.

Table 2.5. Chronologically developed tuberculosis detection methods

Name/ Organization	Name of Method	Year	Technique	Detection Time	Ref.
Robert Koch	Culture	1892	Culture	6-8 weeks	[97]
Seibert and Glen	Tuberculin skin test	1941	Serological test by agglutination observations	1-4 weeks	[73, 83]
Konno K	Culture	1956	Niacin extraction	3-4 weeks	[60]
Engvall and Perlmann	ELISA	1972	Serological test using enzyme- linked immunosorbent assay	1hr-14 days	[83]
Smithwick	Acid Fast Staining	1976	Acid fast staining the bacteria with phenol red	1-2 hr	[73, 98]
Middlebrook	Semi automated liquid culture	1977	Radiometric and fluorescence	1-4 weeks	[60]
Maxam and Gilbert	Nucleic Acid	1977	Discovered chemical sequencing of DNA	2 weeks	[99]
Kary Mullis	PCR	1985	Amplification of DNA	1hr -14 days	[83]

Table 2.6. Comparison between several detection methods

Method	Sensitivity (%)	Specificity (%)	Cost (S\$)	Min. no. (cells/ml)	Ref.
<i>Smear</i>	50	100	2-3	5000	[87, 100]
<i>Culture</i>	95-100	100	2-3	10	[56, 87]
<i>Semi-automated liquid culture</i>	84-97	64-93	50	4000	[58, 75]
<i>Molecular Methods</i>	60-84	97-100	85-170	10	[87]

Table 2.7. Advantages and disadvantages of *Mycobacterium tuberculosis* detection methods

Methods	Advantages	Disadvantages
<i>Culture methods and Smearing</i>	Specific and sensitive Relatively inexpensive Able to detect drug resistance	Long detection period Laborious
<i>Molecular methods</i>	Suitable for patients that cannot produce sputum samples. Fast result	Pure samples required Expensive Trained people to operate the equipment

2.5. TUBERCULOSIS DETECTION IN DEVELOPING COUNTRIES

Ninety five percent of the tuberculosis cases occur in developing countries. In general, tuberculosis can be treated quite easily; but because of ignorance, the disease is wide spread. The first ignorance case is due to lack of education, people are not aware of the lethal impact of this disease to the infected person [101-103]. People then become ignorant to this disease, therefore they do not seek medical treatment to cure the tuberculosis infection they have.

The second ignorance case is from health centres and doctors [104-108]. Because proper health centres are rare and located far from villages, infected people are not able to seek treatment. Travelling trips will take days and some of the people cannot afford to pay the travelling cost. In some cases, doctors that work in rural hospitals do not have any training in chest medicine; as a result, other pulmonary diseases (pneumoconiosis, lung cancer, bronchitis) are mistakenly diagnosed as tuberculosis. This leads to unnecessary treatment and waste of resources [101].

The most frequently used method for tuberculosis detection in developing countries is the acid-fast smearing method [54, 57, 104]. This is the preferred method because it gives a rapid and cheaper detection compared to other advanced detection methods. Molecular methods can give a rapid response, but these methods are seldom used in developing countries because of their inability to purchase or to maintain the equipment, and also because of lack of trained personnel to operate the system [57, 101]. Culturing method is not frequently done because of its long detection period, which takes 6 to 8 weeks. Within the culturing period, the patient does not obtain proper treatment that is required because of the unknown results.

CHAPTER 3

LITERATURE REVIEW (PART 2)

SPECTROSCOPY AND OPTICAL SENSORS

Optical methods are the forms in this investigation of detecting *Mycobacterium tuberculosis* metabolites. Optical methods have played a dominant role in various fields of analytical sciences. Colourimetric and photometry tests have been used qualitatively and quantitatively to determine chemical and biochemical substances. Analyzed samples are tested with chemical reagents, fluorescence or their own optical properties for optical identifications. This chapter describes spectroscopy background, fibre optics and optical sensors applications.

3.1. SPECTROSCOPY

Spectroscopy is a study related to light and its detection method. The classic theory describes light to be a combination of electric and magnetic waves, which spreads out or propagates from one place to another. In the quantum theory, light is described to consist of **photons**. Photons carry energy and momentum but they do not carry mass. The number of photons present represents the brightness of light; while colour of light is described as the amount of energy contained in each photon. Optical spectroscopy includes all analytical methods based on interactions of analytes (the substance being measured in an analytical procedure). Spectroscopy allows non-invasive, non-destructive, continuous and simultaneous monitoring of many analytes at once.

Hence, optical techniques offer the potential for rapid and selective measurements combined with the required specificity and sensitivity.

Colour

Visible light ranges from 380 to 740 nm. Light within this range can be seen and is interpreted by the eyes as colour. Colour ranges from red at the long wavelength (740 nm) through orange, yellow, green to violet (380 nm), the wavelength of each colour are tabulated in Table 3.1. Light with a wavelength below 380 nm is ultra-violet light and above 740 nm is infrared. Light of shorter wavelengths has higher energy.

Table 3.1. Visible light wavelengths

Wavelength (nm)	Colour
380-430	Violet
430-500	Blue
500-520	Cyan
520-565	Green
565-580	Yellow
580-625	Orange
625-740	Red

If light is shined through a sample, there are particular wavelengths being absorbed by the sample. The absorption depends on the optical properties and colour of the sample. For example, a yellow colour solution absorbs all visible colour wavelengths except the yellow wavelengths (565-580 nm). This is the reason why we see the sample as yellow colour.

Colour measurements by human eyes are subjective to the viewer. Therefore, **spectrometers** have been developed to provide standard colour indications. The spectrometer measures the intensity of a sample at various wavelengths. It consists of a prism that diffracts and separates incoming light based on the light wavelengths. The detector then scans and measures the intensity received from the

incoming light. **Spectral power distribution** is the plotted intensity versus wavelength.

Absorbance and Transmittance

As light passes through a sample, there are interactions between the photons and absorbing particles of the sample. Transmittance T of the sample is the ratio between the amount of light intensity detected I_1 and incident intensity I_0 (equation 3.1) [109].

$$T = \frac{I_1}{I_0} \quad \dots (3.1)$$

Absorbance, A , is the logarithm to base 10 of the inverse of transmittance [109].

$$A = \log\left(\frac{1}{T}\right) \quad \dots (3.2)$$

Absorption is caused by presence of absorption centres. These might be, for example, dye molecules, ions or small particles. When absorption centres are uniformly distributed throughout a bulk object (e.g. solid objects), the amount of light absorbed is given by [109]:

$$I_1 = I_0 e^{-\alpha x} \quad \dots (3.3)$$

where I_1 is the intensity after passing through a length of sample l , I_0 is the incident intensity, x is the thickness of the medium (m) and α is the linear absorption coefficient (m^{-1}). This expression is Lambert's Law [109]. The degree of absorption will vary significantly across different wavelengths and maximum absorption will occur at a particular wavelength λ_{max} .

If the absorption medium is a liquid solution, the amount of absorption is a function of the concentration of the absorbing centres throughout the sample. This is taken into account in the Beer-Lambert Law [109]:

$$I_1 = I_0 e^{-\alpha x c} \quad \dots (3.4)$$

The quantity c is called the **absorption coefficient** and has a unit of g/m^3 and therefore the unit of α has a unit of m^3/g .

3.2. FIBRE OPTIC

Fibre optic has cylindrical thin-hair structure. It is able to transmit light signals at high rates and with extremely low signal losses. Fibres are available in a bewildering variety of sizes and materials. Fibre optic functions as a “light pipe” because it guides light rays from a source to the designated detector.

Fibre optic consists of three sections, namely jacket, cladding and core (Figure 3.1a). The core has a higher refractive index than the cladding. Therefore, **total internal reflection** occurs in the fibre, allowing light to travel from one end of the fibre to the other. The total reflection phenomenon is based on **Snell’s Law** (Figure 3.1b). Snell’s Law states that as light travels from a higher refractive index medium to a lower one with an incident angle greater than the critical angle, it will be reflected at the boundary of the two mediums [110].

Figure 3.1. Schematic diagrams of (a) Fibre structure (b) Internal reflection phenomenon [111]

Advantages of using fibre optics are that they are light, very small in size, flexible, immune to electromagnetic interference. Fibre optics eliminates chances of sparking and they have good compatibility with modern solid-state devices [110, 112].

Fibre optic can be categorized based on its mode and material. Fibre type selection is crucial in obtaining optimum results.

Modes

Fibres can be divided into three different categories based on their mode, namely multimode step-index, multimode graded-index and single mode step-index fibres. Their construction, refractive index profiles and geometries are shown in Figure 3.2.

FIBER TYPE	MULTIMODE STEP-INDEX FIBER	MULTIMODE GRADED-INDEX FIBER	SINGLE MODE STEP-INDEX FIBER
PROPAGATION MECHANISM			
GEOMETRY			
REFRACTIVE INDEX MODE			

Figure 3.2. Fibre optic modes [111]

Multimode step-index fibre has a thin cladding layer and a large diameter core. This fibre is normally used in bundles, where light enters from an end and exits from the other. Light falling in the core will be transmitted, but light falling in the cladding will be lost. Thus, it has a thin cladding to increase transmission efficiency [113].

With large core diameter, many light modes are able to pass through. However, this fibre suffers from intermodal dispersion (each mode travels in different velocities),

causing modes to reach fibre-end at different timing. Because of this situation, the light combines to produce a wider pulse than the one originally launched.

Multimode graded-index fibre is designed so that short-path pulses travel with the same velocity as the long-path pulses. This was obtained by modifying the fibre's core profile. Refractive index of the core changes gradually with its radius, causing light to bend gradually. Rays travelling in long paths are accelerated and those travelling in short paths are slowed down [111].

Single-mode step-index fibre has a very small core diameter. Single-mode fibre is best used with laser light source because it only transmits one mode, so that there is no dispersion at the output. However, its small core diameter makes coupling light into the core more difficult. Tolerances for single mode connectors and splices are also more demanding compared to multimode fibres.

Material

All-plastic fibre is made from polymethyl methacrylate core and fluoropolymer cladding. This fibre has large core diameters (from 100-1000 μm) and large numerical apertures ($\text{NA}=0.5$), meaning that the maximum angle that a light ray outside the fibre can have and still be captured and guided by the fibre is large. Since light that passes through this fibre has extremely high optical losses, all-plastic fibre is best used for transmission in over short distances.

Plastic-clad fibre has glass (silica) core with plastic cladding (silicone or Teflon plastic). Although it has the same diameter with the all-plastic fibre, this type has lower NA of 0.3 to 0.4; and therefore couples less light into its core.

All-glass fibre is all made from glass. It has the lowest optical losses but possesses good transmittance for visible and near infrared light. This fibre is frequently used in image transfer analysis because it produces clear images [113]. They are also able to withstand harsh conditions.

Evanescent Wave

Internal reflection in an optical fibre occurs at the core-cladding interface. Light is not reflected instantaneously; rather, it penetrates to some extent into the fibre cladding. This phenomenon is referred to as the **evanescent wave** [114]. This phenomenon is illustrated in Figure 3.3. Evanescent wave has a maximum electromagnetic energy distribution at the core-cladding interface. The energy in the cladding falls off exponentially as the distance from the fibre optic core increases [114].

Figure 3.3. Evanescent wave principle [114]

As shown in Figure 3.3, the standing wave decreases exponentially from higher refractive index medium (n_1) to lower one (n_2). This decay is given by the equation [114]:

$$E_T = E_2 e^{\alpha(-z)} e^{\beta(y)} \quad \dots (3.6)$$

E_T is the electric field portion of a non-uniform wave propagating in the $+y$ direction. The evanescent wave component is in the $-z$ direction. E_2 is the attenuated electric field in n_2 . The attenuation constant α determines the spatial rate of decay of the field in n_2 . The phase propagation constant is β .

Some fibre optic sensors utilize this evanescent wave principle as their detection method. In some sensors, the fibre cladding is stripped off and replaced by a layer of reagents promoting interaction between the reagents and the evanescent wave [115-120].

The evanescent wave is also used in fluorescence excitation. When a fluorescence molecule comes in contact with the sample, the fluorescence molecule excites. This excitation will then be captured by the fibre and transmitted to the detector.

3.3. LIGHT SOURCES

There are several kinds of light sources available. Each type is used in different applications, depending on what the optical system requires. Several light sources are described as follows:

a. Lasers

Laser light is generated in an optical cavity (**optical resonator**) composed of one total and one partially reflecting mirrored surface. The design of the optical resonator controls the laser's output characteristics. Most laser resonators are designed to operate in the lowest order transverse electromagnetic mode. This mode generates a circular beam with an intensity profile that decreases radically from a central maximum. A laser gives a specific spectral band output, normally of single wavelength [121]. The most important characteristic of laser light is that it is coherent and very nearly monochromatic. It is also possible to obtain light pulses in a short duration (e.g. femto second pulses) [122]. Lasers are normally used for sensor applications that require high output power [123].

b. Lamps

Lamp sources are distinguished by their relatively broad spectral emission and incoherent output. They are generally less expensive and often require less utilities support for routine applications. There are two types of lamps, those are

incandescent (or filament) and **gas discharge** lamps. Although incandescent and gas discharge lamps are quite different in design, they share one common feature that is they are all extended or finite light sources [121, 124].

Incandescent lamp consists of a current carrying filament. This filament is enclosed in a glass or quartz envelope. Electric current is directed, through filament, to the incandescence and light is generated. The operating characteristics of the lamp are primarily determined by the length, diameter and surface area of the filament.

One example of the incandescent lamp is the tungsten-halogen lamp. It gives a spectrum ranging from 380 to 2000 nm (visible to near-infrared region). Halogen gas, e.g. iodine, is added to the bulb atmosphere. Iodine is free to combine with the tungsten that has been deposited on the bulb surface. The reaction will form a volatile tungsten-iodine compound. This light source is very stable, provided it is operated at a high temperature to activate the tungsten-halogen clean-up cycle.

Gas discharge lamp consists of two electrodes (anode and cathode) and a gas of neutral atoms that are contained in an envelope of suitable material (glass, quartz or synthetic quartz). Free electrons in the gas are accelerated to high velocity by a potential difference across the electrodes. These free electrons collide with the gas atoms. The bounded electrons are elevated to excited state. Examples of gas discharge lamps are photographic electronic flashes, neon signs and continuous arc lamps.

Lamps are frequently used when a broad range of wavelengths are observed. For example wavelength changes that occurs in the visible region a particular chemical is added to a sample [116, 125-128]. Disadvantages of lamps are they take a long time to stabilize and often become very hot.

c. Light Emitting Diode (LED)

LED generates photons when electron-hole pairs recombine inside a pn-junction. In LED, photons are generated via spontaneous emission process. LED is fabricated from a wide variety of materials that are commercially available and they operate at wavelengths ranging from 480 nm to the near infrared region [129]. Because of its working principle, LED is simpler and cheaper, but more rugged and has lower tolerance requirement. Due to its small size and low power consumption, LED is frequently used for development of portable sensors because of its small size, small input power required, good stability and low cost [130-134].

3.4. SENSOR CONFIGURATIONS

Fibre optic instrumentation can be categorized into two types. The first type is where the sample directly affects the light-guiding properties of the waveguide (known as **intrinsic fibre optic sensor**). The second type is where the fibre is used to guide the light from source to sample. The light interaction occurs outside the fibre optic (coined as **extrinsic fibre optic sensor**).

3.4.1. INTRINSIC FIBER OPTIC SENSOR

In sensors of this type, sample will affect the waveguide property inside the fibre. The sensed samples can cause an increase in strain of the cladding, change of refractive index (by light absorption or swelling) [111].

Sometimes the sample of interest has its own specific optical property that can be detected by the fibre. However, in chemical and biochemical application, not many targeted samples have specific optical properties that can be distinguished from other samples. Hence, addition of chemical reagents is used.

In intrinsic fibre optic sensors, chemical reagents are placed as a part of the fibre optic. One method of immobilizing the reagents is by using **sol gel**. Evanescent

wave principles are applied in this sol gel configuration. The fibre optic cladding is removed and replaced by a layer with similar properties. Chemical reagents are added into this new layer (sol gel) [135]. The sol gel is coated onto a decladded portion of a fibre optic cable (Figure 3.4). To allow sample detection, the sensing reagents are placed into this new sol gel layer.

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Figure 3.4. Sol gel immobilized onto a fibre

The sol gel has a glass-like porous structure made out of rigid silica network. Varying the sol composition, gel treatment and the gelation condition can easily change its properties. The porous structure of the sol gel allows the sample to penetrate and interact with the reagent inside the sol gel. Tetra-ethyl-ortho-silicate (TEOS) is one type of silica that is commonly used for the sol gel fabrication. TEOS is mixed with ethanol and water to form the porous matrix [136].

When TEOS is introduced into water, it will react in different ways depending on the hydrolysis conditions. Their chemical reactions are as follows [135]:

The pH level of the mixture can enhance the hydrolysis reaction. In fact, the final form of the hydrolyzed silica is very dependent on the pH of the solution. Solidification reaction is accompanied by production of alcohol and water.

At low pH levels (acidic), silica tends to form linear molecules that are occasionally porous. Oxygen atoms in silica on the alkaline group will attract H^+ protons. Linear chains are formed, causing the sol gel to have a low density [118].

In high pH levels (alkaline), the amount of water plays an important role in the sol gel fabrication. Extensive hydrolysis and polymerization will occur in high pH condition. Cross-link chains depolymerised rapidly. Small segments dissolve and redeposit onto larger chains. Hence, smaller molecules decrease in number but assist the larger ones to grow. This process is called **Oswald ripening** [135]. The effects of acid and base catalyst in the formation of the sol gel are shown in Figure 3.5.

Figure 3.5. Sol gel matrix in acid and base catalyst [135]

Sol gel technique is frequently used because of its ability to encapsulate molecules or chemical reagents in its porous silicate matrix. Sol gel can also be easily fabricated because the reaction takes place in room temperature [137].

3.4.2. EXTRINSIC FIBRE OPTIC SENSOR

In extrinsic fibre sensors, light leaves the fibre and interacts with the sample. Light then returns to the same or second fibre that is connected to a measuring device [138]. There are a few methods to immobilise reagents onto fibres. The most common method is to place the reagents onto the tip of the fibre (presented in Figure 3.6).

Figure 3.6. Immobilized reagents on an extrinsic fibre optic configuration [111]

A unique fibre configuration, which cannot be classified into any of the two explained fibre classifications, was described by Smela and Aviles [139]. They invented a hybrid between the intrinsic and extrinsic fibre configurations. Two fibres were used for their sensor configuration. Light enters the first fibre, passes through the sample, and then captured by the second fibre (Figure 3.7). The sample medium influences the interaction in several ways: (1) change in refractive index, (2) absorption through the sample, (3) scattering by the suspended particles, or (4) physical interaction with the fibre.

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Figure 3.7. Twisted fibres configuration [139]

3.5. APPLICATION OF OPTICAL SENSORS

The beneficial features of optical sensors have led it to wide variety of applications, e.g. physics analysis, food industries, medical, chemical fields, etc. Since the aim of this project is to develop a novel sensor to detect microbial growth, this section focuses on two main optical applications: **biosensors** and **chemical sensors**. Optical applications in both fields are attractive because optical methods can detect minute changes that occur in an analyzed sample.

3.5.1. BIOSENSORS

Optical sensors are ideal systems for monitoring and controlling bioprocesses. Optical systems do not interfere with metabolism of cells, permitting non-invasive monitoring and offering intracellular information that is nearly impossible to obtain with other methods [140]. Despite all the advantages, optical measurements are not yet routinely used in biotechnological industry because of difficulties that arise from the number of complex interactions taking place inside the cells and in the surrounding medium. For instance, turbidity (which can change drastically during the process) and bubbles that occur in stirred and aerated culture broth often

interfere with the optical measurements. Multiphase systems with medium, cells, bubbles and other liquid or even solid phases cause difficulties because optical measurement cannot define the distribution of a component in these different phases.

Fibre Optic Biosensors (FOBS)

Biosensors are analytical devices that use biological molecules to detect other biological molecules or chemical substances and provide either qualitative or quantitative results. Typically, the detector molecule must be interfaced to a signal transducer, which may be optical, electrochemical, thermometric, piezoelectric or magnetic. In this report, the explanation will focus more on optical biosensors.

In fibre optic biosensors, the biological molecules are interfaced to the fibre optic. The fibre optic detects/measures optical changes that are caused by the biological response and transfers this information to a transducer that will convert these optical signals to electronic signals that would then be interpreted. The output signal is proportional to sample's concentrations to which the biological elements react [141]. Based on the biological elements used for sample identifications, FOBS can be categories into: (1) bio-affinity recognition and (2) bio-metabolic recognition.

a. Bio-affinity recognition

Bio-affinity recognition involves binding of two different biological species. This biological species have complementary structures and specific bindings. Nucleic acid and hormone receptors are used as the recognition material, but most of the bio-affinity sensors use antibody-antigen bindings as their detection methods. Antibodies are products of the cell immune system whenever they are exposed to antigenic substances or molecules. These bindings have specific key-lock configurations [142].

Commonly, bio-affinity sensors available are immunoassay sensors. These sensors are irreversible. Furthermore, disadvantages of this method are: (i) there

is a necessity in obtaining highly efficient fluorescent labels [143] (ii) the use of a specific laser and (iii) the system is bulky.

For the above reasons, the interest has been shifted to fibre optics [144]. The sensitivity and accuracy of the fibre optic methods do not differ much from conventional optical methods [143].

b. Bio-metabolic recognition

Bio-metabolic recognition utilizes binding of analyte with any other co-reactants, such as enzyme-substrate reactions with the metabolism product of specific molecules by organelles, tissue and cells [145].

Applications of FOBS in detecting presences of bacteria are frequently used in food industries. A rapid, small size and accurate sensor is required to detect bacteria and at the same time does not affect the food quality. *Salmonella* is one type of bacteria that causes food poisoning. Most FOBS is developed using a sandwich-binding format, in which a primary antibody is immobilized on the surface of the fibre optic and a secondary or tracer antibody is added into the bulk solution. If antigen is not present, the tracer antibody will remain unattached (moving freely in the solution).

Upon addition of antigen (e.g. bacteria cells), the tracer and antigen will bind to the primary antibody. When this binding occurs, fluorescence is excited; and then detected by evanescent wave in the fibre optic cable. The fluorescence intensity increases with increasing analyte concentration. Letcher et al [146] showed that this fibre optic configuration was able to detect the presence of *Salmonella* with a concentration as low as 10^4 colony forming units (CFU)/ml.

An enhanced fluorescent fibre optic serological biosensor system using an ultrasonic trapping for detecting *Salmonella typhimurium* has been reported by Zhou et al [147]. Conventional serological biosensor systems have bacteria cells scattered all around in the solution sample, preventing most of them from binding with antibody [142, 143, 148]. By using ultrasonic wave, this problem was

solved. The ultrasonic wave was able to trap particles and cells so that the system has a controlled cell distribution. An acoustic chamber, with a fibre probe along its axis, was designed to allow target particles to form parallel layers perpendicular to the fibre. The cells were concentrated along the fibre, giving a better sensitivity. This setup was reported to be able to detect a concentration that is 400 lower than the usual amount that is able to be detected by a normal immunoassay fibre optic sensor [147].

Another immunoassay fibre optic sensor application was to detect *Escherichia coli* (*E. coli*). It used cyanine 5 dye-labelled polyclonal anti *E. coli* O157:H7 antibodies [149, 150] to generate a specific fluorescent signal. De Marco et al. [149] attached the antibodies to a 600 μm fibre optic core by applying the Biotin-avidin interaction. They used a 635 nm LED as the light source. As antigen-antibody binding occurred, the detector antibody gave a fluorescent luminescence peak. Evanescent field excited these fluorescent molecules, and this excitation was then reflected back into the fibre. A photodiode at the other end of the fibre quantified the light intensity, which ranged in between 670 and 710 nm wavelengths. This biosensor was able to detect 3 to 30 CFU/ml of *E. coli* in seeded ground beef samples in only 20 minutes, instead of 24 hours using current culturing method. When combined with PCR, this system was able to detect lower concentration of *E. coli* O157:H7 in 10 hours [150].

Staphylococcus aureus (*S. aureus*) is another common pathogenic bacteria. It causes human infections and intoxication. When living in a nutrient media, *S. aureus* produces a specific toxic called Staphylococcal enterotoxin B (SEB). Therefore, SEB is used as a sensing target in fibre optic biosensors. The decladded region of the fibre optic cable is coated with rabbit anti-SEB antibodies. A sensor developed by Tempelman et al. [151] used a 600 μm silica core fibre. The fibre had a sensing area of 12.5 cm; it was tapered from 600 to 400 μm in the first centimetre and then gradually tapered to 230 μm in the remaining 11.5 cm. This region was coated with anti-SEB antibodies. The results were available in 45 minutes.

A portable *S. aureus* fibre optic sensor has been developed by King et al. [152]. A self-modified polystyrene fibre optic was used in the setup. Polystyrene was chosen as the fibre material because it had the ability to bind with protein. The fibre was coated with anti-SEB antibodies. At the distal end of the fibre, a lens reflector was attached to give a higher sensitivity for the photodiode. The probe was mounted inside an assay cartridge. Samples were then inserted into the “Man–Portable Analyte Identification System” (MANTIS).

FOBS have also been applied in detecting protein A, which is uniquely secreted by *S. aureus*. The detection principle involves a sandwich immunoassay of fluorescein isothiocyanate and anti-(protein A) immunoglobulin G. The concentration limit was one nanogram of protein A per millilitre of sample [153], resulting in 24 hours detection time.

Non-Fibre Biosensors

Most frequently used and available method is a sensor to measure cell growth and biomass concentration based on optical density (OD) of the sample. Its detection is based on turbidity changes in visible or near infrared wavelengths.

This method has problems because of different cell concentrations, types and morphologies. In addition, its reducibility and durability are not always satisfactory. A further disadvantage of these devices is that they cannot distinguish between viable cells and dead cells or non-cellular particles inside the medium. High turbid media can cause problems and the turbidity can change drastically during cultivation. Scattering and absorption is non-linearly dependent on cell density, which makes it difficult to find the correct regression and calibration procedures. However, these sensors are the most universally applicable devices for online biomass monitoring [154, 155].

Infrared (IR) is also used for bacteria identifications [156-159]. Bacteria are smeared onto an IR sensor. The infrared absorbance by the bacteria would show the bacteria cells' chemical composition. However, the main limitation of IR

spectroscopy is that the molecular chemical composition is similar for each bacterium. Hence, false-positive results are frequently obtained.

3.5.2. CHEMICAL SENSORS

A chemical sensor is a device used to measure concentration or chemical activity in a sample of interest. Ideally, this device should be able to operate in a continuous and reversible manner. An ideal chemical sensor should be able to provide real time information on the spatial and temporal distribution of a particular molecular or ionic species. Fibre optics is inexpensive and is able to provide good results. This has led to a new subclass category in chemical sensor called the fibre optic chemical sensors (FOCS).

In FOCS designs, fibre optics are used to transmit electromagnetic radiation to and from the sensing region that is in direct contact with the sample. Concurrently, a chemical recognition phase is used to generate an analyte-dependent spectroscopic detectable signal within the sensing region of the fibre optic. Chemical changes may occur because of interaction between analyte and immobilized indicator. This changes are measured optically by analyzing the electromagnetic radiation that returns from the sensing region [160].

Most commercial FOCS available in the market nowadays are the pH sensors. They are widely used in chemical analysis and in medical applications. The selection of pH indicators depends on the application of the fibre sensor. Based on the location, there are two immobilizing methods, those are reagents are coated onto a specific surface [131, 134, 161] or directly onto a fibre optic [118, 133, 162].

The sensitivity and the pH range of each sensor depend on the composition of the reagent and the membrane used for the immobilization. To have a larger pH range, more than one pH indicator are used [127, 162, 163]. By immobilizing three different pH indicators, a wide-range pH sensor (pH 3 to 10) could be developed. These indicators were phenol red, bromine phenol blue or bromothymol blue [164]. They were enveloped with a hydrogen-ion-permeable membrane onto the fibre.

Gupta and Sharma [162] developed another wide-range pH sensor, ranging from pH 4.5 to 13. Instead of immobilizing the reagents with a permeable membrane, they used sol gel.

The application of pH fibre optic sensors has extended to detect the CO₂ level in a sample. This CO₂ fibre optic sensor has the same sensing element as the pH sensors; only these fibres are added with a selective membrane. This membrane allows only CO₂ to enter the sensing region. There are different kinds of membranes that have been used for the sensor fabrication, e.g. hydrophobic polymer membranes [165], silicon rubber membranes [166] and ethyl cellulose coated with silastic [167]. Other fibre optic sensors are hydrogen peroxide [115], ammonia [168], oxygen [136], and nitrogen dioxide sensors [116]. An optical chemical sensor for nitrate detection was developed by Lumpp et al. [169]. The sensor was composed of poly-vinylchloride membrane containing a nitrate-selective ionophore and a protein selective chromoionophore.

Kishen et al [160] and John et al [125] applied the FOCS principles in FOBS applications. They developed a sensor to monitor growth of *Streptococcus* mutants. The principle of the sensor was to monitor the difference of acidic level in the bacteria culture. *Streptococcus* converts sucrose into glucan and fructan polymers. This process involves formation of lactic acid, which alters the acidity level of the saliva sample. Acidity changes in the investigated sample were detected using a sol gel coated fibre optic. Sol gel was used to coat the pH indicator onto the fibre. Reaction between environment acidity produced by the bacteria and pH dye in the sol gel resulted in spectrum changes. Selectivity of this sensor was dependent on the selectivity of the culture medium being used to grow the bacteria [170].

Another bacterial sensor that is able to detect the presence of bacteria was based on monitoring the CO₂ levels. Bacteria metabolism activity would result in an increased of CO₂ concentrations, which affected emulsion of an aqueous colorimetric pH indicator [126, 170]. Acid level changes affect the absorption intensity of a pH indicator-dye embedded to the fibre optic.

There is also a fibre optic based on evanescent wave to detect *E. coli* [171]. The sensing mechanism of the sensor relied upon the attenuation of a guided light that took place inside the fibre. In the experiment, a fibre was etched leaving 0.5 to 1 μm of cladding over the fibre's core. This decladded region was then placed in a selective culture medium that only allows *E. coli* to grow. As bacteria grew in the fibre surroundings, light intensity changed.

The sensor response relied on the interaction between the light wave and the bacteria, causing optical attenuation at the probe fibre. The optic interaction arose from the evanescent-field coupling that has physical contact with the bacteria. The detection is 5-10 times faster than conventional bacteriological techniques [171].

CHAPTER 4

METHODOLOGY

There are various metabolites excreted by *Mycobacterium tuberculosis* (*M. tuberculosis*) during their growth and multiplication as previously elaborated in Chapter 2. From the described metabolic products, detection of nicotinic acid (niacin) is used for the sensor development. This is because niacin has a specificity of 95% compared to other metabolites produced by *M. tuberculosis*. Detection of niacin also enables differentiation of *M. tuberculosis* from other types of mycobacteria. The only other mycobacteria species that has positive niacin test is *Mycobacterium simiae*, but it has lower specificity of 63%. However, this mycobacteria type is not associated with pulmonary diseases. A table that indicates the sensitivity/specificity for the metabolite detection is attached in Appendix A [50].

Niacin can be detected by colourimetric methods. Cyanogen bromide is a chemical substance that breaks up niacin's pyridine ring bond. Addition of aniline to this new compound will develop a yellow reaction product. The solution's yellowness intensity depends on the quantity of niacin present. The principle of the chemical reaction is explained by the **Koenig reaction** [65] and this colourimetric method is still commonly used. Colourimetric method applications have been expanded not only to detect niacin, but also to detect the presence of *M. tuberculosis*. Current colourimetric identification to detect niacin in *M. tuberculosis*' culture is conducted only after 3 to 4 weeks of incubation, with a minimum number of 50 bacteria colonies. This is to ensure that sufficient amount of niacin is produced by *M. tuberculosis*, resulting in visible colour changes.

Optical sensors are sensitive to detect minute optical changes that occur in a sample. It was expected that at very low niacin concentrations, these colour changes could be detected by the optical sensor. Hence, optical methods were then applied in order to detect low concentrations of niacin in the early *M. tuberculosis* incubation period, leading to a rapid means to detect the presence of cultured *M. tuberculosis* patient samples.

This chapter is divided into three main sections, as illustrated in the following diagram (Figure 4.1). The first two sections elaborate the development process of the sensor, which involves initial trials of comparing current niacin detection methods and comparing four different optical setups. The four different optical setups were tested to investigate the most sensitive setup to detect small niacin concentrations. The best niacin detection method and optical setup were combined and used as the basic principle for the development of the new sensor. The last section elaborates three different culture media used to grow suspected tuberculosis patient's samples. Since each media has different characteristics, the best media that facilitates *M. tuberculosis* growth and its niacin production was selected. The developed sensor and media were then tested to investigate the sensor's sensitivity and specificity.

Figure 4.1. Flow chart diagram of the experiments conducted

4.1. DEVELOPMENT OF SENSOR

Mycobacterium tuberculosis is a pathogenic bacterium; therefore its metabolite production was simulated to avoid frequent contact. Niacin solution was prepared and used as a substitute. It was obtained by diluting niacin control discs (REMEL Co, Singapore) or niacin powder (Sigma Co, Singapore) in distilled water. Both forms are commercially available. In order to detect niacin concentrations with the aid of optical methods, addition of chemical reagents were required. The reagents were available in forms of strips or in chemical solutions. Optical properties of niacin solution change with addition of these specific chemical reagents and these changes were detected by the optical configurations setups.

The first section compares the sensitivity and consistency of two current niacin identification methods. The best method was then applied in further tests that were conducted in order to determine the best optical setup principle for the sensor development, which is presented in the second section.

4.1.1. INITIAL TRIAL: COMPARISON OF NIACIN DETECTION

METHODS

There are two ways of applying the colourimetric method to detect niacin's presence in *M. tuberculosis* cultures; those are using: chemical reagent strips and chemical reagent solutions. The sensitivity and the repeatability of both methods were visually analyzed and compared based on the colour changes that occurred in the investigated samples.

Chemical Reagent Strips

Niacin reagent strips (REMEL Co, Singapore) are strips that are incorporated with specific chemical reagents for detecting presence of niacin in a sample. By utilizing these strips, exposures to hazardous and toxic reagents are minimized. Table 4.1 tabulates chemical composition of each chemical reagent strip [172].

Niacin solution was obtained by diluting niacin disks into distilled water. Each niacin disk contains 5 µg of niacin. Following the instruction's manual, each disk was diluted into 0.6 ml of water. This is equal to a standard concentration of 8.33 µg/ml, which is equivalent to the amount of niacin that should be present in the investigated *M. tuberculosis* culture sample [172]. Since the amount of distilled water used to extract niacin from the *M. tuberculosis* culture is 1 ml [60], the amount of niacin produced by a 3 to 4 weeks *M. tuberculosis* culture is 8.33 µg.

Table 4.1 Chemical concentration of niacin reagent strips [172]

CHEMICALS	CONCENTRATION
Potassium Thiocyanate	60 g
Chloramine T	25 g
Citric Acid dehydrate	8 g
Para-aminosalicylic acid	10 g
Ethyl alcohol	100 ml
Deionised Water	150 ml

Niacin control disc was inserted to a 15 ml test tube filled with distilled water. Different niacin concentrations were obtained by varying the amount of distilled water to dilute the niacin disk. Variations in the concentration are shown in Table 4.2. The high concentrations have an increment of half niacin disc (equivalent to 0.25 mg of niacin) diluted in 0.6 ml of water. Five different concentrations with an interval of 1.25 µg/ml were chosen for the low concentrations.

Table 4.2. Niacin concentration variations in initial trials

	Niacin Variation (µg/ml)
High concentration	10.84, 12.5, 16.67, 25
Standard	8.33
Low concentration	0, 1.25, 2.5, 3.75, 5, 6.25

The niacin disc was left for 30 minutes with occasional agitation to ensure niacin extraction from the disc. The water volume in the test tube was maintained to be 0.6 ml, and any excess was removed using a pipette. Reagent strip was then inserted into the test tube. It was sealed immediately to avoid excessive exposure to oxygen, which might interfere the reaction. After 15 minutes, the niacin disc and reagent strip were removed from the test tube and colour changes were visually observed.

Chemical Solutions

All chemical reagents were purchased from Sigma-Aldrich Co, Singapore. SAHealthInfo [43] indicates that cyanogen bromide 10% and aniline 4% solutions are sufficient to detect niacin in *M. tuberculosis* cultures. A glass beaker that contained 50 ml of distilled water was added with 10 mg of cyanogen bromide crystals. The beaker was then covered with aluminium foil and kept in a fume cupboard at room temperature. It approximately took 36 hours until all crystals were dissolved. Afterwards, the solution was transferred to an amber bottle and kept refrigerated at 4 °C.

The second reagent required for this experiment was aniline 4%. It was prepared by adding 4 ml of aniline into 96 ml of ethanol 95%. The mixed solution was kept in an amber bottle and refrigerated at 4 °C. Before these chemicals were used for experiments, they were allowed to come to room temperature.

In this experiment, 2.5 mg of niacin powder was added into 100 ml of distilled water. Effects of different niacin concentration levels to the samples' colour changes were visually observed. Different niacin concentrations were obtained by diluting initial niacin solution (25 µg/ml) to the desired concentrations, as presented in Table 4.2.

Consistency and sensitivity of both methods were compared. The best method was selected and used for the subsequent experiments those are elaborated in the next section.

4.1.2. COMPARISON OF EXISTING OPTICAL SETUPS

There are many optical sensor configurations, but the most common ones were selected. The tested optical configurations are as follows: (1) optical probe setup, (2) extrinsic optical setup, (3) single decladded fibre optic setup, and (4) double decladded fibre optic setup. All experiment setups required a light source, a spectrometer, a processor and a sample holder. Spectrum changes in the visible light wavelength region occurred when the chemical reagents reacted with niacin solutions. To comply with this situation, tungsten halogen lamp (LS1, Ocean Optics Co, Singapore) was chosen because it has the capability to supply light in the visible (VIS) to near infrared (NIR) wavelength region, i.e. 360-2000 nm. Spectrum changes were detected using a spectrometer (USB2000, Ocean Optics Co, Singapore). Its detection range is in the region 350-850 nm with a resolution of 1.5 nm. Specifications of the light source and spectrometer are attached in Appendix B. The spectrometer was linked to a processor and the OOIBase32 (Ocean Optics Co, Singapore) software program was used for the spectroscopic analysis.

Existence of niacin in a solution was sensed spectroscopically with specific chemical reagents. The chemical reagents have two forms: incorporated in strips or in liquid solutions. The most sensitive and consistent method was selected based on the experiment results elaborated in Section 4.1.1. The following tests were conducted to analyse optical changes associated with different niacin concentrations, after reacting with relevant chemical reagents.

When the sample is illuminated with a range of wavelengths, particular wavelengths are absorbed. This amount of light intensity and wavelength detected by the spectrometer is referred as **transmittance**.

In each experiment setup, two reference data were recorded before the sample readings were taken. Distilled water was used as the first reference (*R*) and a dark room (*D*) reading as the second reference. A dark room reading is necessary to be recorded because in any optical instrument, even in dark room conditions,

interference maybe present. Interference can be caused by stray light (from the surrounding) or because of current leakage through the device (known as dark current).

Transmittance (T) is the ratio between intensity measured after passing the sample (I_1) and initial intensity (I_0), which is shown as the following [109]:

$$T = \frac{I_1}{I_0} \quad \dots(4.1)$$

Spectrum of niacin sample (S) minus the dark room spectrum (D) is equivalent to I_1 , and the initial intensity (I_0) is the reference spectrum (R) subtracted by dark room spectrum (D). Hence equation 4.1 can be represented as [109]:

$$T = \frac{S - D}{R - D} \quad \dots(4.2)$$

4.1.2.1. Setup 1: Optical Probe

The first experiment was conducted using a reflection/backscattering probe (R400-7, Ocean Optics Co, Singapore). The probe is able to measure reflection and fluorescence from solid surfaces, or backscattering and fluorescence in liquid and powders. As shown in Figure 4.2, one end of the probe is connected to the spectrometer, while the other to the light source. A volume of 0.5 ml of niacin solution was transferred into the test tube. Equal amounts of cyanogen bromide and aniline solution were added afterwards. The probe was then inserted into the test tube and adjusted to be directly in contact with the investigated sample. The test tube was covered with a black box so no ambient light would interfere with the readings. Different niacin concentrations were tested with variations shown in Table 4.3. The data were recorded by the processor and then analysed.

Figure 4.2. Experiment setup 1: Optical Probe

Table 4.3. Niacin concentration variations in experiment setup 1

	Niacin concentration ($\mu\text{g/ml}$)
High Concentration	12.5-25 with an increment steps of 4.17
Standard Concentration	8.33
Low Concentration	0-6.25 with an increment steps of 1.25

4.1.2.2. Setup 2: Extrinsic Optical Sensor

The working principle of this setup is that the sensor is not in contact with the sample and thus named **extrinsic optical sensor**. As shown in Figure 4.3, two fibres optics were placed in series. Instead of using a test tube, a cuvette was used to contain the sample (Ocean Optics Co, Singapore). A volume of 0.5 ml of niacin solution was transferred into the cuvette and the chemical reagents were added right after. Variations of niacin concentrations are shown in Table 4.4. This cuvette was then placed in the sample holder. One side of the sample holder was connected to a tungsten halogen lamp and the other to a detector (spectrometer) by fibre optic cables. These connecting fibres had a core size of 400 μm in diameter.

Figure 4.3. Experiment setup 2: Extrinsic Optical Sensor

Table 4.4. Niacin concentration variations in experiment setup 2

	Niacin concentration ($\mu\text{g/ml}$)
High Concentration	12.5-25 with an increment steps of 4.17
Standard Concentration	8.33
Low Concentration	0-6.25 with an increment steps of 1.25

Once the reference spectra were recorded, series of experiments were conducted. The spectrometer was used to detect any spectrum changes that occurred in the sample. These changes were present because of the different intensities and

wavelengths absorbed by different niacin levels. A processor was linked to the spectrometer, and the OOIBase32 (Ocean Optics Co, Singapore) software program was used for the spectroscopic analysis. The spectral data of the samples were recorded and analysed.

4.1.2.3. Setup 3: Single Decladded Fibre Optic Sensor

This experiment setup was based on evanescent wave principles. Samples were tested to observe the occurrence of total internal reflection incident in the fibre. Based on literature review explained in Chapter 3, a multimode silica fibre optic (Thorlabs Co, Singapore), with a core diameter of 550 μm , was selected for the sensor fabrication (Appendix B). This fibre type was chosen so that more light energy can be transferred from the light source to the sample.

Initially, the protective jacket of the fibre was detached mechanically with a fibre stripper. As conducted by John et al. [125], a 4 cm length of fibre cladding was etched by immersion in hydrofluoric acid 35% (Sino Chemical) for 15 minutes so that the core of the fibre is exposed. The final fibre diameter was around 530 μm . After etching, the fibre was cleaned with distilled water and followed by acetone.

Figure 4.4 presents the experimental configuration. One end of the etched fibre was connected to a tungsten halogen light source, while the other to a spectrometer. The decladded region of the fibre was placed in a sample holder so that it was in direct contact with the sample. A computer was connected to the spectroscopy detector so that readings could be recorded and analysed.

Figure 4.4. Experiment setup 3: Single Decladded Fibre Optic Sensor

Five different concentrations (0, 5, 10, 15 and 25 $\mu\text{g/ml}$) were tested. Using a syringe, niacin solutions were injected into the sample holder. The chemical reagents were added to react with niacin that was present in the sample holder. The mixed solution was then covered with a black box to minimize external interference. The decladded fibre region detected the spectrum changes that occurred in the sample. Spectrum data were recorded and analysed thereafter.

4.1.2.4. Setup 4: Double Decladded Fibre Optic Sensor

This experimental setup was similar to the single decladded fibre optic sensor configuration. The only difference was two fibres were used instead of only one. Both decladded fibre regions were attached parallel to each other. One fibre was connected to a tungsten halogen light source and another to a spectrometer (Figure 4.5).

A spectrometer was connected to a personal computer to allow data acquisition. The working principle of this setup was based on the evanescent wave theory. Escaped light from the first fibre optic was collected by the second fibre, which was

connected to the spectrometer. Each of the multimode fibres (core diameter of 550 μm) was decladded at one end with a length of 4 cm [125]. The decladding process has been explained in Section 4.1.3.

Figure 4.5. Setup 4: Double Decladded Fibre Optic Sensor

The decladded portions of the attached fibre optics were placed inside the sample holder. Firstly, niacin solution was injected into the sample holder, followed by the reagents. Different niacin concentrations were tested (Table 4.5.).

Table 4.5. Niacin concentrations variation in experiment setup 4

	Niacin concentration ($\mu\text{g/ml}$)
High Concentration	10-25 with an increment steps of 5
Standard Concentration	8.33
Low Concentration	0-6.25 with an increment steps of 1.25

The length of decladded region played an important role in determining sensitivity of the sensor. Longer decladded length causes the fibre to have more contact with the sample, causing the sensor to be more sensitive to changes. The fibre is able to capture more optical changes because there is more evanescent light being in

contact with the sample. However, there is a trade off, because with longer decladded region, more light is lost from the luminance fibre. In order to know the best length, two different lengths were tested: 3 and 4 cm. A length of more than 4 cm was not possible because the sample holder was not long enough.

In order to increase the sensors' sensitivity, the fibre connection was modified. By decladding only half portion of the fibre (Figure 4.6a), the sensitivity was expected to increase because light intensity would focus more onto the exposed portion. This also reduces losses of light to its surroundings. The second variation was done by twisting a fully decladded fibre around each other (shown in Figure 4.6b). This configuration was adopted from Smela's work [139]. She claims that by doing this, sensitivity of the sensor will increase. These fibres had a decladded length of 4 cm.

Figure 4.6. Schematic drawing of configuration variations in double decladded fibre optic sensor

- (a) front view of the half decladded fibre configuration
- (b) side view of the twisted fibre configuration

4.1.2.5. Data Analyses

Each experiment was conducted three times in order to determine the repeatability of each optical setup. These three sets were averaged and its standard deviation was calculated. The resulting graphs appeared to have base line shifts. This unavoidable situation was caused by many reasons, i.e. slight shifts in the fibre's position, power fluctuations of the light source, and changes in dark room spectrum. Data normalization was done to overcome this problem. Each graph (data) was divided by the maximum value so that the highest value was one. The spectrum fingerprint of niacin solution was investigated and the absorption wavelength range was recorded.

Results of the four experiment setups were compared in terms of sensitivity, cost and safety for the user. The best configuration was chosen and was used as the basis for the development of the new *M. tuberculosis* sensor.

4.1.3. DEVELOPMENT OF NEW TUBERCULOSIS SENSOR

Every optical sensor has a light source, a detector and a sensing region. In this project, the aim was to develop a sensor that is relatively inexpensive, portable and easy to use. Therefore, an LED was chosen to operate as the light source. Its operating wavelength region is selected based on the absorbance spectrum of the chemical reacted niacin sample. A photodiode was used to measure the amount of absorbed light by the sample. The sensing region configuration was selected based on the “experiment setup comparison” results.

Since it was not practical to conduct several tests in order to know the minimum niacin concentration that could be detected by the developed sensor, a mathematical equation was derived. Its derivation was based on the Beer-Lambert equation [109], which is shown in the following equations:

$$A = e^{-\alpha x} \quad \dots(4.3)$$

Where A is the absorbance level (Optical Density), x is the path length (mm) that the light is travelling through, and α is the absorbance coefficient (m^{-1}).

The absorption of a solution depends on the concentration of the sample (c_N); hence, the absorption equation is modified to be:

$$A = e^{-\alpha x c_N} \quad \dots(4.4a)$$

or it can be represented as:

$$A = 0.4343 \alpha c_N x \quad \dots(4.4b)$$

c_N has a unit of $\mu\text{g}/\text{ml}$. The unit of α in a solution is m^2/g .

Absorbance can also be represented as:

$$A = -\log\left(\frac{I_1}{I_0}\right) \quad \dots(4.5)$$

Here I_1 and I_0 is the intensity after and before the light passed the sample, respectively.

Hence, the smallest niacin concentration changes detectable by the sensor could be calculated. By calculating the time required by *M. tuberculosis* to produce that niacin level, the time detection could be estimated. Derivation of the niacin production is described in the next paragraph.

Standard protocol for niacin tests requires the test to be performed in culture samples that have been incubated for 3 to 4 weeks and with a minimum 50 numbers of colonies present [60]. Accordingly, the isolate is estimated to produce $8.33 \mu\text{g}$ of niacin. If a cell is assumed to grow to become 1 colony; then, 50 colonies means that there are initially 50 bacteria present in the sputum sample. In one fully-grown colony, there are around 10^6 of bacteria present [41]. When *M. tuberculosis* from sputum samples is cultured on egg media, it has a lag phase of 0 to 5 days [39, 40]. Based on the *exponential growth equation* (equation 2.1) and the above-elaborated information, the amount of niacin produced by each bacterium was calculated to be $3.1 \times 10^{-10} \mu\text{g}$.

Sputum samples incubated in broth has a lag phase of around 2 days [39, 40]. BACTEC systems claim they can detect the presence of *M. tuberculosis* after 2 to 3 weeks of incubation. It was then assumed that in the second week of incubation period, the amount of bacteria in broth is as many as the amount of bacteria after 3 to 4 weeks in the solid media. Calculation results indicated that in broth *M. tuberculosis* has a faster generation time of 4.5 to 10.5 hours. This calculation is supported by the basis that growth of *M. tuberculosis* in broth is faster compared to growth on solid media [173]. Assuming that *M. tuberculosis* in broth has the same niacin synthesis power as on egg media, the capability of each cell to produce niacin will still be the same (3.1×10^{-10} μg); however, the amount of niacin built up to its surrounding is much quicker because of a shorter generation time.

The expression of niacin production by *M. tuberculosis* can then be expressed as:

$$N_c = 3.1 * 10^{-10} \times a \times (2^x - 1) \quad \dots (4.6)$$

$$x = \frac{i_t}{g_t} \quad \dots (4.7)$$

N_c is the amount of niacin produced by *M. tuberculosis* at a given incubated period; a is the initial number of bacteria present in the sample; x indicates the number of dividing cycle that the initial number of bacteria have done. x is calculated by dividing the incubation period (i_t) by the generation time (g_t). As explained above, the generation time of *M. tuberculosis* is dependent on the environment conditions and the type of culture media used to grow the sample.

If sputum samples went through smearing test before being cultured, the initial number of bacteria can be estimated. For acid fast bacilli level 1 (AFB+), there are 10^3 - 10^4 AFB/ml of sample. For AFB++, the number of bacteria is 10 times more than AFB+. If sample smear results were negative, the culture was assumed to have 1 - 10^3 bacteria/ml of sample [60].

4.2. CLINICAL EXPERIMENTS

This section elaborates methods of applying the developed sensor to detect the presence of *M. tuberculosis* in suspected tuberculosis cases. Clinical trials were conducted to test the workability of the developed sensor in detecting the presence of *M. tuberculosis* in sputum samples that were obtained from patients those were diagnosed by medical doctors to be infected by *M. tuberculosis*.

4.2.1. PREPERATION OF REAGENTS

Reagents used in this section were the same as explained in the previous section, those are: cyanogen bromide 10% and aniline 4%. However, due to the availability of the reagents, the preparation of the cyanogen bromide solution was different. All chemical reagents were bought from Merck Co, Indonesia.

Potassium cyanide solution was prepared by diluting 4 g of potassium cyanide (KCN) powder into 100 ml of distilled water. Bromine-water was prepared by mixing 50 ml of bromine solution with 150 ml of distilled water in a 1000 ml amber flask. Afterwards, two layers were formed. One millilitre was taken from the bottom layer of bromine water and transferred into an Erlenmeyer flask. KCN solution was added drop by drop until the solution became colourless. This mixture formed the 10% cyanogens bromide solution.

The process of preparing aniline was the same as explained in Section 4.1. In an amber bottle, 4 ml of aniline was mixed with 96 ml of ethanol 95%. All reagents were stored in a refrigerator at 4 °C.

4.2.2. FIRST CLINICAL TRIAL: COMPARISON OF MEDIA CULTURE

Literature has stated that egg media is the most consistent media for niacin tests [46]. There are three kinds of egg media, but the most frequently used for *M. tuberculosis* culturing are Lowenstein-Jensen (LJ) and Ogawa media. Broth media is currently used by BACTEC system for its rapid detection, because in broth, *M. tuberculosis* grows faster. However, no one, to date, has reported using broth for niacin tests.

LJ medium contains potassium dihydrogen phosphate (KH_2PO_4), magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), magnesium citrate, asparagine, glycerol, and malachite green [46]; while Ogawa contains potassium dihydrogen phosphate, magnesium citrate, sodium glutamate, glycerol, and malachite green [43]. Ogawa is then cheaper because it does not contain asparagine, and due to its acid buffered composition, samples cultured on this medium need not to be centrifuged.

Agar base medium such as Middlebrook 7H10 or 7H11, are also frequently used for *M. tuberculosis* isolations. However, the media requires additional 0.1 % L-potassium aspartate or 0.25% L-asparagine in order to provide consistent niacin results, [174]. Another method to ensure proper extraction is by flooding the media culture for 2 hours at 37 °C [174]. This method was not convenient to conduct; hence, it was not used in this project. Also, agar media are seldom used in developing countries because of its higher cost compared to the egg media.

Hence, this section elaborates preparations conducted in order to choose the most suitable media used for niacin detection. There are three media chosen, i.e. two egg media (LJ and Ogawa) and Middlebrook 7H9 broth.

Preparation of Culture Media

Both egg media used in this experiment were commercially available. LJ media was purchased from Biomerc Co (Singapore), and Ogawa from Persahabatan Hospital (Jakarta). Middlebrook 7H9 broth, however, was prepared based on the CDC procedure [60].

Middlebrook 7H9 broth powder base (0.47 g) (REMEL Co, Singapore) was mixed into distilled water. The suspension was heated to boiling with agitations until all powder was dissolved. Then, it was sterilized by autoclaving at 121 °C for 10 minutes and was left to cool. When temperature of the broth was around 45-50 °C, 1 ml of ADC enrichment (REMEL Co, Singapore) was added and mixed thoroughly. One millimetre of prepared broth was then dispensed to sterilized test tubes. All culture media were refrigerated at temperature between 2 and 10 °C.

Preparation of Sample Culturing

Four sputum patient samples were collected in sterile containers; of these one sample was obtained from a non-tuberculosis patient (*negative sample*) and the other three (Sample 1, 2 and 3) were specimens collected from patients that were diagnosed to have tuberculosis infections. Sample 1 (*S1*) contained 1-10 acid fast bacilli in 1 microscope field (AFB++), which correlates to 10^4 - 10^5 AFB cells/ ml of sputum sample [60]. Sample 2 (*S2*) had one tenth of *S1* (AFB+). The last sample (*S3*) was obtained from a tuberculosis patient that has been treated for 2 months but was still AFB positive. All of these patient samples were decontaminated before they were inoculated onto the culture media.

The four sputum specimens were decontaminated with sodium hydroxide (NaOH 4%). The amount of NaOH added was equal to the sputum specimen's volume. The suspension was agitated until it became homogeneous. They were placed in a 37 °C incubator for 15 minutes. Four drops of each sample (around 0.13 ml) were transferred to the culture media. Each sample was cultured onto 5 of each LJ,

Ogawa and Middlebrook 7H9, so that the niacin production could be observed at 5 different days.

Two fully-grown *M. tuberculosis* colonies were scraped from the surface of an Ogawa media. The cells were suspended in 2.5 ml distilled water. This suspension acted as *positive* control. The suspension (0.13 ml) was then cultured onto the three different culture media (LJ, Ogawa solid media and Middlebrook broth).

Positive and *negative* controls were used for references. Niacin powder was diluted in distilled water to obtain a concentration of 1 µg/ml. It was used to observe the quality and reactivity of the chemical reagents. If colour of niacin solution, after adding respective reagents, turned yellow, it would mean that the reagents were still in good conditions and were ready to be used for testing. On the other hand, when no colour developed, a new reagent solution is prepared.

Data Collection

The time required for data collection was determined based on the absorption and niacin production calculation. Five different days were selected to test the cultured samples. In solid media cultures, niacin was extracted by flooding the cultures (LJ and Ogawa media) with approximately 0.3 ml of distilled water. The culture tubes were hold in a near horizontal position for about 15 minutes. This amount of distilled water was chosen based on the consideration that it was difficult to extract the liquid out from the culture when less liquid was used. If more than 0.3 ml was used, niacin concentration would be less. The standard amount of distilled water used is 1 ml [60], but by decreasing the amount of water to 0.3 ml, niacin concentration extracted in will be 3 times higher. Only 0.1 ml of the extracted liquid was collected using a syringe and then transferred into cuvette. The remaining 0.2 ml of water was left in the culture tube. The extracted liquid in the cuvette was then added with 0.1 ml of each CNBr and aniline. For broth samples, it was assumed that niacin would be dissolved in the broth solution itself. Therefore, in order to collect niacin from broth samples, sample tubes were

agitated and 0.1 ml of broth was transferred into the cuvette. Equal volume of reagents was added afterwards.

Intensity of the light source (I_0) was checked and recorded every time before the sample was placed inside the sample holder. The filled cuvettes were placed in the sensor's sample holder and intensity displayed on the screen was recorded (I_1). For each sample, 5 readings were taken.

Intensity shown by the sensor indicated the amount of light detected by the photodiode. The absorbed intensity was calculated based on Beer-Lambert's equation [109], which was presented in equation 4.5.

After readings were recorded, the cuvette, containing the extracted liquid, was submerged in a container filled with NaOH. This was meant to normalize the reagents' toxicity and to kill any bacteria that might be accidentally collected during the extraction of niacin from the media culture.

Results of the three different tested media were compared. The comparison was based on the ability of the media to recover *M. tuberculosis* strains.

4.2.3. SECOND CLINICAL TRIAL: CONFIRMATION TEST

The selected best media was tested for further clinical trials. These trials were conducted in order to determine the sensitivity and repeatability of the sensor. Results of the sensor were compared to 15 acid fast smearing test and 150 cultures.

Acid Fast Smearing

Sputum specimens from patients, who were diagnosed by doctors to be infected by *M. tuberculosis*, were collected. These samples were acid fast smeared before inoculated onto the culture media. Ziehl Neelsen staining method was used.

The acid fast smearing chemicals were prepared before the tests were conducted. In a beaker, 0.3 g of fuchsin was dissolved in 10 ml of 95% ethanol. Phenol crystals (5 g) were dissolved in 100 ml of distilled water. Fuchsin solution was then mixed with 90 ml of phenol solution to form **carbol fuchsin** solution. **Acid alcohol** was prepared by adding 3 ml of concentrated hydrochloric acid to 97 ml of 95% ethanol. In 100 ml of still water, 0.3 g of **methylene blue chloride** was dissolved.

The sputum specimen was smeared on a microscope slide, air-dried and passed through a burner flame to kill the bacteria. It was then covered with filter paper and then flooded with carbol fuchsin. The slide was heated again with a burner flame and was left stand for 5 minutes. After removing the filter paper, the slide was rinsed with running water. Acid alcohol was then flooded over the slides and was left for 2 minutes. The smear was washed again with water. Then, the slide was flooded with methylene blue and counterstained for 1 to 2 minutes. After rinsing it with water and being air dried, the smear was examined under the microscope with a magnification of 1000x. [60].

Fifteen patient samples were selected to be cultured; of these 5 samples were acid fast negative (AFB-), 5 were acid fast level 1 (AFB+), and the other 5 were acid fast level 2 (AFB++).

Inoculation of Samples

Samples inoculated on the selected culture media were the 15 acid fast smeared patient samples (elaborated previously), 5 *negative* control samples and 1 *positive* control sample. *Negative* control samples were samples obtained from five healthy people and the *positive* control samples were obtained by suspending 6 fully grown *M. tuberculosis* colonies in 6 ml of distilled water.

The acid fast smeared patient samples and *negative* control samples were decontaminated according to the procedure as elaborated in the Section 4.2.2. Each sample was cultured onto 6 media culture tubes, where each tube was inoculated with 0.1 ml of the decontaminated sputum sample. The *positive* control sample was transferred to 30 culture tubes and each tube contained 0.1 ml of the bacteria suspension. All of the 150 culture tubes were then incubated at 37 °C.

Data Collection

Niacin was extracted by flooding the slant cultures (LJ or Ogawa media) with approximately 0.3 ml distilled water and the tubes were held almost horizontally for 15 minutes. The extracted liquid of 0.1 ml was transferred into the cuvette and then the chemical reagents were added. Before inserting the cuvette into the sample holder, intensity of the light source was recorded and used as reference (I_0). Intensity of the sample was tested and recorded 5 times to ensure the repeatability of the sensor (I_I). Absorption of each samples were calculated based on equation 4.5, and results were analysed afterwards.

Data collection was conducted on 6 different days: day 1, 3, 4, 5, 7 and 15. Each day, one of each 15 acid fast smeared samples, one of each 5 *negative* control samples and 5 *positive* culture were tested.

The absorbance profile of sample was analysed based on the number of bacteria present in the collected sample. Because of conditions in the field during the clinical trial, it was not possible to calculate bacteria numbers by the usual plate count method. Instead, estimates of bacteria numbers were made from the

smearing results. AFB+ (S_{34}) correlates to a number of 10^3 - 10^4 acid fast bacteria cells in 1 ml of sputum sample, and 10^4 - 10^5 cells in AFB++ (S_{45}) samples. AFB- (S_{03}) indicates that there might be a chance that the specimen sample does not contain any acid fast bacteria or it might contain 1 - 10^3 number of bacteria. The acid fast smearing technique cannot sense samples that contain less than 10^3 bacteria cells [60].

Results detected by the sensor were compared to culture results. *M. tuberculosis* culture growth were examined after 6 weeks of incubation. Sensitivity, specificity, positive (PPV) and negative predictive value (NPV) of the developed sensor were calculated. These parameters were determined according to the following equations [175]:

$$\text{Sensitivity} = \frac{\text{TruePositives}}{\text{TruePositives} + \text{FalseNegatives}} \quad \dots (4.8)$$

$$\text{Specificity} = \frac{\text{TrueNegatives}}{\text{TrueNegatives} + \text{FalsePositives}} \quad \dots (4.9)$$

$$\text{PPV} = \frac{\text{TruePositives}}{\text{TruePositives} + \text{FalsePositives}} \quad \dots (4.10)$$

$$\text{NPV} = \frac{\text{TrueNegatives}}{\text{TrueNegatives} + \text{FalseNegatives}} \quad \dots (4.11)$$

CHAPTER 5

RESULTS AND DISCUSSION (PART 1)

DEVELOPMENT OF SENSOR

5.1. INITIAL TRIALS: COMPARISON OF CURRENT NIACIN DETECTION METHODS

Two current colourimetric methods to detect niacin as an indicator of *Mycobacterium tuberculosis* (*M. tuberculosis*) presences were compared; they are the use of **chemical reagent strips** or **chemical reagent solutions**. Commercially available chemical reagent strips (REMEL Co, Singapore) for *M. tuberculosis* identification are designed to detect niacin concentrations of 8.33 µg/ml or higher. Different niacin concentrations were tested and colour changes were visually observed. Solutions with a concentration of 8.33 µg/ml and above had obvious colour changes from transparent to yellow. Generally, as the concentration increases, the reacted sample becomes more yellow. This result supports the chemical reaction property explained by Koenig [65]. However, during the experiment, it was observed that there were some inconsistencies on the intensity of yellow colour. In some cases, smaller concentrations appeared to be more yellow compared to high concentrations. Addition to that, colour changes for samples with concentrations less than 8.33 µg/ml were not visibly obvious.

The second method to detect presence of niacin in the interested sample was done using chemical reagents in solution forms. Cyanogen bromide and aniline solutions were used as reactants. When niacin solution was added with the reagents, but not

mixed, a yellow ring appeared at the interface between the two reagents. If the solution was well mixed, the whole solution turned yellow. These conditions were as described by Fourier [43]. Figure 5.1 compares the colour developed for samples of 0 and 8.33 $\mu\text{g/ml}$ of niacin solutions. It can be seen that there is a colour difference between the two concentrations. Colours developed, using the chemical reagents in solution form, appeared to be consistent and were visible for small niacin concentrations from 2.5 $\mu\text{g/ml}$ and above.

Figure 5.1. Photographs of colour changes in different niacin concentration after it has been added with cyanogens bromide and aniline solutions
(a) 0 $\mu\text{g/ml}$, (b) 8.33 $\mu\text{g/ml}$

Results from the initial trials have suggested that better sensitivity and consistency were obtained using chemical reagents liquid solutions, compared to the niacin chemical reagent strips. Hence, the chemical reagent liquid solutions were chosen as the niacin reactant in conducting the next series of experiment, which is presented in the next section.

5.2. COMPARISON OF OPTICAL SETUPS

Four experimental setups were tested in order to investigate the most suitable optical setup for the novel *M. tuberculosis* metabolite sensor's development. The first experiment used an optical probe; followed by an extrinsic optical setup, single decladded fibre optic setup and lastly the double decladded fibre optic setup. Based on the previous results, different niacin concentrations were reacted with chemical reagents in the form of liquid solutions. Then, the change in the samples' optical properties and the ability of the different setups to sense these optical changes were observed and analysed. The results are presented in the following sections.

5.2.1. SETUP 1: OPTICAL PROBE

The optical probe (*Setup 1*) was tested for its ability to differentiate various levels of the yellow colour in the reacted niacin sample. The setup was also tested if it could sense any spectrum changes that were not visually obvious. Figure 5.2 is a typical transmittance graph of this setup. It shows that each concentration starts at different baselines. Possible reasons for these fluctuations will be elaborated further in Section 5.2.3. Because of these baseline shifts, normalizing was applied to facilitate the analysis. Figure 5.3 shows the graph for each concentration now having the same baseline, with a maximum value of unity.

There are two trend lines presented in the transmitted graphs (Figure 5.3). The first trend line shows transmittance losses in 400 to 550 nm wavelength regions, which is clearly represented by 8.33 $\mu\text{g/ml}$ niacin concentration spectrum. Intensity drop in this region is a resultant of wavelength absorption by the sample. All of the other wavelengths are transmitted; hence, the sensor detects high intensity level for other wavelengths. At 0 $\mu\text{g/ml}$ niacin concentration, the spectrum graph is a straight-line (second trend line).

Figure 5.2. Transmittance graph based on *Setup 1: Optical Probe*

Figure 5.3. Normalized transmittance graph of different niacin concentrations based on experiment *Setup 1: Optical Probe* .

From Figure 5.3, it was noted that inconsistencies in the spectrum results occurred. As an example, at niacin concentration of 16.67 $\mu\text{g/ml}$, the graph is virtually linear, showing that no wavelength is absorbed. This should not be the case because at this niacin level, the reacted solution appears yellow; therefore absorption should occur in the 400-550 nm region. The reason for this occurrence is the sensitivity of the sensor to detect colour changes was poor. Even though there were visible colour changes obviously seen by the observer, the sensor was not able to pick up any wavelength shifts. This is probably due to the insufficient light intensity reflected from the sample to be picked up by the probe.

From the normalized transmittance graph (Figure 5.3), the base-peak difference in 400-550 nm wavelength regions was calculated for each concentration. Results from the three experiments were averaged as presented in Figure 5.4. The absorptive values for concentrations below 8.33 $\mu\text{g/ml}$ were independent to niacin concentrations. Therefore, it can be represented as a horizontal line.

An increase in absorbance is only detectable for concentrations of 8.33 $\mu\text{g/ml}$ and above. For these concentrations, the trend line shows that there is a 0.009 Optical Density (OD) increase every $\mu\text{g/ml}$ of niacin. These results support the theory that absorbance, measured at the wavelength of maximum absorption, is proportional to the concentration of the component of interest [64]. Applications of this sensor configuration are frequently used to detect light intensity from objects that have unscattered reflection properties [176]. When light is passed through liquid samples, it will be scattered. The probe then picks some of this scattered light up, but most of it will be lost to the surroundings. This could be the caused of the low sensitivity of the system.

Based on three experimental results, the repeatability of this setup was not very satisfactory either because of the high deviation levels. For example in Figure 5.4, the deviation for niacin concentration of 6.25 $\mu\text{g/ml}$ is more than 100% of the Full Scale Output (FSO). As explained previously, the colour changes for niacin concentration below 8.33 $\mu\text{g/ml}$ was not enough to be detected by the sensor. The

sensor was sensitive to detect the spectrum changes of niacin concentration of 8.33 $\mu\text{g/ml}$ and above. The ability of the sensor to detect spectrum changes in this region is indicated by the sudden jump in the normalized transmittance level.

Other researchers have overcome this limitation by immobilizing the reagents at the tip of the fibre [112]. Another way is to place a mirror at the bottom of the sample's container so that the incident light can be reflected back and the captured the probe efficiently.

Figure 5.4. Average of the base-peak normalized intensity based on *Setup 1*: Optical Probe

5.2.2. SETUP 2: EXTRINSIC OPTICAL SENSOR

Figure 5.5 shows the normalized transmittance intensity for different niacin concentrations detected by the extrinsic optical sensor (*Setup 2*). The graph shows that it has similar spectrum signature to the standard transmittance graph of the yellow colour (Figure 5.6). Yellow has a high transmittance level in between 540 and 700 nm wavelength region [129].

Figure 5.5. Normalized transmittance graph of different niacin concentrations based on *Setup 2*: Extrinsic Optical Sensor

Figure 5.6. Transmittance graph of medium yellow colour [129]

In between 530 and 550 nm wavelengths range, wavelength shifts occur. It clearly shows that as niacin concentration increases, curves reach the maximum intensity (normalized transmittance value 1) at longer wavelengths. For example, at 25 $\mu\text{g/ml}$ niacin solution (Figure 5.5), the graph reached the maximum intensity at 549.3 nm, and 541.6 nm for 5 $\mu\text{g/ml}$. Since colour of the sample is yellow, the entire yellow wavelengths are transmitted and other visible light wavelengths are absorbed. When yellow colour becomes more visible, the graph shifts more to higher yellow wavelengths (550-580 nm). When niacin is not present (0 $\mu\text{g/ml}$), the entire wavelengths are transmitted (the spectrum graph is presented as a straight line).

In Figure 5.5, a downward intensity shift in the 400-550 nm wavelength region is more obvious compared to the wavelength shift. As niacin concentration increases, more light intensity is absorbed. This relates to the basic theory that absorbance and sample concentration is linearly proportional [110]. Maximum absorption occurs between 440 and 450 nm wavelengths. For concentrations above 5 $\mu\text{g/ml}$, it is quite obvious that the spectrum peak for each concentration has shifted. As niacin concentration increases, the peaks shift from longer to shorter wavelengths.

The amount of absorption increment is affected by two factors: (1) number of molecules, and (2) colour changes in the sample. Molecules inside the sample absorb light intensity at a particular wavelength. Large numbers of molecules present in the sample will absorb/scatter more light. Thus, this graph indicates that

this setup can be used to sense a solution's density. As the sample becomes denser, less light is transmitted to the detector. At the same time, increasing concentrations will change colour of the solution from transparent to darker yellow.

Figure 5.7 presents the average and standard deviation values of three experiment sets. Points plotted are the base-peak differences at an interval between 400 and 550 nm. The graph demonstrates a logarithmic relation between the base-peak differences of the transmitted intensity and niacin concentration, which satisfies the Beer-Lambert's Law [109].

Figure 5.7. Average niacin concentrations vs. transmittance intensity based on Setup 2: Extrinsic Optical Sensor.

Experimental results reveal that standard deviations at low concentrations (<8.33 µg/ml) are larger than higher ones. Maximum standard deviations at low and high concentrations are 9.8% and 2.3% FSO, respectively. This indicates that the setup is more consistent for high niacin concentrations. These variations might be because the samples were not placed directly in contact with the fibre; hence, there are possibilities of interference by other parameters on the intensity readings. The absorption variations from different cuvettes and slight variations of niacin solution

prepared could be the cause of these deviations. However, variations in the findings are those which one would expect from one experiment to another or from any single experiment setup in duplicate.

5.2.3. SETUP 3: SINGLE DECLADDED FIBER OPTIC SENSOR

The principle of this setup is based on alteration of the evanescent wave principles that was set to be in contact with the sample (refer to Section 3.3). A number of developed biosensors have shown that optical sample changes can be detected by evanescent waves [125, 160]. However, as shown in Figure 5.8, there was no particular wavelength absorbed by any niacin concentration tested. This indicates that *Setup 3* was not able to detect any spectrum changes, even though there were visually obvious colour changes in the tested samples.

**Figure 5.8. Transmittance graph based on *Setup 3*:
Single Decladded Fibre Optic Sensor**

Light travels in the fibre optic by internal reflection. Internal reflection occurs when light passes from a high refractive index medium to a lower one, and then hits the latter medium with an incidence angle larger than the critical angle (Snell's Law) [110]. The fibre used in this application was made out of silica. Niacin and chemical reagent sample mixture has a lower refractive index compared to the core of the fibre. This ensures that total internal reflection phenomenon would occur in this setup. However, the graph shows that there are no spectral changes detected. This indicates that the reflected light-wave from the sample back into the fibre did not affect the signal output. The incapability of the sensor to detect these changes could be attributed to high light intensity from the source that has masked the small evanescent wave changes.

Adjusting the refractive index of the sample to be as large as the refractive index of the cladding might overcome this problem. This could be done with additions of buffer solutions; however, it was avoided because it might introduce an additional chemical reaction with niacin or the chemical reagents. Another alternative is by reducing the intensity of the input light. However, the setup will be more complex, because it then requires additional items, such as filters or couplers.

Another method to apply the evanescent principle is by replacing a portion of the cladding with a new layer [120, 142]. This new layer (sol gel) has similar optical properties as the cladding and at the same time contains the respective reagents. The chemical reaction between sample and reagents will occur in the sol gel region and then detected by the evanescent wave. Sol gel means had been tested in this research, but the results were unsatisfactory. Large shrinkage occurred, which caused an uneven coating. Also, the chemical reagents lost its reactivity because of exposure to light, oxygen or reaction with other chemicals that were required to form the sol gel.

From Figure 5.8, it is also observed that there is no correlation between the intensity and niacin concentration. There are a few possibilities why this condition occurred. Firstly, it might be caused by the continuous changes of the samples' refractive

indices. Secondly, it may be due to unavoidable shifts of the fibre's position, fluctuation of electrical power that leads to variation in the source's intensity or loose connections between the fibre and the detector.

This indicates that the raw data, for every experiment conducted, cannot be directly analyzed. Thus, normalization or a feedback sensing techniques need to be applied [177]. In this project, normalization was applied instead. Normalizing will transform the graph to fluctuate at the same baseline.

5.2.4. SETUP 4: DOUBLE DECLADDED FIBER OPTIC SENSOR

In this setup, two decladded fibre optics were attached parallel to each other. Cores of these two identical multimode fibres were exposed to allow direct contact with the sample. The luminance fibre facilitated the light to travel from the light source to the niacin solution sample. The light was then attenuated by the sample. If the gap between the two fibres was less than the evanescent penetration depth, the attenuated light could be captured by the second fibre and transferred to the detector.

Figure 5.9 shows the normalized transmittance graph at different niacin concentrations. Peaks occurred between 400 and 550 nm wavelengths. The graph profile has similar shape to *Setup 2* (extrinsic fibre optic sensor) results (see Figure 5.5). Experiment results shows that as niacin concentration increases, more blue wavelengths (450-500 nm) are absorbed by the sample (niacin) particles. In the range from 450 to 485 nm wavelengths, the peaks shift towards shorter wavelengths with a ratio of 1.94 nm per 1 $\mu\text{g/ml}$ increment of niacin (Figure 5.10). Shifts occurred because of density and colour changes in the sample.

Intensity shifts were more obvious compared to wavelength shifts. Figure 5.11 shows that as niacin concentration increases, more light intensity is absorbed by the sample (downward shift). The difference between the baselines and peaks were calculated for each concentration. The data from three experiments were then averaged (Figure 5.11).

Data points for 10 and 15 $\mu\text{g/ml}$ niacin concentrations did not lie within the expected graph line. Displacement of the fibres' position while data was recorded or inaccurate sample mixture might have been the cause of these deviations.

**Figure 5.9. Normalized transmittance graph based on *Setup 4*:
Double Decladded Fibre Optic Sensor**

Figure 5.10. Average graph of wavelength vs. niacin concentration **Figure 5.11. Average niacin concentrations vs. transmittance based on Setup 4: Double Decladded Fibre Optic Sensor.**

For concentrations between 1.25 and 3.75 $\mu\text{g/ml}$, the maximum standard deviation is 8.6% FSO. This indicates that the setup is best used for identifying small sample concentrations. Niacin concentrations between 8.33 and 25 $\mu\text{g/ml}$ have larger standard deviations compared to the lower concentrations. In this region the maximum error is 23% FSO.

Wavelength shifts also occurred between 530 and 550 nm wavelengths (Figure 5.9). It was previously described that as the niacin level increases there are colour changes ranging from yellow to red [67]. Results from this experiment are consistent with the stated findings, i.e. as the sample becomes more yellow; there are peak shifts toward longer wavelengths. Since colour of the samples are only in the yellow range, the wavelength shifts occurred only in the 550-580 nm

wavelengths region. The average shifts of the wavelength are around 1.2 nm for an increment of 1 μg of niacin in 1 ml of water.

Wavelength shifts also occurred in the region below 380 nm. However, any observation made in this region is not valid because the tungsten halogen lamp used only emits VIS-NIR spectrum. The spectroscope showed some readings, but they are inaccurate.

A few variations had been made in order to improve the sensitivity of the *Setup 4*. These were variations of decladded length and connection between the two fibres.

Decladded Length Variation

The effects of varying the length of the decladded fibre are discussed in this section. Three (f_3) and four (f_4) centimetres of fibre decladded lengths were tested. Results comply with the statement made by Ferreira [171], i.e. longer decladded fibre region would increase the sensitivity of the sensor. This is shown in Figure 5.12 that f_4 is three times more sensitive than f_3 .

Figure 5.12. Normalized intensity detected by different decladded lengths

$f_3 = 3 \text{ cm}$, $f_4 = 4 \text{ cm}$.

For concentrations less than 5 $\mu\text{g/ml}$, it can be seen that at concentrations 1.25 and 2.5 $\mu\text{g/ml}$, there are no differences in intensity detected by f_4 . This shows that if the decladded region is less than 3 cm, the fibre is not well suited for detecting low niacin concentrations.

Fibre's Connection Variation

Based on Smela's work [139], a twisted fibre configuration was also tested to detect different niacin concentrations. Another configuration tested in this experiment was half decladded fibres. Results from each test are shown in Figure 5.13. It suggests that the twisted fibre configuration is apparently less sensitive compared to the half and fully decladded fibre. The half decladded fibre configuration indicates an increase of 66.7% in sensitivity at 1.25 $\mu\text{g/ml}$.

Figure 5.13. Comparison between three different connection variations (half decladded , fully decladded and twisted fibre configuration)

Table 5.1 shows that sensitivity of detection is highly dependent on the gap distance between the input and output fibre. When fibres are too tightly attached, less spectrum changes occur. The reason behind this is because only a small amount of

sample solution is able to pass through the gap, and causes only a small spectrum change to occur. It might also be because light density from the source is too intense; thus, obscuring the detection of spectrum absorbed by the sample. On the contrary, the gap between the two fibres cannot be too wide. There is a maximum distance for the escaped light from the luminance fibre to be detected by the detection fibre. This is known as **penetration depth**.

Table 5.1. Gap variation in between the two connected fibres

Distance (mm)	Normalized Intensity (OD)
0	0.12
0.8	0.40
1	0.16

Based on Table 5.1, it is concluded that the twisted fibre configuration is not functional (Figure 5.13). The distance between the two fibres was too small, causing difficulties for the light to interact with the sample.

The half decladded fibre has higher sensitivity of 66.7% compared to the fully decladded fibres. As schematically illustrated in Figure 5.14, decladding half of the fibre has minimized light losses to the surroundings.

Figure 5.14. Representation of light losses: (a) fully, and (b) half decladded fibre.

Note: the arrows indicate the light travelling path.

5.2.5. COMPARISON OF SETUP CONFIGURATIONS

Three average curves from experiment *Setup 1* (optic probe), *Setup 2* (extrinsic fibre optic sensor) and *Setup 4* (double decladded fibre optic sensor) are plotted in Figure 5.15. Symbols z and x in the presented in the graph represent the normalized intensity and niacin concentrations, respectively. It is shown that *Setup 1* was not able to detect any intensity changes for low niacin concentrations ($<8.33 \mu\text{g/ml}$). It also has a poor repeatability, compared to *Setup 2* and *Setup 4*.

Figure 5.15. Comparison graph of the three experiment setups

Sensitivity of *Setup 2* to detect low niacin concentrations is the highest among the other setups. It has a sensitivity of 0.07 normalized intensity/niacin concentration, which is 2.5 two times higher compared to *Setup 4*. The sensitivity of *Setup 4* could be improved by modifying the distance between the two fibres and/or by decladding only half portion of the optical fibre. Experiment result has shown that by decladding half of the fibre, the sensitivity would increase by 66.7%.

Besides its sensitivity, *Setup 2*'s repeatability has shown to be the best among the others as well. Comparing *Setup 2*'s maximum standard deviation to the others showed that its repeatability was the best among the others. Its maximum standard deviation was 20 and 2.3 times lower compared to *Setup 1* and *Setup 4*.

Several aspects that were considered to be essential for the development of the sensor are tabulated as shown in Table 5.2. From the table, *Setup 2* shows that it has the best sensitivity and the setup minimizes the probability of contamination from one sample to another. This is because the probe is not directly in contact with the sample. Based on these results, *Setup 2* was further developed and tests were conducted with patients sample. The sensor's development and clinical trials are explained in Chapter 5.2 and 6, respectively.

Table 5.2. Comparison of different setups

No.	Criteria	Setup 1	Setup 2	Setup 4
1	Sensitivity			
	Good sensitivity for low niacin concentrations	No	Best	Yes
	Good repeatability for low niacin concentrations	No	Yes	Best
2	Contamination			
	Probe must not contaminate one sample to the other samples	No	Yes	No
3	Safety for the user			
	Minimum exposure to the analyst	No	Yes	No
4	Easy to use			
	Analyst need not to go for special training	Yes	Yes	Yes
5	Cost			
	Affordable for developing countries	Yes	Yes	Yes

CHAPTER 6

RESULTS AND DISCUSSION (PART 2)

CLINICAL EXPERIMENTS

6.1. FIRST CLINICAL TRIAL: COMPARISON OF CULTURE MEDIA

Lowenstein-Jensen, Ogawa solid media, and Middlebrook 7H9 broth are the three most commonly used media for culturing *Mycobacterium tuberculosis* (*M. tuberculosis*). Each of these culture media is made out of different ingredients and chemical compositions that would affect bacteria growth profile. As an example, Lowenstein-Jensen and Ogawa suppress growth of contaminants, and broth facilitates a faster growth of *M. tuberculosis* [179]. Therefore, these three media were tested to compare which was the most suitable media used with the SC-Sensor. The sensitivity of the sensor is 2.25×10^{-4} Optical Density (OD)/bit.

6.1.1. LOWENSTEIN-JENSEN SOLID MEDIA

Lowenstein-Jensen media (LJ) contains Malachite green [60]. This egg medium is mostly used in developed countries [180]. However, it is seldom used for drug susceptibility tests because egg media change the drug compositions [44].

In conducting the experiments, it was observed that a greenish liquid existed in some of the incubated culture tubes (Figure 6.1a). Its existence is suspected due to one or all of the following conditions:

1. Liquid Separation

Liquid separation appeared to have happened during the media shipment from the storage (Singapore) to the working area (Indonesia). Temperature fluctuations during transportation might have caused some of the medium to liquefy.

Figure 6.1. LJ culture

(a) Excess liquid, (b) Colour variation in the LJ culture media:
(i) Contaminated sample, and (ii) Positive sample

2. Absorption capability of the LJ media

Based on the Centre of Disease and Control (CDC) culturing procedure, the amount of sample inoculated is 0.1 ml [60]. Logically, if a larger sample volume is inoculated, there will be more bacteria present in the culture. As the amount of bacteria increases, the amount of niacin produced will increase as well. Therefore, in this experiment, 0.13 ml of sample was

inoculated. A larger volume would certainly culture more bacteria, but the amount of cultured sample is limited by the amount of sputum produced by the patient.

Solid culture media has certain the capability to absorb liquid that is inoculated onto it. In this case, LJ might not have been able to absorb excess inoculated sample of 0.03 ml. This could be overcome by increasing the area/volume of the LJ media, or by using less amount of sample.

3. Contamination

Contamination by proteolytic organisms might contribute to the said condition. In the presence of these organisms, egg based medium tends to liquefy [46]. Figure 6.1(b_i) shows that contamination caused a change in the colour of media and the presence of excess liquid in the culture tube.

This excess liquid appeared greenish since the Malachite green dye (one of the LJ ingredients) was diluted in it. Because the amount of liquid present could not be controlled, there were colour variations in the extracted liquid.

Figure 6.2a shows the absorbance of *negative* and *positive* cultures. A *negative* culture is a sputum specimen that was obtained from a healthy patient, and a *positive* culture is fully-grown *M. tuberculosis* colonies that was suspended in distilled water and re-cultured. Both *negative* and *positive* samples showed an increase in absorbance as they were incubated for a longer period. The *positive* sample showed an increase in light absorbance of 55% on day 8, but dropped by 50% on day 11.

Results and Discussion: Clinical Experiments

(a)

(b)

Figure 6.2. Intensity detected by SC-Sensor on LJ media
(a) Negative and positive samples, (b) Negative, S1, S2 and S3 samples

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Results and Discussion: Clinical Experiments

The *negative* sample had an increase of 70% in light absorbance after 6 days of incubation and decreased afterwards by 25% then remained stable. Theoretically, *negative* samples will not show any increase in their absorbance level because niacin is not produced. However, absorption increment in this experiment may have occurred because of the presence of the greenish extracted liquid. Results have shown that the sensor is not able to differentiate between *negative* and *positive* samples. Both cultures have an increase in absorbance, and their absorbance differences are not significant.

Absorbance levels of *S1*, *S2* and *S3* are compared against *negative* cultures' (Figure 6.2b). *S1* is acid fast bacilli positive level 2 (AFB++) specimen, *S2* is AFB+ and *S3* is an AFB+ specimen obtained from a tuberculosis patient who has gone through treatments. AFB++ is sample that contains 1-10 acid fast bacilli in 1 microscope field (which correlates to 10^4 - 10^5 AFB cells/ml of sputum) and AFB+ is sample that contains 1-10 acid fast bacilli in 10 microscope fields. *S1* had 74% increase in absorbance on day 11, but the absorption level then decreased by 50% on day 13. Absorbance of *S2* on day 2 was 55% higher compared to *S1*, but it then dropped drastically by 53-60% as cultures were incubated for longer time. *S3* had a 74% increase in intensity on day 13 compared to intensity at day 8. The results from LJ media showed that the results had no particular trend line.

After 2 months of incubation, bacteria growth in the culture tubes was visually observed. There were confluent growth of *M. tuberculosis* present in the *positive* and *S1* culture, but none appeared in *S2* and *S3* culture tubes.

6.1.2. OGAWA SOLID MEDIA

According to the literature, Ogawa media reduces the probability of contamination and gives more consistent niacin test results compared to the agar-based media (e.g. Middlebrook 7H10 agar) [46]. Unlike in LJ media, condensation did not appear in the Ogawa culture.

Extracted liquid from the media appeared colourless, but not green. However, after reagents were added, the sample mixture became green. *Negative* samples appeared dark green and *positive* samples were less green compared to the *negative* ones. Difference in the greenness level was quantified by the light absorption values presented in Figure 6.3a. A trend line was drawn in this graph. All results (except for day 4) of *positive* samples had lower absorbance level compared to the *negative* ones (Figure 6.3a). Since, *negative* reacted samples (extracted liquid that had been added with the chemical reagents) were greener than the *positive* ones; the former had higher absorption level of 0.25 OD with a 20% variation. For *positive* samples, as they were incubated longer, the reacted samples appeared less green in colour. This caused a drop from 0.225 OD to 0.17 and 0.007 OD at day 6 and 8, respectively. On day 13, there was an increase in the absorption level, because the reacted sample was yellow.

S1 and *S2* samples had the same absorption profile as the *positive* sample (Figure 6.3b). The trend lines for *S1* and *S2* from day 2 to 8 show that there was an absorbance difference of 0.5 and 0.55 OD compared to the *negative* sample, respectively. There was further intensity drops for both samples at day 11. However, at day 13, there was an increase of 58% and 30% for *S1* and *S2*, respectively. Reason of the increase of absorption level on day 13 for *positive* sample, *S1* and *S2* is explained in Section 6.2. *S3* absorption level fluctuated at 0.25 OD with a 20% variation. *S3*'s absorbance level and profile was similar to the *negative* ones. This indicates that *S3* was not a *M. tuberculosis* culture sample.

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Figure 6.3. Absorption intensity detected by SC-Sensor on Ogawa media
(a) Negative and positive samples, (b) Negative, S1, S2 and S3 samples

After two months of incubation, *S1* and *S2* had visible *M. tuberculosis* colonies (Figure 6.4a), but in *S3* (Figure 6.4b) a different type of bacteria grew. As shown in Figure 6.4b, *S3* colony has similar morphology as that of *M. tuberculosis*; those are rough, raised, and thick with a nodular surface and irregular thin margin. The only difference is that the colony was orange in colour. It was later identified morphologically that the *S3* colony was *Mycobacterium scrofulaceum* [73]. According to the diagnostic table, attached in Appendix A, *M. scrofulaceum* is not a niacin producer. Hence, this has shown that the developed system was able to differentiate between different *M. tuberculosis* and *M. scrofulaceum*.

Figure 6.4. Growth of incubated patient sample on Ogawa media
(a) Positive and (b) S3 samples

Light absorption results showed that the sensor was able to detect the presence of *M. tuberculosis* in *positive*, *S1* and *S2* samples, by showing a difference in absorbance level against the *negative* samples. The sensor could also distinguish between *M. tuberculosis* and other acid-fast bacilli (*S3* samples).

6.1.3. MIDDLEBROOK 7H9 BROTH

M. tuberculosis growth in broth is faster compared to that on solid media. In broth, cells have better nutrient absorptions [173] and at the same time, each cell is able to absorb equal amount of nutrient from its surroundings. In solid media, nutrient those are available for bacteria are only from its surrounding area, which in many is shared with other neighbouring bacteria colonies. This will cause different multiplication factors for each colony.

Figure 6.5a shows that there is an increase in absorbance level for both *negative* and *positive* samples. In *negative* samples, the increase of absorbance may be caused by increment of other bacteria present in the media, which would increase the turbidity. As a result, the absorbance level would increase as well. *Positive* samples would have an increase in turbidity too, but with the increment of niacin level by *M. tuberculosis*, they were expected to have higher absorbance levels. Apparently, the sensor could not detect these differences. It was perhaps due to the amount of niacin produced by *M. tuberculosis* was diluted in the broth, which was used to provide nutrients for bacteria. If the amount of broth was reduced, niacin concentration would have increased; but it might not be enough to support the bacteria growth.

Figure 6.5b compares the absorbance levels of *S1*, *S2* and *S3* to *negative* samples. On day 2, the absorbance of *S1*, *S2* and *S3* were respectively 2, 3, and 3.1 times higher than the *negative* samples. Variations in the absorbance level could be attributed to:

1. Significant amount of niacin that have been produced by *M. tuberculosis*.
2. Different numbers of bacteria present in the media, which caused different turbidity levels.
3. Variations in the colour of broth after sputum samples were added.

Some of the broth turned yellow, but some remained clear. Other detection systems, such as BACTEC, have overcome colour variation by processing sputum specimens before culturing. The specimens are centrifuge so that sediments are built up. These sediments are then cultured [60].

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(a)

(b)

Figure 6.5. Absorption intensity detected by SC-Sensor in broth
(a) Negative and positive samples, (b) Negative, S1, S2 and S3 samples

Different numbers of bacteria that were present in the broth could be the cause of the decrease in absorbance of *S2* to 0.3 OD (day 8 onwards). *S3* showed that there was an increase of 66% in absorption level from day 6 to day 13, which could be attributed to the increase of bacteria present in the cultured specimen. *S3*'s Ogawa culture (refer to Section 6.1.2) showed that *S3* was not *M. tuberculosis*. Thus, the increase in absorbance was a resultant of the increase in turbidity (due to increase in bacteria numbers) and not because the increase in niacin level.

Similar absorbance level of *positive* and *negative* samples showed that the sensor was not able to differentiate these samples. *S1* and *S2* absorption graph have indicated no trend either. The increase in absorbance level of *S3* samples demonstrated that with broth, the sensor was not able to differentiate *M. tuberculosis* from other bacteria.

6.1.4. COMPARISON OF THE THREE MEDIA

Experiments revealed that the Ogawa media has shown to give the most consistent and sensitive results compared to LJ and Middlebrook media. Culture on Ogawa media showed significant absorbance difference of 0.36 Optical Densities (OD) between *positive* and *negative* samples on day 5. No trend lines could be observed for specimens that were grown on LJ and broth.

Sensitivity and specificity of smearing test and each culture media are tabulated in Table 6.1. These figures are based on the growth of bacteria in each medium, not on the ability of the developed sensor to detect presence of *M. tuberculosis*. It appeared that bacteria growth was more consistent on Ogawa compared to LJ media. *S1* showed growth of *M. tuberculosis* on both Ogawa and LJ media, but *S2* only appeared on Ogawa cultures. *S3* had no bacteria present on LJ media, but on Ogawa media, it turned out to be *M. scrofulaceum* instead. This has shown that Ogawa gave a better correlation between the absorption and culture results. It also had a sensitivity and specificity of 100%.

Table 6.1 Sensitivity and specificity of smearing and SC-Sensor on different media
(Total sample size (n)= 5)

Method	True Positive (n)	True Negative (n)	False Positive (n)	False negative (n)	Sensitivity (%)	Specificity (%)
Smear	3	1	1	0	100.0	50.0
LJ	2	1	0	2	50.0	100.0
Ogawa	3	2	0	0	100.0	100.0
Broth	2	1	0	2	50.0	100.0

Turbidity levels in broth can be used as bacteria growth indicator. However, it was difficult to distinguish the growth of *M. tuberculosis* to other types of bacteria. Although broth allows a faster multiplication rate, the *SC-Sensor* was not able to pick up any absorbance difference between the *negative* and *positive* samples. Further more, it appeared that the sensor was not able to differentiate between *M. tuberculosis* and other mycobacteria species either (i.e. *S3* results).

Based on these experiment results, Ogawa was selected to be the most suitable media to be used with the sensor application. In addition to its consistency in the experimental results, Ogawa is more commonly used in developing countries for the following reasons:

1. It is more stable and seems to recover more resistance strains compared to LJ, although the numbers are not very significant [44].
2. It is cheaper because it does not use asparagines as LJ does [181].
3. It is good for samples that do not go through centrifugation process. By reducing this process, it reduces the exposure risk of clinicians to *M. tuberculosis*. Developing countries quite often do not have the facilities to perform centrifugations. This shows why Ogawa media is more preferable for the application in developing countries [181, 182].

Ogawa results showed that *positive* samples had their sample mixture's colour shifted from dark green to yellow as time of incubation increased. These colour changes were different from what was expected, which was from transparent to yellow (refer to Chapter 5). Based on Jones and Falkinham's [183] work, a number

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of mycobacteria were capable of bleaching malachite green. This might have affected the different colour changes during the experiment. Another possibility is that it could be due to another chemical reaction that occurred between the extracted liquid (that contain niacin and Malachite green) and the chemical reagents. Section 6.2 discusses the results of experiments that were conducted to confirm if there was any relation between the chemical reactions and the level of greenness in the sample mixture.

Graphs presented in this section were plotted based on single points because only one sample was tested on each experiment day. More samples should be tested in order to justify the results. The next clinical trials were tested with the latest modified sensor (*RC-Sensor*). These results are elaborated in Section 6.3.

6.2. REACTION OF MALACHITE GREEN AND NIACIN

Ogawa results in the previous experiment (see Section 6.1.2) have demonstrated that absorbance level decreased as *positive* samples were incubated longer. The older the culture was, the reacted sample (extracted liquid that has been added with chemical reagents) became less green and then turned yellow. Older culture means that more niacin will be produced by *M. tuberculosis* in the culture.

These results differ from the chemical reaction explained by Koenig's theory [65]. Koenig explains that a yellow colour will develop when cyanogen bromide and aniline react with a sample solution that contains niacin. This different outcome is probably due chemical reaction between niacin and its reagents together with an additional substance, i.e. Malachite green, a chemical substance used as one of the media's ingredient. It was suspected that Malachite green was extracted along with the niacin extraction process from the culture media.

Malachite green's absorption spectrum is shown in Figure 6.6a [184]. The graph shows that it absorbs visible light in the range of 380-450 and 550-650 nm. For comparison, absorption spectrum of niacin, which was added with cyanogen bromide and aniline, is presented in Figure 6.6b. This spectrum fingerprint was obtained from experiment results elaborated in Section 5.2. Its absorption appears to be in the 380-550 nm wavelengths region.

In order to confirm if there was any relation between Malachite green, niacin and chemical reagents to the colour development after they were reacted, series of tests were conducted. Different niacin concentrations were incubated for three days on Ogawa media. The absorbance profile was recorded using a spectrometer (USB2000, Ocean Optics Co, Singapore).

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(a)

(b)

(c)

Figure 6.6. Absorption spectrum in the visible light wavelength region

(a) Malachite Green, (b) Niacin (c) Niacin incubated in Ogawa

Figure 6.6c shows that the spectrum fingerprint of the mixture sample occurred in 400-550 nm and 600-650 nm wavelengths. Since the developed sensor has a light source that operates in the wavelength range of 430-500 nm, analysis is focused in the 400-550 nm wavelengths region.

The absorption intensity level changed as different niacin concentrations were tested (Figure 6.7). With the increase of niacin concentration, from 1 to 5 $\mu\text{g/ml}$, the absorption level decreased. However, when the niacin concentration was very high, in this case it was 15 $\mu\text{g/ml}$, it was noted that a sudden increase in the absorption level occurred.

Figure 6.7. Absorption graphs of niacin when incubated in Ogawa medium

This result proves that Malachite dye affects the colour of the reacted sample. It was also noted that as niacin concentration in the media increases, there was a colour shift from green to yellow. This result verifies the hypothesis with the addition of Malachite green in the sample; the chemical reaction differs from what is explained by Koenig's theory. None of the literature research conducted by the author has reported such findings.

If a sputum sample is *negative* (does not contain *M. tuberculosis*), the extracted liquid added with the reagents will appear dark green causing a high absorption level. It is because there will be no niacin present in the sample. If *M. tuberculosis* is present in the media, there will be an increase in niacin level causing a decrease in absorbance. However, if the level of niacin produced by *M. tuberculosis* is a very high, the sample will again have a high absorption level because the reacted sample will be yellow in colour. This conclusion is illustrated in Figure 6.8.

**Figure 6.8. Niacin in Ogawa vs. absorbance level
based on experiment results**

Figure 6.8 is plotted based on the normalized intensity level of different niacin concentrations at 472 nm. Due to the minimum number of experiments conducted, the point of deflection was estimated mathematically based on the fit curve (Figure 6.8) to be around 7 μg/ml. Further experiments are required to justify this deflection point.

Figure 6.8 gives an indication in what time frame should a culture be tested. Since non-*M. tuberculosis* cultures will have high absorbance level, an absorbance difference will indicate that the investigated sample contains *M. tuberculosis*. When more niacin is produced, the absorbance difference will be larger until the point of deflection (region 1 of the graph). After the deflection point (region 2), as *M. tuberculosis* is incubated longer, the absorbance difference against negative cultures reduces. This figure indicates that the test needs to be conducted before the amount of extracted niacin solution reaches 7 µg/ml.

Since the amount of liquid required to extract niacin, using *RC-Sensor*, is 0.3 ml, the quantity of niacin required to be present in the culture is 2.1 µg. Time for *M. tuberculosis* to produce 7 µg/ml of niacin can be calculated using equation 4.6 and 4.7. Thus, it was calculated that based on the number of *M. tuberculosis* cells present, the test should be conducted before the days as indicated in Table 6.2.

Table 6.2. Testing time for different amount *M. tuberculosis* cells

Number of <i>M. tuberculosis</i> Present (cells/ml)	Testing Time (day)
1-10 ³	20
10 ³ -10 ⁴	17
10 ⁴ -10 ⁵	15
10 ⁵ -10 ⁶	13

In relating equation 5.2 and 5.3 to graph 6.8 (in the first region), the trend noted was the opposite. This was suspected due to an additional chemical reaction that occurred between niacin, its reagents and malachite green that was present in the extracted liquid. However, due to the lack of resources and opportunity at the field site, further experiments to verify the equation and graph correlation could not be carried out.

6.3. SECOND CLINICAL TRIAL: CONFIRMATION TESTS

This section elaborates results of the improved developed sensor, Round Cuvette Sensor (*RC-Sensor*), when it was tested with various patients' sputum samples, which were cultured on Ogawa media. *RC-Sensor* has a larger cuvette size compared to the previous setup, the Slide Cuvette Sensor. The *RC-Sensor* is able to contain a larger amount of sample, resulting in a longer path length and a higher absorbance level. It has also a better resolution of 5.35×10^{-4} OD/ bit, 2.4 times more sensitive compared to the Slide Cuvette Sensor. Based on the results from the media comparisons, presented in Section 6.1, Ogawa gave the most consistent results. Hence, in this second clinical trial, samples were cultured on this media.

The cultured samples consisted of *negative* control samples collected from five healthy people, a *positive* control sample that was obtained by re-culturing fully-grown *M. tuberculosis* colonies, and 15 patient's sputum samples which were acid fast smeared before cultured. These 15 patient samples were separated into 3 groups; those are S_{03} (5 samples), S_{34} (5 samples), and S_{45} (5 samples). They were classified based on the number of bacteria present in the sputum sample. The number of bacteria present was estimated based on the acid fast smearing results. Negative acid fast smearing test is assumed to contain $0-10^3$ acid fast bacilli (AFB)/ml of sputum sample (S_{03}), AFB+ has 10^3-10^4 cells/ml (S_{34}) and AFB++ has 10^4-10^5 cells/ml (S_{45}) [60].

6.3.1. COMPARISON OF NEGATIVE AND POSITIVE CONTROL CULTURES

After 6 weeks of incubation, all *negative* control cultures did not have any bacteria growth. Extracted liquids for *negative* samples appeared colourless. When chemical reagents were added to the extracted liquid, about 2 minutes after, the mixture appeared dark green (Figure 6.9a). Based on previous experiment results, when small niacin concentrations were incubated, absorption levels of the reacted samples were high (see Section 6.2). Therefore, hypothetically, since *negative* cultures do not produce any niacin, their absorbance levels are high and independent to the incubation time. Results of this experiment, shown in Figure 6.10a, support this finding. The absorbance level of these samples fluctuates around an average value of 0.9 Optical Density (OD) (Figure 6.10b). This value is the average of the *negative* samples' absorbance from day 1 until 15. All of points lie in $\pm 5\%$ of 0.9 OD range.

In *positive* control cultures, positive *M. tuberculosis* colonies were already visible after 7 days of incubation. Even though *positive* cultures came from the same bacteria suspension, different colony numbers grew in each culture tube. This was attributed to the unavoidable inability in preparing homogenous bacteria suspension. Due to the similar environmental conditions from where they lived, *positive* control colonies were visible faster than other culture samples. This is because the *positive* control samples require less time to adapt to the new media environment.

Figure 6.9. Colour developed for extracted liquid after added with reagents

(a) *Negative Culture*, (b) S_{03} Culture, (c) S_{34} Culture,
(d) S_{45} Culture, (e) *Positive Culture*

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(a)

(b)

Figure 6.10. Absorbance level of *negative* and *positive* culture using RC-Sensor

(a) Averaged graph, (b) Trend line graph

As with *negative* control samples, extracted liquid from *positive* ones appeared colourless. However, the reacted samples (extracted liquid that has been reacted with chemical reagents) on day 1, 3 and 4 turned from colourless to green. The reacted samples from *positive* after 5 incubation days appeared to be less greenish compared to those of day aforementioned. On day 7, some of the reacted samples were light green or slightly yellow (Figure 6.9e). After 15 days of incubation, all reacted samples for *positive* cultures became yellow. Different greenish levels and colour changes that occurred correlates to the absorption levels detected by the sensor (Figure 6.10). Figure 6.10 is the average data of five samples for each testing day.

Absorption value for *positive* samples on day 1, 3 and 4 presents differences of 0.1, 0.14 and 0.1 OD from the *negative* samples, respectively (Figure 6.10a). These changes are considered significant because they do not lie within the standard deviation range of the *negative* samples. This absorption differences indicates that niacin has already been present in the extracted liquid detected since day 1. The presence of niacin in the early incubation period may have been related to the amount of niacin that was transferred from the previous culture when the *positive* sample suspensions were prepared.

Absorbance levels on day 5 and 7 decreased by 20 and 50%, respectively, compared to the absorption level on the first three data points. The drop in absorbance levels are related to the increase of niacin concentration produced by *M. tuberculosis*. On day 15, *M. tuberculosis* cultures had synthesized a significant amount of niacin that was large enough to cause the reacted sample to be yellow in colour. Thus, the absorbance level increased as detected by the sensor.

A trend line for *positive* samples data points is plotted to assist the observation (Figure 6.10b). Niacin levels for the first three points, on day 1, 3 and 4, are assumed to have the same value because standard deviation of both points are within the same Optical Density range of 0.7-0.81 OD. Based on the niacin production calculation (equation 4.6 and 4.7), the amount of niacin produced on day

3 and 4 has a difference of 5×10^{-8} μg . This niacin concentration difference is not significant enough to be picked up by the *RC-Sensor*. The trend line shows that the result of this experiment has the same profile as the previous experiment results (presented in Section 6.1.2 and Section 6.2). It also shows that the ideal operating time of the system is up to day 11 (indicated by the deflection point in the graph). Hence, the experimental results of day 4 to 5 days are valid.

Variations of the absorption level occurred because of the following reasons:

1. Different number of bacteria that was present in each test tube.

The different numbers of bacteria present in each culture could not be controlled due to the lack of facilities to really dilute colonies in the distilled water. There were small bacteria colonies floating that might cause the different number of bacteria present in each culture tubes.

2. Inconsistency of reagents' volume.
3. Inconsistency in extracted liquid volume.

Volume inconsistencies could be overcome if the experiments were conducted with apparatus with high precision and accuracy. For example the use of a scaled pipette will limit volume variations. However, results have shown that even without advanced equipments, the sensor was still able to provide good sensitivity to differentiate the *positive* and *negative* cultures.

6.3.2. COMPARISON OF NEGATIVE, S_{34} AND S_{45} CULTURES

Based on the acid fast smearing results, S_{34} and S_{45} contained 10^3 - 10^4 and 10^4 - 10^5 acid fast bacilli in every millimetre of sputum sample, respectively. The bacteria colonies in S_{34} and S_{45} cultures were not visible when the experiment data were collected from day 0 until day 15. Limited positive *M. tuberculosis* colonies were visible after 6 weeks of incubation period. However, standard negative/positive results still require 6 to 8 weeks of incubation for further results confirmation. Plate counts should have been conducted in order to reconfirm the amount of bacteria cells present in the sputum samples, but it could not be performed because of limited resources and facilities. The colour of the extracted liquid of S_{34} and S_{45} are presented in Figure 6.9c and 6.9d, respectively.

Comparison of S_{34} , S_{45} and *negative* data is presented in Figure 6.11. On day 1, 3 and 4, the absorbance levels of S_{34} and S_{45} did not differ much from the *negative* samples. On day 5, S_{34} had a slight decrease of 5% from the previous days' absorbance levels. However, this drop is not reliable since its standard deviation still intersects with the *negative* ones. On the contrary, absorption level for S_{45} on day 5 (a decrease of 33%) was distinctive, and its standard deviation is not within the *negative* range. As the samples were incubated longer, absorbance intensity of both S_{34} and S_{45} decreased further to 0.58 and 0.42 OD, respectively, on day 15. This absorption decline is an indication of *M. tuberculosis* growth.

The absorption rate of S_{34} was 0.27 times lower than of S_{45} . However, the gradient of both samples were about the same (Figure 6.11b), which means that niacin was produced at the same rate. This result is related to the number of bacteria that is initially present in the cultured specimen. According to the results of acid fast smearing test, the number of bacteria in S_{34} samples are roughly 10 to 100 times less than in S_{45} ones [60].

Results and Discussion: Clinical Experiments

(a)

(b)

Figure 6.11. Absorbance level of *negative*, S_{34} and S_{45} cultures using *RC-Sensor*

(a) Averaged graph, (b) Trend line graph

Results of this investigation demonstrate that the developed *RC-Sensor* is able to detect of *M. tuberculosis* in 5 days, but this is only valid for sputum samples those are S_{45} . S_{34} sputum samples might be able to be detected in 5 days, but it has a lower confidence level. Its standard deviation is 1.6 times larger than S_{45} . Thus, if S_{34} tests are conducted on the fifth day, it is more likely to have false negative results. As such, *RC-Sensor* is estimated to be able to detect *M. tuberculosis* in S_{34} samples in 7 days.

6.3.3. COMPARISON OF NEGATIVE AND S_{03} CULTURES

Based on literature reviews, acid fast smearing technique would give negative results if the amount of acid fast bacteria present in 1 ml of sputum sample is less than 10^3 cells; therefore, leading to false negative results. This would mean that there are high chances that the investigated specimen appears to be negative by acid fast smearing, but positive by culturing method. Hence, AFB negative samples were tested in order to test the sensitivity of the sensor to detect the presence of *M. tuberculosis* in negative acid fast tests but culture positive specimens. The samples were assumed to have a bacteria range of 0 to 10^3 cells.

After 6 weeks of incubation, none of the S_{03} cultures showed any presence of *M. tuberculosis*. This finding indicates that there is not any *M. tuberculosis* present in the investigated sputum samples; therefore, hypothetically, they could be treated as if the samples were negative samples. This means that there would be no increment of niacin level. For that reason, there would be no changes in the absorption level. The extracted liquid after adding chemical reagents appeared to have almost the same colour as the *negative* samples (Figure 6.9b).

Results and Discussion: Clinical Experiments

(a)

(b)

Figure 6.12. Absorbance level of *negative* and S_{03} cultures using *RC-Sensor*

(a)Averaged graph, (b) Trend line graph

Figure 6.12a presents data collected for S_{03} samples. Even after 15 days of incubation, no data points showed any significant difference in absorbance between S_{03} and *negative* samples. All data points fell within the $\pm 5\%$ error range for *negative* samples. Absorption results detected by *RC-Sensor* tallied with the culture growth observations. Because of the non-existence of *M. tuberculosis*, the trend line of this batch of S_{03} samples is shown to be a horizontal line (constant ordinate) at 0.91 OD (Figure 6.12b). This value is the average of all S_{03} data points with a variation of $\pm 10\%$. The average line of S_{03} lies within the *negative* sample range.

6.3.4. COMPARISON OF RC-SENSOR TO CURRENT DETECTION METHODS

Two important parameters of the developed sensor are compared to other systems/methods currently available for *M. tuberculosis* detection. These parameters are: **detection time** and **sensitivity** of the sensor. Detection time of *RC-Sensor* is compared with several other commercial culture systems, i.e. BACTEC, MGIT and conventional culturing method using LJ solid media. BACTEC and MGIT are culturing systems that monitors the level of carbon dioxide produced by incubated samples. BACTEC utilizes radioactive substance, while MGIT does not. Sensitivity of the developed sensor is compared to smearing, PCR and BACTEC detection methods.

Time required for other several culture systems to detect the growth of *M. tuberculosis* is presented in Figure 6.13. The graph shows detection time required to detect $1-10^3$, 10^3-10^4 , 10^4-10^5 and 10^5-10^6 numbers of bacteria present per ml of samples. Graphs for BACTEC, MGIT and LJ are based on Kanchana's work [185]. He compared the detection time of BACTEC 460 TB, BACTEC MGIT 960 and the conventional LJ solid medium for the recovery of mycobacteria from human clinical specimens.

RC-Sensor detection time for negative smear specimens (samples that contains less than 10^3 bacteria cells) and 10^5 - 10^6 is not presented in this graph, because clinical trials were only conducted on 10^3 - 10^4 and 10^4 - 10^5 samples. The AFB negative specimens experimented turned out to be culture negative; hence, the results cannot be used to justify the time needed to detect negative smear (but culture positive) samples. However, *RC-Sensor* detection time can be calculated using the developed niacin production equation (equation 4.6 and 4.7).

Experiment results of 10^3 - 10^4 (S_{34}) and 10^4 - 10^5 (S_{45}) showed that the results could be detected in 7 and 5 days, respectively; which is equivalent to 1.5×10^{-5} μg of niacin. Samples that contain 1 - 10^3 and 10^5 - 10^6 cells produce 1.5×10^{-5} μg of niacin in 11 and 5 days, respectively. Even though the amount of bacteria present in 10^5 - 10^6 samples is 10 times larger than 10^4 - 10^5 , both of their detection time is 5 days. This is to compensate the lag phase of *M. tuberculosis* samples, which is around 5 days. Within this phase, *M. tuberculosis* might not metabolise causing no increase in niacin. Overall, the graph indicates that the sensor is able to reduce the time from a few weeks to a few days.

Figure 6.13. Detection time of mycobacteria samples

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (PPN) of the developed sensor is tabulated based on the clinical results obtained from conducted experiments (Table 6.3). The sensitivity and specificity of the results are overall better compared to the other detection methods. Figures of smear [175], PCR [175], BACTEC [179] were based on the number of cultures that contains mycobacteria, not only *M. tuberculosis* in particular. This is the reason why the specificity of smear is 100%. On the other hand, the data for *RC-Sensor* is specific for *M. tuberculosis* bacteria only.

Table 6.3. Sensitivity, specificity, positive predictive and negative predictive values of several methods

Method	Sample Size (n)	True Positive (n)	True Negative (n)	False Positive (n)	False Negative (n)	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)
SMEAR ^a	192	37	128	0	27	57.8	100.0	100.0	82.6
PCR ^a	183	54	126	2	1	98.2	98.4	96.4	99.2
BACTEC ^b	1685	151	1441	56	37	80.3	96.3	72.9	97.5
RC-SENSOR	25	15	10	0	0	100.0	100.0	100.0	100.0

^a Results obtain from Albay et al [175]

^b Results obtain from Badak et al [179]

RC-Sensor's sensitivity is 1.7 times better compared to acid-fast smearing method. Its sensitivity and specificity are also higher compared to PCR and BACTEC method. More tests are required to validate the *RC-Sensor*'s results, particularly test on smear negative (but culture positive specimens). Despite the minimum numbers of samples tested, results have demonstrated that the developed sensor is able to provide high sensitivity and specificity.

Testing cost is another factor to be considered if the sensor is to be used in developing countries, where tuberculosis cases are most prevalent. The *RC-Sensor*'s single testing cost was estimated based on the cost for sample processing (e.g. decontamination), culture media and chemical reagents, but it does not include the cost of setting up the equipment and making the sensor (Table 6.4). The cost was

estimated based on Singapore's standards. A single sample testing cost was calculated to be S\$3-5.

Table 6.4. RC-Sensor's testing cost breakdown

Item	Cost (S\$)
Sample processing and culturing	2-3
Chemical reagents for the niacin test	1-2
Total cost	3-5

Table 6.5 tabulates cost required by different methods in order to test one sample. Cost for smearing test and molecular methods was obtained from a study conducted by Schulger [186]. Culture and semi-automated liquid culture costs were based on the testing fees required to conduct these test in the RSPT hospital, Indonesia [187]. Although *RC-Sensor* is twice more expensive compared to smearing technique, but it is approximately 1/10 to 1/17 of the cost needed for the semi-automated liquid culture and molecular methods, respectively.

Table 6.5. Testing cost of several methods

Method	Total Cost (S\$)
Smear	2-3
Culture	4-5
Semi-automated liquid culture	50
Molecular Methods	85-170
RC-Sensor	5-6

Based on the time detection, sensitivity results and cost estimation, the developed sensor demonstrates another very promising alternative to aid tuberculosis cases detection in developing countries.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1. CONCLUSIONS

Niacin metabolite was the target of choice for the novel *Mycobacterium tuberculosis* (*M. tuberculosis*) sensor development because of reasons given in the report. Niacin production is reported to give 95% specificity to the presence of viable *M. tuberculosis* cells in the sample [50]. This developed optical sensor for detecting *M. tuberculosis* metabolite was novel in that it applies optical system-cuvette to facilitate rapid detection in niacin identification from patient's sputum specimens.

Four different experiment setups were conducted to investigate the most suitable method for detecting *M. tuberculosis* (i.e. optical probe, extrinsic optical sensor, single decladded fibre sensor and double decladded fibre sensor). The results have shown that only the extrinsic optical sensor and double decladded fibre optic sensor were able to quantify concentrations of niacin in samples with repeatable accuracy. Both setups were able to detect 1/6th of the usual required concentration when using conventional techniques. However, the extrinsic optical sensor had better sensitivity compared to the double decladded fibre optic. Hence, the extrinsic optical sensor configuration was chosen as the basis of the development of the new sensor.

Since niacin spectrum signature varies between 400 and 550 nm wavelengths, the developed sensor had a light source that was operating in the blue wavelength

region, with a maximum intensity at 472 nm. A photodiode was used to detect the absorbance of the sample being tested. The development process of the novel sensor went through three phases: Fibre Optic Sensor (*FO-Sensor*), Slide Cuvette Sensor (*SC-Sensor*) and Round Cuvette Sensor (*RC-Sensor*). Shape of the cuvette differentiates each prototype. An algorithm was developed to correlate the minimum niacin concentration for the sensor to be able to sense spectrum changes and time required for *M. tuberculosis* sample to produce that required amount.

Two clinical trials were performed. The first clinical trial was done to investigate the best culture media that gives the best sensitivity and using the *SC-Sensor* was shown that Ogawa media gave the best and most consistent results compared to Lowenstein-Jensen solid media and Middlebrook broth media.

The second clinical trial was done to evaluate the derived algorithm with the results consistency and also to determine the sensitivity and specificity of the developed sensor. *RC-Sensor* was used to perform the trials because it has the best sensitivity compared to the previous two setups (*FO-Sensor* and *SC-Sensor*). Fifteen acid fast sputum specimens, five negative control sputum specimens and one positive control of *M. tuberculosis* suspension, were tested. Results show the presence of *M. tuberculosis* could be detected within 5 days for positive samples and for samples containing 10^4 - 10^5 *M. tuberculosis* cells and 7 days for samples containing 10^3 - 10^4 *M. tuberculosis* cells.

An experiment protocol was established to assist the sensor's operation (refer to Appendix D). Cost of testing was calculated to be two times higher than that for the smearing process, but it was 17 times cheaper compared to current molecular methods.

Results of this project have demonstrated that the developed sensor is able to detect *M. tuberculosis* 10 times faster compared to the current culture methods. The developed sensor is a novel and promising method to detect tuberculosis. Low

operating cost and maintenance of the portable unit make this new developed sensor affordable for applications in developing countries.

7.2. RECOMMEDATIONS

The following are recommended for future work:

1. Conduct experiments to correlate the incubated niacin in Ogawa media with absorbance levels using the developed *RC-Sensor*.

This experiment will also identify the point of deflection shown in Figure 6.8.

2. Conduct more clinical trials to further confirm the specificity and sensitivity of the sensor.

The sensitivity and specificity of the sensor has only been tested for 15 clinical samples. For a new system to be used clinically, it needs to be tested with hundred samples. Thus, more tests are required to be conducted before the developed sensor can actually be commercialized and be applied as part of *M. tuberculosis* diagnostic method. The sensor should be tested for acid fast smear negative specimens but culture positives.

3. Conduct test on extra pulmonary samples and on patients who went for treatment.

Published studies reported that current detection systems have lower sensitivity for extra pulmonary specimens [188] and samples from patients whom already went under treatment [24]. In this project, all samples are sputum specimens, and only one sample from a treated patient was tested. Hence, for a complete study, the sensor must also be tested with extra pulmonary samples, such as gastric washing and urine.

4. Conduct test to detect multi drug resistance *M. tuberculosis*.

Detection of drug resistance *M. tuberculosis* will take additional 3 to 4 weeks of isolation. Resistant *M. tuberculosis* strains would have the same ability as normal strains to synthesis niacin [189]. Since the sensor has aided a faster detection method, it is not impossible if the sensor is also able to detect drug resistance.

5. Incorporate chemical reagents to be part of the cuvette.

Incorporating the chemical reagents into the cuvette will help to reduce the exposure of the user to the toxicity of reagents. People have successfully incorporated the reagents onto paper strips for *M. tuberculosis* niacin detection [190]. This principle will then be applied for further development of the sensor.

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