MICROCANTILEVER-BASED BIOSENSOR FOR DETECTION OF *Vibrio cholerae* O1

USA SUNGKANAK

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.....

Miss Usa Sungkanak
Candidate

.....

Assoc. Prof. Sarisak Soontornchai,
D.Sc.
Chair

.....

Asst. Prof. Chamras Promptmas,
Ph.D.
Member

.....

Dr. Adisorn Tuantranont,
Ph.D.
Member

.....

Asst. Prof. Auemphorn Mutchimwong,
Ph.D
Acting Dean
Faculty of Graduate Studies
Mahidol University

.....

Assoc. Prof. Virapong Prachayasittikul,
Ph.D.
Dean
Faculty of Medical Technology
Mahidol University

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.....

Miss Usa Sungkanak

Candidate

.....

Asst. Prof. Chamras Promptmas,

Ph.D.

Major-Advisor

.....

Asst. Prof. Kesara Wat-Aksorn

Ph.D.

Co-Advisor

.....

Dr. Adisorn Tuantranont,

Ph.D.

Co-Advisor

.....

Asst. Prof. Auemphorn Mutchimwong,
Ph.D
Acting Dean
Faculty of Graduate Studies

.....

Lect. Ratana Lawung,
Ph.D.
Chair
Master of Science Programme in Medical Technology

Faculty of Medical Technology

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Usa Sungkanak

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OF *Vibrio cholerae* O1

USA SUNGKANAK 4837280 MTMT/M

M.Sc. (MEDICAL TECHNOLOGY)

THESIS ADVISORS: CHAMRAS PROMPTMAS, Ph.D., ADISORN TUANTRANONT, Ph.D., KESARA WAT-AKSORN, Ph.D.

ABSTRACT

Vibrio cholerae O1 is a bacterium which causes severe diarrhea or cholera in humans. Rapid detection is necessary for effective control of this disease but the conventional method for *V. cholerae* O1 detection is time consuming, laborious and has low sensitivity. In this study, a new microcantilever-based biosensor was developed for detection of this food and waterborne pathogen. This biosensor was constructed by immobilizing the anti-*V. cholerae* O1 monoclonal antibody on microcantilever surface using the self-assembled monolayers (SAMs) method.

The 3-Mercaptopropionic acid (MPA) was used as alkanethiol to form a monolayer and to leave the carboxyl group on the microcantilever surface. The antibody was immobilized on this surface by activation of 1-ethyl-3-(3-di-methylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). The empty surface was blocked with bovine serum albumin (BSA) to prevent nonspecific binding.

For the sensing experiment, antibody and antigen (*V. cholerae* O1 cells) binding induced mass loading of *V. cholerae* O1 cells bound onto the cantilever beam surface and caused the shift of resonance frequency of the cantilever beam, which was then measured by atomic force microscope (AFM). The microcantilever-based biosensor was able to detect *V. cholerae* O1 in concentrations ranging from $1x10^3$ to $1x10^6$ CFU/ml. The limit of detection was $1x10^3$ CFU/ml and the mass sensitivity, $\Delta m/\Delta F$, was approximately 146.5 pg/Hz. A scanning electron microscope (SEM) was used to evaluate topography of microcantilever with *V. cholerae* O1 antigen-antibody binding. The new biosensor provides fast and uncomplicated detection steps with high sensitivity, and offers the protection required for public health protection.

KEY WORDS: MICROCANTILEVER / IMMUNOSENSOR / CHOLERA /
VIBRIO CHOLERAE O1 / ATOMIC FORCE MICROSCOPY

95 P.

ไบโอเซนเซอร์ชนิดไมโครแคนติลิเวอร์สำหรับการตรวจวัดเชื้อ *Vibrio Cholerae* O1

(MICROCANTILEVER-BASED BIOSENSOR FOR DETECTION OF *Vibrio Cholerae* O1)

อุษา สังขนาค 4837280 MTMT/M

วท.ม. (เทคนิคการแพทย์)

คณะกรรมการควบคุมวิทยานิพนธ์: จำรัส พร้อมมาศ, ปร.ด., อดิสร เตือนตราสนนท์, ปร.ด., เกศรา วัดอักษร, ปร.ด.

บทคัดย่อ

Vibrio cholerae O1 คือเชื้อแบคทีเรียที่เป็นสาเหตุของการเกิดโรคอุจจาระร่วงอย่างรุนแรงหรืออหิวาตกโรค การตรวจวิเคราะห์เชื้อก่อโรคอย่างรวดเร็วมีความสำคัญต่อการควบคุมการแพร่ระบาดของเชื้อ *V. cholerae* O1 แต่วิธีการตรวจวิเคราะห์เชื้อแบคทีเรียในห้องปฏิบัติการปัจจุบันใช้ระยะเวลายาวนาน มีกระบวนการหลายขั้นตอนแต่ให้ความไวในการตรวจวิเคราะห์ต่ำ ในการศึกษาครั้งนี้ได้พัฒาไบโอเซนเซอร์ชนิดไมโครแคนติลิเวอร์สำหรับการตรวจหาเชื้อ *V. cholerae* O1 กระบวนการทดลองเริ่มจากการตรึงสารแอนติบอดีที่มีความจำเพาะกับเชื้อที่ต้องการศึกษาด้วยวิธีการที่เรียกว่า self-assembled monolayers (SAMs)

สารเคมีที่ชื่อว่า 3-mercaptopropionic acid ซึ่งเป็นสารอินทรีย์ประเภทอัลเคนไธอัลที่มีความสามารถในการเรียงตัวเป็นชั้นเดียวอยู่บนผิวทองและให้หมู่ฟังชั่นชนิดคาบอกซิลิก (-COOH) สำหรับใช้ในการสร้างพันธะชนิดโควาเลนส์กับโมเลกุลของแอนติบอดี ซึ่งในขั้นตอนนี้ต้องการการกระตุ้นจากสารเคมีที่ชื่อว่า 1-ethyl-3-(3-di-methylaminopropyl)carbodiimide hydrochloride (EDC) และ *N*-hydroxysuccinimide (NHS) หลังจากนั้นพื้นที่ที่ยังวางอยู่บนพื้นผิวไมโครแคนติลิเวอร์จะถูกปิดด้วยโปรตีนซีรั่มจากวัว (bovine serum albumin)

ไมโครแคนติลิเวอร์จะถูกนำไปทดสอบกับสารแขวนลอยของเชื้อ *V. cholerae* ปฏิกิริยาระหว่างแอนติเจนและแอนติบอดีจะเกิดขึ้นและสร้างคอมเพลกซ์ที่มีมวลกดทับลงบนไมโครแคนติลิเวอร์ ซึ่งมีผลทำให้ความถี่ในการสั่นของไมโครแคนติเวอร์ลดลงซึงตรวจวัดได้ด้วยเครื่อง Atomic force microscope (AFM) ไบโอเซนเซอร์ชนิดไมโครแคนติลิเวอร์มีความสามารถในการตรวจวัดเชื้อ *V. cholerae* O1 ในช่วงค่าความเข้มข้นที่ $1x10^3$-$1x10^6$ CFU/ml ค่าความเข้มข้นต่ำสุดที่วัดได้คือ $1x10^3$ CFU/ml และความไวในการตรวจวัดมีค่าประมาณ 146.5 พิโคกรัม/เฮิร์ต ไมโครแคนติลิเวอร์ที่ผ่านการทดสอบกับเชื้อแบคทีเรียแล้วจะถูกนำไปถ่ายภาพพื้นผิวด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบสแกนนิ่งเพื่อยืนยันการเกาะของเชื้อ *V. cholerae* O1 บนผิวของไมโครแคนติลิเวอร์ จากผลการทดลองพบว่าไบโอเซนเซอร์ชนิดใหม่นี้ให้การทดสอบที่รวดเร็วด้วยวิธีการตรวจที่ง่ายและมีความไวสูง เหมาะสมสำหรับการนำไปใช้เป็นเครื่องมือในการควบคุมการแพร่ระบาดของเชื้อ *V. cholerae* O1 ตามความต้องการของกระทรวงสาธารณสุข

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LIST OF ABBREVIATIONS

Ab	=	antibody
AFM	=	atomic force microscope
Ag	=	antigen
ATR	=	attenuated total reflection
BSA	=	bovine serum albumin
CFU	=	colony forming unit
^{o}C	=	degree Celsius
DI	=	deionized water
DW	=	distilled water
DNA	=	deoxyribonucleic acid
EDC	=	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride
ELISA	=	enzyme-linked immunosorbent assay
Fab	=	fragment antibody
F_c	=	fragment crystallizable
g	=	gram
h	=	hour
Hz	=	hertz
H_2O_2	=	hydrogen peroxide
Ig	=	immunoglobulin
IgG	=	immunoglobulin G
kHz	=	kilohertz
L	=	liter
µl	=	microliter
mg	=	milligram
ml	=	milliliter
mm	=	millimeter

LIST OF ABBREVIATIONS

(Continued)

mM	=	milimolar
min	=	minute
MIP	=	molecularly imprinted polymer
MHz	=	megahertz
MPA	=	3-mercaptoproprionic acid
ng	=	nanogram
NHS	=	*N*-hydroxysuccinimide
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction
pg	=	picogram
psi	=	pound per square inch
QCM	=	quartz crystal microbalance
RNA	=	ribonucleic acid
SAM	=	self-assembled monolayer
SAW	=	surface acoustic wave
SEM	=	scanning electron microscope
SPE	=	screen printed electrode
spp.	=	species (plural)
SPR	=	surface plasmon resonance
µL	=	micrliter
µm	=	micrometer
V	=	voltage
V/V	=	volume by volume

CHAPTER I
INTRODUCTION

This thesis address challenges at the intersection between microelectromechanical system (MEMS) and biotechnology for developed a new *Vibrio cholerae* O1 biosensor.

Vibrio cholerae O1 is a gram-negative bacterium, which is identified as a food and waterborne pathogen that causes life-threatening cholera or severe diarrhea in tropical region. Enterotoxin or cholera toxin produced by *V. cholerae* O1 is a causative of watery diarrhea when human intake of food contaminated with *V. cholerae* O1. The toxin acts on mucosal epithelium of intestine and is responsible for the characteristic of severe stool rice water diarrhea. Rapid lose of water and electrolyte bring the infected person get dehydration and consequence to other symptoms such as acidosis, volumetric shock and eventually become to death if no treatment provided. Cholera has been seven times explored to the world, including Thailand and other Asian countries. Nowadays, the sanitation of Thailand (safe water and sewage treatment system) under controlling of the public health organization has been developed, affected to decreasing a number of cholera reported cases. A report of cholera is rarely from any part of Thailand. However, it is still remain and has possibility to spread at any time. Rapid detection of the actual vehicles of cholera and block them immediately is a good way to prevention and control the epidemic and endemic outbreak of this disease.

The conventional method of bacteria detection in clinical microbiology laboratories relies on microscopic examination and biochemical identification. Although, these techniques are inexpensive and give both quality and quantitative information, they are gently restricted by assay time which is requiring at least 2-3 days, laborious, insensitive and initial pre-enrichment was needed. Other monitoring techniques have been developed for detection bacteria including enzyme-linked

immunosorbent assay (ELISA) and polymerase chain reaction (PCR) technique. These techniques have provided high sensitivity detection of pathogens in food products but they are still requiring pre-enrichment and the process of detection is complexity. An alternative way for bacteria detection is using new technologies of biosensor. Many portable biosensors have been developed and used for detection several of biological substances including blood glucose, enzyme, and also microorganisms. The biosensor is providing high sensitivity and specificity of detection by easily to handle. Moreover, biosensor in portable design can possibly used in field study or in remote area.

Microcantilever-based biosensor is a new system of biosensor has been explored in the past decade. Utilizing of microcantilever as a microelectronic transducer was reported in 1994 where Gimzewski and colleague form IBM published a paper on detection of heat produced by the catalytic conversion of H_2 and O_2 to H_2O over a platinum coated cantilever surface. Currently, many of biosensor-based biosensor has been proposed for detection of biomolecules including proteins, nucleic acid sequences and microorganisms. Combining of micocantilever with atomic force microscope (AFM) can be operated into two modes; static mode and dynamic mode. In static mode, the static bending of the cantilever is observed by detection of cantilever deflection of surface stress. In dynamic mode, the mass loading related to vibration properties, for example changes in resonance frequency.

This thesis work presents the development of a new biosensor system for detection of *V. cholerae* O1 which consists of microcantilever sensing layer immobilized with a monoclonal antibody. The antibody will be immobilized on gold-coated microcantilever surface by using self-assembled monolayers (SAMs) method. The antibody-immobilized microcantilever will be operated in dynamic mode that can be measured the resonance frequency by using optical method of the atomic force microscope (AFM). The resonance frequency shift of microcantilever after bacteria detection relates to the mass of *V. cholerae* O1 bound on the microcantilever. Finally, characterization of the sensing layer will be examined by scanning electron microscope (SEM). This sensing device will give advantages to clinical microbiology laboratories which is good for prevention and control endemic of cholera.

CHAPTER II
OBJECTIVES

1. To find out the appropriate method for immobilization of *Vibrio cholera* O1 specific antibody on microcantilever surface.
2. To develop microcantilever-based immunosensor for detection of *Vibrio cholerae* O1.
3. To investigate the effected factors to microcantilever-based sensor system.

CHAPTER III
LITERATURE REVIEW

This chapter is divided into six main sections. The first to the third section were explained about biosensor and biosensor technologies, component of the biosensor and immobilization technique use in biosensor development. The forth section is the usage of microcantilever in biosensor applications. In the next section is described biosensor for detection of microorganisms and the last section is the usage of microcantilever based-biosensor for detection of *Vibrio cholerae* O1.

1. Biosensor and Biosensor Technologies

Biosensor is an analytical device that utilized the biochemical reactions mediated by molecule of the biological recognition element to detect the analyte target molecules. The principle of biosensor was shown in Figure 1. The biochemical reactions are affected to the changes of transducer physical properties and provide measurable signals, usually, electrical signal. Thus, the biochemical signal, immeasurable signal could be converted into the measurable signal by the transducer. There are two main components of biosensor; the first one is a sensing material of the biological recognition elements (e.g. enzymes, antibodies and antibody fragments, nucleic acids, tissues, receptors or microbial cells), and the other component is a transducer (e.g. optical, acoustic, electrochemical or thermal). These two components are combined or integrated into one compact unit by immobilization technique [1]. The International Union of Pure and Applied Chemistry (IUPAC) definite the word biosensor as [2]:

"*Biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with a transduction element.*"

The term of biosensor is also often used in order to determine the concentration of biological substances and other parameters of biological interest even where they do not utilize a biological system directly.

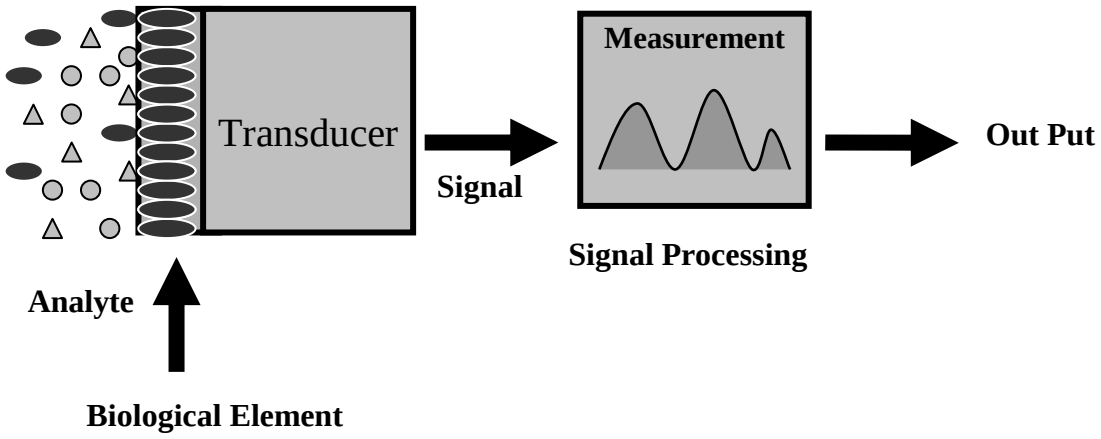


Figure 1. Principle of biosensor; when the target of analytes binds to the biological recognition element (receptor) molecules on the transducer, the transducer will convert the biological signal into the measurable electrical signal.

In 1956, Professors Leland C. Clark Jr., who is known as the father of the biosensor, was developed the first sensor, the Clark's oxygen electrode. He described the fabrication of electrochemical biosensors. For the Clark's electrode, the glucose oxidase (GOD) enzyme was entrapped in a dialysis membrane. The method of detection is based on decreasing of the oxygen concentration which is proportional to the glucose concentration. His idea of the glucose sensor based on amperometric detection became the commercial reality in 1975. Based on this experience, he expand the development of biosensor for measuring several biomolecules in the human body such as glucose, hormone and vitamin in the blood sample [3]. Guilbault and Montalvo were the first who provide a detail description of potentiometric enzyme electrode. The usage of thermal transducer for biosensor has been purposed in 1974. In 1985, Vadgama and Davis suggested that bacteria could be used as biological element for biosensor application and Lubbers described the using of fiber optic in biosensor [4].

Biosensor technologies have been developed for over 35 years. The technology should meet several criteria; the biosensor should be user friendly, inexpensive, easy to use, sufficiently sensitive and specificity, accurate, reliable, non-destructive, real-time measurement, rapid turn around time, high throughput and easily manufactured with high rates [5, 6].

Applications of biosensors are applied more and more in different fields. They are expected to play an increasingly important role in the improvement of life quality. [7]. The following list describes some of the current applications [8]:

- Clinical diagnosis and biomedicine
- Farm, garden and veterinary analysis
- Process control: fermentation control and analysis
- Food and drink production and analysis
- Microbiology: bacterial and viral analysis
- Pharmaceutical and drug analysis
- Industrial effluent control
- Pollution control and monitoring
- Mining: industrial and toxic gases
- Military: biological warfare

The prevalence of commercial biosensor was summarized in Table 1. Biosensors are involved in the area of the commercial area and R&D activities at academic, private or public centers. Most of them are concerned in medical clinical markets. In hospital, there is a continued need for medical biosensor based device that are used for real-time point-of-care testing or monitoring of glucose, blood gases, and other vital signs. Johnson & Johnson Company is currently the market leader in blood glucose self-testing, launched its first product, the Fast*Take*™ system, in April 1998. BAYER company (Leverkusen, Germany) is also launched a glucose sensor, the Glucometer™. Abbott Laboratories (Abbott Park, USA) market launched the ExacTech™ and Precision QID™ for detection of blood glucose and ketone, and Roche Diagnostics (Basel, Switzerland), launched the ACCU-CHEK™ [9]. Future market of clinical biosensor includes the screening of genetic diseases and oncogenes. In addition, exploring of microfabrication technology will be developed miniaturized biosensor for variety of biosensor applications [5].

Table 1. Commercial biosensor products

Company	Product Name	Biological Targets
Abbott Laboratories	Precision QID™ Precision Xtra™ ExacTech™	Blood glucose and blood ketones (β-hydroxybutyric acid, the predominant ketone body)
Bayer	Glucometer™	Glucose
BIACORE	BIACORE3000	General purpose SPR
Cygnus, Inc.	Glucowatch	Glucose
Genoptics Plasmonic	SPRiLab™	General purpose SPR
HemoCcue AB	Hemocue	Glucose, hemoglobin, urine
Johnson & Johnson	Induo	Glucose
MediSense	Optium	Glucose, Ketone
Nissin Electric	BOD-3000	BOD
Polymer Technology System Inc.	BioScanner2000, CardioCheck	Glucose, total cholesterol, HDL cholesterol, triglycerides and blood ketones
Roche	ACCU-CHEK™	Glucose
Smiths Medical MD	The Deltec CoZmonitor	Glucose sensor with insulin pump
Syntron Bioresearch Inc.	Urige-8	Bilirubin

2. Biosensor Components

As describes in the first section, the biosensor consists of two main components; the sensing materials or biological recognition elements and the transducers. These two components are combined or integrated into one compact unit by helping of immobilization techniques. Many kinds of biological recognition elements used in biosensor. They can be classified into two systems; the biocatalytic recognition system and the affinity recognition system. The biosensor transducers also have several types. There are different in detection systems and they have their own properties that providing both good and bad advantages. To chosen of suitable type of the biological

recognition elements and the transducers will conducted the good sensitivity and specificity biosensor.

2.1 Biological Recognition Elements

The biological recognition elements can be sub-classified into two systems; the biocatalytic recognition systems and the affinity recognition systems (Figure 3.). The biocatalytic recognition system element is the origin of the sensing element used in biosensor area which consists of the molecules that have catalytic activity including, enzymes, microbial cells and tissues. The affinity recognition system is the next generation of biological recognition element. However, they are providing effective used in biosensor applications. This system is including antibodies, nucleic acids, and other synthetic molecules that containing affinity property, such as aptamers, and molecularly imprinted polymers.

2.1.1 Biocatalyic Recognition Systems

The biocatalytic recognition system is consisted of enzymes, microbial cells and tissues which are described as the following paragraphs;

2.1.1.1 Enzymes

The enzyme-based biosensor provides a wide variety of measurable reaction products arising from the catalytic process including protons, electron, light and heat [10]. Glucose biosensor is the most widely used the enzyme as recognition element and it has successful for clinical relevance of diabetes. Currently, most glucose biosensor utilizes glucose oxidase as their recognition element that catalyzes the oxidation of glucose to gluconolactone as shown in reaction (1).

$$\text{Glucose} + O_2 \rightarrow \text{gluconolactone} + H_2O_2 \qquad (1)$$

In above reaction scheme, the dominant reaction approach is electrochemical in nature. The hydrogen peroxide (H_2O_2) is a product of enzymatic reaction, which is detectable by the using of intermediary compounds, so called mediators, e.g. organic dye such as Prussian Blue and inorganic redox couples. The mediators serve as an electron sinks [10]. The electrochemical mediators shuttles redox equivalents between the recognition element and the transducer. This supplementary step in the biosensing chain usually results in the increase of the

biosensor's selectivity and sensitivity [7]. In additional, the enzymatic biosensors are providing wide variety of sensing devices in quantitative information.

2.1.1.2 Microbial Cells and Tissues

The whole cell microorganisms (bacteria and yeasts) were suggested as a biological substance for biosensor in 1975. The viable microbial cell localized on the transducer is a terrific sensor and offers the information on cell signaling. The signal from microorganisms have been transduced include the assimilation of organic compounds, changes in respiration activity, the production of electrochemically active metabolites, bioelectric responses and metabolically-related pH or thermal responses which is unobtainable by other method. The whole cell biosensor became commercial microbial biosensor devices such as a microphysiometer [11]. Many of genetically modified to produce a recombinant microorganisms that exhibits a number of important traits, e.g. expression of cellular degradative enzymes, specific binding protein and receptor enzymes which is induced in the presence of the target analytes [12].

The higher eukaryotic, plant and animal cell lines have been typically used in and intact tissue slide from later than as single cell populations. For example, plant tissue utilized for prototype biosensors thus far include banana, beet corn kernel, squash jack bean potato, cucumber and eggplant tissue, as well as, *in vitro* cultured tobacco callus tissue. In the first animal tissue based biosensors, kidney, mucosal and lung were exclusively utilized. More recently, olfactory receptor and rabbit thymus have been reported.

Since microbial cells and tissues continuously monitor and respond to their constantly changing extracellular, it was a reason that the intact microbial cells and tissues may eventually be proven to be the ultimate biological sensing elements for hybrid biosensor design.

2.1.2 Affinity Recognition System

The affinity biosensors were used for monitoring various biological recognition elements. This kind of sensor is explored after biocatalytic biosensors. There is generated real-time information about the binding of antibodies to antigens, cell receptors to their ligands, DNA and RNA to nucleic acid with a complementary sequence [1].

2.1.2.1 Antibodies

Antibodies or immunoglobulin (Ig) are protein produced by the immune system. They have the function of high specifically recognizing specific structure of a target protein, the antigens (molecules, hormone, virus, bacteria etc.). Antibody-based recognition element combined with optical or electrochemical provide immunosensors that can be detecting analytes in sub-nanomolar concentration [8]. The subclass IgG produces in great numbers against foreign intruders in the body of a mammal which represented schematically as Y-shape structure (Figure 2). The IgG molecule composes of two identical F_{ab} (Fragment antibody) portion hinged to the F_c (Fragment crytallizable) portion. The binding sites for specific antigen are located on variable region of F_{ab} fragments, two binding sites per antibody.

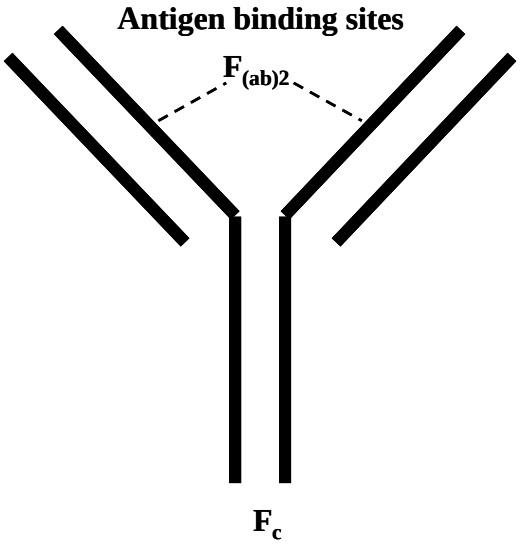


Figure 2. Schematic drawing basic structure of an IgG molecule

The F_c fragment contains carboxy-terminal amino acid which allows linkage to solid substrates like transducer, and this fragment does not combine with the antigen. Currently, antibody-based biosensors (immunosensors) are applied in two different formats: small-sized devices for a single defined analyte and simultaneous detection of vary large number of different proteins with protein chips [9]. The major advantages of immunosensor are high specificity, versatile, and bind strongly and stably to targets (antigens). However, it is difficult to produce large quantities of

antibodies to multi-target biosensor applications where many ligands are needed. In deed, the lack of easily synthesized high-affinity ligands is a major limiting factor for development of high throughput biosensor based detection system [10, 13, 14].

2.1.2.2 Nucleic Acids

Utilizing of nucleic acids have been conducted by the complementary relationships between adenosine (A) and thiamine (T) and cytosine (C) and guanine (G) bases in DNA which is form the basis of specificity in nucleic acid-based biosensors. These sensors are capable of detecting trance amount of targets by comparing a target strand and complementary strand DNA. By unwinding the target DNA strand, adding the DNA probe, an annealing the two strands, the probe will hydrolyze to the complementary sequence on the adjacent strand. If the probe is tagged with a fluorescent compound, then this annealing can be visualizes under the microscope. For accurate analysis, polymerase chain reaction (PCR) is often used to create multiple copies of the same DNA [15]. The detection of specific DNA fragments by hybridization with complimentary strands has gained considerable interest because of its important to the early diagnosis of diseases, such as cancer, hypercholesteremia, and so on [9]. However**,** there is offers several drawbacks including the requiring of sample preparation, and the process of detection should be well-controlled of the contaminations.

2.1.2.3 Synthetic Molecules

Recently, two new classes of semi-synthetic and fully synthetic binding molecules: high affinity RNA aptamer and molecularly imprinted polymers (MIPs) have been started [16]. There are offer and alternative approach involving the use of artificial biomimetic recognition systems [17]. The aptamer generated by random synthesis of RNA nucleotide. The first biosensor using an aptamer as the recognition element was described by Kleinjung *et al* [18]. The MIPs can be synthesized by forming a polymeric network around the template and then created structure complementary of the analyte [19]. Application of MIPs is increasing number of analyte in biosensor, e.g. D- and L-amino acids, atrazine, cholesterol, ephedrine, diazepam, and morphine [20]. However, both of these two kind of synthetic molecules are still exhibit drawbacks with regard to affinity, cross-reactivity, and unspecific binding.

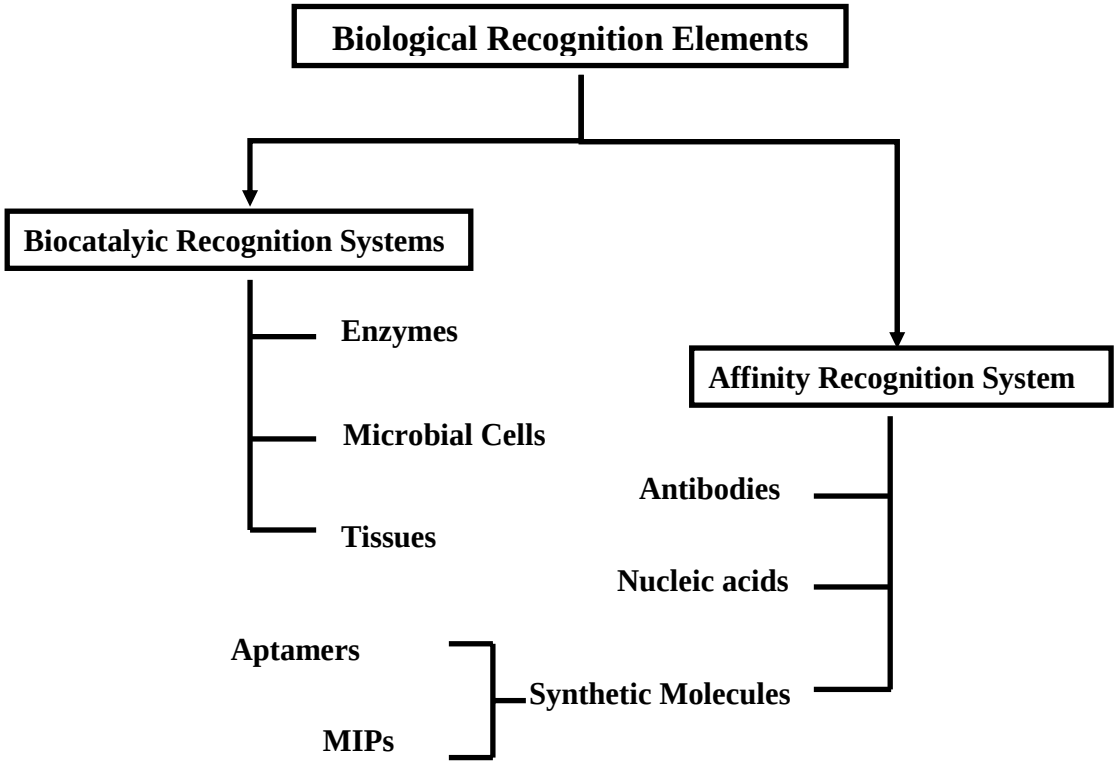


Figure 3. Biological recognition element in biosensor applications

2.2 Biosensor Transducers

2.2.1 Electrochemical

Electrochemical-based biosensor has been used to determine the concentration of various analytes in the testing samples. Electrochemical sensor is a normally based on enzymatic catalysis of a reaction that produces or consumes electrons. The sensor substrate usually contains three electrodes, a reference electrode, an active electrode and a sink electron. The sensor measure the change in current produced from oxidation and reduction reactions. This current produced is correlated to either the concentration of the electro-active species present [15]. Several electrochemical techniques can be applied for analytical purpose, including potentiometric, amperometric, and conductimetric. The system of amperometric detection has been demonstrated to be the most suitable means for immunosensor construction due to their high sensitivity, low cost and possibility of instrument miniaturization [21]. Electrochemical sensors are frequently used in medical and environmental analysis.

2.2.2 Optical

Optical-based biosensor systems are the most diverse class of biosensors. They employed a number of techniques to detect the presence of the target analyte and are based on well-founded method including chemiluminescence, light absorbance, fluorescence, phosphorescence, light polarization, and rotation [15]. Among optical methods, surface plasmon resonance (SPR) is currently the most used technique (Figure 4). The SPR sensor system was used based on the Kretschmann geometry of the attenuated total reflection (ATR) method and spectral investigation of SPR condition. Others optical-based techniques are fiber optics and evanescent wave.

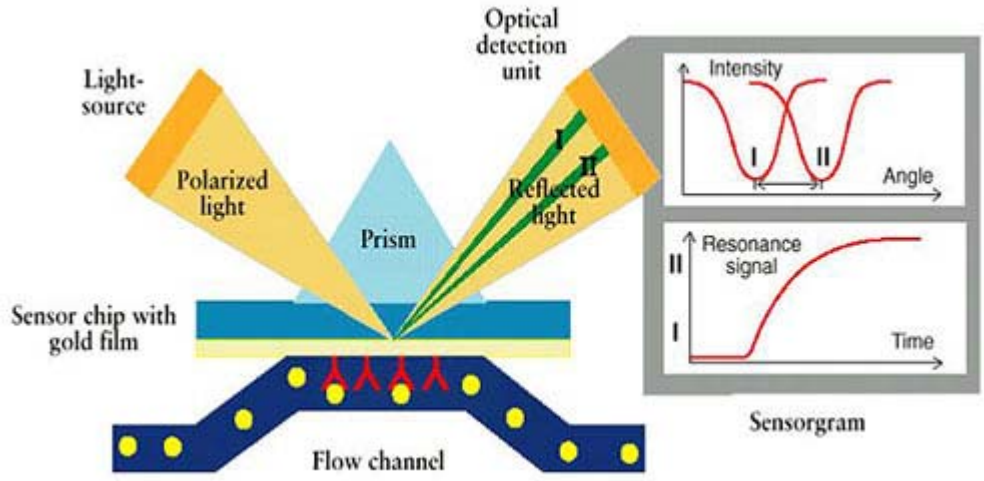


Figure 4. Principle of surface plasmon resonance (SPR)**;** the light from the light source is focused through the prism on sensor surface, giving an incident light angles that monitored by a sensitive diode, and computer interpolation algorithms determine the angle of the SPR.

2.2.3 Piezoelectric

Piezoelectric-based biosensors are containing piezoelectric crystals of quartz materials. Detection of target analytes can be detected by detection of the small mass changes caused by biochemical binding to a piezoelectric crystal. Initially, a specific electrical signal can be applied to the crystal to cause them to oscillate at its resonance frequency. This frequency of oscillation depends on the electrical signal frequency and

the mass of the crystal. As such the binding of the target of analytes will increase the mass of the crystal and subsequently change its resonance frequency, which can then measured electrically and used to determine the mass of the analyte of interest bound to the crystal. The example of piezoelectric biosensor is quartz crystal microbalance (QCM) sensor as shown in Figure 5. The QCM has been introduced by Sauerbrey in 1959 as a new method for mass sensing. Starting from the gas sensor, nowadays it is a transducer that widely used in sensor applications for detection of various chemical and biological species.

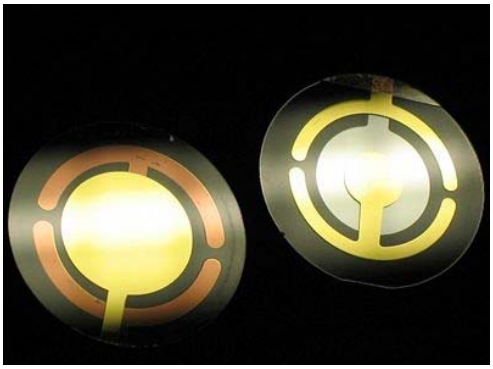


Figure 5. Quartz crystal for piezoelectric biosensor

3. Immobilization Techniques

Immobilization is an important step used to bind or integrate the biological recognition element with the transducer surface to generate a sensing unit. Two main types of immobilization techniques are physical adsorption and chemical adsorption. For choosing technique of immobilization, it should be considered to the type of transducer or substrate surfaces used (gold, silver, silicon or polymeric surface) and the types of biological elements want to immobilize. Some kind of biomolecules, such as protein, can be immobilized in both physical adsorption and chemical adsorption method but some molecules, such as nucleic acids and enzymes, need helping of organic solvents or supporting polymer. Silicon or silicon nitride coated with gold is most of material used in biosensor. Gold surface provides sulhydryl group (-SH group) that available for simultaneous packed of alkanethiols and provide the amide groups available for covalent bonding with protein. Chemically binding of protein

onto silica or silicon dioxide surface need organic silanes treated with activation of the cross-linking agent. The microbial cells can also immobilized by physical adsorption, chemical binding and entrapment. Moreover the information of biomolecules about size, pI, amino acid composition, pH stability, and possible site for oriented coupling also has to know for potential immobilization. These following paragraphs show the information of each method.

3.1 Physical Adsorption

Physical adsorption is a simplest way to immobilize molecule of protein onto the solid substrate surface. This technique is relied on non-specific physical interaction of the protein and the substrate surface without any reagent treated, causes little or no conformation change of protein structure. The binding is mainly cooperated with the hydrogen bonds, multiple salt linkages, and van der Waal's forces that similarity to the situation found in biological membrane *in vivo* system. However, it has the disadvantage that the adsorbed molecules may leak from the substrate or the material during use due to the weak binding forces between the protein and the substrate, and immobilization by nonspecific adsorption may require a long incubation time (up to 12 h)

3.2 Entrapment and Encapsulation

Entrapment of biological recognition element within 3-D gel matrix is a common method of immobilization of microorganisms and enzymes. Numerous entrapment media have been employed but the most favored are polyacrylamide, calcium alginate and gelatin. Entrapment is a simplest way of immobilization and can be applied for any enzymes. The method of protein entrapment is conducted by mixed the protein molecules with monomers/polymers and cross-linking agent in solution. The solution is then exposed to polymerization promoters to start the process of gel formation.

The encapsulation of biological recognition elements in microenvironment, especially in liposome, has developed to greatly improve protein stabilization against unfolding, denaturation, dilution effects. Liposomes are microscopic, fluid-filled pouches whose walls are made of layers of phospholipids identical to the phospholipids that make up cell membranes as shown in Figure 6. There are several technique to form a microcapsule of liposome such as centrifugation.

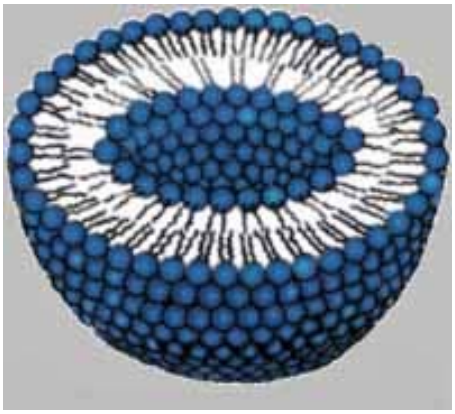


Figure 6. Structure of phospholipid liposomes

3.3 Chemical Adsorption

Covalent binding techniques provide high stability form of immobilization, based on chemically modification between the reactive functional groups of on both the substrate and the biomolecules needed to immobilize. If the biomolecules are protein, there have varieties of functional groups for covalent binding such as lysine amino groups, cycteine thiol groups, tyrosine phenolic groups, arginine guanidine groups, histidine imidazole groups, cysteine disulfide groups, tryptophan indole groups, methionne thioester groups and serine and threonine hydroxyl groups. The main idea is that the reactive groups for covalent binding must be outside the active biological activity or the binding site of immobilized protein and the limited of the covalent binding is the reaction that has to be done under the conditions that do not cause loss of biological activity. Immobilization methods for biological recognition element molecules on the transducer surface using covalent binding are described in the following paragraph in the section of self-assembled monolayers. The critical point is the substrate surface must be chemically modified to provide the reactive groups available for subsequent immobilization steps.

3.3.1 Self-Assembled Monolayers (SAMs)

The self-assembled monolayer (SAM) is one kind of immobilization by the covalent bonding which consists of a single monolayer form of chemisorbed organic substances on solid substrate by spontaneous organization. The self assembled monolayers have been composed from different types of simple organic molecules,

such as alkanethiols and silane compounds on different substrate such as on metal oxide and gold surface substrate. The SAM system is extreme versatile and can provide a method for *in vitro* development of bio-surface [22].

3.3.1.1 Silanization

Silanization is a method to form a monolayer of silane compounds on glass or silicon oxide surface. Silane compounds are formed a monolayer on hydroxyl (-OH) functional groups bearing oxide surfaces through chemical reaction of silane compounds. Silanization is promising for practical applications, because its monolayer is markedly stable due to the strong immobilization through siloxane (–Si-O-Si-) bonding**.** Three main groups of silane compounds are aminosilanes (i.e, 3-aminopropyl-triethoxysilane (APTES), 3-aminopropyl-diethoxy-methylsilane (APDEMS), 3-aminopropyl-dimethyl-ethoxysilane (APMES)), glycidosilanes (i.e. 3-glycidoxypropyl-dimethyl-ethoxysilane (GPMES)), and mercaptosilanes (i.e. 3-mercaptopropyl-methyl-dimethoxysilane (MPDMS)). Mica, glass, and metal oxide surfaces can all be silanized, because they contain hydroxyl groups which attack and displace the alkoxy groups on the silane compounds and form a covalent bond of silanized surface as shown in Figure 7 [23].

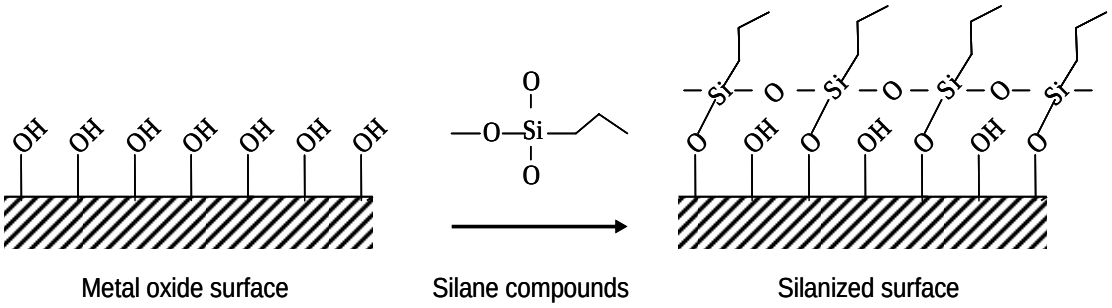


Figure 7. Silanization method of the silane compounds on the metal oxide surface.

3.3.1.2 Self-assembled of Alkanethiol

The self-assembly process on gold is initiated by the strong chemical interactions between the sulfur and gold surface caused monolayer depicted as perfectly aligned closely packed alkane chains, attach to a smooth surface. This interaction is considerate a result of chemisorption that forced a thiolate molecule to adsorbed with a gold lattice. Then the tail-to-tail interaction of the molecules created

by lateral interchain non-bonded interactions, such as by van der Waal's forces steric, repulsive and electrostatic forces, is strong enough to align the molecule parallel on the gold surface and create a monolayer. A self-assembled monolayer forms very rapidly on the substrate but it is necessary to use adsorption time of 15 h or more to obtained well-ordered [24].

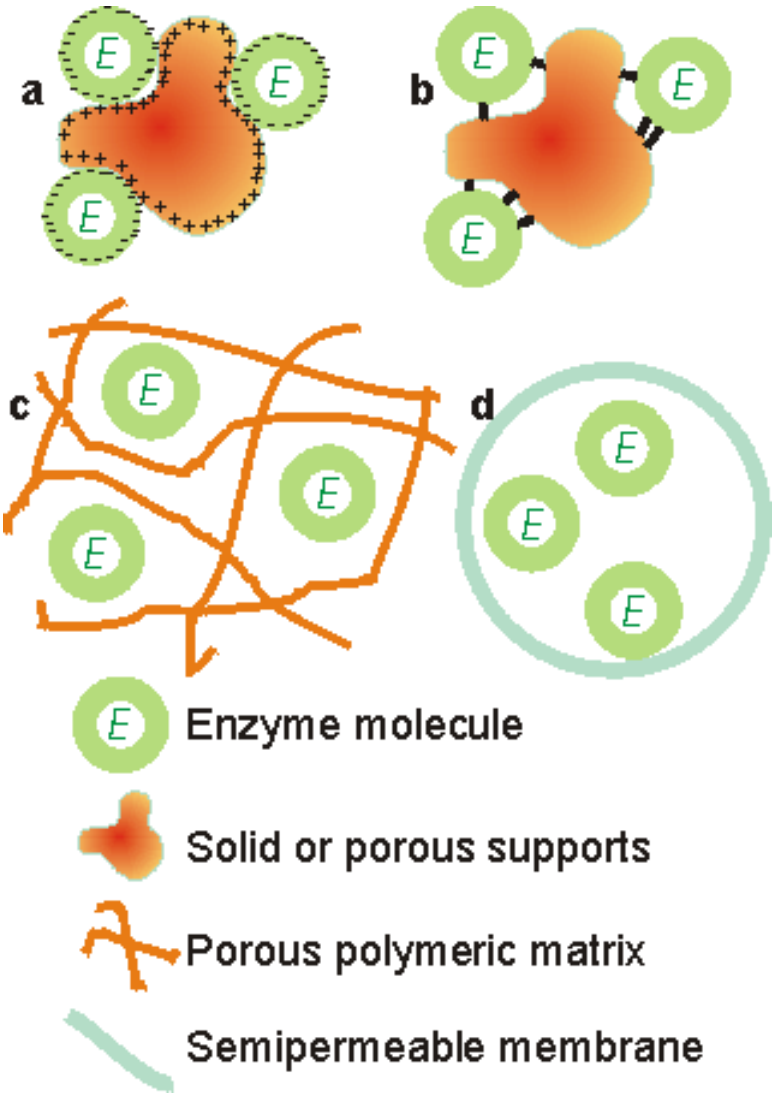


Figure 8. Enzyme immobilization by physical and chemical adsorption; (a) enzyme adsorption on insoluble particle surface, (b) chemical adsorption by covalent bonding, (c) enzyme entrapped within an insoluble particle by a cross-linking polymer (d) enzyme encapsulated within a semi-permeable membrane.

4. Microcantilever-based Biosensor

In the mid-1980s, microcantilever first introduced as a touches element of atomic force microscopy (AFM) to image the surfaces both in air and under liquids [25]. Microcantilever is a particularly of microelectromechanical (MEMS) structure that fabricated as a long beam of silicon, silicon nitride or silicon oxide in micrometer scale [26]. Silicon nitride cantilevers can be produce thinner than other materials which gives them more flexibility and lower stiffness [27]. Several shapes and dimensions of microcantilever have been reviewed as shown in Figure 9. but the common shape is a rectangular [28]. The dimensions of microcantilever are a few hundred micrometers in length, 50-100 micrometers in width and the thickness can be less then a micrometer. Nowadays, microcantilevers have demonstrated their potential to be used as high sensitive sensors [29].

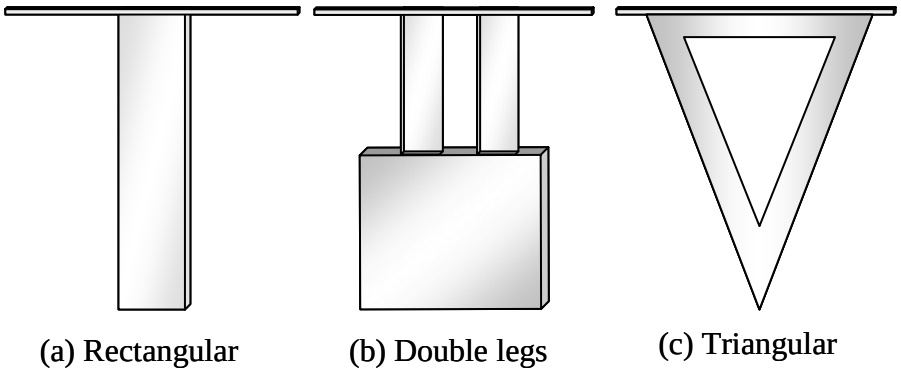


Figure 9. Different designs of microcantilever shape for various applications

Very sensitive cantilever-based biosensors can be constructed from cantilevers coated with biological recognition molecules which are capable to bind target of interest. The binding causes the cantilever beam to bend or change in oscillation property. These changes can be monitored as a resistance change and resonance frequency shift, responsively.

4.1. Operation Modes

Microcantilever is a device that can act as physical, chemical or biological sensor by detecting change in cantilever bending or cantilever oscillating. There are two

different operation modes remain common for all different applications, the static mode and the dynamic mode**.** Figure 11 shows the detection modes using cantilever sensors. Two main of operation mode: static mode and dynamic mode.

4.1.1 Static Mode

In static mode, external influences and chemical/physical reactions on one side of microcantilever surface caused microcantilever bending in nanometer scale. The asymmetric coating with a sensing layer on one side of the cantilever favors preferential adsorption of molecules on this surface. The intermolecular forces in the adsorbed molecules layer produce a compressive stress, i.e. the cantilever bend down if its sensing surface is its upper one. The opposite case, if the intermolecular forces produce a tensile stress, giving rise to the cantilever bend up [30]. The bending of microcantilever can be detected by detecting the deflection of cantilever beam, usually, by optical. Besides, it can be detected by observe surface stress change that occurred when microcantilever bending [31].

4.1.2 Dynamic Mode

In dynamic mode, the microcantilever is driven by its resonance frequency using piezoelectric actuator. If the target molecules adsorbed onto microcantilever, its resonance frequency changes due to changing mass of the oscillating cantilever by additional mass deposited on the cantilever. The resonance frequency of the cantilever also changes, if the mass is removed from the cantilever [30]. Thus, the mass changes on the cantilever are derived from shift in resonance frequency and this shift is proportional to the amount of adsorbed molecules [31].

4.2 Detection Systems

Two modes of microcantilever operation can de detecting the microcantilever signal using three detection systems; detection the change of surface stress, light deflection change and resonance frequency change as shown in Figure 11. In static mode, the deflection of microcantilever undergoes a stress change to the piezoresistor that can be detected the resistance directly by electronic means [28]. This technique is requiring microelectromechnical system which is the piezoresistor is intrigrated in the microcantilever. Figure 10 shows a scanning electron microscope (SEM) image of the front of piezoresistive microcantilever chip which is design by MEMS lab, NECTEC, Thailand.

The surface stress change of microcantilever can be observed using the optical leverage method by commercial atomic force microscope (AFM), a laser beam is reflected of the free end of the microcantilever and the movement of the reflected beam is detected by a photosensitive detector device. Atomic force microscope provides sub-angstrom resolution in the cantilever deflection and it allows the readout of deflection signal in a second [32]. Detection of the resonance frequency changing is also detected by using the optical technique of AFM.

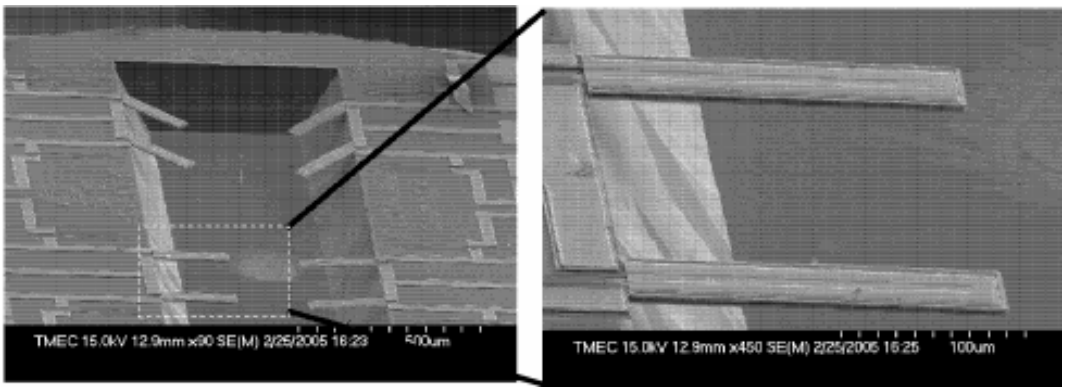


Figure 10. Scanning electron micrograph of the piezoresistive microcantilever chip

4.3 Application of Microcantilevers for Sensor Applications

Microcantilever-based sensors have enormous potential for the detection of various analytes in gaseous, vacuum and liquid medium. Microcantilevers have got potential applications in every field of science ranging from physical, chemical and biological sensing. Heavy metal detections were reported by microcantilever-based sensor [33]. Polymer layer on microcantilever have been examined to artificial nose, volatile organic compounds (VOCs) and explosives detection [34-36]. Microcantilevers have been applied for detection DNA specific sequences [37-41]. Moreover, in medical fields, microcantilevers can be employed for the monitoring and diagnosis of large number of biological analytes such as blood glucose and protein biomarkers such as creatin kinase, myoglobin and prostate specific antigen (PSA) [42, 43]. The various applications of microcantilever based sensors are summarized in Figure 12. They were interested transducer because of their high specificity, high sensitivity, simplicity, low cost, low amount of analyte requirement, quick response and low power requirement [28], [44].

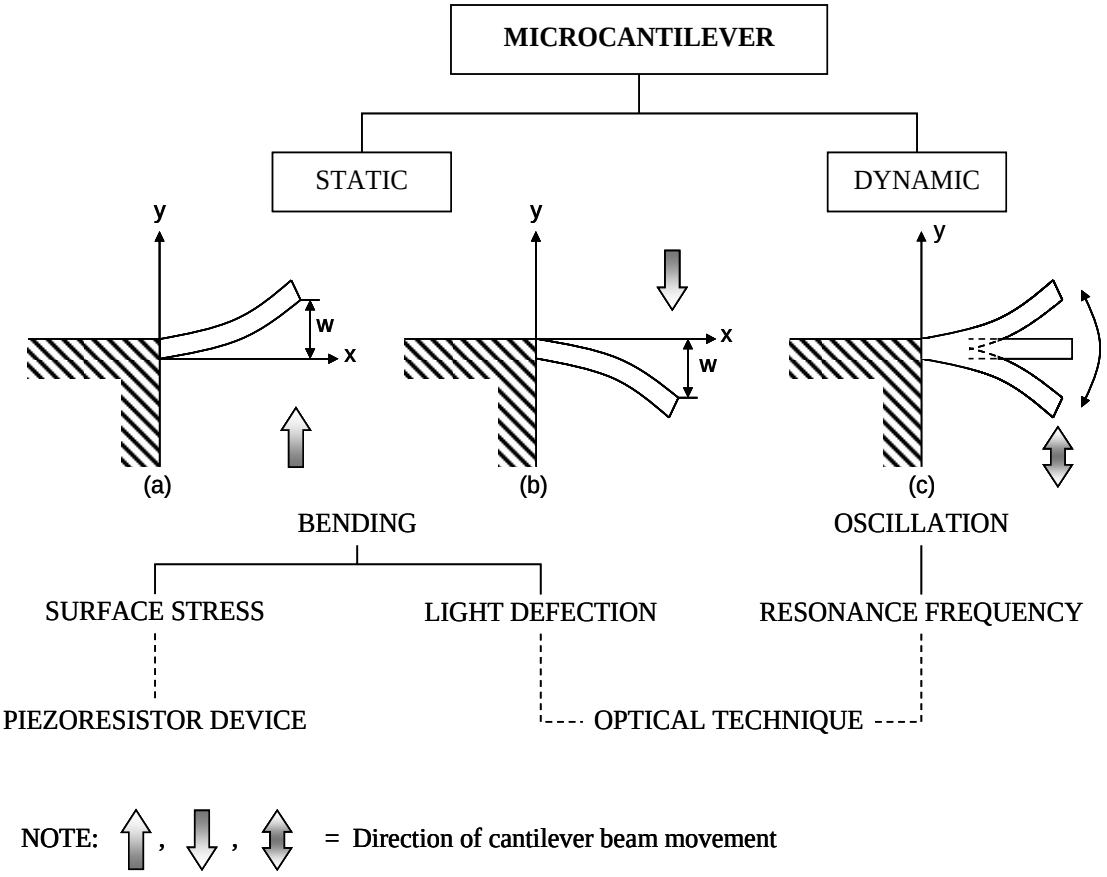


Figure 11. Mode of operation of the microcantilever

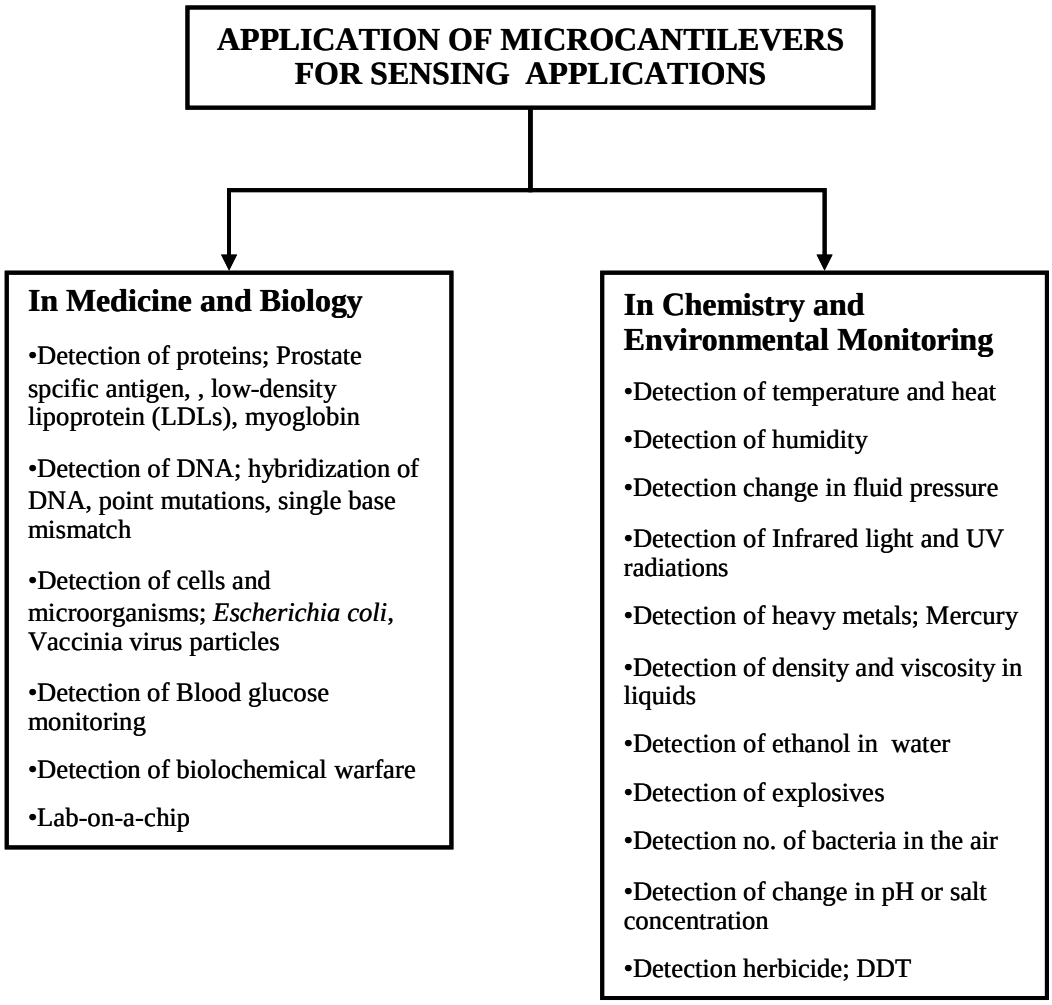


Figure 12. Microcantilever in sensor applications

5. Biosensor for Microbial Detection

Microorganisms such as bacteria, viruses, are found widely throughout nature and environment such as in marine and estuarine waters, soil, intestinal tract of humans and animals, water contaminated with fecal matter, and in food products. The majority of microorganisms carry out essential activities in nature, and many are closely associated with plants or animals in beneficial relation. However, certain potentially harmful microorganism can have profound effects on animals and humans and may be cause of different infectious disease [45].

Bacterial infections are common and involved in many forms of disease, ranging from inflammation to food poisoning. A growing number of bacterial pathogens have been identified as important food- and water-borne pathogens including *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureaus*, *Campylobacter jejuni*, and *Bacillus cereus*.

5.1 Conventional Technique for Microbial Detection

The conventional method for detection and identification of microorganisms mainly rely on specific microbiological and biochemical identification. While these method can be sensitive, inexpensive, and give both quality and quantitative information on the number and the nature of the microorganisms test, they are gently restricted by assay time, with initial enrichment needed in order to detect pathogens with typically occur in low number in food and water.

Other techniques have been developed for microbial detection including immunoassay techniques, enzyme/substrate based technique and molecule-specific probes, such as enzyme-linked immunosorbent assay (ELISA), nucleic acid detection methods target specific nucleic acid sequences of bacteria, viruses, or protozoa. These include polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR), quantitative PCR (Q-PCR), nucleic acid sequence based amplification (NASBA), and microarray [46].

5.2 Biosensor for Microorganism Detection

One approach for rapid microbial detection is the use of biosensor devices. In specific area of microorganism sensors mostly used the antibody and nucleic acid as a biological recognition element. Several different techniques have been used to produce biosensor for pathogen detection including surface plasmon resonance (SPR) [47-57],

optical waveguide [58-60], impedance-based techniques [56, 57, 61], electrochemical-based immunosensor [62], and magnitoelastic sensor [63]. Moreover, mass-sensitive biosensor using piezoelectric transducers are also reported.

In this study, microcantilever-based biosensor as a mass sensitive sensor for detection of *Vibrio cholerae* O1 was studied. The principle of piezoelectric microcantilever is the same as in quartz crystal microbalance but they are different in the operation and detection of the change in resonance frequency signal. These following paragraphs are described the QCM and the microcantilever based biosensor for detection of microorganism detection.

5.2.1 Quartz Crystal Microbalance (QCM)-based Microorganism Biosensor

Most of the quartz crystal microbalance (QCM) biosensor for microbial detection is based on highly specific of antigen-antibody reaction. The capture antibodies are immobilized on AT-cut quartz crystal microbalance surface. The adsorption of microbial cells onto the immobilized antibodies results in an increase in the mass loading of the crystal, and this will decrease the sensor resonance frequency [64]. The principle relies on the principle of the microgravimetric quartz crystal microbalance, as described by the Sauerbray equation, given as follow [65]:

$$\Delta F = -2.3 \times 10^6 F_0^2 \Delta m/A \qquad (1)$$

where ΔF is the change in frequency of the crystal (Hz), F_0 is the resonance frequency of the crystal (MHz), Δm is the mass deposited on the electrode surface (g) and A is the area coated (cm^2). Many of QCM-based microbial biosensors have been reported and the major are the biosensor for bacterial detection and food-borne pathogen detection.

5.2.1.1 *Escherichia coli* Detection

E. coli is a food-borne pathogen cause urinary tract infections, inflammations and peritonitis in immunosuppressed patients, children and elder people [66]. Detection of *E. coli* O157:H7 by QCM was described by Xiao-Li Su and Yanbin Li. They immobilized Anti-*E. coli* 157:H7 antibody onto QCM electrode surface by self-assembled monolayers (SAMs) method using 16-mercaptohexadecanoic acid

(MHDA) as an alkanethiol to form a monolayer. The resonance frequency of antibody-immobilized electrode surface was measured and observed the resonance frequency shift after target detection. The biosensor can be detected *E. coli* O157:H7 in the range of 10^3-10^8 CFU/ml within 30-50 min [64].

Other report of *E. coli* detection-based on QCM was described by Nora Adanyi and Maria Varadi *et al*. They are also performed the experiment by immobilize the antibody specific to *E. coli* onto the gold electrode using SAMs method, but the different was an alkanethiol used to formed a monolayer [67]. The QCM-based DNA biosensor was developed as *E. coli* sensor. This technique is provide high sensitivity of mass detection which reported to be 2.67 x 10^2 CFU/ml [68].

5.2.1.2 *Salmonella species* Detection

Salmonella spp is also a food-borne pathogen that causes infection disease in human. In 1997s, Jainming YE *et al.*, was developed a flow injection analysis (FIA) system based on QCM for detection of *Salmonella typhimurium*. The sensor is an immunosensor that constructed by immobilized the anti-*S. typhimurium* antibody onto the gold electrode by covalent binding with functionalized polymer electrode surface of polyethylenimine (PEI) by helping of glutaraldehyde cross-linker molecule. The detection limit of this technique was reported to be 5.3 x10^5 CFU/ml [69]

Y.S. Fung and Y.Y Wong was reported detection of *S. parathyphi* A using gold electrode of QCM. The sensing electrode surface was conducted by immobilizing the antibody to *S. parathyphi A* on the gold electrode using self-assembled monolayers method. The 3-mercaptopropionic acid (MPA) was used as an alkanethiol chemisorptions on the gold electrode surface and formed chemical linkages with antibody by activating of 1-ethyl-3-(3-dimethylaminopropyl) carbodiinide (EDC) and *N*-hydroxysuccinimide (NHS). The detection limit of this system was 1.7x10^2 CFU/ml [65]. Y.Y Wong et al. was also reported the using of silver electrode as a transducer for detection of *Salmonella* species serogroup A, B, and D. Monoclonal antibodies specific to Salmonella antigen cells were immobilized by SAMs method. The detection range of this sensor was ranging from 1x10^5-5x10^8 CFU/ml [70].

5.2.2 Microcantilever-based Microorganism Biosensor

The microcantilever-based biosensors for detection of microorganisms have been purposed. Piezoelectric microcantilever is based on piezoelectric effect like the QCM-based sensor. The oscillating microcantilever can be detected the microorganisms by detection of the decreasing of resonance frequency due to mass loaded on its surface.

5.2.2.1 *Escherichia coli* Detection

Microcantilever-based biosensors for *E. coli* detection have been detected by Gossett A. Campbell and Raj Mutharasan. They were demonstrated real-time detection of a millimeter-sized lead zirconate titanate (PZT) glass cantilever for detection of *E. coli* O157:H7. An anti-*E. coli* O157:H7 was immobilized on the glass surface by silanization method. The antibody-immobilized cantilever was measured the change of resonance frequency under liquid environment that can detect *E. coli* O157:H7 in real-time, with a high detection limit of 700 cells/ml without enrichment or PCR method [71-73]. Growth detection of *E. coli* cells was demonstrated Karin Y. Gfeller *et al*. by coated nutritive layer and observed the mass change due to bacterial growth with measuring resonance frequency shift by atomic force microsope (AFM). This sensor system was able to detect active growth of *E. coli* cells within 1 h and the calculated mass sensitivity of the sensor was 50 pg/Hz.

5.2.2.2. *Salmonella species* Detection

Qing Zhu *et al*. was investigated the using of gold-coated glass microcantilever as a biosensor device for *S. typhimurium* detection. The experiment was done in dynamic mode of microcantilever and the results shown the sensor mass sensitivity of this sensor as 50 pg/Hz. The limit of detection was found $1x10^3$ CFU/ml in 2 ml of the sample [74].

5.2.2.3 *Bacillus anthrais* Detection

The lead magnesium niobate-lead titanate/tin (PMN-PT/Sn) piezoelectric microcantilever was developed by John-paul McGoven *et al*. to test with *Bacillus anthracis* spores in real-time detection [75]. The mass sensitivity of *B. anthracis* Strene spore detection was reported both in air and liquid, it was 9.23 fg/Hz in the air and 0.1 fg/Hz in liquid [76]. Moreover, *B. anthrais* can be detected by piezoelectric excited millimeter-size cantilever (PEMC) sensor consisting of a

piezoelectric and borosilicate glass layer. The lowest concentration that can be detected *B. anthrais* by this technique was 300 spores/ml [77, 78].

5.2.2.4 Detection of Other Microorganisms

Detection of *Aspergillus niger* spore was examined by Natalia Nugeva *et al*. The method of preparation is the same as others method in pervious described. Quantitative information of the target fungi was derived from resonance frequency shift when the antibody-immobilized cantilever was tested with the suspension of the fungi. The detection limit of this sensor was $1x10^3$ CFU/ml and mass sensitivity was reported to be 53 pg/Hz [79].

Ultrasensitive detection system for vaccinia virus was reported by Amit Gupta *et al.* The microcantilever was immobilized with antibody specific to vaccinia virus was a sensing layer for vaccinia viral detection. The sensor can be detected small mass of this viral particle which has average mass equal to 9.5 fg by detecting the resonance frequency changes. The mass sensitivity of this technique was 6.3 attogram(ag)/Hz. [80]. Amit Gupta *et al.* is also demonstrated the usage of microcantilever for detection of *Listeria innucua* bacteria [81].

6. Microcantilever-based Biosensor for *Vibrio cholerae* O1 Detection

To construct a new biosensor for detection *Vibrio cholerae* O1 (*V. cholerae* O1) bacteria, the sensor component and biological recognition element should be determined. In this work, the microcantilever was used as a transducer and the monoclonal antibody that specific to *V. cholerae* O1 used as a biological recognition element. The microcantilever was operated in dynamic mode which is detection of the resonance frequency change of microcantilever by atomic force microscope (AFM).

6.1 Bacterial Target: *Vibrio cholerae* O1

Vibrio cholerae is a “curve rod” or “comma-shaped” bacterium which is a causative agent of cholera and belongs to a group of organisms whose natural inhabitant in the aquatic environment [82]. It is a gram negative bacterium with a signal polar flagellum for its movement (Figure 13) [83]. The size of *V. cholerae* O1 is varies from 1-3 μm in length and 0.5-0.8 μm in diameter. The optimum of pH and temperature for *V. cholerae* O1 growth is 7.6 and 37^oC, respectively. It is very sensitive to the acid conditions and dies rapidly in the solutions at below pH 6, but

tolerant to alkaline conditions. In coastal regions it may persist in shellfish and plankton and it has been claimed that they can exist in a viable but non-culturable (VBNC) state.

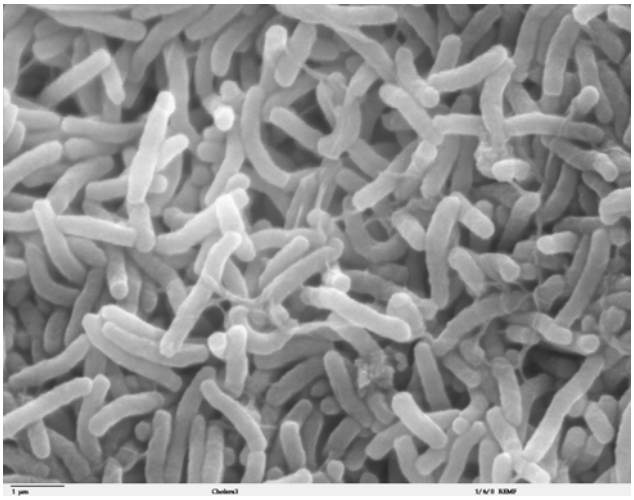


Figure 13. Scanning electron micrograph of *Vibrio. cholerae* O1

Vibrio cholera species is divided into serogroups on the basis of the O antigen. Almost 200 *V. cholerae* serogroups have been identified to date, but only two serogroups O1 and O139, are associated with epidemic cholera due to their ability to produce cholera toxin (CT) [84]. *V. cholerae* O1 can be sub-classified into two biotypes, classical and El Tor on the basis of several phenotypic characteristics. Both of these biotypes contain two major serotypes, Inaba and Ogawa. The cholera group has a common antigen; antigen A and each serotype are different by type specific antigens, antigen B (Ogawa) and antigen C (Inaba). Another rare serotype, Hikojima, has both specific antigens (Figure 14).

Cholera is a food and waterborne gastroenteric infection, remains a significant treat to public health in the developing countries [85]. In the nineteenth century, pandemic waves of cholera spared to many parts of the world and affects more than 75 countries in every continent [86]. There have been seven pandemics of cholera; almost of pandemics of cholera are known to be due to *V. cholerae* serogroup O1 and only the seventh pandemic of cholera caused by *V. cholerae* O139 [87]. The first pandemic was began in Southeast Asia and spread rapidly to South Asia, the Middle East, and

southeastern Europe. John Snow, a doctor who known as the father of epidemiology, did a study during the London cholera epidemic was concluded that cholera was passed by contaminated water, and discovered that a well that provided the water to the public was collecting the leaching of a bacteria. After removed the water pump, the epidemic began to subside. At present cholera is endemic in some regions of Asia and Africa with a few cases in America and Australia [88]. Currently, the El Tor biotype is responsible for virtually all of the cholera cases throughout the world, and classical isolates are not encountered outside of Bangladesh.

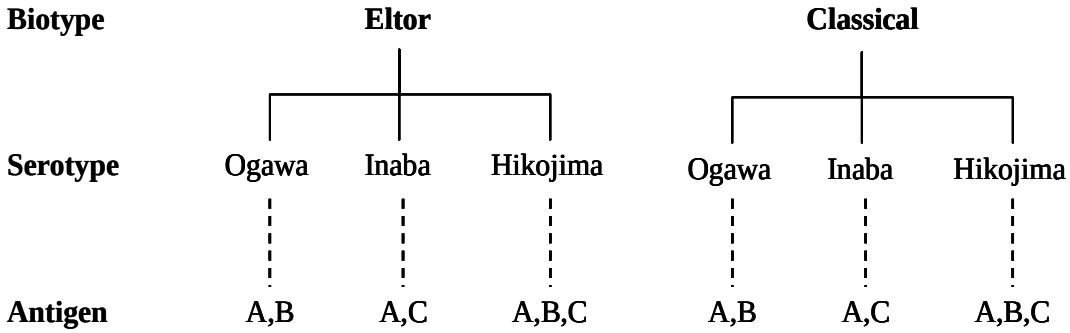


Figure 14. Classification of *Vibrio cholerae* O1, there are two biotypes; classical and El tor. These two serotypes can be sub-divided into three serotypes; Ogawa, Inaba and Hikojima.

Cholera disease is particularly noticeable after natural disasters due to contaminated water supplies. In 2004, estimated 225,000 deaths from the Indian Ocean tsunami devastated parts of Thailand, India, Sri Lungka, Maldives, Malaysia, Myanmar, Somania, and much of Indonesia. There are several areas were cholera is endemic and the spread of *V. cholera* increased the number of additional deaths in aftermath of the tsunami [89]. Cholera is characterized by numerous, voluminous watery diarrhea with rapid loss of body fluids, often accompanied by nausea and vomiting, resulting in hypovolumic shock and acidosis. The human is the only one of natural host for *V. cholerae* O1. Connection between human and *V. cholerae* O1 is directly through food and water by ingestion of foods and water contaminated this

organism. Direct person-to-person is not common. When *V. cholerae* O1 get into the body it will produce and release the toxin called cholera toxin. The toxin has been characterized and contains 5 binding (B) units of 11,500 daltons, an active (A1) subunit of 23,500 daltons, and a bridging piece (A2) subunit of 5,500 daltons that links A1 to the 5B subunits.

Vibrio cholerae O1 contains several characteristics of pathogenic which important determinant of the colonization process includes adhesins, neuraminidase, motility, chemotaxis, and toxin production. It is resisted to bile salts and penetrates the mucous layer of the small intestine, aided by the secretion of neuraminidase and protease. Once the cholera toxin has entered the cell, the A1 subunit enzymatically transfers ADP ribose from NAD to a protein (called Gs protein), that regulates the adenylate cyclase system which is located on the inside of the plasma membrane of the calls, leading to increase levels of intracellular cAMP. High rate of cAMP is stimulating secretion of Cl^-, H_20, Na^+, K^+, and HCO_3^- into the lumen of intestine. The result is watery diarrhea with electrolyte concentrations isotonic to those of plasma. The effect depends on a specific receptor, monosialosyl ganglioside (GM1 ganglioside) present on the surface of intestine mucosa cells. Treatment of cholera includes rehydration and electrolyte replacement and antimicrobial therapy.

6.2 *Vibrio cholerae* O1 Detection

6.2.1 Conventional Isolation Methods

Isolation and identification of *V. cholerae* O1 is based on conventional culture method and genotypic method. In conventional method, pre-enrichment by alkaline peptone water (APW) is needed to enhance the isolate of *V. cholerae* O1 in specimen that few vibrio cells are present such as in case of the specimen from asymptomatic infected persons. Alkaline peptone water can be inoculated with liquid stool, fecal suspension, or rectal swab. The processes are done by adding the specimen into APW broth and incubate at 35^o to 37 ^oC for 6 to 8 hours. A single colony of bacteria growth on the APW will inoculate onto a thiosulfate citrate bile salts sucrose (TCBS) agar plate, a selective medium for *V. cholerae*. Incubate at 35^o to 37 ^oC for 18-24 hours and then observe colonies on the plate. Colonies suspicious for *V. cholerae* O1 will appear on TCBS agar as yellow, shiny colonies, 2-4 mm in diameter. Then, select one of colony from TCBS agar inoculates on a heart infusion agar (HIA) or

another non-selective medium. Incubate the HIA agar at 35^{o} to 37 ^{o}C for up to 24 hours, the colonies growth on this medium will use to further screening test for *V. cholerae* O1.

6.2.1.1 Oxidase Test

Place 2-3 drops of oxidase reagent (1% *N,N,N',N'*-tetramethyl-*p*-phenylenediamine) on a piece of filter paper in Petri dish. Smear the fresh colony from HIA agar across the wet paper. In positive reaction, the bacterial growth becomes dark purple immediately. Oxidase negative will remain colorless.

6.2.1.2 String Test

The string test perform on a glass microscopic slide by suspended a few colonies bacteria growth from HIA agar in a drop of 0.5% aqueous solution of sodium deoxycholate. The bacterial cells will be lysed by sodium deoxycholate in positive result, the suspension will lose turbidity, and DNA will be released from the lysed cells, causing the mixture to become viscous. This test is useful for ruling out non-*Vibrio* spp.

6.2.1.3 Klingler Iron Agar and Triple Sugar Iron Agar

Klingler iron agar (KIA) and triple sugar iron agar (TSI) can be used to rule out *Pseudomonas* spp. and certain Enterobacteriaceae. The reaction of *V. cholerae* on KIA is alkaline (red) slant, acid (yellow) butt, no gas, no H_2S. However, on TSI, *V. cholerae* O1 produce an acid (yellow) slant and acid (yellow) butt, no gas and no H_2S. The LIA reaction for *V. cholerae* O1 is typically an alkaline slant (purple), alkaline butt (purple), no gas and no H_2S.

6.2.1.4 Gram Stain

Gram strain is based on the structure of the bacterial cell wall. The *Vibrio cholerae* O1 is a gram negative bacterium containing the outer membrane, which the pink safranin is trapped by the peptidoglycan layer of the outer membrane and demonstrate typical small, curved gram negative rods.

6.2.1.5 Wet Mount

Dark-field and phase-contrast microscopy have been used for screening suspected *V. cholerae* O1. With this technique, saline suspensions are microscopically examined for the presence of organisms, curved rod, and darting (“shooting star”) motility.

6.2.2 Serologic Identification

6.2.2.1 Presumptive Identification Using O1 Antisera

For slide agglutination test, colonies of suspected *V. cholerae* O1 growth from HIA agar will test with polyvalent O1

6.2.2.2 Confirmation of *V. cholerae* O1 Using Inaba, Ogawa, and Hikojima Antisera

The positive O1 serogroup of *V. cholera* has been further monovalent antisera to type specific O antigens (Inaba, Ogawa, and Hikojima)

These conventional techniques are time-consuming, laborious, requiring prolong incubation, lack the necessary sensitivity, and this method can not detect the *V. cholerae* O1 in a viable but not culturable (VBNC) state [88].

6.2.3 Other Techniques for *V. cholerae* O1 Detection

Cholera SMART^{TM}, a rapid, colorimetric immunodiagnostic kit was reported by Jafrul A.K. Hasan *et al*. The test is used a colloidal-gold-labeled monoclonal antibody. If *V. cholerae* O1 antigen is present in the specimen, it will form complex to the anti-*V. cholerae* O1 and the complexes diffuses and is subsequently captured and concentrated by a polyclonal antibody-antibody-coated solid phase matrix and appears to the naked eyes as a pink to red test spot developing from the deposition of colloidal gold. The Cholera SMARTTM is an easy technique that can be used directly in the field by untrained or minimal skilled personnel to detect *V. cholerae* O1 in less than 15 min. However, it will give only qualitative information and the sensitivity of detection is quite low [90, 91].

Other immunoassay such as Enzyme-linked immunosorbent Immunoassay (ELISA) is also interesting and available for *V. cholerae* O1 detection within 4 hours. The minimum detection limit of standard ELISA was found to be $1x10^6$ CFU/ml [92].

Biosensors for detection of *V. cholerae* O1 have been developed. Ampermetric immunosensor is one that combined electrochemical immunosensor and specific of traditional immunochemical method. Screen-printed electrode (SPE) was immobilized with antibody for detection of *V. cholerae* O1. This technique provide higher sensitivity compared with standard ELISA ($1x10^5$ CFU/ml) within 55 min [92]. Immunosensor-based on SPR is another biosensor developed for *V. cholerae* O1 detection. In principle of SPR-based technique, a surface Plasmon is a bound

electromagnetic wave propagating at the metal-dielectric interface. The external laser field drives the free electron gas on metal surface. The spatial change distribution creates an electric field which is localized at the metal-dielectric interface. Detection range of this technique is $10^5 – 10^9$ CFU/ml [93].

Many of polymerase chain reactions (PCR) for *V. cholerae* O1 detection were developed, such as standard PCR, real-time PCR, and multiplex PCR [94-98]. There are provide high sensitivity of detection but requiring sample DNA extraction and needed well-trained personnel to process the detection.

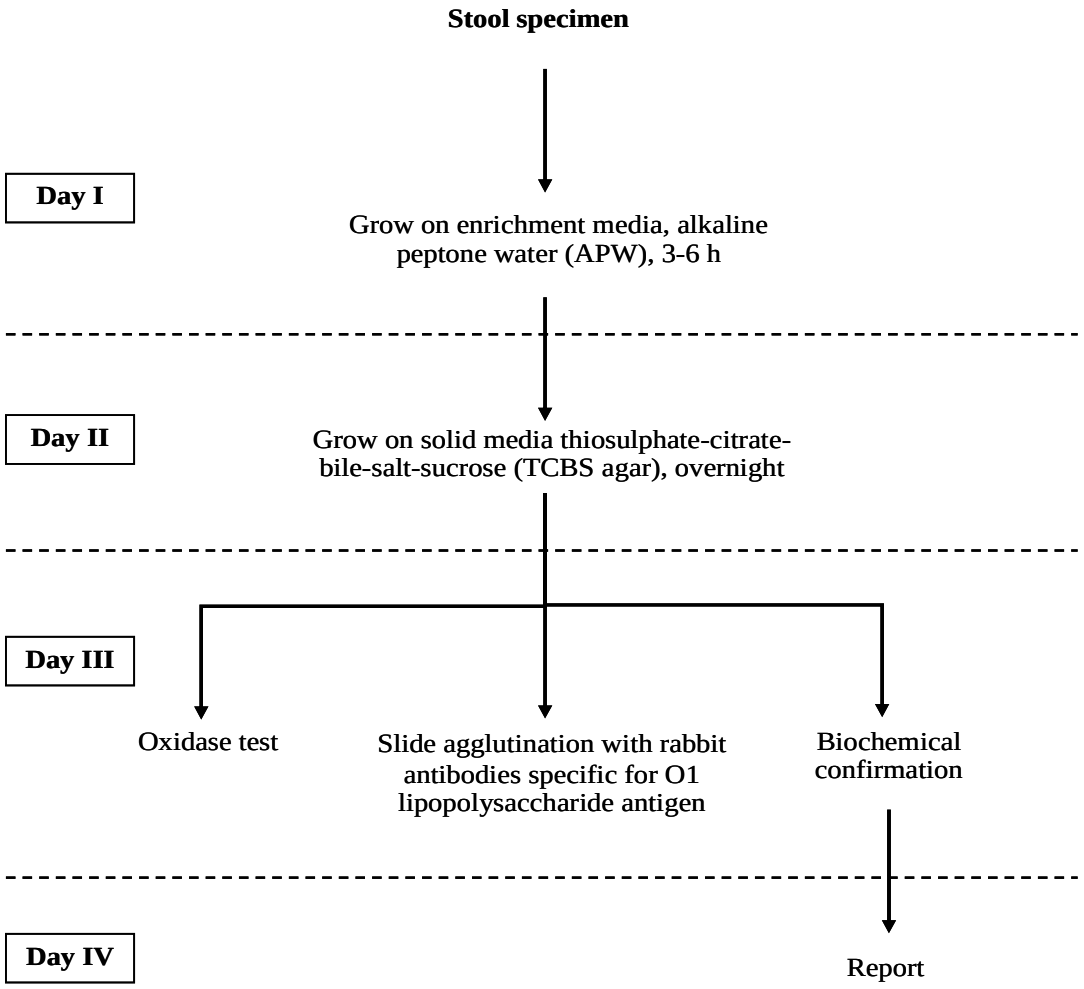


Figure 15. Conventional technique for *Vibrio cholerae* O1 detection

6.3 Biological Recognition Element: Monoclonal Antibody

Antibody is a specific molecule that can attach at one end to the antigen which is produced by B lymphocytes in mammalian systems. Knowledge of antigen-antibody reaction is essential in developing the technique for pathogen detection such as enzyme-linked immunosorbent assay (ELISA) and western blotting. The first used of antibody to capture their antigen was first described as a biological recognition element in early 1970s by Kronic and Lattle, Giaever, and Tromberg *et al.* [99]. Figure shows one of ELISA technique for antigen detection**.** Figure 12 shows pathogen detection by ELISA technique.

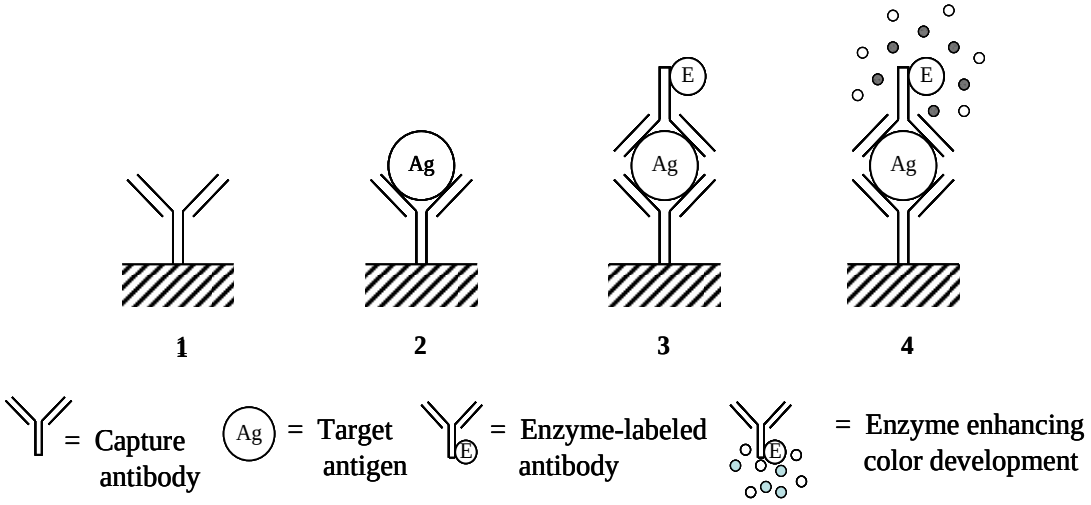


Figure 16. Antibody-based antigen detection; Sandwich-ELISA technique. This system exploits the antibodies attached to the slid phase to capture antigen. This is then detected using an enzymes-labeled serum specific for antigen. The detecting antibody is labeled with enzyme.

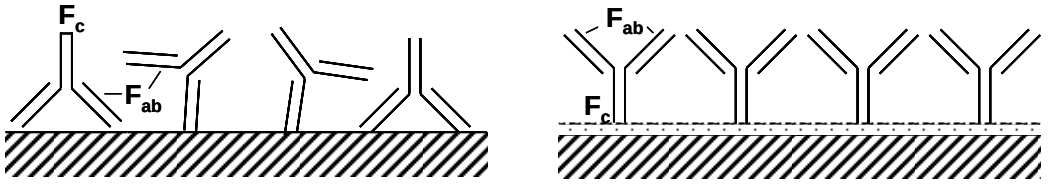
The top end of the "Y" or Fab fragments contain the antigen-binding site, they retain specificity of the antibody. The assay technique derived from antigen-antibody reaction is called "immunoassay" or called as "immunosensor" in the scope of biosensor. To develop the immunosensor for detection of *V. cholerae* O1 bacteria, it is needed to immobilize the anti-*V. cholerae* O1 on the transducer surface to generated the sensing layer that available for *V. cholerae* O1 cell captured.

6.4 Immobilization of Antibody on the Microcantilever Surface

Immobilization of antibody on various solid-phase surfaces have been widely used in many field such as purification of materials, diagnostic immunoassays, and immunosensors [100]. For development of an immunosensor, the immobilization of IgG on the solid substrate is very important step. Several methods for the immobilization of IgG were reported. These methods relay on physical adsorption and chemical adsorption [101].

6.4.1 Physical Adsorption

The most straightforward procedure of analyte detection use antibodies to capture analyte antigen by simply absorbed antibody directly on solid substrate without using any chemical reagent treated, but the bindings of antibody adsorbed are weak, and their molecules are oriented on the solid substrate by random adsorption as shown in Figure 17 (a) [25]. To increase ability to bind the antigen, the antibody molecule should be immobilize in a manner which F_{ab} fragments pointing away from the surface, in order to allow for easy antigen-antibody binding as shown in Figure 17 (b) [102]. The way to conducted that requirement is using protein linkers; protein A or protein G. Protein A and protein G are antibody-binding proteins to F_c portion of antibody molecule that have been used to capture the antibody on sensor surfaces with properly orientations and maintaining antibody functionality as shown in Fig 17 (b) [103]. The direct immobilization of protein molecules onto a metal substrate has several disadvantages; reversibility of adsorption process, unspecific, random and multi-oriented immobilization of the protein [22].



(a) Antibodies directly adsorbed on solid surface.

(b) Controlled orientation antibodies on a layer of protein A or protein G

Figure 17. Schematic drawing of antibody attachment on solid surface (a) Random attachment of antibody molecules on the solid substrate surface, (b)**,** Antibody molecules in a controlled orientation**,** remain fully functional, and potentially improve the sensitivity of the immunoassay.

6.4.2 Chemical Adsorption

An alternative to physical adsorption is offered by chemical modification which has been found to show good reproducibility and coverage because the protein is covalently immobilized on the substrate. The self-assembly technology applied to protein modification are described as simplicity and adaptability of self-assembled monolayers (SAMs) and control over biomolecule surface orientation [22]. Sulfur-containing compounds e.g. alkanethiols, dialkyl disulfides and dialkyl sulfides have a strong affinity for noble metal surfaces. In the case of alkanethiols, the mechanical of binding is considerate to be an oxidative addition of the S-H bond followed by reductive elimination of a thiolate species as shown in equation (2). The eliminated of the hydrogen is though to combine to form molecular hydrogen [22].

$$R\text{-}S\text{-}H + Au_n^0 \rightarrow R\text{-}S\text{-}Au^+ Au_n^0 + \tfrac{1}{2} H_2 \qquad (2)$$

6.5 Blocking Agents

Biosensor technologies utilizing the high specificity between an antibody and antigen have to ensure that binding occurs under conditions where non-specific interactions are minimized [15]. Blocking agents have been used for blocking substrate surface after antibody immobilization to avoid non-specific binding with other molecules. A typical blocking agents would be a neutral macromolecule that largely enough to establish a stable attachment to the surface and small enough to find their way between antibodies. Commonly of blocking agents used in this case are bovine serum albumin (BSA) and casein.

6.6 Atomic Force Microscope (AFM)

Initial design of atomic force microscope (AFM) was described by Binning *et al.* in 1986 [104]. It is a powerful device to measure the topography of sample surface [27]. Qualitative information by numerous imaging a mode of AFM is also provides quantitative information for sensor applications performed by operating the sensing layer of microcantilever as described in section 4.3.

The AFM used in this study is SPA400 from Seiko, Japan (Figure18), operated in dynamic mode. The SPA400 has been developed aiming at higher resolution, easy operation, and quick scan speed. The working principle to measure the resonance

frequency is shown if Figure 19. Firstly, the microcantilever has to immobilize with the capture molecules, molecules specific to the target molecules. In case of *V. cholerae* O1 detection, monoclonal antibody specific to *V. cholerae* O1 was immobilized. The immobilized microcantilever is subsequently placed into an environment containing the target. The interaction between the binding sites of the capture and the target molecules changes the microcantilever system; mass change and bending.

Figure 18. SPA 400 atomic force microscope

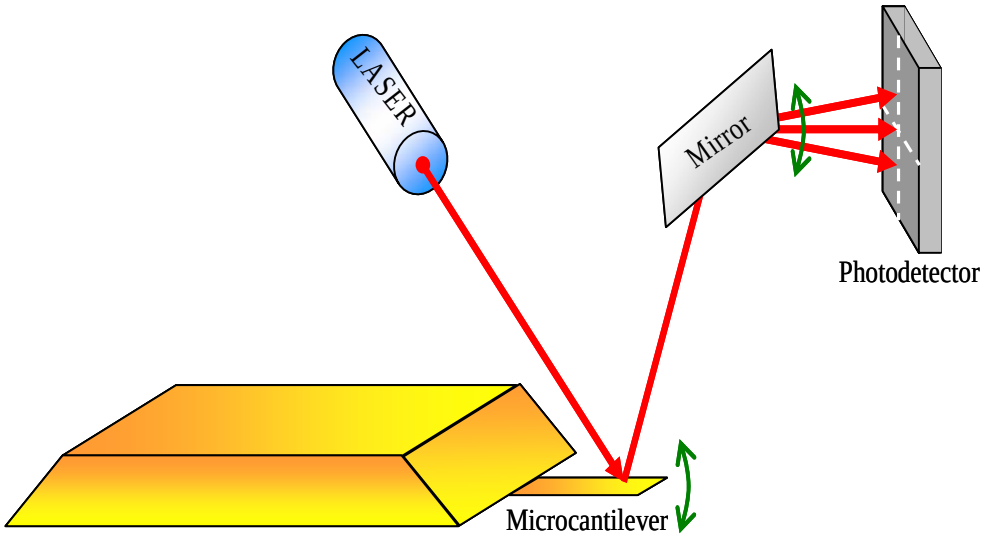


Figure 19. Optical method for frequency measurement by atomic force microscope

The way of detection is the visible light from a lower (few mW) laser diode focused on the free end of the cantilever. In order to increase reflectivity, commercial cantilever for AFM are often coated by a thin layer of gold [27]. The reflected beam hits a position sensitive photodetector. The distance moved by the laser beam is proportional to the cantilever deflection [105]. The signal from photodetector is pre-amplified by amplifier and the summarized signal transferred to analyzer program. The oscillation signal is presented on personal computer.

CHAPTER IV
MATERIALS AND METHODS

1. Materials

1.1 Chemical Reagents, Microorganisms and Antibody

1.1.1 Chemical Reagents

Chemical reagents used in this study were analytical and molecular biological grades.

Table 2. Lists of chemicals

Chemical Name	Molecular Weight	Company
Absolute ethanol	46.07	Merck
Bacto-tryptone	-	Difco
Bacto-yeast extract	-	Difco
Bovine serum albumin, BSA	6,600	Sigma
1-ethyl-3-(3-dimetylaminopropyl) carbodiimide hydrochloride, EDC	155.24	Sigma
Hydrogen peroxide, H_2O_2	34.1	Merck
N-hydroxysuccinimide, NHS	115.09	Sigma
3-Mercaptoproprinoic acid, MPA	106.14	Sigma
Potassium chloride, KCl	74.55	BDH
Protein A	45,000	Sigma
Sodium chloride, NaCl	58.44	BDH
Sodium phosphate monobasic, KH_2PO_4	136.10	Sigma
Sodium phosphate dibasic, Na_2HPO_4	142.00	Sigma
Sulfuric acid, H_2SO_4	98.08	Merck

1.1.2 Microorganisms and Antibody

1.1.2.1 Microorganisms

Microorganisms used in this study were obtained from Department of Medical Technology, Mahidol University, Thailand. *Vibrio cholerae* O1 was preparation into suspension form in varies of concentrations (the preparation method was shown in section 1.3.13) and used to test as positive controls. *Vibrio parahemolyticus* was used as other strain of bacteria to check cross-reaction of the sensor system.

1.1.2.2 Monoclonal Antibody

Antibody against *V. cholerae* O1 common antigen (clone 27E10, Ig G3, kappa) was kindly supplied from Prof. Dr. Wanpen Chaicumpa, Department of Immunology, Faculty of Medicine, Mahidol University, Thailand. This antibody can specifically react with both Ogawa and Inaba serotypes of *V. cholerae* O1.

1.2 Equipments and Instruments

Table 3. Lists of Equipments and Instruments

Instrument Name	Company	Country
Autoclave	Tomy Seiko Co., LDT	Japan
Automatic pipettes	Gilson	France
Atomic force microscope, SPA 400	Seiko	Japan
Balance	Precisa	Switzerland
Cantilever, NSG10	NT-MDT Co., LDT	Russia
Fume hood, FH 1200	Official Equipment Manufacturing Co., LDT	Thailand
Incubator	Termarks	Norway
Magnetic Stirrer and Shaker	MEMS Lab, NECTEC	Thailand
pH meter	Orian research	England
Water bath	PolyScience	USA
Scanning Electron Microscope, S-3400N	Hitachi-High Technologies Ltd.	USA
Spectrophotometer, UV-120-20	Shimadzu Corporation	Japan
Temperature and Humidity Meter	MEMS Lab, NECTEC	Thailand

1.3 Reagent Preparations

1.3.1 Stock 10X Phosphate Buffer Saline (0.1 M PBS), pH 7.4

The solution of 10X PBS, pH 7.4 was prepared by dissolving 80 g of sodium chloride (NaCl), 2 g of potassium chloride (KCl), 11.5 g of sodium phosphate, dibasic ($NaHPO_4 7H_2O$), and 2 g of potassium dihydrogenphosphate (KH_2HPO_4) in 800 ml of distilled water (DW). The pH of solution was adjusted to obtain pH 7.4 and then filled up with DW water to 1000 ml. This solution was autoclaved at 15 psi for 30 min. Kept the solution at room temperature.

1.3.2 Working 1X Phosphate Buffer Saline (0.01 M PBS), pH 7.4

One volume of 10X PBS buffer solution was added into 9 volumes of DW water. This solution was kept at room temperature.

1.3.3 Stock 10 mg/ml Protein A Solution

Pipette 19.23 µl of protein A solution (52 mg protein) into 980.77 µl of PBS buffer solution (pH 7.4). Kept at -20^{o}C.

1.3.4 Working 1 mg/ml Protein A Solution

One volume of 10 mg/ml protein A solution was added into 9 volumes of PBS buffer solution (pH 7.4) and kept the solution at -20^{o}C.

1.3.5 The 10 mM 3-Mercaptoproprionic acid (MPA)

Pipette 9 µl of MPA solution into 9.99 ml of absolute ethanol. This solution was kept at 4^{o}C.

1.3.6 The 200 mM 1-ethyl-3-(3-dimetylaminopropyl)carbodiimide hydrochloride (EDC)

Pipette 3.5 µl of EDC into 100 µl of DI water. This solution must be freshly prepared prior performing the experiment.

1.3.7 The 50 mM *N*-Hydroxysuccinimide (NHS)

Weigh 0.0024 g of NHS and dissolved into 200 µl of DI water. This solution must be freshly prepared before use.

1.3.8 Mixture Solution of 200 mM 1-ethyl-3-(3-dimetylaminopropyl) and 50 mM *N*-Hydroxysuccinimide (NHS)

One volume of 200 mM EDC solution was added into one volume of 50 mM NHS solution. The solution must be prepared immediately before the use to avoid lost of activity.

1.3.9 The 1 mg/ml Monoclonal Antibody

The concentration of monoclonal antibody was determined by spectrophotometric technique. The wavelength was preset at 260 nm and PBS buffer solution was used as a blank reagent to correct the spectrophotometer. Measure the optical density (OD) of the antibody solution and then add the PBS buffer solution (pH 7.4) into antibody solution until reached the O.D. of 1.4 which is approximately equal to the concentration of antibody (IgG) of 1 mg/ml. Kept at -20^{o}C.

1.3.10 The 0.5 mg/ml Monoclonal Antibody

One volume of 1 mg/ml monoclonal antibody was dilute with one volume of PBS buffer solution (pH 7.4). Aliquoted 100 µl to microtubes and kept at -20^{o}C.

1.3.11 The 3 mg/ml Bovine Serum Albumin (BSA)

The powder of bovine serum albumin 0.003 g was added into 1 ml of 1X PBS buffer solution, pH 7.4. Mixed by gently until the solution is homogeneous and then kept this solution at 4^{o}C up to one week.

1.3.12 Piranha Solution

One volume of conc. sulfuric acid (H_2SO_4) was added into one volume of 30% Hydrogen peroxide (H_2O_2). This solution should be used immediately after preparation.

1.3.13 Bacteria Suspension

Vibrio cholerae O1 and *Vibrio hemolyticus* were grown on Luria-Bertani (LB) agar medium plates, incubated at 37^{o}C for 18-24 h. A few of bacterial colonies grown on LB agar plate was picked up into PBS buffer solution. Mixed until reached homogeneous and then adjusted the concentration of the bacterial suspension to 1x10^{8} CFU/ml using spectrophotometric determination. The wavelength was preset at 625 nm and the PBS buffer solution (pH 7.4) was used as a blank to standardize the spectrophotometer. Adjust the O.D. of bacterial suspension with PBS buffer solution (pH 7.4) until obtained 0.08-0.1 which is approximately equal to 1x10^{8} CFU/ml.

The bacterial suspensions were boiled in 80^{o}C water bath for 20 min to inactivate the bacteria. These bacterial suspensions were diluted with PBS buffer solution (pH 7.4) in serially ten-fold dilution in range of 1x10^{2} to 1x10^{7} CFU/ml. One milliliter of the test bacterial suspensions was aliquoted into 1 ml sample container and kept at -20^{o}C until use.

2. Methods

2.1 Microcantilever Surface Cleaning

In this studies, the commercially rectangular-shaped microcantilever, series number NSG10 (NT-MDT Co., LDT.) was used. It was constructed from silicon nitride which coated at the bottom side with gold (Figure 20). The typically dimension of the microcantilever is approximately 100 μm long, 35 μm wide and 1 μm thick. Table 4 is described the characteristics of this cantilever.

Table 4. Characteristics of microcantilever, NSG10 from NT-MDT Co, LDT

Cantilever series	Length L±5μm	Width W±3μm	Thickness, μm	Resonant Frequency, kHz	Force Constant (N/m)
NSG10	100	35	2	190-325	11.5

Before usage, the gold-coated microcantilever was thoroughly cleaned with piranha solution (conc. H_2SO_4 in 30 %H_2O_2; 1:1 v/v) for 5 min to remove organic substances from the microcantilever surface. The microcantilever was subsequently rinsed with DI water. The fresh cleaned microcantilever was immobilized with monoclonal antibody both in physical adsorption and chemical adsorption by self-assembled monolayer technique. The process of immobilization was shown in Figure 21-22.

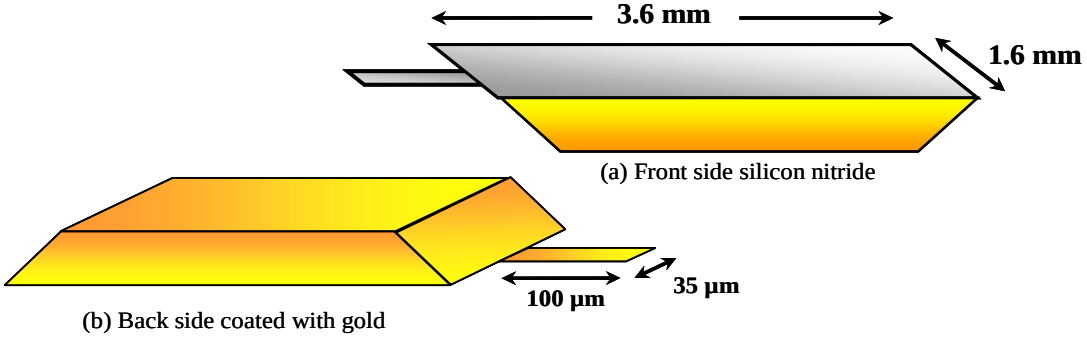


Figure 20. The front and bottom side of gold-coated microcantilever chip (a) Front side of silicon nitride microcantilever (b) Back side of microcantilever coated with gold.

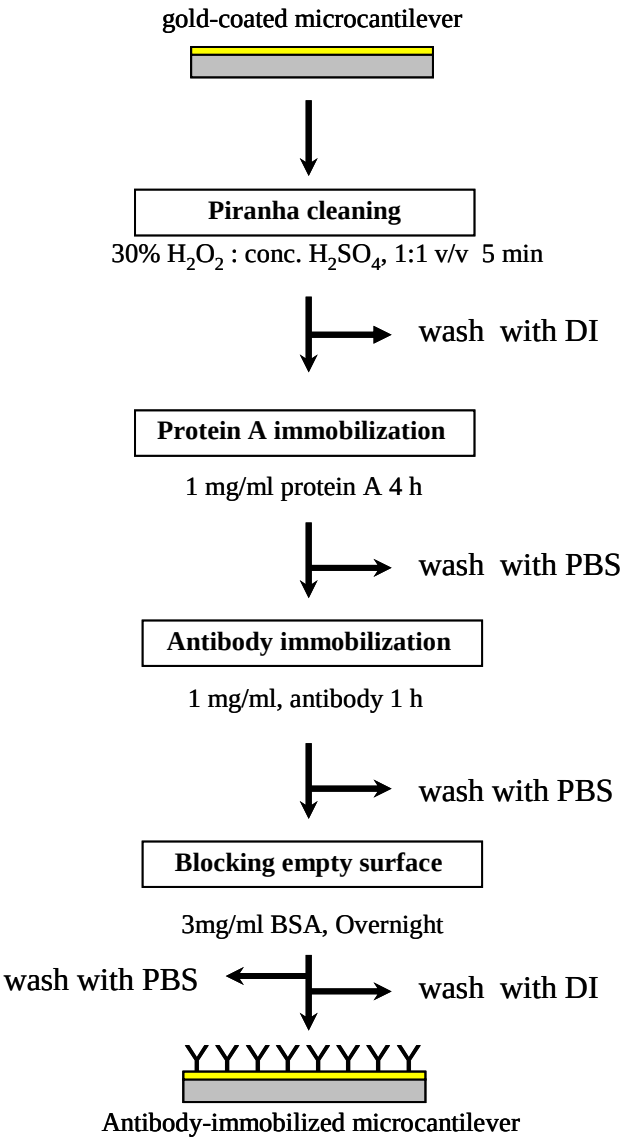


Figure 21. Process of antibody immobilization by physical adsorption. Monoclonal antibody was immobilized on microcantilever surface using a linker protein, protein A, to orient the direction of antibody molecule.

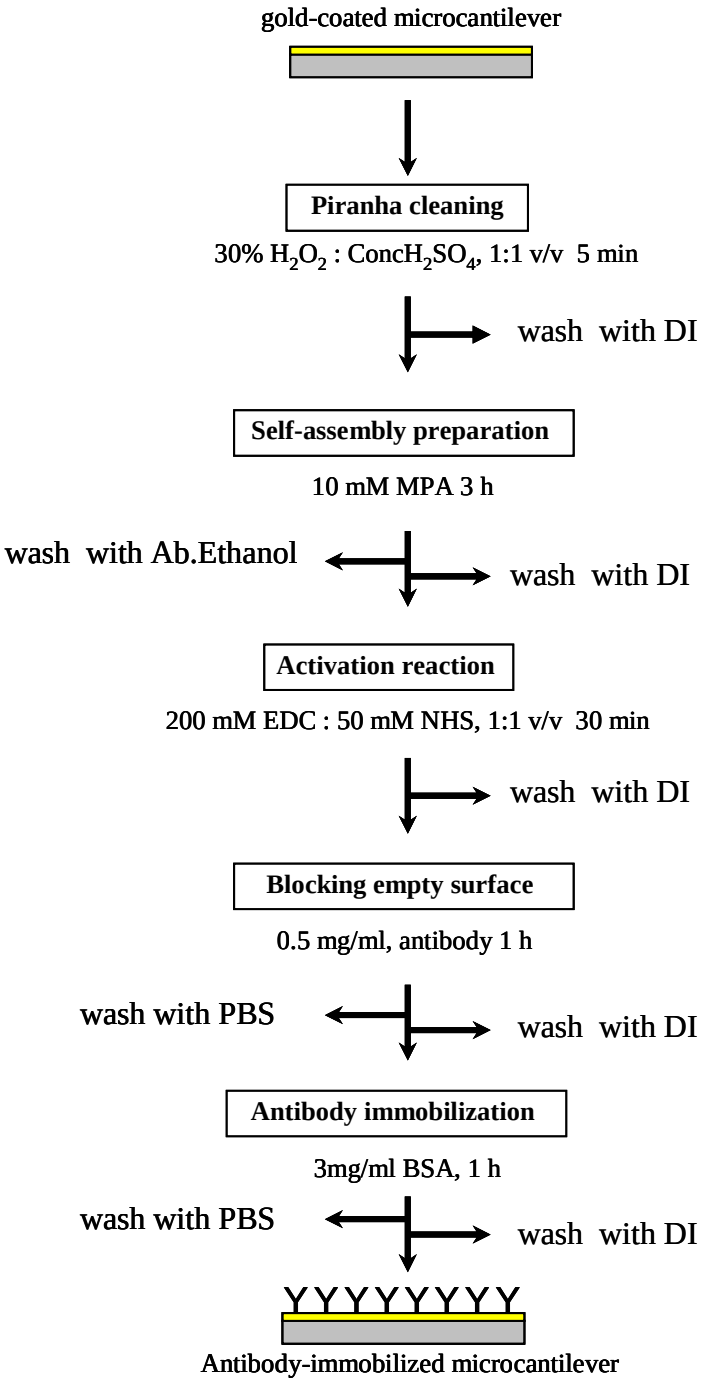


Figure 22. Process of antibody immobilization by self-assembled monolayer (SAMs) technique. The 3-Mercaptoproprionic acid (MPA) was adsorbed on the gold surface, provide the functional group with antibody molecule.

2.2 Antibody Immobilization by Physical Adsorption

Solution of 1 mg/ml protein A was spread onto the cleaned microcantilever. After immobilization for 4 h, the microcantilever was washed with PBS buffer solution (pH 7.4). The protein A immobilized microcantilever was dipped into 1 mg/ml monoclonal antibody solution for 1 h at room temperature and then washed with PBS buffer solution (pH 7.4). The microcantilever was immersed into 1 mg/ml bovine serum albumin (BSA) solution to block the empty surface to prevent unspecific binding signal by the other molecules, which would give rise to false positive signal. Incubate the microcantilever for overnight and then washed with PBS buffer solution pH 7.4 followed by DW water. The scheme of antibody immobilization by physical adsorption is illustrated in Figure 21.

2.3 Antibody Immobilization by Self-Assembled Monolayers (SAMs)

The freshly cleaned microcntilever surface was modified by simply dipped into the ethanol solution containing 10 mM 3-mercaptoproprionic acid (MPA) for 3 h at room temperature to form a self assembled monolayer. The excess of MPA solution was rinsed with absolute ethanol solution and then followed by DI water. In this step, the molecules of MPA were adsorbed onto the gold surface and form a monolayer, leaving free carboxylic groups for further reaction.

The microcantilever was then immersed in the 1:1 (v/v) mixture of 200 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 50 mM *N*-hydroxysuccinimide (NHS) for 30 min at room temperature to activated the carboxylic groups to reactive NHS-ester groups which available to form covalent bond with primary amine of antibody molecules. After reached incubation period, the microcantilever was washed with DI water and then the 0.5 mg/ml monoclonal antibody (Anti-*V.cholerae* O1) in PBS buffer solution, pH 7.4 was spread over the microcantilever surface. This step was carried out for 1 h at room temperature. The excess antibody solution was then washed by PBS buffer solution (pH 7.4). Finally, the 3 mg/ml bovine serum albumin (BSA) in PBS buffer solution (pH 7.4) was added onto the antibody-immobilized microcantilever surface. The scheme of antibody immobilization is illustrated in Figure 22-23. After washing with PBS buffer solution (pH 7.4) and DI water, the microcantilever was mounted into the microcantilever holder and dried directly in a stream of nitrogen as shown in Figure 24.

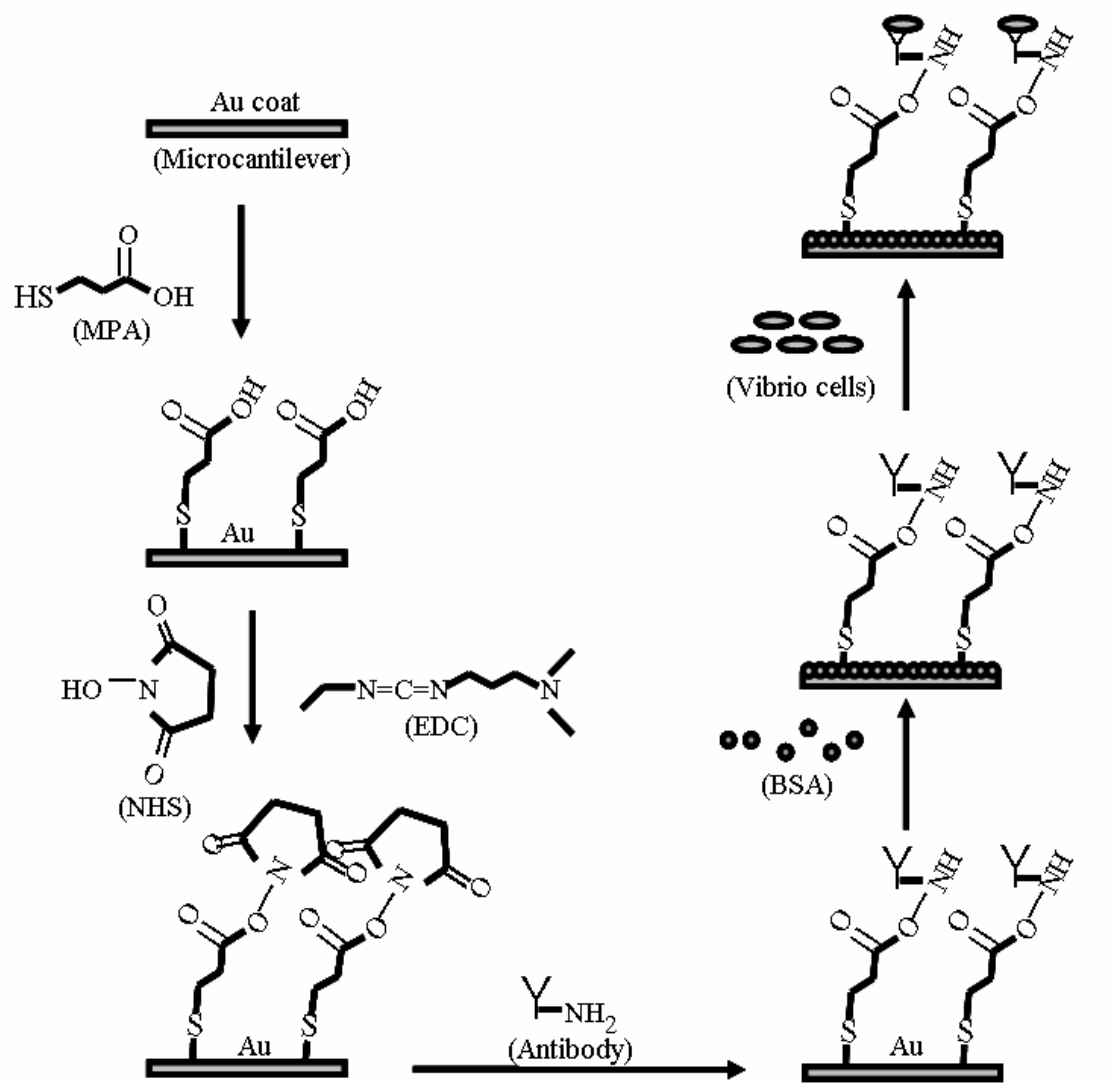


Figure 23. Schematic representation of self-assembled monolayers (SAMs) for antibody immobilization on the gold surface of microcantilever; The 3-mercaptoproprionic acid (MPA) was adsorbed and provided the carboxyl group on the microcantilever surface. The 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were used to activate the MPA to form covalent bond with monoclonal antibody.

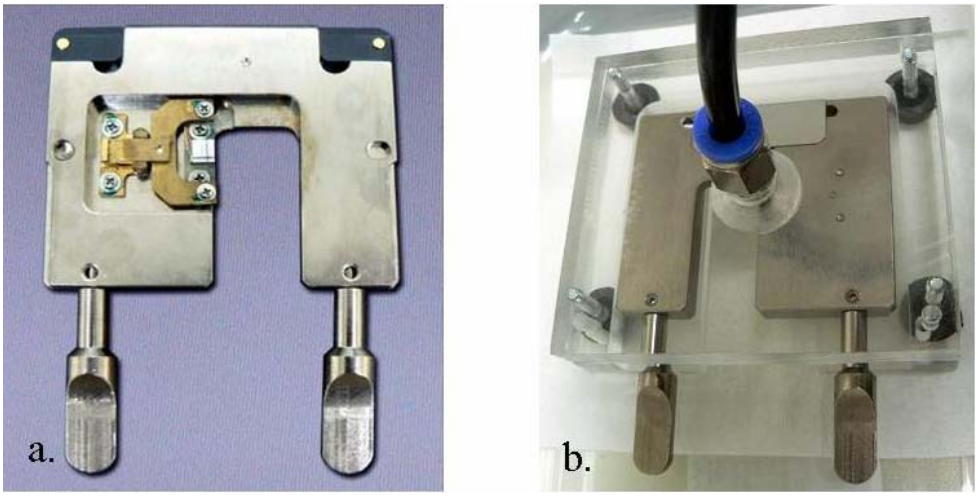


Figure 24. Microcantilever holder (a.) microcantilever holder used for connects the microcantilever to the atomic force microscope (AFM), (b.) microcantilever mounted into the microcantilever holder and drying with stream of nitrogen.

2.4 Bacteria Binding Measurement

2.4.1 Study the Effect of Shaking for Antigen-Antibody Reaction

Antibody-immobilized microcantilever was immersed in the suspension of *Vibrio cholerae* O1 under 2 conditions.

Case 1: The antibody-immobilized microcantilever was immersed in suspension of *V. cholerae* O1 without shaking for 30 min. The resonance frequency was measured before and after bacteria binding.

Case 2: The antibody-immobilized microcantilever was immersed in suspension of *V. cholerae* O1 with shaking for 5 min. The resonance frequency was measured before and after bacteria binding.

2.4.2 *Vibrio cholerae* O1 Detection

Antibody-immobilized microcantilever was dried with nitrogen gas for 10 min and then measured the resonance frequency (F_0). The microcantilever was immersed in the suspension of *Vibrio cholerae* O1 in concentration ranging from $1x10^2$-$1x10^7$ CFU/ml and the PBS buffer solution (pH 7.4) was used as negative control. The immersion time used was 5 min to allow forming of antigen-antibody complex. Shaking during the time of immersion was required for the antigen-antibody reaction. Finally, the microcantilever was measured the resonance frequency (F_1).

2.5 Cross-reactivity Testing

The *Vibrio parahemolyticus* in concentration of $1x10^8$ CFU/ml was used as a test to check the cross-reaction of the sensor. The antibody-immobilized microcantilever was immersed in *V. parahemolyticus* under the same condition as *V. cholerae* O1 detection.

2.6 Resonance Frequency Measurement

The experiments were performed using atomic force microscope (AFM, SPA400, Seiko), operated in DFM (Dynamic force microscope) mode. The resonance frequency was measured before (note the signal as F_0; baseline resonance frequency signal) and after antigen-antibody binding (note the signal as F_1; test resonance frequency signal). Before performing any measurement, the microcantilever was mounted to the microcantilever holder which the metal electronic wire contacts to the cantilever chip. Placed the cantilever holder with detecting cantilever on the AFM and the cantilever was driven by mechanical-acoustic excitation using an actuator in close proximity to the cantilever holder. Figure 25 shows schematically of the resonance frequency measurement.

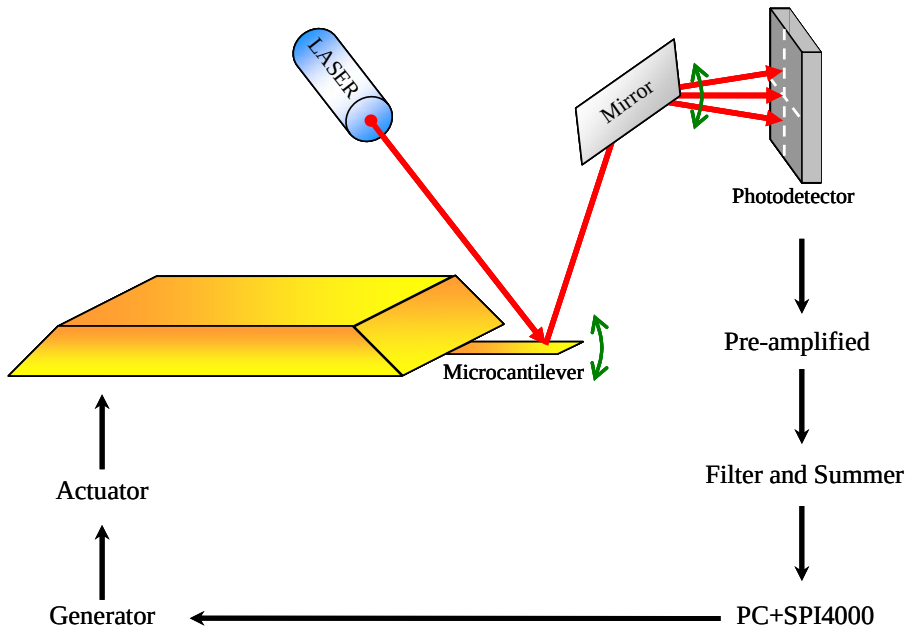


Figure 25 Schematic drawing of the resonance frequency measurement by optical leverage method of atomic force microscope (AFM)

The cantilever oscillation was measured by using the optical beam deflection method, in which an incident beam from laser diode focused on the free end of cantilever as shown in Figure 26. The reflected light from the cantilever surface was allowed to fall on a four quadrant photo-sensitive detector. The cantilever deflection gave the signal which is proportional to the difference in the photocurrents generated in the upper and lower segments. The photocurrents of the upper and lower segments were pre-amplified by the amplifier. The signal was connected to a data acquisition program (SPI 4000, NT-MDT Co., LDT) for processing and analysis the resonance frequency of the microcantilever and shown the result in a personal computer (PC). The resonance frequency shift is the different value of the resonance frequency F_0 and F_1.

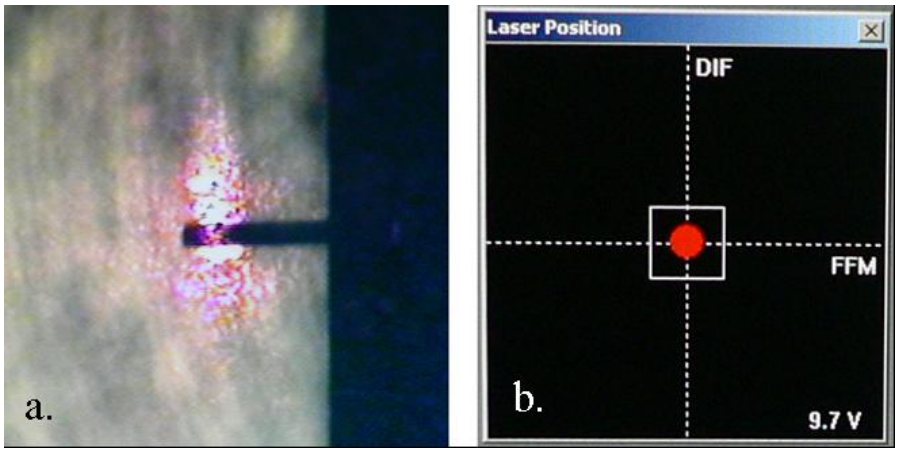


Figure 26. Alignment of the laser on the microcantilever surface, (a) the light beam from the laser diode was focused on the free end of microcantilever surface, (b) the reflected light was adjusted at the center of the position sensitive detector (PSD).

2.7 Study the Effect of Temperature

To study the effect of the temperature, the hot air was blown to the environment surrounding the atomic force microscope (AFM). The resonance frequency of the microcantilever was measured before and after blowing the hot air. The resonance frequency was fine observed in every temperature that changing. The temperature of the environment surrounding the AFM was measured by the temperature and humidity meter as shown in Figure 27.

Figure 27. Temperature and humidity meter created from Nanoelectronics and MEMS laboratory, National Electronics and Computer Technology Center (NECTEC), Thailand.

2.8 Surface Analysis

After bacteria detection, the microcantilever surface was directly examined by scanning electron microscope (SEM, S-3400N, Hitachi) to confirm the bacteria binding by imaging the *Vibrio cholerae* O1 cells bound on the microcantilever surface.

CHAPTER V
RESULTS

This chapter presents the results from the study of microcantilever-based biosensor for detection of *Vibrio cholerae* O1. Gold-coated microcantilever was immobilized with monoclonal antibody to *V. cholerae* O1 and tested with *V. cholerae* suspensions. The antibodies on microcantilever was captured the *V. cholerae* O1 cells and formed immunocomplex on the microcantilever and affected to increasing of microcantilever mass and decreasing of resonance frequency of the microcantilever. The first section shows the results of the resonance frequency response of the microcantilever that coated with monoclonal antibody by physical adsorption and chemical adsorption (self-assembled monolayers or SAMs) both in liquid and air environment. Next, the result of the limit of detection, the mass sensitivity and the calibration curve were shown. The third section shows the result of antibody-immobilized microcantilever tested with other strains of bacteria to check cross-reaction. Moreover, in the last section of this chapter shows the scanning electron (SEM) micrographs of *V. cholerae* O1 cells bound on microcantilever surface.

1. The resonance Frequency Response of Microcantilever

1.1 Characteristic of Microcantilever under Liquid and Air Environment

On the experiment the resonance frequency of microcantilever can be operated under the condition of liquid and air environment using the atomic force microscope (AFM). The experimental results have shown the decreasing of resonance frequency when the mass adsorbed on the microcantilever surface. However, the characteristic of the resonance frequency conducted under the liquid condition was not shaped like the signal conducted under the air environment. Figure 28 shows the resonance frequency response under liquid and air environment. Lower of resonance frequency signal was observed under the liquid condition due to the damping effect of the water.

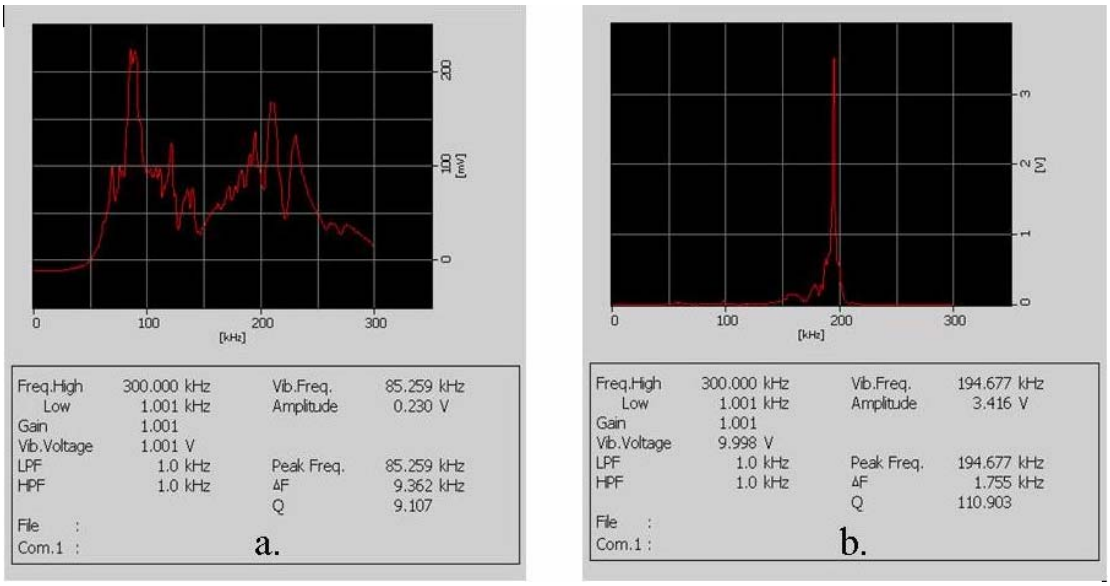


Figure 28. Frequency of the microcantilever under liquid and air environment; (a.) the resonance frequency measured in liquid environment shows many of noise signals due to damping effect, (b.) the resonance frequency measured in air environment shows good characteristic of resonance frequency with low noise of signal.

1.2 Resonance Frequency Response due to Mass Loading

Before study the resonance frequency response due to mass loading, the microcantilever was measured the resonance frequency under air environment to observed the signal of resonance frequency response. The resonance frequency was measured in every minute until reached 10 min and the results show stable signal of resonance frequencies as shown in Table 5. The same microcantilever was immersed in DI water and measured the resonance frequency again. The results also gave the same of resonance frequency signal.

To study of resonance frequency response due to mass loading, the experiment was done by measured the resonance frequency of microcantilever before and after protein A adsorption. The experimental results show resonance frequency of microcantilever was shifted to the lower value after protein A adsorption onto the microcantilever surface. The shift of resonance frequency due to the protein A adsorption was found approximately 585 Hz as shown in Table 6. It can be assumed that the resonance frequency was shift due to the mass loaded on the microcantilever.

Table 5. Stability of the resonance frequency signal; the resonance frequency was measured in every minute for 10 min (Measure 1). The microcantilever was immersed in DI water, dried with nitrogen and measured the resonance frequency again (Measure 2). The results show the same result with the first measurement.

Time (min)	Resonance Frequency (kHz)	
	Measure 1	Measure 2
1	121.537	120.952
2	120.952	120.952
3	120.952	120.952
4	120.952	120.952
5	120.952	120.952
6	120.952	120.952
7	120.952	120.952
8	120.952	120.952
9	120.952	120.952
10	120.952	120.952

Table 6. Resonance frequency shifts of microcantilever due to protein A adsorbed on microcantilever surface. The resonance frequency shift was found 585 Hz.

Cantilever	Resonance Frequency (kHz)		ΔF(kHz)	ΔF(Hz)
	Baseline (F_0)	Protein A (F_1)		
1	195.848	195.263	0.585	585
2	195.263	194.677	0.586	586
3	222.763	222.718	0.585	585
4	155.474	154.889	0.585	585
5	166.006	165.421	0.585	585
Average	**Resonance Frequency (kHz)**		**0.5852**	**585.2**

2. *Vibrio cholerae* O1 Detection

2.1 Antibody Immobilization by Physical Adsorption

The microcantilever that coated with monoclonal antibody was tested against suspension of *Vibrio cholerae* O1 concentration. The result shown the shift of resonance frequency response both in higher and lower value compared with the resonance frequency at baseline (data not shown). The non-specific signal can be occurred on the physical adsorption processing by loss of some molecules of protein (protein A, antibody and BSA) adsorbed on the microcantilever. The loss of the molecule on the microcantilever reduced the mass and leading to increase of the resonance frequency of microcantilever.

2.2 Antibody Immobilization by Chemical Adsorption

The microcantilever that immobilized with monoclonal antibody by self-assembled monolayer (SAMs) was tested with different known concentration of *Vibrio cholerae* O1 suspension. The results show the resonance frequency of microcantilever decreased when increased the concentration of *V. cholerae* O1 suspension as shown in Table 7-8.

Figure 29 shows the resonance frequency response of the antibody-immobilized microcantilever when tested with *V. cholerae* O1 concentrations ($1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$ and $1x10^7$ CFU/ml. The PBS buffer solution was used as negative control. From the experimental results, it is reasonable to assume that antigen-antibody reaction formed immuno-complex of antigen and antibody that induced mass adsorbed on the microcantilever surface.

The calibration curve for *V. cholerae* O1 detection was shown in Figure 30. The curve shows relationship of the resonance frequency shift versus the concentration of *V. cholerae* O1 suspension in log scale. It can be seen that the resonance frequency shift is linearly proportional to the log of *V. cholerae* O1 suspension in concentration ranging from $1x10^3$ to $1x10^6$ CFU/ml.

From the experiment, the limit of detection of the sensor for *V. cholerae* O1 detection was found at $1x10^3$ CFU/ml. The antibody-immobilized microcantilever was tested with PBS buffer solution (pH 7.4) in the control experiment, in which bacterial cells were not presented. The result shows no significant of the resonance frequency shift (~14 Hz).

Table 7. Resonance frequency shift of antibody-immobilized microcantilever after tested with $1x10^3$ CFU/ml *V. cholerae* O1 suspension. Phosphate buffer saline (PBS, pH 7.4) was used as a blank test (No bacterial cell) for baseline resonance frequency signal. The Resonance frequency was measured every minute until reach stable signal, mostly 5 minutes.

Time	**Resonance Frequency (kHz)**			
(min)	**Baseline (F_0)**	**10^3 CFU/ml *V. cholerae* O1 (F_1)**	**ΔF(kHz)**	**ΔF(Hz)**
1	260.172	259.973	0.199	199
2	260.178	259.973	0.205	205
3	260.172	259.973	0.199	199
4	260.166	259.973	0.193	193
5	260.172	259.973	0.199	199
Average	**260.172**	**259.973**	**0.199**	**199**

Table 8. Resonance frequency shift of antibody-immobilized microcantilever after tested with $1x10^4$ CFU/ml *V. cholerae* O1 suspension. Phosphate buffer saline (PBS, pH 7.4) was used as a blank test for baseline resonance frequency signal. The experiment shows increased of resonance frequency shift to 260.4 Hz when compared with the pervious experiment.

Time	**Resonance Frequency (kHz)**			
(min)	**Baseline (F_0)**	**10^4 CFU/ml *V. cholerae* O1 (F_1)**	**ΔF(kHz)**	**ΔF(Hz)**
1	238.709	238.241	0.468	468
2	238.709	238.247	0.462	462
3	238.709	238.253	0.456	456
4	238.714	238.253	0.461	461
5	238.709	238.259	0.450	450
Average	**238.710**	**238.2506**	**0.4594**	**459.4**

Table 9. Resonance frequency shift of antibody-immobilized microcantilever after tested with $1x10^5$ CFU/ml *V. cholerae* O1 suspension Phosphate buffer saline (PBS, pH 7.4) was used as a blank test for baseline resonance frequency signal. The experiment shows increased of resonance frequency shift to 258.2 Hz compared with the pervious experiment.

Time	**Resonance Frequency (kHz)**			
(min)	**Baseline (F_0)**	**10^5 CFU/ml *V. cholerae* O1 (F_1)**	**ΔF(kHz)**	**ΔF(Hz)**
1	250.149	249.423	0.726	726
2	250.137	249.423	0.714	714
3	250.143	249.423	0.720	720
4	250.137	249.429	0.708	708
5	250.149	249.429	0.720	720
Average	**250.143**	**249.4254**	**0.7176**	**717.6**

Table 10. Resonance frequency shift of antibody-immobilized microcantilever after tested with $1x10^6$ CFU/ml *V. cholerae* O1 suspension. Phosphate buffer saline (PBS, pH 7.4) was used as a blank test for baseline resonance frequency signal. The experiment shows increased of resonance frequency shift to 384.8 Hz when compared with the pervious experiment.

Time	**Resonance Frequency (kHz)**			
(min)	**Baseline (F_0)**	**10^6 CFU/ml *V. cholerae* O1 (F_1)**	**ΔF(kHz)**	**ΔF(Hz)**
1	281.033	279.932	1.101	1101
2	281.033	279.932	1.101	1101
3	281.039	279.932	1.107	1107
4	281.039	279.932	1.107	1107
5	281.033	279.937	1.096	1096
Average	**281.0354**	**279.933**	**1.1024**	**1102.4**

Table 11. Resonance frequency shift of antibody-immobilized microcantilever after tested with 1x10^7 CFU/ml *V. cholerae* O1 suspension Phosphate buffer saline (PBS, pH 7.4) was used as a blank test for baseline resonance frequency signal. The experiment shows increased of resonance frequency shift to 1,610.2 Hz when compared with the pervious experiment.

Time	**Resonance Frequency (kHz)**			
(min)	**Baseline (F_0)**	**10^7 CFU/ml *V. cholerae* O1 (F_1)**	**ΔF(kHz)**	**ΔF(Hz)**
1	243.022	240.301	2.721	2721
2	243.022	240.301	2.721	2721
3	243.022	240.313	2.709	2709
4	243.022	240.313	2.709	2709
5	243.022	240.319	2.703	2703
Average	**243.022**	**240.3094**	**2.7126**	**2 712.6**

The additional mass, Δm, which is due to antigen-antibody binding, can be straightforwardly related to the shift in the resonance frequency of microcantilever that given by

$$1/f_1^2 - 1/f_0^2 = \Delta m/(4n\pi^2 k) \quad (2)$$

where *k* is the spring constant of the cantilever, f_0 is the initial resonance frequency prior to the mass loading, f_1 is the resonance frequency after mass addition (mass of bacteria bound on the microcantilever surface) and *n* is a geometry-dependent correction factor (*n* = 0.24 in the case that assumed that the additional mass is uniformly distributed over a rectangular shaped microcantilever). From the experimental, the results of the resonance frequency were obtained. The additional mass of the bacteria cells bound on the microcantilever surface can be estimated by equation (2) and the results are listed in Table 12.

The detection mass sensitivity of the sensor was determined by Δm/ΔF, which was found approximately to be 146.5 pg/Hz.

Table 12. Estimation of mass adsorbed on microcantilever surface

Resonance Frequency (kHz)				
Concentration (CFU/ml)	**1x10^3**	**1x10^4**	**1x10^5**	**1x10^6**
ΔF (Hz)	199	459.4	717.6	1,100.9
Δm (ng)	30.4	82.6	104.4	121.5
Δm/ΔF (pg/Hz)	152	179	145	110
Average	**146.5 pg/Hz**			

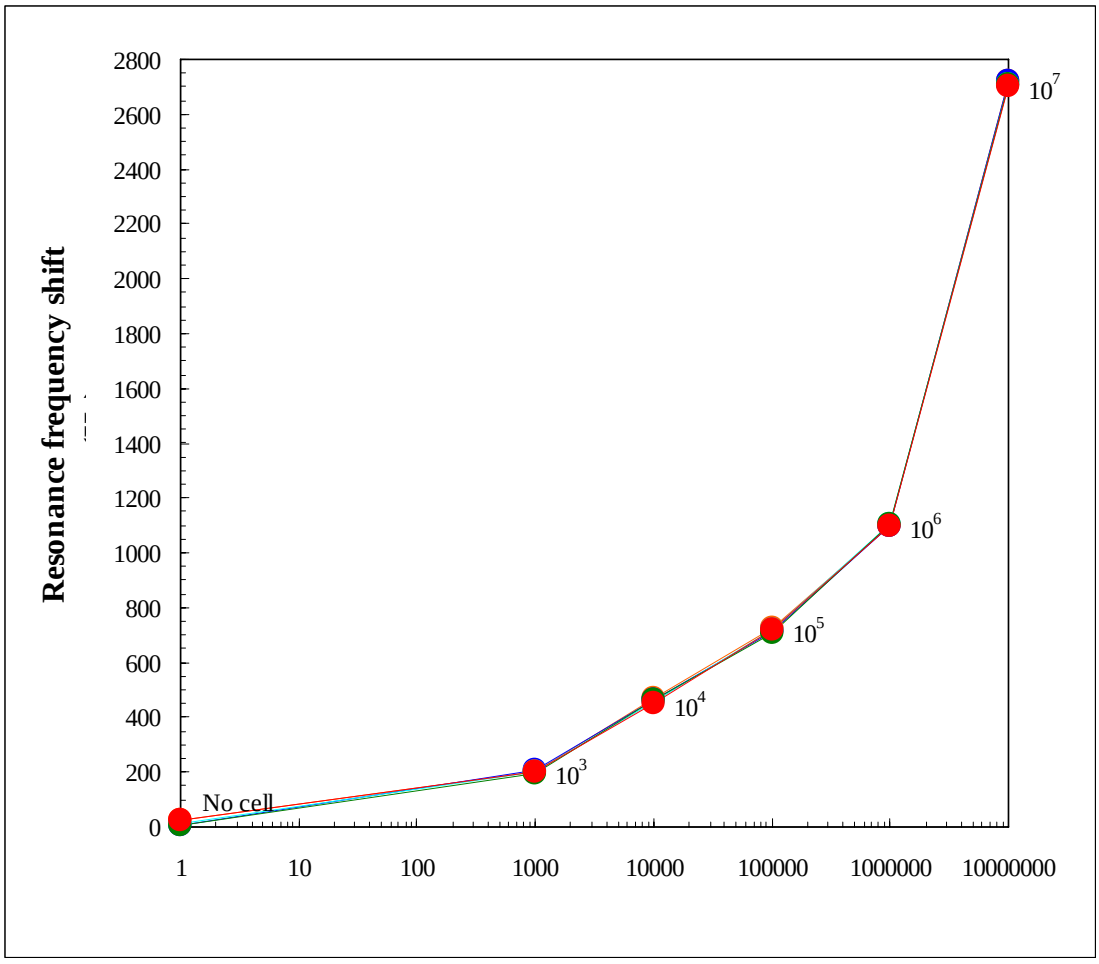


Figure 29. Relationship of resonance frequency shift *versus V. cholerae* O1 concentration. The control experiment was an antibody-immobilized microcantilever tested in PBS buffer solution.

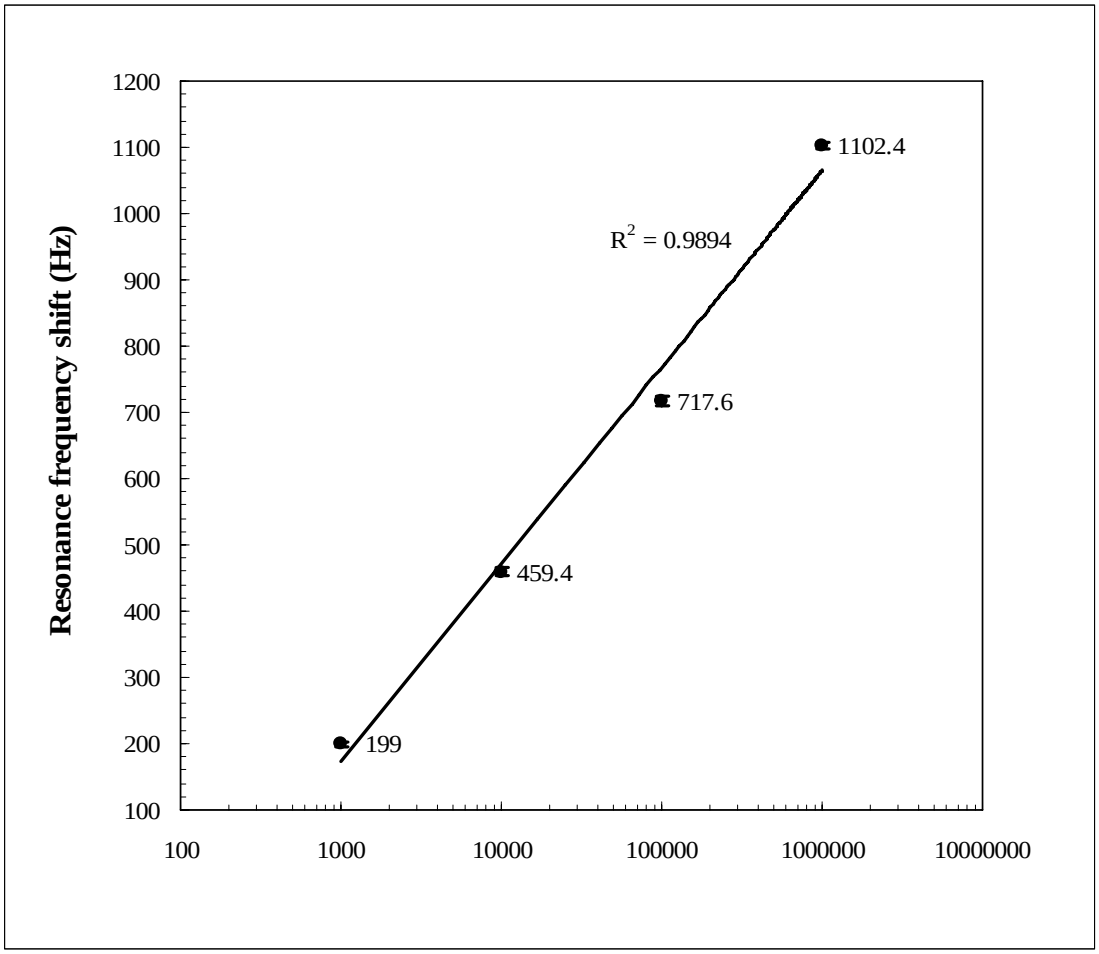


Figure 30. Calibration curve of the resonance frequency shift versus *V. cholerae* O1 concentration. The result the relationship of the resonance frequency and *V. cholerae* O1 concentration in linearity ranging from 10^3-10^6 CFU/ml.

3 Cross-Reactivity Tests

Vibrio parahemolyticus bacteria were used as a test to check the cross-reactivity performance of the sensor. The test was done under the same condition used for *Vibrio cholerae* O1 detection. The antibody-immobilized microcantilever was immersed into 1x10^8 CFU/ml *V. parahemolyticus* with shaking for 5 min. After washed with PBS buffer solution (pH 7.4) and DI water. The microcantilever was measured the resonance frequency after dry with nitrogen gas, the resonance frequency of microcantilever was measured before and after immersion in suspension of *V. parahemolyticus*.

The resonance frequency shift of the antibody-immobilized microcantilever tested with *V. parahemolyticus* suspension shown no significant resonance frequency response (~24 Hz). It can be assumed that it has no cross-reactivity by other strain of bacteria. Figure 31 is the comparison of the resonance frequency shift of antibody-immobilized microcantilever tested with PBS buffer (pH 7.4), $1x10^8$ CFU/ml *V. parahemolyticus* suspension and *V. cholerae* O1 with the concentrations of $1x10^3$ and $1x10^4$ CFU/ml. The resonance frequency shifts were 14.4, 26.6, 199.0 and 459.4, respectively.

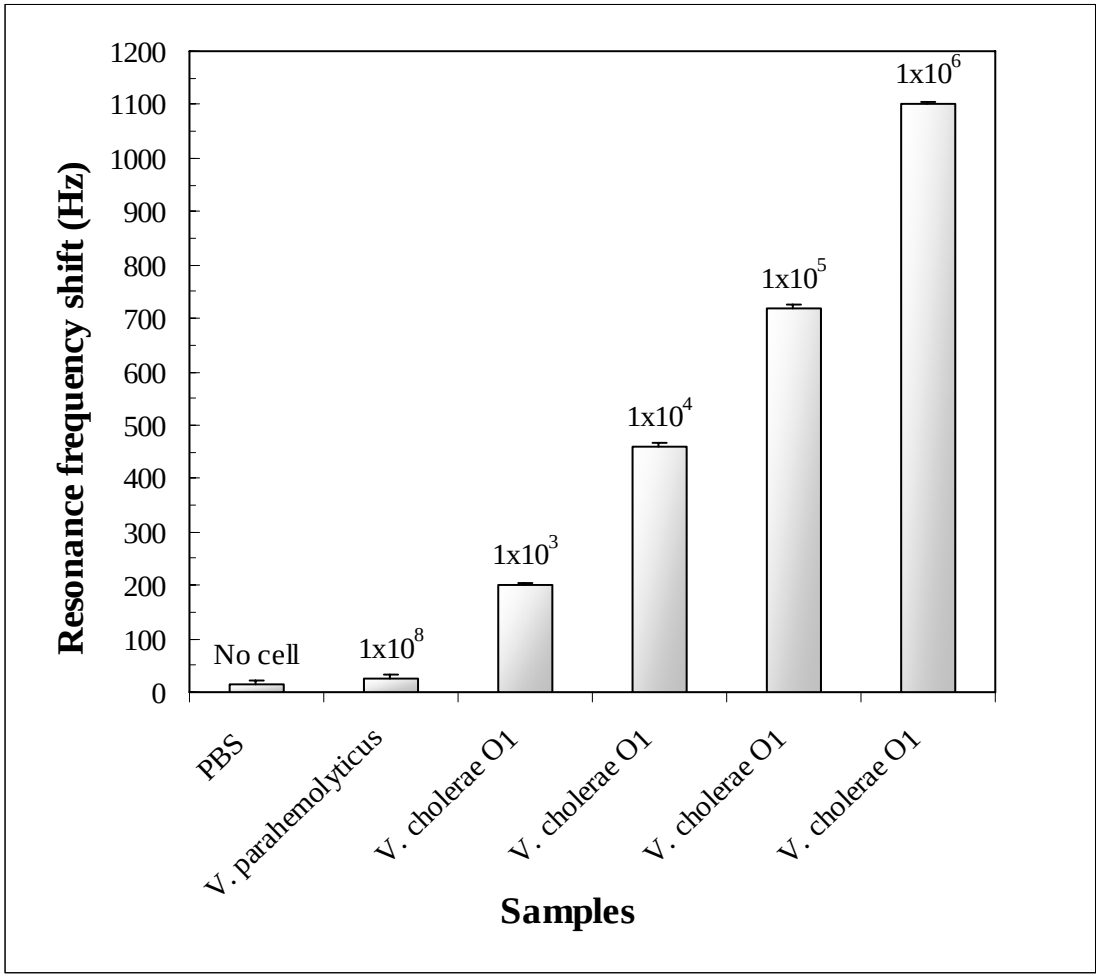


Figure 31. Cross-reactivity of the sensor; the graph shows comparison of resonance frequency shift response when the antibody-immobilized microcantilevers were tested with suspension of *Vibrio parahemolyticus*, *Vibrio cholerae* O1, and PBS buffer solution.

4. Study the Effect of Shaking

The antibody-immobilized microcantilever was immersed in *Vibrio cholerae* O1 suspension for 30 min to allow the antigen-antibody interaction. The resonance frequency was shifted 120 Hz from the baseline. The same microcantilever was immersed in *V. cholerae* O1 suspension for 5 min with shaking. The resonance frequency of the microcantilever after binding with bacterial cell was shown the shift of resonance frequency around 1,100 Hz as shown in Figure 32.

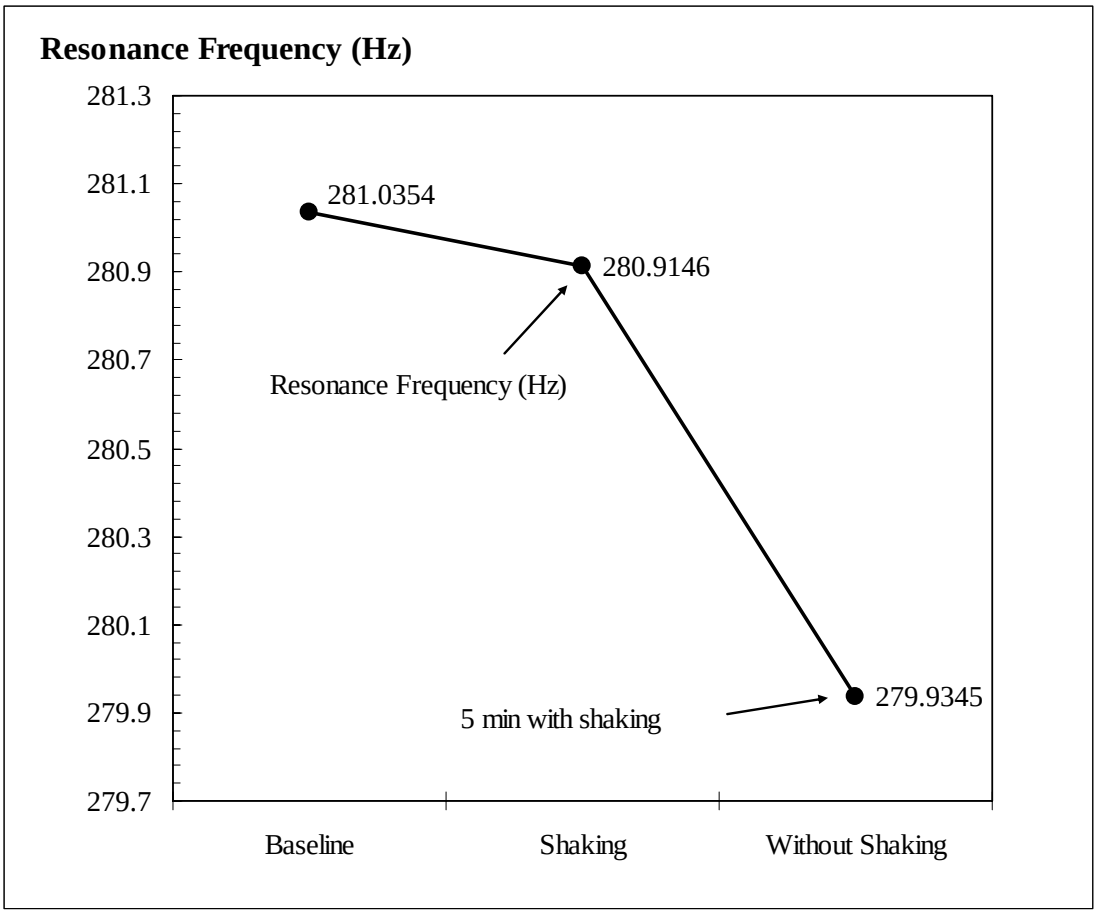


Figure 32. Plots of resonance frequency responses with shaking and without shaking of antibody-immobilized microcantilever. The resonance frequency shift was shift 120 Hz after immersion in *V. cholerae* O1 suspension for 30 min without shaking and the resonance frequency shift was 1.1 kHz within 5 min when the shaking was added.

5. Study the Effect of Washing

On the experiment, the washing step is a critical step which affected to the signal gained from the experiments. The purpose of washing is to separate bound and unbound (free) protein molecules from the microcantilever surface. Figure 33 shows the affect of insufficient washing to resonance frequency responses. The antibody-immobilized microcantilever was measured the resonance frequency after washed with PBS buffer solution (pH 7.4) for 30 sec and DI water for 15 sec. The resonance frequency was recorded and then repeated the step of washing. The resonance frequency was measured again and the result shown the resonance frequency was increased for 2,085 Hz. The resonance frequency of microcantilever was reached the stable signal after washed for three times.

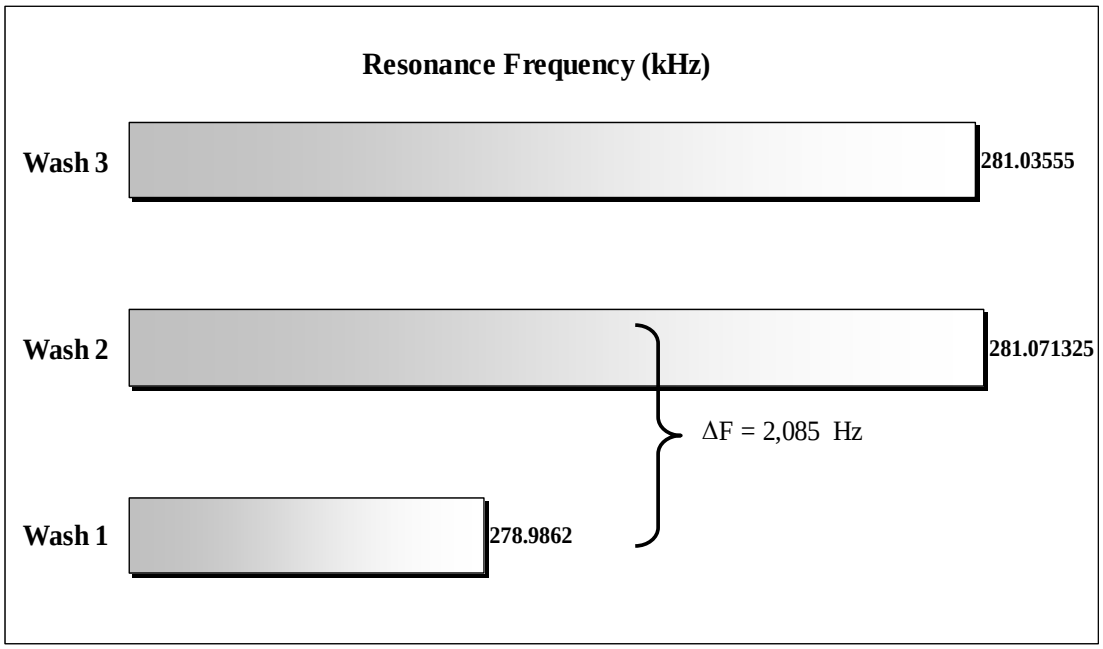


Figure 33. Non-specific signal gain from insufficient of washing; the microcantilever was measured the resonance frequency after washing (Wash 1), resonance frequency measured to be 278.9862 kHz. Wash the microcantilever again (Wash 2). The resonance frequency was 281.0713 Hz in second wash which is changed to the first wash to 2,085 Hz. It the third wash, no significant change in resonance frequency was observed.

6. Effect of Temperature

Temperature is a factor that may be affected to resonance frequency of the microcantilever. In this study the experiment to examine this effect was done by blowing the hot hair to the environment surrounding the main body of AFM (SPA 400). Figure 34 show the results of the experiment. The temperature was increased after blowing the hot air and decreased when the temperature came down. Figure 35 shows the result of resonance frequency response measured at each temperature. The experimental result had shown that no significant of resonance frequency change of the resonance frequency when the temperature changed ±3 ^{o}C. Thus, if control the experiment of measurement at room temperature (22-26 ^{o}C), it has no significant change of the resonance frequency.

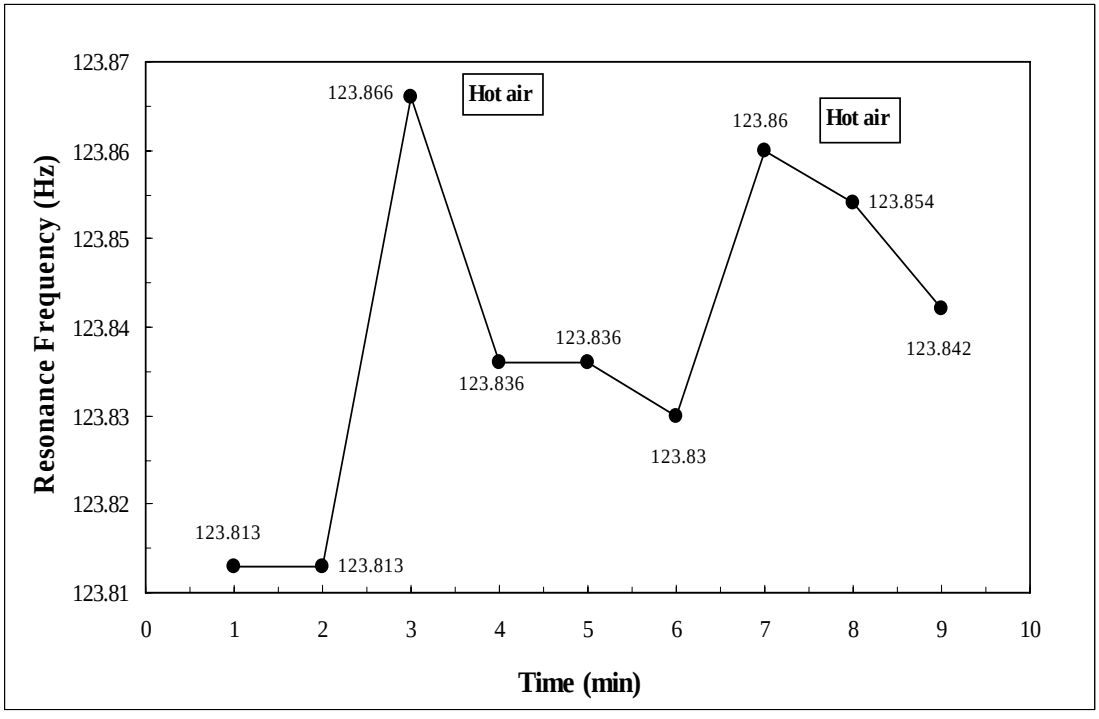


Figure 34. Resonance frequency response after hot air exposure; the resonance frequency was increased after exposed to the hot air and decreased when the temperature of the environment surrounding atomic force microscope (AFM) came down.

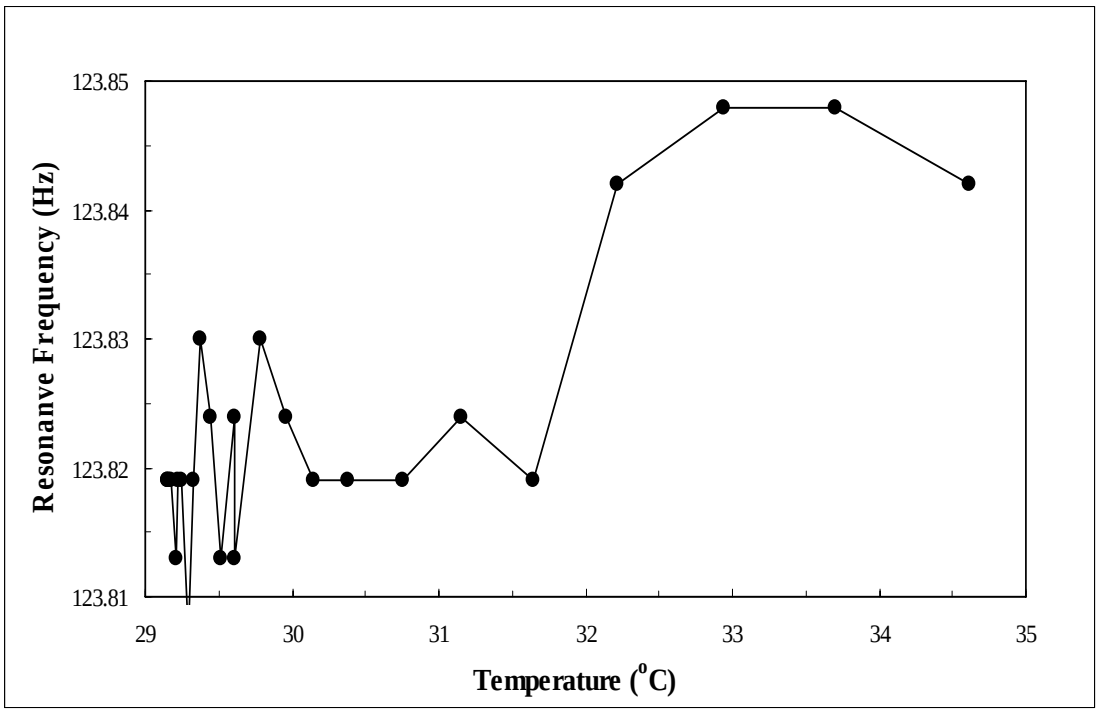


Figure 35. Effect of temperature variation to the resonance frequency shift; no significant change of the resonance frequency measured by the atomic force microscope when the temperature was changed in range of ± 3 °C.

7. Surface Characterization

The tested microcantilever was examined by scanning electron microscope (SEM) to characterized the *V. cholerae* 01 cells bound on microcantilever surface and confirmed the resonance frequency shift was generated from the mass of bacteria cells bound on the microcantilever. Figure 36 (a) shows the SEM image of the antibody-immobilized microcantilever beam before antigen (*V. cholerae* O1 cells) binding. It can be seen that the cantilever surface is smooth and the anti-*V. cholerae* O1 protein immobilized on the gold can not be observed because its small size scale. Figure 36 (b) and (c) demonstrates the SEM micrograph of the microcantilever beam after *V. cholerae O1* binding with different concentrations. It can be observed that thin and long dark cells are now scattered on the cantilever surface. The magnification view (Figure 36 (d)) reveals that the cells have curve-rod shaped, which is a typical characteristic of *Vibrio cholerae* bacteria.

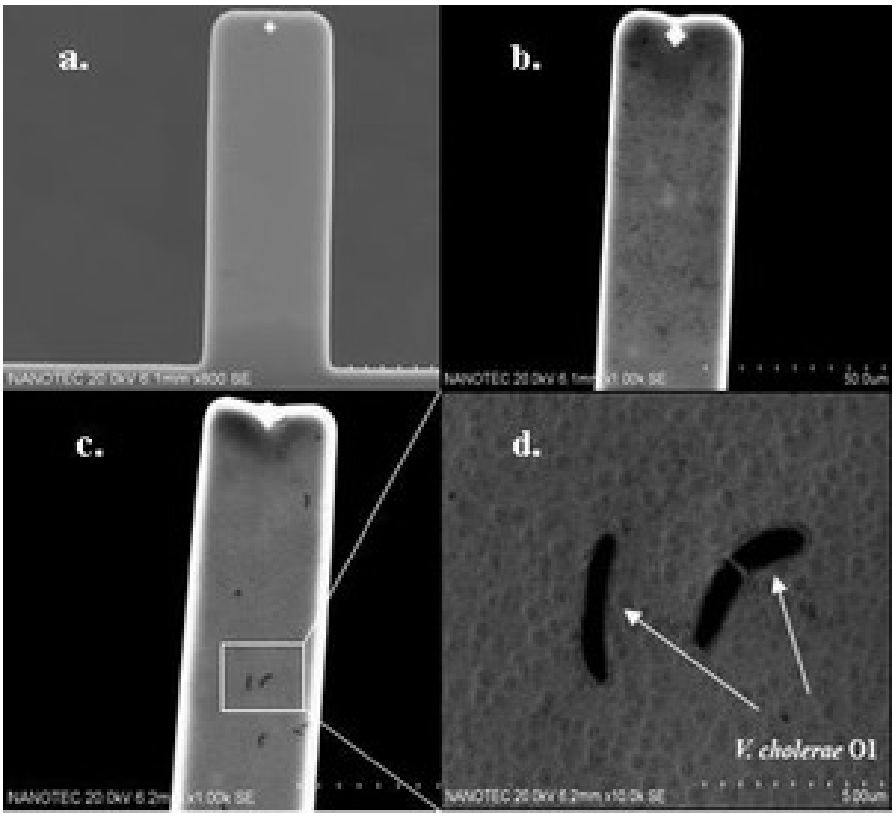


Figure 36. Scanning electron microscope (SEM) images of antibody-immobilized microcantilever surface (a) before and (b) - (c) after bacteria detection at different concentrations and (d) higher magnification view of *V. cholerae* O1 cells on gold surface.

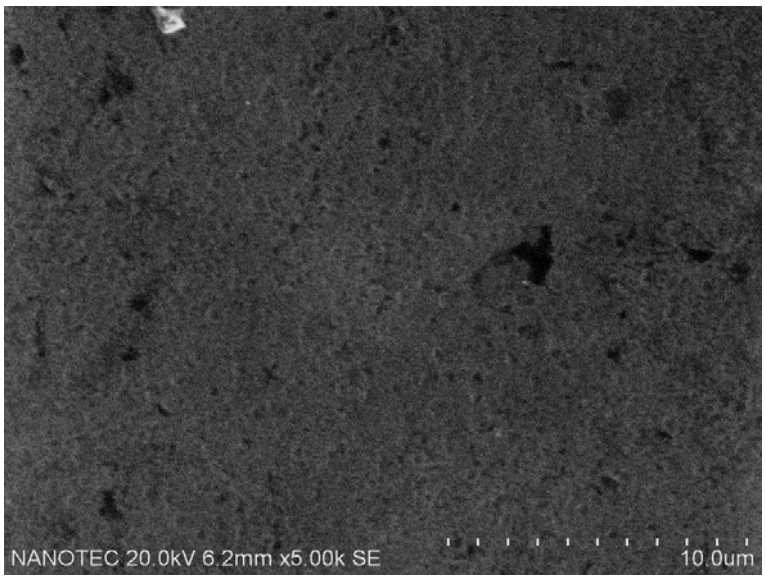


Figure 37. Scanning electron micrograph of antibody-immobilized microcantilever after applied PBS buffer solution

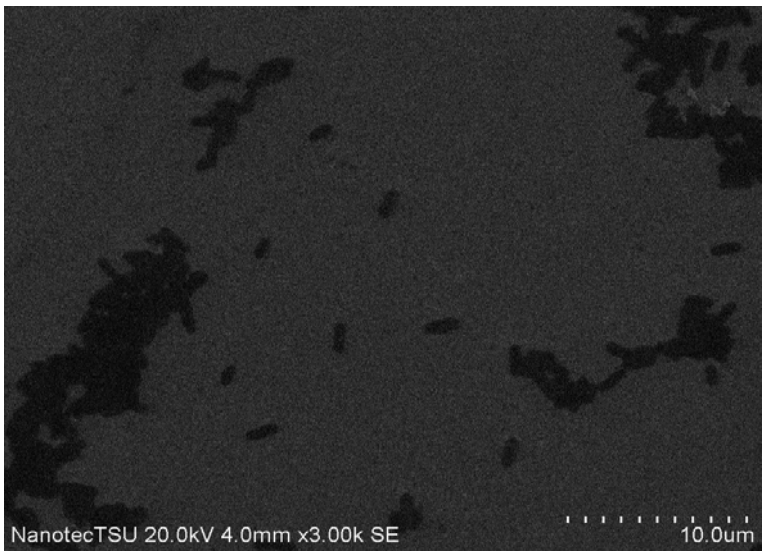


Figure 38. Scanning electron micrograph of antibody-immobilized microcantilever surface after applied with *V. cholerae* O1 concentration $1x10^4$ CFU/ml

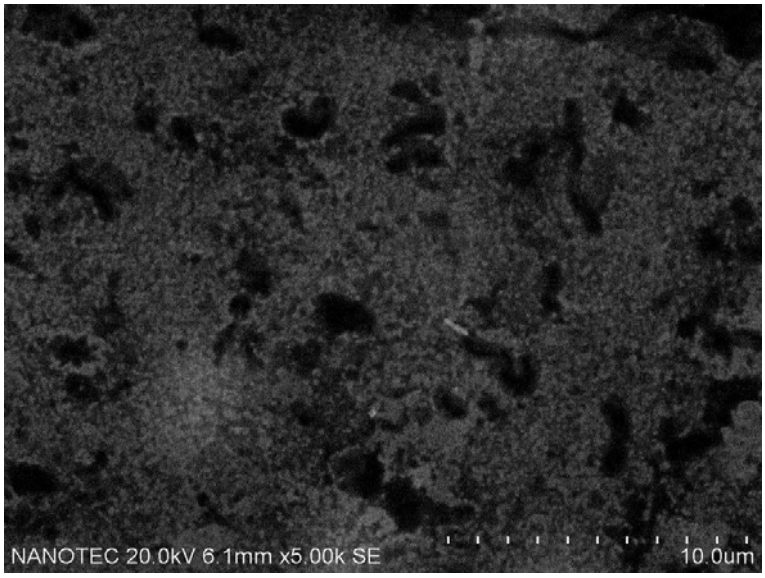


Figure 39. Scanning electron micrograph of antibody-immobilized microcantilever surface after applied with *V. cholerae* O1 concentration $1x10^8$ CFU/ml

CHAPTER VI
DISCUSSION

There are several ways for detection of *Vibrio cholerae* O1. Basically, the conventional culture method combined with biological reaction for screening and confirmation testing which are gold standard methods used in routine work of bacterial isolation and identification in microbiological laboratory. Though, these techniques provide good qualitative and quantitative information, they are also having several disadvantages including time-consuming, laborious, low sensitivity, and requirement of pre-enrichment step.

An alternative way for *V. cholerae* O1 detection is an immunological method. Enzyme liked-immunosorbent assay (ELISA) is one of preferable technique for microorganism detection. Detection of *V. cholerae* O1 by ELISA technique provides quantitative information with good detection sensitivity ($1x10^6$ CFU/ml) which is suitable for clinical specimen. The ELISA is enzyme-labeled technique that needed multiple steps of reagent addition. The reagent involved in this technique is expensive comparing to the conventional method. The pre-enrichment process of the sample is still required when it is containing very low amount of *V. cholerae* O1.

Genetic-based techniques are providing the highest of detection sensitivity. Polymerase chain reaction (PCR) technique has capability to detect *V. cholerae* O1 in very low amount of 8 CFU/ml, but it has drawbacks of complexity steps and requirement of well-trained personnel to handle the process. Moreover, the efficient separation space (area for pre-PCR, PCR and post-PCR processes) is a critical requiring for prevention of contaminations.

The investigation of microcantilever-based biosensor for detection of *V. cholerae* O1 in this study is consists of the using of microcantilever as a transducer in biosensor applications, characteristics of microcantilever, the factors effected to the sensitivity of the sensor, the limit of detection, the antibody immobilization by physical adsorption

and chemical adsorption of self-assembled monolayers (SAMs) technique, cross-reactivity of the sensor, and the environmental factors affected to the sensor.

The microcantilever-based immunosensor for detection of *Vibrio cholerae* O1 is consisted of a microcantilever as a transducer and monoclonal antibody as a biological recognition element. The sensitivity of this *V. cholerae* O1 biosensor was found approximately to be 146.5 pg/Hz. It is nearly the same as another work of microcantilever sensor. Karin Y. Gfeller *et.al*. has been reported the sensitivity of the microcantilever-based microcantilever for *Escherichia coli* growth detection approximately of 140 pg/Hz [66]. The limit of detection of this *V. cholerae* O1 sensor was found to be $1x10^3$ CFU/ml which is higher than the limit of detection of the quartz-crystal microbalance (QCM)-based biosensor for detection *V. cholerae* O1 as described in pervious work [106]. This limit of detection is much more enough to cover the infectious dose of *V. cholerae* O1 ($1x10^4$ CFU/ml).

There are several factors concerned with the sensitivity and detection limit of the biosensor, including the size of microcantilever, the material used to construct the microcantilever, the operation modes, the immobilization techniques, the volume of the sample, and the incubation time for antigen-antibody reaction.

In this study, the microcantilever was used as a transducer. It is very small, in scale of micrometer. Most of commercial microcantilevers are constructed with silicon and silicon nitride. The dimension of microcantilever and material used to construct the microcantilevers are also involved with the sensitivity of the sensor by changing their force constant. The force constant *k*, defines the stiffness of the cantilever. The cantilever with *k* less than 1 N/m are designed as soft cantilever and the cantilever with *k* higher that 1 N/m are designed as rigid cantilever [27].

$$1/f_1^2 - 1/f_0^2 = \Delta m/(4n\pi^2 k) \quad (1)$$

Equation (1) shows the relationship of resonance frequency shift and additional mass (Δm) [107]. The resonance frequency shift of the cantilever relates to the additional mass and force constant (*k*). The parameter *k* depends on the thickness, density, and the modulus of the cantilever material [108]. Thus, scale down of microcantilevers will increase the resonance frequency [109].

Commercial silicon nitride microcantilever used in this study provide lower of sensor sensitivity (pictogram/Hz) compared with Aluminium-based microcantilever sensor (the sensitivity was found in scale of attogram/Hz) because the lower of the force constant [110].

Microcantilever-based biosensor for *V. cholerae* O1 detection was developed as a mass sensor device based on piezoelectric effect like the QCM-based sensor. However, the microcantilever-based technique has superior of mass sensitivity. This high sensitivity is consequence of the cantilever properties itself [107]. The microcantilever is resonating at 20-200 kHz while QCM is resonating in range of 50-500 MHz [107]. That is the reason why the microcantilever can weigh the same amount of bacteria by the lower of signal. The basic principle of piezoelectric effect to weigh the mass of molecules adsorbed on its surface is changing of the resonance frequency of microcantilever after mass adsorbed on its surface. In case of detection of *V. cholerae* O1, the resonance frequency of microcantilever is an indicator to tell the result of *V. cholerae* O1 cells bound on the microcantilever. From the shift of resonance frequency, it can be calculated back to the number of bacteria bound on the microcantilever. The resonance frequency of the microcantilever was measured by the atomic force microscope or AFM which is operated in DFM (Dynamic Force Mode) mode. Atomic force microscope is integrated with optical leverage unit that provides ability to measure both in the microcantilever deflection and the resonance frequency of the microcantilever with high sensitivity. The AFM can detect the deflection of microcantilever in sub-angstrom resolution which is derived ultrasensitivity of the sensor. It has been demonstrated that the resonating microcantilevers can detect added mass at the level of zeptograms [111].

Comparation of the operation modes of microcantilever; the static mode and the dynamic mode, there are also give high sensitivity of measurement using the AFM. However, there are different in the design of protein immobilization. Immobilization of the biological recognition elements on one side of the microcantilever is required when operate the microcantilever in static mode [29, 44]. More techniques are needed to control the biological recognition element located only on one side of microcantilever, such as evaporating, sputtering, or spraying [112]. The limitation of immobilization was excluded if the microcantilever is operated in dynamic mode

which the biological elements or receptor molecules can be immobilized on both side of the microcantilever [30].

Beside of good advantages, there are several disadvantages of using the dynamic mode of the AFM for detection the change of resonance frequency. Measuring the resonance frequency by the AFM in dynamic mode is requiring the external device, such as the laser assembly. Moreover, it has restricted to measure the resonance frequency of microcantilever in liquid environment due to damping effect and low quality (Q) factor that leading to gain the poor of signal-to-noise ratio [29, 113]. The Q factor of microcantilever is low in liquid environment, high in air environment and highest in vacuum environment. The high Q-factor leading to improve the mass sensitivity signal [114]. If the experiment need to be done in the liquid environment, it would be better to using the microcantilever that operated in static mode (Measure the change of surface stress change).

The immobilization technique is also a critical step for biosensor construction. In this study, the molecules of monoclonal antibody that specific to *V. cholerae* O1 cell have to immobilized on the gold-coated microcantilever surface. In this case, there are three possible way for antibody immobilization. First, the antibody is a protein molecule that can be adsorbed directly onto the microcantilever surface. It is a simplest way of physical adsorption technique. Another two ways are based on chemical adsorption; the self-assembled monolayer of an antibody molecule on the gold surface of microcantilever and silanization of an antibody molecule on silicon surface of microcantilever using the helping of a cross linker, such as glutaraldehyde.

Both of physical adsorption and chemical adsorption was performed on the experiment. In physical adsorption, the molecule of antibody was bonded with protein A molecule that immobilized on the microcantilever surface. There is no need of any chemical reagent for immobilization. Using of protein A, the F_c portion of antibody molecule will bind specifically to the protein A and leaving the F_{ab} portion pointing away from the microcantilever surface. The experimental results have shown the non-specific signal gain from the microcantilever immobilized with antibody by physical adsorption. The reasonable caused of non-specific adsorption might be the loss of protein molecule, especially bovine serum albumin (BSA) molecules that adsorbed on the microcantilever surface by weak interaction.

In chemical adsorption, the monoclonal antibody was immobilized by self-assembled monolayers (SAMs) method. The 3-mercaptoproprionic acid (MPA) was used as alkanethiol to form a monolayer on microcantilever surface. This technique has been successfully reported in many works [65, 67, 75]. Alkyl chain of MPA is rather short which is easily to adsorb on gold (Au) surface and reach stable in short time of process. The SAMs method is using of chemical bonding leading antibody molecules to form covalent linkage on solid substrate surface. The microcantilever immobilized with antibody by SAMs method was tested with *V. cholerae* O1 suspension. The results show good relationship between the number of *V. cholerae* O1 and resonance frequency shift of microcantilever. The non-specific signal was not presented like in case of using microcantilever immobilized with antibody by physical adsorption. However, the antibody molecules were randomly immobilized on the microcantilever surface. To improve the orientation of antibody, the solution of protein A or protein G should be added in the process of immobilization. Protein A and protein G have specifically to bind F_c portion of IgG (antibody) molecule and leave F_{ab} portion for binding to antigen.

The condition used for antibody immobilization in this study is the condition that had already been reported in the pervious studies [67, 106]. However, this condition was optimized for other kind of transducer, surface plasmon resonance and quartz crystal microbalance. It should be better if the experiment was done with the optimized microcantilever and immobilize the antibody by optimized-chemical condition to obtain the highest sensitivity of the sensor.

From the experimental results, the antibody-immobilized microcantilever can detect the *V. cholerae* O1 cells after immersed into *V. cholerae* O1 suspension only for 5 min compared with 30 min for standard incubation time of antigen-antibody reaction. This condition was used for *V. cholerae* O1 detection. The limit of detection was found to be $1x10^3$ CFU/ml. It should be improved if the time for antigen-antibody reaction was extended until reach the complete of the reaction. The incubation time can be shorter by using of additional shaking step to enhance to immunological reaction. Shaking of the microcantilever during antigen-antibody reaction can improve the rate of reaction and reduce the time for *V. cholerae* O1 detection. This incubation

time (5 min) is the lowest of detection time reported by other technique of immunoassays and immunosensors [66, 92].

In this study, the resonance frequency shift was calculated by using the initial frequency (F_0) of baseline signal and the frequency after bacteria binding (F_1) gain from the experiment as follow;

$$\Delta F = F_0 - F_1 \quad (1)$$

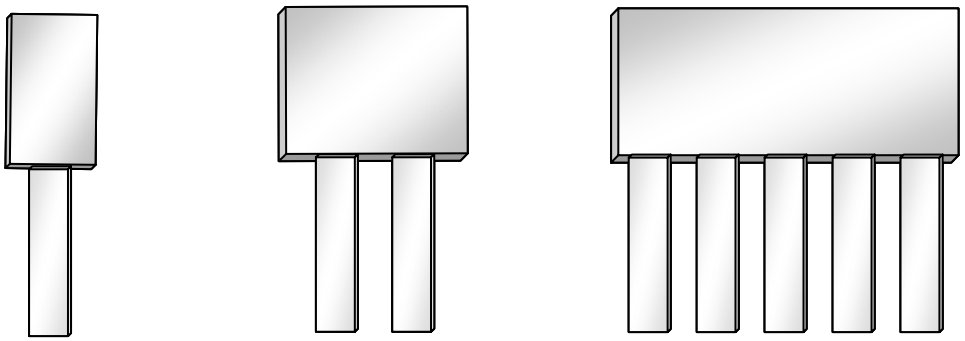
The non-specific signal from the resonance frequency should be avoided. The washing step is a crucial step that can caused non-specific signal, especially in case of immobilization by physical adsorption. The purpose of washing is to isolate the specific bound and unbound (includubg non-specific bound) of protein molecules on the microcantilever surface. The protein molecules used in the experiment, such as the protein A or bovine serum albumin, adsorbed on microcantilever surface by weak interaction, it can be stripped out of the microcantilever by vigorous conditions. Thus, it is necessary to wash the antibody-immobilized microcantilever until reaching the stable resonance frequency signal before testing with bacterial suspensions.

Specificity of the sensor is depending upon the specificity of the antibody used in the experiment. In this study, the monoclonal antibody specific to *V. cholerae* O1 cell was used. This monoclonal antibody is containing the binding sites specific to only one epitope on *V. cholerae* O1 cell. Another study had already confirmed the specificity of this monoclonal antibody by ELISA technique. The result had shown no cross-reactivity with coliform bacteria and specific to *V. cholerae* O1 cells. However, the microcantilever-based biosensor was also test with another of bacteria named *Vibrio parahemolyticus* to check cross-reactivity of the sensor in this study. The result also showed no cross-reaction of the sensor with *V. parahemolyticus.* Thus, it could assume that the microcantilever-based biosensor containing high specificity for detection of *V. choerae* O1 without cross-reactivity.

In the experiment that study about the effect of temperature on microcantiever sensor, the result has shown no significant effect of the temperature to the resonance frequency when operate the range of temperature within 3^oC of the ambient

temperature. Thus, it is necessary to measure the resonance frequency at room temperature (22-26 ^{o}C).

Microcantilever has microelectromechnical structure provide useful in miniaturized biosensor devices. Very small working area (μm^2) of microcantilever permits very low amount of chemicals and samples used for detection, picoliter of biological sample was reported in microcantilever-based system [116]. Micro-sized of microcantilever contains posibility to perform multiple detection in form of microcantilever array or lab-on-a-chip. Figure 37 shows schematic drawing of microcantilever in different forms.



(a) Single cantilever (b) Dual cantilever (c) Cantilever array

Figure 40. Schematic drawing of microcantilvers (a) single cantilever (b) dual cantilever; one cantilever is use as a sensing layer whereas the other is reference cantilever (c) cantilever array available for multi-detection

The experimental results gain from this study, give more information for further study with piezoresistive microcantilever that designed by MEMs Laboratory, NECTEC. A new design of piezoresistive microcantilever sensor is in form of microchip containing multi-cantilevers and measures the resistance change by using the piezoresistive system combined with the microfluidic system. Development of multi-cantilevers on a chip is leading to apply the microcantilever-based sensor for various food-borne pathogens in real-time detection.

CHAPTER VII
CONCLUSION

In this paper, we have demonstrated the development of microcantilever based biosensor for detection of *Vibrio cholerae* O1, a food and waterborne pathogen that caused cholera disease in human. The sensor system was developed for fast and easy for *V. cholerae* O1 detection compared with the conventional method. This research found that concentration of bacterial target was related to the mass loading of antigen-antibody complex that caused microcantilever change in resonance frequency.

The sensitivity of this microcantilever sensor offers high sensitivity up to 146.5 pg/Hz. The high sensitivity of detection has the possibility to detect *V. cholerae* O1 without any pre-enrichment step. The limit of detection of the sensor was found $1x10^3$ CFU/ml which is covered the infectious dose of *V. cholerae* O1 to caused cholera disease. The microcantilever-based biosensor is suitable for detection *V. cholerae* O1 cells in clinical specimen and useful to the microbiological laboratory. Highly selective layer of the sensor was formed by monoclonal antibody binding on microcantilever surface. The physical adsorption and chemical adsorption by self-assembled monolayers (SAMs) method can be used to immobilize antibody molecules on the microcantilever surface. However, the SAMs method is better and gave more stable form of antibody layer on microcantilever.

Linear relationship between the resonance frequency and the *V. cholerae* O1 concentration in log scale is obtained in concentration ranging from $1x10^3$ to $1x10^6$ CFU/ml. The microcantilever-based biosensor for detection of *V. cholerae* O1 can offer the protection of *V. cholerae* O1 spreading, required by public health protection. However, it is needed for further improvement of detection sensitivity in order to provide the sensitivity higher enough for detection of *V. cholerae* O1 in food samples.

REFERENCES

1. Turner APF. Biosensor-Sense and Sensitivity. Science 2000;290:1315-7.
2. Thevenot DR, Toth K, Durst RA, Wilson GS. Electrochemical biosensors: Recommended definitions and classification Biosens Bioelectron 2001;16:121-31.
3. Heineman WR, Jensen WB. Obituary:Leland C. Clark Jr. (1918-2005). Biosens Bioelectron 2006;21:1403-4.
4. Chauhan S, Rai V, Singh HB. Biosensors. Resonance 2004:33-43.
5. Weetall HH. Biosensor Technology What? Where? When? and Why? Biosens Bioelectron 1996;11:I-IV.
6. Kissinger PT. Biosensors: A perspectives. Biosens Bioelectron 2005;20:2512-6.
7. Castillo J, Gaspar S, Leth S, Niculescu M, Mortari A. Biosensor for life quality design, development and applications. Sens Actuators B 2004;102:179-94.
8. Collingsy AF, Caruso F. Biosensors: Recent advances. Rep Prog Phys 1997;60:1397-445.
9. Scheller FW, Wollenberger U, Warsinke A, Lisdat F. Research and development in biosensors. Curr Opin Biotechnol 2001;12:35-40.
10. Chambers JP, Arulanandam BP, Matta LL, Weis A, Valdes JJ. Biosensor recognition elements. Curr Issue Mol Biol 2008;10:1-12.
11. Eklund SE, Taylor D, Kozlov E, Prokop A, Cliffel DE. A microphysiometer for simultaneous measurement of changes in extracellular glucose, lactate, oxygen, and acidification rate. Anal Chem 2004;76:519-27.
12. Patel PD, (Bio)sensors for measurement of analytes implicated in food safety. Trends Anal Chem 2002;21:96-115.
13. Rasooly A. Biosensor technologies. Methods 2005;37:1-3.
14. Gooding JJ. Biosensor technology for detecting biological warfare agents: Recent progress and future trends. Anal Chim Acta 2006;559:137-51.

15. Gonsalves KE, Laurencin CL, Halberstadt CR, Nair LS, editors. Biomedical nanostructure. New York: John Wiley & Sons; 2008.440-442.
16. Piacham T, Josell A, Arwin H, Prachayasittikul V, Ye L. Molecularly imprinted polymer thin films on quartz crystal microbalance using a surface bound photo-radical initiator. Anal Chim Acta 2005;536:191-6.
17. Velasco-Garcia MN, Mottram T. Biosensor technology addressing agricultural problems. Biosyst Eng 2003;84:1-12.
18. Kleinjung F, Klussmann S, Erdmann VA, Scheller FW, Bier FF. High-affinity RNA as a recognition element in a biosensor. Anal Chem 1998;70:328-31.
19. Haupt K, Mosbach K. molecularly imprinted polymers and their use in biomimetic sensors. Chem Rev 2000;100:2495-504.
20. Scheller FW. Biosensor and their applications. TRANSDUCERS' 97 International conference on solid-state Sens Actuators, Chicago, June 16-19. 1997:899-901.
21. Ricci F, Volpe G, Micheli L, Palleschi G. A review on novel developments and applications of immunosensors in food analysis. Anal Chim Acta 2007;605:111-29.
22. Ferretti S, Paynter S, Russell DA, Sapsford KE. Self-assembled monolayers: a versatile tool for the formulation of bio-surfaces. Trends Anal chem 2000;19:530-40.
23. Halliwell CM, Cass AEG. A factorial analysis of silanization conditions for the immobilization of oligonucleotides on glass surfaces. Anal Chem 2001;73:2476-83.
24. Wink T, Zuilen SJ, Bult A, Bennekom WP. Self-assembled monolayers for biosensors. Analyst 1997;122:43R-50R.
25. Baselt DR, Lee GU, Hansen KM, Chrisey LA, Colton RJ. A high-sensitivity micromachined biosensor. IEEE 1997;85:672-80.
26. Frometa NR. Cantilever biosensors. Biotechnologia Aplicada. 2006;23:320-3.
27. Aksu SB, Turner JA. Calibration of atomic force microscope cantilevers using piezolever. Rev Sci Instrum 2007;78:043704-8.

28. Vashist SK. A review of microcantilevers for sensing applications. Nanotechnol 2007;3:1-15.
29. Ramos D, Calleja M, Mertens J, Zaballos A, Tamayo J. Measurement of the mass and rigidity of adsorbents on a microcantilever sensor. Sensors 2007;7:1834-45.
30. Lang HP, Hegner M, Meyer E, Gerber C. Nanomechanics from atomic resolution to molecular recognition based on atomic force microscopy technology. Nanotechnol 2002;13:R29-R36.
31. Lang HP, Hegner M, Gerber C. Cantilever array sensors. Mater Today 2005;8(4):30-6.
32. Alvarez M, Tamayo J. Optical sequential read out of microcantilever arrays for biological detection. Sens Actuators B 2004;106:687-90.
33. Ji H-F, Zhang Y, Purushotham VV, Kondu S, Ramachandran B, Thundat T, et al. 1,6-Hexanedithiol monolayer as a receptor for specific recognition of alkylmercury. Analyst 2005;130:1577-9.
34. Baller MK, Lang HP, Fritz J, Gerber C, Gimzewski JK, Drechsler U. A cantilever array-based artificial nose. Ultramicros 2000;82:1-9.
35. Pinnaduwage LA, Thundat T, Hawk JE, Hedden DL, Britt PF, Houser EJ. Detection of 2,4-dinitrotoluene using microcantilever sensors. Sens Actuators B 2004;99:223-9.
36. Porter TL, Eastman MP, Macomber C, Delinger WG, Zhine R. An embedded polymer piezoresistive microcantilever sensor. Ultramicros 2003;97:365-9.
37. Marie R, Jensenius H, Thaysen J, Christensen CB, Boisen A. Adsorption kinetics and mechanical properties of thiol-modified DNA-oligos on gold investigated by microcantilever sensors. Ultramicros 2002;91:29-36.
38. Calleja M, Nordstrom M, Alvarez M, amayo J, Lechuga LM, Boisen A. High sensitive polymer-based cantilever-sensors for DNA detection. Ultramicros 2005;105:215-222.
39. Tian F, Hansen KM, Ferrell TL, Thundat T. Dynamic microcantilever sensors for discerning biomolecular interactions. Anal Chem 2005;77:1601-6.

40. Su M, Li S, Dravid VP. Microcantilever resonance-based DNA detection with nanoparticle probes. Appl Phys Lett 2003;82:3562-4.
41. Gunter RL, Zhine R, Delinger WG, Mnygoats K, Kooser A, Porter TL. Investigation of DNA sensing using piezoresistive microcantilever probes. IEEE 2004;4:430-3.
42. Arnz Y, Seelig JD, Lang HP, Zhang J, Hunziker P, Ramseyer JP. Label-free protein assay based on nanomechanical cantilever array. Nanotechnol 2003;14:86-90.
43. Yan X, Lvov Y, Ji H-F, Singh A, Thundat T. A general microcantilever surface modification method using a multilayer for biospecific recognition. Org Biomol Chem 2003;1:460-2.
44. Battiston FM, Ramseyer J-P, Lang HP, Baller MK. A chemical sensor based on a microfabricated cantilever array with simultaneous resonance-frequency and bending readout. Sens Actuators 2001;77:122-31.
45. Leonard P, Hearty S, Brennan J, Dunne L, Quinn J. Advances in biosensors for detection of pathogen in food and water. Enzyme Micro Technol 2003;32:3-13.
46. Noble RT, Weisberg SB. A review of technologies for rapid detection of bacteria in recreational waters. J Water Health 2005;3:381-92.
47. Koubova V, Brynda E, Karasova L, Skvor J, Homola J. Detection of food borne pathogens using surface plasmon resonance biosensors. Sens Actuators B 2001;74:100-5.
48. Bokken G, Corbee RJ, Knapen F, Bergwerff AA. Immunochemical detection of *Salmonella* group B, D, and E using and optical surface plasmon resonance biosensor. FEMS Microbiol Lett 2003;222:75-82.
49. Oh B-K, Kim Y-K, Park KW, Lee WH, Choi J-W. Surface plasmon resonance immunosensor for detection of *Salmonella typhimurium*. Biosens Bioelectron 2004;19:1497-504.
50. Taylor AD, Yu Q, Chen S, Homola J, Jiang S. Comparasion of *E. coli* O157:H7 preparation methods used for detection with surface plasmon resonance sensor. Sens Actuators B 2005;107:202-8.

51. Subramanian A, Irudayaraj J, Ryan T. A mixed self-assembled monolayer based surface plasmon immunosensor for detection of *E. coli* O157:H7. Biosens Bioelectron 2006;21:998-1006.
52. Subramanian A, Irudayaraj J, Ryan T. Mono and dithiol surfaces on surface plasmon resonance biosensors for detection of *Staphylocuccus aureus*. Sens Actuators B 2006;114:192-8.
53. Taylor AD, Ladd J, Yu Q, Chen S, Homla J, Jiang S. Quantitative and simultaneous detection of four food borne bacterial pathogens with a multi-channel SPR sensor. Biosens Bioelectron 2006;22:752-8.
54. Mazumdar SD, Harfmann M, Kampfer P, Keusgen M. Rapid method for detection of *Salmonella* in milk by surface plasmon resonance (SPR). Biosens Bioelectron 2007;22:2040-6.
55. Nanduri V, Bhunia AK, Tu S-I, Paoli GC, Brewster JD. SPR biosensor for the detection of *L. monocytogenes* using phage-displayed antibody. Biosens Bioelectron 2007;23:248-52.
56. Varshney M, Li Y. Interdigitatated array microelectrode based impedance biosensor coupled with magnetic nanoparticle-antibody conjugates for detection of *Escherichia coli* O157:H7 in food sample. Biosens Bioelectron 2007;22:2408-14.
57. Varshney M, Li Y, Srinivasan B, Tung S. A label-free microfluidics and interdigitated array microelectrode-based impedance biosensor in combination with nanoparticles immunoseparation for detection of *Escherichia coli* O157:H7 in food sample. Sens Actuators B 2007;128:99-107.
58. Demarco DR, Lim DV. Detection of *Escherichia coli* O157:H7 in 10- and 25-gram ground beef samples with an evanescent-wave biosensor with silica and polystyrene waveguides. J Food Prot 2002;65:596-602.
59. Horvath R, Pedersen HC, Skivesen N. Optical waveguide sensor for on-line monitoring of bacteria. Opt Lett 2003;28:1233-5.
60. Zhu P, R.Shelton D, S.Karns J, Sundaram A, Li S, Amstutz P, et al. Detection of water-borne *E. coli* O157 using the integrating waveguide biosensor. Biosens Bioelectron 2005;21:678–83.

61. Varshney M, Li Y. Double interdigitated array microelectrode based impedance biosensor for detection of *Escherichia coli* O157:H7 in growth medium. Talanta 2008;74:518-25.
62. Cho EC, Choi J-W, Lee M, Koo K-K. Fabrication of an electrochemical immunosensor with self-assembled peptide nanotubes. Colloid Surface Physicochem Eng Aspect 2008;313:95-9.
63. Huang S, Pang P, Xiao X, He L. A wireless, remote-query sensor for real-time detection of *Escherichia coli* O157:H7 concentrations. Biosens Bioelectron B 2008;In Press.
64. Su X-L, Li Y. A self-assembled monolayer-based piezoelectric immunosensor for rapid detection of *Escherichia coli* O157:H7. Biosens Bioelectron 2004;19:563-74.
65. Fung YS, Wong YY. Self-assembled monolayers as the coating in a quartz piezoelectric crystal immunosensor to detect *Salmonella* in aqueous solution. Anal Chem 2001;73:5302-9.
66. Gfeller KY, Nugaeva N, Hegner M. Micromechanical oscillators as rapid biosensor for the detection of active growth of *Escherichia coli*. Biosens Bioelectron 2005;21:528-33.
67. Adanyi N, Varadi M, Kim N, Szendro I. Development of new immunosensors for determination of contaminants in food. Curr Appl Phys 2006;6:279-86.
68. Mao X, Yang L, Su X-L, Li Y. A nanoparticle amplification based quartz crystal microbalance DNA sensor for detection of *Escherichia coli* O157:H7. Biosens Bioelectron 2006;21:1178-85.
69. Y.E. J, Letcher SV, Rand AG. Piezoelectric biosensor for detection of *Salmonella typhimurium*. J Food Sci 1997;62:1067-72.
70. Wong YY, Ng SP, Ng MH, Si SH, Yao SZ, Fung YS. Immunosensor for the differentiation and detection of *Salmonella* species based on a quartz crystal microbalance. Biosens Bioelectron 2002;17:676-84.
71. Zuehlke J. Rapid detection of foodborne *E. coli* O157:H7 using piezoelectric excited millimeter-size cantilever sensors. Microsc Microanal 2007;3:14-9.

72. Campbell GA, Mutharasan R. *Escherichia coli* O157:H7 detection limit of millimeter-sized PZT cantilever sensor is 700 cells/ml. Anal Sciences 2005;21:335.
73. Detzel AJ, Campbell GA, Mutharasan R. Rapid assessment of *Escherichia coli* by growth rate on piezoelectric-excited millimeter-sized cantilever (PEMC) sensors. Sens Actuators B 2006;117:58-64.
74. Zhu Q, Shih WY, Shih W-H. In situ, in-liquid, all-electrical detection of *Salmonella typhimurium* using lead titanate zirconate/gold coated glass cantilevers at any dipping depth. Biosens Bioelectron 2007;22:3132-8.
75. McGovern J-P, Shih WY, Shih W-H. In situ detection of *Bacillus anthracis* spores using fully submersible, self-exciting, self-sensing PMN-PT/Sn piezoelectric microcantilever. Analyst 2007;132:777-83.
76. Davila AP, Jang J, Gupta AK, Walter T. Microresonator mass sensors for detection of *Bacillus anthracis* Strene spores in air and water. Biosens Bioelectron 2007;22:3028-35.
77. Campbell GA, Mutharasan R. Piezoelectric-excited millimeter-sized cantilever (PEMC) sensors detect *Bacillus anthracis* at 300 spores/mL. Biosens Bioelectron 2006;21:1684–92.
78. Campbell GA, Muthrasan R. Detection of *Bacillus anthracis* spore and a model protein using PEMC sensors in a flow cell at 1 ml/min. Biosens Bioelectron 2006;22:78-85.
79. Nugaeva N, Gfeller KY, Backmann N, Duggelin M, Lang HP. An antibody-sensitized microfabricated cantilever for the growth detection of *Aspergillus niger* spores. Microsc Microanal 2007;13:13-7.
80. Gupta A, Akin D, Bashir R. Single virus particle mass detection using microresonators with nanoscale thickness. Appl Phys Lett 2004;84:1976-8.
81. Gupta A, Akin D. Detection of bacterial cells and antibodies using surface micromachined thin silicon cantilever resonators. J Vac Sci Technol B 2004;22:2785-91.

82. Louis VR, Russek-Cohen E, Choopun N, Rivera ING, Gangle B, Jiang SC, et al. Predictability of *Vibrio cholerae* in Chesapeake Bay. Appl Environ Microbiol 2003;69:2773-85.

83. Heidelberg JF, Eisen JA, Nelson WC, Clayton RA. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature 2000;406:477-83.

84. Yamai S, Okitsu T, Shimada T, Katsube Y. Distribution of serogroups of *Vibrio cholerae* non-O1 and non-O139 with specific reference to their ability to produce cholera toxin, and addition of novel serogroups. J Jpn Assoc Infect Dis 1997;71:1037-45.

85. Jensen MA, aruque SM, Mekalonos JJ, Levin BR. Modeling the role of bacteriophage in the control of cholera outbreaks. PNAS 2006;103(12):4652-7.

86. Zo Y-G, Rivera ING, Russek-Cohen E, Islam MS, Siddique AK, Yunus M, et al. Genomic profiles of clinical and environmental isolates of *Vibrio cholerae* O1 in cholera endemic areas of the Bangladesh. PNAS 2002;99(19):12409-14.

87. Albert MJ. *Vibrio cholerae* O139 Bengal. J Clin Microbiol. 1994;32:2345-9.

88. Tantillo GM, Fontanarosa M, Pinto AD, Musti M. Updated perspectives on emerging vibrios associated with human infections: A Review. J Appl Microbiol 2004;39:117-26.

89. Richards GP, Watson MA. A simple fluorogenic method to detect *Vibrio cholerae* and *Aeromonas hydrophila* in well water for areas impacted by catastrophic disasters. Am S Trop Med Hyg 2006;75:516-31.

90. Hasan JAK, Huq A, Tamplin ML, siebeling RJ, Colwell RR. A novel kit for rapid detection of *Vibrio cholerae* O1. J Clin Microbiol 1994;32:249-52.

91. Villalpando-Guzman S, Eusebio-Hernandez MG, Aviles-Ruiz D. Detection of *Vibrio cholerae* O1 in oysters by the visual colorimetric immunoassay and the culture technique. Rev Latinoam de Microbiol 2000;42:63-8.

92. Rao VK, Sharma MK, Goel AK, Singh L, Sekhar K. Amperometric immunosensor for the detection of *Vibrio cholerae* O1 using disposable screen-printed electrodes. Anal Sciences 2006;22:1207-11.

93. Jyoung J-Y, Hong S, Lee W, Choi J-W. Immunosensor for the detection of *Vibrio cholerae* O1 using surface plasmon resonance. Biosens Bioelectron 2006;21:2315-9.

94. Fykse EM, Skogan G, Davies W, Olsen JS, Blatny JM. Detection of *Vibrio cholerae* by real-time nucleic acid sequence-based amplification. Appl Environ Microbiol 2007;73:1457-66.

95. Chow KH, Ng TK, Yuen KY, Yam WC. Detection of RTX gene in *Vibrio cholerae* by PCR. J Clin Microbiol 2001;39:2594-7.

96. Singh DV, Matte MH, Matte GR, Jiang S, Sabeena F, Shukla BN, et al. Molecular analysis of *Vibrio cholerae* O1, O139, non-O1, and non-O139 strains: Clonal relationships between clinical and environmental isolate. Appl Environ Microbiol 2001;67:910-21.

97. Lyon WJ. Taq Man PCR for detection of *Vibrio cholerae* O1, O139, Non-O1, and Non-O139 in pure cultures, raw oysters, and synthetic sea water. Appl Environ Microbiol 2001;67:4685-93.

98. Singh DV, Isac SR, Colwell RR. Development of a hexaplex PCR assay for rapid detection of virulence and regulatory genes in *Vibrio cholerae* and *Vibrio mimicus*. J Clin Microbiol 2002;40:4321-4.

99. Cass T, Ligler FS, editors. Immobilized Biomolecules in Analysis. New York: Oxford University Press;1998.35-52

100. Kanno S, Yanagida Y, Haruyama T, Kobatake E. Assembling of engineered IgG-binding protein on gold surface for highly oriented antibody immobilization. J Biotechnol 2000;76:207-14.

101. Demirela G, Caykara T, Akaoglu B, Cakmak M. Construction of a novel multilayer system and its use for oriented immobilization of immunoglobulin G. Surf Sci 2007;601:4563-70.

102. Bae YM, Oh B-K, Lee W, Lee WH, Choi J-W. Study on orientation of immunoglobulin G on protein G layer. Biosens Bioelectron 2005;21:103-10.

103. Jung Y, Kang HJ, Lee JM, Jung SO, Yun WS. Controlled antibody immobilization onto immunoanalytical platforms by synthetic peptide. Anal Biochem 2008;374:99-105.

104. Binning G, Quate CF, Gerber C. Atomic force microscope. Phys Rev Lett 1986;56:930-3.
105. Raiteri R, Grattarola M, Butt H-J, Skladal P. Micromechanical cantilever-based biosensor. Sens Actuators B 2001;79:115-26.
106. Chongpermpornwattana W. Development of immunosensor based on piezoelectric quartz crystal microbalance for *V. cholerae* O1 detection [MS Thesis in Medical Technology]. Article in Bangkok: Faculty of Graduates Studies, Mahidol University; 2006.
107. Sepaniak M, Datskos P, Lavrik N. Microcantilever Transducers: A new approach in sensor technology. Anal Chem 2002;74:568A-75A.
108. Campbell GA, Mutharasan R. Detection and quantification of proteins using self-excited PZT-glass millimeter-sized cantilever. Biosens Bioelectron 2005;21:597-607.
109. Hwang KS, Eom K, Lee JH, Chun DW, Cha BH. Dominant surface stress driven by biomolecular interactions in dynamical response of nanomechanical microcantilevers. Appl Phys Lett 2006;89:173905-7.
110. Davis ZJ, Boisen A. Aluminum nano-cantilevers for high sensitivity mass sensors. Nanotechnol 2005; 126; 126-9.
111. IIic B, Craighead HG, Krylov S, Senaratne W, Ober C. Attogram detection using nanoelectromechanical oscillators. J Appl Phys 2004;95:3694-703.
112. Raiteri R, Butt H-J, Grattarola M. Change in surface stress at the liquid/solid interface measured with a microcantilever. Electrochim Acta 2000;46:157-63.
113. Oden PI, Chen GY, Steele RA, Warmack RJ, Thundat T. Viscous drag measurements utilizing microfabricated cantilevers. Appl Phys Lett 1996;68:3814-6.
114. Burg TP, Godin M, Knudsen SM, Shen W, Carlson G, Foster JS, et al. Weighing of biomolecules, single cells and single nanoparticles in fluid. Nature 2007;446:1066-9.
115. Belaubre P, Guirardel M, Leberre V, Pourciel J-B, Bergaud C. Cantilever-based microsystem for contact and non-contact deposition of picoliter biological samples. Sens Actuators A 2004;110:130-5.

APPENDIX

OPERATION OF SPA 400 ATOMIC FORCE MICROSCOPES

Operation of DFM (Dynamic Force Mode) of SPA400

Figure 41. SPA 400 main unit

1. Start up the System

1.1 Turn on the main circuit breaker under the rear panel of the SPI4000N Probe station controller.

1.2 Turn on the power at the center of the front panel of SPI4000N Probe station controller. The switch lights up.

1.3 Turn on the computer and the display. The Windows startup menu will appear.

1.4 Double click the Selector icon on the desktop. [SPI4000N Program Selector] will appear.

1.5 Select [DFM] in SPA400 frame and click [OK]. The main program (SPIWin) will start.

2. Set the Cantilever

2.1 Prepare the DFM cantilever and the cantilever holder

2.2 Press down the release key to raise the wire which holds the cantilever.

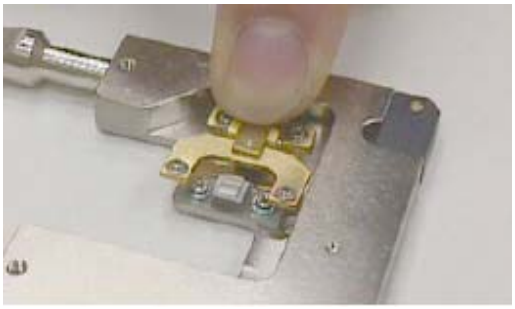


Figure 42. Cantilever setting step 1: Press down the release key to raise the wire

2.3 Place the cantilever on the cantilever mount.

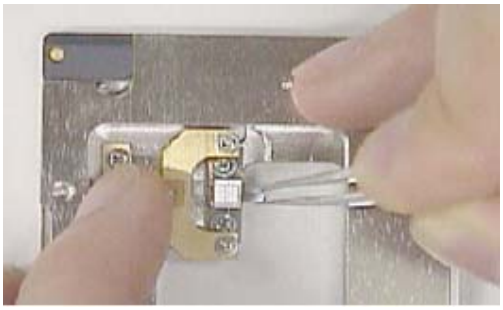


Figure 43. Cantilever setting step 2: Place the cantilever on the cantilever mount

2.4 Release the release key to hold the cantilever by the wire.

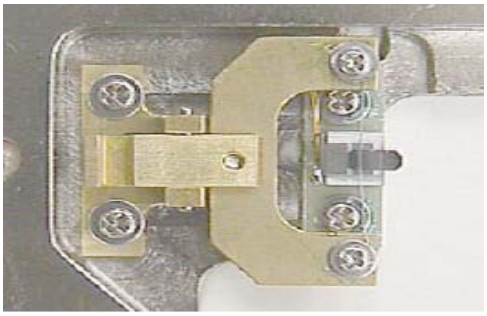


Figure 44. Cantilever setting step 3: Cantilever holded

2.5 Installation the cantilever holder into the unit.

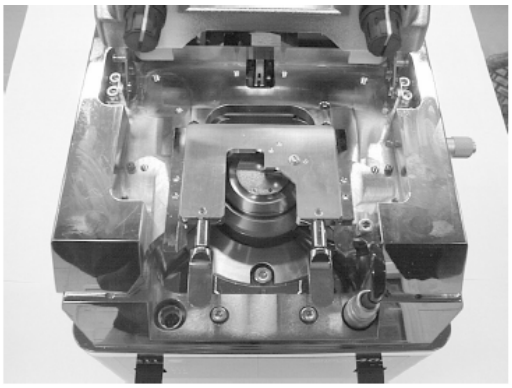


Figure 45. Installation of the cantilever holder into the SPA 400 unit.

3. Adjustment the Laser Light Axis

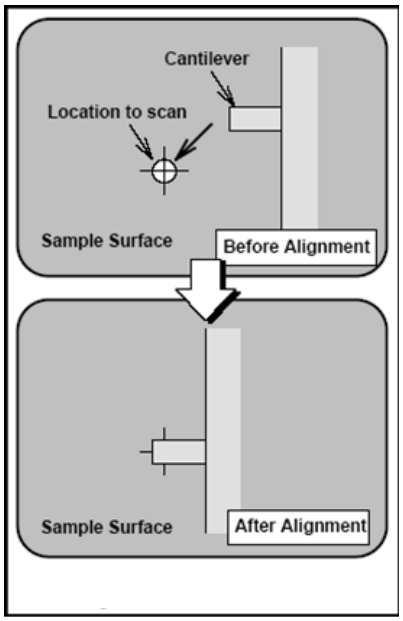


Figure 46. Adjustment of the laser light axis: before and after laser alignment

3.1 Set the optical head onto the main unit

3.2 Select [CCD Monitor] in [Setup] menu to display [CCD].

3.3 Check the measuring position by focusing on the sample surface while observing the CCD Image.

3.4 Focus on the cantilever while observing at the CCD image.

3.5 Select [Approach] in [Scan] menu to display [Approach].

3.6 Select [Laser Position] in [Setup] menu to display [Laser Position].

3.7 Rotate adjustment knobs LASER X and LASER Y to adjust the laser light to the cantilever while observing the CCD image.

3.8 Rotate adjustment knobs LASER X and LASER Y to maximize the ADD output (8 ~ 13V).

4. Adjustment of the Photodetector

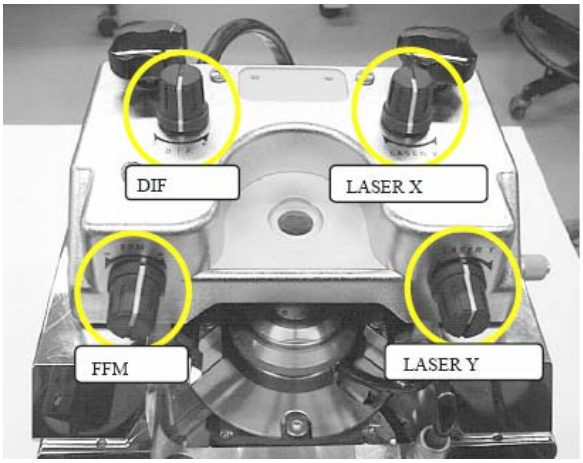


Figure 47. Knobs for photodetector adjustment

4.1 Rotate adjustment knobs FFM and DIF to ensure that the spot (●) moves smoothly across the range (fill area) in Fig. 47 and 48 of [Laser Position].

4.2 Rotate adjustment knob FFM to bring the spot (●) into the frame □ at the center of [Laser Position].

4.3 Rotate adjustment knob DIF to bring the spot (●) into the frame □ at the center of [Laser Position].

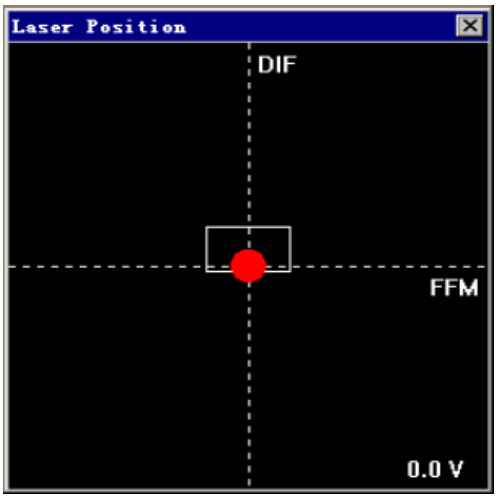


Figure 48. Adjust the photodetector: rotate adjustment knob FFM and knob DIF to bring the spot (●) into the frame □ at the center of [Laser Position].

5. Measure the Resonance Frequency

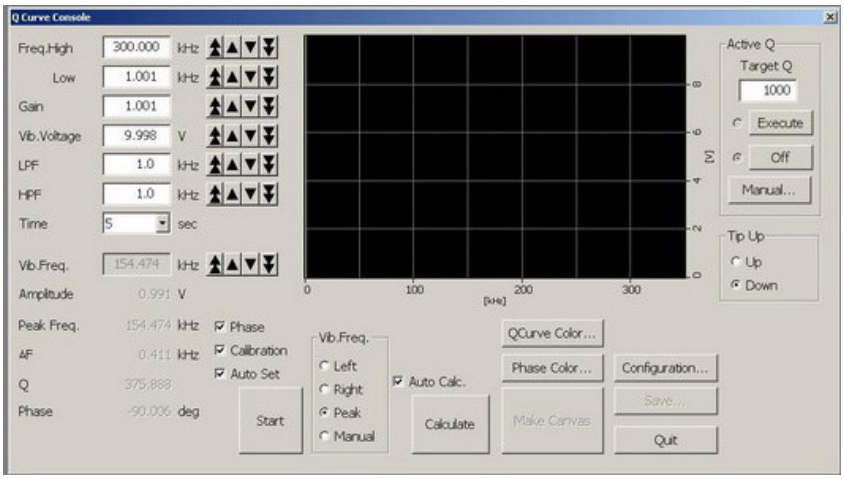


Figure 49. Parameter setting for resonance frequency measurement

5.1 Set the frequency range

5.2 Set the vibration voltage “Vib. Voltage”

5.3 Set the time scan

5.4 Set the Vibration Frequency “Vib. Freq”

5.5 Click “Start”

6. Saved Measured Data

6.1 Use the mouse to activate the canvas of the image to be saved. “Make Canvas”

6.2 Select [Save] in [File] menu to display [Save File].

6.3 Select the destination drive with [Drive] and the destination folder with [Folders].

6.4 Input the file name into [File Name] text box.

6.5 Click [OK] to save.

BIOGRAPHY

NAME	Miss Usa Sungkanak
DATE OF BIRTH	11 March 1982
PLACE OF BIRTH	Nakhon Phanom, Thailand
INSTITUTIONS ATTENDED	Thammasat University, 2000-2003 Bachelor of Science (Medical Technology) Mahidol University, 2005-2008 Master of Science (Medical Technology)
FELLOWSHIPS	Thesis is partly supported by; Thailand Graduate Institute of Science and Technology (TGIST)
ORAL PRESENTATION	Asia-Pacific Conference on Transducers and Micro-Nano Technology 2008 (APCOT 2008), Taiwan. 22 - 25 June 2008.
HOME ADDRESS	99 Sonthonvichit Road, Muang, Nakhon Phanom, Thailand 48000
E-MAIL ADDRESS	katemedtech@yahoo.com