OPTICAL BIOSENSOR FOR
MICROALBUMIN DETERMINATION

PALAKORN TANTILIPIKARA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF ENGINEERING
(BIOMEDICAL ENGINEERING)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2005

ISBN 974-04-5886-6

Thesis
Entitled

OPTICAL BIOSENSOR FOR MICROALBUMIN DETERMINATION

Palakorn Tantilipikara
Mr. Palakorn Tantilipikara
Candidate

Asst.Prof. Chamras Promptmas,
Ph.D.(Biochemistry)
Major-Advisor

Theeraporn Rubcumintara
Asst.Prof. Theeraporn Rubcumintara,
Ph.D.(Materials Engineering & Science)
Co-Advisor

Assoc.Prof. Rassmidara Hoonsawat,
Ph.D.
Dean
Faculty of Graduate Studies

Theeraporn Rubcumintara
Asst.Prof. Theeraporn Rubcumintara,
Ph.D.(Materials Engineering & Science)
Chair
Master of Engineering Programme
in Biomedical Engineering
Faculty of Engineering

Thesis
entitled

OPTICAL BIOSENSOR FOR MICROALBUMIN DETERMINATION

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Engineering (Biomedical Engineering)
on
April 8, 2005

Palakorn Tantilipikara
Mr. Palakorn Tantilipikara
Candidate

Asst.Prof. Chamras Promptmas,
Ph.D.(Biochemistry)
Chair

Theeraporn Rubcumintara
Asst.Prof. Theeraporn Rubcumintara,
Ph.D.(Materials Engineering & Science)
Member

Assoc.Prof. Sudarat Manochiopinij,
Ph.D.(Biochemistry)
Member

Asst.Prof. Udom Tipayamontri,
Ph.D.(Physiology)
Member

Assoc.Prof. Rassmidara Hoonsawat,
Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

Asst.Prof. Piya Rattanasuwan,
M.Eng.
Dean
Faculty of Engineering
Mahidol University

ACKNOWLEDGEMENT

I would like to thank Asst. Prof. Chamras Promptmas for being a kind advisor who gave me suggestion, guidance and support. I am grateful to a co-advisor Asst. Prof. Theeraporn Rubcumintara. My sincere thanks are also extended to the committee, Asst. Prof. Udom Thipayamontri and Assoc. Prof. Sudarat Manochiopinij for their valuable comments and suggestions.

Special appreciations are expressed to Dr. Pattira Piumngam for her kindness and valuable suggestions. Special appreciations are also reached to Dr Boonsong Sutapun and all of the staffs in the electro-optics laboratory at National Electronic and Computer Technology Center (NECTEC) for their suggestions and supports, including work place and equipments.

This accomplished achievement would not be that much worth without sharing it with the whole of my family especially my beloved one Ray who must be recognized for being the wind beneath my wing. Thanks for everything that you have devoted to me. I am definitely indebted in their loves, encouragement, understanding and great supports.

Palakorn Tantilipikara

OPTICAL BIOSENSOR FOR MICROALBUMIN DETERMINATION

PALAKORN TANTILIPIKARA 4336838 EGBE/M

M.Eng.(BIOMEDICAL ENGINEERING)

THESIS ADVISORS: CHAMRAS PROMPTMAS, Ph.D. (BIOCHEMISTRY), THEERAPORN RUBCUMINTARA, Ph.D. (MATERIALS ENGINEERING AND SCIENCE)

ABSTRACT

Biosensor is an analytical device comprising of a specific biorecognition element directly interfaced to a signal transducer, which together reveal the concentration of an analyte into a measurable response. The requirements for a biosensor are high specificity and sensitivity. The specificity is a function of the biological sensing element and its ability to interact with the analyte. The high degree of specificity of biosensors is best typified by the antibody-antigen interaction.

Albumin was used in this study to represent as a biomolecule. The optical biosensor for detecting the human albumin based on the evanescent wave excitation had been demonstrated. The sensor chip consisted of two parts, the cover plate (60x15x2mm) with the inlet and outlet for fluid injection and the trapezoidal shaped prism (20x15x6mm) for the anti-albumin immobilization. The fluorescent dye labeled albumin competed with the albumin in the sample to bind with antibody and the measurement was performed until the end point of the reaction. The detected fluorescent signal was inversely proportional to the albumin concentration in the sample as the assay format was the competitive binding. The calibration curve of signal vs albumin concentration ranging from 0, 50, 100, 200, 500 and 1,000 µg/mL was 119.7, 109, 103, 88.7, 73, 70.2 mV respectively with SD in repeated measurements (n = 3) ranging from 3.56 to 8.4. The assay time was completed within 5 minutes. Sample volume of 5 µL was required in each assay. The results obtained from the study revealed the capability for detecting the microalbumin.

KEY WORDS : OPTICAL BIOSENSOR/EVENESCENT WAVE/MICROALBUMIN

60 p. ISBN 974-04-5886-6

ออปติคอลไบโอเซ็นเซอร์สำหรับการตรวจวิเคราะห์ไมโครอัลบูมิน (OPTICAL BIOSENSOR FOR MICROALBUMIN DETERMINATION)

พลากร ตันติลีปิกร 4336838 EGBE/M

วศ.ม. (วิศวกรรมชีวการแพทย์)

คณะกรรมการควบคุมวิทยานิพนธ์: จำรัส พร้อมมาศ, Ph.D. (Biochemistry), ธีรพร รับคำอินทร์ Ph.D. (Materials Engineering and Science)

บทคัดย่อ

ไบโอเซ็นเซอร์เป็นเครื่องมือวิเคราะห์ซึ่งประกอบด้วยสารชีวโมเลกุลจำเพาะที่ติดโดยตรงกับตัวส่งสัญญาณเพื่อที่จะสามารถวัดค่าของสารที่ต้องการรู้ค่าความเข้มข้น การมีความจำเพาะและความไวสูงเป็นสิ่งที่ต้องการในการทำไบโอเซ็นเซอร์ ความจำเพาะคือความสามารถของสารชีวโมเลกุลจำเพาะในการทำปฏิกิริยากับสารที่ต้องการรู้ค่าความเข้มข้น การมีความจำเพาะสูงของไบโอเซ็นเซอร์สามารถพบได้จากปฏิกิริยาของแอนตีบอดีและแอนติเจน

ในการศึกษาไบโอเซ็นเซอร์ครั้งนี้ได้นำอัลบูมินมาใช้เป็นตัวแทนของสารชีวโมเลกุลเนื่องจากมีความคงสภาพสูง ออปติคอลไบโอเซ็นเซอร์สำหรับการตรวจไมโครอัลบูมินโดยการกระตุ้นจากคลื่นหายตัวอย่างรวดเร็วได้ถูกแสดง เซ็นเซอร์ชิพประกอบด้วยสองชิ้นส่วน แผ่นประกบขนาด 60x15x2 มิลลิเมตรซึ่งมีช่องเปิดเข้าและออกสำหรับการฉีดของเหลวและส่วนปริซึมรูปสี่เหลี่ยมคางหมูขนาด 20x15x6 มิลลิเมตรสำหรับการตรึงแอนตีบอดีต่ออัลบูมิน อัลบูมินที่ติดฉลากด้วยสารเรืองแสงจะแย่งกับอัลบูมินที่อยู่ในตัวอย่างตรวจเพื่อจับกับแอนตีบอดีต่ออัลบูมินและการวัดกระทำจนถึงจุดสิ้นสุดปฏิกิริยา สัญญาณจากสารเรืองแสงที่วัดได้เป็นปฏิภาคผกผันกับความเข้มข้นของอัลบูมินในสารละลายตัวอย่างโดยรูปแบบการตรวจวัดเป็นแบบการแย่งจับ เส้นโค้งมาตรฐานระหว่างสัญญาณและค่าความเข้มข้นของอัลบูมินตั้งแต่ 0, 50, 100, 200, 500, 1000 ไมโครกรัมต่อมิลลิลิตรมีค่าเฉลี่ยเท่ากับ119.7, 109, 103, 88.7, 73, 70.2 มิลลิโวลท์ตามลำดับ ค่าเบี่ยงเบนมาตรฐานที่ 3 การทดลองมีค่าในช่วง 3.56 ถึง 8.4 เวลาที่ใช้ในการวัดอยู่ที่ห้านาที ปริมาณของสารตัวอย่างที่ใช้ในการตรวจวัดน้อยกว่าห้าไมโครลิตร เส้นโค้งมาตรฐานที่ได้จากการทดลองบ่งชี้ว่าสามารถใช้วัดระดับความเข้มข้นของไมโครอัลบูมินได้

60 หน้า. ISBN 974-04-5886-6

CONTENTS

CONTENTS (Cont.)

CONTENTS (Cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Cont.)

LIST OF ABBREVIATIONS

BCG = Bromocresol green
BCP = Bromocresol purple
BSA = Bovine serum albumin
˚C = Degree Celsius
Cy3 = Cyanine 3
Cy5 = Cyanine 5
DNA = Deoxyribonucleic acid
dsDNA = Double stranded DNA
DW = Distilled water
ENFET = Enzyme field effect transistor
g = Gram(s)
hr = Hour(s)
Ig = Immunoglobulin
ISFET = Ion-sensitive field effect transistor
kDa = Kilodalton
L = Liter(s)
M = Molar(s)
mg = Milligram(s)
min = Minute(s)
ml = Milliliter(s)
mM = Millimolar(s)
mm = Millimeter(s)
nm = Nanometer
PBS = Phosphate buffered saline
PMMA = Polymethyl methacrylate
PMT = Photo multiplier tube
QCM = Quartz crystal microbalance

LIST OF ABBREVIATIONS (Cont.)

RI	=	Refractive index
RNA	=	Ribonucleic acid
rpm	=	Revolution per minute
SAW	=	Surface acoustic wave
SPR	=	Surface plasmon resonance
ssDNA	=	Single stranded DNA
TIR	=	Total internal reflection
TSM	=	thickness-shear mode
µg	=	Microgram(s)
µL	=	Microliter(s)
µm	=	Micrometer(s)

CHAPTER I
INTRODUCTION

Biosensor is an analytical device that combines the specificity of a biological sensing element for the analyte of interest with a transducer to produce a signal proportional to target analyte concentration. The transducer converts this signal into a measurable response. This signal can be further amplified, processed, or stored for later analysis. With this principle, any biomolecule can be combined with any suitable transducer to produce an operational biosensor.

The requirements for a successful biosensor are high specificity and sensitivity. The specificity is a function of the biological sensing element and its ability to interact with the analyte. High sensitivity is achieved when there is sufficient interaction between the recognition element and the analyte to be efficiently detected by the transducer. The high degree of specificity of biosensors is best typified by the antibody-antigen interaction. The binding between these molecules is highly specific, even in the presence of interferences. Biosensors, including immunosensors, have several advantages over conventional biological assays. Biosensors can directly detect analyte molecules, thus avoiding the addition of various reagents, which requires operator skill.

In clinical diagnostics and biomedical applications, the use of biosensors has become more versatile due to several advantages over the conventional procedure, which require the experienced technician to perform the assay. For achieving the model of biosensor, albumin has been used in this study to represent as a biomolecule due to its stability and commercially available.

Albumin is a major plasma protein also found in urine where its presence may be an indication of some types of kidney or liver dysfunction. Several analytical methods have been developed to quantify albumin. Among these methods, the dye-binding methods, especially, those employing bromocresol green (BCG) and bromocresol purple (BCP) are widely used in the clinical laboratory. However, BCG and BCP methods have some disadvantages, such as poor selectivity and sensitivity, when the specimen is a protein mixture. Furthermore, the micro-determination of albumin is important since an increase in the urinary excretion of albumin has been seen in diabetic patients and is considered to be an early sign of diabetic nephropathy.

Therefore, the use of biosensing from immunological interaction in combination with the suitable optical transduction technique along with the use of fluorescence dye was studied to determine the microalbumin concentration.

OBJECTIVES

The objectives of the study are;

1. To develop the biosensor based on the evanescent wave excitation for detecting the microalbumin.

2. To optimize the assay for microalbumin determination.

CHAPTER II

LITERATURE REVIEW

2.1 Biosensors

2.1.1 Definition and principle of biosensors

Biosensor is an analytical device that combines the specificity of a biological sensing element for the analyte of interest with a transducer to produce a signal proportional to target analyte concentration. The transducer converts this signal into a measurable response. This signal can be further amplified, processed, or stored for later analysis (1-6). With this principle, any biomolecule can be combined with any suitable transducer to produce an operational biosensor. Therefore, a biosensor can be generally defined as a device that consists of two components connected in series: a biological recognition system, often called a bioreceptor, and a transducer.

The basic principle of a biosensor is to detect the molecular recognition and to transform it into another type of signal using a transducer. The main purpose of the recognition system is to provide the sensor with a high degree of selectivity for the analyte to be measured. The interaction of the analyte with the bioreceptor is designed to produce an effect measured by the transducer, which convert the information into a measurable effect. Figure 2.1 illustrates the conceptual principle of the biosensing process (3).

2.1.2 Classification of biosensors

Biosensors may be classified according to the biological specificity conferring mechanism or to the mode of signal transduction, or alternatively a combination of both.

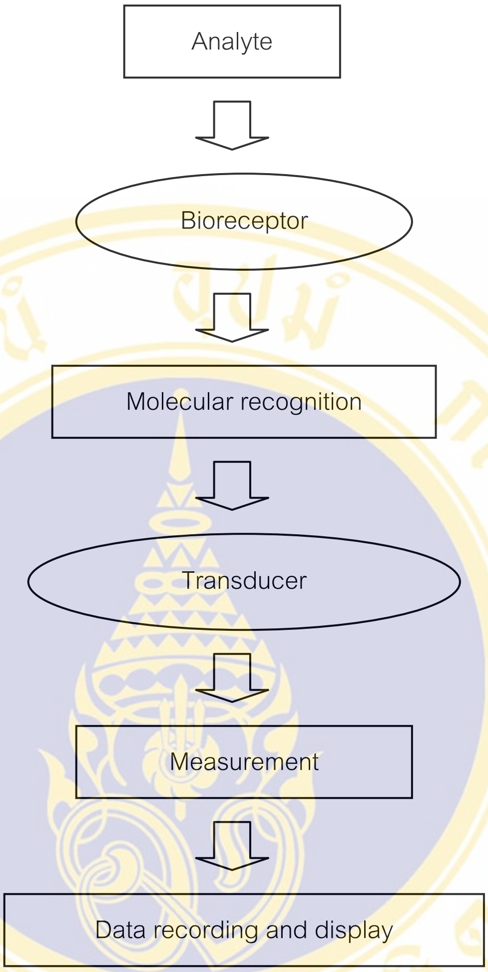

Figure 2.1 Conceptual diagram of the biosensing principle (3).

2.1.2.1 Classification by bioreceptors

A bioreceptor is a biological molecular species or a living biological system that utilizes a biochemical mechanism for recognition. The sampling component of a biosensor contains a biosensitive layer that can contain bioreceptors or made of bioreceptors covalently attached to the transducer (3, 4). Depending upon the mechanism of biochemical interaction between the receptor and the analyte, two basic types of biosensors can be classified: to biocatalytic sensors and bioaffinity sensors (2-5), shown in Table 2.1 (4).

Table 2.1 Biosensor specification according to the nature of the primary sensing reaction (4).

Biocatalytic sensors		**Bioaffinity sensors**	
Indicating reaction: 			
Analytes	**Receptors**	**Analytes**	**Receptors**
substrate	enzyme	protein	dye
cosubstrate	enzyme system	glycoprotein	lectin
cofactor	organelle	cells	antigen
prosthetic group	microorganism	antigen/haptan	antibody
	tissue slice	antibody	RNA/DNA
		neurotransmitter	receptor/protein
		pesticide	
		enzyme (system)	

2.1.2.1.1 Biocatalytic recognition element

Biocatalytic sensors are based on the recognition and binding of an analyte followed by a catalyzed chemical conversion of the analyte from a non-detectible form to a detectible form, which are detected and recorded by a transducer (2, 4, 5). Biocatalysts include:

(1) mono-enzyme or multi-enzyme,

(2) microorganisms, (such as bacteria, fungi, yeast), or sub-cellular organelles, and particles (mitochondria, cell walls),

(3) animal or plant tissue slice.

2.1.2.1.2 Bioaffinity recognition element

This biosensor operation is based on the interaction of the analyte with biological components, such as antibodies, nucleic acids, lectins, cell membrane receptors and hormone receptors, that have been isolated or engineered. The detection and measurement of substance can be achieved at very low concentrations by immunological techniques. Bioaffinity sensors primarily depend on the use of antibodies due to the availability of antibodies directed towards a wide range of analyte as well as their relative affinity and selectivity (1, 3, 4, 6).

2.1.2.2 Classification by transduction systems

The transducing element of a biosensor is used to convert the biological recognition step into the measurable signal that can be detected and displayed. The transduction can be accomplished through several of methods (1, 2, 4, 6).

2.1.2.2.1 Electrochemical transduction

An electrochemical biosensor is a biosensor with electrochemical transducer (Table 2.2) (2). Electrochemical detection is usually based on the chemical potential of a particular species in solution (the analyte), as measured by comparison to a reference electrode. Therefore, the electrochemical response is dependent on the activity of the analyte species, not their concentration. This technique has been extensively used in biosensors. Electrochemical sensors include:

(1) Potentiometry

Potentiometric measurement involves the determination of the potential difference between either, an indicator and a reference electrode, or two reference electrodes separated by a selectively permeable membrane (1, 2, 6). A classical example of this device is the pH electrode where hydrogen ions are measured at an ion selective glass membrane. The transducer may be an ion-selective electrode (ISE) which is an electrochemical sensors based on thin films or selective membranes as recognition elements. Several other ion (F^-, K^+, Cl^-, Ca^{2+}, I^-, CN^-, Br^-, etc.) and gas (CO_2, NH_3) selective electrodes are available. An advantage of potentiometric sensor is the simplicity of operation, which can be used for automation and the small size of the solid-state FET sensors (6).

Table 2.2 Type of electrochemical transducers for classified type of measurements, with corresponding analytes to be measured (2).*

Measurement type	Transducer	Transducer analyte
1. Potentiometric	ion-selective electrode (ISE)	K^+, Cl^-, Ca^{2+}, F^-
	glass electrode	H^+, Na^+…
	gas electrode	CO_2, NH_3
	metal electrode	Redox species
2. Amperometric	metal or carbon electrode	O_2, sugars, alcohols…
	chemically modified electrode (CME)	oligonucleotides, sugars, alcohols, phenols, …
3. Conductometric, impedimetric	Interdigitated electrode, metal electrode	urea, charged species, oligonucleotides…
4. Ion charge or field effect	ion-sensitive field effect transistor (ISFET), enzyme FET (ENFET)	H^+, K^+…

*non electrochemical transducers are also used within biosensor: (a) piezoelectric (shear and surface acoustic wave); (b) calorimetric (thermistor); (c) optical (planar wave guide, fiber optic, surface plasmon resonance)

(2) Amperometry

Amperometry is based on the measurement of the current flow resulting from the electrochemical oxidation or reduction of electroactive compounds at constant voltage (2, 6). The generation of these compounds typically relies on an enzyme system that catalytically converts electrochemically non-active analytes into products that can be oxidized or reduced. The current is proportional to the concentration of electroactive product that is proportional to the amount of tracer.

(3) Conductometry

Conductometric transducers rely on the alteration of the electrical conductivity in the solution, at the constant voltage, caused by biochemical reaction (1, 2, 6). The capacitance changes are measured using electrochemical system, in which the bioactive element is immobilized onto a pair of noble metal electrodes (mostly, Au or Pt) (6).

(4) Surface charge using field effect transistor (FET)

An important variation of the systems used to determine ion concentrations are the ion-sensitive field-effect transistors (ISFETs). An ISFET is composed of an ion-selective membrane applied directly to the insulated gate of the FET. When such ISFETs are coupled with a biocatalytic or biocomplexing layer, they become biosensors. These operating devices are strongly related to those of the ISE based biosensors (2).

2.1.2.2.2 Optical transduction

Optical tranducers in biosensors are applied to measure the responses to illumination or to light emission. These biosensors are constructed from electronic as well as standard optical components. Light-intensity measurement can be read directly or interpreted by standard analogue or digital circuitry (1, 5, 6). Optical biosensors offer advantage in terms of miniaturization, low cost, disposability and no electrical interference (5). The parameters to be measured in the optical transduction are the changes in the absorbance, refractive index or fluorescence.

In the optical transduction technique, light from a suitable source travels along the optical fiber to the distal end where the sensing material is immobilized. The changes in the light characteristic such as reflection, scattering, or emitted light returns from the sample can determine the concentration of the analyte.

2.1.2.2.3 Microgravimetric transduction

Microgravimetric or mass sensitive transduction relies on the use of piezoelectric crystals that can be made to vibrate at a specific frequency with the application of an electric signal. The frequency of oscillation is dependent on the electrical frequency applied to the crystal as well as crystal’s mass. When the mass increase due to the binding of the analyte, the oscillation frequency of the crystal changes and the resulting change can be measured electrically and used to determine the mass added to the crystal (1, 3, 6). The microgravimetric sensor devices are divided into quartz crystal microbalance (QCM) devices applying a thickness-shear mode (TSM) and devices applying surface acoustic wave (SAW) detection (6).

2.1.3 Immunoassay and immunosensor

Immunosensors are affinity ligand-based biosensor solid-state devices in which the immunochemical reaction is coupled to a transducer. The fundamental basis of all immunosensors is the specificity of the molecular recognition of antigens by antibodies to form a stable complex (7).

2.1.3.1 Antibody

Antibodies are complex biomolecules made up from amino acids arranged in a highly ordered sequence. The antibodies are actually produced by immune system cells when these cells are exposed to substances or molecules called antigens. The property of antibodies is the ability to recognize molecular structures and bind specifically to antigens. The antigen specificity and binding affinity originate from the variations in amino acid sequence at the antigen binding site. Antibodies are immunoglobulins and can be categorized into five classes: IgG, IgA, IgM, IgD, and IgE. These classes are structurally related glycoproteins that differ in size, charge, amino acid composition, and carbohydrate content (8, 9). Antibodies are often chosen as the biological recognition element because they have a high degree of specificity that allows them to recognize the appropriate analyte even in the presence of the interference.

2.1.3.2 Immunoassay

The specificity of the molecular recognition of antigens by antibodies to form a stable complex is the basis of the analytical immunoassay (7). Immunoassays use antibodies or antibody-related reagent for the determination of sample analytes (10). The selectivity of ligand-biding of antibodies allows these biomolecules to be employed in analytical methods that are highly specific even in complex biological matrices (6). Biomolecular interaction can be classified into two categories according to the test format performed: direct or indirect. In a direct format the immobilized target molecule interacts with a ligand molecule or the immobilized ligand interacts with target molecule. The simplest situation involves the incubation of antigen and antibody followed by direct measurement or development of fluorophor-labeled second antibody. The resulting antibody produced a fluorescence signal directly proportional to the amount of bound antigen. The indirect format involves competition between fluorophor-labeled and unlabeled antigen. The unlabeled analyte competes with the labeled analyte for a limited number of antigen binding sites (1, 3). By combining the selectivity of antibody-analyte interactions with vast array of antibodies and the availability of numerous detectable labels, immunoassay can be designed for a wide variety of analytes (6).

2.1.3.3 Immunosensor format

The simplest biochemical format for antibody-based biosensors sometimes termed "immunosensors" or "affinity-based biosensors" consists of an antibody or antigen immobilized to a transducer and results in a signal generated from the binding of an analyte to an antibody at the sensor surface (1, 6). The general immunosensor design is depicted in Figure 2.2 (6). With the combination of transduction system, there are four types of immunosensor detection devices: electrochemical, optical, microgravimetric and thermometric (Figure 2.2). All types can either be run as direct or indirect immunosensors. There are a great variety of different labels that have been applied in indirect immunosensors. Among the most valuable labels are enzymes and a series of fluorescent labels (rhodamine, fluorescein, Cy5).

Conventional immunoassays require skill of the operator and time to get reliable analytical results. Immunosensors are the way to provide scientists and clinicians with precise measurements of a variety of analytes in complex mixtures of clinical specimens. Advantages over other immunoassays include the convenience of not having to accurately pipette various reagents in a multitude of steps, the possibility of designing a portable unit, the ability to measure more than one analyte simultaneously, and a decrease in the time between sample collection and obtaining results.

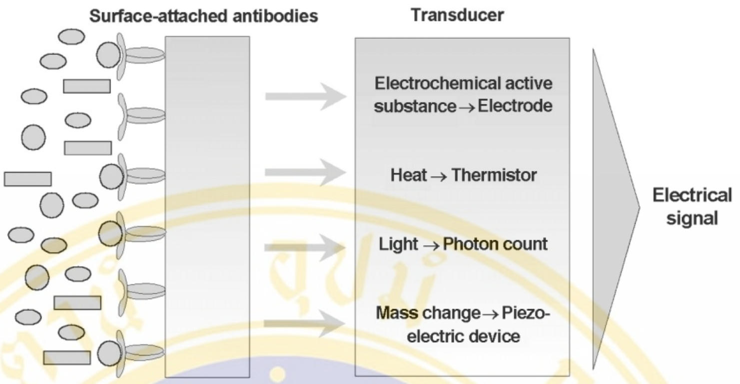

Figure 2.2 Scheme of the general immunosensor design depicting the intimate integration of immunological recognition at the solid-state surface and the signal transduction. (6)

The immunoassay can be categorized into three main formats which are the direct assay format, the competitive assay format and the sandwich assay format. In the case of **direct assay format**, the analyte binds to the recognition site of the bioaffinity element and accumulates on the sensor surface. The sensor signal in the direct assay format is directly proportional to the analyte concentration. Because many sensors cannot detect the binding of an analyte, **competitive formats** are often used in combination with the optical or electrochemical transduction. Competitive immunoassay formats can be described using the following relationship:

$$\mathrm{Ab} + \mathrm{Ag} + \mathrm{Ag}^* \Longrightarrow \mathrm{AbAg} + \mathrm{AbAg}^*$$

where Ab is the antibody, Ag is the analyte, Ag* is the analyte tracer, AbAg is the antibody-antigen complex, and AbAg* is the antibody antigen-tracer complex. There are several types of formats typically used in competitive assay biosensor. Either the antibody is immobilized on the sensor surface and the analyte and analyte-tracer compete for available binding sites (Figure 2.3), or the antigen is immobilized on the sensor and free analyte competes with the analyte immobilized to the sensor surface for binding sites on the labeled antibody. In either case, the transducer can differentiate the relative amount of antibody binging sites that are occupied by analyte. This results in a sensor signal that is inversely proportional to the analyte concentration (1). The advantage of this format is the high sensitivity and low potential for interference from matrix components from other molecules. A disadvantage is the need to add a reagent other than analyte of interest. Because the signal is inversely proportional to the concentration of analyte, the lowest concentration is measured as a small change in the signal detected which would increase the relative noise (1).

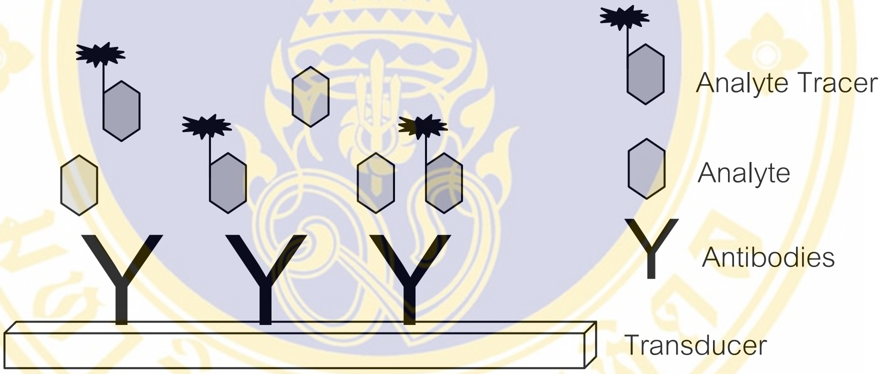

Figure 2.3 Competitive assay format, the antibody is immobilized on the sensor surface and the analyte and analyte-tracer compete for available binding sites.

Another type of biosensor format is the **sandwich assay** (Figure 2.4). For this assay format, the analyte target is "sandwiched" between a surface-immobilized antibody and an antibody tracer. Because this assay requires the target analyte to have at least two antigenic sites, this format is typically used for large molecules or for microorganisms. Sandwich assay format is particularly useful with mass-sensitive acoustic transducers (1).

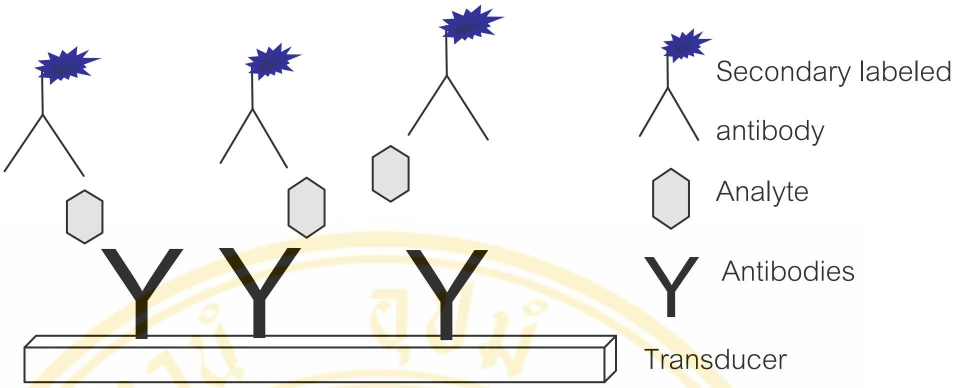

Figure 2.4 Sandwich assay format, the analyte target is sandwiched between a surface-immobilized antibody and an antibody-tracer.

2.1.4 Optical biosensor

As the consequence of the rapid development in the telecommunications field, the biosensors which utilize the optical transduction are one of the most popular for the bioanalysis. This is due to the advantages of applying visible radiation compared to other transducer techniques (6). The parameters to be detected in optical biosensor can be the changes in the adsorption, fluorescence, or refractive index (3, 5). Optical biosensor is constructed from electronic as well as standard optical components as light sources, lasers and detectors (photodiodes or PMT), filters and lenses. Light-intensity measurements can be read directly or interpreted by standard analogue or digital circuitry (5). The simplest optical biosensor utilizes absorbance measurement to determine the changes in the concentration of the analyte that absorb the given wavelength of light. This optical system works by transmitting light through the optical fiber to the sample. The amount of light absorbed by the analyte is detected through the same fiber. The biological molecule is immobilized at the distal end of the optical fiber and either produce or extracts the analyte that absorb the light.

2.1.4.1 Fluorescence measurement

Another method used in the optical biosensor is the fluorescence measurement. Since there are few analyte which can be directly detected in this manner, the labeling with fluorescence dye is used in order to monitor the biomolecular interaction. Fluorescent measurement can also be applied to monitor the binding events occurring on the surface of optical biosensors (5). When light traveling through the optical waveguide excites the fluorophores within the evanescent field, the fluorescent signal is propagated back and detected by the detector. By using the detection of fluorescence-emitting labels, specific antibody/antigen immunocomplex can be monitored as illustrated in Figure 2.5.

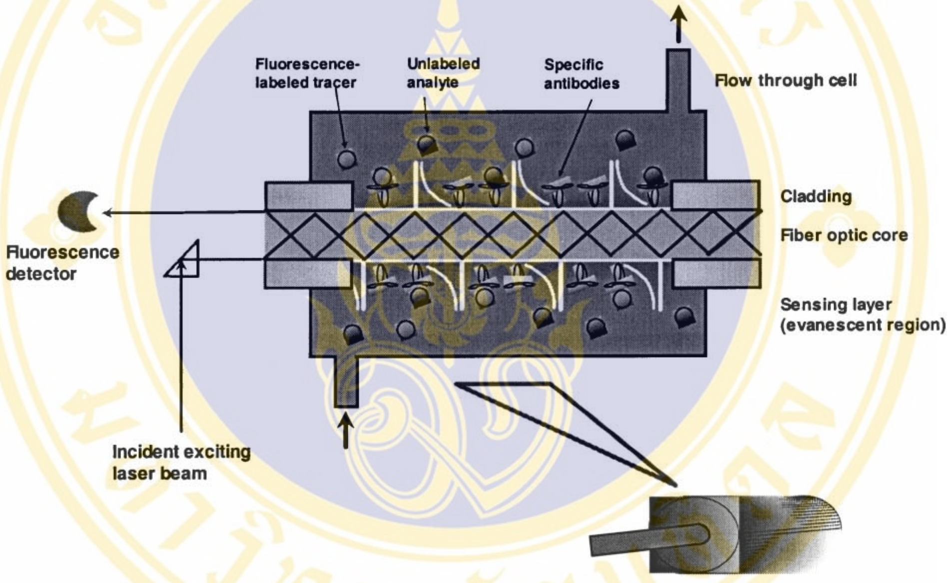

Figure 2.5 Fluorescence measurements by fiber optic biosensor (11).

2.1.4.2 Surface plasmon resonance

Surface plasmon resonance (SPR) is a unique optical transduction. SPR is used to monitor the changes of refractive index (RI) that occur on the sensor surface when one of the interacting partners binds to its ligand immobilized on (1, 3, 5, 6). A surface plasmon is basically an evanescent electromagnetic field generated at the surface of a metal conductor, usually silver or gold, excited by incident light of appropriate wavelength. The transduction principle involved in SPR is based on the arrangement of thin film of metal coated on the prism or deposited glass slide, that is optically contacted on prism by using a refractive index matching fluid (3, 12). The sample is attached to the layer of low RI. At the interface between the two different media, a thin (approximately 50 nm) gold film is interposed. Although light does not propagate into the low RI medium, the interfacial intensity is not equal to zero. The physical requirement of continuity across the interface is the reason for exciting the surface electrons in the metal film by the light energy. As a result, the electrons start oscillating. This produces an exponentially decaying evanescent wave penetrating a defined distance into the low RI medium, which is accountable for a characteristic decrease in the intensity of the reflected light. The insight in changes of the RI at the surface interface is made possible by monitoring the intensity and the resonance angle of the reflected light, caused by the biospecific interactions (6).

2.1.5 Evanescent wave

Biosensor based on the evanescent wave is one of the techniques applied from the characteristic of the light. Light waves are propagated along the media by the law of total internal reflection (TIR). This law states that incident light striking nearly parallel to the interface between two media of differing refractive indices is reflected according to Snell's Law (13):

$$n_1 \sin \theta_1 = n_2 \sin \theta_2 \tag{1}$$

Where n_1 is the higher refractive index of the first medium, θ_1 is the incident ray angle through the first medium, n_2 is the lower refractive index of the second medium and θ_2 is the angle of internal reflection back into the first medium. TIR occurs when the angle of incidence is greater than the critical angle.

The critical angle is defined as:

$$\theta_c = \sin^{-1}\left(\frac{n_2}{n_1}\right) \tag{2}$$

Even though the light is totally internally reflected, the intensity does not abruptly fall to zero at the interface. The intensity exponentially decays with distance, starting at the interface and extending into the medium of lower refractive index. The evanescent wave is the electromagnetic field created in the second medium. It is characterized by the penetration depth defined as the distance from the interface at which it decays to 1/e of its value at the interface. The wavelength of light, ratio of the refractive indices, and angle of the light at the interface determine the penetration depth. The penetration depth (d_p) is related to these factors by:

$$d_p = \frac{\lambda}{4\pi(n_1^2 \sin^2\theta_1 - n_2^2)^{1/2}} \tag{3}$$

where θ_1 is the incident ray angle with the normal to the n1/n2 interface, and λ is the wavelength of light. Penetration depths are typically in the range of 100 nm for visible light ($d_p < \lambda$), thus the evanescent wave is able to interact with many biomolecules attached at the surface of the first medium.

With this advantage, many biosensor applications utilize this principle in conjunction with the fluorescence dye to monitor the biological interaction bound to the surface of the first medium as shown in Figure 2.6

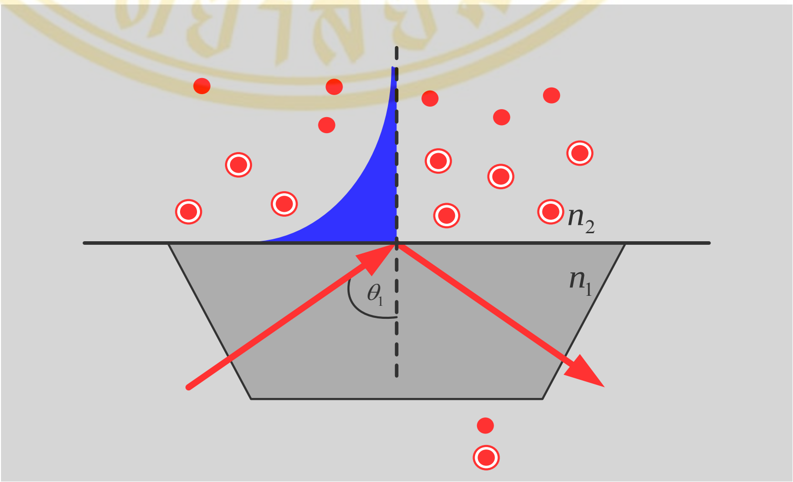

Figure 2.6 Evanescent wave excitation

2.1.6 Fluorescence dye

Many applications in biosensing require the fluorescence dye to be bonded to the protein, DNA, drug, or other biomolecules. The fluorescence dyes provide the means to detect the interaction of these molecules. There are several fluorescence dye used in biosensing applications. One of the most commonly known fluorescence dye is the cyanine dye.

Cyanine Dye is the one of the most popularly used fluorescence dye utilized in the optical biosensor application due to its low non-specific-binding, high photo stability, and insensitive to pH. This dye is intensely fluorescent and highly water soluble providing significant advantages over the existing fluorophores

Cyanine 3

The fluorescence of Cy3-fluorophore is the near red region. This dye has the maximum absorption at 550 nm and the maximum fluorescent at 560 nm.

Cyanine 5

The fluorescence of Cy5-fluorophore is in the red region of the spectrum. The dye absorption is at 649 nm and the emission at 670 nm. The spectrum of the Cy5-fluorophore is shown in Figure 2.7.

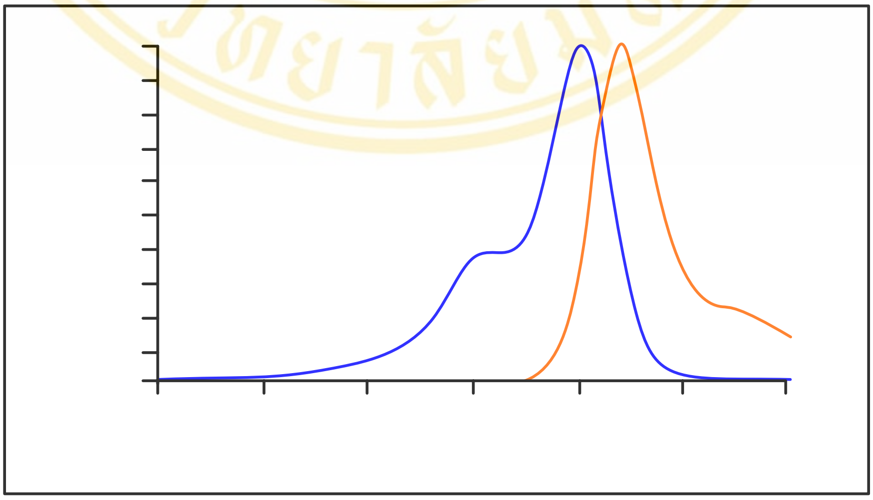

Figure 2.7 The absorption and emission spectra of Cy5 florophore (14).

2.1.7 Applications for biosensors

Compared with traditional sensors and classical methods of analysis, biosensors offer an attractive alternative for the rapid, selective and highly sensitive assay for a wide variety of analytes. They have been potentially applied in clinical diagnostic, food industry, environmental monitoring and defense (1, 15). Receptor and nucleic acid-based biosensors show potential for future development, antibodies are currently the most highly developed recognition element for these devices. One of the critical issues with respect to biosensors is whether or not the direct interface of these assays to signal transduction technologies will significantly improve currently available methods as well as generate new applications. The interface of immuno-chemicals to microsensor arrays is generating a considerable amount of interest. The receptor-based biosensors show considerable potential involves biological parameters such as interference with receptor function, the identification and enumeration of pathogenic microorganisms. The receptor-based biosensors have primarily focused on two mechanisms; immunochemical recognition of antigens expressed on the surface of the microorganism and identification of DNA sequences that are unique to the interested organism (1).

The utilization of the optical fiber has been brought into the biosensing application. Hirshfeld and Block (16) had demonstrated that evanescent wave sensing excites fluorophores bound to the fiber than to those in the bulk solution. Fluorescent radiation propagates back through the fiber in high order modes. As the fluorescent light enters the cladded portion of the fiber it is susceptible to loss because the higher refractive index of the cladding does not support higher order modes (17). To improve excitation and recovery of the fluorescent signal, Thompson and Villarruel (18) patented tapered optical fibers. When uncladded fiber is immersed in water, which has a lower refractive index than the cladding, the fiber’s modal capacity is increased (19). To efficiently couple the returning fluorescence into propagating modes, the modal capacity of the probe portion must match the cladded portion. Tapering the probe region acts as a mode converter and improves fluorescent signal propagation.

In recent years, success has come to fluorescent biosensors. Immunosensors for a variety of targets have been experimented. A fluorescent-based fiber optic biosensor for the detection of ricin, a potently toxic protein, has been reported (20). The immobilization of the anti-ricin IgG on the fiber used the direct immobilization onto silanized surface via a crosslinker and avidin coated fibers incubated in biotinylated anti-ricin IgG. The fiber was tapered before immobilization. After anti-ricin attachment, the fiber was incubated with ricin, and then exposed to Cy5-labeled anti-ricin antibody. It has been demonstrated that avidin-biotin immobilization prior to the sandwich assay gave a signal response 100-fold greater than for direct immobilization (20).

2.1.7.1 Biomedical applications

Biosensers offered increase versatility for many clinical applications, especially in the diagnosis of diseases. The evaluation of potential application in bacteriology and virology is a fast-growing area. The use of biosensors for detecting specific viruses in biological specimens has been targeted (21). Identifying viruses in clinical materials during the acute phase of infection could give necessary information for better treatment. Some of these viruses include herpes simplex virus (22), dengue virus (23) and also human immune deficiency virus (HIV) (24). A real-time detection system for viruses in general has been developed using an optical biosensor and herpes simplex virus type 1 as a model (22). A serotype-specific RNA biosensor was developed for the rapid detection of dengue virus in blood samples (23). SPR optical biosensor assays developing for distinguishing HIV-1 inhibitors from noninteracting reference drugs used immobilized HIV-1 protienase on the sensor surface (24).

Several biosensor technologies have been developed for applications in bacterial detection (25). An evanescent wave optical sensor was developed for the detection of protein A of *Staphylococcus aureus* (26). A sandwich immunoassay with fluorescein isothiocyanate conjugated with anti-protein A IgG was used to monitor Ag-Ab reaction.

An antibody-based biosensor has also been applied for detection of food allergens, such as proteins from eggs and milk (27-28). It has provided a sensitive, specific, and rapid method required by the food industry. It was successfully demonstrated for toxin, bacteria, and virus detection in food, clinical samples, and environmental matrixes.

2.1.7.2 Environmental applications

The advancement in miniaturization and microfabrication technology has led to the development of sensitive and selective electrochemical devices for field-based environmental monitoring. Electrochemical sensing devices have a major impact upon the monitoring of priority pollutants. They are portable and capable of continuous monitoring (29-30). Such devices can perform automated chemical analyses in complex matrices and provide rapid, reliable and inexpensive measurements of a variety of inorganic and organic pollutants.

Due to the increased threats of chemical and biological agents by terrorist organizations, a significant effort is underway to develop tools that can be used to detect and effectively combat chemical and biochemical toxins. The novel sensors, such as autonomous cell-based toxicity monitoring, 'Lab-on-a-Chip' devices and conventional environmental analytical techniques have been designed in order to meet the success in the prevention, early detection and the efficient and timely response (31).

2.1.8 New technology

2.1.8.1 DNA biosensor

As already known that deoxyribonucleic acids (DNA) are the most important biochemical molecules, DNA is composed of four repeating nucleotides: Adenine (A), Guanine (G), Cytosine (C), and Thymine (T). DNA is coiled to form a double helix (double-stranded DNA [dsDNA]) composed of two strands held together by hydrogen bonds that can be broken by heat or high pH. The unique complementary structure of DNA between the base pairs adenine/thymine and cytosine/guanine has been the basis for genetic analysis.

In the DNA techniques, including hybridization, amplification and recombination, are all based on the feature of the double helix structure of a DNA molecule. The principle of developing DNA biosensors is the kinetics of nucleic acid hybridization. The single-stranded DNA (ssDNA) is relatively stable, but on removal of the heat source or pH extreme, the DNA molecule will re-form (reanneal) into the double-stranded configuration. When the ssDNAs are from different sources, the reannealing process is called hybridization. The reannealing of the dsDNA is possible because nucleotide bases will re-form hydrogen bonds only with specific complementary bases; adenine pairs with thymine, and cytosine pairs with guanine. In RNA, the nucleotide base uracil replaces thymine and pairs with adenine. The stability of the hybridization depends on the nucleotide sequences of both bonds. A perfect match in the sequence of nucleotides produces the stable dsDNA.

Recently, an optical DNA-sensor was developed which is based on an evanescent-field excitation of fluorophors and allows real-time analysis of the hybridization event. The system consists of two components, the disposable sensor chip and the optical read-out device. In the sensor chip the oligonucleotide probes are immobilized on the surface of a glass plate. In the device, an evanescent-field is generated by total internal reflection of a laser beam. When the sample with the corresponding target is added, the evanescent field causes an excitation of fluorescence light only at those targets in very close proximity to the surface. As the fluorophore in the bulk solution are not excited, washing or separation steps are not required. After the start of the measurement, the binding of the labeled targets to the immobilized probes can be observed in real-time (32).

2.1.8.2 Micro array

Nowadays, there is the new interest in the high throughput assay for the clinical and medical application, the microarray technique. The microarray technique has first been described by Ekins (7, 33-34) for an antibody microspot array. This type of array differed form DNA chip type in that, DNA array based on nucleic acid recognition on a surface-immobilized template where as antibody microarray is capable for ultrasensitive multi-analyte determinations. The small amount of antibody packed as the monolayer on the array's surface 10^4-10^5 molecules per 1 μm^2 is able to sense the analyte concentration. By using two different fluorescence labels and the confocal laser microscope to scan on the microspot area of 100-1000 μm^2 for determining the α and β fluorescence emitted from each individual spot as shown in Figure 2.8

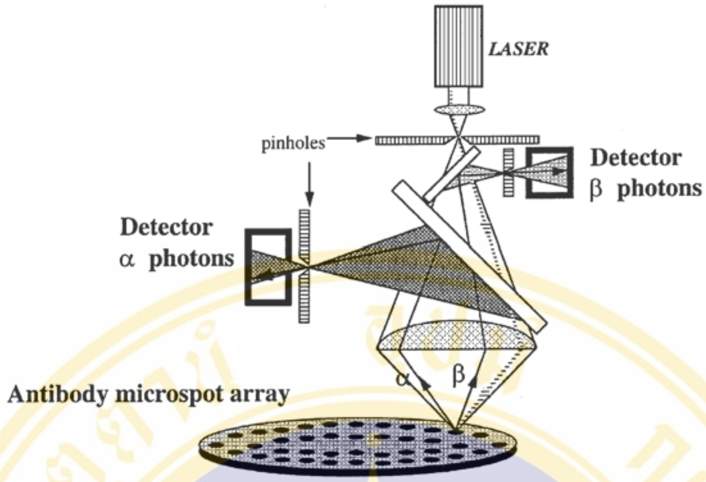

Figure 2.8 Schematic for multianalyte antibody microarray (7)

2.1.8.3 Nanosensor

Advancement in nanotechnology helps for further development of biosensors, particularly in proteomics and cellomics. Optical fiber-based nanosensors depend on the construction of nanometer-sized optical fibers. Two different techniques are already available to produce fibers' tips: a CO_2 laser-based micropipette-puller or the chemical-etching technique with hydrofluoric acid (35). Miniaturization allows the integration of all steps of analytical process into single-sensor devices, so called "Lab-on-a-chip. The fluidic parts of the sensor allow a reduction of sample volume less than 1 µL (36). However, pumps, valves and electrical control circuitry are also probable building parts of a miniaturized and integrated sensor (37).

2.2 Albumin

Albumin is a major blood plasma protein and normally filtered back by the kidneys by the glomerular filtration. When the kidneys work properly, there is almost no albumin present in the urine. If the kidneys are damaged, small amount of albumin will leak into the urine. This condition is called microalbuminuria. The microalbuminuria is defined as the persistent elevation of albumin between 30-300 mg/day (38), which indicate high probability of damage of the glomerular filtration capacity of the kidney and may lead to renal failure.

2.2.1 Clinical significance

Microalbuminuria is a powerful predictor for the future development of diabetic nephropathy, retinopathy and cardiovascular complications due to diabetes (39-42). Patients with hypertension have increased risk of cardiovascular disease if microalbuminuria is present (41).

2.2.2 Microalbumin detection

Several analytical methods have been developed to quantify albumin. Microalbuminuria results may also be dependent on the type of urine collection. A microalbumin urine test can be done on a sample of urine collected randomly (usually after the first time in the morning), on a sample of urine collected over a 24-hour period, or on a sample of urine collected over a specific period of time, such as 4 hours or overnight.

2.2.2.1 Microalbumin dipstick

The dipstick test is based on the change in color of an acid-based indicator. At a constant pH, the development of any blue color is due to the presence of albumin. A screening test performed by using a strip read with a device, and leading to a semi-quantitative result. Positive results from dipstick tests should be confirmed with more specific methods (43). Dipsticks may read falsely positive in the presence of alkaline urine, penicillins, radiocontrast agents, and sulfonamides.

2.2.2.2 Immunoturbidimetry

This method depends on the formation of a turbid solution. In the reaction, albumin combines with specific antibody (anti-albumin antibodies) to form insoluble antigen-antibody complexes. The system monitors the change in absorbance. Change in absorbance is proportional to the concentration of albumin in the sample and is used to calculate urine albumin concentration based upon a multi-point, non-linear calibration curve by standard albumin solutions (44).

2.2.2.3 Radioimmunoassay (RIA)

RIA is performed with double antibody technique. Albumin in the sample competes with a fixed amount of ^{125}I-labeled albumin for binding sites of specific antibodies. After incubation for a fixed time, separation of bound from free is achieved by polyethylene glycol (PEG)-accelerated double-antibody method. Finally the antibody-bound fraction is precipitated and counted. Albumin concentration in the sample was inversely proportional to the radioactivity.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals/substances

The chemicals used in this experiment were analytical grade.

Chemicals/substances	**Manufacturer**
Absolute alcohol	Merck
Bovine serum albumin (BSA)	Sigma
Disodium hydrogen phosphate anhydrous (Na_2HPO_4)	Merck
Dimethylether	Merck
Ethylenediaminetetraacetic acid (EDTA.Na_2.$2H_2O$)	USB
FluoroLink™ Cy5™ (PA25001)	Amersham life science
Human serum albumin (HSA)	Sigma
Rabbit anti-human albumin, A3293	Sigma
Potassium chloride (KCl)	Merck
Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Sephadex® G-50	Pharmacia LKB
Sodium chloride (NaCl)	Merck
Tris (Hydroxymethyl aminomethane)	Sigma

3.1.2 Instruments/Equipments

Instruments/Equipments	Model	Manufacturer
Acrylic sheet (PMMA)	In-house	-, Thailand
Autoclave	MLS-3000	Sanyo Labo, Japan
Automatic pipettes	P2, 20, 200,1000	Gilson, France
Digital camera	C470	Olympus, Japan
Double coated tape	655	3M, Thailand
Electronic analytical and precision balance	B3105	Sartorius, USA
Freezer	SF-C992NG	Sanyo, Japan
Interference filter	670-10	Thorlab Inc., USA
Laser diode	HL3412G	Laser Max Inc., USA
Lock-in amplifier	LIA100	Thorlab Inc., USA
Optical chopper	MC1000	Thorlab Inc., USA
pH meter	520 A	Orion, USA
Photo multiplier tube	PMT-3	Optomatrics INC.
Plano-convex lens	LA1131	Thorlab Inc., USA
Plano-convex lens	LA1027	Thorlab Inc., USA
Microcentrifuged tube	1.5 mL	Treff, Switzerland
Microcentrifuged tube	0.6 mL	Axygen, USA
Refrigerator	New touch	Sanyo, Japan
Rocker	PR 55	Hoefer Scientific instruments, USA
Separation column	In-house	-, Thailand
Spectrometer	In-house	NECTEC, Thailand
Vortex-mixer	Vortex Genie2	Scientific Industries, USA
Water distiller	Milli-Q plus	Millipore, USA

3.2 Methods

3.2.1 Anti-human albumin preparation

The anti-human albumin was used as the biorecognition molecule to be immobilized on the sensor surface in order to form the recognition event of the antigen-antibody interaction.

The anti-human albumin of 2.5 mg/mL initial concentration was pipetted from the container and diluted 500-fold with phosphate buffer saline (PBS) to be used as the working solution. The working anti human albumin was prepared by adding 2 μL of the anti-human albumin into 1000 μL of PBS and mixed thoroughly in the room temperature. The solution of the 1:500 anti-human albumin dilution was kept at 4ºC until used.

3.2.2 Conjugation of Cy5 dye and albumin

In order to monitor the accumulation of antigen-antibody complex, the fluorescence dye (Cy5) was used to conjugate with the albumin. The conjugation was done by weighing five milligrams of human albumin and dissolved in 1 mL of 0.1M sodium carbonate buffer (pH 9.3). The protein solution was mixed thoroughly and added into the vial of Cy5 dye. The solution of protein and dye were then incubated for 30 minutes with additional mixing every 10 minutes in the dark at room temperature. After the incubation time was reached, the dye-protein solution was separated from free dye by column gel filtration. The column was prepared with the filtration gel (Sephadex G50) and phosphate buffer saline was used as the eluting buffer. The dye-protein solution was then added into the column. Two blue bands were developed during the procedure. The faster moving band was Cy5-labeled albumin while the slower moving band was the free dye. The Cy5-labeled albumin solution was then collected into the microtube for further estimation of final dye /protein ratio and protein concentration.

3.2.3 Estimation of final dye/protein ratio

After the separation of labeled albumin from free dye was achieved, the absorbance of labeled albumin solution was measured by the spectrometer at λ=280 nm for protein and λ=650 nm for the dye to estimate the molar concentration of the dye and protein. The ratio of these values was the average number of the dye molecules coupled to each albumin molecule. It was recommended by the manufacturer protocol to label 1mg of protein with the dye to a final dye /protein ratio between 4-12.

3.2.4 Sensor chip preparation

The sensor chip was used as the solid phase to immobilize the anti-albumin and used as the laser mediated material which leads to the total internal reflection for the fluorescence emission induced by the evanescent wave. The sensor chip was made from the transparent acrylic sheet, chemically called polymethyl methacrylate (PMMA) with the known refractive index of 1.49. The PMMA sheet was manually cut into the designed configuration.

The sensor chip consisted of two parts, the cover plate (60x15x2 mm) with the inlet and outlet for fluid injection. And the trapezoidal shaped prism (20x15x6 mm) for the antibody immobilization with angled cut for the laser light path at critical angle which was calculated by $\theta_c = \sin^{-1}(1.33/1.49) = 63.204^{o}$.

Between these two parts, the double coated adhesive film of 75 μm in height was used to attach both parts together and to form the channel of 4x13 mm for sample delivering. Hence, the sample volume used in each measurement was less than 5 μL. The configuration of the sensor chip is shown in figure 3.1.

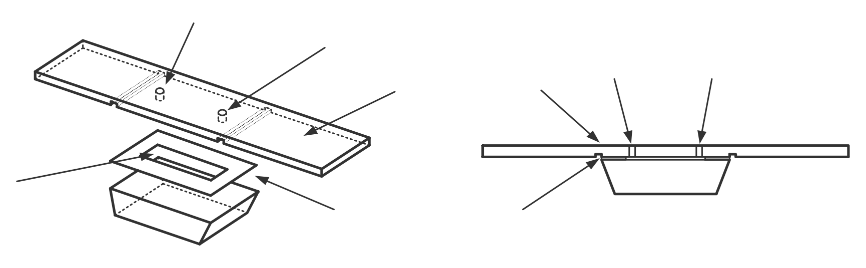

Figure 3.1 The configuration of sensor chip

3.2.5 Immobilization of anti-albumin

The prism was cleaned prior to the immobilization. The prism cleaning was performed by washing with the detergent and rinsing with tap water, followed by wiping with deionized water and alcohol. The adhesive film of the same size of the prism was attached onto the surface of the prism with the inner surface area of 4x13mm peeled off to form the immobilization area. Fifty microliters of anti-albumin was pipetted onto the immobilization area as shown in Figure 3.2. The prism was then incubated in the moist chamber for 2 hours at room temperature to let the antibody to be physically absorbed on the prism surface. After the incubation time was completed, the prism was rinsed by 100 µL of phosphate buffer saline for three times and left dried at room temperature. The possible area that may be unoccupied on the prism surface was then blocked with 0.5% bovine serum albumin (BSA) in phosphate buffer saline pH 7.4 and stood for 1 hour at room temperature followed by washing with 100 µL of PBS for three times and left until dry at room temperature. The anti-human albumin immobilized chips were stored in the container at 4ºC until use.

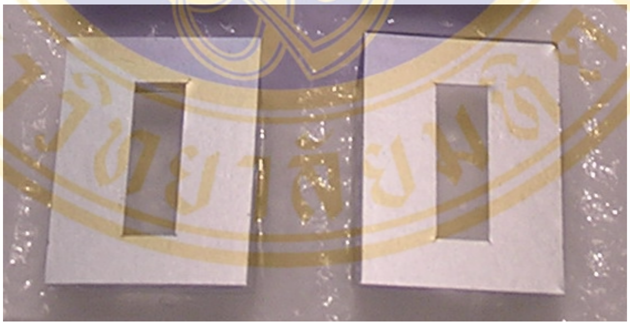

Figure 3.2 Preparation for anti-albumin immobilization on the prism surface

3.2.6 Optical set up for fluorescence detection

In order to detect fluorescence emission of the Cy5 excited by the evanescent wave, the fluorescence detection system was developed. The detection system comprised of the laser source for generating the excitation light at λ=635 nm. The light was passing through the lens for the beam focusing. The laser beam entered perpendicular through the prism under the predetermined angle of 70 degree leading to the total internal reflection at the interface of the prism surface (n_1) and the solution in the flow channel (n_2) as shown in figure 3.3 resulting in the evanescent wave.

The penetration depth according to the given angle, refractive index of the medium (n_1= 1.49, n_2= 1.33) and excitation light of 635 nm was calculated from the penetration depth relationship stated in chapter 2.

For the PMMA prism with 70 degree cutting, the penetration depth was in the range of 115.43 nm. The Cy5 attached in the close proximity of the prism surface were excited and released the energy as the fluorescence emission at λ=670 nm.

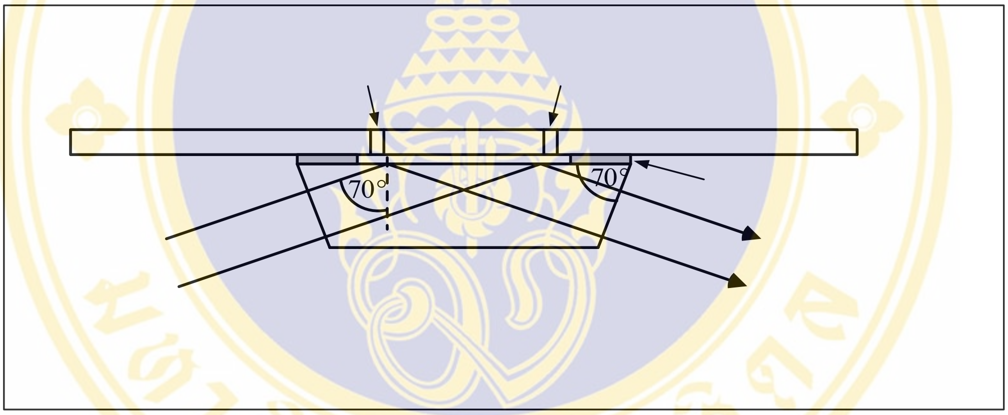

Figure 3.3 Configuration of sensor chip showing excitation light path

The fluorescent emission induced by the evanescent wave at λ=670 nm was focused by the lens and filtered by the bandpass filter in the light protected optical module. The fluorescence emission was then detected by the photomultiplier tube connected at the distal end of the optical module. The fluorescence signal detected by the PMT was amplified by the lock-in amplifier and displayed continuously by the digital multimeter. All the data were collected for further analysis in the personal computer. The block diagram of optical setup for florescence detection is shown in Figure 3.4.

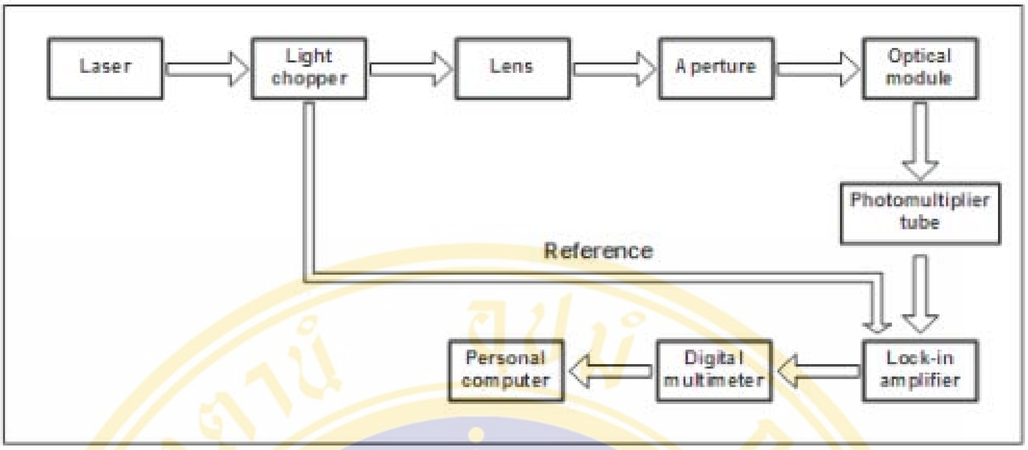

Figure 3.4 Block diagram of optical set up for fluorescence detection

3.2.7 Fluorescence detection of Cy5 labeled albumin

In the primary study for the fluorescent excitation induced by evanescent wave, the Cy5 labeled albumin was directly immobilized on the prism surface to investigate the fluorescence produced by the dye itself, not from the binding event of antigen-antibody complex. The surface of the prism was cleaned prior the Cy5 labeled albumin immobilization. The immobilization area was filled with the Cy5 labeled albumin for the volume of 50 µL. The prism was then incubated in the moist chamber for 2 hr at room temperature to let the Cy5 labeled albumin to be absorbed on the prism surface. After the incubation time was completed, the prism was rinsed by 100 µL of phosphate buffer saline for three times to remove the excess and left dried at room temperature.

For the measurement, the fluorescently coated prism was attached with the cover plate by the double coated adhesive film. The two components of the sensor chip were pressed together. The sensor chip was the inserted into the measuring chamber and the buffer of 10 µL was then added into the inlet channel as shown in Figure 3.5. Then the measuring process was started to monitor the fluorescence emission.

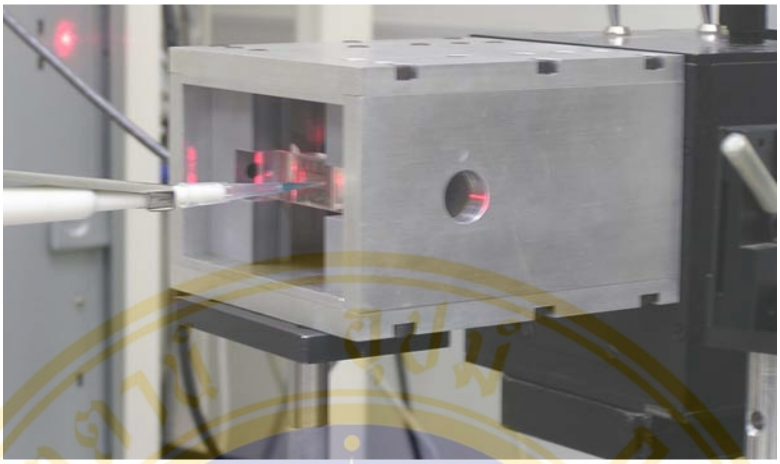

Figure 3.5 Sample delivered into the sensor chip

In order to ensure that the fluorescent emission was from the Cy5 within the range of the evanescent field, not from the emission of Cy5 in the bulk solution beyond the evanescent field, therefore the immobilization was also done on the surface of the cover plate instead of on the prism surface with the same procedure as for the prism immobilization.

3.2.8 Optimization of anti-human albumin

In order to find the optimum condition for the albumin assay, the concentration of the anti-albumin to be immobilized on the chip was determined.

The concentrations of the antibody to be immobilized were varied into 1:500, 1:1000, 1:2000, 1:3000, 1:5000 dilutions. Each of the anti-albumin concentration was immobilized on the chip as previously described. The undiluted Cy5 labeled albumin was used as sample to obtain the maximum fluorescence signal from the chip with various antibody concentration. Then, the antibody concentration of the highest signal was selected for the microalbumin assay.

3.2.9 Optimization of Cy5-labeled albumin

After the optimum antibody concentration for the assay was chosen, the Cy5 labeled albumin concentration was varied to find the concentration to competitively bind with the albumin. The Cy5 labeled albumin was varied by diluting with PBS ranging from 954 µg/mL, 476 µg/mL, 190 µg/mL, and 95 µg/mL respectively. These varied concentrations were tested to bind directly to the selected antibody concentration for the production of fluorescence signal. The selected concentration of Cy5 labeled albumin was then used for the competitive assay to be performed.

3.2.10 Competitive assay format

After the anti-albumin and the Cy5 labeled albumin concentrations were optimized, the assay for determining the albumin was then performed. Five concentrations of human albumin were prepared from the stock solution of 2 mg/mL by diluting with PBS to the various concentrations ranging from 50 µg/mL, 100 µg/mL, 200 µg/mL, 500 µg/mL, 1000 µg/mL respectively. All five concentrations of human albumin were stored in the microtube and kept at 4ºC until use.

The assay for microalbumin determination was performed by using the 1:2000 dilution of anti-human albumin immobilized chip. Cy5 labeled albumin and the varied concentration albumin were pipetted for the volume of 10 µL for each one and mixed in the microtube. The sample was then delivered into the inlet channel of the sensor chip. Both forms of albumin competed to bind for the limited binging site of the anti-albumin attached on the prism surface. The fluorescence signal detected was inversely proportional to the concentration of the albumin in the sample. The more albumin in the sample, the less fluorescence signal to be detected. While the sample with maximum signal of fluorescence detected was considered as negative result.

The measurement for the micro-scale albumin assay was performed in triplicate and the end point signal was recorded and calculated for the means value and standard deviation. The calculated values were then plotted as the calibration curve for microalbumin determination.

CHAPTER IV
RESULTS

4.1 Estimation of dye to protein ratio

After the albumin was conjugated with the Cy5 dye, it was necessary to determine the ratio of the dye molecule per protein molecule. The calculation of dye to protein ratio was performed by measuring the absorbance of the dye-protein solution with the spectrometer at λ =280 nm and 650 nm. The values obtained from the absorbance measurement were calculated for the dye to protein ratio by the following relationship

[Cy5 dye] = A650/250,000
[Protein] = A280/170,000
Dye/protein ratio = [Dye]/[protein]

Where, [Cy5dye] is the concentration of the Cy5 dye
[Protein] is the concentration of the albumin

The absorbance value obtained at λ 280 nm was 0.107 and at λ 650 nm was 0.666 respectively.

By substituting the absorbance values in the above relationship, the final dye to protein ratio was then equal to 4.235, which was in the optimum value given by the manufacturer for the protein labeling.

4.2 Calculation of the final albumin concentration

According to the conjugation of the albumin to the Cy5 dye procedure, the initial concentration of the albumin was 5mg/mL. As the consequence of the column gel filtration, the final concentration of albumin was decreased by the separation step. Therefore, the recalculation of the final albumin concentration was necessary.

The calculation was done by using the relationship for estimating the protein concentration by measuring the absorbance of the protein solution at λ=260 nm and λ=280 nm as the following relationship.

Concentration (mg/ml) = (1.55 × A280) – (0.76 × A260)
The absorbance value obtained at λ260 nm =0.927
The absorbance value obtained at λ280 nm =1.070

By substituting in the relationship stated above, the estimation of final albumin concentration was 954 µg/mL. And this amount of albumin labeled with Cy5 was used in the experiment for microalbumin assay.

4.3 Fluorescence signal of Cy5 labeled albumin

The sensor chip with Cy5 labeled albumin immobilized on the surface of the prism was measured for the fluorescent emission. And to ensure that the signal detected was from the excitation at the prism surface, the same immobilization was also done on the surface of the cover plate. The result revealed that the signal detected from the cover plate immobilization was at the same magnitude of the blank chip (buffer filled) as shown in Figure 4.1. This demonstrated that only Cy5 labeled albumin at the close proximity of the prism was excited by the evanescent wave. The Cy5 labeled albumin immobilized on the cover plate was beyond the range of the evanescent wave.

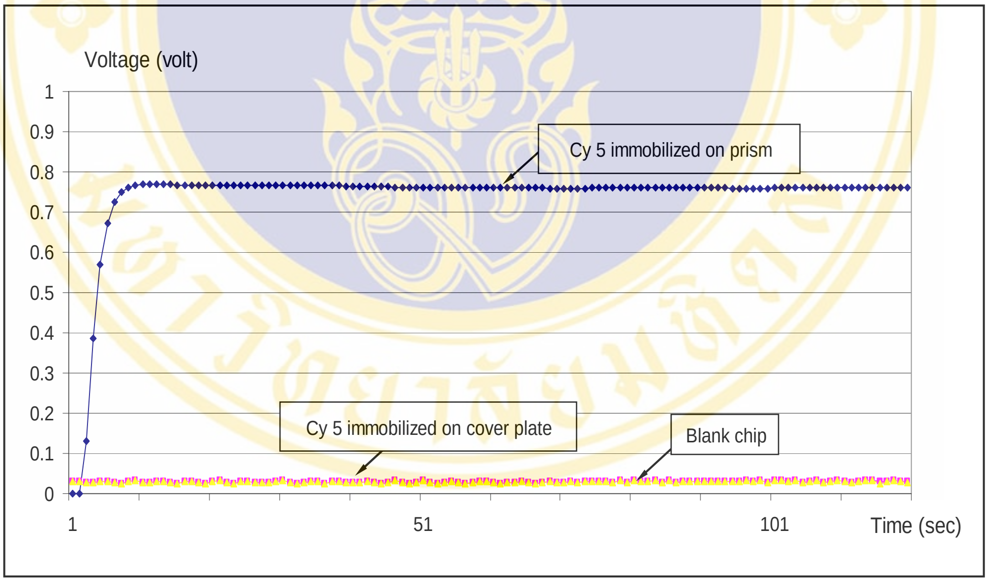

Figure 4.1 Signal of the prism immobilization comparing with cover plate immobilization and blank chip.

The immobilization area was filled with the Cy5 labeled albumin for the volume of 50 μL and then incubated in the moist chamber for 2 hr at room temperature. After the incubation, the immobilization area was rinsed with PBS to remove the excess and left dried at room temperature.

4.4 Optimization of anti albumin concentration

The optimization of anti albumin concentration was done by varying the amount of antibody to be immobilized on the prism and determining the maximum fluorescent signal obtained from the direct binding with the undiluted Cy5 labeled albumin (954 µg/mL). The highest signal was obtained from the 1:2000 dilution antibody concentration as illustrated in figure 4.2. This optimized antibody concentration was then used to immobilize on the prism for the assay to be followed.

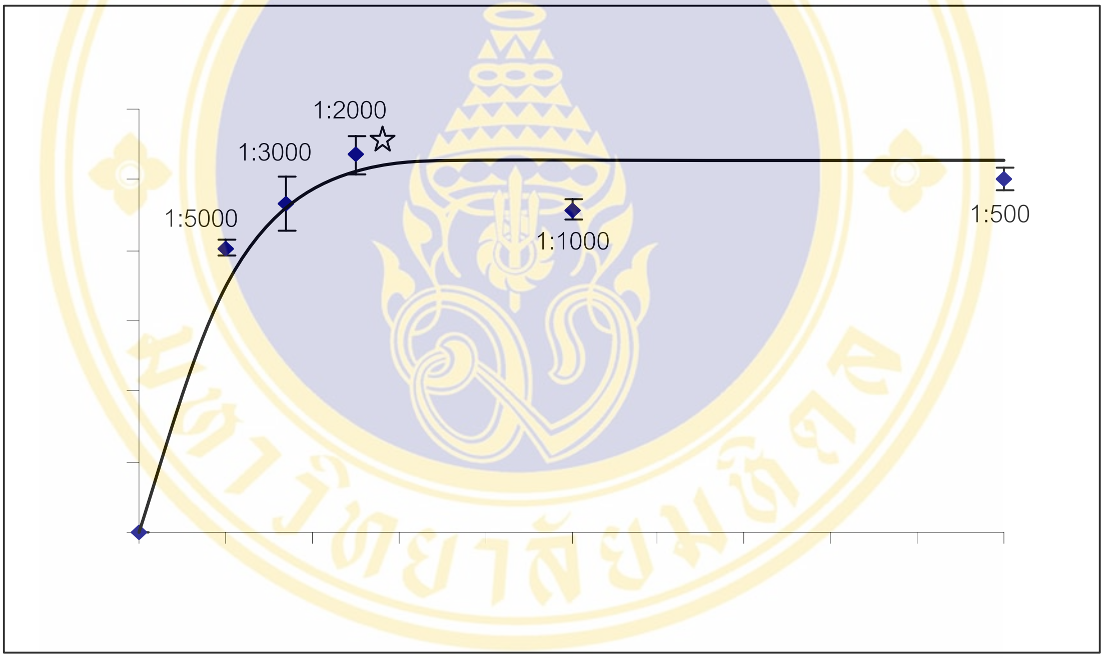

Figure 4.2 Curve of the varied antibody concentration.

The concentrations of the antibody to be immobilized were varied into 1:500, 1:1000, 1:2000, 1:3000, 1:5000 dilutions. Each of the anti-albumin concentration was immobilized on the chip as previously described in the materials and methods. (Each data points represent the mean ± SD of 3 experiments) Asterisk indicates the antibody concentration of the highest signal selected for the microalbumin assay.

4.5 Effect of BSA as blocking agent

According to the antibody immobilization procedure, the possible unoccupied area that may be left on the prism after the antibody attachment was then filled with 0.5% BSA in phosphate buffer saline pH 7.4 to block the space left on the prism surface.

The prisms with BSA blocked and unblocked were tested to compare for the fluorescence signal. The fluorescence signals obtained from both BSA blocked and unblocked prism were shown in Figure 4.3

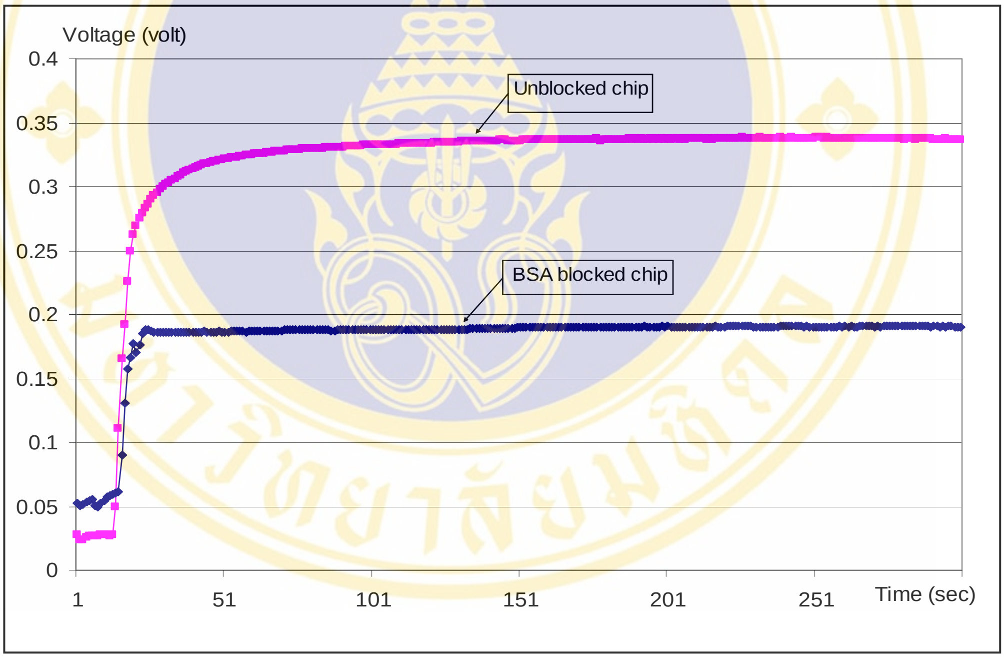

Figure 4.3 Signal of BSA blocked chip and unblocked chip.

The prism was immobilized with 50 μL of 1:2000 dilution of anti-human albumin with or without blocking with 0.5% BSA as described in the materials and methods. The signals obtained as the direct binding with 954 μg/mL Cy5 labeled albumin.

4.6 Signal of varied Cy5 labeled albumin concentration with constant antibody concentration

After the optimization of the anti-albumin concentration was completed, the step for selecting the suitable Cy5 labeled albumin concentration was performed. By using the four different concentrations starting from 954 µg/mL, 476 µg/mL, 190 µg/mL, and 95 µg/mL of albumin concentration to bind directly to the antibody immobilized on the prism. The signals of varied albumin concentration were shown in Figure 4.4.

As shown in Figure 4.4, the sensor chip was inserted in the measuring chamber and the measurement was started, followed by the adding of buffer at approximately 60 seconds delayed. All of the signals from the four chips were dropped at the same baseline. Then the sample was introduced into the chip at around 180 seconds delayed, and the fluorescence emission from differing albumin concentration can be distinguished from the one another easily. The signal magnitude detected from the varied concentration chip was decreased proportionally with the decreased in the albumin concentration.

The optimized Cy5 labeled albumin concentration for the microalbumin determination was selected at 476 µg/mL albumin concentration as the consequence of the micro scale determination with the competitive assay format which the small amount of albumin in the sample can hardly compete with the large amount of Cy5 labeled albumin to give the distinguishable signal for the detection system.

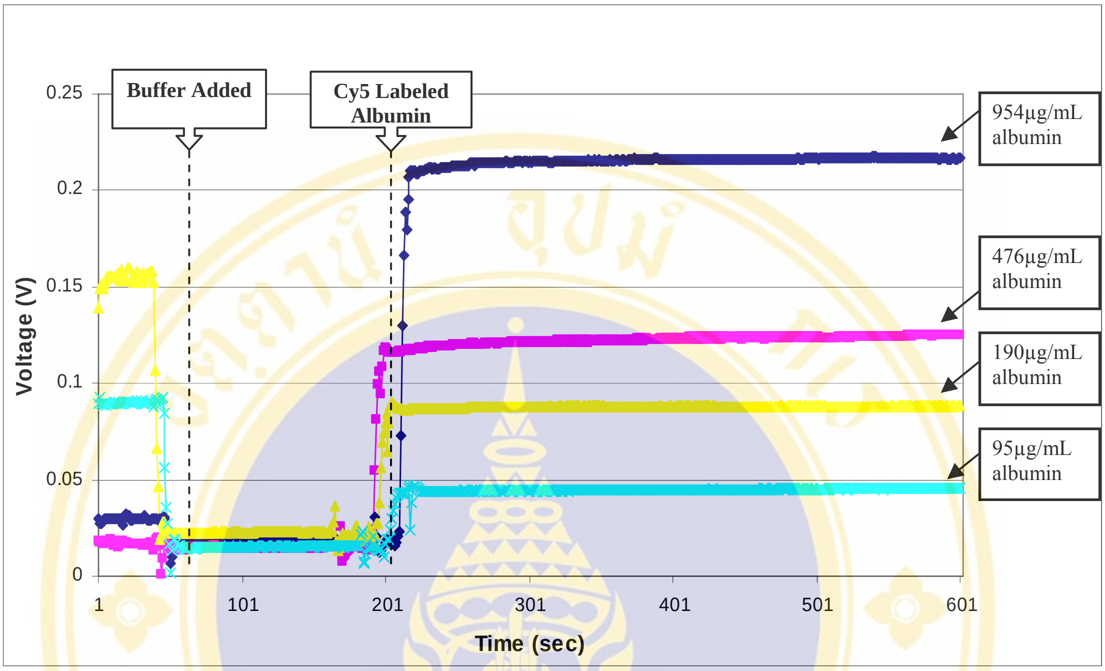

Figure 4.4 Signal of varied concentration of Cy5 labeled albumin.

The assay was performed by using the 1:2000 dilution of anti-human albumin immobilized chip. The Cy5 labeled albumin was varied by diluting with PBS ranging from 954 µg/mL, 476 µg/mL, 190 µg/mL, and 95 µg/mL. These varied concentrations were tested for the production of fluorescence signal.

4.7 Signal of competitive binding with varied albumin concentration

Since the optimization of the Cy5 labeled albumin was achieved, the test for competitive assay was performed. The Cy5 labeled albumin of the selected concentration (476 µg/mL) was used as the negative control for the assay.

After the negative control for the albumin assay was achieved, the solutions containing 50 µg/mL, 100 µg/mL, 200 µg/mL, 500 µg/mL and 1000 µg/mL albumin concentration and the 476 µg/mL albumin conjugated with Cy5 were used as the sample. The signals of competitive binding between the sample of varied albumin concentration and the Cy5 labeled albumin were decreased with the increasing in albumin concentration as illustrated in Figure 4.5.

The first signal shown on the top of the figure was obtained from the negative control, the second one was the 50 µg/mL albumin, the third was from the 100 µg/mL albumin, the fourth was from the 200 µg/mL albumin, the fifth was from 500 µg/mL albumin and the lowest signal obtained was from 1000 µg/mL albumin concentration. The measurement for varied albumin concentration was done repeatedly and the data obtained from the measurement was recorded and set for the calibration curve.

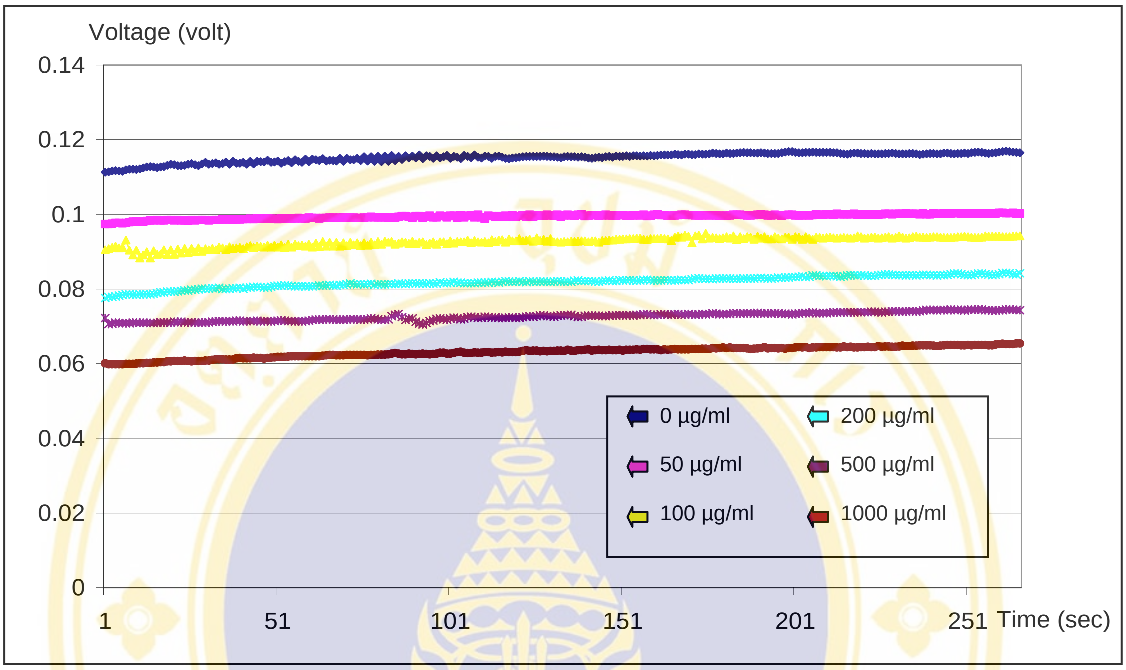

Figure 4.5 Signal of competitive binding with varied albumin concentration ranging from 0-1000 µg/mL albumin.

The assay for microalbumin determination was performed by using the 1:2000 dilution of anti-human albumin immobilized chip. Ten µL of 476 µg/mL Cy5 labeled albumin and varied concentration albumin were pipetted and mixed in microtube before delivering into the inlet channel of the sensor chip.

4.8 Calibration curve for microalbumin assay

The signals of fluorescence emission detected from six different concentrations ranging from 0, 50, 100, 200, 500, and 1000 µg/mL albumin concentration were measured repeatedly. The data of the end point signals for each concentration were recorded and calculated for the mean value and standard deviation. The calibration curve for microalbumin determination was shown in Figure 4.6

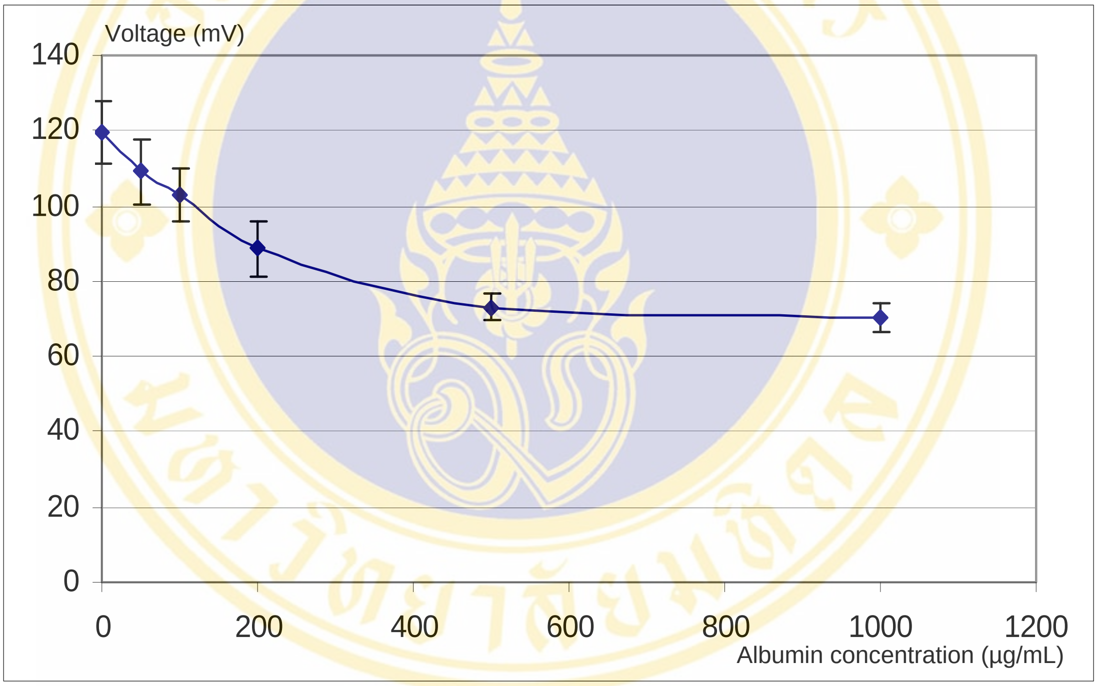

Figure 4.6 Calibration curve for microalbumin assay.

The assay for microalbumin determination was performed by using the 1:2000 dilution of anti-human albumin immobilized chip. Ten µL of 476 µg/mL Cy5 labeled albumin and varied concentration albumin were pipetted and mixed in microtube. Both forms of albumin competed to bind for the limited binging site of the anti-albumin attached on the prism surface. The measurement was performed in triplicate and the end point signal was recorded. [Each data point represents the mean ± S.D. of 3 experiments]

4.9 Repeatability of the microalbumin assay

After the calibration curve for the assay was accomplished, the sensor chips were tested for the repeatability of the chip itself since both of the cover plate and the prism were produced manually in limited amount. The sensor chips had to be reusable after the measurement was performed.

The used sensor chips were cleaned by washing in the detergent and rinsing with tap water, followed by wiping with deionized water and alcohol. And the immobilization of antibody process was performed again. The signal obtained from repeated measurement with various albumin concentration were illustrated from Figure 4.7 - 4.12.

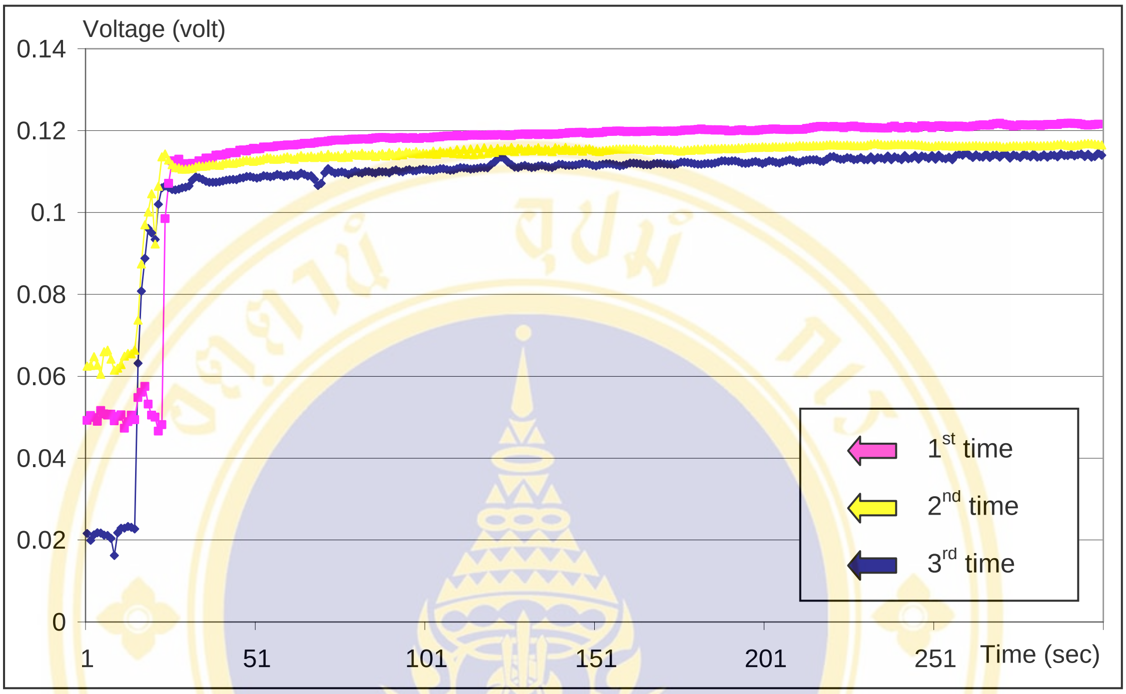

Figure 4.7 Signals of direct binding of Cy5 labeled albumin.

The assay for microalbumin determination was performed by using the 1:2000 dilution of anti-human albumin immobilized chip. Ten μL of 476 μg/mL Cy5 labeled albumin was measured for the fluorescent emission. The signal obtained from the three sensor chips were tested on different chip and date of antibody immobilization.

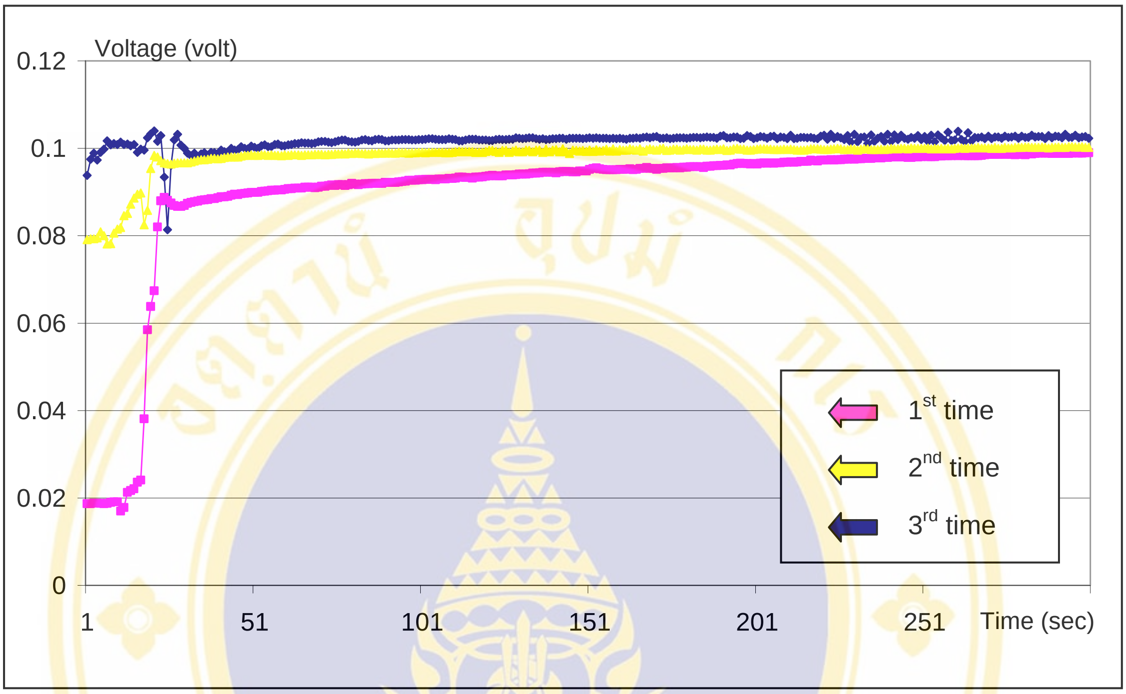

Figure 4.8 Signals of competitive binding of 50 µg/mL albumin.

The assay for microalbumin determination was performed by using the 1:2000 dilution of anti-human albumin immobilized chip. Ten µL of 476 µg/mL Cy5 labeled albumin and 50 µg/mL albumin were pipetted and mixed in the microtube. Both forms of albumin competed to bind for the limited binging site of the anti-albumin attached on the prism surface. The signal obtained from the three sensor chips were tested.

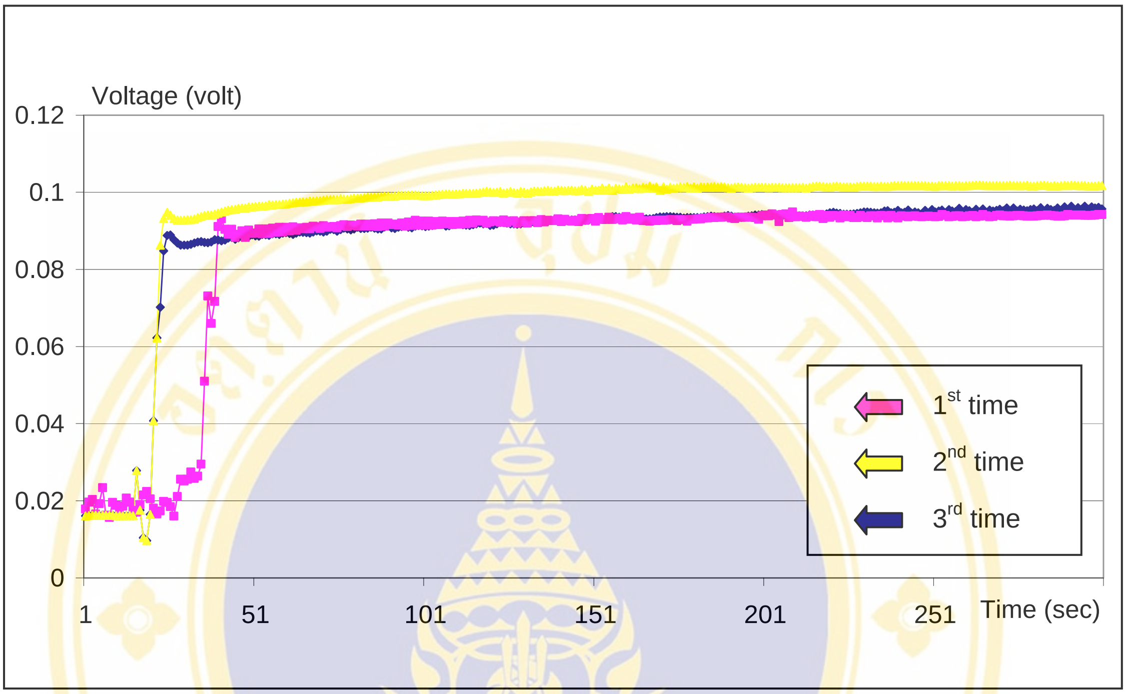

Figure 4.9 Signals of competitive binding of 100 µg/mL albumin.

The assay for microalbumin determination was performed by using the 1:2000 dilution of anti-human albumin immobilized chip. Ten µL of 476 µg/mL Cy5 labeled albumin and 100 µg/mL albumin were pipetted and mixed in the microtube. Both forms of albumin competed to bind for the limited binging site of the anti-albumin attached on the prism surface. The signal obtained from the three sensor chips were tested.

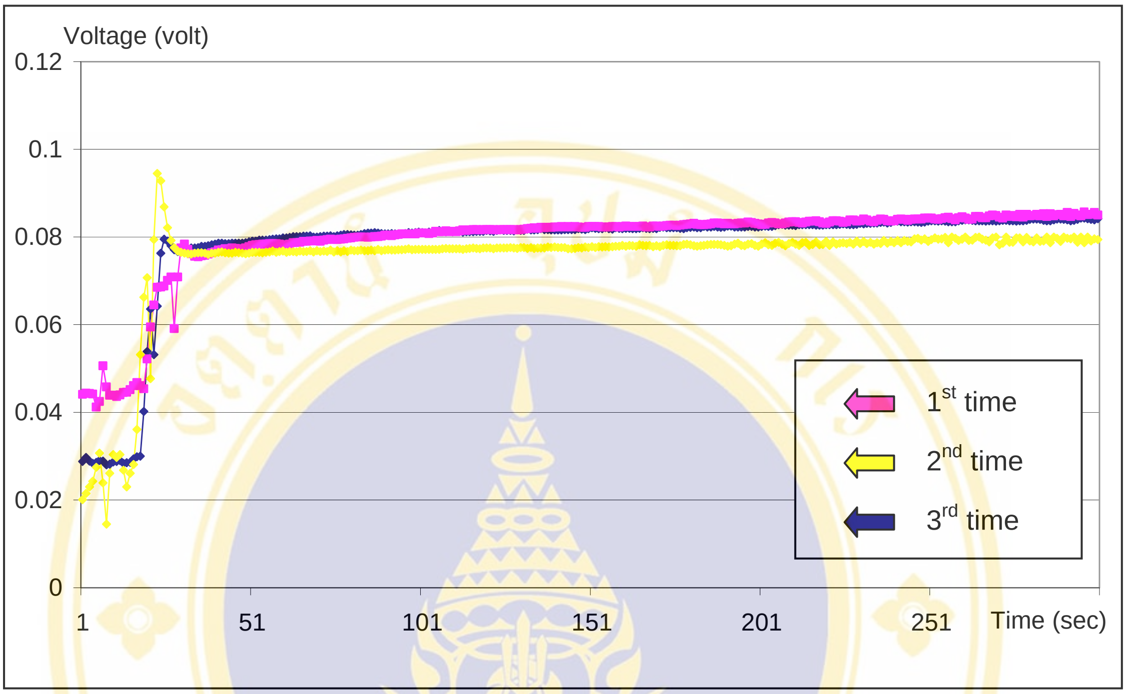

Figure 4.10 Signals of competitive binding of 200 µg/mL albumin.

The assay for microalbumin determination was performed by using the 1:2000 dilution of anti-human albumin immobilized chip. Ten µL of 476 µg/mL Cy5 labeled albumin and 200 µg/mL albumin were pipetted and mixed in the microtube. Both forms of albumin competed to bind for the limited binging site of the anti-albumin attached on the prism surface. The signal obtained from the three sensor chips were tested.

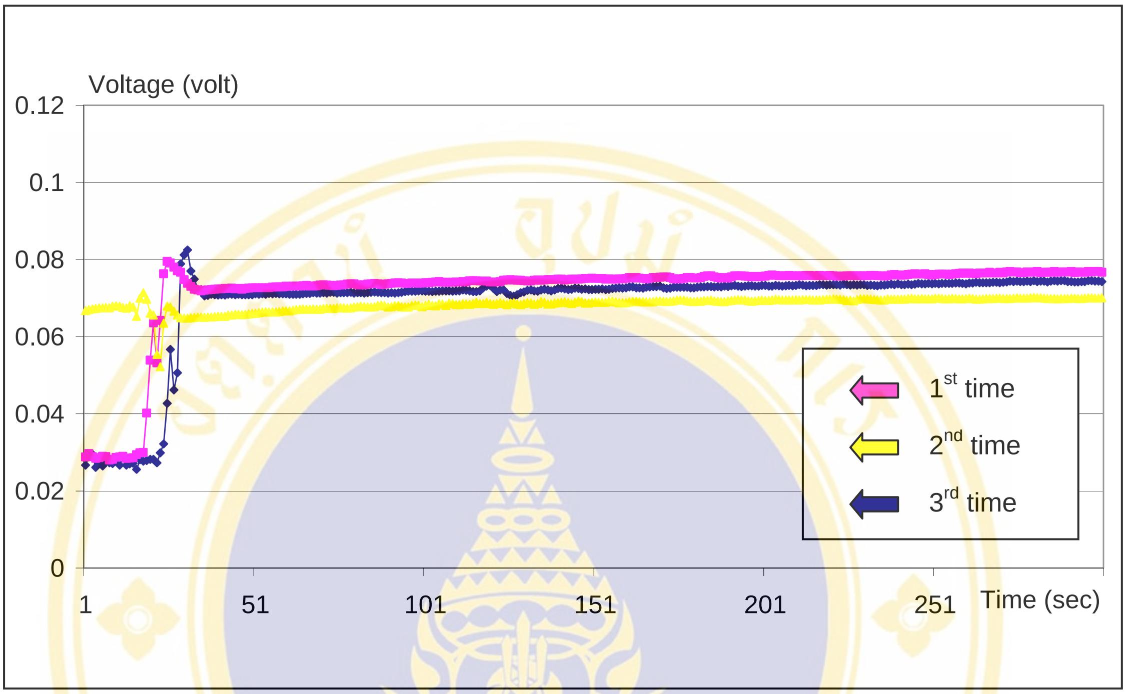

Figure 4.11 Signals of competitive binding of 500 μg/mL albumin.

The assay for microalbumin determination was performed by using the 1:2000 dilution of anti-human albumin immobilized chip. Ten μL of 476 μg/mL Cy5 labeled albumin and 500 μg/mL albumin were pipetted and mixed in the microtube. Both forms of albumin competed to bind for the limited binging site of the anti-albumin attached on the prism surface. The signal obtained from the three sensor chips were tested.

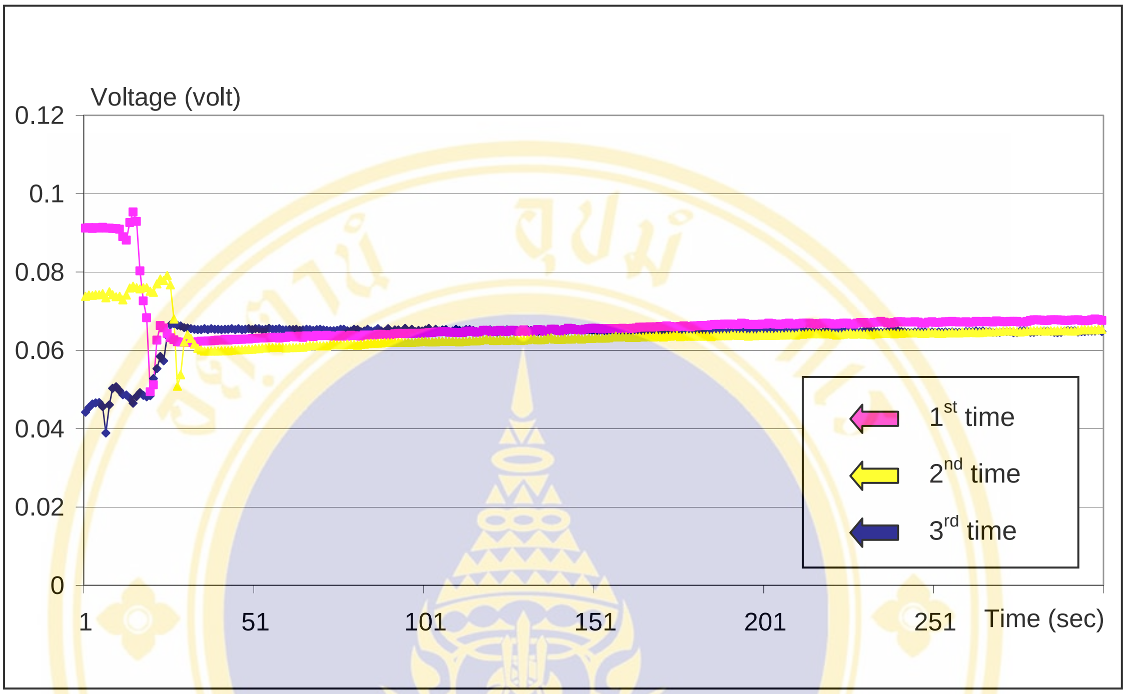

Figure 4.12 Signals of competitive binding of 1000 µg/mL albumin.

The assay for microalbumin determination was performed by using the 1:2000 dilution of anti-human albumin immobilized chip. Ten µL of 476 µg/mL Cy5 labeled albumin and 1000 µg/mL albumin were pipetted and mixed in the microtube. Both forms of albumin competed to bind for the limited binging site of the anti-albumin attached on the prism surface. The signal obtained from the three sensor chips were tested.

CHAPTER V
DISCUSSION

The sensor chip configuration with the predetermined angle of 70 degree was used instead of 63.204º, the value calculated from the Snell's law because of the convenience of the alignment for cutting and polishing the PMMA prism at 70 degree was easier than the calculated value. And the penetration depth resulting from the evanescent wave at that angle was enough to excite the fluorophore at close proximity of the prism surface. The penetration depth can also be slightly altered by the making of prism since the prism has to be manually sanded and polished until no scratchy surface was observed on both sides of the prism where laser light path entered.

The estimation of dye to protein ratio was corresponding to the ratio given by the manufacturer within the interval of 4-12 dye molecules per albumin molecule. The over labeling of the dye can resulting in the reduced fluorescence intensity due to the self quenching of the dye. Quenching is the process that causes the reduction of the fluorescence emission process.

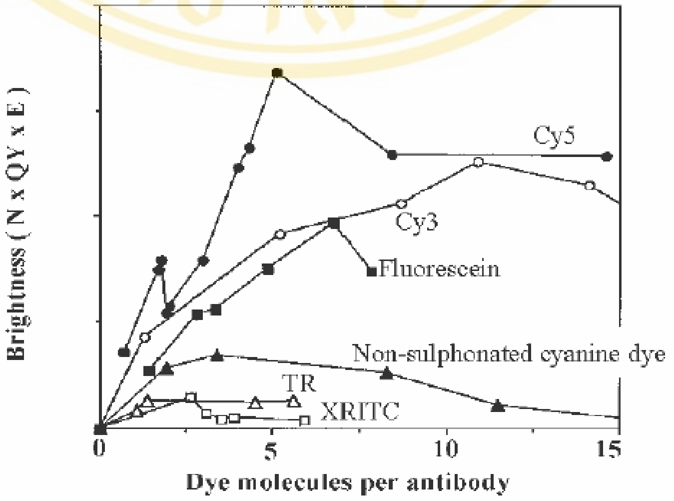

Figure 5.1 Fluorescent brightness and quantities of dye per antibody relationship (14)

The use of multiple labeling of a molecule with the fluorescent dye does not always leads to the increase in fluorescent intensity as seen in Figure 5.1. The optimum number of the Cy5 dye per antibody is around 5 dye molecules per antibody.

The fluorescent emission signal measured from the sensor chip with Cy5 labeled albumin immobilized on the surface of the cover plate was almost equal to the signal obtained from the blank chip. This can be concluded that only Cy5 labeled albumin at the close proximity of the prism was excited by the evanescent wave. The Cy5 labeled albumin immobilized on the cover plate was beyond the penetration depth of the evanescent wave and was not influenced by the evanescent field. This is the advantage of the surface specific detection for monitoring the biomolecular interaction.

The signal detected from the sensor chip blocked with 0.5% BSA in phosphate buffer saline after the antibody immobilization was apparently lower than the signal obtained from the unblocked chip according to the Figure 4.3. This could possibly due to the Cy5 labeled albumin in the bulk solution filled up the unoccupied space on the prism which was left after the immobilization of the antibody. Since the immobilization of antibody was performed by the adsorption, the antibody molecules attached on the prism surface in random. There could be somewhere on the prism surface where the antibody was not attached on. The Cy5 in the sample was coming to fill the unoccupied space and was then excited by the evanescent wave which resulting in the elevated fluorescent signal than the BSA blocked chip. Another reason that could be possible in this issue was the blocking agent used in the assay. There could be the cross reactivity between the anti-human albumin and the BSA which could lower the fluorescent signal due to the occupied binding site of the anti-human albumin on the prism surface prior the sample injection.

The antibody concentration at 1:2000 dilution was selected to immobilize on the prism because the fluorescence signal measured was more than that of other concentration. After the optimum antibody concentration was immobilized on the prism, the sample of differing albumin concentration was introduced. Although the initial signals obtained from the four chips were varied due to the chip production step, all the signals were at the same baseline after the buffer was delivered into the channel.

The signals after sample injecting were directly proportional to the albumin concentration. As can be seen from the Figure 4.4, the signal of 954 µg/mL albumin concentration was more than the signal of 476 µg/mL albumin concentration at nearly double scale and also for the signal of 190 µg/mL albumin and 95 µg/mL albumin concentration which the obtained signal were in the same manner.

Another aspect for selecting the Cy5 labeled albumin at 476 µg/mL albumin concentration was that the study was focused on the micro-scale detection and the assay format was in competitive manner. The small amount of albumin in the sample was not be able to compete with the large amount of Cy5 labeled albumin to bind with the immobilized antibody and resulting in the indistinguishable signal detected from the reaction if the 954 µg/mL albumin concentration was chosen as the competitor.

The signals of competitive binding between the sample of varied albumin concentration and the Cy5 labeled albumin were decreased with the increasing in albumin concentration as can be seen in Figure 4.5. The signal of negative control was the highest magnitude since there was no albumin in the sample to compete with the Cy5 labeled albumin. The second signal from the top represented the 50 µg/mL albumin competed with the Cy5 labeled albumin, a slightly decreased in signal magnitude was found since the small amount of albumin in the sample could hardly compete with the 476 µg/mL albumin which was labeled with Cy5. The third and the fourth signal obtained from the measurement represented the 100 µg/mL and 200 µg/mL albumin. The more distinguishable signal was detected from the experiment. The albumin of this scale in the sample was able to compete easier than the 50 µg/mL albumin. The last two signals from figure 4.5 were from the 500 µg/mL and 1000 µg/mL albumin. The amount of albumin in the sample was large enough to make the markedly decreased in the signal magnitude.

As can be seen in Figure 4.6, the signals of fluorescence emission detected from six different concentrations ranging from 0, 50, 100, 200, 500, and 1000 µg/mL albumin concentration were measured repeatedly. The calibration curve of the microalbumin assay was plotted. The signal showed the working range for the albumin concentration from negative control (0 µg/mL) up to 200 µg/mL albumin concentration. The concentration of albumin at 500 and 1000 µg/mL showed a slightly changes in the signal.

The repeatability of the microalbumin assay revealed that even though there was some background signal detected at the beginning of the measurement which was due to the prism production or some scratches on the surface after the repeated regeneration of the prism, the signal obtained from the same concentration was almost at the same magnitude for all five albumin concentrations. This phenomenon could be avoided by the use of newly prepared chip for the assay to reduce the initial background signal. The production of prism for the immobilization could be performed by the mold injection in order to reduce the irregular background signal occurred during the measurement.

Other immobilization technique may be applied to improve the binding efficiency of the antibody since there was no pretreatment on the prism surface except the surface cleaning procedure. And the technique used in the study was the physical adsorption. The bonding between the antibody and the chip surface was not so strong since it was only physically adsorbed through non-covalent bonding. The surface modification for the antibody immobilization would be another aspect to be considered in achieving the chip production.

Since the assay format for the microalbumin determination was in competitive format, the signal detected from the binding event was inversely proportional to the albumin concentration. Small amount of albumin revealed slightly decreased in fluorescent signal when comparing with the signal obtained from the negative control. The remedy should be the selection of the 190 µg/mL of Cy5 labeled albumin instead of 476 µg/mL of Cy5 labeled albumin in order to let the small amount of albumin to be able to compete with the Cy5 labeled albumin. The fluorescent signal detected could be more distinguishable than the signal obtained in the study.

The clinical trial with the urine sample should be performed since the sensor chip was initially designed to detect the albumin in urine. There could be some interference in the urine which interfere the measurement such as the color variation of the urine, the turbidity of the sample or the pH variation. These issues are the aspect to be concerned in the development of this optical biosensor.

CHAPTER VI

CONCLUSION

1. The optical biosensor for detecting the human albumin based on the evanescent wave excitation has been demonstrated.

2. The human albumin was used as the model to study the immunological reaction along with the application of the fluorescent dye and evanescent wave excitation to monitor the immunological reaction.

3. The measurement was performed at the end point of the reaction. The fluorescent signal detected was inversely proportional to the albumin concentration in the sample as the assay format was the competitive binding.

4. The calibration curve for microalbumin assay was achieved. The assay time was completed within 5 minutes and only small amount of sample volume (<5 µL) was required in each assay.

5. From the result of the presented work, it could be possible for other biological interaction format to apply for establishing the optical biosensor to be used as the rapid detection tool for other biomedical applications.

REFERENCES

1. Rogers KR. Principle of affinity-based biosensors. Mol Biotechnol 2000;14(2):109-29.
2. Thevenot DR, Toth K, Durst RA, Wilson GS. Electrochemical biosensors: recommended definition and classification. Biosens Bioelectron 2001;16(1-2):121-31.
3. Vo-Dinh T, Allain L. Biosensors for medical applications. In: Vo-Dinh T, editor. Biomedical Photonic handbook. CRC Press; 2002. p. 20/1-20/40.
4. Katerkamp A, Grundig B, Renneberg R. Chemical and biochemical sensors. Biochemical Sensors. In: Bailey JE, editor. Ullmann's Encyclopedia of Industrial Chemistry, 6th Edition. John Willey & Sons, New York, NY. 2002.
5. Mehrvar M, Bis C, Scharer JM, Moo-Young M, luong JH. Fiber optic biosensors-Trends and advances. Anal Sci 2000;16:677-92.
6. Luppa PB, Sokoll LJ, Chan DW. Immunosensors-principles and applications to clinical chemistry. Clin Chim Acta 2001;314:1–26.
7. Ekins RP. Immunoassay and other ligand assays: from isotopes to luminescence. J Clin Ligand Assay 1999;22:61–77.
8. Parslow TG. Immunoglobulins and immunoglobulin genes. In: Stites DP, Terr AI, Parslow TG, editor. Medical immunology. 9th Edition. Appleton & Lange, Stamford, Connecticut; 1997. p. 95-114.
9. Davies DR, Padlan EA. Antibody-antigen complexes. Annual Review Biochem 1990;59:439-73.
10. Price CP. The evolution of immunoassay as seen through the journal clinical chemistry. Clin Chem 1998;44:2071-4.
11. Mahoney WC, Luderer AA, Brier RA, Lin JN. Real-time immunodiagnostics employing optical immunobiosensors. In: Chan DW, editor. Immunoassay automation. An updated guide to systems. Academic Press, San Diego. 1996.

12. Mullett WM, Lai EPC, Yeung JM. Surface Plasmon Resonance-Based Immunoassays. Methods 2000;22:77-91.

13. Keiser G. Optical fiber communications. 2nd Edition, McGraw-Hill. 1991.

14. Amersham Life Science, Inc. Product specification. FluoroLink™ Cy5™ Monofunctional Dye 5-pack PA25001. Arlington -Heights, IL. Available at: http://www1.amershambiosciences.com. (accessed June, 2003)

15. Taylor RF. Chemical and biological sensors: markets and commercialization. In: Taylor RF, Schultz JS, editor. Handbook of Chemical and Biological Sensors. IOP Publishing, Philadelphia, PA.; 1996. p. 553-9.

16. Hirshfeld TE, Block MJ. Fluorescent immunoassay employing optical fiber in a capillary Tube. *United States Patent Number 4,447,546* 1984.

17. Ligler FS, Golden JP, Shriver-Lake LC, Ogert RA, Wijesuria D, Anderson GP. Fiber-optic biosensor for the detection of hazardous materials. Immunomethods 1993;3:122-27.

18. Thompson R, Villaruel C. Waveguide-binding sensors for use with assays. *United States Patent Number 5,061,857* 1991.

19. Anderson GP, Breslin KA, Ligler FS. Assay development for a portable fiberoptic biosensor. ASAIC Journal. 1996;42:942-6.

20. Narang U, Anderson GP, Ligler FS, Burans J. Fiber optic-based biosensor for ricin. Biosens Bioelectron 1997;12(9-10):937-45.

21. Richalet-Secordel PM, Poisson F, Regenmortel MH. Uses of biosensor technology in the development of probes for viral diagnosis. Clinical and diagnostic virology 1996;5:111-9.

22. Inoue K, Arai T, Aoyagi M. Real time observation of binding of herpe simplex virus type 1 (HSV1) to vero cells and neutralization of HSV1 by sulfonated human immunoglobulin. J Biochem 1997;121:633-6.

23. Baueumner AJ, Schlesinger NA, Slutzki NS, Romano J, Lee EN, Montagna RA. Biosensor for dengue virus detection: sensitive, rapid, and serotype specific. Anal Chem 2002;74:1442-8.

24. Markgren PO, Hamalainen M, Danielson UH. Screening of compounds interacting with HIV1 protienase using opyical biosensor technology. Anal biochem 1998;265:340-50.

25. Ivnitski D, Abdel-Hamid I, Atanasov P, Wilkins E. Biosensors for detection of pathogenic bacteria. Biosens Bioelectron 1999;14:599-624.
26. Chang YH, Chang TC, Kao EF, Chou C. Detection of protein A produced by *Staphylococcus aureus* with a fiber optic based biosensor. Biosci Biotechnol Biochem 1996;60:1571-8.
27. Williams KM, Westphal CD, Shriver-Lake LC. Determination of egg proteins in snack food and noodles. J AOAC Int 2004;87(6):1485-91.
28. Shriver-Lake LC, Taitt CR, Ligler FS Applications of array biosensor for detection of food allergens. J AOAC Int 2004;87(6):1498-502.
29. Hanrahan G, Patil DG, Wang J. Electrochemical sensors for environmental monitoring: design, development and applications. J Environ Monit 2004;6(8):657-64.
30. Dennison MJ, Turner AP. Biosensors for environmental monitoring. Biotechnol Adv. 1995;13:1-12
31. Sadik OA, Wanekaya AK, Andreescu S. Advances in analytical technologies for environmental protection and public safety. J Environ Monit 2004;6(6):513-22.
32. Peter C, Meusel M, Grawe F, Katerkamp A, Cammann K, Borchers T. Optical DNA-sensor chip for real-time detection of hybridization events. Fresenius J Anal Chem. 2001;371(2):120-7.
33. Ekins RP, Chu FW. Ligand assays: from electrophoresis to miniaturized microarrays. Clin Chem 1998;44:2015–30.
34. Ekins RP. Microarrays: their origins and applications. Tibtech 1999;17:217–8.
35. Cullum BM, Vo-Dinh T. The development of optical nanosensors for biological measurements. Trends Biotechnol 2000;18:388–93.
36. Song MI, Iwata K, Yamada M, et al. Multisampling analysis using an array of microreactors for an alternating-current field-enhanced latex immunoassay. Anal Chem 1994;66: 778–81.
37. Felder RA, Kost GJ. Automation: Part 1. Modular stepwise automation and the future of diagnostic testing. MLO Med Lab Obs 1998;30(4):22–4, 26–7.

38. Sacks DB, Bruns DE, Goldstein DE, Maclaren NK, McDonald JM, Parrott M. Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. Clin Chem 2002;48:436–72.

39. Shihabi Z, Konen J, O'Connor M. Albuminuria vs. urinary total protein for detecting chronic renal disorders. Clin Chem 1991; 37(5):621–4.

40. Viberti GC, Hill RD, Jarret RJ, Argyropoulos A, Mahmud U, Keen H. Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. Lancet 1982; 1:1430–2.

41. West JN, Gosling P, Dimmitt SB, Littler AW. Non-diabetic microalbuminuria in clinical practice and its relationship to posture, exercise and blood pressure. Clin Sci 1991;81:373–7.

42. Meinhardt U, Ammann RA, Fluck C, Diem P, Mullis PE. Microalbuminuria in diabetes mellitus efficacy of a new screening method in comparison with timed overnight urine collection. J diabetes and its complications 2003;17:254-7.

43. Kutter D. A chemical test strip to determine low concentrations of albumin and creatinine in urine. Chemistry 1998; 29:769-73.

44. Lloyd DR, Hindle EJ, Marples J, Gatt JA. Urinary albumin measurement by immunoturbidimetry. Ann Clin Biochem 1987; 24:209-10.

BIOGRAPHY

NAME	Mr. Palakorn Tantilipikara
DATE OF BIRTH	5 March 1971
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTION ATTENDED	Mahidol University, 1995 : Bachelor of Science (Medical Technology) Mahidol University, 2005 : Master of Engineering (Biomedical Engineering)
ADDRESS	50/3 Mu 3 Saenanikom 1, Phahonyothin Rd., Ladprao, Bangkok, Thailand E-mail: palakorn56@yahoo.com