STUDY OF LONG-RANGE SURFACE PLASMON RESONANCE BIOSENSORS FOR ABO BLOOD TYPING

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Wanida Tangkawsakul

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Ph.D. (ELECTRICAL AND INFORMATION ENGINEERING)

ABSTRACT

In this study, I would like to propose new techniques for blood grouping application. Due to the advantage of optical biosensors such as high sensitivity, specificity, simple setup and rapid detection, they have been applied to a variety of biomolecules. Optical biosensors have also been interested in blood grouping application. In this work, I divided in two parts of feasibility studies which were developed as an optical biosensor for blood grouping based on two techniques.

In the first study, I demonstrate a long-range surface plasmon resonance (LR-SPR) biosensor for the detection of whole cell by captured antigens A and B on the surface of red blood cells (RBCs) as a model. The LR-SPR sensor chip consists of high-refractive index glass, a Cytop film layer, and a thin gold (Au) film, which makes the evanescent field intensity and the penetration depth longer than conventional SPR. Therefore, the LR-SPR biosensor has improved capability for detecting large analytes, such as RBCs. The antibodies specific to blood group A and group B (Anti-A and Anti-B) are covalently immobilized on a grafting self-assembled monolayer (SAM)/Au surface on the biosensor. For blood typing, RBC samples can be detected by the LR-SPR biosensor through a change in the refractive index. I determined that the results of blood typing using the LR-SPR biosensor were consistent with the results obtained from the agglutination test. I obtained the lowest detection limits of 1.58×10^5 cells/ml for RBC-A and 3.83×10^5 cells/ml for RBC-B, indicating that the LR-SPR chip has a higher sensitivity than conventional SPR biosensors (3.3×10^8 cells/ml). The surface of the biosensor can be efficiently regenerated using 20 mM NaOH.

The second study, a transmission surface plasmon resonance (T-SPR) technique for ABO blood typing was proposed based on the immunological interaction

between surface antigen of the red blood cell (RBC) and immobilized antibody. The antibody specific to blood group A (Anti-A) was covalently immobilized on carboxylic acid on gold grating surface. The RBC samples were detected on the immobilized anti-A where the blood typing was determined from the change in the intensity of the transmission peak by TSPR technique. The maximum TSPR excitation was at an incident angle 35°. All the results of blood typing was all consistent with the results obtained from agglutination test.

In summary, as the performance, sensitivity, simply experimental setup and utility of LR-SPR and T-SPR technique, it can easily be applied for ABO blood group typing.

Keywords: Long range-surface plasmon resonance; Transmission surface plasmon resonance; Red blood cell; ABO blood grouping; Optical biosensor;

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LIST OF ABBREVIATIONS

11-MUA	11-mercaptoundecanoic acid
16-MHA	16-mercaptohexadecanoic
AFM	Atomic Force Microscopy
Anti-A	monoclonal antibody for blood group A
anti-A IgG	immunoglobulin G against blood group A
Anti-B	monoclonal antibody for blood group B
Anti-mouse IgM HRP	horse-radish peroxidase-conjugated goat anti-mouse IgM mAb
ATR	attenuated total reflection
CPWR	coupled plasmon-waveguide resonance
C-SPR	conventional SPR
DVD-Rs	digital versatile disc-recordable
EA-HCl	ethanolamine hydrochloride
EDC	1-ethyl-3-(3-dimethylaminopropy) carbodiimide
ELISA	enzyme-linked immunosorbent assay
EM	electromagnetic wave
Gal	galactose
GalNac	acetylgalactosamine
IgM	immunoglobulin M antibodies
LOD	limit of detection
LR-SPR	long-range surface plasmon resonance
mAb	monoclonal antibody
MgCl2	Magnesium chloride
NaOH	Sodium hydroxide
NHS	N-hydroxysuccinimide
PAA	poly (acrylic acid)
PAD	Paper-based analytical device
RBC-A	Red blood cell group A
RBC-B	Red blood cell group B
RBC-O	Red blood cell group O
RBCs	Red blood cell
RI	reflective index
SAMs	Self-assembled monolayer
SPP	surface plasmon polaritrons
SPR	Surface plasmon resonance
SPs	surface plasmons
TSPR	transmission surface plasmon resonance
WBCs	white blood cells
WCSPR	waveguide-coupled SPR

CHAPTER 1
INTRODUCTION

Blood group typing is necessary in the treatment of patients with massive blood loss. Matching the donor's blood group with the patient's blood group is required before transfusion to avoid a blood incompatibility event. The ABO blood typing system is tested first for all blood transfusions, because it can cause serious damage in all other blood systems due to its strong specific antigen-antibody interactions. The ABO blood group system is classified based on the inherited properties of red blood cells (RBCs). It is determined by the presence or absence of certain proteins and oligosaccharides called antigens, including A and B, which are presented on the surface of RBCs. A, B, and O RBCs structures are similar, but the difference is that A-type RBCs have N-acetylgalactosamine (GalNac) and B-type RBCs have galactose (Gal), while O-type RBCs have neither. The blood group is associated with Anti-A and Anti-B immunoglobulin M antibodies (IgM), which are the body's natural defense against foreign antigens. The ABO blood typing system breaks blood types down into four groups: A, B, AB, or O. Each group specifies the antigens and antibodies found in that individual, such as the A antigens and B antibodies found in blood group A, the B antigens and A antibodies found in blood group B, both A and B antigens found in blood group AB, and both A and B antibodies found in blood group O [1–5].

The conventional technique of the blood grouping is agglutination technique. This technique is based on the interaction between antibody and its specific antigen leading to the agglutination of RBCs. Although agglutination is very easy to use in clinical but there are some limitations including laborious processing, subjective interpretation, qualitative results, and low sensitivity. Nowadays, there is the automatic instrument for testing agglutination that is high performance in the routine use. However, this method is expensive [5].

Recently, many groups have reported an analytical technique called surface plasmon resonance (SPR), which gives high sensitivity, real-time monitoring, and label free sensing systems. SPR is an optical sensitive technique that can be applied for the real-time monitoring of the interaction between biomolecules in solution-phase [6-9]. The method of SPR is based on changes in the optical reflectivity on gold surface. When biomolecules are adsorbed onto the SPR-active gold surface, they can be detected with high sensitivity and short response time [10-12].

In this work we are interested to apply other types of SPR like a long-range surface plasmon resonance (LR-SPR) and a transmission surface plasmon resonance (T-SPR) to increase efficiency in the detection and classification of ABO blood grouping. LR-SPR has evanescent field intensity and the penetration depth longer than conventional SPR (C-SPR), so it has ability for detecting large analytes. T-SPR is a simple in situ sensing systems because it is prism-less, convenient, and propagating SPR excitation method. So, it has high possibility to apply T-SPR in real fields.

Objective

1. To use long-range surface plasmon resonance (LR-SPR) biosensor for increasing sensitivity in the detection of antigen on red blood cell (RBC) surface.
2. To investigate immobilized anti-A antibodies on gold grating surface for detection of red blood cell ABO typing via T-SPR technique.

CHAPTER 2
Relevant Theory

2.1 Optical Biosensor

Sensor is a device that responds to a physical stimulus (such as heat, light, sound, pressure, magnetism, or motion) and transfer a resulting impulse. In the 1960s, a sensor seems to be a probe of some category, probably due to a vision inextricably linked to pH, ion selective or oxygen electrodes [13]. In addition, sensor can classify many types according to things to measure or type of interaction or reaction that is interesting. For example, temperature sensors, light sensor, gas sensor, chemical sensor, biosensor and so on. The basic principle of sensors in every type are consist of three main part; input (part that reaction occurred), sensor (signal conversion part) and output. In this work focus on optical biosensor type, which will be described in the following:

Biosensor is one type of sensor that vary useful in now a day. Bioelectrodes, enzyme electrodes or biocatalytic membrane electrodes are recently well known as biosensors. Biosensor was defined by IUPAC Recommendations 1992 as a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals. Biosensors can be classified into various category depend on the procedure of signal such as transduction optical, electrochemical, thermometric, piezoelectric, acoustical, mechanical, colorimetric, electronic or magnetic and then compare with the concentration of analyte. Biosensor also can be categorized according to biorecognition elements include enzymes, antigen antibodies, cell, microorganisms, biological tissue, and organelles. Biosensors may be differentiated according to other option is a combination of the two [13-15].

The signal detection methods in biosensor are importance is explained follow this

Amperometry is performed at an applied potential between the working electrode and the reference electrode, and the generated signal is related with the concentration of target compounds. This method the current signal is generated as a function of the reduction or oxidation of an electro-active product on the substrate of a working electrode.

Potentiometry connects the determination of the potential difference between the working electrode and the reference electrode that is dependent on concentration associated behavior.

The most versatile technique in electrochemical analysis is *Voltammetry*. It measured both the current and the potential. The position of peak current is related to the specific chemical and the peak current density is proportional to the concentration of the corresponding species [16].

Conductometry is depended on the conductivity change in the solution by reason of the production or consumption of ions.

Optical detection is based on the measurement of luminescent, fluorescent, colorimetric, or other optical signals produced by the interaction of micro-organisms with the analytes and correlates the observed optical signal with the concentration of target compounds [17-19].

Optical biosensors represent the most common type of biosensor. They have massive advantages more over other type of biosensor is direct detection, real-time monitoring, rapid analysis and label-free detection of many biological their also high specificity, sensitivity, and low cost. Optical biosensor can be separate into two modes. The fist mode is label-free, which detected signal is originate directly by the interaction of the analyzed material with the transducer. The second mode is label-based. It involves the use of a label and the optical signal is then generated by a colorimetric, fluorescent or luminescent method.

Normally, biosensor is composed of four elements as seen in figure 2.1. First element is a bioreceptor or biological recognition. This part is immobilized sensitive biological element (e.g. antigen/antibodies, microorganism, DNA, enzyme) recognizing the analyte (whatever antibodies/antigen, complementary DNA, enzyme substrates). The second part is transducer that is used to convert chemical or bio signal resulting from the interaction between analyte and bioreceptor into electronic part. The

example of reaction that can input to transducer electroactive substance, pH change, heat, light, mass change by types of transducers that related is electrode, semiconductor/pH electrode, thermistor, photo diode/photon counter, quartz electrode/piezoelectric device. Electronic amplifier is the third part which is an electronic device that enhance the power of signal. The latest element is a data processing system. In this part, it was combined the control input processes and defined set of output. The output can be as data, number, intensity peaks, graph, spectrum depending on the interpreter's relation to the system [16,18-19].

There are many types of optical transducers like fluorescence, chemiluminescence, light absorption, reflectance, Rama scattering, refractive index. Optical biosensor is widely known such as fiber optic, SPR, planer guide wave, interferometer, colorimetric, Raman, Nano materials.

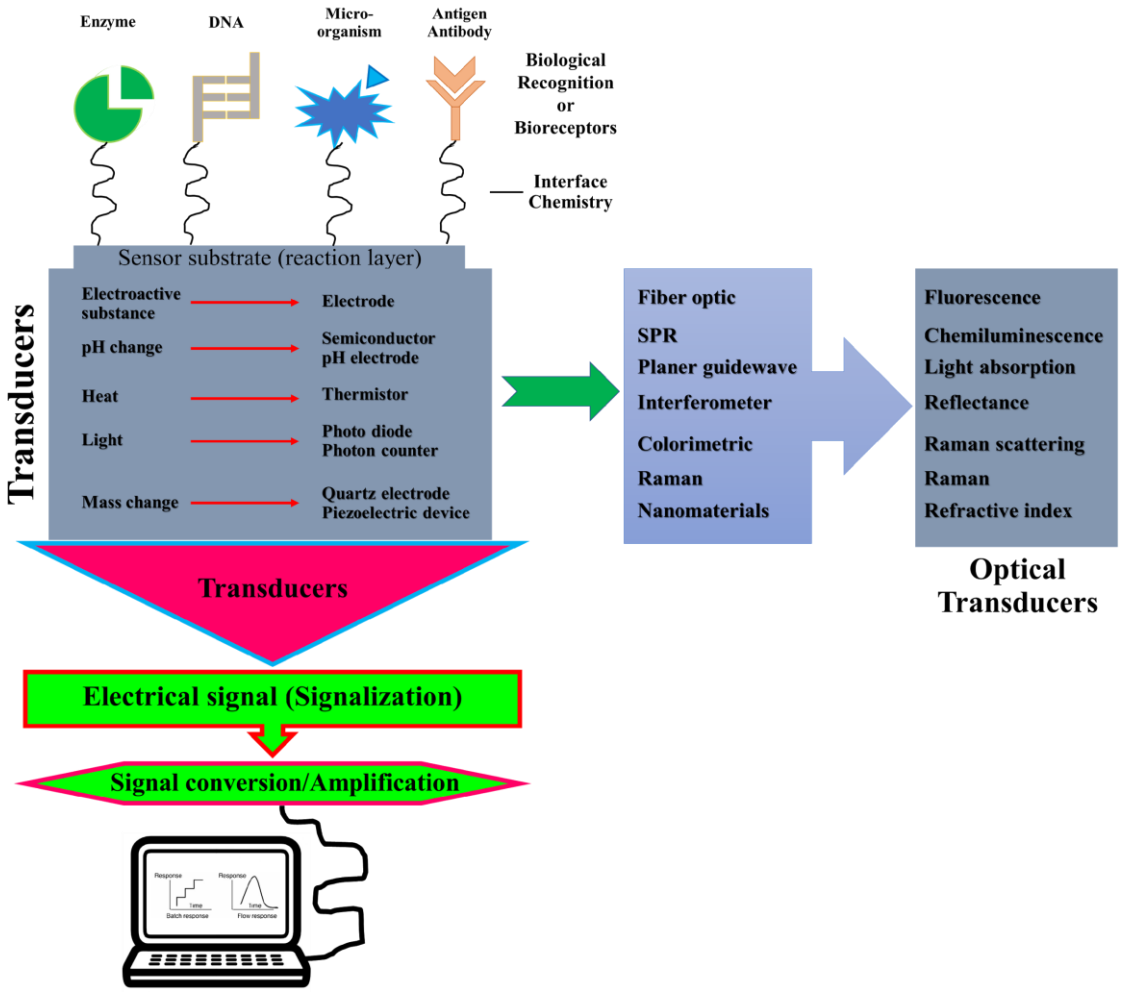


Figure 2. 1 The principle of optical biosensor

The acceptable biosensor must be determined by the characteristics in terms of

Selectivity means the sensor can detects a complex analyte by does not action with contaminants or added mixtures.

Precision is quality in repeated measurements under unchanged any conditions by provided same result.

Sensitivity is known in terms of detection limit also. It is indicative the minimal amount or concentration of analyte that can be detected.

Regeneration time is time required to return the biosensor to working state after previous interaction with the sample.

Signal stability is signal drift under constant conditions that causes an error in measured concentration.

Working range is the range of analyte concentrations in the sensor can operate.

Optical biosensor has more advantages over other types of sensor because it is multi-target sensing and continuous monitoring. Due to the multitudinous advantages, optical biosensor was useful in many applications like environmental concerns, food testing and especially medical application in terms of multiplex diagnostics. Optical biosensor already has been commercially developed to test for infectious disease, alcohol, drugs of abuse and heart attack [17].

2.2 Surface plasmon resonance (SPR) technique

2.2.1 Surface plasmon

Plasmon is a quasiparticle of free electron or charge density oscillation in volume of conducting media as metal. Plasmon is stimulated when charge particles obtain energy and momentum matching with their oscillation state. They can be excited by shooting an electron into conducting media. In case of plasmon generated at the interface between dielectric and conducting media, it is called surface plasmons (SPs) or surface plasmon polaritrons (SPP) as well as seen in figure 2.2. It is a surface bound electromagnetic wave (EM) that propagates along the interface of metal and dielectric [20-22].

However, for SPR technique, not only SPs is excited but also evanescent filed. Evanescent filed occurs under total internal reflection. It means that

the light is totally reflected at an interface between a high refractive index media like prism and lower refractive index media at incident angles larger than the critical angle. Under total internal reflection, there is evanescent field propagating above metal surface and decay exponentially as seen in figure 2.2.

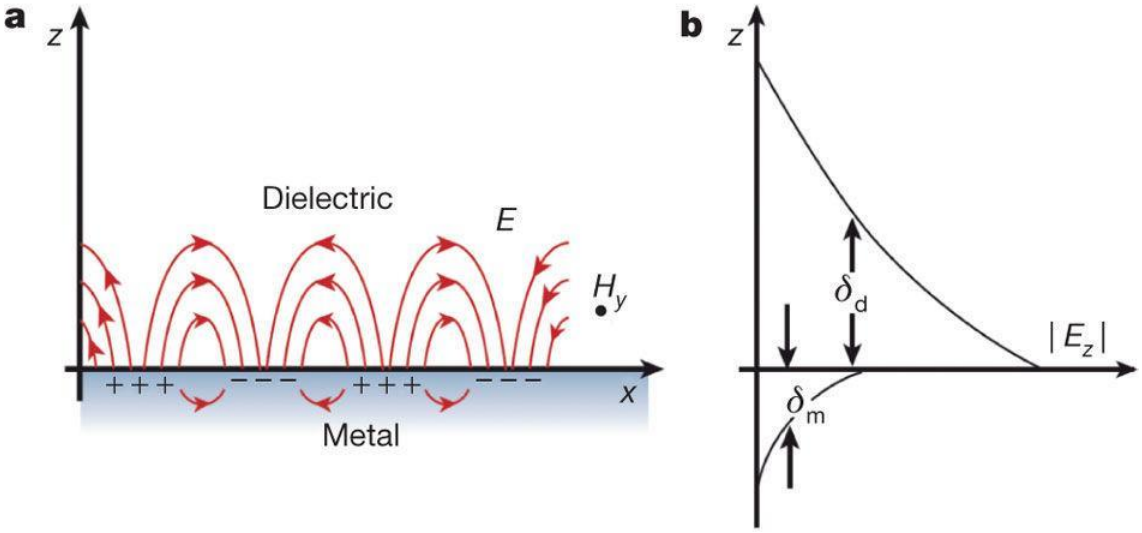


Figure 2.2 a, An SPP as a collective excitation at a metal–dielectric interface. The electromagnetic field is drastically enhanced when E is electric field that plotted in the z–x plane; Hy is magnetic field which sketched in the y direction. b, The electric field Ez which perpendicular at the interface of metal and dielectric layers. [20]

In 1986, the excitation of SPs using attenuated total reflection (ATR) was reported by Otto [21] and Kretschmann [22]. SPR techniques widely use Kretschmann geometry in figure 2.3 to exited SPs. Kretschmann geometry of ATR method was done by a high refractive index prism with refractive index n_p is interfaced with a metal-dielectric wave guide consisting of a metal thin layer by q is thickness and has permittivity is ε_m, n_d refer to refractive index of dielectric. When light wave propagating in the prism is made incident on metal film, a part of the light is reflected back into prism and part propagates in the metal is EM. The SPs was observed in form of exponential decay, and the direction is perpendicular to prism-metal interface which referred as an evanescent wave [23].

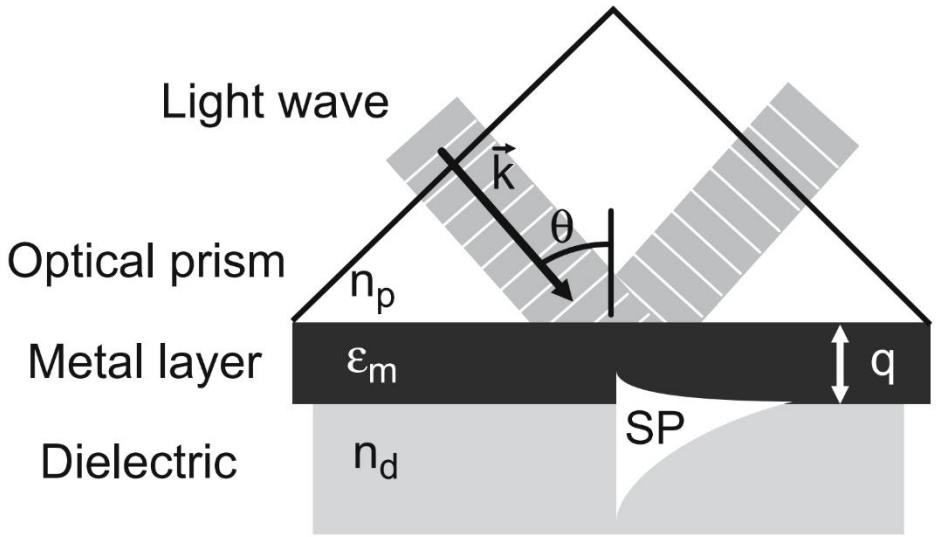


Figure 2.3 The Kretschmann geometry of ATR mode for excitation SPs [23].

2.2.2 Surface plasmon resonance (SPR) [23-28]

SPR was started to be applied in biosensor filed in 1980 on wide variety of applications including biomedical, agricultural, enzymatic system. But they were first observed in 1902. SPR is phenomenon takes places at the interface of metal and dielectric substance. SPR phenomena occur when the energy and momentum of light match with the EM of free electron oscillation in conductor media. Kretschmann configuration was used to excite free electron for SPR phenomena. This situation utilized the total internal reflection phenomena to generate evanescent wave and then to find the condition giving SPR phenomena. It is controlled by the momentum of this wave matching with the momentum of electron oscillation via change of the incident angle or wavelength. This is shown in the diagram in figure2.4. The reflectivity is measured with varying the wavelength of light. The SPR phenomena occur at the SPR wavelength that give the minimum reflectivity.

SPR phenomena depend on reflective index (RI) of the structure of the sensor chip. When we change the reflective index in any layer, the characteristic of SPR phenomena change. As shown in figure 2.5, the SPR curve shift if the RI on the sensor change. We can obtain the change of SPR wavelength which is proportional to the change of RI on the sensor.

Fortunately, the SPR instrument is highly sensitive to RI of media that it can be changed by having some different molecules attach on the sensor surface as show in this slide. When small molecules attach on the probe, the SPR signal shift and if small molecules leak off the SPR curve will return to original position.

Basic components of SPR instrument biosensing compose the metal film is evaporated onto the glass and has prism [25]. The light is illuminating from the glass, and the evanescent wave penetrates through the metal film. The plasmons are excited at outside the film. So, when wave vector of evanescent wave is equal to wave vector of surface plasmon wave in equation 2.1

$$k_x = k_{sp} \ldots\ldots (2.1)$$

it occurs the surface plasmon resonance and this is the SPR condition that shown in equation 2.2,

$$k_x = \mathrm{ksin}\,\theta_{sp} = k \sin\theta_i \ldots\ldots (2.2)$$

when

$$k = \frac{2\pi}{\lambda} = \frac{n\omega}{c} \ldots\ldots (2.3)$$

it can rearrange to equation 2.4.

$$k_x = \frac{n\omega}{c} \sin\theta_i \ldots\ldots (2.4)$$

And in case of metal surface the wave vector of the surface plasmon can expand following this equation

$$k_{sp} = \frac{\omega}{c}\sqrt{\frac{\varepsilon_m \varepsilon_s}{\varepsilon_m + \varepsilon_s}} \ldots\ldots (2.5)$$

For a dielectric material

$$n = \sqrt{\varepsilon} \ldots\ldots (2.6)$$

where the refraction index square is equal to dielectric constant, so the wave vector of surface plasmon was show in this equation.

$$k_{sp} = \sqrt{\frac{\varepsilon_m n_s^2}{\varepsilon_m + n_s^2}} \ldots\ldots (2.7)$$

When wave vector and energy matching following this equation,

$$n \sin \theta_i = \sqrt{\frac{\varepsilon_m n_s^2}{\varepsilon_m + n_s^2}} \ldots\ldots (2.8)$$

that mean the SPR angle will change when the media change the refractive index.

In case of the incidence angle is varied, a plot of the reflected intensity as function of incidence angle shows a minimum of the reflected light intensity at the SPR angle. The value $\boldsymbol{k}_x$ can be modulated by changing the incident angle. The value of $\boldsymbol{k}_{sp}$ depends on refractive index of sample, the equation (2.8). Any small change in the refractive index means a change of the $\boldsymbol{k}_{sp}$ as a result; the $\boldsymbol{k}_x$ must be changed for the SPR to take place. By continuously follow the angle at which SPR take place, the information regarding the change in the refractive index at the surface can be obtained.

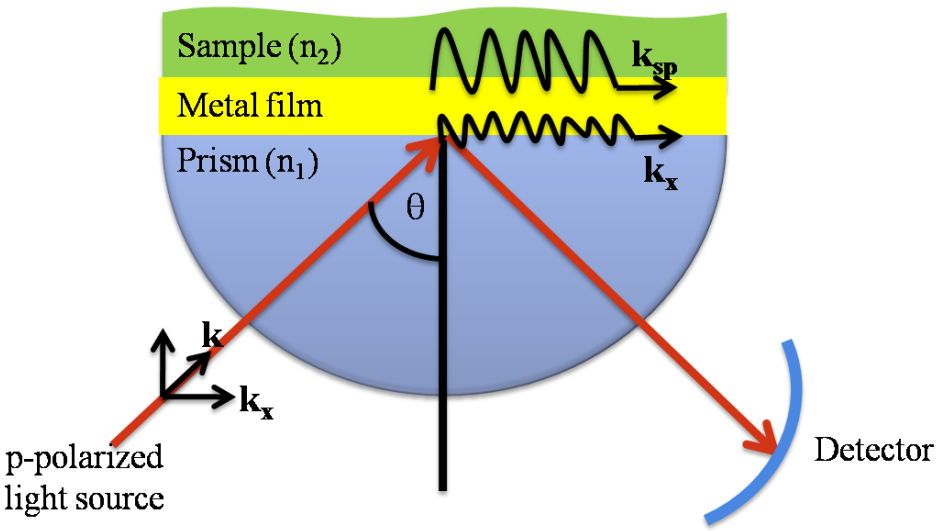


Figure 2.4 Basic component of SPR biosensor setup.

For all equation and figure 2.4, define variables following this;

θ_i /θ_{spr} is incidence angle and SPR angle.

ε is the complex permittivity of metal and dielectric ($\varepsilon = n^2$).

n is the refraction index

λ is incident wavelength

$\boldsymbol{k}_{sp}$ is the wave vector of the surface plasmon

$\boldsymbol{k}_x$ is the wave vector parallel to the prism/metal interface

k is wave vector component of the incident light

c is the speed of light

ω is the angular frequency of the incident wave.

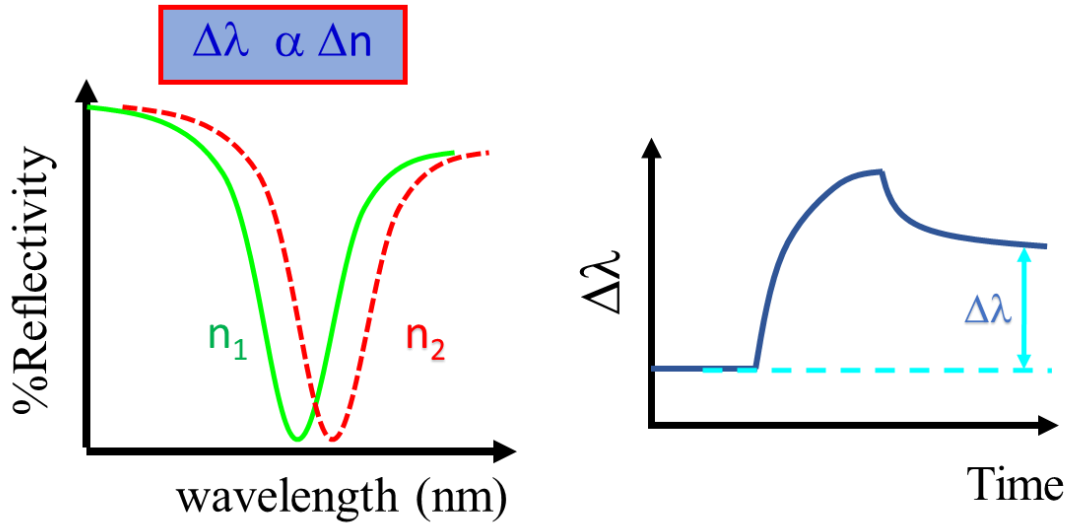


Figure 2.5 schematic of SPR curve and RI change.

2.2.3 Four types of surface plasmon resonance [23,29]

SPR technique which described in previous topic are high sensitive more than other optical technique and label-free devices, but they still cannot detect small molecular interactions or low molecular concentrations. So, various research groups have developed the enhancement of sensitivity and resolution of SPR biosensor by using different detection or SPR mode as seen in figure 2.6. There are four types of SPR. The first mode previouslu described is called conventional SPR or C-SPR biosensor. In this mode, they consist of prism coupler, metal layer and biomolecular layer to generated SPR biosensing phenomena as seen in figure 2.6(a). The second mode is named long range SPR (LR-SPR), which inserts a dielectric buffer layer between the prism and the metal and the metal layer of C-SPR (figure 2.6b). LR-SPR is accomplished when RI of the dielectric buffer layer and the buffer are equal. As a result, the propagation length of the SPs increases more than C-SPR. The energy of incident beam is also treated. LR-SPR device presents a very sharp reflectivity dip which enhances resolution around seven times compared to the C-SPR. The third mode is coupled plasmon-waveguide resonance (CPWR). The configurations of

CPWR mode combine with prism coupler, metal layer, waveguide layer and biomolecular layer represent in figure 2.6c. However, the sensitivity of CPWR is lower than 10 times from C-SPR. The last mode is waveguide-coupled SPR or WCSPR. In this mode comprise prism coupler, metal layer, waveguide layer, metal layer and biomolecular layer according to figure2.6d. There are two metal layers and a waveguide layer. WCSPR retains the sensitivity and yields sharper reflectivity spectra dips which enhance the measurement precision.

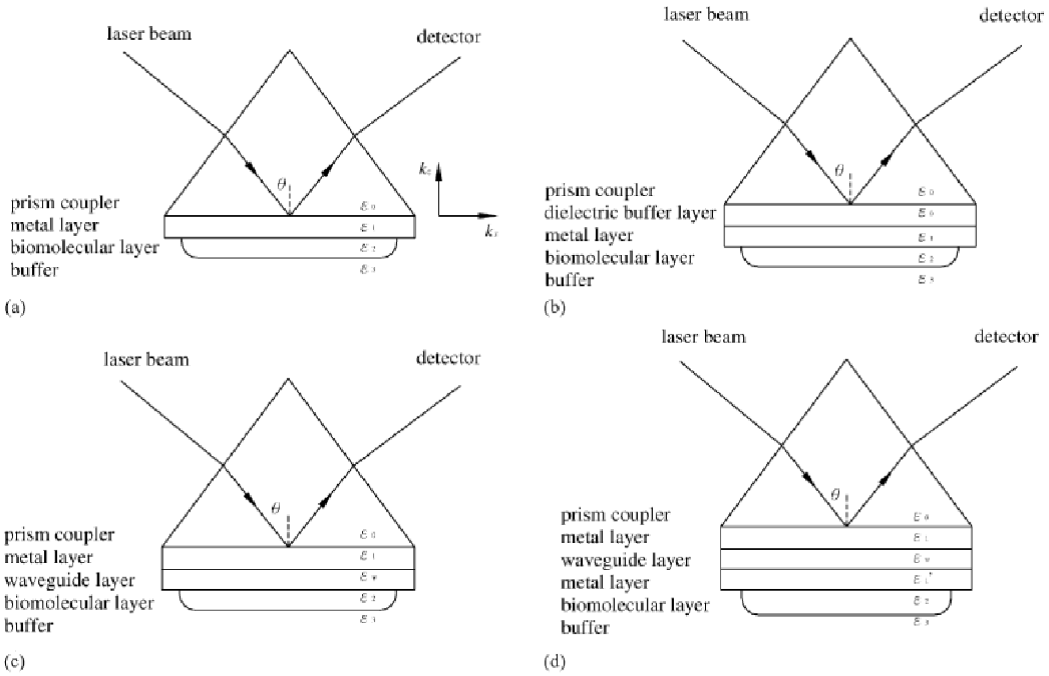


Figure 2.6 Four different SPR biosensor configurations (a) C-SPR, (b) LR-SPR, (c) CPWR and (d) WCSPR [29].

2.2.4 Long-range surface plasmon resonance (LR-SPR)

LR-SPR was first presented in 1981. LR-SPR was developed due to improve the sensitivity of SPR. The setup based on the Kretschmann configuration consist of five basic layers as seen in figure 2.6 that is prism, the dielectric buffer layer, the metal layer, the biomolecular layer and buffer. The plasmon mode on opposing surfaces of the metal film is coupled together via a symmetric configuration. LR-SPR shows a greater sensitivity to bulk refractive index variations in the dielectric medium and has been used to detect thick analytes. These modes are featured by

greater and less loss. The propagation length of the long-range surface plasmon in increased by a factor of approximately 10 times when compared to the C-SPR biosensor. The incident beam energy is more concentrated, and the depth-to-width ratio of the resonance dip is increased. The penetration depth of LR-SPR fields can be up to 1 μm, while C-SPR is around 200 nm. The LR-SPR was widely used to study in application that detected large molecule like cell or bacterial [29-39].

2.2.5 Transmission Surface Plasmon Resonance (TSPR)

In addition to C-SPR and LR-SPR discussed in previous section, other SPR type is explained in this section. This technique utilizes transmission mode for sensing in SPR technique, namely TSPR [40-41]. TSPR is an abbreviation of transmission surface plasmon resonance which was first observed in 1998. This SPR technique was used grating structures for excited SPs field like in figure 2.7. SPR excitation has been shown to enhance light transmission through nanostructures such as grating patterns [42-44], hole arrays [45-46] and grating substrates of commercial DVD-Rs (digital versatile disc-recordable) coated with thin gold films [47-48]. Remarkably, transmission SPR (T-SPR) spectra of light transmitted through such a gold grating substrate show strong, narrow peaks in the visible light region. The T-SPR wavelength depends on the grating pitch and the angle of incidence [47-48]. TSPR phenomena are frequently induced using metal-coated grating structures with highly uniform surfaces. Moreover, a spectrometer or a camera's detector is still needed for the recording of TSPR signals. TSPR is very high selectivity and the simple and flexible setup procedures make this technique highly useful, so T-SPR spectroscopy a productive technique for optical sensing applications.

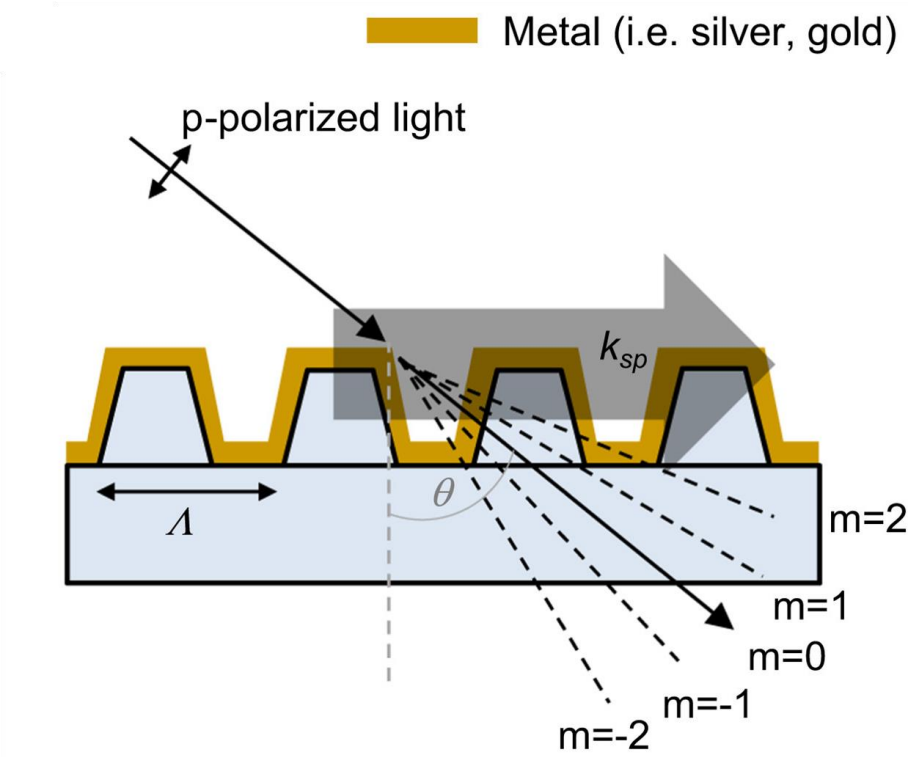


Figure 2.7 TSPR where Λ is the diffraction grating pitch, θ is the incident light angle, and ksp is the sensitive surface plasmon wavevector [40].

2.3 Surface modification, the principle of immobilization and the detection in SPR biosensor applications

In the study and development of biosensor, sensor substrate or reaction area is very importance because it is a sensing area. This part needs substrate for functionalized biological recognition element (biochemical receptor). Surface modification is the platform of modifying the surface by expose physical, chemical or biological characteristics different from originally found on the surface. The modification can be done by various methods with a view to differentiation of characteristics of the surface, like roughness, hydrophilicity surface charge. Normally, biosensor chip is modified in numerous platform such as polymer, hydrogen polymer, hydrazine, glutaraldehyde, carboxy dextran, self-assembled monolayer etc.

Self-assembled monolayer, also known as SAM, is the surface made from a molecular film of biological and is generated by chemical modification. SAMs base on

the concept of self-assembly monolayer of thiol or disulfide molecules on the metal surface. It can be modified by functional groups (both aliphatic and aromatic) containing suitable functional groups like –SH, -CN, -COOH, -NH_2 etc [50] depend on a variety of application. Reactive function on SAMs has been used to be covalently attached to some biomolecule. The application has been used in developing biological sensors, as well as studying protein-protein [51-52], DNA-taget [53] and ligand-receptor interaction [54]. Molecule of SAMs consist with three parts; (1) head group which has the capability to the affinity the regarding substrate, (2) spacer which is alkane chain and (3) terminal function groups (or tail) which is modified in various chemical functional groups to interact with various kind of molecules.

Biomolecules can be attached on the sensor chip via three main approaches. The first one is covalent immobilization, which uses covalent chemical link the molecule with surface. It is the method that was used in this work. High affinity capture is others approaches. The interested molecule is attached with another molecule by non-covalent interaction. They are used as affinity for biomolecule together. Last method is hydrophobic adsorption. This method exploits more and less specific hydrophobic interactions to attach each another.

In this work, we used covalent immobilization because to strength and durability in immobilization and sensor has a stable lifetime. Generally, there are varies common foundations for covalent immobilization i.e., amine coupling, thiol coupling, aldehyde coupling etc.

Amine coupling was used in this work. It was done by activated the amine groups on surface with a mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropy) carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) in water to give reactive succinimide esters. Ligand is then passed over the surface and the esters react spontaneously with primitive amine groups to link the ligand covalently to the dextran matrix (Figure 2.8)

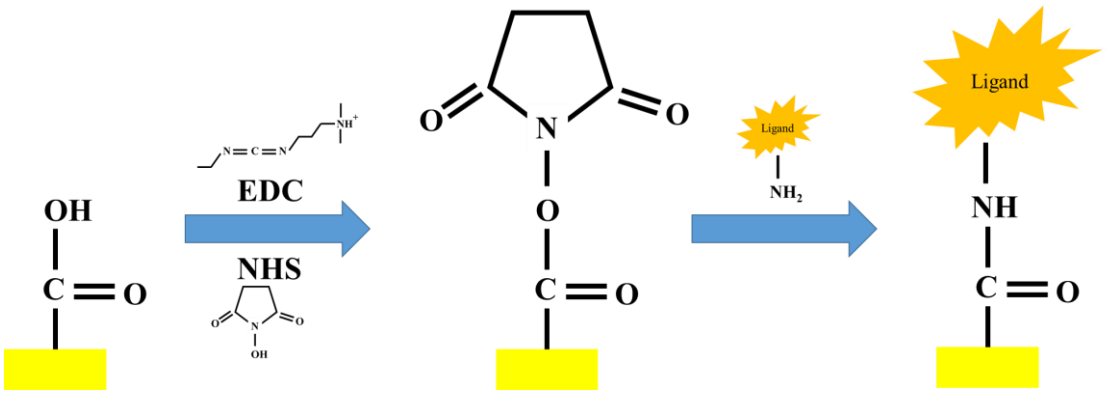


Figure 2.8 Modification of the sensor surface with amine coupling [55]

Factors that should be paid attention to the immobilization ligand on sensor surface is electrostatic pre-concentration of ligands on SAMs surface matrix. Sensor chip surfaces have charged and electrostatic attraction behavior in different situation. Normally, the primary requirement for immobilized ligand on sensor surface is ligand and SAMs surface should opposite in charge. The pH of ligand solution should lie between 3.5 and isoelectric point of ligand as seen in middle situation in figure 2.9. The best recommendation of buffer solution for covalent immobilization is 10 mM buffers at pH 4.5 – 5.5.

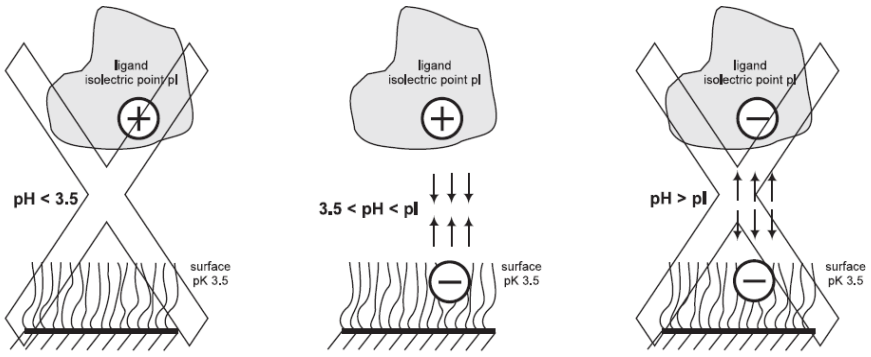


Figure 2.9 The interaction of ligand and surface under electrostatic environment. [23]

SPR biosensor has the basic principle to have ligand, which binds to the surface, the analyte flow over the surface and bind to ligand as seen in figure 2.10. The interaction of biomolecule are measured by detecting the change in refractive index on a metal surface. SPR can observe mass change, reflective index change, thickness change and so on that is occurred on sensor chip, so there is no need for labelling like

other optical technique. SPR can detect interaction by direct detection, which is advantages of SPR technique.

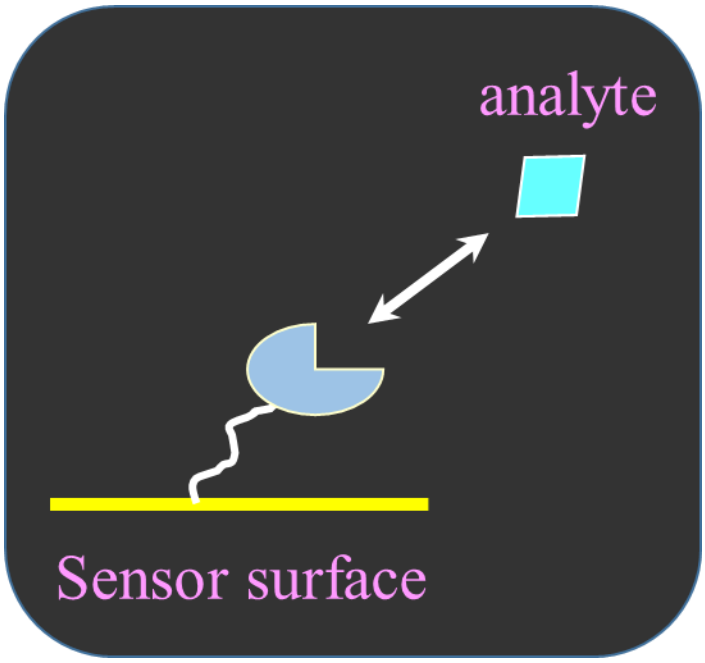


Figure2.10 The bioreceptor or ligand is the interaction couple that is attached to the sensor surface. The analyte is free in solution and binds to the immobilized ligand.

2.4 Blood group system

Blood is the fluid that circulates throughout the body by the blood vessels. On average, normal adult blood volume is about 70 cc / body weight in Kg unit, meanly about 5-6 liters in males and 4-5 liters in females. Blood in the body can separate to two components are blood plasma and blood. The plasma is composed of water, biochemical enzymes, hormones, albumin, immunoglobulin and substances that help blood clotting. In the blood cell, there are red blood cells (RBCs), white blood cells (WBCs) and platelets. The blood in the human body is special by the viscosity is mild and red in color, because hemoglobin in RBCs. Blood in our body has many important functions: transporting oxygen from breathing and lead carbon dioxide from the body when exhaled. It is responsible for transporting food through the absorption of nutrients from the stomach and intestines into the bloodstream and then circulating to the liver. Then send it to the cell tissue of the organ, especially from the gut that can be

transmitted to the organs. In addition, blood is also responsible for maintaining a balance of water and minerals. Adjust the body temperature to a constant flow of blood throughout the body. White blood cells act to prevent infection and the immunity in the body.

RBCs (erythrocytes) are cells that do not have nuclei and the shape is different from the general cell. Size are about 6-8 microns. When looking through the microscope, it was found that the RBCs look like donuts without holes in central. The central portion of the RBCs is thinner over the edge of the cell as seen in figure 2.11. When viewed through a light microscope, the amount of light passing through it is not equal. The image is similar to the donut to see the familiar. The reason that RBCs are not round like other cells is they can be used to increase the surface area of the oxygen diffusion through the cell. Another reason is the spectrin protein can separated from each other by the ventricles within the cell and the RBCs can stretch and survive through the capillaries.

Blood grouping is the blood grouping of an individual person. Group or type according to the type of biochemical substance known as glycoprotein or glycolipid, which are produced in body system and appear on the skin of the RBCs and called antigen, which is specific in each blood group. Currently, more than 300 types of antigens have been discovered, and each has similar or related features. There are more than 35 systems, but important systems that people should know blood group ABO system [56-61].

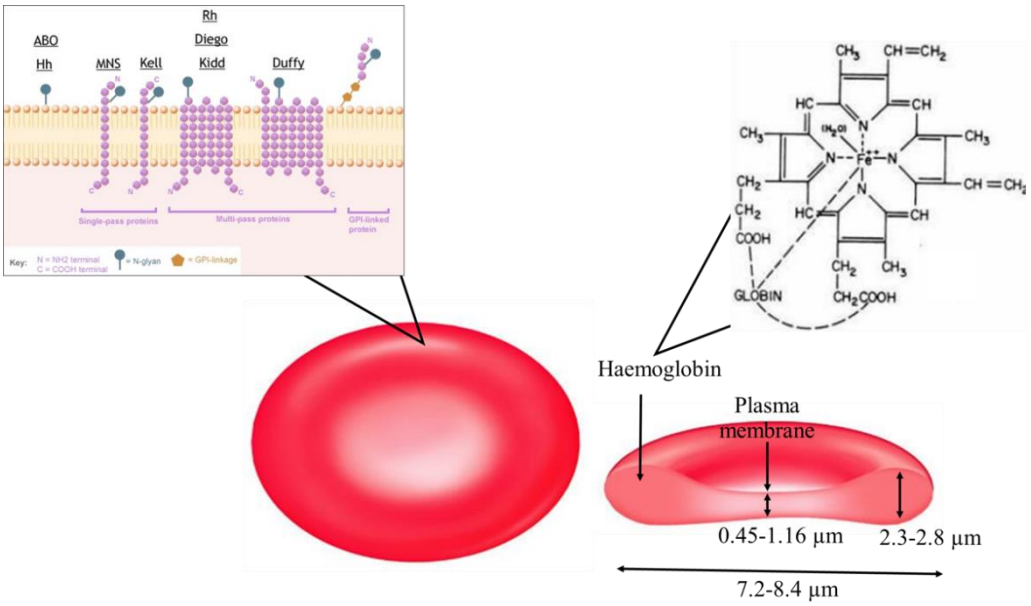


Figure 2.11 Structure and morphology of RBCs [62]

ABO blood group occurs naturally in all people, with different characteristics. It according to the type of gene, it was inherited from father and mother. Gene is a genetic material that is used to make each of the biochemistry that we call antigens and it is named according to the discoverer. Antigens in the ABO system found on RBCs, are also found on platelet-derived leukocytes on tissues of various tissues. The organs of the internal organs (such as the kidneys) and in the secretions of the body such as saliva, milk, etc. The discovery of blood group ABO began in 1900 by Karl Landsteiner, an Austrian biologist and physician. Blood samples of 6 co-workers were then taken to separate the blood and plasma from the blood plasma. Then each RBCs and lymph to each other alternate effect. Some couples react swollen or blood coagulation. Some blood couples are homogeneous without swarming. This phenomenon later in 1901, Carl Landstein. The results of the research indicated the blood is divided into 3 groups: A, B and O. The fourth group is AB, discovered by Alfred von Decastello and Adriano Sturli. They were identified as an associate of the Karl Land steiner in 1902. The A and B antigens share the same structure except for a terminal sugar bound by an α-1,3 glyosidic linkage to galactose. A and B antigens have precursor substrate is the H antigen. The large amounts of H are present on group O RBC's. Both A and B antigens structure are similar, but different in only A antigens has the large sugar is N-acetylgalacosamine (GalNac) while galactose (Gal) is the last

sugar in B antigens like a schematic in figure 2.12. [58-60] The ABO blood group consists of four main blood groups: Group A, B,O and AB. The blood type is determined by the type of antigen present on the surface of the RBCs and antibodies in plasma, which are naturally created. However, it is clear that people do not develop antibodies to their own antigens by mean antibodies are substances that the body produces when foreign substances or antigens enter the body. So, we can tell each type of blood group by detecting the antigen and blood antibody. Each blood group will have the following characteristics and as seen in Table 2.1.

Group A: antigen A on red skin and antibody B in plasma

Group B: antigen B on RBCs surface and antibody A in plasma

Group O: no antigen on RBCs surface but there are both antibodies A and B in plasma.

Group AB: Both antigen and red blood cells. But no antibodies A and B in plasma.

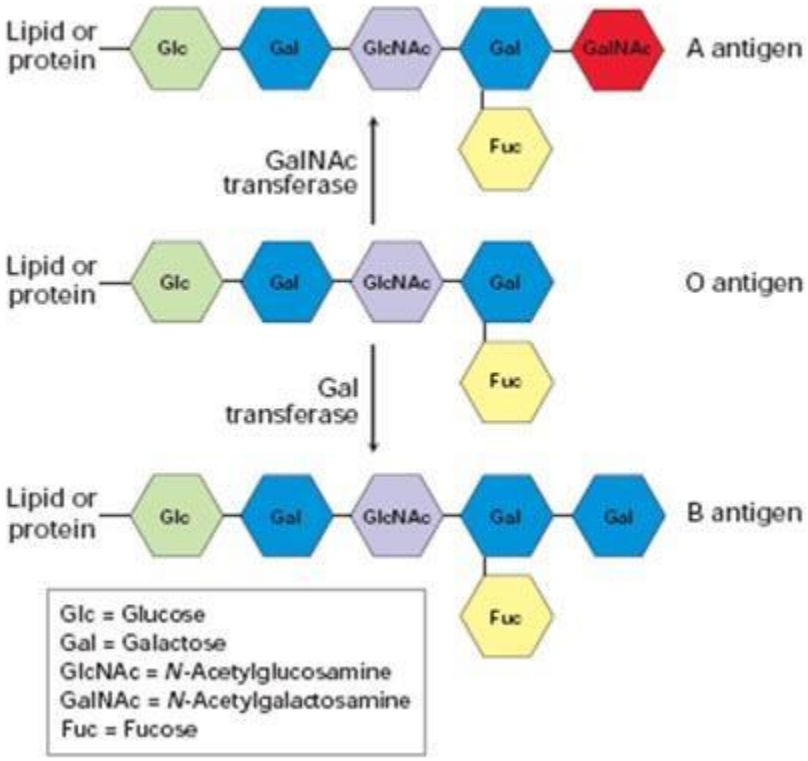


Figure 2.12 ABO antigen specificity. The ABO antigens differ by just one sugar at the antigen terminus. [63]

The benefits of blood transfusion are:

1. It is a specific feature found on the blood, making each person know their blood type.

2. Blood grouping is used to determine appropriate blood and is compatible with patients who require blood for treatment of abnormalities in the body. To avoid adverse reactions or side effects of getting blood.

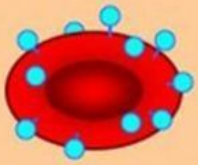
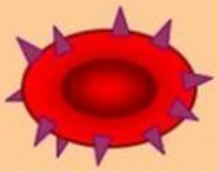
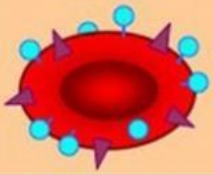
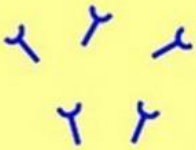
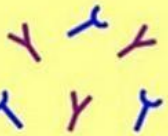
3. The ability to detect and identify the type of blood group ABO is useful in the diagnosis and prevention of maternal and neonatal blood disorders that can cause the red blood cells of the baby to be destroyed by antibodies from the mother.

4. The blood group is inherited by genetic control and is used as evidence. Proof of parenthood is possible and can be used to study the descent of these breeds.

People with blood clusters if they need blood. Doctors will consider blood transfusion among patients first except in case of emergency or cannot find blood match with the patient. The principle is to give blood when the patient into the body. RBCs do not have antigen / antibody-specific antibody / antigen. Because of the interaction between antigens and antibodies, red blood cells are destroyed. Amy's Disease Jane and have antibodies B. Blood transplants, which on red blood cells do not have antigen B, because they react with antibody B of the patient until the red blood cells are destroyed.

Table 2.1 ABO antibodies and antigens. [64]

ABO Blood Groups

	Antigen A	Antigen B	Antigens A + B	Neither A or B
Antigen (on RBC)				
Antibody (in plasma)	Anti-B Antibody 	Anti-A Antibody 	Neither Antibody	Both Antibodies 
Blood Type	**Type A** Cannot have B or AB blood Can have A or O blood	**Type B** Cannot have A or AB blood Can have B or O blood	**Type AB** Can have any type of blood Is the universal recipient	**Type O** Can only have O blood Is the universal donor

The conventional technique that uses to determine the presence or absence of specific agglutination between antibodies and antigens on RBCs's surface is slide agglutination. The mixture of antibody and blood is placed on a glass slide and waiting for a few minutes, the aggregation will occur. Slide agglutination is only practicable when the clumping of substances occurs within a few minutes. ABO blood group can be classified by this technique. Figure 2.13 show the slide agglutination, the agglutination appears when the type of RBCs corresponds to the antibody. Nowadays, there is high throughput automate instrument for testing agglutination, called gel agglutination test, that is high now in the routine use [65].

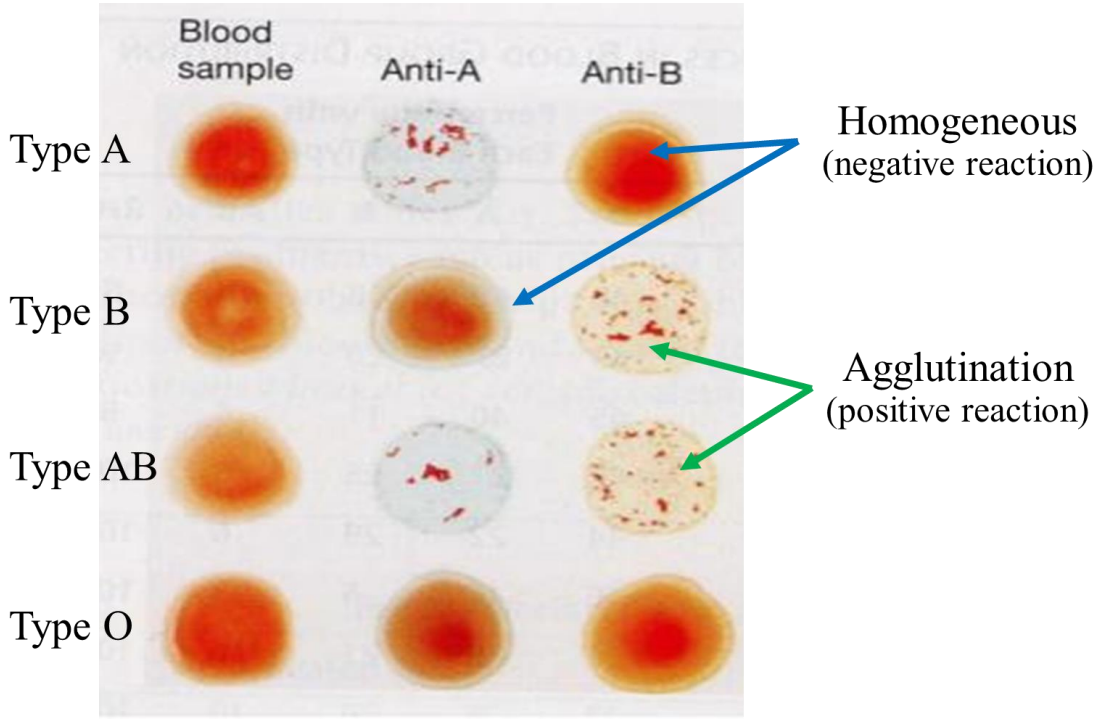


Figure 2.13 The conventional technique for blood typing called slide agglutination technique [65]

CHAPTER 3
Literature Reviews

3.1Conventional technique for ABO blood typing

The general techniques of blood typing are based on agglutination method. This method utilizes the interaction between antibody and its specific RBCs-antigen leading to the agglutination of red blood cell (RBCs). One technique widely used is automated micro-plate agglutination machine [5]. This technique is based on containing antibodies for blood group typing reagent in each well of 96 well plate, which specially designed to be suitable for the precipitation of blood sample. Add blood samples in each well and incubate for agglutination reaction. The positive reaction happed when antigen on RBCs surface linked with antibody reagent by forming network and sedimentation to the bottom of the well. The negative reaction is show nonagglutination character without any reaction between antigen/antibody. It seems like two solutions mixed to gather as seen in figure3.1.

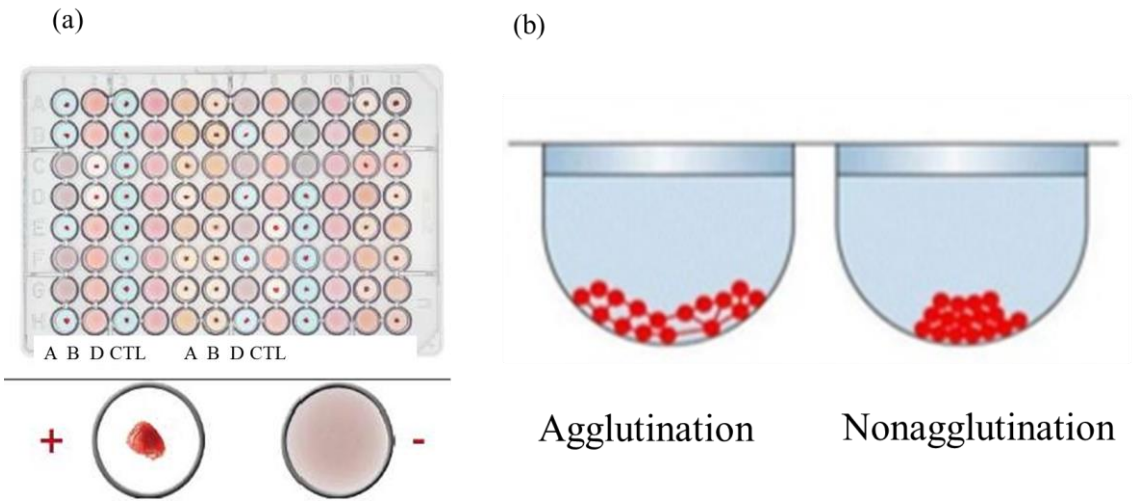


Figure 3.1 (a) microplate agglutination test for blood typing show positive and negative (b) agglutination and nonaaglutination behavior [66].

Another technique that is widely used is column agglutination test. This technique based on the filter effect of small bead microparticles like glass bead, polymer bead. Antibodies for blood typing were functionalized cover bead particles individual in each column. Add blood sample in all column that contained difference type of antibodies. If there are specific reaction, the RBCs agglutinates are trapped in the bead matrix during centrifugation like figure 3.2. RBCs samples unagglutinated will form a pellet at the bottom of the column. This technique can indicate the agglutination strength like figure 3.2c which it is one of the advantage of this technique.

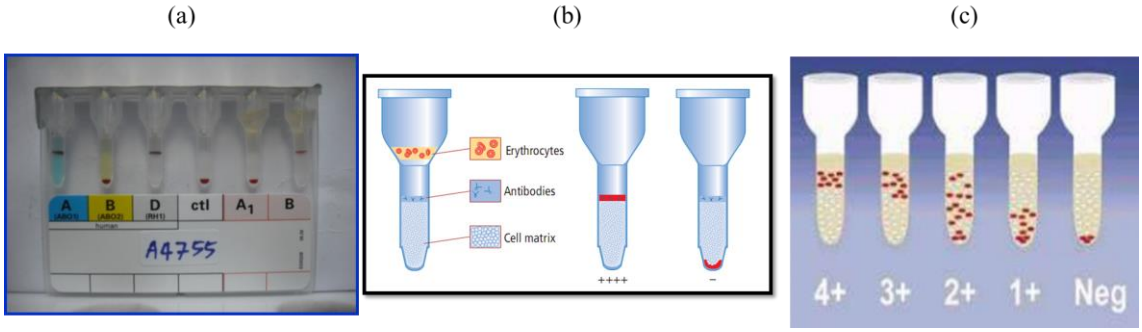


Figure 3.2 (a) Microcolumn agglutination card for detected blood group type A sample (b) the component of column agglutination and how to read positive and negative results (c) agglutination reaction level in 4+, 3+, 2+, 1+ and negative [67].

Both techniques are an automate method, which is expensive instrument and cannot reused. Although the agglutination techniques are easy and accepted in clinical, there are some limitations like qualitative results and low sensitivity [5]. So, there are development of new technologies to meet the needs of the user. It can be summarized as Table 3.1. Briefly, the first method is slide test method that describe in previous chapter. Next, very common technique that used for many years and still used is test tube method. Subsequently, there are transferring to microplate materials according to figure 3.1. Then test tube is centrifuged for short time and bring microparticles to increase performance, named microcolumn agglutination test. Moreover, molecular technique has been used for blood typing. This technique takes a different approach as it investigate the genetic information in the

DNA that encipher the antigen on RBCs surface. However, this technique has some limitation such as low reliably, difficult for blood sample from person who received transfusion, requires specific reliable antisera, expensive, some antibodies are limited in volume and weakly reaction, some antibodies are nor FDA-approved. Recently, there are using SPR, imaging technique, paper based diagnosis for improved blood typing technology, which talked in next topic.

Table 3.1 show recent and future trends in blood group typing [5]

Test	Detection of	Test principle	Reactants	Time per assay (min)	Cost per assay	Comments	Potential for automation
Slide	Blood groups	Agglutination	Patient RBC and antiserum	10-30	++	Insensitive, fast	No
	Isoagglutinnins		Patient serum and indicator RBC				
Tube	Blood groups	Agglutination	Patient RBC and antiserum	10-30	++	Sensitive, time-consuming, centrifugation needed	No
	Isoagglutinnins, antibody screening (indirect Coombs test)		Patient serum and indicator RBC				
Microplate	Blood groups	Agglutination	Patient RBC and antiserum	10-30	+++	Sensitive, fast, centrifugation usually not needed	Yes
	Isoagglutinnins, antibody screening (indirect Coombs test)		Patient serum and indicator RBC				
Micro column agglutination	Blood groups	Agglutination and separation from no agglutinated RBC by centrifugation	Patient RBC and antiserum	10-45	+++	Sensitive, time-consuming, centrifugation needed, easy documentation	Yes
	Isoagglutinnins, antibody screening (indirect Coombs test)		Patient serum and indicator RBC				
Molecular	Blood groups difficult to determine by serological methods	Nucleic acid amplification techniques	Patient leukocytes	Hour	++	Highly sensitive, time consuming, no need for rare antisera, requires special knowledge, does not exclude genes that are not expressed from detection	Yes
SPR	Blood groups	Solid phase immunoassay	Patient RBC	10-15	++	Highly sensitive, real-time monitoring, high throughput with array technology	Yes
		Solid phase DNA probe	leukocytes				
Imaging technique	Blood groups	Solid phase immunoassay	Patient RBC	5-7	+	Real-time monitoring, high throughput with array technology	Yes
Future trends	Blood groups difficult to determine by serological methods	Microarray, single-nucleotide polymorphism detection, quartz crystal microbalance, SPR	Still experimental	Hours?	?	?	Yes

3.2Biosensor for blood grouping

The improvement of the technology for blood typing is still interesting by a lot of research group. Biosensors or immunoassays were used in study the potential to be a high throughput technique for blood typing process. There are both advantages and disadvantages depended on each technique. The following are some examples of interesting techniques.

Indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed for the detection of ABO blood group antigen in human blood stains [68]. The experimental design was concluded in figure 3.3. Blood group antigen probes were coated in well of polystyrene ELISA plates. BSA was used blocked free binding are. Samples are blood stains was incubated with anti-A or anti-B monoclonal antibody (mAb). It was added to well plates and incubated for waiting the reaction. After that, added horse-radish peroxidase-conjugated goat anti-mouse IgM mAb (Anti-mouse IgM HRP). Next, o-Phenylenediamine solution was added in each well, and the colorimetric reaction stopped by the addition of sulfuric acid. Finally, spectrophotometer was used to the measurement. This work tested with blood, saliva and blood stains. The result showed the detection limit for blood, saliva and blood stain are 1:200, 1:32 and 1:16. This work was specific and sensitive for the detection of A and B type antigens, but failed to detect O type antigens. Furthermore, it was tough to test a large number of samples at a time using the conventional method. This technique has high throughput screening for the detection of ABO blood group antigen, especially for blood stains.

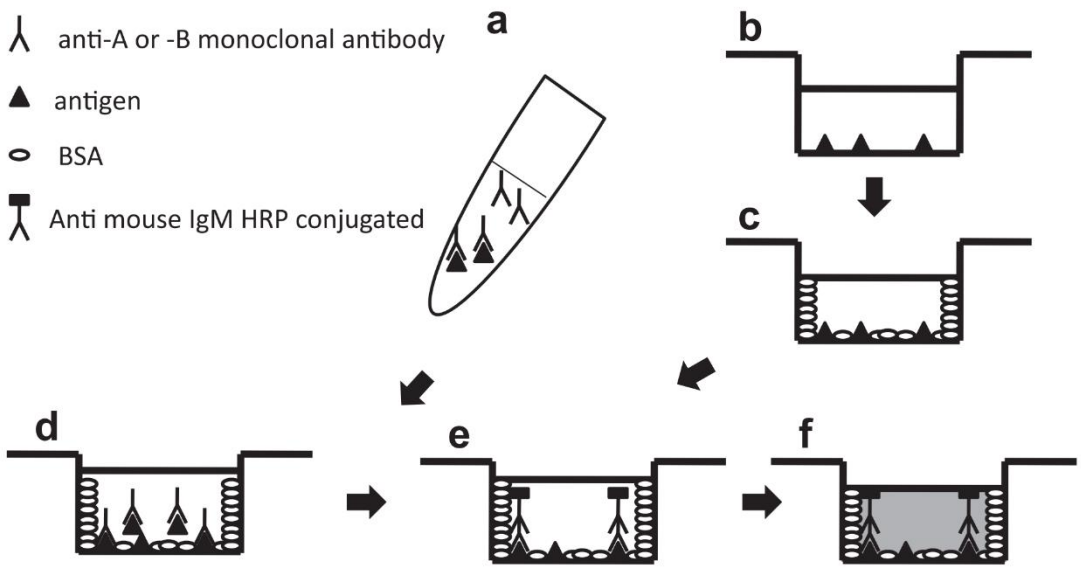


Figure 3.3 The process of an indirect competitive ELISA for ABO blood group antigen screening. (a) Mixed sample with anti-A or anti-B mAb (b) Coating blood antigen in each well. (c) BSA was blocked unbound site (d) Added (a) sample in each well (e) Added anti-mouse IgM-HRP (f) stopped colorimetric reaction with o-Phenylenediamine and sulfuric acid [68].

Low cost materials like bio-active paper were applied to biosensor for blood typing. Jarujamrus and team [69] were study mechanisms of agglutination in antibody-treated-paper with RBCs, to study the feasibility of developing for use. This study evaluated the number of antibodies molecules that are adsorbed on cellulose fibers. The optimal condition for functionalized on paper substrate was study in this work. The principle of this work is separation of agglutinated RBCs by porous matrices of antibodies-treated paper. The method for blood testing in this technique is show in figure 3.4. Testing paper, which antibody-treated doped with blood grouping antibody take place on inspection paper. Small amount of blood sample was drop on testing paper. Interpretation of the results achieved by clean testing paper with saline solution. RBCs agglutination with specific antigen-antibody reaction shown clean inspection paper. Because the agglutination of RBCs is large particles that cannot permeate through filter paper, inspection paper is not bloody. This work showed

potential of using paper- and tread-based microfluidic platforms to develop low cost and friendly user for blood typing biosensor.

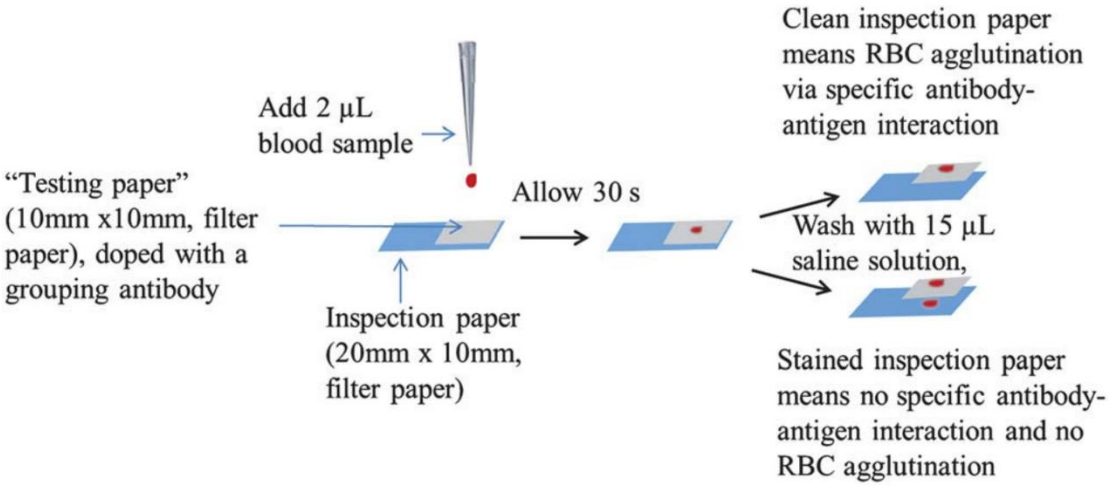


Figure 3.4 The protocol for blood testing based on paper [69].

Paper-based analytical device (PAD) was proposed to new technology for blood typing [70]. This device can detect ABO and Rh blood groups both in forward and reverse grouping. Paper-based was designed for sample loading zone and three channels for test zone as show in figure3.5. PAD was fabricated by combined wax printing and wax dipping method. Left side is forward detection side (F-side), which immobilized Anti-A, Anti-B and Anti-D. Right hand side is reverse checking (R-side). The detection was started with add blood sample in F-side and added whole blood sample in R-side. After that added A/B cells to the R-side. The results were observed from the diffusion distance in each immobilized antibody channel, that is non-agglutinated RBCs were transported longer than agglutinated RBCs. PAD has good reproducibility, which over 90% accuracy in blood group type A, AB and O which Rh positive. But sample group B shown 85% accuracy that indicated quite low reproducibility. The advantages of this work are potable, easy preserved and low cost. There is high potential for using in military medicine or in remote developing regions.

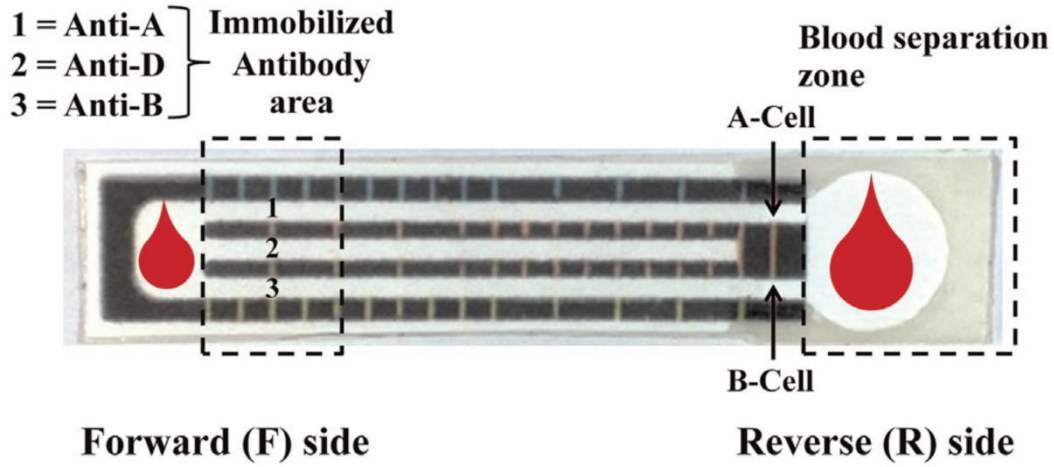


Figure 3.5 The PAD approach for ABO and Rh blood typing [70].

In addition to the three above mentioned examples, there are others example research were concisely discussed in table 3.2. They are indicated that the studies and developed are very interesting and be worthy of medical profession.

Table 3.2 Researches for development of biosensor for blood typing application

Test	Principle behind	Advantage	Disadvantage	Ref.
Monolayer coagglutination microplate	agglutination tests by add titrated sera after that RBCs was added to react. The positive result indicated by layering of RBC over the monolayer by a coagglutination phenomenon	Automation, suitable for large scale	Interpretation of results difficult	71
Faradaic impedimetric immunosensor	immobilized monoclonal IgM for blood typing (anti-A) on gold electrodes modified surface. Observed interaction by impedance change	Success in lysed RBCs	Not clear result due to the mass and size of RBCs.	72
Paper based assay	agglutination of RBCs with their specific antibodies were not move along the porous of paper	potable, easy preserved and low cost	Not clear result due to diffusion of blood samples and color from RBCs	73-75

Test	Principle behind	Advantage	Disadvantage	Ref.
PMMA-based microarray sensor chip combined with microfluidic and imaging analysis	PMMA sheet was modified by 3-aminopropyltriethoxysilane (APTES), and coupled antibodies to the surface via a-mime coupling. This work show advantage in array technique, easy to read result by microscope that seen RBCs captured on sensor chip	Inexpensive, simply setup, good reproducibility and high potential in microarray	Difficult for quantitative result	76

3.3Application of SPR for blood grouping

The specifically detect the A or B antigens on RBCs use of surface plasmon resonance (SPR) was first reported by Quinn et al. They showed high specificity of monoclonal anti-A IgM for A RBCs without non-specific binding to B or O RBCs, and monoclonal anti-B IgM also exhibited specific binding to B RBCs and no non-specific binding with A or O sample [77]. Figure 3.6 shows one result from this work that successfully indicated for develop SPR for blood typing detection. Graph showed four regions that started with baseline of the anti-A IgM-modified sensor chip surface, which was labelled as (1). The enumeration (2) is RBCs adsorbed. (3) is the dissociation phase. Baseline after regeneration using two short pulses of 20 mM NaOH as (4). This result indicated SPR biosensor not only classify blood type but also indicative qualitative. It can be seen from the increasing of SPR signal that related with increasing concentration of blood sample. This work is the beginning of development of SPR technique for blood typing detection which will continue to discussing.

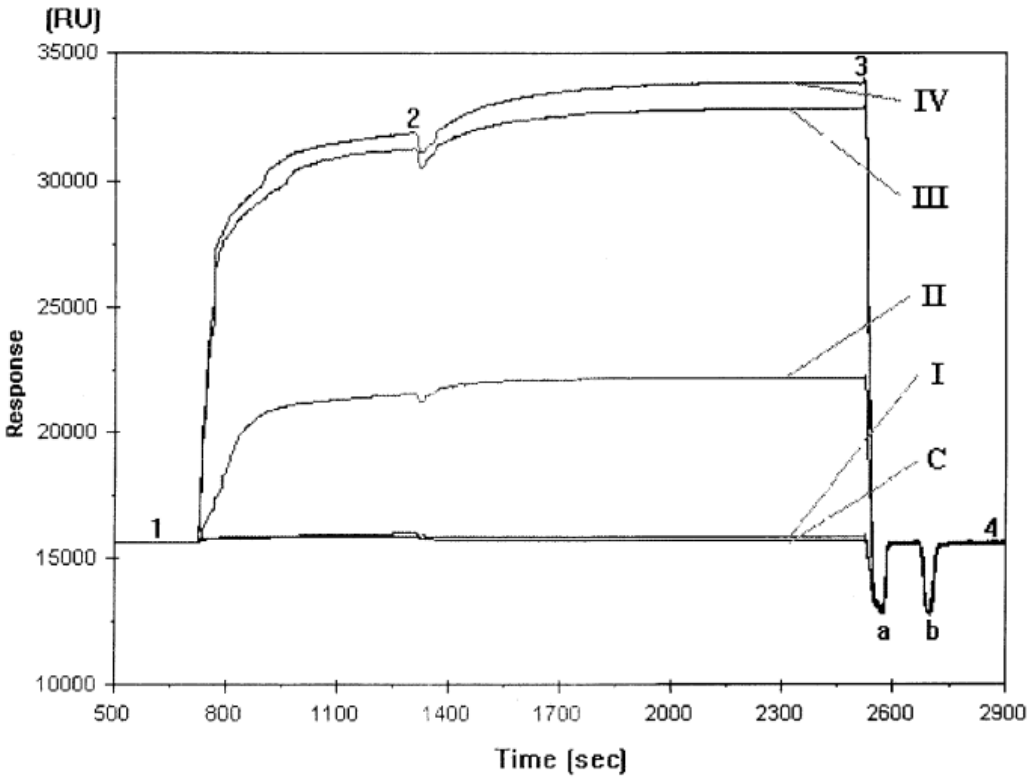


Figure 3.6 Binding of four increasing concentrations of blood group A+ to the anti-A IgM surface when 1.10×10^9, 1.50×10^9, 2.19×10^9 and 2.50×10^9 cells/ml giving response curves I, II, III and IV, respectively and RBCs group B was plot as C curve.

Houngkamhang, et al. [78] reported the multiple detection of ABO blood typing based on SPR technique. This work has the advantage in array technique, which can detect and analytes many samples in one time. This work claims the regeneration sensor surface was completely done, and the suitable for reuse at least 20 times. The accuracy showed 100% by testing in 15 samples for each group.

Sudprasert, Krisda, et al. [79-80] were present assays to quantify the agglutination strength by measurement of RBCs adhesion strengths in three assays that are cell attachment assay, cell movement assay, cell surface separation assay. They are difference in processes and models for explained adhesion strengths. This work shows high potential for using SPR technique study the behavior and characteristics of cell.

SPR was not only used for ABO blood typing but also developed for other blood group system. Duffy blood group in term of Fya and Fyb type was studied by Then et al. [81] This work starts with trial type and concentration of antibodies.

They immobilized anti-human IgG on SPR sensor chip. Detection process requires pre-sensitized RBCs before injection over sensor chip as seen in figure 3.7. Pre-sensitized RBCs is incubation of RBCs sample with selected anti-Fy^a or anti-Fy^b. The positive results are anti-human IgG binds to the Fc region of the blood group IgG antibody like step 2 in figure 3.7. Magnesium chloride ($MgCl_2$) was used regeneration solution. Binding responses of both anti-Fy^a and anti-Fy^b at higher antibody concentration confirmed that the binding interaction response observed was concentration dependent. Detection of the Fy^a and Fy^b antigens was successfully achieved. While not reproducible, with each sample tested in triplicate, the positively detected antigens where always achieved during the first injection. This work reported the SPR platform is not the limiting factor; test reproducibility/sensitivity is limited by the dissociation of the Duffy antigen-antibody complex. In case of stable antibody antigen complex has been achieved, the SPR platform will provide reproducible blood typing detection. the successful instances of binding detection for positive-Duffy antigen RBCs demonstrated that SPR analysis and detection can be achieved once a stronger and more stable antigen-antibody complex can be formed.

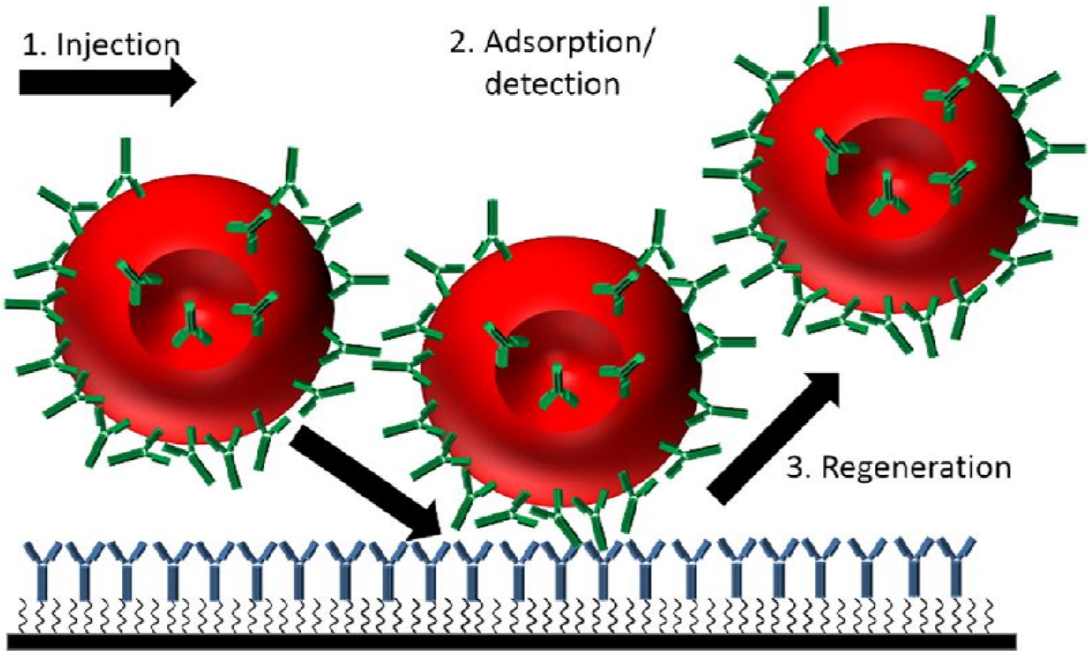


Figure 3.7 The process of RBC detection using SPR requires: 1) Pre-sensitized RBCs injected over the anti-human IgG immobilized surface; 2) if positive (shown), anti-human IgG binds to the Fc region of the blood group IgG antibody for detection; 3) surface regeneration using 3 M $MgCl_2$ desorbing bound RBCs [81].

In addition to C-SPR were used for blood typing application, LR-SPR waveguides also used to selected capture for blood type. This work presented other type of optical biosensor based on immobilized immunoglobulin G against blood group A (anti-A IgG) on gold waveguides [82]. Gold waveguide substrate was embedded in CYTOP by deposit on silicon wafer. Sensor chip was modified by growth 16-mercaptohexadecanoic (16-MHA) be for immobilization antibody. RBCs type A, B, O and AB was used to demonstrate anti-A functionalized waveguide. RBC-A in five different concentration were detected to determine limit of detection (LOD). The slight change signal and increasing proportional to increased concentration, ranging from 1.14 $*10^5$ cells/ml to 1.83$*10^6$ cells/ml (as seen in figure 3.8). This work claimed LR-SPR waveguide is simply, low-cost and compact transducers for selective detection of cells.

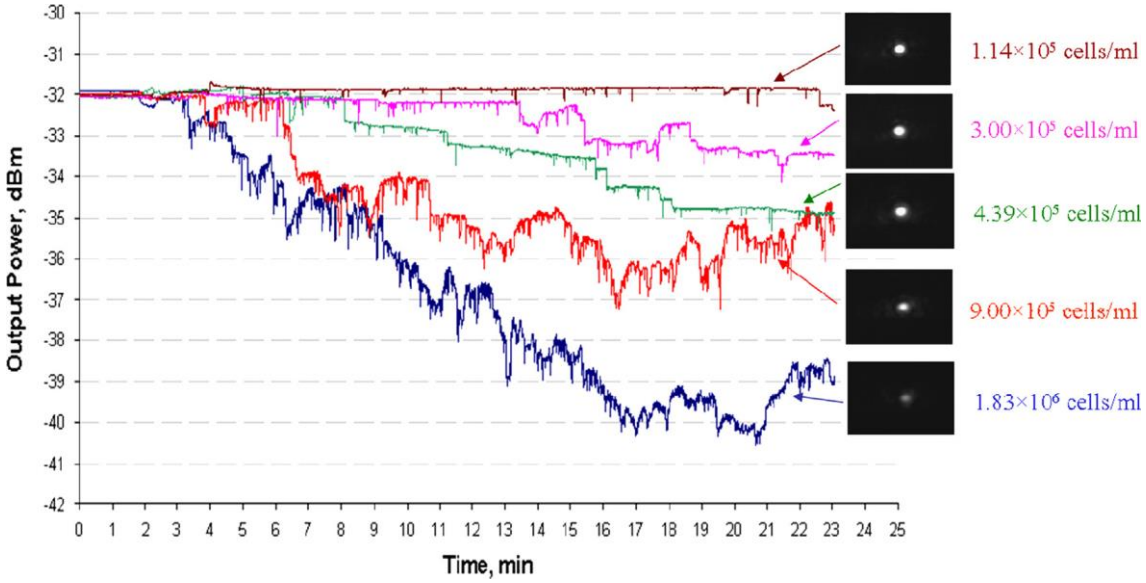


Figure 3.8 The experiments were performed in a sequence of decreasing concentrations [82].

According to various research publications that mentioned above, they are indicated that SPR is the best one candidate in optical biosensor for blood typing application.

CHAPTER 4

APPLICATION OF LONG-RANGE SURFACE PLASMON RESONANCE FOR ABO BLOOD TYPING

Surface plasmon resonance (SPR) technique has become a widely used technique for the detection of biomolecular interactions since the 1990s. Among other applications, SPR sensor is often used in clinical diagnosis for the detection of antigen-antibody binding, protein-ligand interaction, and DNA detection [7, 9, 28-29, 83]. SPR is a sensitive technique that can be applied for real time monitoring of the biomolecular interactions in solution [11, 84]. The SPR biosensor is based on changes of the optical reflectivity by the adsorption of biomolecules on a gold (Au) surface, which cause a change in refractive index near the SPR-active gold surface. Long-range surface plasmon resonance (LR-SPR) involves surface plasmons (SPs) that propagate along a thin metallic film embedded between two dielectric materials with similar refractive indices, for which both the evanescent field intensity and the penetration depth (more than 1 m) are greater than those for conventional short-range SPs (penetration depth is ~200 nm) [30-35]. Hence, it is expected that the sensitivity at longer distances from the metal surface will increase with LR-SPR spectroscopy. Recently, Homola et al. reported that the LR-SPR biosensor could detect large analytes, such as the bacteria Escherichia coli (0.7–1.0 m) due to the large penetration depth of the evanescent wave [36-37]. In addition, the LR-SPs enhanced the optical field wave at the metal-dielectric interface, leading to a higher sensor sensitivity and increased penetration into the analyte solution than that observed for conventional SPR. As a result, a thicker sensor coating with a significantly larger number of binding analyte molecules on the surface could be used [38-39]. We have also previously reported an LR-SPR immunosensor based on the electrospun poly (acrylic acid) (PAA) fibers for the detection of human IgG [35].

The detection of antigens A and B on the surface of RBCs by conventional SPR was reported by Quinn et al. [77, 85]. However, the detection of RBCs via conventional SPR is limited due to the penetration depth of evanescence field

(~200 nm), which is much less than the size of RBCs (i.e., ~2 μm thick and ~7.5 μm in diameter). Recently, various studies have been reported in the applications of SPR technique for the study of RBCs. Houngkamhang et al. [78] reported ABO blood typing via SPR imaging which observed interaction between immobilized Anti-A and Anti-B antibodies array and A and B antigens on RBCs surface. The interaction of antigens around lower RBCs membrane surface and immobilized Anti-A and Anti-B antibodies array was applied to obtain the agglutination strength of the RBCs and immobilized antibody [79]. The SPR imaging, which utilized the shear force generated within the flow cell, was used to measure the rolling speed of red blood cell [79]. The technique can only extract the information at the interface because the limit of the evanescence field makes it impossible to study in whole cell. A number of important pieces of information regarding the property of the cell were undetectable such as cell elasticity and cell deformation. Krupin et al. used long-range surface plasmon waveguides for capturing only blood group antigen A on RBCs by immobilized Anti-A IgG via no comparative studies with Anti-B [82]. To our knowledge, there was no report regarding the detection of RBC by LR-SPR. Because the difference in surface chemistry, experimental set-up, and nature of SPR signal between LR-SPR and SPR, in this work, it is desirable to explore the possibility of applying LR-SPR to detect ABO blood group and understand the nature of the LR-SPR signal. Anti-A and Anti-B will be covalently immobilized on self-assembled monolayer (SAM) surface and A, B, AB, and O will be detected by using LR-SPR. The Cytop (n = 1.34) fluoropolymer is used in the LR-SPR system in order to match the refractive index with the phosphate buffered saline (PBS) buffer. All the results of blood typing were consistent with the results obtained from the agglutination test. The lowest detection amounts of RBC-A and RBC-B were 1.58×10^5 and 3.83×10^5 cells/ml indicating that LR-SPR has much higher sensitivity than that obtained with conventional SPR biosensors [77, 85] and LR-SPR waveguides [82]. We demonstrate that LR-SPR is a good candidate for classification of blood typing and has a high potential in other clinical applications such as bacteria cells, cancer, and rare RBCs.

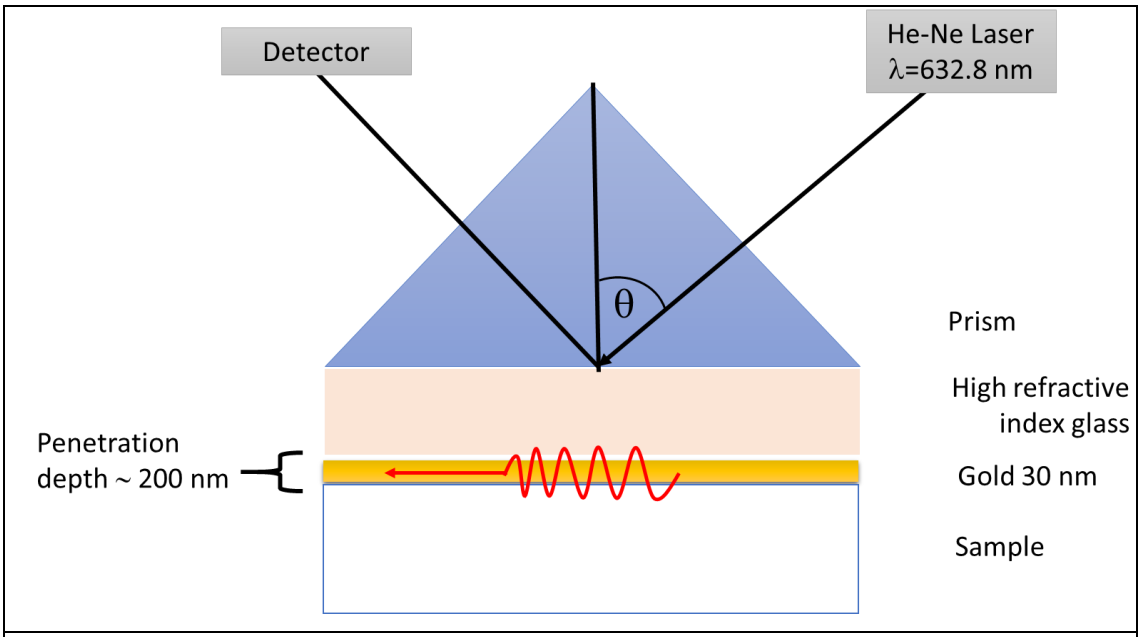
4.1 Materials and methods

4.1.1 Chemicals and materials

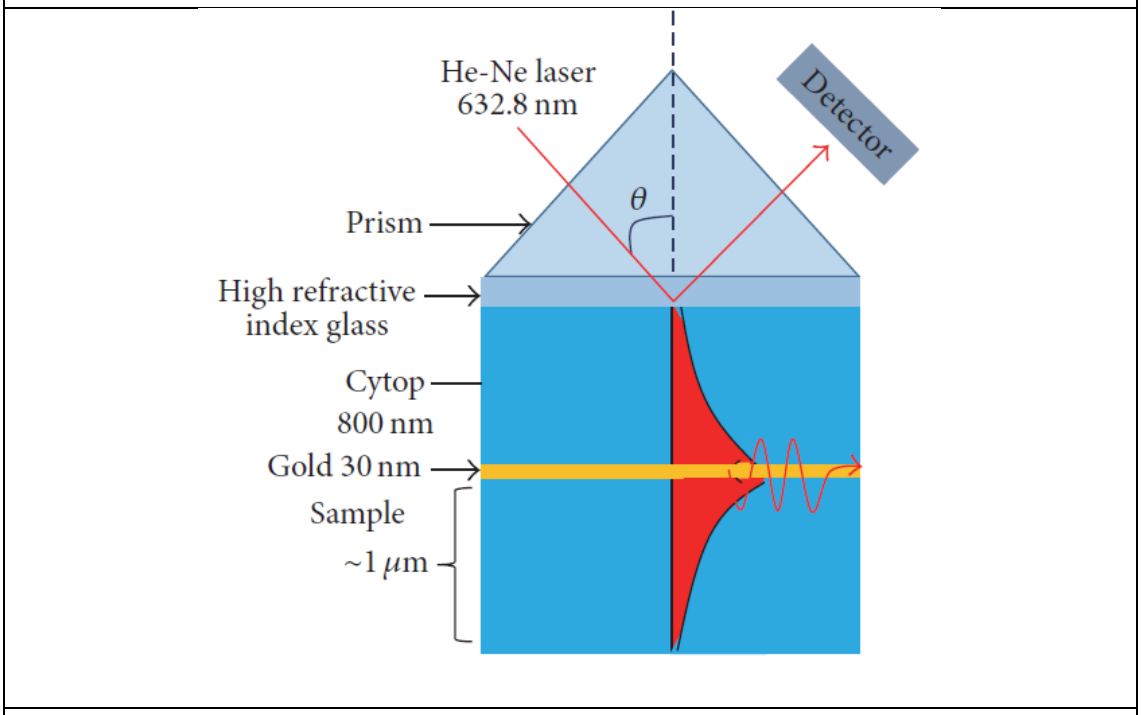
The 11-mercaptoundecanoic acid (11-MUA), phosphate buffered saline (PBS) tablets, and sodium acetate buffer (pH 5) were all purchased from Sigma-Aldrich. The 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and ethanolamine hydrochloride (EA-HCl) were purchased from Tokyo Chemical Industry (TCI). The CTL-809M and CTL-180 solvents for the Cytop solution were purchased from Asahi glass. Mixed clones of monoclonal Anti-A and Anti-B, standard A cells, B cells, and O cells were obtained from the research unit of the Thai Red Cross Society.

4.1.2 Surface Plasmon resonance (SPR) and Long-range surface plasmon resonance (LR-SPR) setup

SPR setup were develop in home-made attenuated total reflection (ATR). The Kretschmann configuration is used for exciting surface plasmons using He–Ne laser with wavelength (λ) of 632.8 nm as seen in figure 4.1 [78,79]. The kinetic experiments were operating at fixed incident angles. The angular measurement was set by scanning the incident angles to obtain reflectivity-incident angle curve. The incident angles were determined at high contrast region that occurred before minimum point of the curve or SPR angle as seen in figure 4.2. This region provided high sensitivity of SPR signal change. The changing of reflective index on SPR sensor chip was change the reflectivity curve, and it obtain reflectivity-time curves, which called SPR signal. LR-SPR was arranged to propagate along a thin metallic film embedded between two dielectrics with similar refractive index (Figure 4.1b). There is the different between angular reflectivity curves of conventional SPR and LR-SPR as seen in figure 4.3. The LR-SPR chip is sharper than that of the conventional SPR chip



(a)



(b)

Figure 4.1. Schematic of (a) surface plasmon resonance (SPR) (b) long-range surface plasmon resonance (LR-SPR) setup.

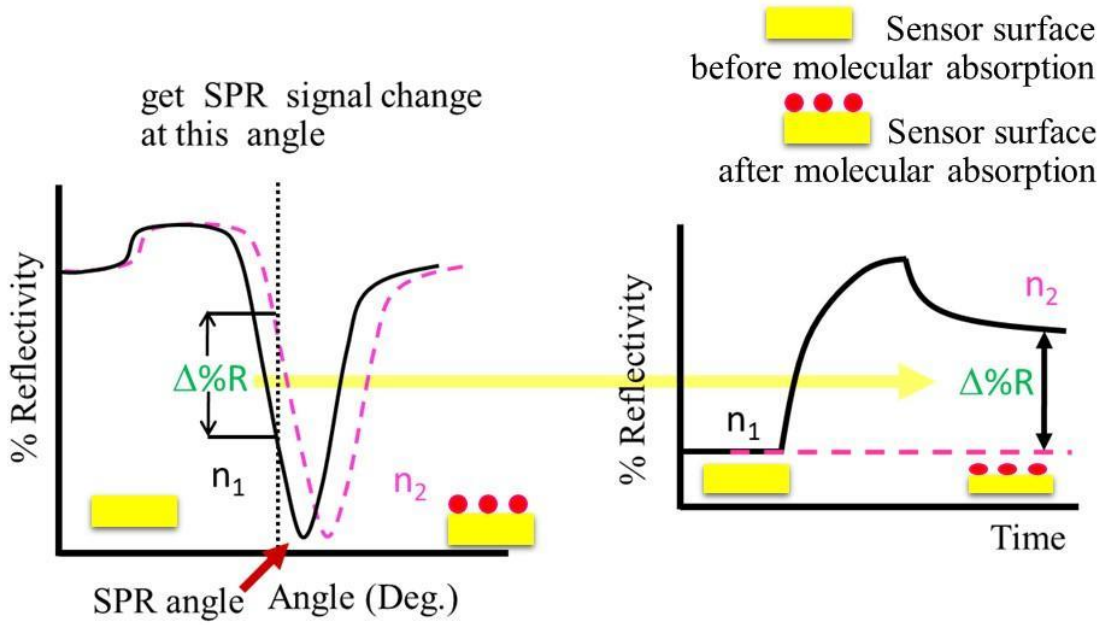


Figure 4.2. Schematic of reflectivity-incident angle curve, which corelated with reflectivity-time curves when n_1 is before molecular absorption and n_2 is after molecular absorption.

4.1.3 Preparation of sensor chip

4.1.3.1 LR-SPR sensor chip fabrication

The fabrication of the LR-SPR sensor chip, a 7% Cytop solution (dissolved 9% Cytop (CTL-809M) in CTL-180 solvent) was spin-coated on a high refractive index glass at a first spin rate of 500 rpm for 10 s and second spin rate of 1,300 rpm for 20 s. The Cytop solvent was dried at 180 °C for 1 h in an oven, and the Cytop film (ca. 800 nm) with a refractive index of 1.34 (similar to a refractive index of water, 1.33) was obtained on a high-refractive index glass. Then, 30 nm of gold (Au) film was deposited on the Cytop film by vacuum evaporation. A chromium layer (1 nm) was used to promote Au adhesion with the Cytop film. LR-SPR chips were gave the similar in reflectivity curve in different lot, which show the quality in preparation of Au film is high quantity in reproducibility. The angular reflectivity curves of the conventional SPR and LR-quantity in reproducibility. The angular reflectivity curves of the conventional SPR and LR-SPR chips (bare Au) indicate that the curve of the LR-SPR chip is sharper than that of the conventional SPR chip (Fig. 4.3).

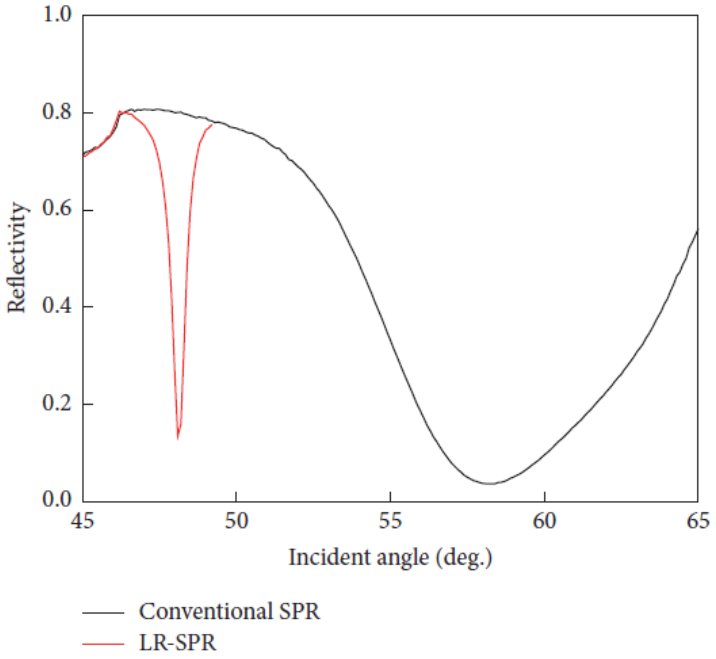


Figure 4.3 The angular reflectivity curves of conventional SPR and LR-SPR.

4.1.3.2 Preparation of self-assemble monolayer

The LR-SPR chip Au surface was covered by a self-assembled monolayer (SAM) of *11*-mercaptoundecanoic (11-MUA). First, clean LR-SPR chip substrate with deionized water and ethanol. Then LR-SPR chip was immersed in 10 mM of 11-MUA, which was dissolve in absolute ethanol for 12 h under dark and dry environment. After that rinse LR-SPR chip with ethanol 3 times and dry it with stream of dry nitrogen gas. The configuration of SAM consists with terminal sulfur group that linked with gold substrate. The length of spacer is depended on amount of carbon. In this work the head group is carboxyl group (-COOH) as seen in figure 4.4 and 4.5.

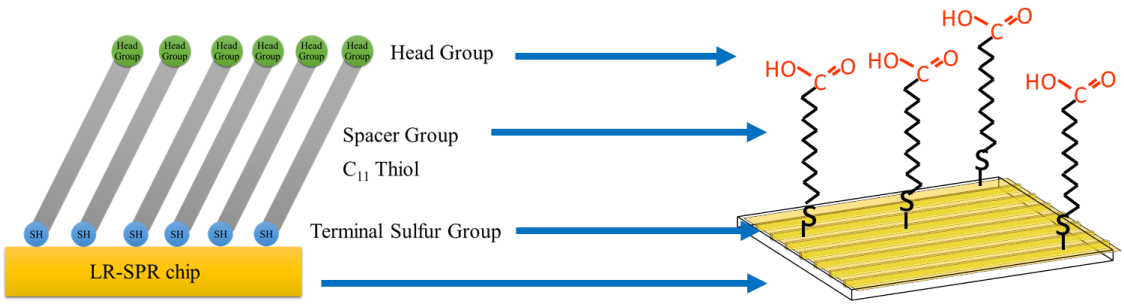


Figure 4.4 configuration of 11-MUA SAM on LR-SPR chip.

4.1.3.3 Immobilization of antibodies and detection of RBCs

The LR-SPR chip Au surface was covered by a self-assembled monolayer (SAM) using 10 mM of *11*-mercaptoundecanoic (11-MUA). Figure 4.5 shown the carboxylic groups of the SAM surface were activated to their ester forms by immersion in 0.4 M EDC and 0.1 M NHS dissolved in deionized water at a ratio of 1:1. The monoclonal antibody for blood group A (Anti-A) and monoclonal antibody for blood group B (Anti-B) were covalently immobilized on the activated sensor surface (after rinsing the sensor chip with PBS buffer*)* by injecting the antibodies in sodium acetate at pH 5 at a 1:10 dilution onto the activated SAM surface [82]. The residual activated surface sites, which did not react with antibodies, were inactivated or blocked with 0.2 M ethanolamine.

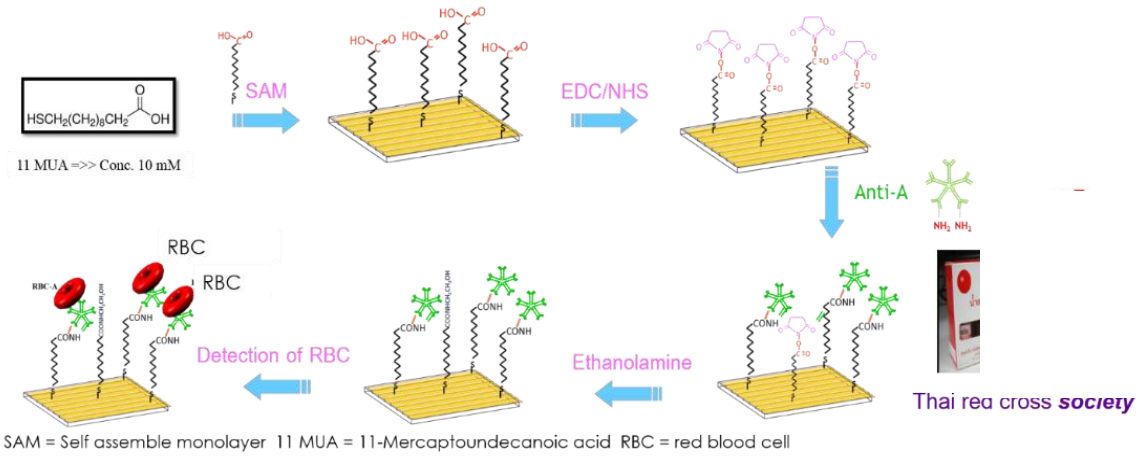


Figure 4.5 Schematic representation of immobilization of antibodies (Anti-A/Anti-B) and detection of red blood cells (RBCs).

4.1.4 Detection of red blood cell (RBCs)

The RBC samples from standard A, B, and O cells were detected on the immobilized antibody by observing the change in the refractive index on the LR-SPR biosensor. Standard RBC-A and RBC-B samples were diluted in the range of 3×10^4 to 3.8×10^7 cells/ml. The number of cell number was counted using the hematocrit test. Regeneration of the LR-SPR chip surface was performed using 20 mM NaOH, followed by rinsing with PBS running buffer. A summary of the antibody immobilization on the biosensor chip and the detection of RBCs is shown in Figure 4.5.

4.1.5 RBCs counting by hemocytometer

In this work we compared %R from SPR detection with the number of RBCs in unit of cell/volume by using the conventional method named hemocytometer. Hemocytometer is a device that counts cells. Normally, it is designed for the counting of blood cells. If the number of cells counts more or less than normal level, it can be indicated some symptoms or various diseases, so blood cell counts are a very common method used in medicine because the information is wide about the health of the patient. Scientists invented the hemocytometer is a Louis-Charles Malassez [86]. Hemocytometer composed of slides and slide sheet made from glass or plastic material, rectangular shape seen in figure 4.6. It looks like a glass slide, but it is thicker. There will be space for sample material along with the area is counting a very small count cell. Being perpendicular grid cell count or particles inside the grid and the exact volume of the sample liquid. It will be possible to calculate the concentration of cells in the liquid. Cell counts will be used to expand the microscope. Then count them directly only the five-red square that shown in figure 4.6. Units used to count cells is Cells per volume

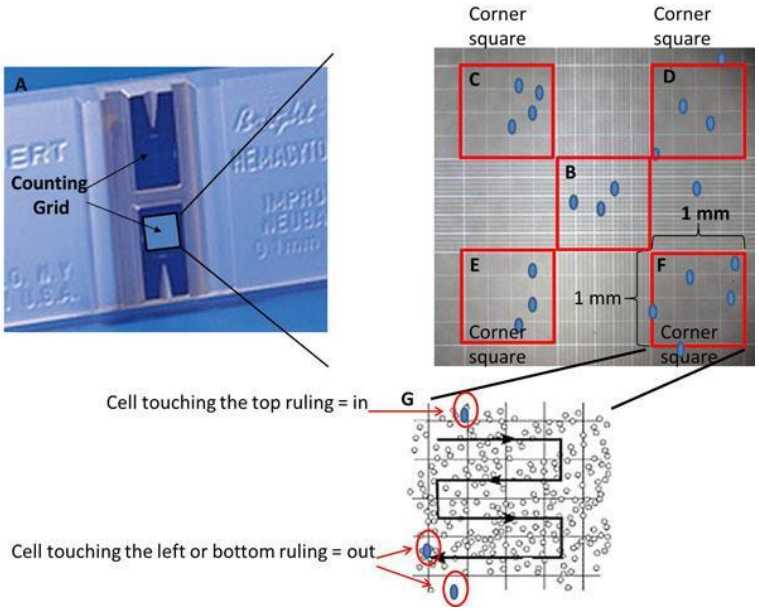


Figure 4.6 the grid of the hemocytometer, red area is where RBCs were counted [86]

4.2 Results and discussion

4.2.1 Immobilization of Antibody and Detection of RBC-A

We compared the sensing ability between conventional SPR and LR-SPR for the detection and classification of RBC typing on grafted SAMs (11-MUA). First, the conventional SPR sensor chip was used for the evaluation of the immobilization of Anti-A/Anti-B on the sensor chip and the detection of RBC-A. Angular reflectivity curves of the conventional SPR were observed before immobilization or after grafting the SAM, after immobilization of Anti-A, and after the detection of RBC-A (Figure 4.7(a)). The corresponding kinetic reflectivity curve is also shown in Figure 3(b). The angular SPR reflectivity curve after immobilization of Anti-A was shifted to higher angle, indicating that Anti-A was immobilized on the sensor chip. The increase in the reflectivity during the immobilization is also shown in the SPR kinetic curve (Figure 3(b)). However, the reflectivity during the detection of RBC-A is almost constant, as shown in the kinetic curve (Figure 4.7(b)). Moreover, the corresponding angular reflectivity curve was almost constant after the detection of RBC-A. This indicates that the conventional SPR sensor is limited to the detection of RBC, presumably because of the fact that the evanescence field of conventional SPR

is much shorter than the thickness of RBCs, and hence the reflectivity change is not sensitive to the adsorption of the surface.

Then, the LR-SPR sensor chip was used for the detection and classification of RBC typing on 11-MUA SAM. The LR-SPR kinetic reflectivity curve for the immobilization of Anti-A and the detection of RBC-A is shown in Figure 4.8(b). The Anti-A was immobilized on activated group on the SAM surface. The remaining available active groups were blocked by ethanolamine. The baseline in the figure is obtained after the activation process by rinsing with PBS buffer. The reflectivity was obviously increased by the immobilization of Anti-A and after the detection of RBC-A (by ~0.008) (Figure 4.8 (b)). In this result, the baseline of the initial reflectivity is relatively high because the experiment was continuously carried out after the activation of the surface. Hence, the reflectivity was saturated by the injection of Anti-A. However, after the detection of RBC-A, we clearly observed the increased reflectivity change compared to the conventional SPR. In order to clearly compare with the conventional SPR reflectivity curve, the -scale was shown by finite difference of reflectivity. Furthermore, the angular SPR reflectivity curve clearly shows that the dip angle shifts to a higher angle after the detection of RBC-A (Figure 4.8 (a)). These results indicate that the LR-SPR is more sensitive for the detection of RBC-A in comparison with the conventional short-range SPR measurements.

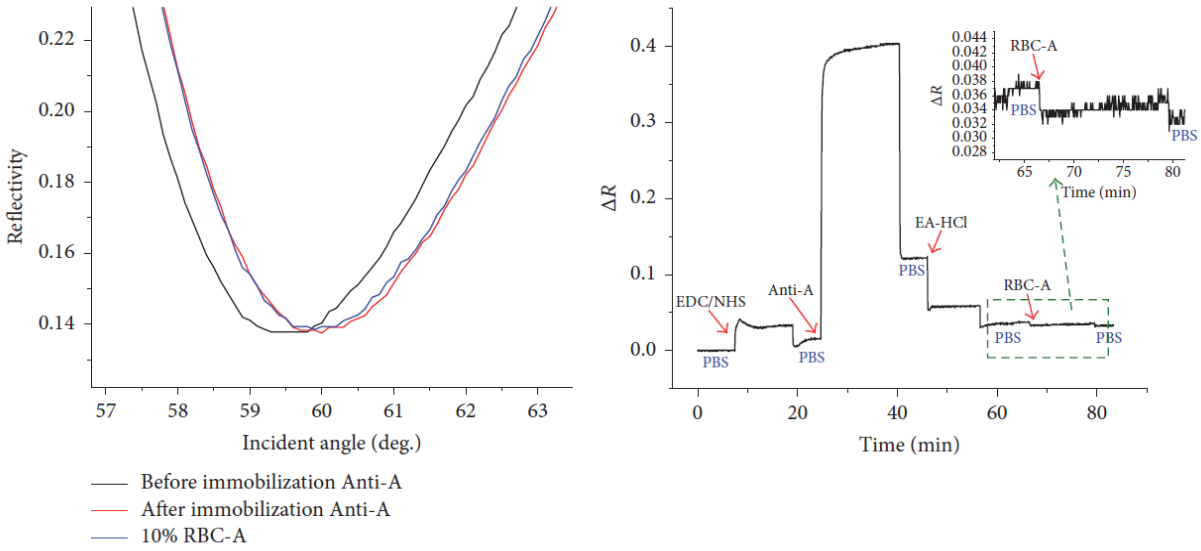


Figure 4.7 (a) Angular reflectivity curves of conventional SPR before and after immobilization and after the detection of RBC-A. (b) Corresponding SPR kinetic reflectivity curve.

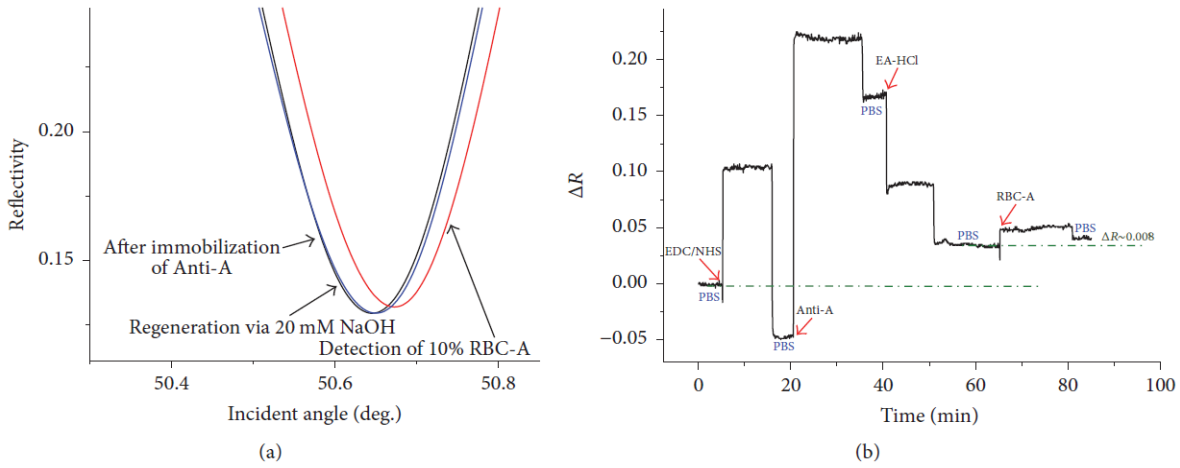


Figure 4.8 (a) Angular reflectivity curves of LR-SPR after immobilization of Anti-A, after detection of RBC-A, and after regeneration with 20 mM NaOH. (b) Corresponding LR-SPR kinetic reflectivity curve.

4.2.2 Immobilization of Antibody and Detection of RBC-B

Next, we immobilized Anti-B on activated 11-MUA SAM surface and detected RBC-B by LR-SPR (Figure 4.9). In this experiment, the sensing procedure was the same as for RBC-A detection described above. Similar to the results for RBC-A, LR-SPR showed an obvious increase at each adsorption step, indicating that it can easily detect RBC-B.

4.2.3 Surface Regeneration

Because one important advantage of the biosensor is reusability, we also studied the regeneration ability of RBC-A on Anti-A. For the regeneration of the RBC detection system, we found that 20 mM of NaOH was a suitable condition to disrupt the antigen on the RBCs, causing the disruption of the RBC-antibody specific adsorption interaction without destroying immobilized antibody on the activated surface. The LR-SPR curve is shifted back to the lower incident angle and is almost the same as the LR-SPR curve of immobilized Anti-A surface, indicating that the adsorbed RBC-A is completely removed from the Anti-A surface without removing the immobilized Anti-A. Figure 4.10 shows the kinetic property of the LR-SPR during the regeneration of RBC-A/Anti-A surface over three times. During the experiment, PBS buffer was used to obtain a baseline reading, which is shown as "Δ" in the figure. After the binding of RBC-A on the Anti-A for about 15 min, the surface with residual unbound RBC-A was rinsed with PBS buffer, which is shown as "⧫" Then, 20 mM NaOH was injected on the RBC-A/Anti-A surface, followed by the injection of PBS buffer (baseline, shown as "Δ"). As shown in the figure, each time the baseline returned to the original baseline values, indicating that the Anti-A surface still remained (i.e., was regenerated), even after three sensing and regeneration processes. From previous reports, regeneration Anti-A and Anti-B sensor surface can reusable around 100 cycles. [87] The reason that antibody surface lost the ability to reuse maybe come from denature or destabilize of antibody or antibody loss from sensor chip during using regeneration solution. [88]

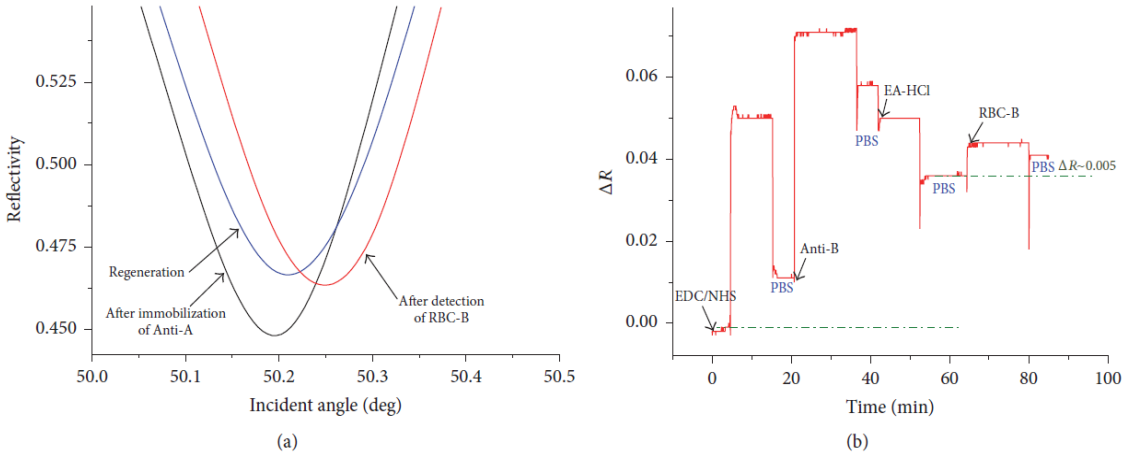


Figure 4.9 Angular reflectivity curves for LR-SPR after immobilization of Anti-B, after detection of RBC-B, and after regeneration with 20 mM NaOH. (b) Corresponding LR-SPR kinetic reflectivity curve.

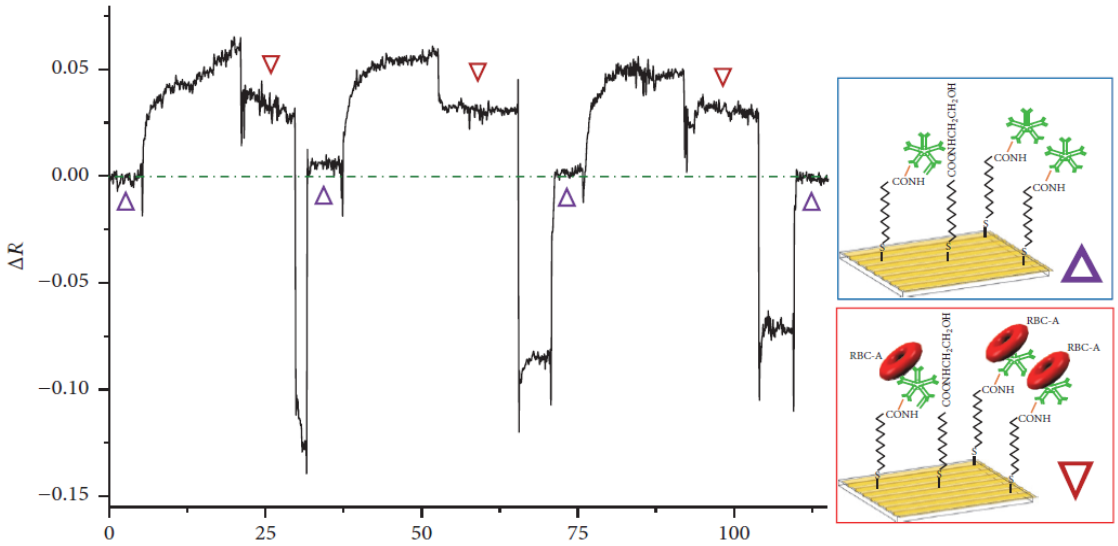


Figure 4.10 LR-SPR kinetic curve showing detection of red blood cells (RBCs) and regeneration of the surface using 20 mM NaOH. PBS buffer was used to obtain a baseline reading and then RBC-A is bound on the Anti-A for ~5 min, before the surface is rinsed with PBS buffer. Then, 20 mM NaOH is injected on the RBC-A/Anti-A surface, followed by the injection of PBS buffer.

4.2.4 Specificity between Antibodies and RBCs

To study the specificity between antibodies and RBCs, Anti-A or Anti-B was immobilized on activated 11-MUA SAM surface, and RBC-A, RBC-B, or RBC-O was detected to determine both specific and nonspecific interactions. The LR-SPR kinetic curves during the detection of RBC-A, RBC-B, and RBC-O on Anti-A (Figure 4.11(a)) and Anti-B (Figure 4.11(b)) show that the reflectivity was increased in all cases. This indicated that the RBCs were adsorbed on the antibodies both by specific and nonspecific interactions. After rinsing with PBS buffer, the LR-SPR reflectivity for RBC-B and RBC-O decreased to around baseline. This is because the nonspecifically or physically adsorbed RBCs were removed from the Anti-A surface. On the other hand, the LR-SPR reflectivity for RBC-A kept higher reflectivity than that of the initial baseline, indicating that the specifically adsorbed RBC-A on Anti-A remained on the surface. The slight decrease by the PBS buffer indicates some physically adsorbed RBC-A was removed from the surface. In the case of the RBCs detection on Anti-B surface, the LR-SPR reflectivity for RBC-A and RBC-O decreased to almost the initial baseline after the adsorption on the surface, while the reflectivity for RBC-B remained higher. These results indicate that the specific and nonspecific adsorption of RBCs can be clearly detected by the LR-SPR sensor chip, showing the ability to classify blood types.

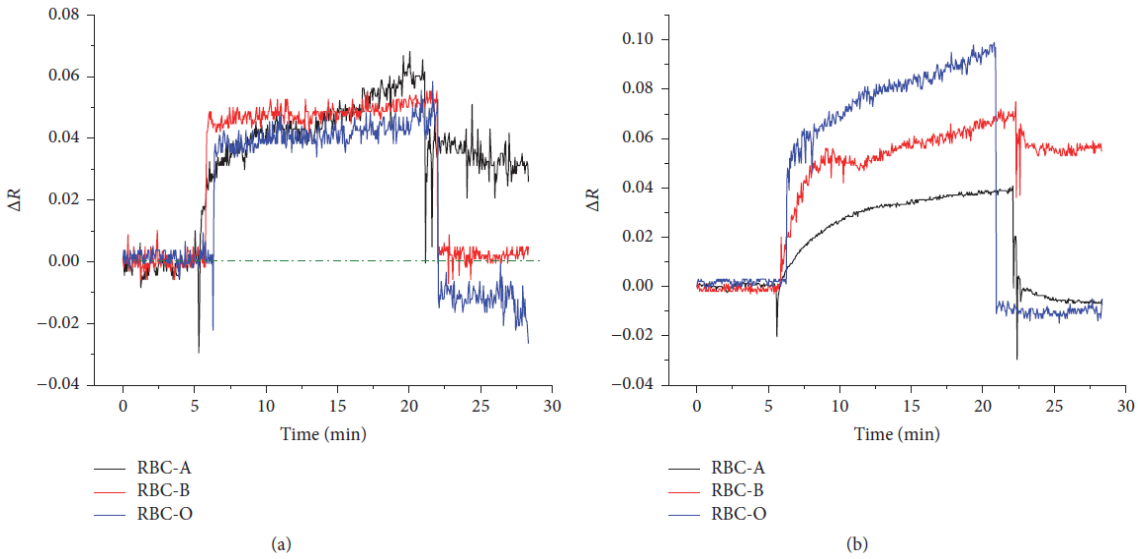


Figure 4.11 LR-SPR kinetic curves during the detection of RBC-A, RBC-B, and RBC-O on Anti-A (a) and on Anti-B (b).

Moreover, RBC-A, RBC-B and RBC-O was test with immobilized Anti-A antibody surface, which observe by optical microscope as seen in figure 4.12. We can observe only RBC-A stick on Anti-A antibody surface but RBC-B and RBC-O not binding on Anti-A surface. This result is correlate and confirmed SPR result in figure 4.11.

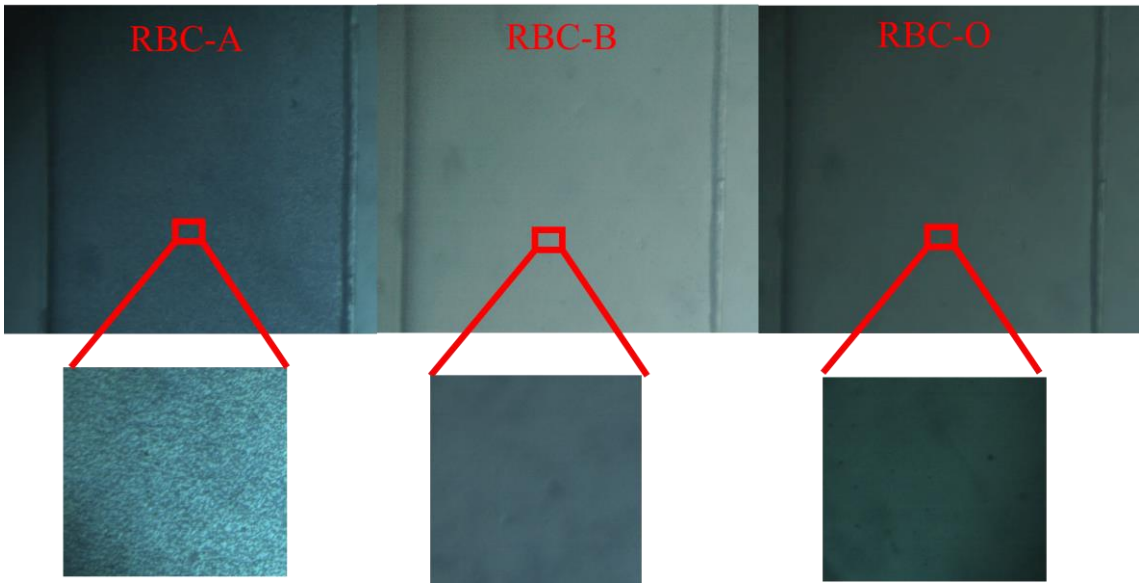


Figure 4.12 The interaction of RBC and immobilized anti-A antibody observe under optical microscope.

4.2.5 Determination of the Detection Limit

Standard RBC-A and RBC-B samples were serially diluted from their original concentration and injected to study the interaction with Anti-A and Anti-B immobilized on 11-MUA SAM. RBC-A and RBC-B were detected on the immobilized Anti-A and Anti-B, respectively, by varying each concentration. RBCs at each concentration were detected for 10 min, followed by rinsing with PBS. The SPR reflectivity change (ΔR) was obtained by the reflectivity difference between the baseline before the injection of RBCs and after rinsing with the PBS buffer. Antibody surfaces were regenerated with 20 mM NaOH for the following experiment. As shown in Figure 4.13, the reflectivity change of LR-SPR increases when the concentration of RBC-A and RBC-B increases. The limit of detection (LOD) of RBC-A and RBC-B

were at 1.58×10^5 cells/ml and 3.83×10^5 cells/ml, respectively. The LOD was defined as three times standard deviation of the blank (PBS buffer) [89]. We found that the obtained LOD using the LR-SPR is lower than that with conventional short-range SPR (i.e., 3.3×10^8 cells/ml) [77, 85] and also lower than that with long-range surface plasmon waveguides that exhibited the LOD less than 3×10^5 cell/ml [90]. This clearly indicates that LR-SPR is a promising technique for the detection of RBCs.

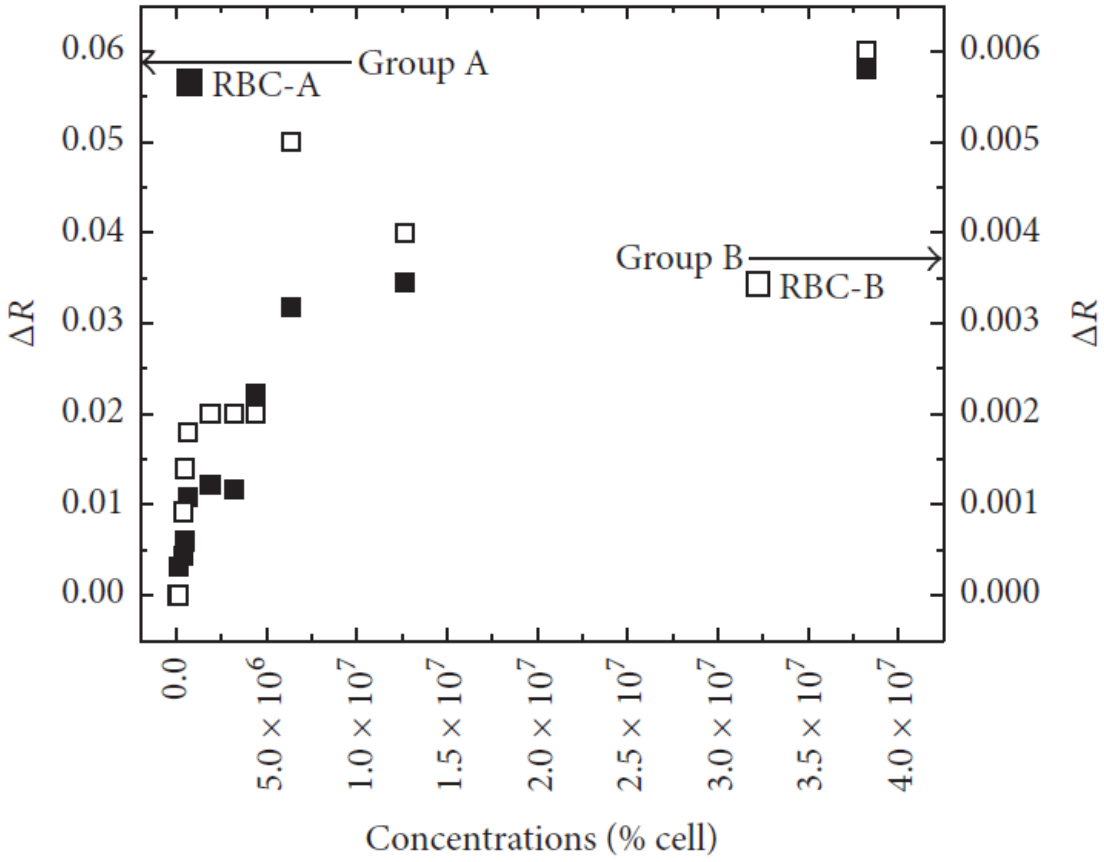


Figure 4.13 LR-SPR reflectivity change after sensing with various concentrations of red blood cells (RBCs).

4.3 Conclusions

We demonstrated that the LR-SPR sensor, consisting of high-refractive index glass, Cytop film layer, and thin gold (Au) film, is capable of detecting large analytes, red blood cell (RBCs). The antibodies of blood group A and group B (Anti-A and Anti-B) are able to be covalently immobilized on a grafting self-assembled monolayer (SAM)/Au surface on the LR-SPR biosensor chip. For blood typing, the RBC samples are detected on the immobilized Anti-A and Anti-B surface by the change in the refractive index. We found that the results of blood typing obtained by the LR-SPR biosensor were consistent with those obtained from the agglutination test. Moreover, the LR-SPR exhibited the lowest detection limits of 1.58×10^5 cells/ml for RBC-A and 3.83×10^5 cells/ml for RBC-B, indicating that the LR-SPR chip has a higher sensitivity than conventional short-range SPR biosensors (3.3×10^8 cells/ml). Finally, the sensor showed a good efficiency of surface regeneration using 20 mM NaOH. Therefore, the LR-SPR technique demonstrates many advantages for the detection of RBCs and could be used to perform ABO blood group typing in the future.

CHAPTER 5

Investigating Immobilized Anti-A Antibodies on Gold Grating Surface for Detection of Red Blood Cell ABO Typing via Transmission Surface Plasmon Resonance (T-SPR) Biosensor

The conventional technique of the blood grouping is agglutination technique. This technique is based on the interaction between antibody and its specific antigen leading to the agglutination of RBCs. Although agglutination is very easy to use in clinical, there are some limitations including laborious processing, subjective interpretation, qualitative results, and low sensitivity.

Nowadays, there is the automatic instrument for testing agglutination that is high performance in the routine use. However, this method is expensive. Therefore, we propose a new method to detect RBC antigen and antibody that is a transmission surface plasmon resonance (T-SPR).

The T-SPR based sensor is an attractive platform in label-free biosensor and immunosensor applications because it is applicable to simple in situ sensing systems. The advantages of grating-based SPR technique include the fact that it is prism-less, convenient, and propagating SPR excitation method.

The principle of detection relies on the transmission spectra of p-polarized light, which is observed when the light passes through the thin gold film coated grating substrate under specific conditions.

5.1 Materials and methods

5.1.1 Chemicals and materials

DVD-Rs were purchased from Taiyo Yuden. 11-mercaptoundecanoic acid (11-MUA), phosphate buffered saline (PBS) tablets, and sodium acetate buffer (pH 5) were all purchased from Sigma-Aldrich. The 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and ethanolamine hydrochloride (EA-HCl) were purchased from Tokyo Chemical Industry (TCI). Mixed clones of monoclonal Anti-A and Anti-B, standard A cells and B cells, were obtained from the research unit of the Thai Red Cross Society.

5.1.2 Preparation of T-SPR sensor chip

DVD-R (Taiyo Yuden) was used as the diffraction grating substrate. DVD-R was cut into a square size 1*1 cm and removed the attached film after washing the substrate with ethanol and DI water and dried by oven. The gold coated grating substrate was prepared by vacuum evaporation of approximately 5 nm Cr and 45 nm gold on the cleaned DVD-R substrate on grating side. The morphologies of the bare gold coated grating surface were observed by AFM. The gold grating surface was covered by SAM of 11-MUA like previous topic (4.1.3.2). The antibody of blood group A (Anti-A) was covalently immobilized on gold grating surface and detected RBC sample by the change of the intensity in the transmission peak of T-SPR signals as seen in figure 5.1.

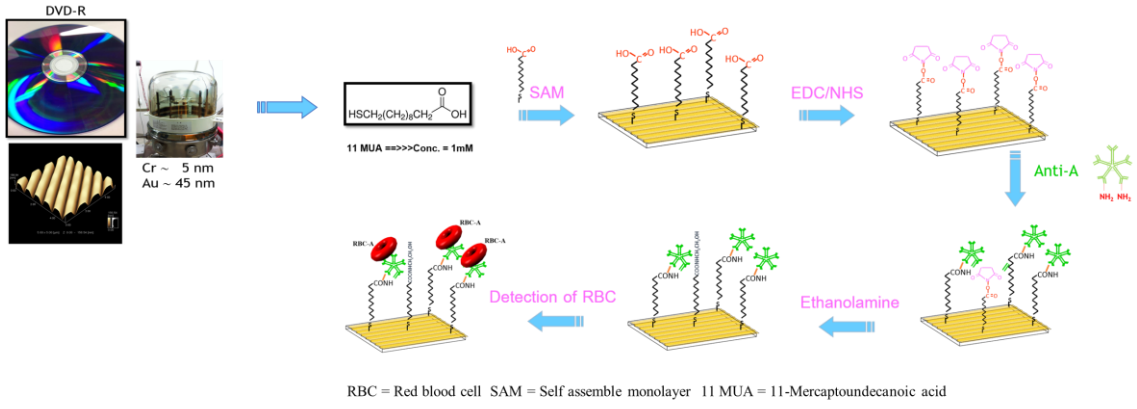


Figure 5.1 Schematic diagram of preparation of gold grating substrate for anti-A antibody sensor chip.

5.1.3 Transmission Surface Plasmon Resonance (T-SPR) setup

The homemade T-SPR setup consisted of a fiber optic spectrometer (HR4000, Ocean optics, Inc.) and Tungsten halogen light source **(**Ocean optics, Inc.), which both were placed on the opposite side. White light was passed through a polarizer to grating substrate. Gold grating substrate was on a rotation stage as seen in figure 5.2. The T-SPR spectrum is the p-polarized light that subtracting by raw spectrum of S-polarized light.

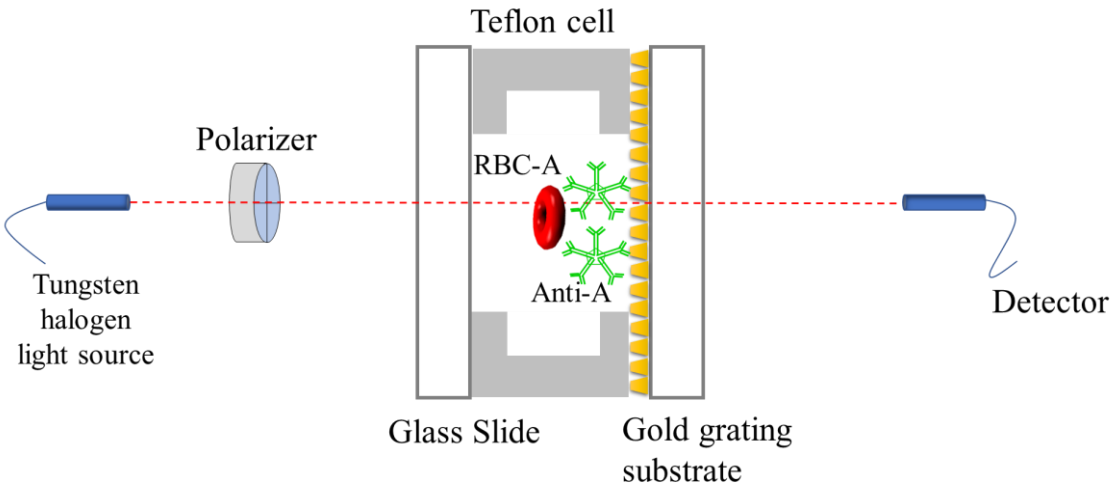


Figure 5.2 Schematic diagram of the T-SPR set-up.

5.1.4 Detection of RBC sample

Standard A cells and B cells were diluted to 5 %v/v from initial concentration via PBS buffer. The anti-A immobilized on the carboxylic acid/gold grating surface was employed as molecular receptor. T-SPR peak intensity change by the immunological interaction between immobilized anti-A and antigens on RBC surface.

5.2 Results and Discussion

First, gold grating was characterized by atomic force microscopy (AFM) to check the integrity of the groove grating. It was found that the grating surface is good homogeneous. The width of groove is around 0.73 µm. The gold film was completely covered the grating surface as seen in figure 5.3. This result confirms the grating surface of DVD-R.

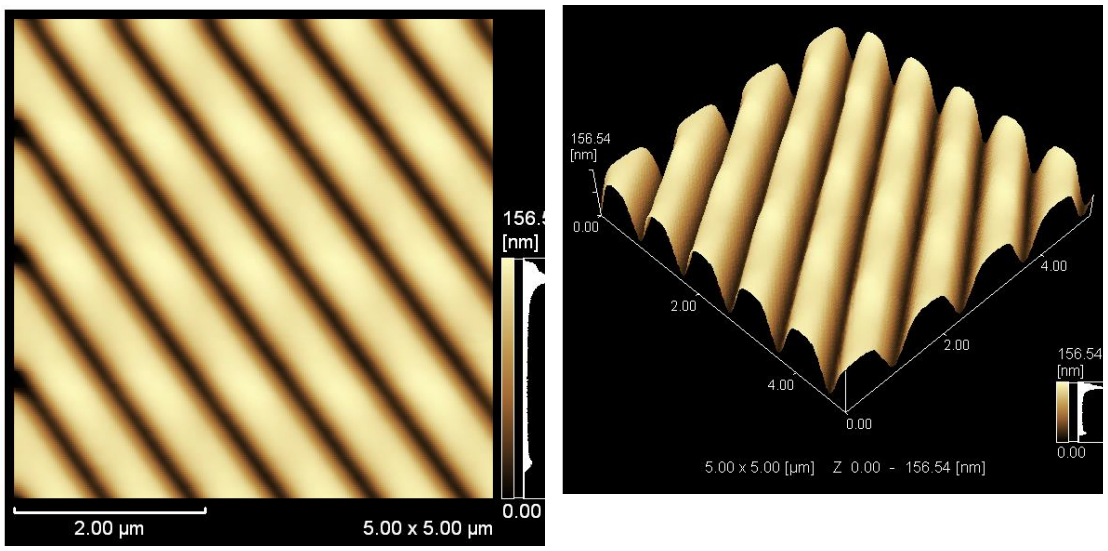


Figure 5.3 The AFM images of bare gold grating surface.

Next, Immobilization of anti-A antibody on the carboxylic acid/gold grating surface was stated with find the optimal concentration of anti-A antibody. This experiment was done via vary concentration of anti-A antibody from initial concentration to 1, 5, 10, 20 and 50 %v/v in pH 5 of sodium acetate buffer. And detected the interaction of anti-A surface and antigen A on RBC surface by fixed the concentration of RBC sample at 5% v/v. Figure 5.4 shows the T-SPR intensity changed by each binding reaction. It was found that the large changing of T-SPR peak occurred at 10 %v/v of anti-A antibody. When we observed the ability in binding of antigen A on RBC sample, it showed the maximum change in the intensity of T-SPR in the immobilized 10%v/v of anti-A. This result indicated that the suitable concentration of anti-A antibody in immobilization on carboxylic acid/gold grating surface is 10 %v/v. This condition was used in next experiment.

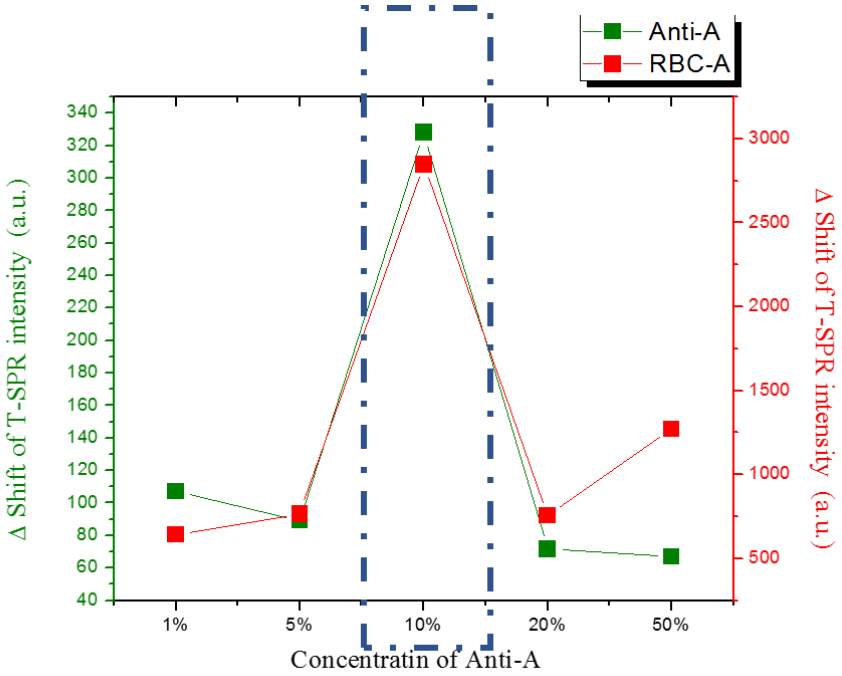


Figure 5.4 Changing T-SPR intensity in each binding reaction according to concentration of anti-A

Figure 5.4 shows T-SPR peak intensity change by growth 11 MUA to be a carboxylic surface on gold grating substrate, the immobilization of anti-A and detection of RBC-A. The intensity increased according to molecule absorption and binding in every layer. Started from the lowest intensity is came from observation of a bare gold grating substrate. Next, the intensity came from growth carboxylic surface layer. Immobilization of anti-A antibody in next layer change T-SPR intensity. The highest intensity came from the interaction of immobilized anti-A antibody and antigen A on RBC samples. This result shows the successfully apply T-SPR technique for ABO detection.

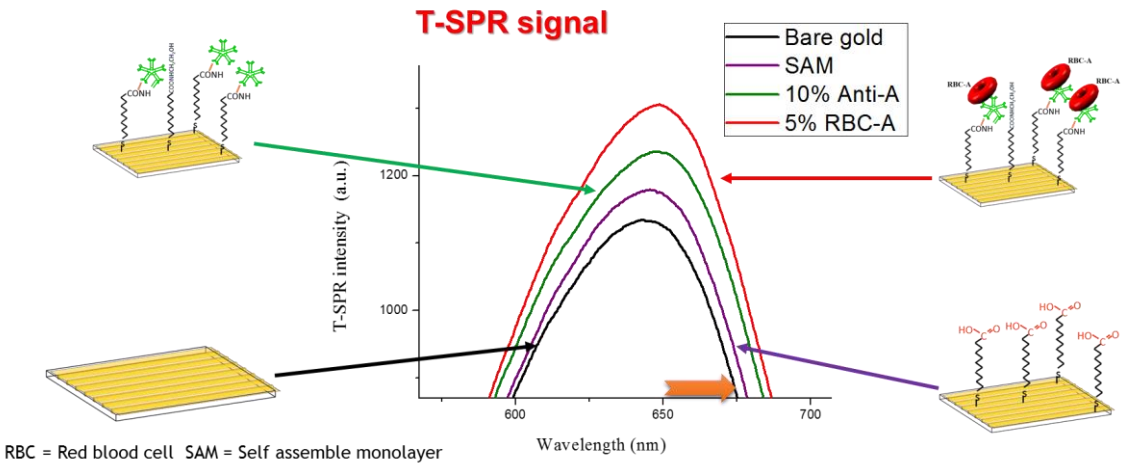


Figure 5.5 TSPR spectra of bare gold grating surface, growth 11 MUA, immobilization anti-A and detection of RBC-A.

The study of non-specific binding was discussed in this part. This study was carried out via immobilized anti-A antibody on carboxylic surface on gold grating substrate. The RBC-B that do not have antigen A on surface were checked the cross reaction and non-specific binding. This pair of antigen-antibodies is not specific with each other, so the binding interaction cannot occur. T-SPR spectra should not increase. This result was reported in figure 5.5, which correlated with the assumption. The T-SPR peak of detection of RBC-B samples were decreased from the peak of immobilized anti-A antibody. This experiment is reinforced gold grating substrate and T-SPR technique have high potential to ABO blood grouping application.

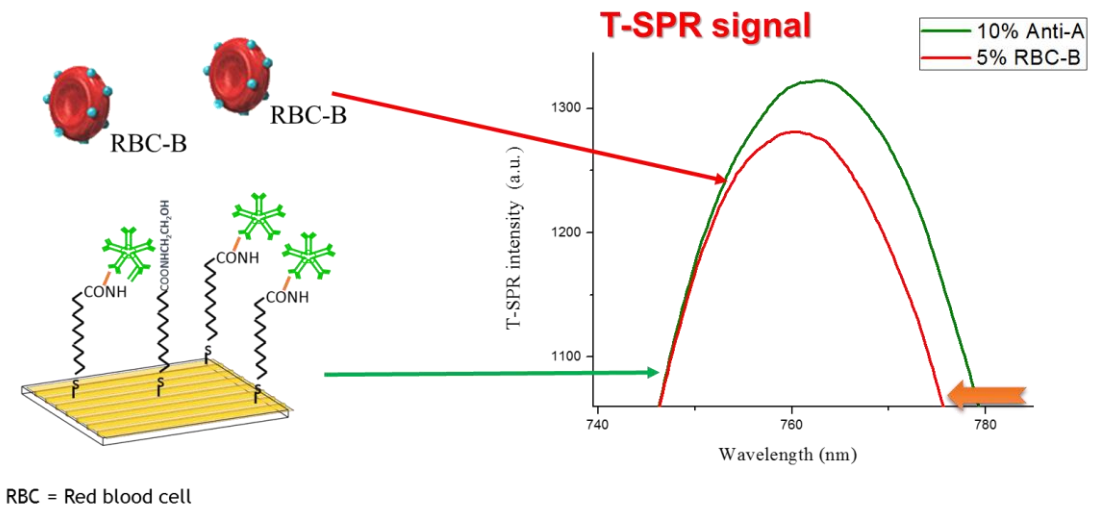


Figure 5.6 T-SPR spectra of testing non-specific binding between immobilized anti-A antibody surface and RBC-B.

The regeneration is one parameter that can increase the value of biosensor. This parameter is testing reusable of sensor chip []. The regeneration was done by using some chemical like week base or acid to destroyed interaction bonding between receptor and analyst but they do not affect to receptor. In this work 5 mM NaOH was used for regeneration reagent. Figure 5.6 is the regeneration result. We found 5 mM NaOH can regenerated RBC-A from immobilized anti-A antibody surface. Even if T-SPR signal after regeneration not completely recovery to original signal but detection of RBC-A sample in second round show very close to the first round. Actually, it could be increase the concentration of NaOH to 10-20 mM for better regeneration but gold grating substrates is a DVD-R, which is the polycarbonate is poor in chemical resistance. So increasing concentration of base is the disadvantage for this work.

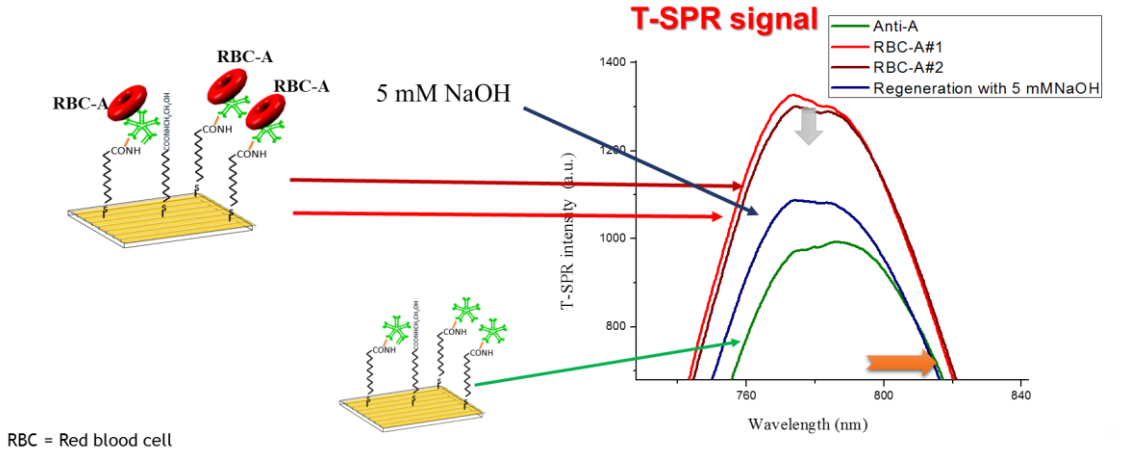


Figure 5.7 T-SPR spectra of regeneration carboxylic acid/gold grating surface after detection of RBC-A with 5 mM of NaOH.

As I mention about the disadvantage of poor chemical resistance of polycarbonate in form of DVD-R substrate to be a gold grating substrate in T-SPR techniques. I would like to propose the preparation of grating structure on high chemical resistance like BK7 glass slide and created the grating surface by Poly (methyl methacrylate) (PMMA). The preparation is shows in figure 5.7, which was started by cut and clean DVD-R in same method in topic 5.1.2. Next, place DVD grating substrate in plastic Petri dish and then pour polydimethylsiloxane (PDMS) cover the DVD-R grating substrate. Heat it to 60 ^{o}C for 4 hour. After that remove DVD-R grating substrate from PDMS silicon. In this step we get silicon grating substrate. Next, coat PMMA on BK7 glass substrate by spinning technique. Then place the silicon grating on BK7 coated PMMA together by grating slide contact with PMMA film. After that compressed they under compressor. Remove it out and oven BK7 in 80 ^{o}C for 4 hours for form PMMA film to be the grating structure. Finally, we will get the grating structure on BK7, which it is good in chemical resistance and high potential to apply to be grating substrate for biosensor applications. To make sure this idea is possible, the grating substrate was observed the morphologies by AFM as seen in figure 5.8 and figure 5.9 shows the T-SPR spectra from grating structure on BK7 glasses substrate compared with grating on DVD-R substrate. It can indicate that this propose can be a good study for future work.

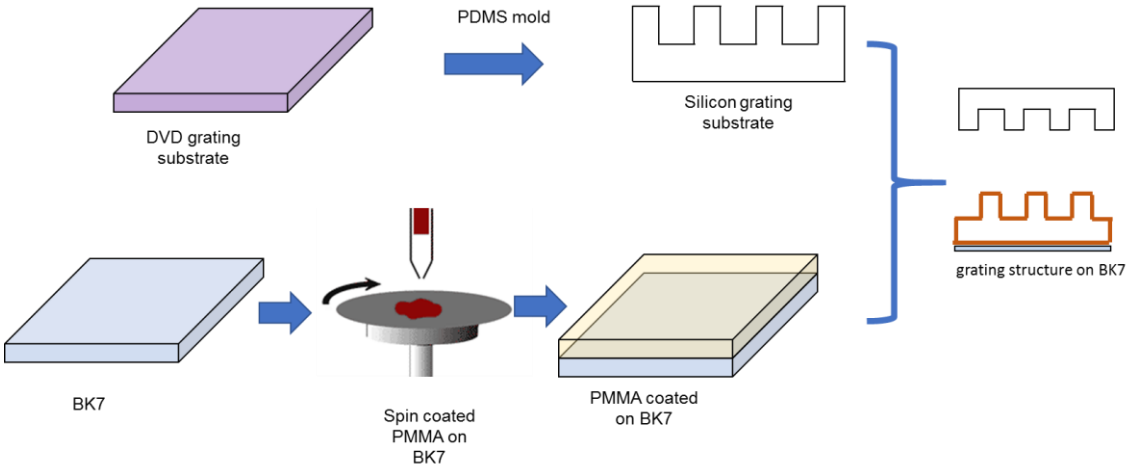


Figure 5.8 The preparation of grating structure on BK7 glasses substrate.

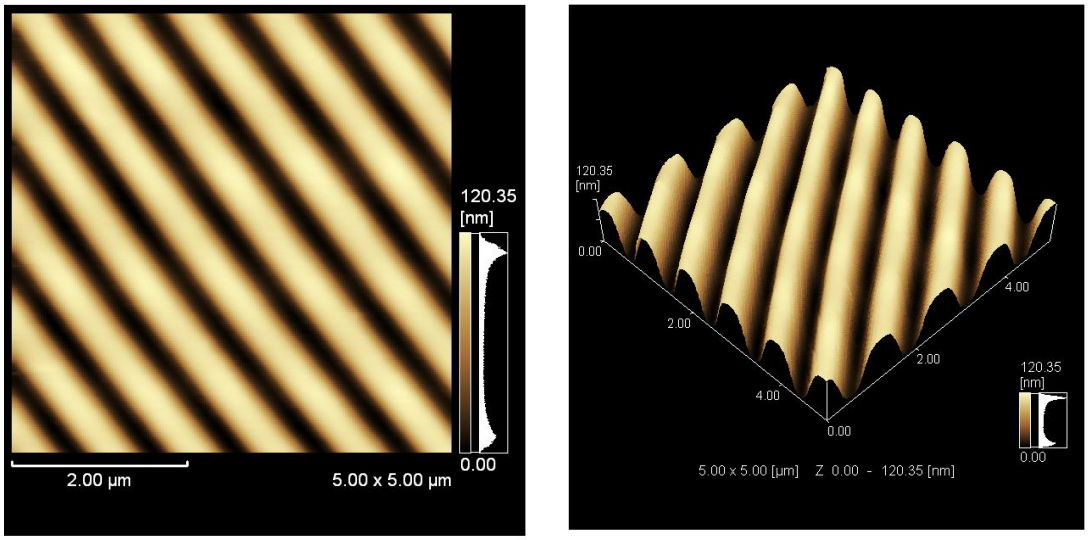


Figure5.9 AFM image-grating pattern from imprinting

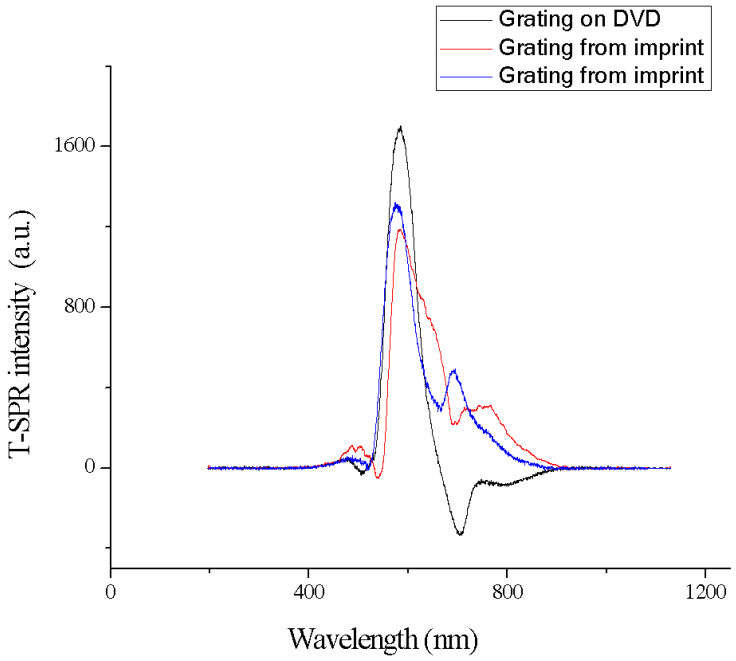


Figure5.10 T-SPR spectra from grating structure on BK7 glasses substrate and grating on DVD substrate.

5.3 Conclusion

Gold grating surface could be used to immobilized Anti-A that the specific and non- specific binding was detected. The performance and utility of T-SPR technique showed obvious advantages for the detection of binding process with the simple experimental setup which should be applied to be the classified ABO blood group typing technique.

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EDUCATION	**Material Science and Engineering Program, Faculty of Science, Mahidol University (Bangkok),** *June 2012 - present: Doctor of Philosophy Programme in Materials and Engineering (International Programme),* ***(GPA: 3.60)*** ***Project:*** *Development of biosensor array for blood group detection* ***Department of Physics, Faculty of Science, Mahidol University*** **(Bangkok),** *June 2009 – May 2012: Master of Science Programme in Physics (International Programme)* ***(GPA: 3.15)*** ***Project:*** *Surface plasmon resonance imaging (SPR imaging) for the detection of IgE antibody in serum on cow's milk allergy disease* ***Department of Physics, Faculty of Science,*** **Naresuan University (Phitsanulok),** *June 2005 - 2009 : Bachelor's degree in Physics,* ***(GPA: 3.09)*** ***Project :*** *Prepration of lead barium zirconate ceramics by combustion technique*
RESEARCH GRANTS	*Doctor degree Scholarships of* ***Japan Student Services Organization (JASSO)****, Japan, May 2013- June 2014 (finished, period of 1 year)* *Master's degree Scholarships of* ***Thailand Graduate Institute of Science and Technology (TGIST)****, NSTDA, Bangkok, Thailand, June 2010- June 2012 (finished, period of 2 year)* *Master's degree Scholarships of* ***Institutional***

RESEARCH GRANTS	***Strengthening Program**, Faculty of Science, Mahidol University, Bangkok, Thailand, June 2009- June 2010 (finished, period of 1 year)* *Bachelor's degree Scholarships of **Human Resource Develop in Science Project**, Science Achiment of Thailand, Bangkok, Thailand, June 2005- June 2009 (finished, period of 4 year)*
CONFERENCE PRESENTATION	*W. Tangkawsakul, T. Srikhirin A. Baba, K. Shinbo, K. Kato and F. Kaneko. ABO blood typing by long-range surface plasmon resonance (LR-SPR) biosensor. The 8th International Symposium on Organic Molecular Electronics (ISOME 2014) (Poster Presentation): May 2014, Tokyo, Japan.* *W. Tangkawsakul, T. Srikhirin A. Baba, K. Shinbo, K. Kato and F. Kaneko. Investigating Immobilized Anti-A Antibodies on Gold Grating Surface for Detection of Red Blood Cell ABO Typing via Transmission Surface Plasmon Resonance (TSPR) Biosensor. 平成 25 年度応用物理学会北陸 (Oral Presentation): November 2013, Kanasawa, Japan.* *W. Tangkawsakul, T. Srikhirin A. Baba, K. Shinbo, K. Kato and F. Kaneko. Investigation on Antigenetic ABO Blood Typing by Transmission Surface Plasmon Resonance Technique. Bioelecrtronics workshop (Poster Presentation): October 2013, Tokyo, Japan.*