ANTIBODY PLATFORM FOR MICROALBUMIN DETECTION AND ABO BLOOD TYPING BY ANTIBODIES ARRAYS BASED SURFACE PLASMON RESONANCE IMAGING

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ANTIBODY PLATFORM FOR MICROALBUMIN DETECTION AND ABO BLOOD TYPING BY ANTIBODIES ARRAYS BASED SURFACE PLASMON RESONANCE IMAGING

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ABSTRACT

In the first part, surface plasmon resonance (SPR) technique was applied to measure the microalbumin in urine. Various combinations of capture antibody and signal enhanced antibody on the mixed self assembly monolayer (mSAM) and carboxymethyldextran (CMD) surface were compared. The most suitable combination was a monoclonal anti-human serum albumin (anti-HSA) as the capture antibody immobilized on CMD surface, providing the specificity of the surface toward HSA binding, and polyclonal anti-HSA as the signal enhanced molecule with the detection limit of 0.001 μg/mL. The amount of the HSA presence in the urine was quantified by using the four parameters fitting (4PL). The detection was in the range of 0.02 to 2.5 μg/mL of HSA in urine sample. The result from the SPR technique was quantitatively consistent with a standard turbidimetric method with coefficient of determination (R^2) 0.94.

In the second part, the application of SPR in ABO blood-typing was investigated. Anti-A, anti-B and anti-AB antibodies were immobilized on CMD surface. A red blood cells (RBCs) sample flowed through the surface using a cross-flow immobilization technique and blood group was determined via the immunological interaction between immobilized antibody and antigens on the RBCs surface. The surfaces were regenerated using 5.0 mM NaOH and can be used a minimum of 20 times. The results from blood-typings were consistent with the results obtained from standard agglutination tests.

In the third part, SPR was applied for Rh (D) blood typing. Monoclonal anti-D antibody (mixed of IgG and IgM type) was covalently immobilized on CMD surface. RBCs samples flowed over the surface and the Rh blood type were determined based on the presence or absence of D antigen on RBCs surface. The optimum flow rate for D antigen detection was 10 μL/min, providing signal of Rh positive RBCs that clearly separate from Rh negative RBCs. All 44 samples of Rh blood typed by SPR were in good agreement with standard agglutination tests.

In the last part, SPR was applied for ABO-Rh blood typing in one sensor chip. The suitable condition for serum grouping using immobilized synthetic blood group A and B antigen on CMD surface was studied. All ligands were immobilized on CMD as integrated platform for cell grouping, serum grouping and Rh (D) typing. The method for ABO-Rh blood typing using a single injection of 1:5 dilution of whole blood in PBST buffer was demonstrated. ABO and Rh blood were typed at the same time in 20 minutes. This technique allows the easy and convenient method for ABO-Rh blood typing with saving time and cost by the surface can be regenerated with 20 mM NaOH. The surface can be used up to 10 times.

KEY WORDS: SPR / MICROALBUMINURIA / ANTIGEN ANTIBODY BINDING / ABO BLOOD TYPING / ANTIBODY ARRAYS

110 pages

การพัฒนารูปแบบของแอนติบอดิสำหรับการตรวจวัดไมโครอัลบูมินและการจำแนกหมู่เลือดชนิดเอบีโอในลักษณะอาร์เรย์โดยเทคนิคเอสพีอาร์แบบภาพ

ANTIBODY PLATFORM FOR MICROALBUMIN DETECTION AND ABO BLOOD TYPING BY ANTIBODIES ARRAYS BASED SURFACE PLASMON RESONANCE IMAGING

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บทคัดย่อ

ในส่วนที่ 1 เทคนิคเอสพีอาร์ถูกประยุกต์ใช้ในการตรวจวัดปริมาณไมโครอัลบูมินในปัสสาวะ (microalbuminuria) โดยการเปรียบเทียบประสิทธิภาพของการวัดปริมาณอัลบูมิน (HSA) โดยการตรึงแอนติบอดิและการขยายสัญญาณด้วยแอนติบอดิแบบต่างๆ บนพื้นผิว mixed self assemble monolayer (mSAM) และ carboxymethyl dextran (CMD) พบว่าพื้นผิวที่เหมาะสมที่สุดคือพื้นผิว CMD ที่ตรึงด้วยแอนติบอดิ anti-HSA ชนิดโมโนโคลนอล ซึ่งมีความจำเพาะเจาะจงต่อการตรวจวัด HSA และการขยายสัญญาณด้วยแอนติบอดิ anti-HSA ชนิดโพลิโคลนอล โดยมีค่าการตรวจวัดต่ำที่สุดอยู่ที่ 0.001 ไมโครกรัมต่อมิลลิลิตร ปริมาณของ HSA ในปัสสาวะถูกคำนวณโดยใช้ 4PL fitting ซึ่งมีความสามารถในการตรวจวัดอยู่ในช่วง 0.02-2.5 ไมโครกรัมต่อมิลลิลิตร ความเข้มข้นของ HSA ในปัสสาวะที่วัดได้จากเทคนิคเอสพีอาร์สอดคล้องกับวิธีมาตรฐานเดิม (การวัดความขุ่น) โดยมีค่าสัมประสิทธิ์การตัดสินใจ (R^2) 0.94

ในส่วนที่ 2 เกี่ยวข้องกับการใช้เทคนิคเอสพีอาร์ในการจำแนกหมู่เลือดชนิดเอบีโอทำโดยตรึง anti-A, anti-B และ anti-AB แอนติบอดิลงบนพื้นผิว CMD เลือดตัวอย่างถูกไหลผ่านพื้นผิวในลักษณะอาร์เรย์ ซึ่งหมู่เลือดถูกจำแนกจากสัญญาณจำเพาะที่เกิดขึ้นระหว่างคู่แอนติบอดิที่ถูกตรึงและแอนติเจนบนผิวเม็ดเลือดแดง พื้นผิวสามารถล้างและใช้ซ้ำได้อย่างน้อย 20 รอบ ด้วย 5 มิลลิโมลาร์ โซเดียมไฮดรอกไซด์ เทคนิคเอสพีอาร์ให้ค่าความถูกต้องเหมือนเทคนิคมาตรฐานเดิม

ในส่วนที่ 3 เป็นการประยุกต์ใช้เทคนิคเอสพีอาร์สำหรับการตรวจวัดหมู่เลือด Rh (D) โดยการตรึงแอนติบอดิ anti-D ชนิดโมโนโคลนอล ผสมระหว่าง IgG และ IgM ลงบนพื้นผิว CMD จากนั้นเลือดตัวอย่างถูกไหลผ่านพื้นผิวและหมู่เลือด Rh ถูกจำแนกตามการปรากฏของแอนติเจนดีบนพื้นผิวเม็ดเลือดแดง พบว่าอัตราการไหลของเม็ดเลือดแดงที่เหมาะสมอยู่ที่ 10 ไมโครลิตรต่อนาที ซึ่งให้ค่าสัญญาณการวัดหมู่เลือด Rh บวก แตกต่างจากหมู่เลือด Rh ลบ อย่างชัดเจน เลือดตัวอย่างทั้งหมด 44 ตัวอย่างถูกนำมาทดสอบด้วยเทคนิคเอสพีอาร์ พบว่าให้ค่าที่สอดคล้องกันกับเทคนิคมาตรฐานเดิม

ส่วนสุดท้ายรวมการตรวจวัดหมู่เลือดชนิดเอบีโอและอาร์เอช บนเซนเซอร์แผ่นเดียวด้วยเทคนิคเอสพีอาร์ รวมถึงศึกษาเพิ่มเติมในส่วนการตรวจหาแอนติบอดิ anti-A และ anti-B ในพลาสมา (serum grouping) ด้วยการตรึงแอนติเจนเอและบีสังเคราะห์บนพื้นผิวเดกทรานซ์ จากนั้นแอนติบอดิและแอนติเจนสำหรับใช้จำแนกหมู่เลือดชนิดเอบีโอและอาร์เอช ถูกตรึงบนพื้นผิว CMD ด้วยสภาวะที่เหมาะสม การจำแนกหมู่เลือดระบบเอบีโอและอาร์เอชในครั้งเดียวโดยการฉีดเลือดตัวอย่างที่ไม่ได้ปั่นแยกที่เจือจาง 1:5 เท่าใน PBST บัฟเฟอร์ หมู่เลือดเอบีโอและอาร์เอช ถูกจำแนกในคราวเดียวกันภายใน 20 นาที เทคนิคนี้ให้วิธีที่ง่ายและสะดวกสำหรับจำแนกหมู่เลือดระบบเอบีโอและอาร์เอช พร้อมทั้งประหยัดเวลาและค่าใช้จ่ายเนื่องจากสามารถล้างพื้นผิวและใช้ซ้ำได้ด้วย 20 มิลลิโมลาร์โซเดียมไฮดรอกไซด์ พื้นผิวสามารถใช้ซ้ำได้อย่างน้อยถึง 10 ครั้ง

110 หน้า

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CHAPTER I
SURFACE PLASMON RESONANCE (SPR) SENSORS

1.1 Introduction to Surface Plasmon Resonance (SPR)

1.1.1 What is SPR?

The first physical phenomenon of surface plasmon resonance (SPR) was discovered by Wood in 1902 [1]. He noticed that the pattern of dark and light bands from reflected light occurred after gleam with polarlized light on a mirror with a diffraction grating on its surface. Incomplete explanation of the phenomenon had originated [2, 3] until a complete explanation by excitation of surface plasmons was reported from Otto [4] and Kretschmann and Raether [5] in 1968.

During the last two decades, SPR sensors have been realized as a useful tool for measuring biomolecular interactions [6, 7]. SPR is an optical technique that can measure the surface mass coverage of adsorbed material [8-11]. SPR is a sensitive technique for studying interactions between immobilized biomolecules and a solution-phase analyte. The sensitivity and versatility of this label-free, real-time method has advantages over conventional methods such as fluorescence or ELISA (enzyme-linked immunosorbent assay). Commercially available SPR systems, such as the Biacore system, are well-known and commonly used in many experiments [12-14].

1.1.2 Basic SPR phenomenon

Surface plasmon resonance is phenomenon that occurs at interface between metal and dielectric material. When a p-polarized light incident on a metal surface through prism at the angle higher than the critical angle, a minimum of reflected light is observed at a certain angle which called “SPR angle” due to the excitation of the surface plasmon [15, 16]. The most common optical configuration of SPR sensor is Kretschmann configuration [5], showed in figure 1.1. Under total internal reflection, the incident light is absorbed and excites electron oscillations onto

the metal surface (usually gold), inducing surface plasmon resonance, resulting in decreasing of reflected light at SPR angle.

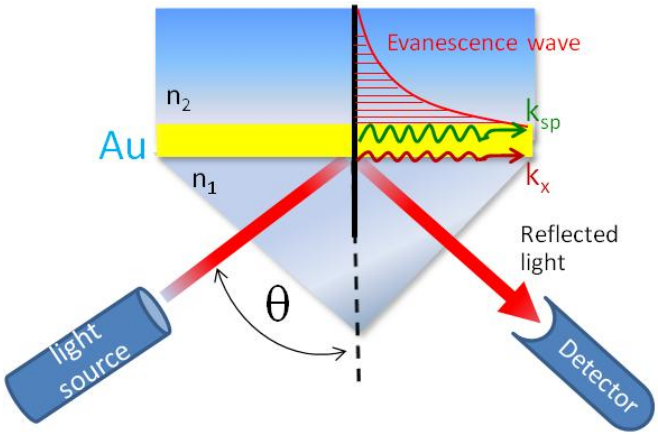
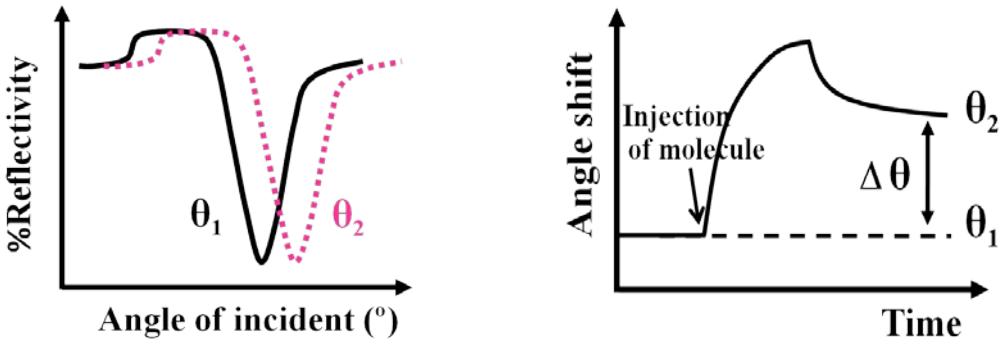


Figure 1.1 Typical configuration of SPR sensors

The position of SPR angle depends on the optical configuration such as refractive index at both side of metal. Refractive index near metal surface will change when biomolecule are adsorbed on it, while refractive index of prism is not change. Then SPR has ability to monitor the adsorption of protein molecule by monitoring the change of the SPR angle (figure 1.2) due to the change of refractive index on the metal surface [17].



(a) (b)

Figure 1.2 Monitoring molecular absorption by SPR; (a) The SPR curve, minimum intensity of reflected light is observed before (θ_1) and after (θ_2) molecular absorption and (b) real time monitoring SPR response against time, after molecule absorb show in $\Delta\theta$.

1.1.3 Total internal reflection and Evanescent wave

Total internal reflection take place when the light incident from material which has high refractive index (prism) to another material which has low refractive index (air or dielectric) at incident angle higher than critical angle. The evanescent wave is generated in material which has low refractive index. When add metal layer between these two materials, amplitude of evanescent wave is increase and penetrate to the dielectric layer [18]. More understanding of SPR can be described here, under condition of total internal reflection and incident light resonance to metal surface, the electromagnetic field (evanescent wave) are leaks across the metal surface into the dielectric layer of lower refractive index without losing net energy. The sensitivity of SPR determines by the penetration depth of evanescent wave which is about 200 nm into the dielectric layer [15, 19] and decaying exponentially with distance from the surface as shown in figure 1.1. Refractive index change in the evanescent field will affect the change of SPR signal. In another word, change in buffer concentration or molecule bind to surface can cause change in refractive index which can be monitored by SPR signal shift.

1.1.4 Surface plasmon

Plasmons are charge-density oscillations of the vicinity free electron in a metal suface [20] . Surface plasmons involved with surface electromagnetic waves that propagate along the metal-dielectric interface (figure 1.3).

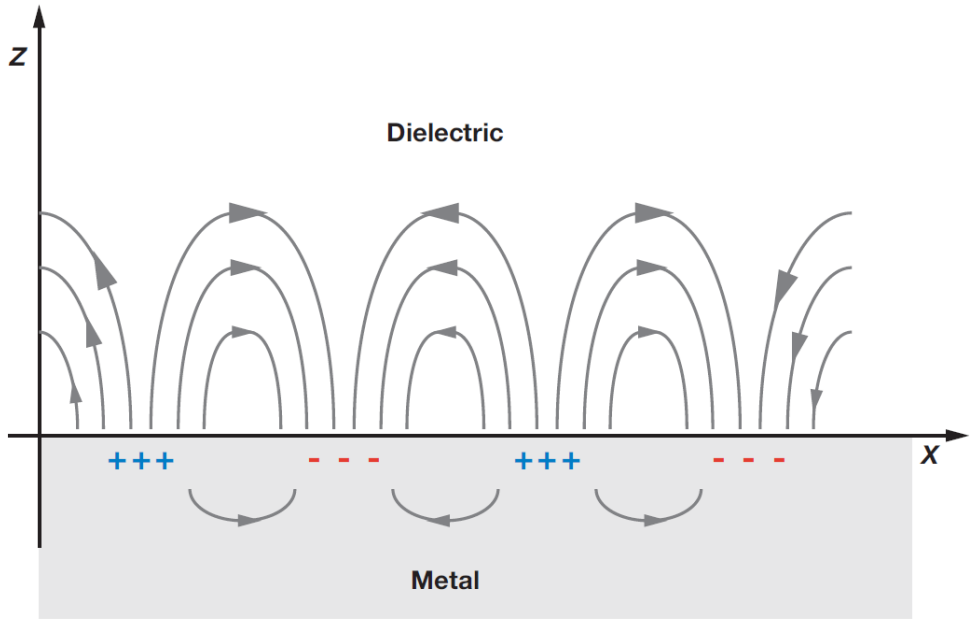


Figure 1.3 Schematic of surface plasmon wave, plasmons propagate along the electron sea of metal. Minus signs (-) is area which have large density of electron and plus signs (+) is area which have low density of electron [21, 22].

The propagation constant of surface plasmon along the metal-dielectric interface is given by [15]

$$k_{sp} = \frac{\omega}{c}\sqrt{\frac{\varepsilon_{metal}\,\varepsilon_{sample}}{\varepsilon_{metal} + \varepsilon_{sample}}} \qquad (1.1)$$

where

k_{sp} is propagation constant (wave vector) of surface plasmon

ω is the angular frequency of incident wave

c is the velocity of light

ε is the dielectric constant of metal and sample layer; ($n = \sqrt{\varepsilon}$)

Incident light can excite surface plasmon when propagation constant of an incident light (k_x) along x axis is equal to propagation constant of surface plasmon (k_{xp}). The propagation of incident light is written by

$$k_x = \frac{\omega}{c} n_1 sin\theta \quad (1.2)$$

where

k_x is propagation constant of an incident light

n_1 is refractive index of prism

θ is incident angle

Hence, condition for generate surface plasmon resonance is when wave vector of an incident light (k_x) is equal to wave vector of surface plasmon (k_{xp}) then the energy from incident light are absorbed onto metal surface that is showed the minimum of reflected light [15, 23, 24]. Condition for SPR is shown below

$$k_x = k_{sp} \quad (1.3)$$

$$n_1 sin\theta = \sqrt{\frac{\varepsilon_{metal}\, n^2_{sample}}{\varepsilon_{metal} + n^2_{sample}}} \quad (1.4)$$

Equation 1.4 shows that when sample layer change, refractive index change so the incident angle (θ) will change to generate surface plasmon resonance.

1.1.5 SPR Imaging Technique

SPR imaging has basic principle same as conventional SPR. Reflectivity change are measured at a fixed incident angle and use a camera (CCD or CMOS) to detect intensity change (figure 1.4). SPR imaging suitable for sensor arrays detection platform.

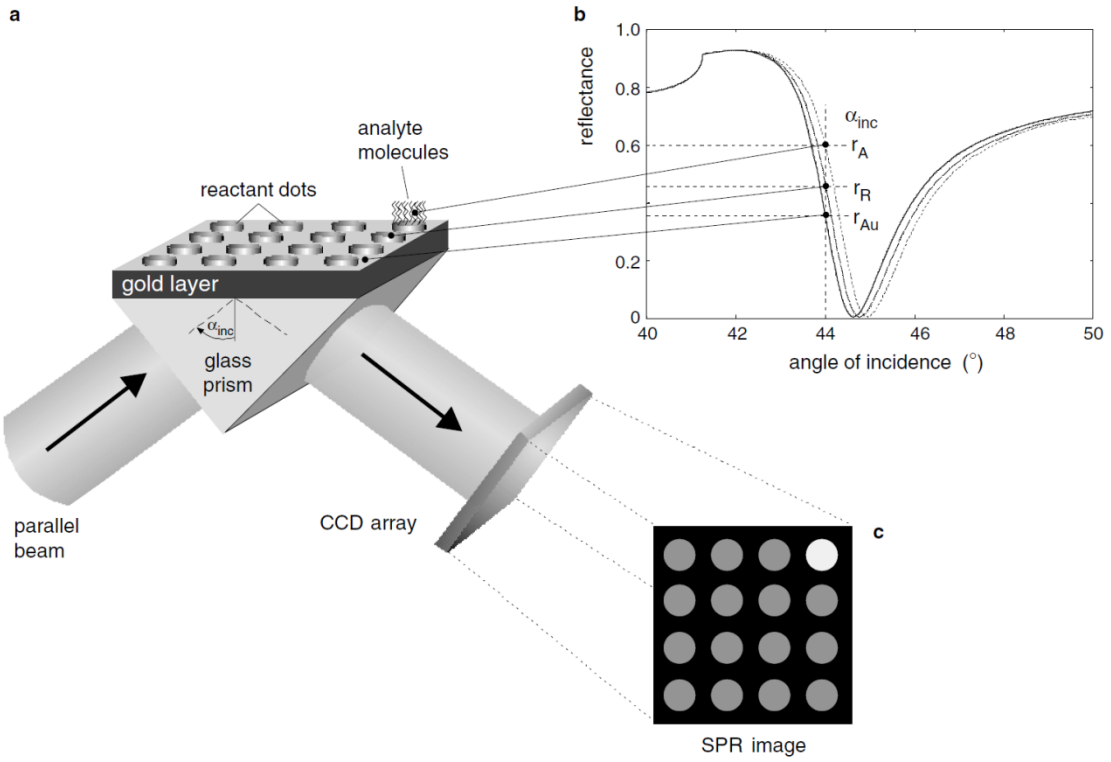


Figure 1.4 SPR imaging; (a) schematic diagram of SPR imaging, (b) calculated SPR reflectance curve for a pure gold surface (solid line) a reactant dot (dashed line) and adsorbed analyte molecules on a reactant dot (dotted line), (c) The contrast of the SPR image is based on the different reflectance rA > rR > rAu [25]

The works in this thesis use 2 set up of SPR imaging machine which constructed by *Photonics Technology Laboratory*, *NECTEC*.

1) Angular scanning SPR imaging system which shows the image result by set angle at high contrast (figure 1.5(a)); this system is applied for microalbumin detection project (see chapter II).

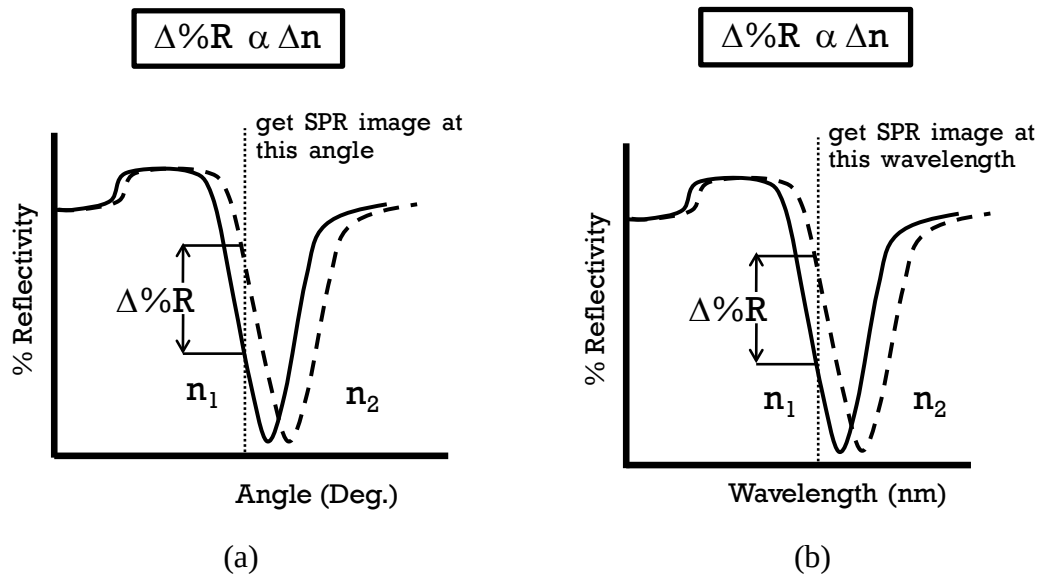


Figure 1.5 SPR imaging mode (a) angle scanning and (b) wavelength scanning

Generating of SPR phenomena based on the Kretschmann configuration and coupling prism. The light emitting diode (LED), a light source, with a centered wavelength at 880 nm and a CCD camera (Sony, XC-EI50) is used as a detector. The SPR sensor chip is attached on glass prism along with an index matching gel (Cargille Labs.). The 7-channels flow cell is made of polydimethylsiloxane (PDMS) covered on the top of sensor surface via mechanical clamps. The volume of each flow cell channel is 5 μL. Buffers and samples are pumped into the flow cell by a multi-channels peristaltic pump (Ismatec), the set up shown in figure 1.6.

The reflected images from the SPR chip were collected by the CCD camera at the imaging angle which was adjusted in a linear region of the SPR curve to get the highest image contrast. The selected incident angle was kept constant throughout the experiment. The relationship between the reflectivity change and the refractive index unit (RIU) was established just before the experiments, by using the solutions with known refractive indices. The term “reflectivity” in this report was defined as the ratio of the p-polarized light intensity to the s-polarized light intensity Sample concentrations were carefully chosen to ensure that all succeeding SPR signals were well within a linear respond curve [26].

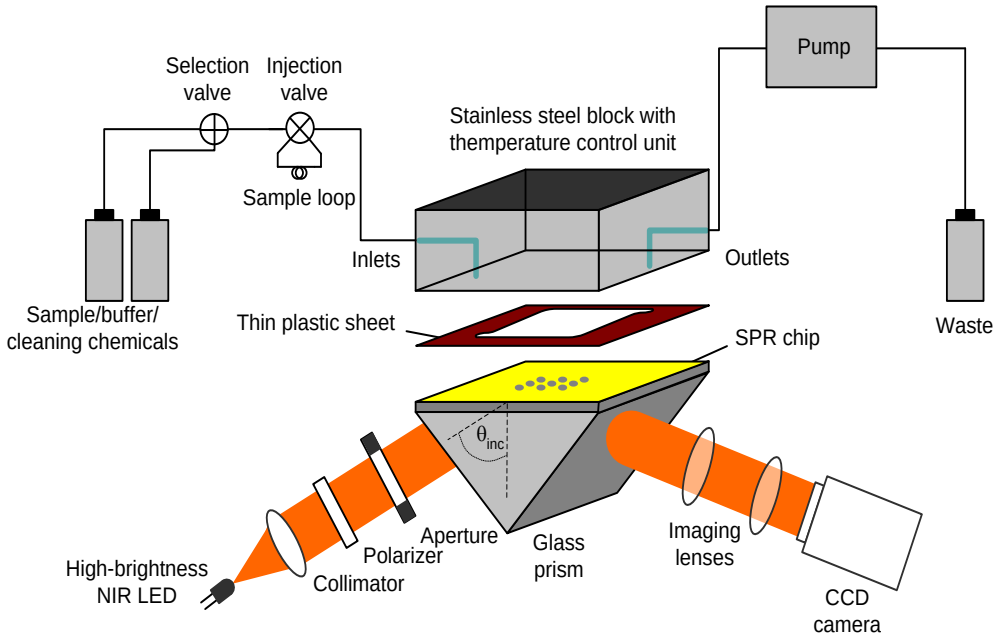


Figure 1.6 Schematic of angular scanning SPR imaging set up by NECTEC

2) Wavelength scanning SPR imaging system which shows the image result by fix incident angle and set angle at high contrast (figure 1.5(b)); this system is applied for ABO and Rh blood typing project (see chapter III-V).

The wavelength-tunable SPR imaging system (figure 1.7), details of the SPR imaging apparatus are similar to the previously-reported intensity-based SPR imaging systems with some modification [27]. Intensity-based SPR imaging technique uses a monochromatic light source and measure the reflectivity changes at the fixed incident angle that has a maximum contrast and thus it requires a precise angular positioning device to locate the maximum-contrast angle. In this work, however, a wavelength-tunable light source designed based on a tungsten-halogen lamp (ZiNir, 20W) and a linear variable filter (Edmund Optics, 620-1080 nm) was used to obtain the reflectivity spectrum plotted as a function of wavelength at a fixed predetermined incident angle. From the reflectivity spectrum, the high-contrast wavelength was selected and the reflectivity measurement was performed.

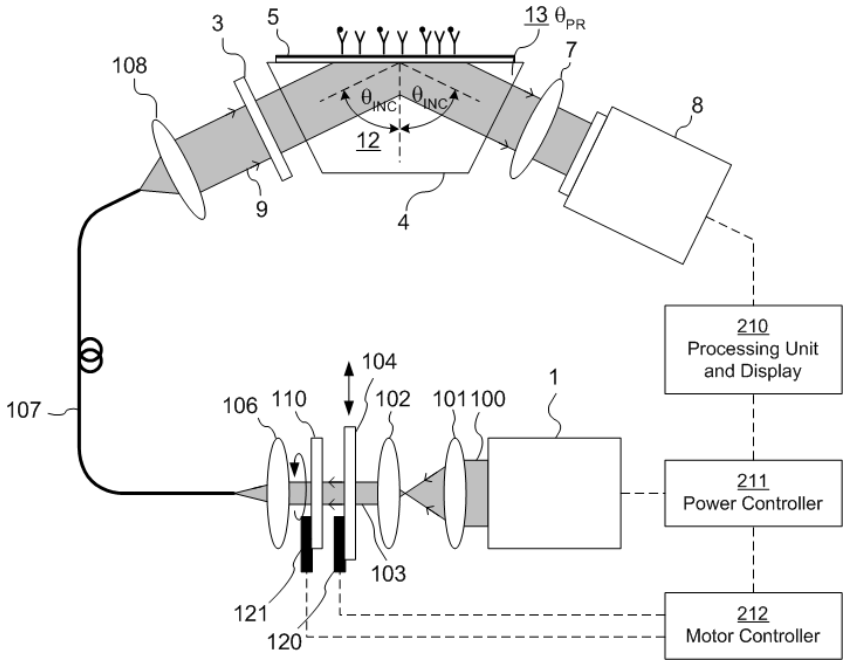


Figure 1.7 Wavelength-tunable SPR imaging set up by NECTEC

The SPR sensor chip was put on top of the prism surface using an index matching liquid (Cargille Labs.). The p-polarized light beam was arranged to illuminate the sensor chip through the coupling prism in a Kretschmann configuration in order to excite surface plasmon waves. The reflected light was focused onto a charge-coupled device (CCD) camera and an imaging lens for intensity measurement. A 7-ch polydimethylsiloxane (PDMS) flowcell made by a precision aluminum molding technique was then put on top of the SPR sensor chip and mechanically clamped firmly to the prism holder. Each flowcell chamber has a volume of about 5 μL and dimensions of 17 mm × 0.5 mm × 0.5 mm ($l \times w \times h$). Fluid flow to the sensor surface via tubing was controlled by a multichannel peristaltic pump (Ismatec) and samples were manually introduced to the fluidic flow using injection valves (Upchurch Scientific) with a sample loop of 100 μL.

Before starting an experiment, the full reflectivity spectrum was obtained by scanning the tunable light source in the range of 650 – 900 nm and then the high-contrast wavelength was determined and kept constant throughout the experiment. The

SPR signal was defined as the reflectivity difference before and after sample introduced to the sensor surface. The calibration curve between the reflectivity change, ΔR, and the refractive index unit (RIU) was established just before the experiment using the solutions with known refractive indices. The term "reflectivity" in this report is defined as the ratio of the p-polarized light intensity to the s-polarized light intensity. SPR signal can also be visually observed from the different image which is the reflectivity difference of the images captured before and after the experiment.

1.2 SPR Biosensors

If the surface of the metal is designed to have a specific interaction without interference from the non-specific binding by immobilized the antibody on the surface, the antigen in the analyte can be detected by flowing through the sensor surface. The interaction of antigen and antibody occur on metal surface, SPR angle will change depend on the refractive index of molecule on the surface. The interaction of molecule is monitor by plotting SPR signal against time which shown in figure 1.8. The advantage of SPR is a rapid sensitive technique, label free and real time monitoring of the analyte interaction.

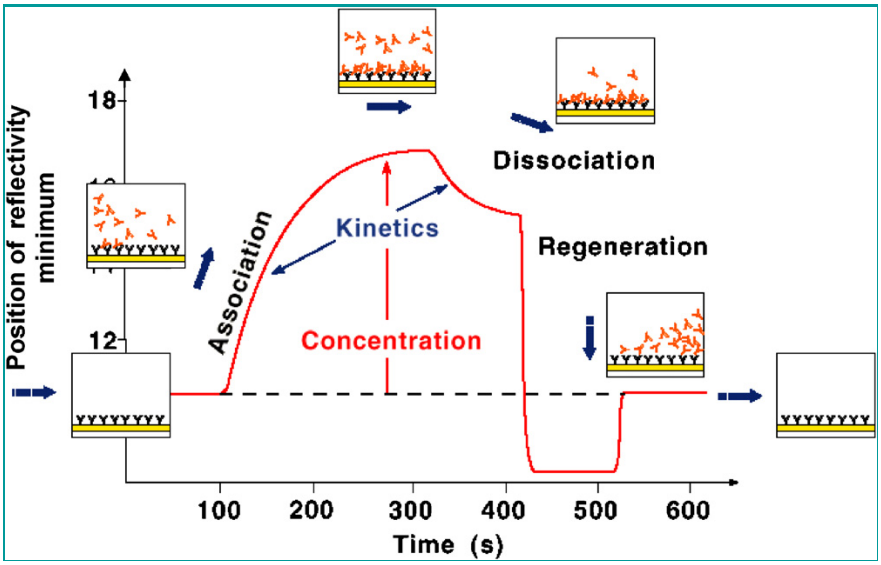


Figure 1.8 Typical SPR sensorgram [28]

Figure 1.8 is the SPR sensorgram which performs the SPR signal shift against time. At beginning graph is baseline signal of the surface, no change in SPR signal. After injection of sample, molecule adsorb on the surface then SPR signal increase. This step is called the association phase. Next, buffer is passed over the surface cause the loosely bound molecules to be flushed off, defined as dissociation phase. Finally, to get rid of the target molecule from the surface, a regeneration solution is injected to break the specific binding between them so the signal come back to base line.

There are several applications of SPR biosensors due to its ability to measure interaction in real time, quantitative determine kinetic parameters and concentration or qualitative characterize the binding of ligands and targets molecule. For example, SPR biosensors for environmental monitoring [29-31], for food safety [32-34], and for medical diagnostic [35-38].

1.2.1 SPR sensor surface

Sensor surface is an important part where the biomolecular interaction takes place. The quality of data effects by the suitable sensor surface for biomolecule interaction. SPR sensor chip is made from BK7 coat with 50 nm of gold layer which provide the characteristic of SPR. Gold has a higher stability than other metal, allow the well-defined minimum of reflectivity and can be functionalized with matrix layer to improve the capacity of immobilized ligand [39]. Immobilization of ligand directly onto gold substrate is not well performed because the molecules often loss its binding function due to the change in the conformation of immobilized specied. Decreasing ability may be come from conformational change, inactive of binding site from contacting to surface or unsuitable orientation. Modification of gold surface is needed to prevent the biomolecule directly contact to gold substrate. There are many type of surface modification which is used in SPR biosensors classified from the immobilization method [40] . Each method has own advantage and disadvantage which effect to ligand, analyte, molecule binding, functional group for covalent bonding and application. Example of immobilization method are physical adsorption [41], covalent binding [42], self assemble [43], sol-gel [44] and Langmuir–Blodgett deposition[45].

In this thesis focused on a covalent binding method to immobilize ligand molecule onto the surface. The advantage of covalent protein is stable when reaction occurs in harsh solution such as in regeneration step [46]. Furthermore, they may control orientation of the immobilized molecule [47, 48] and increase amount of ligand on surface [49, 50]. Two type of sensor surface were used in this research including mix self assemble monolayers and carboxymethyl dextran surface.

1.2.1.1 Mixed self assemble monolayers (mSAMs)

Self assemble monolayers is a widely use functionalized surface method for immunosensor. This technique is convenient and provides well-order monolayer of alkanethiol onto metal surface. No effect of mass transport on SAMs which allow faster of molecule binding to their specific probe. SAMs is flexible due to the producing a wide variety of surface from incorporation of thiol of different chain lengths with the same or different functional group [51]**.** Monolayer can be prepared by immerse metal surface onto alkanethiol solution at several hour (about 12 hr). The mechanism of alkanethiol on gold substrate can be considered as oxidative addition of S-H bond then follow by reductive elimination of the hydrogen as shown in equation 1.5 [51, 52].

$$R-S-H + Au_n^{\circ} \rightarrow R-S-Au^{+} \cdot Au_n^{\circ} + 1/2\ H_2 \qquad (1.5)$$

The previous work reported kinetic studied of self assembly onto gold substrate [53]. Two distinct kinetic of self assembly can observed, fast step is occur in a few minutes explained by sulfur is assembled onto substrate, and slower step which take several hour due to rearrangement of alkyl chain (different chain-chain interaction; van der Waals) to form closely pack. The alkyl chain will tilt form the vertical to gold surface which shown in figure 1.9.

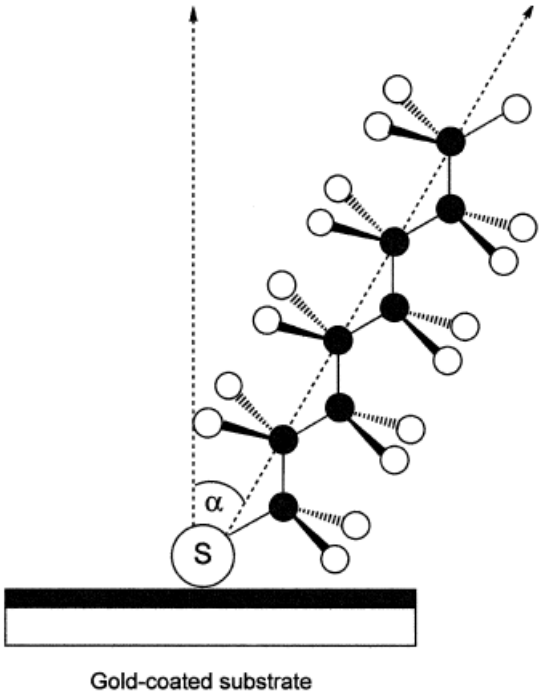


Figure 1.9 Show a self assembled alkanethiol molecule, a tilt of alkyl chain away from vertical to the gold substrate [51]

The mixture of two types of alkanethiol with different chain length provides comfortable surroundings for immobilization of molecule. Mixed SAMs (mSAMs) can reduce the steric hindrance from terminate group of SAMs by making a spacer [54, 55]. There were many reported that compared the mSAMs surface with different molar ratio to find which one provide the highest sensitivity for immobilization of biomolecule [55, 56].

1.2.1.2 Carboxymethyl dextran surface

Carboxymethyl dextran (CMD) surface is a common immobilized surface for the commercial Biacore® SPR chip. In this thesis, CMD surface was chose for SPR sensor surface due to its ability in increasing the capacity of immobilized protein. CMD is a 3 dimensional (3D) surface from long chain of dextran which coated on the gold surface. Dextran is an α-1,6 glycosidic linked of glucose molecules and branching out from the backbone units at α-1,3 linked, along with its hydroplilicity that causes swelling and forming into 3D structure [40]. The CMD was prepared according to the previous report [57]. The gold substrate was

cleaned with piranha solution (30:70 v/v of 30% hydrogen peroxide (H_2O_2) and concentrated sulfuric acid (H_2SO_4)). After cleaning, the surface was reacted with 11-hydroxy-1-undecanethiol in ethanol/water (80/20) solution. A monolayer was formed with the complete wetting surface. The epichlorohydrin was attached to the terminal hydroxy group yielding the epoxy group that will react with dextran molecular weight 500 kDa. The dextran was converted into carboxydextran via reacting with bromoacetic acid (figure 1.10).

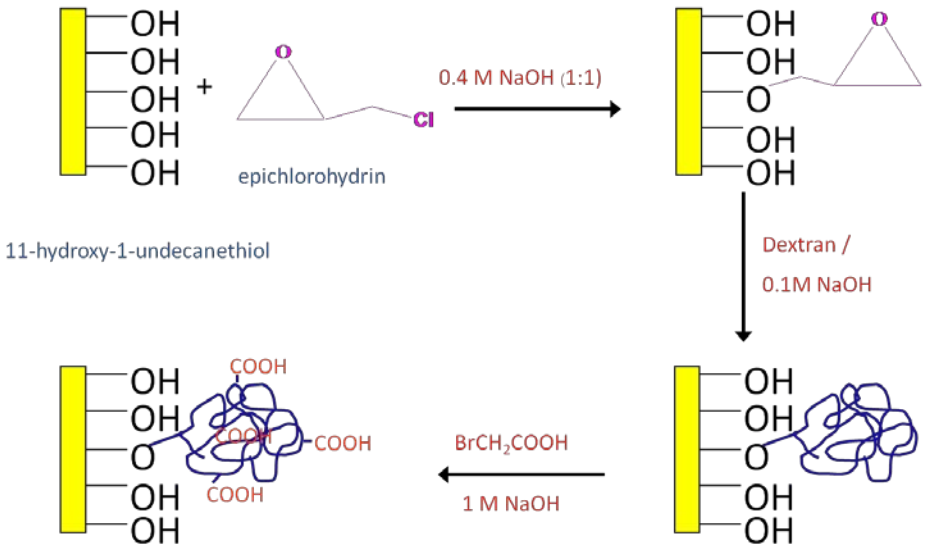


Figure 1.10 Preparation of carboxymethyl dextran surface [57]

To immobilize protein on CMD surface is achieve by covalent binding method. The electrostatic force has effect for protein immobilization in this surface. In addition, the negative charges from carboxyl group on dextran react with positive charges from molecule of protein that induce molecule of protein come to bind on the surface (figure 1.11). This situation occur when pKa of surface > pH buffer > pI of protein [17].

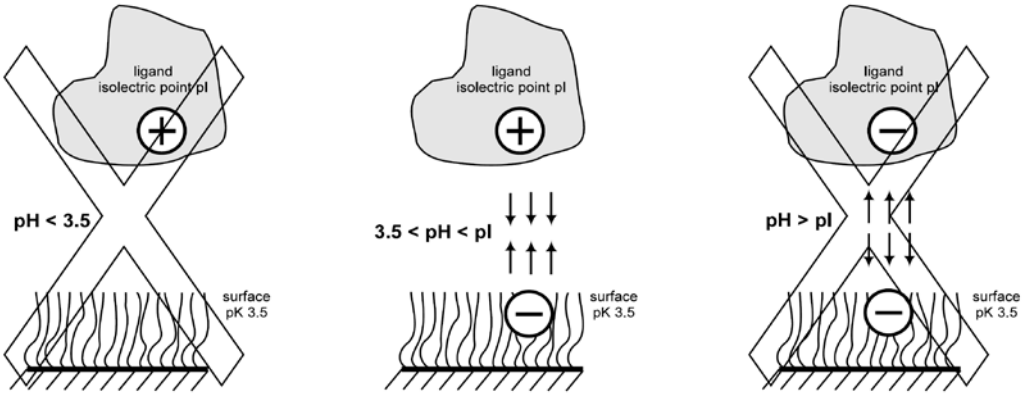


Figure 1.11 Protein concentrate to CMD surface [58]

1.2.2 Coupling procedure

Immobilization of biomolecule onto mSAMs and CMD surface perform by covalent binding method. Common method is amine coupling because most of protein molecules contain amine groups. The reaction occurs via active carboxylic group on mSAMs or CMD and amine groups on protein molecule with less affect the activity of molecule. A method to immobilize ligand onto surface, the carboxilic group was activated by amine coupling reagent; 0.4M EDC (N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride) / 0.1M NHS (N-hydroxysuccinimide), for about 10 min to form reactive N-ester group. Protein molecule dissolved in a low ionic strength buffer at pH less than isoelectric point (pI) of molecule is flowed over the activated surface where they were linked to the surfaces via amide linkage. 1 mg/mL casein was used as the blocking agent to block the rest of the active groups on mSAMs while ethanolamine pH 8.5 was used as the blocking agent for the CMD (figure 1.12).

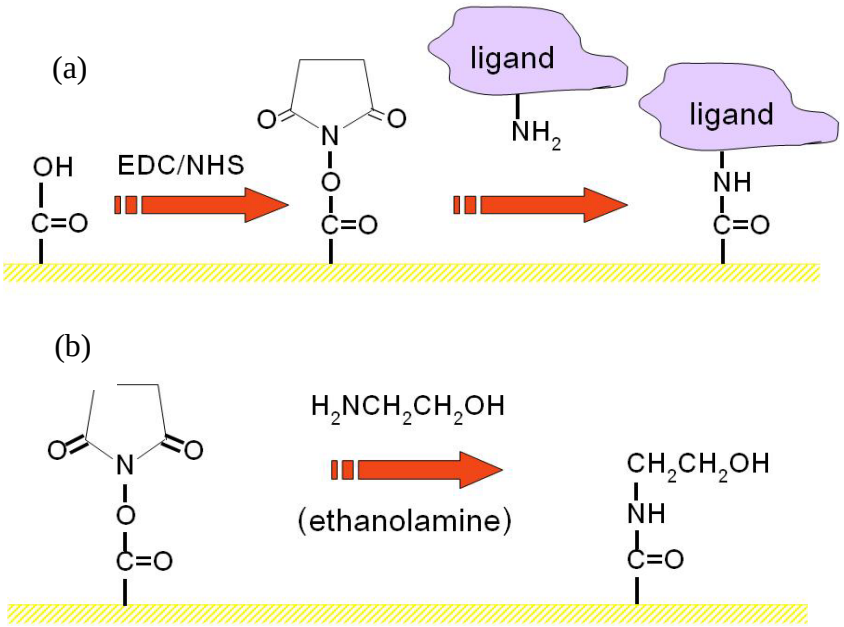


Figure 1.12 Coupling procedure; (a) activation of surface via amine coupling [58] and (b) blocking of CMD surface with ethanolamine pH 8.5

1.3 SPR immunoassays

There are several assays for measuring interaction of biomolecule depend on different situation. Direct assay (direct detection and sandwich assay) is suitable for macromolecule (MW > 5000 Da) and indirect assay (inhibition and competitive assay) is proper for small molecule (MW < 5000 Da) [59].

1.3.1 Direct assay

a) Direct detection (figure 1.13) use to measure macromolecule which can easily detect although has low concentration. By immobilize ligand onto the surface and flow analyte pass over the surface. Specific interaction of analyte to their probe will occur and sense in SPR signal shift.

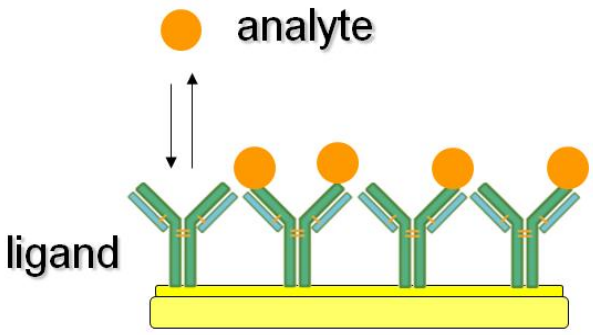


Figure 1.13 Illustration of direct detection which analyte attached to the sensor surface

b) Sandwich assay (figure 1.14) use to enhance the binding signal from direct detection. After the analyte bind to their specific probe, second molecules for enhancement are flow over the surface and they will bind to the analyte resulting in increasing the binding signal. Hence, sandwich assay can improve limit of detection and can provide specificity. For example, analyte is a mixture of IgG antibody then can use second molecule (anti-IgG antibody) for identify the interesting analyte from the mixture. The use of sandwich assay in SPR technique has advantage due to no need to label the second molecule, unlike the other technique (ELISA or RIA).

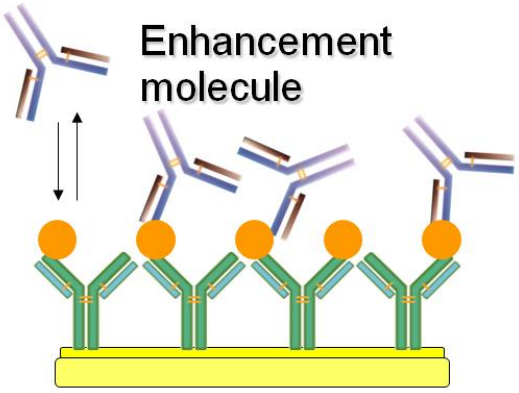


Figure 1.14 Illustration of sandwich assay; an extension of single step direct approach (enhance the analyte response)

1.3.2 Indirect assay

a) Competitive assay (figure 1.15) suitable for the small molecules which are difficult to attach to the sensor surface. Native analyte and analyte conjugated to a larger protein compete to bind to an immobilized ligand on the surface. Inverse relationship between concentration of analyte and sensor response could be observed.

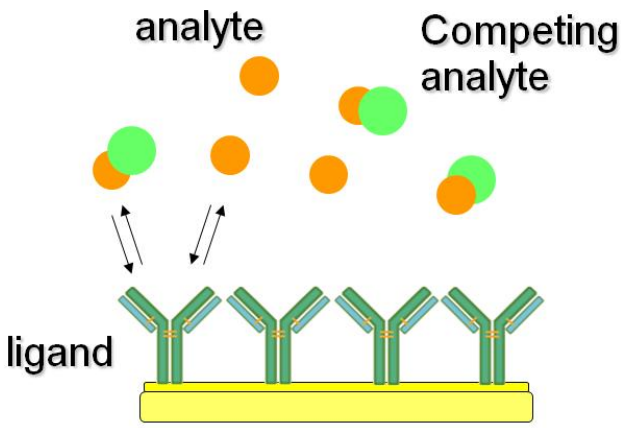


Figure 1.15 Illustration of competitive assay

b) Inhibition assay (figure 1.16), analyte is incubated with a fixed concentration of antibody. This incubation solution is then passed over a surface. Free antibody binds to the sensor surface giving the inverse relationship between concentration of analyte and signal response [59].

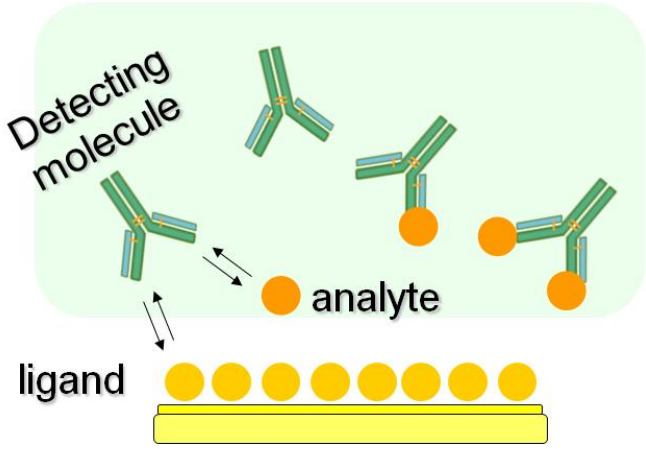


Figure 1.16 Illustration of inhibition assay; the analyte attached to the surface and detecting molecule in solution

CHAPTER II
DETECTION OF MICROALBUMIN BY SPR TECHNIQUE

2.1 Introduction

Surface plasmon resonance (SPR) has gained a wide spread interested for the studying of ligand and the analyte binding because it is a sensitive technique, labeling free and real time monitoring [20, 40]. The signal detection is based on the monitoring of changed in refractive index on the sensor surface. SPR in immunoassay has been broadly used in many biomolecular detections where the affinity of binding (strong or weak) and the amount of binding (large or few) can be monitored [12-14, 60-62]. However, the binding property on the surface does not solely depend on the interactive properties between the ligand and the analyte but the physical and chemical properties of the surface are also interplay. Self assemble monolayer (SAM) is the molecule which has thiol functionality and can be spontaneously adsorbed from solution onto gold substrate and formed a monolayer [63]. The mixed SAM (mSAM) consisting of two thiol with different chain length or functionality that promote the immobilization. Mixed SAMs can reduce the steric hindrance from terminate group of SAMs by making a spacer [54, 55]. However, the mSAM, considered as 2-dimensional surface (2D), has lower ligand immobilization capacity when compared to the 3 dimensional (3D) surface, as dextran surface [40, 55, 56] . Dextran is an α-1,6 glycosidic linked of glucose molecules and branching out from the backbone units at α-1,3 linked, along with its hydroplilicity that causes swelling and forming into 3D structure [40] . In this paper, 2D sensor surface (mSAM) was compared to 3D sensor surface (CMD) for the detection of human serum albumin (HSA) in urine.

HSA is a 67 kDa protein and is the most abundant protein in human serum. HSA has many functions such as, transports biomolecules (hormones, fatty acids, unconjugated bilirubin, drugs) [64]. For the healthy person, the HSA is rarely filtered through glomerular basement and usually undetected in the urine, called albuminuria. The amount of albuminuria is divided in to two major category, based upon the level

of the albumin, which are (a) microalbuminuria; where the level of albuminuria is between 20-200 μg/mL or 30-300 mg/24h and (b) macroalbuminuria; where the level of albuminuria is higher than 200 μg/mL or higher than 300 mg/24h [65-68]. To evaluate the presence of microalbuminuria in diabetic patient is very important because it is the marker of diabetic nephropathy, also as the independent risk factor of hypertensive cardiovascular disease [69-73]. The abitlity to quantify the amount of albuminuria at the early stage is very crucial for the management of diabetic patient in order to improve the disease prognosis.

There are several techniques to determine albumin level in the urine [66]. Many immunoassays are widely used in clinical laboratory for measurement of microalbuminuria especially particle enhanced technique, turbidimetry and nephelometry, but still having many constraints such as the detection limit above 20 μg/mL, prozone effect, turbid sample, low throughput and unreusability. Applying the SPR technique for detection of microalbuminuria is presumed to get more sensitivity, reusability and high throughput. It is also has the possibility to combine with some other marker to form into the array detection where various analyte can be monitored at one time. In this work, two sensor surfaces, mSAM and CMD, immobilized with mAb and pAb were compared. The signal enhancement and the assay technique were evaluated in order to improve the detection of the albuminurea. The amount of the albuminurea was measured by SPR technique and compared to the standard turbidity test.

2.2 Literature reviews

The present of albumin protein in urine is the marker that indicated the diabetic nephropathy and also important risk factor of hypertensive cardiovascular disease. In normal condition the concentration of albumin is lower than 20 mg/L in urine but in patient who has the problem with renal dysfunction albumin present increase in urine. Microalbuminuria is term of albumin excrete in urine 20-200 mg/L [73, 74]. So quantitative measurement of albumin in urine is important to an early diagnostic it can prevent the nephropathy patient form the renal dysfunction [75].

Screening of urine albumin excretion is the routine work for the nephropathy patient who has a risk of renal disease and hypertension. The high sensitivity technique is needed for microalbumin detection due to the low concentration of albumin cannot detect by standard laboratory technique (dipstick test) [76].

There are several techniques which can be used for microalbumin detection such as radioimmunoassay (RIA) [77], ELISA [77-79], immunoturbidimetric assay (ITA) and immunonephelometric assay (INA) [80-82]. The two screening method for microalbuminuria are dipstick technique and immunoassay method [83]. Dipstick test is rapid and convenient method; nevertheless, it is unable to measure the low level of albumin in urine and the reported result is semi-quantitative. The immunoassay based technique can measure low level of albumin in urine and they are quantitative interpretation the details are shown in table 2.1.

Table 2.1 Screening method for microalbuminuria [83]

	Dipstick	Direct Measurement (Albumin:Creatinine Ratio)
Method	Colorimetric test	Immunoturbidiometric, radioimmunoassay, high-performance liquid chromatography
Quantitative	No	Yes
Units	mg/L	mg albumin/g creatinine
Sample requirement	Random urine	Random urine
Advantages	Office measurement	Quantitative, preferred method for confirmation of microalbuminuria
	Easy to perform	More accurate
	Immediate result	
Disadvantages	Not a quantitative test	Not immediate
	Office staff time	Cost (see below)
	Not definitive (albumin:creatinine ratio required for quantification and confirmation)	
	Less accurate	
Cost per test	$4–$6	$10–$20

Therefore the high sensitivity technique is needed for microalbumin detection. The previous work was reported the comparison of three methods that use for quantifying microalbuminuria which perform in table 2.2. INA and RIA allow the similar results even though RIA has quite more sensitivity than INA with detection limit 0.1-0.3 mg/L. RIA have disadvantage because it is expensive, time consuming and required a use of radioactive substance. INA is suitable for routine work with a convenient and rapid method. ITA method is similar to RIA with the detection limit

around 5-8 mg/L which is higher than RIA. ITA is suitable for the large-scale screening because it is rapid, easy and reliable. RIA and NIA are proper for detection of albumin in normal condition [74].

Table 2.2 Comparison of immunoassay technique for microalbuminuria

Immuno assay	Details	Advantage	Disadvantage	Detection limit range (mg/L)	Assay time
Radioimmuno assay (RIA)	Use the radioactive tracer molecule to detect the amount of albumin	-Sensitive	-Expensive -Time consuming -Not suitable for the assay of small number of samples - radioactive reagent	0.1-0.3	Approximately 40 samples in 2-3 h
Immunonephelo-metric assay (INA)		-Similar accuracy and comparable result with RIA - suitable for routine - easier and faster than RIA	- Sensitivity and accuracy lower than RIA	0.5-1.0	160-180 samples in 1 h
Immunoturbidi-metric assay (ITA)	When interaction of albumin and its antibody occur it form the immuno-complex then cause turbidity. The albumin concentration will measure by spectroscopic technique	-Suitable for large scale screening - tight correlation with RIA - suitable for routine	-Sensitive lower than RIA and INA - accuracy lower than RIA	5-8	Approximately 20 samples in 30-60 min

The other works was published by using piezoelectric immunosensor for detect human serum albumin (HSA). Monoclonal anti-HSA were immobilized on a quartz-crystal sensor and HSA solution concentration range 0-20 ppm were flow pass the surface. Polyclonal anti-HSA was used to enhance signal by sandwich assay. This technique showed sensitivity about 20 Hz ppm^{-1} and the sensor surface able to repeat up to 30 times [84]. The detection of HSA by scanning tunneling microscopy (STM) was reported to be able to detect the HSA at the level of 100 fg/mL [85].

This research work proposes SPR technique which is the new tool for measurement low concentration of albumin (lower than 30 μg/mL). This is low cost technique which suitable for screening and follow up people who may have an abnormal with renal function by measurement of albumin in urine. SPR is an immunoassay which have the advantages of label free system, high sensitivity, reusable and high throughput as an array platform. The standard method that are used to compare with SPR technique is immunoturbidimetric method which is the clinical method that use in Ramathibodi Hospital.

2.3 Objective

1. To find the suitable surface and proper assay for detection of human albumin (HSA) by using SPR technique.
2. To detect microalbumin or human albumin in urine by SPR technique and correlate the signal with standard turbidimetric method.

2.4 Experiment

2.4.1 SPR Instrument

The SPR imaging setup is a custom made unit equipped with a 7-channels flow cell [86]. Generating of SPR phenomena based on the Kretschmann configuration and coupling prism. The light emitting diode (LED), a light source, with a centered wavelength at 880 nm and a CCD camera (Sony, XC-EI50) is used as a

detector. The SPR sensor chip is attached on glass prism along with an index matching gel (Cargille Labs.). The 7-channels flow cell is made of polydimethylsiloxane (PDMS) covered on the top of sensor surface via mechanical clamps. The volume of each flow cell channel is 5 μL. Buffers and samples are pumped into the flow cell by a multi-channels peristaltic pump (Ismatec).

The reflected images from the SPR chip were collected by the CCD camera at the imaging angle which was adjusted in a linear region of the SPR curve to get the highest image contrast. The selected incident angle was kept constant throughout the experiment. The relationship between the reflectivity change and the refractive index unit (RIU) was established just before the experiments, by using the solutions with known refractive indices. The term “reflectivity” in this report was defined as the ratio of the p-polarized light intensity to the s-polarized light intensity Sample concentrations were carefully chosen to ensure that all succeeding SPR signals were well within a linear respond curve [26].

2.4.2 Equipment and Reagents

Glass BK7 substrate coated with 50 nm layer of gold (Ssens, Netherlands), mSAM: 11-mercaptoundecanoic acid, (11-MUA) and 3-mercapto-1-propanol, (3-MPOH), CMD: Dextran MW 500 kDa (Pharmacosmos A/S, Denmark), *Amine coupling reagents:* N-hydroxysuccinimide (NHS), N-ethyl-N-(3-diethyl-aminopropyl) carbodiimide (EDC), *Blocking reagents:* 1 mg/mL Casein, pH 8.5 Ethanolamine, *Regenerative reagents:* pH 2 Glycine (Research Organics, Ohio, USA), 10 mM and 50 mM NaOH, 10 mM and 100 mM HCl, *Running buffers:* 0.05% and 0.005% Phosphate Buffer Saline with Tween 20 pH 7.4 (PBST), *Antibodies and Albumin:* anti-HSA monoclonal (mAb) and polyclonal (pAb), Standard Human Serum Albumin (HSA) (Serotec), *Combur 10 urine test strip:* (Roche), *Patient urine samples:* Samples and the measured value (Cobas c111® Turbidimetry) at the microscopy unit, pathology department, Ramathibodi hospital.

2.4.3 Preparation of sensor surfaces

2.4.3.1 Preparation of mixed SAM surface

The mSAM sensor surface was prepared by cleaning the gold substrate with piranha solution (70:30 ratio of H_2SO_4/ H_2O_2), rinsed with DI water, dried with nitrogen gas and followed with plasma cleaner [PDC-32G (115v), Harrick Plasma, USA.] for 5 min. The formation of mSAM was carried out by placing a gold substrate in the mixtures of 1:10 mole ratio of 11-mercaptoundecanoic acid (11-MUA) and 3-mercapto-1-propanol (3-MPOH) in ethanol then incubated at least 12 hrs. Finallly, the substrate was rinsed with ethanol, DI water and dried with nitrogen gas [56] .

2.4.3.2 Preparation of CMD sensor surface

The CMD was prepared according to the previous report [57]. The gold substrate was cleaned with piranha solution as described previously. After cleaning, the surface was reacted with 11-hydroxy-1-undecanethiol in ethanol/water (80/20) solution. A monolayer was formed with the complete wetting surface. The epichlorohydrin was attached to the terminal hydroxy group yielding the epoxy group that will react with dextran molecular weight 500 kDa. The dextran was converted into carboxydextran via reacting with bromoacetic acid.

2.4.3.3 Immobilization of anti-HSA on mSAM and CMD

The antibody immobilization was carried out within the flow cell and the running buffer was PBST 0.05% for mSAM and PBST 0.005% for CMD, respectively. The carboxilic group was activated by amine coupling reagent (150 μl of 0.4M EDC/0.1M NHS) for about 10 min to form reactive N-ester group. Monoclonal anti-HSA (mAb-HSA) and polyclonal anti-HSA (pAb-HSA) at different concentrations in sodium acetate buffer pH 5 were flowed over the surface where they were linked to the surfaces via amide linkage. One mg/mL casein was used as the blocking agent to block the rest of the active groups on mSAM while ethanolamine pH 8.5 was used as the blocking agent for the CMD. Glycine pH 2.0 volume 100 μL was injected for washing out non-covalently bound anti-HSA from the surfaces.

2.4.4 Detection and Regeneration

2.4.4.1 Direct HSA detection assay

After completion of the immobilization step, the running buffer was flown over the surfaces at rate 15 μL/min. Concentration of HSA at 0.1-5.0 μg/mL volume 100 μL were injected into the flow channels while monitoring the SPR signal. The signal was left to stabilize for 15 minute prior to the SPR signal evaluation.

2.4.4.2 Enhancing HSA detection assay

Surfaces were immobilized by 40 μg/mL of mAb-HSA or pAb-HSA, then the HSA at 0.1-10.0 μg/mL concentrations were injected in the separated flow channel. The enhancing step was done by injecting 100 μL of 5.0 μg/mL mAb-HSA or pAb-HSA. The PBS buffer was immediately passed over the surface followed the antibody. The binding signal was obtained after the PBS buffer was on the sensor surface. The SPR signals were compared among each of the HSA concentration. The notation used for this work was described in order of the interaction from the sensor surface from bottom to the top and they were separated by slash which were surface platform (mSAM or CMD)/immobilized antibody (pAb or mAb)/HSA antigen (HSA or urine albumin u-HSA)/signal enhanced antibody (pAb or mAb).

2.4.4.3 Urine HSA (u-HSA) detection assay

The urine samples were centrifuged at 3,000 rpm (1,000 g) for 2 minutes to precipitate the sediment. Only the supernatant was used and diluted with PBST 0.005% at 1:10 and 1:100 dilutions prior to the experiment. The interference in the urine was evaluated by Combur 10 urine strip (Roche, Germany) to see the effect of interference to microalbumin detection by SPR technique. The CMD/mAb /HSA/pAb platform was chosen to measure the SPR signals. The signals were converted into the albumin concentration (μg/mL) by interpolation on the calibration curve and correlated with the value from standard turbidimetric assay.

2.4.4.4 Regeneration of surface

The pH 2 Glycine, 10 mM and 50 mM NaOH as the regenerate reagents were compared with each other on the CMD, only pH 2 Glycine was used on the rest of the experiment.

2.4.4.5 Turbidimetric assay

Turbidity is produced by an immune complex of HSA in urine and anti-HSA. When HSA in urine sample is mixed with anti-HSA the aggregation occurred by antigen-antibody reaction. Turbidity is measured by spectrophotometer due to the reduction of light that scattered from an immune complex which proportion to their size, shape and concentration. Albumin in the sample is quantitatively determined. The turbidity test for all urine samples were received from Ramathibodi hospital.

2.5 Results and Discussion

2.5.1 Immobilized antibodies signals

The optimum amount of the immobilized antibody surface density was obtained from immobilized both mAb and pAb onto mSAM and CMD following by passing the HSA over the immobilized antibody surface to evaluate the SPR signal. The surface that yielded the highest respond was chosen for further study. The mAb- and pAb volume 100 μL at different concentration 5, 20, and 40 μg/mL in sodium acetate buffer pH 5 were injected into each flow channel. The optimum pH 5 was obtained from the scouting experiment [39, 87]. The immobilized of all antibodies on both mSAM and CMD were reported in μRIU, as shown in figure 2.1.

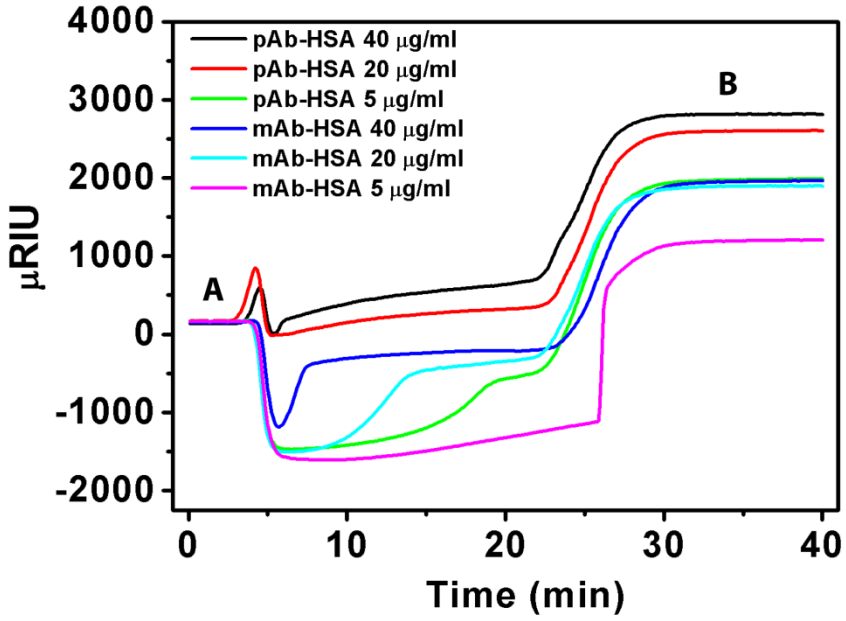


Figure 2.1 The SPR sensorgram of immobilization of anti-HSA on mSAM surface. At A starting injection of antibody and at B the signal reach plateau which mean antibody bound on surface

On the CMD, the anti-HSA was immobilized via amine coupling reaction through carboxylic group of dextran. The amount of immobilized signal reflects the amount of anti-HSA per surface area (antibodies density) and the signals were also increased consistently with the anti-HSA concentration. On the mSAM surface, the amount of immobilized antibodies was increased and saturated at about 10 μg/mL for both of mAb and pAb. On the CMD, the signals saturated about 30 μg/mL for pAb but still increasing for mAb, as shown in figure 2.2. The signals on the CMD were greater than the mSAM at the same anti-HSA concentration due to more available coupling sites of the CMD branched-chain.

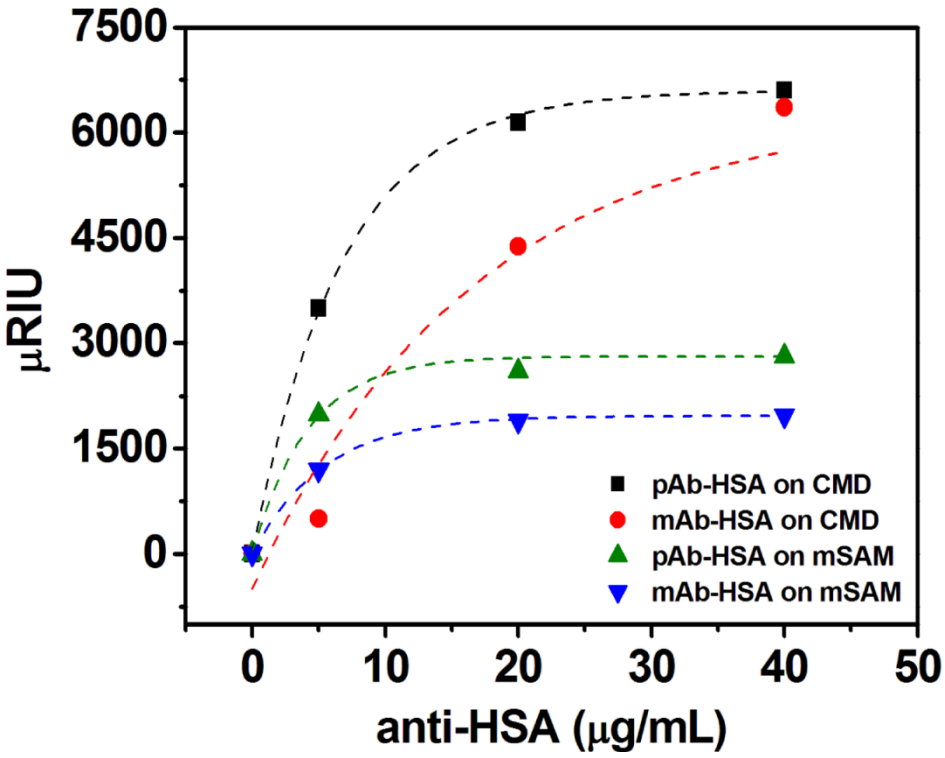


Figure 2.2 The immobilized anti-HSA signals on mSAM and CMD surfaces

The mSAM of 11-MUA and 3-MPOH at the 1:10 mole ratio has an estimated area for the active carboxylic site at around 200 $\mathring{A}^2$ which is suitable for the size of IgG type antibody [14, 52, 88, 89]. The area for immobilization of the mSAM is in the 2D planar in contrast with the CMD is extended into 3rd dimension along its grafted chain providing a higher number of the immobilizing site for the antibody. At the excess antibodies concentration, the signals would not increase and the surface was fully covered by the antibodies. The mSAM had less available immobilizing sites and the immobilization signal reached saturation before the CMD. The saturated point was starting at the plateau line of each curve, as shown in figure 2.2. The surfaces could be roughly divided into two cases, the low coverage surface (before plateau) and the full coverage surface (beyond plateau). The immobilized signals of the pAb were greater than the mAb at any given concentrations on both surface types and the lot variation might be explained; however, the further investigation, such as changing the lot, using antibody from other animal types were needed.

2.5.2 Direct HSA detection assay

2.5.2.1 Direct detection on mSAM surface

The direct HSA detection on the mSAM was performed by using the diluted standard HSA in PBST 0.05% at the concentration range 0.1-5 μg/mL and injected through the set of immobilized anti-HSA surfaces.

The result of anti-HSA above 20 μg/mL, the signals were equal on the same type antibody surface but obviously different from the other type of antibody, as shown in figure 2.3 (a and b). On the low coverage surface, the signals were depended on two factors, (i) the anti-HSA surface density and (ii) the HSA concentration. The amount of bound HSA on the low coverage surface is related directly to the surface density of anti-HSA. The signal obtained in this case is rather straight forward where the lower the concentration, the lower the signal obtained. The saturation of the signal was observed when the amount of HSA was increased until all the available anti-HSA sites were fully occupied. A similar result was observed for both the immobilized surface of mAb and pAb antibody.

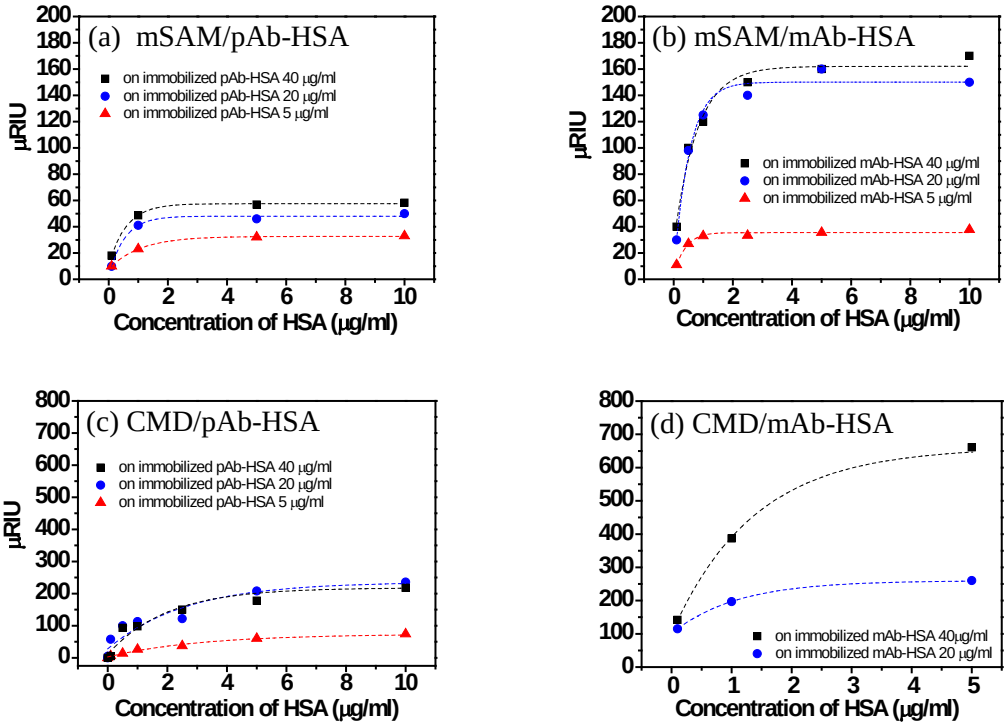


Figure 2.3 Direct HSA detection signals on mSAM surface immobilized with; (a) polyclonal anti-HSA (pAb-HSA), (b) monoclonal anti-HSA (mAb-HSA) and on CMD surface immobilized with; (c) pAb-HSA, (d) mAb-HSA.

On the full coverage surface, the signals were depended on (i) the type of anti-HSA (mAb or pAb) and (ii) the HSA concentration. For the full coverage surface, the distinctive property between the mAb and the pAb was observed. The each of mAb recognizes the same epitope of the antigen while the each of pAb recognizes multiple epitope of the antigen. On the full coverage surface, the pAb molecules were closely enough to bind the same HSA molecule, called multiple site binding, but this was not happened to the mAb (one anti-HSA for one HSA), as shown in figure 2.4 (b). The additional reason was that the mAb possesses a higher affinity toward the HSA than the pAb and causing higher amount of bound HSA molecules.

The SPR signal is based upon the refractive index change and the refractive index change is based upon the additional mass on the sensor surface. Considering of the HSA mass that is around 67 kDa and the anti-HSA mass that is around 150 kDa (IgG type). The IgG type antibody has two binding arms and if both

arms are occupied by two HSA, the detected signal will be estimated around the immobilized signal. Raising the full coverage surface as the example, the immobilized signal of mAb was 1,500 μRIU but the detected signal was only 170 μRIU, the immobilized signal of pAb was 2,250 μRIU but the detected signal was only 60 μRIU. This indicated that not all immobilized antibodies was participating in the HSA binding and the loss of binding capacity was assumed from the two reasons. First, the randomly amine coupling that impaired some of the binding sites. Second, the steric hindrance that prevented anti-HSA molecule to bind two HSA molecules at the same time, especially on tightly immobilized antibody surface [88, 90, 91], as shown in figure 4. Moreover for the pAb surface the signal was further affected by the multiple site binding, as previous described.

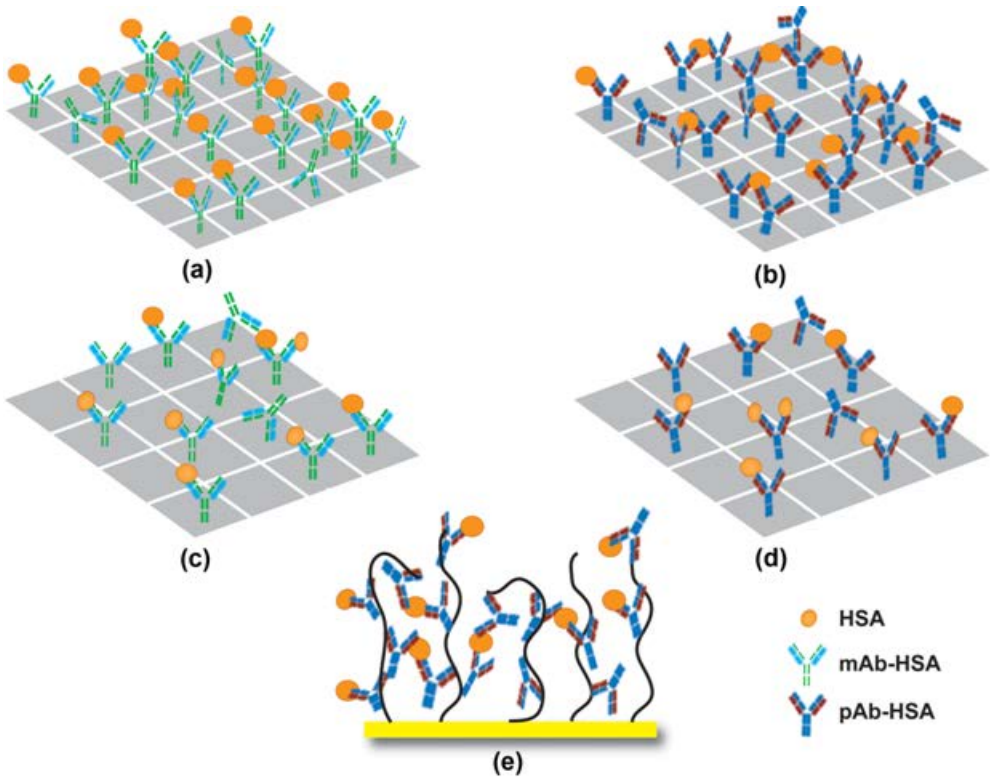
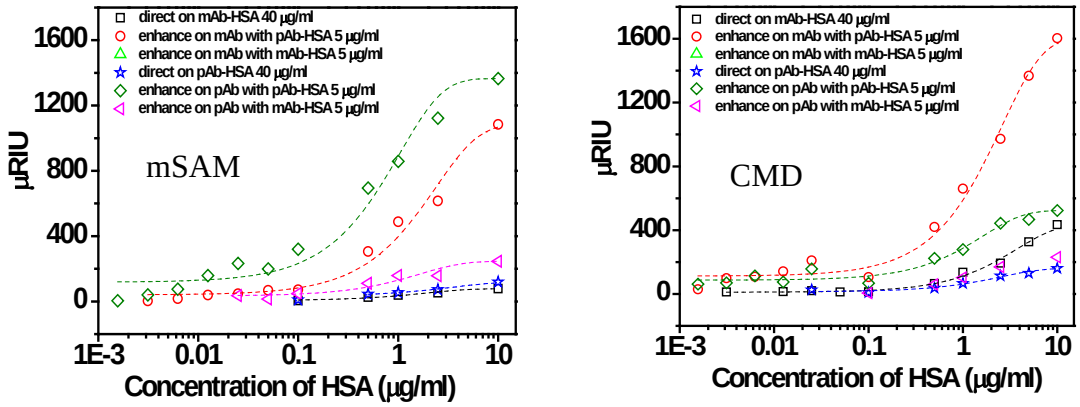


Figure 2.4 Distribution and orientation of the immobilized antibodies images on mSAM surface. (a,b) Full coverage surface, (c,d) Low coverage surface, (a,c) mAb-HSA and (b,d) pAb-HSA. And (e) the multiple site binding of pAb-HSA on the CMD.

Quantification of the HSA concentration use the characteristic of S-curve fitting to determine lower limit of detection (LOD) and resolution (see figure 2.5 (a)). The LOD was the lowest HSA concentration to give the significant signal (3 times of standard deviation, 3SD, above noise signal) and the resolution was calculated from the total signal gained per HSA concentration (slope). For the immobilized mAb, the LOD was 0.1 μg/mL and the resolution was 37.09 μRIU per μg/mL. For the immobilized pAb, the LOD was 0.1 μg/mL and the resolution was 57.58 μRIU per μg/mL, as shown on table 2.3.



(a) (b)

Figure 2.5 Characteristic S-curve fitting of HSA binding signal by direct detection on immobilized with mAb-HSA and pAb-HSA 40 μg/mL and enhance signal with mAb-HSA and pAb-HSA 5 μg/mL on (a) mSAM and (b) CMD surface

Table 2.3 LOD, Resolution, Improved factor and Applicability of each platform

Surface type	Immobilized Ab	Enhancing Ab	LOD (μg/mL)	Resolution (μRIU per μg/mL)	Improved factor	Applicability
mSAM	mAb-HSA	no	0.1	37.09	-	+
		mAb-HSA	0.1	37.09	0	
		pAb-HSA	0.006	496.28	13.38	++++
	pAb-HSA	no	0.1	57.58	-	+
		mAb-HSA	0.03	94.83	1.65	+
		pAb-HSA	0.003	533.34	9.26	+++++
CMD	mAb-HSA	no	0.01	279.60	-	+++
		mAb-HSA	0.01	279.60	0	
		pAb-HSA	0.001	929.44	3.32	++++++
	pAb-HSA	no	0.1	95.08	-	+
		mAb-HSA	0.1	132.94	1.40	++
		pAb-HSA	0.01	241.17	2.54	+++

Note : ++++++ = best, +++++ = good, ++++ = fair, +++ = poor, ++ = bad, + = worst

2.5.2.2 Direct detection on CMD surface

The direct HSA detection on the CMD was performed as the mSAM, excepted for PBST 0.005% as diluents and running buffer. For a given type of immobilized antibody, the signals for HSA binding to the immobilized antibody on CMD were higher than those of mSAM due to the higher number of the immobilized antibodies on the CMD (figure 2.2).

The amount of immobilized 40 μg/mL mAb, 6,360 μRIU, was higher than those of 20 μg/mL, 4,380 μRIU. As the result, the signal of HSA bound to immobilized antibody was higher due to a larger number of the binding site available on the sensor surface, figure 2.3 (c and d). The number of HSA bind to immobilized mAb was in a direct proportion to the amount of the immobilized mAb because each mAb can only bind to one HSA molucule due to its monoclonal nature. This was different from the case of the immobilized polyclonal antibody where each of the immobilized pAb can bind to more than one epitope of the HSA molecule. As a result, the same HSA molecule may be bound with more than one immobilized pAb if the immobilized pAb were in close proximity, figure 2.4 (e). This can be seen clearly that the amount the immobilized pAb at 20 μg/mL, 6,145 μRIU, and 40 μg/mL, 6,600 μRIU, were slightly higher than the immobized mAb at the same concentration, figure

2.2. However the amount of the HSA bound to the immobilized pAb were far lower than those of mAb. This result was supported the concept of the "multiple site binding" of the HSA molecule to the immobilized pAb. The CMD can also provide the flexibility to the CMD/pAb-HSA due to the long branched-chain which may facilitate the binding.

About the LOD and resolution, for the immobilized mAb, the LOD was 0.01 μg/mL and the resolution was 279.60 μRIU per μg/mL. For the immobilized pAb, the LOD was 0.1 μg/mL and the resolution was 95.08 μRIU per μg/mL, as shown on table 2.3. For the direct detection platform, the better LOD and resolution were obtained from the CMD over the mSAM and the mAb over pAb. This was concordant to the HSA binding capacity on each surface type.

2.5.3 Enhancing HSA detection assay

2.5.3.1 Enhancing HSA detection on mSAM surface

The signals were enhanced by concomitantly injected 5 μg/mL of mAb or pAb after finishing the direct detection step then the additional and total signals were recorded. The 5 μg/mL anti-HSA was found to be an optimum amount for enhancing signal in the range of detection. The concept of the signal enhancement was to increase the amount of the signal detected by increasing the mass, or refractive index, of the detected molecule. The direct and enhancing signals at each HSA concentration were converted into plotted equation, along with the improved factor that was calculated from the division of the enhancing detection slope by the direct detection slope, as shown in figure 2.7 (a). The improved factor implied to the enhancing effectiveness of each assay type.

For the mAb/HSA/pAb surface, the improved factor was 13.38 but for mAb/HSA/mAb was no additional signal, as shown in figure 2.6 (a). The signals were enhanced by pAb but not by mAb. Because the several pAb bound to the different epitopes on the one HSA molecule while the one mAb can bind only to specific epitope on the one HSA molecule. As a result, if HSA molecule was bound to immobilized mAb, there would be no binding site left for any additional binding of the additional mAb, the enhanced molecule.

For the pAb/HSA/pAb surface, the improved factor was 9.26 and for pAb/HSA/mAb was 1.65, as shown in figure 2.6 (c). The reason for pAb enhancement as described above but the enhancement from mAb was due to the one additional mAb molecule to the altered epitope not already occupied by the surface pAb.

About the LOD and resolution, for the mAb/HSA/pAb, the LOD was 0.006 μg/mL and the resolution was 496.28 μRIU per μg/mL. For the mAb/HSA/mAb, the LOD and the resolution were same as the direct detection. For the pAb/HSA/pAb, the LOD was 0.003 μg/mL and the resolution was 533.34 μRIU per μg/mL. And for the pAb/HSA/mAb, the LOD was 0.03 μg/mL and the resolution was 94.83 μRIU per μg/mL, as shown on table 2.3.

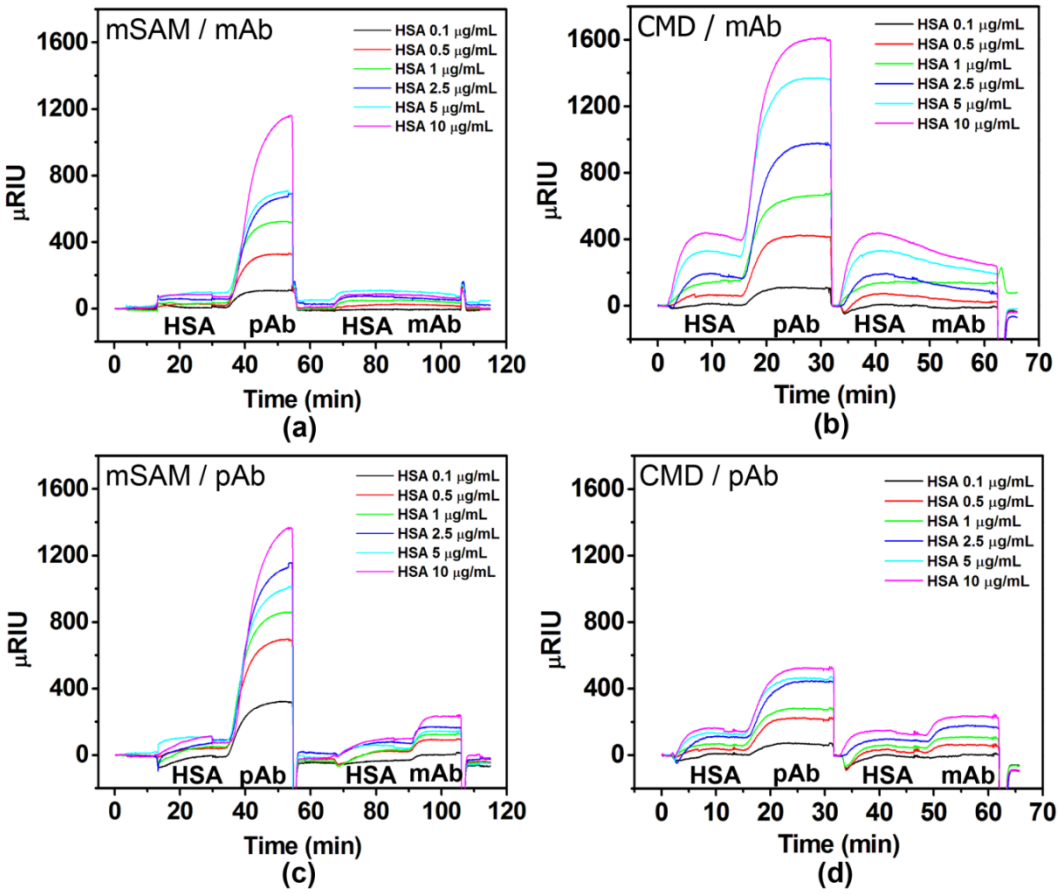


Figure 2.6 Enhancing HSA detection signals on (a,c) mSAM surface, (b,d) CMD surface.

2.5.3.2 Enhancing HSA detection on CMD surface

The enhancement was done in the same way as on the mSAM. For the mAb/HSA/pAb surface, the improved factor was 3.32 but for mAb/HSA/mAb was no additional signal as shown in figure 2.6 (b). For the pAb/HSA/pAb surface, the improved factor was 2.54 and for pAb/HSA/mAb was 1.40. The trend of enhancement also depended on the antibody type as occurring on the mSAM, excepted for pAb/HSA/pAb surface that the signals were prominently low due to the multiple site binding effect on HSA molecule, as shown in figure 2.6 (d). The long branched-chain of CMD increased the immobilizing power but forfeit for the pAb suface because of easily multiple site binding phenomenon, as shown in figure 2.4 (e).

About the LOD and resolution, for the mAb/HSA/pAb surface, the LOD was 0.001 μg/mL and the resolution was 929.44 μRIU per μg/mL. For the mAb/HSA/mAb, the LOD and the resolution were same as the direct detection. For the pAb/HSA/pAb, the LOD was 0.01 μg/mL and the resolution was 241.17 μRIU per μg/mL. And for the pAb/HSA/mAb, the LOD was 0.1 μg/mL and the resolution was 132.94 μRIU per μg/mL, as shown on table 2.3.

The improved factors could be ranked as, mSAM/mAb/HSA/pAb, mSAM/pAb/HSA/pAb, CMD/mAb/HSA/pAb, CMD/pAb/HSA/pAb, mSAM/pAb/HSA/mAb and CMD/pAb/HSA/mAb. No improvement on the mSAM/mAb/HSA/mAb and on the CMD/mAb/HSA/mAb, as shown in table 2.3. The mSAM had more potential for enhancement (improved factor) than the CMD because of the less steric hindrance and more sparing space to additional antibodies. Moreover the mAb-HSA surface had the benefit over the pAb-HSA surface, in the aspect of more specificity toward the HSA and more possibility for further enhancement.

The LOD and resolution of each platform depended on the total signal gained at the certain HSA concentration. On the direct detection, the LOD and resolution were concordant to the HSA binding capacity, the CMD over the mSAM and the mAb-HSA over pAb-HSA. On the enhancing detection, the improved factor was included to obtain the final LOD and resolution then the mSAM refined its LOD and resolution via enhancing method over the CMD. Overall the

CMD/mAb/HSA/pAb-HSA was chosen as the platform for urine HSA detection assay because of the surface specificity with the best LOD and resolution.

2.5.4 Urine HSA detection assay

The calibration curve was plotted by the standard HSA solution at 0.02–5 μg/mL against the SPR signal on the CMD/mAb/HSA/pAb platform. The range for quantification was defined by the linear range at HSA 0.1–1.5 μg/mL on semi-log scale (HSA concentration in log scale, ΔRIU in normal scale) and the curve was fitted along with 4 parameter logistic (4PL) [92] for data analysis. A 4PL nonlinear regression model is commonly used for curve fitting analysis in bioassay such as enzyme-labeled antibody; ELISA, labeled-antibody assay; sandwich assay, assay involved with radioimmunoassay; RIA or dose response curve [93-95]. The equation can be wrote as F(x) = [(A-D)/(1+((x/c)^B))] + D. By x is concentration of analyte, F(x) is SPR signal, A is minimum SPR signal (-0.41), B is slope or steepness of curve (0.86), c is inflection point or concentration producing 50% the maximum SPR signal (0.17), D is maximum SPR signal (3.74). The urine samples, concentration in the range of 1–130 μg/mL, total 31 samples were centrifuged at 3000 rpm (1000 g) to obtain the supernatant for further analysis. Each of the supernatant was diluted into 1:10 and 1:100 dilution then both were analyzed on the platform. The two dilutions were required in order to cover the concentration range of the samples. The remained supernatants were evaluated for the interference by Combur 10 urine strip test.

Most of the samples had the HSA concentration around 1–40 μg/mL and the interferences were found in most of the samples, mostly were contaminated with nitrite, glucose, bilirubin and leukocyte, as shown on table 2.4. The interference had no impact on the SPR measurement. The SPR value was compared with the turbidimetric value and showed no statistical different (pair t-test) along with the good correlation with $R^2 = 0.94$, as shown in figure 2.7 (b).

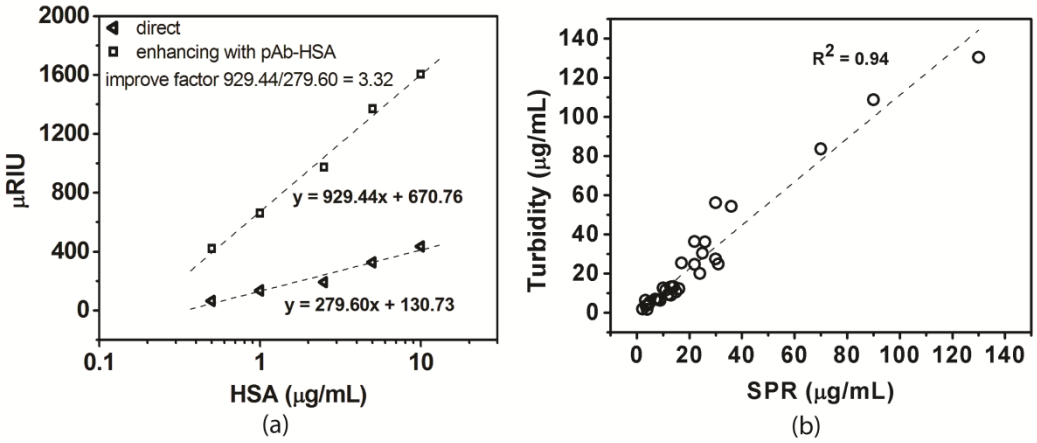


Figure 2.7 (a) Direct and enhancing detection plot of the CMD/mAb/HSA/pAb and (b) the correlation of HSA concentration in urine between turbidimetric and SPR technique.

Table 2.4 The HSA concentration in urine samples and the interference

HSA in Urine (μg/ml)	Sample (n)	Interference (n)							
		LEU	NIT	PRO	GLU	KET	UBG	BIL	Hb
1 - 9.99	12	5	10	-	4	-	-	2	-
10 – 19.99	7	1	5	-	4	-	-	3	1
20 – 29.99	4	-	2	-	1	-	-	-	1
30 – 39.99	3	-	2	-	-	1	1	2	1
50 – 59.99	2	2	2	1	-	-	-	1	-
100 – 109.99	1	-	-	-	-	-	-	-	-
130 – 139.99	1	-	-	1	1	-	-	1	-
Total	30	8	21	2	10	1	1	9	3

Note : LEU = Leukocyte, NIT = Nitrite, PRO = Protein, GLU = glucose, KET = ketone bodies, UBG = Urobilinogen, BIL = Bilirubin, Hb = Hemogobin & Red blood cell

2.5.5 Regeneration of sensor surface

To evaluate for the suited regenerative reagent and to study the effect against the immobilized CMD/anti-HSA surface, the pH 2 Glycine, 10mM HCl, 100mM HCl, 10mM NaOH and 50 mM NaOH were among the candidates. The violence of regenerate solution was sorted by stability of surface after regenerate 15

cycles which shown in figure 2.8. All reagent showed the negative baseline shifting except 10 mM HCl. Glycine pH 2 gave the lowest negative baseline shifting (-141.68 μRIU). The others were too harsh due to more drifting of baseline which were 10 mM NaOH (-206.08 μRIU) , 100 mM HCl (-287.22 μRIU) and 50 mM NaOH (-468.83 μRIU). But for 10 mM HCl, base line signal increase for each cycle this may be due to there were still something on the surface (not clearly washed). The signal detection of HSA for 15 cycles were considered as % coefficient of variation (%CV) value which is a measure of dispersion of a probability distribution. It is the ratio of standard deviation to *mean and multiply by 100*. The sequence of %CV from low to high is 10 mM HCl 7.39%, 10 mM NaOH 8.16%, glycine pH2 12.27%, 100 mM HCl 37.04% and 50 mM NaOH 40.23% respectively. From considering both baseline stability and %CV value, the 100 mM HCl and 50 mM NaOH was so strong and deteriorated the surface (negative shifting of the base line) and more fluctuation of binding signal. The 10 mM NaOH was amiable; however it also disrupts the surface (decreasing of base line). The Glycine pH 2 and 10mM HCl was the choice because of the more stable baseline and preserved binding capacity in several cycles run. The average used of the sensor surface was around 20 cycles.

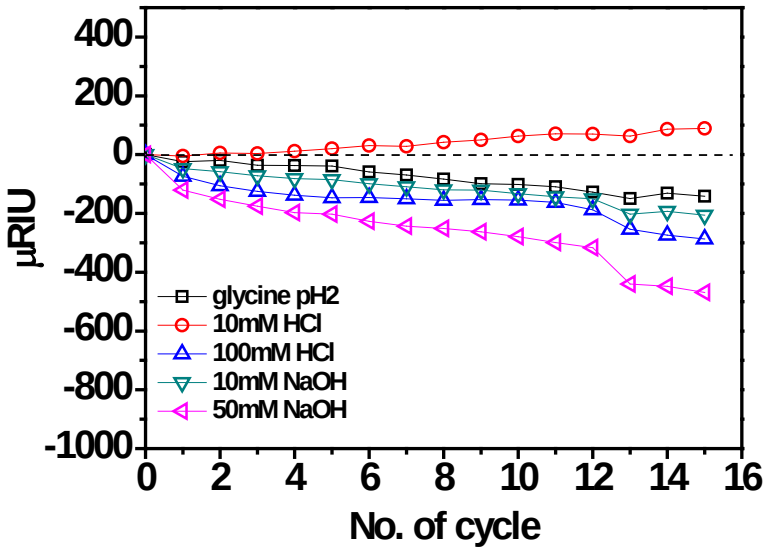


Figure 2.8 Stability test of immobilized anti-HSA on CMD surface; base line signal after regenerate with different reagent for 15 cycles

2.6 Conclusion

Both the mSAM and CMD surfaces served the immobilization of the anti-HSA for the specific HSA detection. The long branched-chain of the CMD promoted the greater binding capacity than the mSAM, and also resulted in a better LOD and resolution. However the monoclonal antibody was suitable for the CMD immobilization to prevent multiple site binding phenomenon that would cause the poor signaling. The enhancing assay improved the LOD and resolution especially, on the mSAM and also maximized the LOD to 6 ng/mL level. The selected platform for urine HSA measurement was the CMD/mAb/HSA/pAb-HSA on the reasons of specificity and the best signaling. Using the SPR technique to quantify the urine HSA concentration, the result correlated well along with the standard turbidimetric assay and this was very convincing to apply in microalbuminuria measurement. The average used of the mAb-HSA platform was around 20 cycles by using glycine pH 2 as regenerate solution. Moreover the SPR technique is a promising analysis platform in the near future, based upon the good sensitivity, reusability and high throughput (in array platform).

CHAPTER III
SPR APPLICATION IN ABO BLOOD TYPING

3.1 Introduction

Surface plasmon resonance (SPR) is an optical technique that measures the surface mass coverage of adsorbed material [8-11, 96] SPR is a sensitive technique for studying interactions between immobilized biomolecules and a solution-phase analyte. The sensitivity and versatility of this label-free, real-time method has advantages over conventional methods such as fluorescence or ELISA (enzyme-linked immunosorbent assay). Commercially available SPR systems, such as the Biacore system, are well-known and commonly used in many experiments [11, 12, 14, 60-62, 92]. The ABO blood system was the first blood-type system [97] and is also the most important system when considering blood transfusion. Conventional ABO blood-typing is based on the agglutination technique, which has limitations including laborious processing, subjective interpretation, qualitative results, and low sensitivity. In the near future, conventional blood-typing may be superseded by new analytical techniques, such as automated gel agglutination or SPR [98]. The SPR technique can specifically detect A or B antigens on whole red blood cells (RBCs) using immobilized monoclonal IgM antibodies [99] . A complete investigation has not been shown regarding SPR blood-typing for clinical applications. The development of SPR imaging (SPRI) is a promising platform for use as a high-throughput bioanalyzer [25, 27, 100]. Applying the SPRI technique for ABO blood-typing has the potential to replace the conventional ABO blood-typing.

The main objective of this study was to develop the real-time antibody array technique for blood-typing based on immunological interaction by SPRI. Specific antibodies to each blood group were immobilized onto a carboxydextran surface via covalent binding as a linear array. The determination of the ABO blood group was done by observing bright spots on specific immobilized antibodies on the

surface. The results obtained from SPR were compared to the conventional agglutination test in order to check the reliability of the technique.

3.2 Relevant principle and literature reviews

A blood group could be defined as an inherited character of the red cell surface detected by alloantibody. The ABO blood group system was discovered by Landsteiner in 1900 [97]. The ABO blood grouping is the most significant for transfusion. The blood compatibility is listed in table 3.1. Blood type will determine by antigen presence on red blood cell surface and antibody in serum. Normally people have antibodies which not bind to their antigen on red cell surface such as a person who has blood type A will has A antigen on red blood cell surface and anti-B in serum.

Table 3.1 ABO blood group: antigen on red cell surface and antibody in serum

Blood Type	Cell Antigen	Serum Antibodies
A	A	B
B	B	A
AB	A and B	None
O	None	A and B

A and B antigens are oligosaccharide. The structures on red cells hold ABO activity are the N-linked oligosaccharides of red cell surface glycoprotein (band 3) and also glycolipids (figure 3.1). Oligosaccharide constructed from monosaccharides sugar. The H antigen present on red cell surface of almost people is a biosynthetic precursor of A and B antigen. The fucosylated Gal structure (H substance) that is converted to the A-active trisaccharide by addition of GalNAc (N-acetyl-D-galactosamine) or to the B-active trisaccharide by addition of Gal (D-galactose) (figure 3.2). In blood group O, H antigen expressed strongly due to it remains unconverted [101].

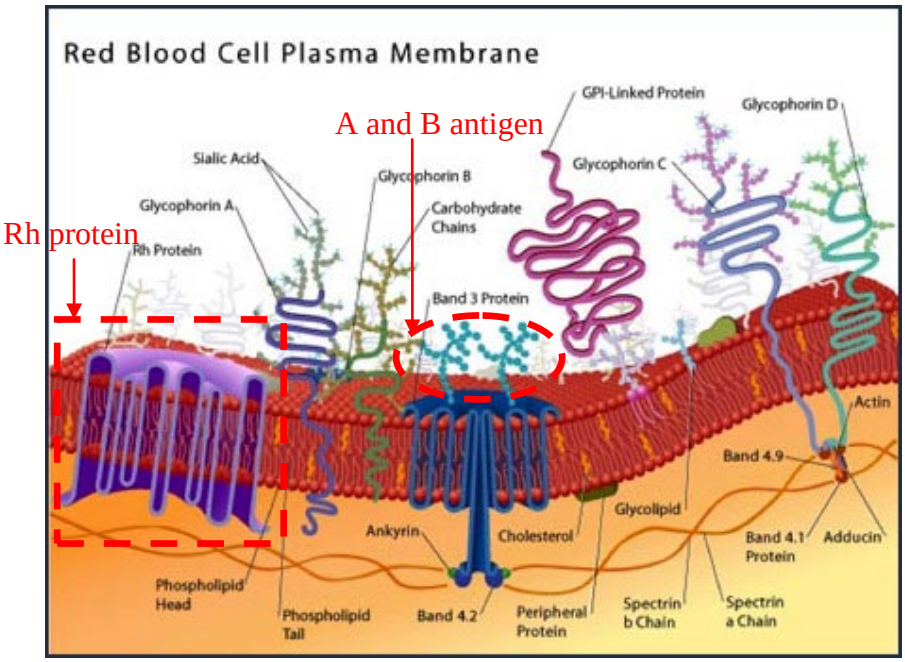


Figure 3.1 Red blood cell membrane; A and B antigen present on the carbohydrate chain of band 3 protein [102]

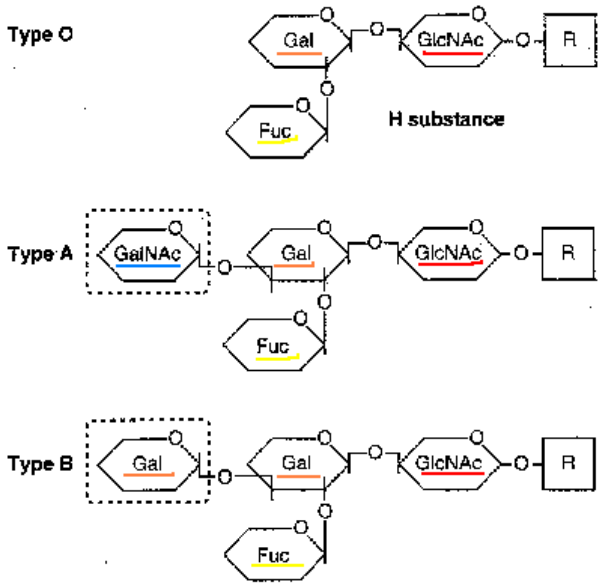


Figure 3.2 Represent A and B oligosaccharide and H substance (type O), the precursor of A and B [103].

The incompatibility of the blood transfusion can cause the death due to the agglutination of the blood. It is necessary to carry out the blood grouping on the blood donation and prior to the use for the treatment.

There are many methods that are used to perform the blood typing. The technique based on the immunological interaction between the surface antigen on the RBC and the IgM antibody. Each technique has its own advantage and disadvantage and they are listed in table 3.2 which reference from Malomgré and Neumeister in 2009 [98] and some modification [104].

Table 3.2 Method for blood typing

Test	Test principle	samples	Time per assay (min)	Cost per assay	Comments	Potential for automation
Slide	Agglutination	Fresh blood	2-3	+	Fast but insensitive, limit only cell grouping, high error	✘
Tube	Agglutination	Blood sample and anti-sera	10–15	++	Moderate sensitivity, Time-consuming	✘
Micro-plates	Agglutination	Blood sample and anti-sera	10–15	++	Moderate sensitivity	✓
Micro column agglutination	Agglutination	Blood sample and anti-sera	10–15	+++	Sensitive than other agglutination technique and easy documentation	✓
Molecular	Nucleic acid amplification techniques (PCR-RFLP, PCR-SSP, PCR-SSO)	Leukocytes	Hours	++++	Highly sensitive, time consuming, no need for rare antisera, requires special knowledge, does not exclude genes that are not expressed from detection	✘
SPR	**Solid phase immunoassay, solid phase DNA probe**	**Blood sample, anti-sera, leukocytes**	**10-15**	**Varied depend on platform**	**Highly sensitive, real-time monitoring, High throughput with array technology, Promising but still confined to experimental laboratories**	✓

From literature, the solid phase techniques that use in ABO blood grouping include beads, test tube, sheets, strips and microtiter plates all made of polystyrene or polyvinylchloride. Forward blood grouping is carry out by immobilizing antibody on solid surface and enzyme activated blood sample undergo immune reaction which indicated by the color. Reverse blood grouping is performed by absorb known antigen onto the surface and enzyme treat is needed. Antibody is determine by the color that presence from immune reaction [105].

Gel agglutination technique will define blood group from complex of red blood cell with their antibody in gel medium [106, 107]. Other gel agglutination technique was reported by using microparticle coated with ligand (antigen or antibody) in the container. Sample solution is added and the container is centrifuged for reading agglutination [108].

There is hemaagglutination assay using monoclonal antibody panel to distinguish different types of blood group A antigen in extreme details. The results were performed by macroscopic and microscopic methods. This is important because cancer cells can express a various type of A antigenic determinants on their surfaces which cannot be detected by conventional reagents. A antigen can be distinguish between normal and tumor sample [109].

In this work, we will focus on SPR imaging technique because it is the solid phase immobilization technique and can be developed into the high throughput and the surface can be reused. Quinn et.al. [99] demonstrated the use of solid phase SPR biosensors for the blood typing using carboxydextran surface**.** Immobilized monoclonal anti-A IgM antibody can specifically bind to blood group A erythrocytes without the cross reaction with blood group B or O erythrocytes. Monoclonal Anti-B IgM was specifically bind to group B erythrocytes and was not bind with blood group A or O erythrocytes. The surface regeneration was carried out by using 20mM NaOH to elute the bound erythrocytes on the sensor surface. It was found that the sensor surface can be used repeatedly. Quantitative measurement gives a detection limit of $< 0.33\times10^{9}$ RBC/ml.

3.3 Objective

To explore the possibility of applying SPR imaging technique for ABO blood typing by cell grouping using unpurified antibody prepared by National Blood Center, Thai Red Cross Society.

3.4 Experiment

3.4.1 SPR apparatus

SPRI used in this work have been developed by NECTEC, Thailand. The set-up consisted of a polychromatic light source that is irradiated on the sensor surface, a collimator, polarizer, and prism. The sensing principle is based on wavelength scanning and measuring reflectivity of incidental light at a fixed angle (figure 3.3). Signal intensity was measured by a CCD camera. Flowing of solution into 7-channels flow cell was done by peristaltic pump. For the array detection platform, antibodies were immobilized onto the surface, and then the surface was rotated 90° to flow the samples through. Matching samples to specific antibodies surface produce bright spots on the intersection area, which are measured to calculate % change of reflectivity (%ΔR). The conversion to refractive index units (RIUs) was calculated.

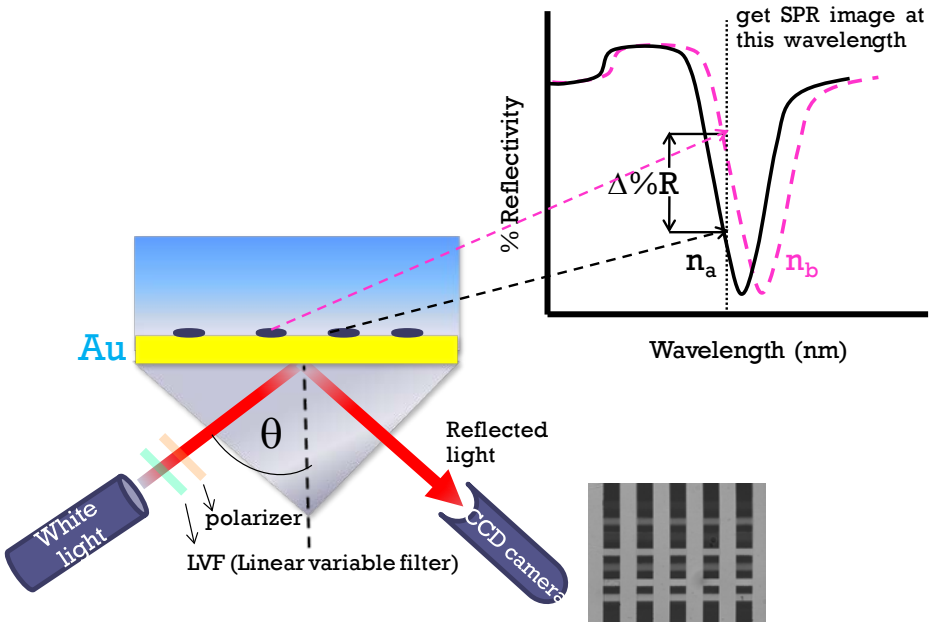


Figure 3.3 Wavelength-tunable SPR imaging

3.4.2 Reagents

Two types of monoclonal antibodies were used: (a) mixed clones of monoclonal antibodies including anti-A, anti-B, and anti-AB (total protein content: 284, 382, and 321 mg/dL); and (b) subclones of monoclonal antibodies specific to RBC A and B including anti-A clone 3C4 and anti-B clone 18F8 (total protein content: 324 and 279 mg/dL). Antibodies were obtained from the research unit of the Thai Red Cross Society. All antibodies were type IgM. Blood samples were left-over samples from the Blood Bank, Ramathibodi Hospital (Bangkok, Thailand). This work was approved by the Ramathibodi Hospital Ethics Committee. Dextran molecular weight 500 kDa was purchased from Pharmacosmos A/S, Denmark. Phosphate buffer saline (PBS) solution was prepared from NaCl and KCl (LabScan, Thailand) sodium phosphate dibasic (Na_2HPO_4) (Sigma), potassium dihydrogen orthophosphate KH_2PO_4) (Fisher Scientific, UK) in deionized water with 0.005% of Tween20 and was adjusted to pH 7.4 by 1M HCl. N-ethyl-N-(3-diethyl-aminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) and ethanolamine were used in coupling step [99].

3.4.3 Sample preparation

Fresh EDTA blood samples were collected, and centrifuged. Plasma was removed, and RBCs were washed three times with PBS buffer. Alsever; the preservative reagent was added, and samples were kept at 4^0C. The total number of RBC samples was sixty (n=15 for each group A, B, AB, and O). 5%RBC (v/v) was prepared by 1:20 dilution of 100%RBC in PBS pH 7.4. All procedures followed AABB guidelines [110].

3.4.4 Agglutination typing (test tube technique)

3%RBC was added with standard antibodies (mixed antibodies, 1:2 by volume), centrifuged and reading for agglutination. All procedures and interpretation of results followed AABB guidelines [110].

3.4.5 Antibody immobilization and sample detection

The carboxydextran surface was prepared followed Lofas [57] . The immobilization was done by activating the carboxydextran with 0.4M EDC and 0.1M NHS followed by injection of the antibodies in sodium acetate pH 5 at 1:10 dilution through the flow cell. The remaining available active groups were blocked by ethanolamine pH 8.5. The adsorbed antibodies on the surface were washed out by 5 mM NaOH. Experiments were performed by injecting 100 µL 5% RBC solution at a flow rate of 10 µL/min. After the SPR signal reached a plateau, running buffer was passed over the surface. The blood type was determined from the changed in intensity at the region of interest (ROI). Regeneration of surfaces was done using 5 mM NaOH, followed by running buffer. Measurements were made in the seven parallel microchannel made of polydimethylsiloxane; PDMS (SYLGARD 184, Dow Corning Corporation, USA.) molded with in-house molding with a channel width of 500 µm and height of 500 µm. Six channels were used for the measurement, while one channel was used as the reference channel.

3.5 Results and discussion

3.5.1 Antibodies immobilization

Five types of probing antibodies were immobilized via covalent binding onto the carboxydextran surface. Other than the three mixed monoclonal antibodies, the addition of two monoclonal antibody subclones of anti-A and anti-B were used in order to increase the accuracy of the typing. The mixed antibodies are multivalent IgM molecules that were optimized for the agglutination blood-typing. All the antibodies contained bovine serum albumin (BSA) that was immobilized onto the sensor surface along with the probing antibodies. The presence of immobilized BSA did not disturb blood-typing by the SPRI technique. The optimum concentration for the antibody immobilization was at 1:10 dilution in acetate buffer pH 5. The use of more concentrated antibody on the sensor surface resulted in lower binding of the RBC. The optimum amount of the immobilized antibodies was comparable for all antibodies at 1:10 dilution. Mixed and separate clone antibodies were used simultaneously for the purposes of confirming the results, searching for suitable antibodies, and evaluating affinity between antigens and antibodies.

3.5.2 Qualitative analysis for blood typing

Agglutination–based blood-typing was used as the standard method to check the blood group. The result is reported in a semi-quantitative manner, i.e., positive (1+, 2+, 3+, and 4+) and negative (-). All samples in the experiment were 4+ (strong agglutination) or negative (no agglutination) according to their ABO blood group (table 3.3); for example, A-RBC gave 4+ to anti-A and negative to anti-B. These were used for cross checking the blood grouping with the results from SPRI. Bound RBCs to immobilized antibodies were observed as bright spots on the monitor, and brighter signals referred to there being more RBCs bound on surfaces (figure 3.4 (a)). The RIU was calculated from the average of sum signal intensity in selected ROIs (figure 3.4 (b)).

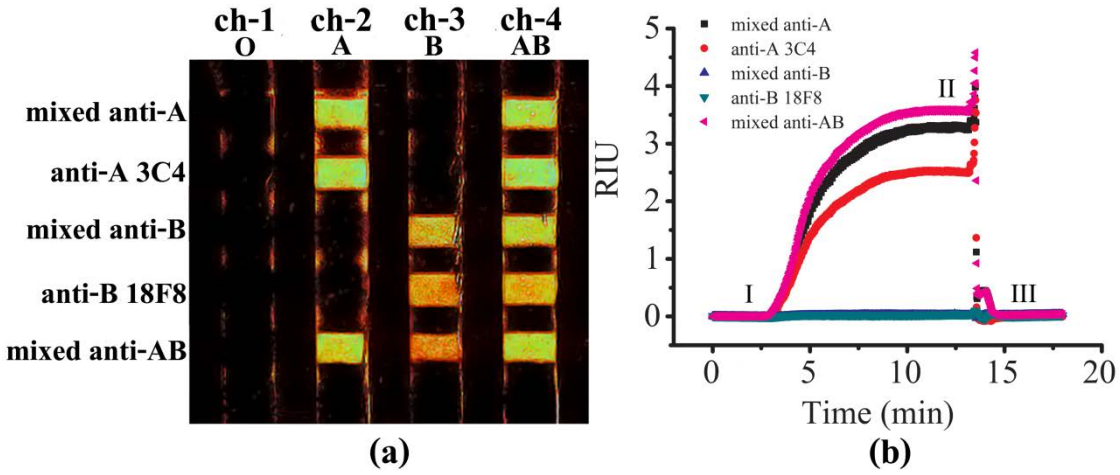


Figure 3.4 SPRI data readout. (a) SPR image signals on array detection platform. Each row was immobilized by specific antibodies and each column was flowed by different ABO-RBC. O-RBC no signal with any antibodies, A-RBC show signal on anti-A and anti-AB, B-RBC show signal on anti-B and anti-AB, and AB-RBC show signal on all antibodies. (b) SPR sensorgram; I) baseline signals II) A-RBC signal to anti-A 3C4, anti-A mix, and anti-AB but not to anti-B 18F8, anti-B mix and III) Signals returned to baseline after regeneration with 5mM NaOH.

Each row from top to bottom are immobilized antibodies in the order : anti-A mix, anti-A 3C4, anti-B mix, anti-B 18F8, and anti-AB. Each column from left to right were injected with the blood samples: O-RBC (ch-1), A-RBC (ch-2), B-RBC (ch-3), and AB-RBC (ch-4). Intersecting areas of each row and column were the ROI for observing bright spots and measuring signals. By observing bright spots only, all RBC samples were typed into the ABO blood system correctly without cross-reactivity (figure 3.4 (a)). A-RBC gave bright spots on anti-A surfaces, anti-A mix, and anti-A 3C4, and anti-AB-mix surface. B-RBC gave bright spots on anti-B surfaces, anti-B-mix, and anti-B 18F8, and anti-AB. AB-RBC gave bright spots on all surfaces. The O-RBC gave no bright spots on any surface. By measuring signals in RIU, the results were reported as a quantitative number and the interpretation was similar to observing bright spots, for example A-RBC gave signals on anti-A and anti-AB surfaces when compared with the baseline signal (figure 3.4 (b) and table 3.3).

Table 3.3 Comparison between agglutination and SPR technique for ABO blood grouping.

n=15	Agglutination		SPR signal (μRIU)				
	anti-A mix	anti-B mix	anti-A mix	anti-A 3C4	anti-B mix	anti-B 18F8	anti-AB mix
O-RBC	-	-	28±15*	20±13*	17±11*	19±13*	20±12*
A-RBC	4+	-	2789±795#	1998±489#	38±9*	42±11*	2330±818
B-RBC	-	4+	41±11*	39±12*	2060±425Ⓑ	1223±405Ⓑ	1571±770
AB-RBC	4+	4+	2687±648#	2026±538#	1078±531Ⓑ	720±371Ⓑ	2064±680

n=15 for each group, Anti-AB signals to all RBC except O-RBC

* No signal when compared with baseline noise.

#,Ⓑ Signals different of each specific antibodies between mixed and single clone (pair t-test, $p<0.005$).

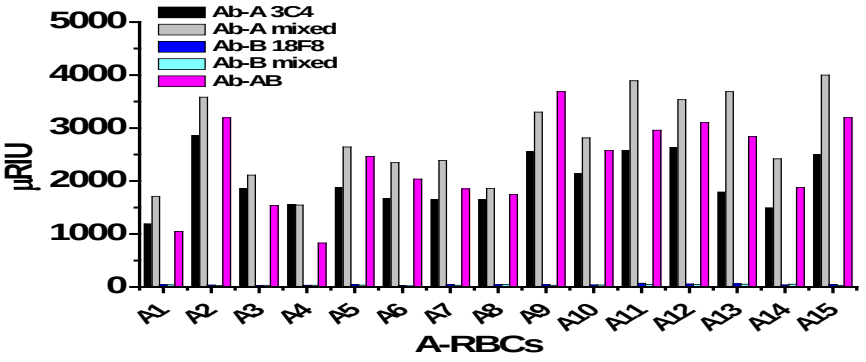
Figure 3.4 (b) showed the sensorgram for A-RBC. Point I was the baseline signal after being immobilized with antibodies. Point II was the plateau signal after being injected with A-RBC. SPR signals were observed only on specific surfaces (anti-A, anti-A 3C4, and anti-AB). Point III was the baseline signal after being regenerated with 5 mM NaOH; all signals returned to baseline and were ready to use in subsequent detections. The re-usability of each surface was around 20 times.

3.5.3 Signals analysis and interfering factors

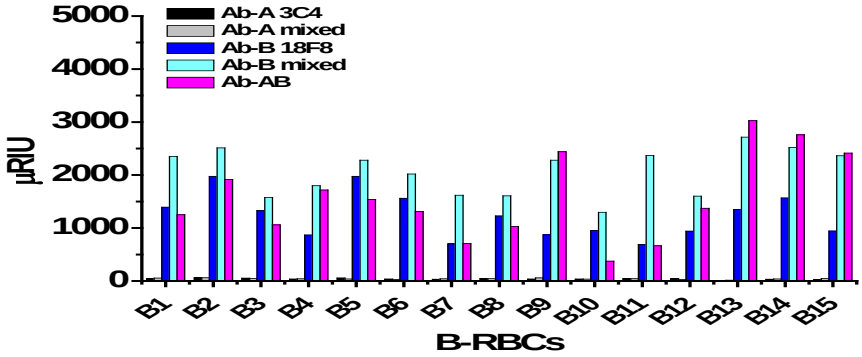
Each μRIU value in table 3.3 was an average signal with SD (standard deviation) from 15 samples (n=15) and each sample was measured twice (in duplicate). Duplicate RIU values showed no statistical difference (paired t-test, $p>0.05$, data not shown). However, some duplicate values with statistical difference were found which may be due to a non-uniform distribution of the bound RBC on the sensor surface due to a relatively large sample size. It should be noted that the signal from specific binding—e.g., anti-A with A-RBC—was distinct from the non-specific binding (baseline signal)—e.g., anti-A with B-RBC which shown in figure 3.5. The weakly reacted sub-group RBC is expected to show a weak reaction with the immobilized antibody due to the low affinity between antigen and antibody. The array

platform including antibodies specific to sub-groups of RBC should be immobilized to realize the true potential of the antibody array technique.

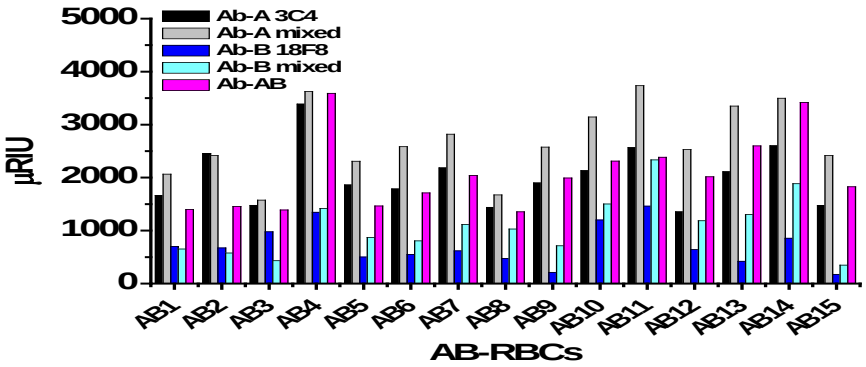
(a) Blood group A



(b) Blood group B



(c) Blood group AB



(d) Blood group AB

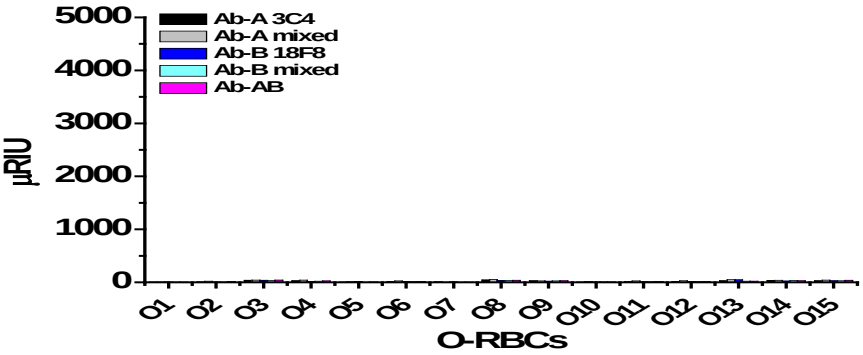


Figure 3.5 Signal of ABO blood typing by SPR technique; the signal from blood group (a-d) A, B, AB and O respectively.

For quantitative interpretation, there are three factors that should be noted. First is the antigen factor. %RBC concentrations were directly proportion to the observed RIU values [99]. The concentration of the RBCs should be kept constant throughout the experiment. The amount of antigens present on the RBC surface. RBCs of the same blood group but different donors showed unequal signal intensity towards the same antibody (figure 3.6). AB-RBC, which has more A antigens than B antigens, showed an increased signal on anti-A compared to anti-B (table 3.3). In this context, SPR signals are related to the total number of antigens on the RBC surface, and make it possible for ABO typing into subgroup populations based on number of antigens on the RBC [111]. An in-depth investigation of the individual antibodies with different types of RBC is still needed.

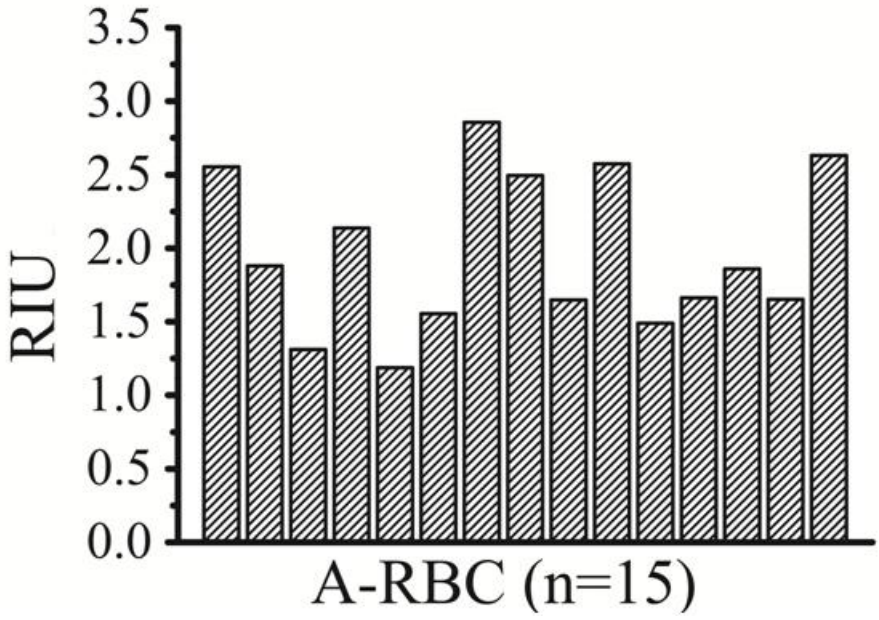


Figure 3.6 Signal intensity on anti-A 3C4 surface among RBC-A samples (n=15). This might indicate the variation of A-antigens presented on the RBC surface of individual donors.

Second is the antibody factor. The amount of immobilized antibodies affected the signal intensity. Too low or too high surface concentration resulted in decreased signal, while the optimal concentration would give maximal signal (at 1:10 dilution). High antibody affinity towards antigens gives strong antigen-antibody

bonding and results in more bound RBC on the surface. Each clone of antibody would give a different signal intensity to the same sample. For example RBC-A, gave different signals for the anti-A mix compared with anti-A 3C4 (table 3.3). It is clear that the signal obtained from the specific binding is distinct from the non-specific binding. The accessibility to antigenic sites, or stearic hindrance, has an effect on the binding. The antigenic site of A-RBC, B-RBC, and AB-RBC is the branching sugar molecules that stretch out from the surface of the red blood cell. This makes them easily recognized by antibodies when compared with other blood group systems such as Rh antigen.

The third factor is the analytical factor. The flow-rate was found to have directly direct influence on the observed signal. RBCs are a biconcave shape of longitudinal diameter 6-8 μm and vertical diameter ~2 μm. In the SPR detection platform, the longitudinal diameter of RBCs was proposed to lay horizontally on the sensor surface [99]. RBCs would be captured only on the edge of the biconcave and might slip through with high flow-rate. The higher shear force exerted by the flow in the microchannel might explain why the high flow-rate produced a decreased signal compared with the low flow-rate, which will have a lower shear force. Flow cell diameter, a larger diameter caused less bound RBC on surface, too small diameter caused occlusion by microclotting. Both effects would lower the observed signal. The optimal size diameter must be especially designed for the system. The optimal pH for antigen-antibody complexes are around pH 7, while extremely low or high pHs cause denaturation of antibodies. The dissociation of complexes will occurring weak base solution (5mM NaOH) without losing their properties. PBST pH 7.4 was used for running buffer while 5mM NaOH was used for regeneration. The main two types of antibodies will react at different temperature: IgG is a warm antibody, while IgM is a cold antibody [110]. The IgM used in these experiments performed well at 25 ^{0}C (room temperature).

3.6 Conclusion

An SPR imaging technique was successfully applied for ABO blood-typing. All samples were correctly grouped, and were consistent with the standard

agglutination technique. Three main factors that effect the signal variation were: (a) RBC factor (RBC concentration and nature of antigenic site on the RBC surface); (b) antibody factor (amount of immobilized antibody, affinity, accessibility to antigenic sites; and (c) analytical factors (flow rate, flow cell diameter, pH, temperature). The surface can be regenerated by 5mM NaOH at least 20 times.

CHAPTER IV
DETECTION OF RH (D) ANTIGENS ON RED BLOOD CELL SURFACES BY SPR TECHNIQUE

4.1 Introduction

The Rh blood group is one of the most complex systems and the most important blood group systems for blood transfusion, second to the ABO. There are over 50 antigens in the Rh blood system [101]. However, only 5 antigens are clinically significant which are D, E, C, e and c. The Rh positive (Rh+) is defined as having D antigens on the red blood cell and lacking anti-D in the serum. On the other hand, the Rh negative (Rh-) blood does not contain either D antigen on red blood cell or antibody D (anti-D) in serum. There are more complicate about Rh (D) antigen, for a recipient who has weak D or partial D is labeled as Rh negative but for a donor who has weak D or partial D will be defined as Rh positive. In general, anti-D does not occur naturally but can be produced either by (a) receiving Rh positive blood from a blood transfusion or (b) gaining from the pregnancy in the case of the baby having the Rh positive blood. Thai people have about 99.7% Rh positive blood and 0.3% Rh negative blood while the Caucasian population has about 15% Rh negative blood [112].

The detection of D antigens is important for blood transfusions and for pregnant woman in order to prevent anti-D immunization and the immune hemolytic transfusion reaction. Humans with Rh negative blood can only receive Rh negative blood to avoid production of anti-D. In pregnant women with Rh negative, if the fetus has Rh positive, the woman body will produce anti-D which is dangerous in the next pregnancy because anti-D can bind to the fetus red blood cells causing the hemolysed, called hemolytic disease of the fetus and newborn (HDFN) [113].

This work is aiming to develop the SPR technique for the detection of D antigens on red blood cells based on solid phase immobilization of antibodies. Anti-D was immobilized on the sensor chip and the red blood cells flowed over the surface to

measure the interaction signal of D antigen with the immobilized antibody. The SPR technique can save costs because its surface can be regenerated. Moreover, this technique has ability to be automated and can be combined with the ABO blood typing by a crossing array format. The ABO-Rh blood typing system can then be used in a blood bank.

4.2 Relevant principle and literature reviews

In 1939, Levine and Stetson [114] reported about hemolytic disease of newborns. They found antibodies in serum of maternal and the mom had a hemolytic reaction after receiving a blood transfusion from her husband, but the cause of hemolytic disease was not clear. In 1940, Landsteiner and Wiener [115] immunized antibodies by using red blood cells from Rhesus monkeys injected into rabbits and guinea pigs. They found that immunized antibodies agglutinated with 85% human red blood cells, they named anti-Rh. In the same year, Levine and Katzine [116] tried to find the relationship between antibodies that cause hemolytic disease of newborns and anti-Rh. In 1945, the original Rh factor had been renamed D and other antigens in Rh system were found.

Rh antigens are expressed on polypeptides. These polypeptides span over the red cell membrane exposing six extracellular [110, 111, 117, 118]. The complexity of Rh antigens is due to the polymorphic genes that encode the RhD, RhCE and genes which encode the Rh-associate glycoprotein is RhAG which is shown in figure 4.1.

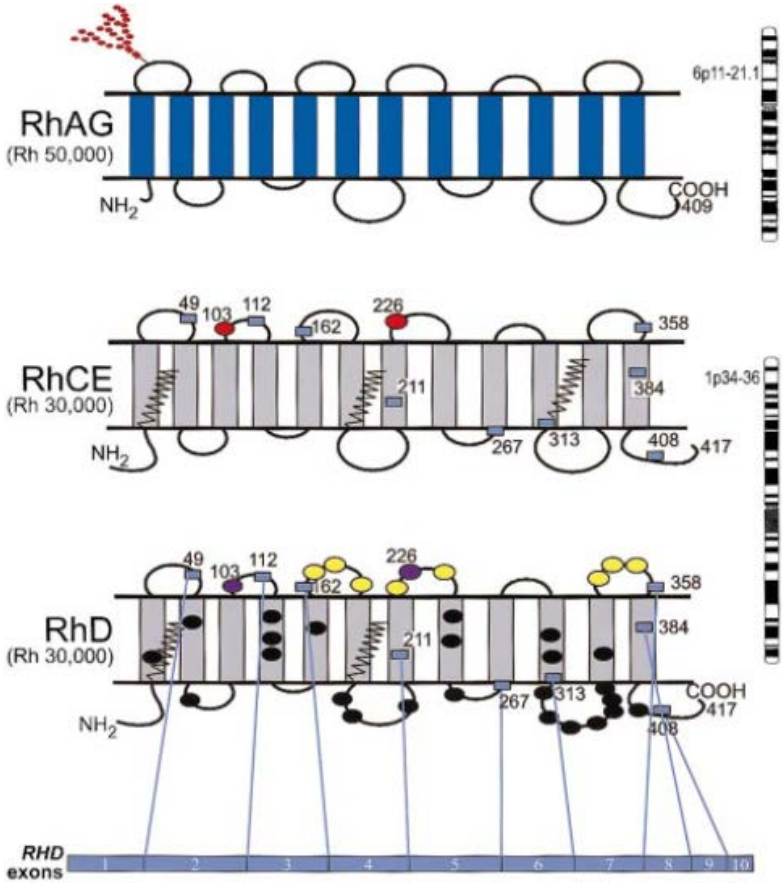


Figure 4.1 Topological model for RhAG, RhCE, and RhD proteins in the RBC membrane. RhAG consists of 409 amino acids and is encoded by RHAG on chromosome 6p11-p21.1. RhCE and RhD are predicted to have a similar topology and are encoded by RHCE and RHD, which are on chromosome 1p34-p36 [117].

Figure 4.1 is the model of the RhD protein which is the main focus in this chapter. The domain of RhD protein encoded by each exon, shown by numbered boxes. D-specific amino acids, 8 are on the exofacial surface (yellow ovals), and 24 are predicted to reside in the transmembrane and cytoplasmic domains (black ovals). Red ovals represent amino acids that are for C/c and E/e antigens [117]. An Rh negative person will lack the RHD gene which is encoded for D antigen.

The standard method for detection of the D antigen is using the same principle as ABO blood typing which is the agglutination test, as discussed in chapter III in table 3.2. Anti-D was used directly for the agglutination test where the

interaction between D antigen on the red blood cells and anti-D was observed. The agglutinated sample means Rh positive while no agglutination may indicate Rh negative or weak D. An extra test is needed in the case of no agglutination. The method for weak D detection is carried out by incubating the mixture of anti-D and red blood cells at 37ºc for 30 mins then washing and adding coomb's reagent. If the result shows agglutination, that blood unit is labeled as Rh positive (weak D or patial D). If red blood cells are non-reactive by direct agglutination or weak D method, they will be defined as Rh negative [110]. The agglutination tube test for Rh D antigen is shown in figure 4.2.

(a) (b)

Figure 4.2 Agglutination tube test of Rh D antigen (a) no agglutination and (b) agglutination of red blood cell and anti-D antibody.

Although the standard agglutination method is simple, reliable, and versatile, there is a downside because it is not an objective method and requires considerable expertise and technical skills for interpretation. Instead of using tubes, the large amount of blood typing has been carried out in microplate which is a 96 wells by the same principle as the standard method [119]. The blood typing in microplate had the advantage because it can easily be applied to large scale samples and are more objective with the use of an instrument and computer. There were previous works involved with Rh typing which are more sensitive than standard agglutination method and they are described as follow.

In 1985, Sinor and et.al. developed the solid-phase adherence method (SPAM) for ABO grouping and Rh typing [120]. The solid-phase for Rh typing was prepared by coating polystyrene microplate wells with goat anti-human IgG and then with anti-D. The brolemain-treated RBCs were added to the antibody-coated wells and were subsequently centrifuged. The positive and negative reactions were easily distinguished by a microplate spectrophotometer and interpreted into Rh types by a computer. They reported the correlation of 99.9 % for Rh typing of 1586 donor RBC samples by the SPAM and microplate agglutination method. The SPAM had increased sensitivity, eliminated the need for an antiglobulin test and provided an objective endpoint which could be read spectrophotometrically.

Flow cytometric methods for simultaneous detection of ABO and Rh (D) antigens in human RBC was presented in 1998 [121]. The invention provides a method for a quick and efficient way of analyzing blood samples and can be automated. The mouse's monoclonal anti-A, anti-B and human monoclonal anti-D (IgG) antibodies were used to react with blood samples. Unbound antibodies were washed out. The secondary labeled antibodies with different dye types were subjected in flow cytomerty to indicate blood type. A parallel testing of over 300 donor blood specimens from the blood bank including selected weaker variants of A_2 and A_2B showed no discrepancies with conventional blood typing.

Both SPAM and flow cytometry still need the labeling method. SPAM still needs enzyme treated RBCs and flow cytometry requiring the labeling of antibody molecules for analysis. The main purpose of this work is to develop SPR technique for detection of D antigen on red blood cell based on solid phase immobilization of antibodies without the need of a labeling molecule.

4.3 Objective

To apply the SPR imaging technique for detection of D antigens on red blood cell surfaces.

4.4 Experiment

4.4.1 Reagents

Monoclonal mixed anti-D (IgG and IgM type) was purchased from the Thai Red Cross Society. This antibody contains about 90% BSA. The blood samples were left-over samples from the Ramathibodi Hospital Blood Bank (Bangkok, Thailand). This work was approved by the Ramathibodi Hospital Ethics Committee. All blood samples were labeled Rh type from the gel agglutination technique. The Dextran surface was prepared as described in chapter II and III.

4.4.2 Sample preparation

Fresh EDTA blood samples were centrifuged at 4500 rpm about 3 minutes by using centrifuge TGW16 (Changsha Yingtai Instrument Co ltd). Plasma was removed and RBCs were washed three times with PBS buffer. Alsever preservative was added and samples were kept at 4^0C. Sample of 5% RBCs (v/v) was prepared by 1:20 dilution of 100% RBCs in PBS buffer. All procedures followed AABB guidelines [110].

4.4.3 Antibody immobilization

The carboxydextran on the gold coated sensor surface was prepared following the guidelines in Lofas [57]. The immobilization was done by activating the carboxydextran with 0.4M EDC and 0.1M NHS followed by injection of the antibodies in sodium acetate pH in the range of 4-5.5 at 1:5 dilution through the flow cell to find the optimum pH. The remaining available active groups were blocked with ethanolamine pH 8.5. For the array measurement, one channel was immobilized with BSA for use as the reference. After crossing, the 7 channel array was formed. This allows the measurement of 7 blood samples in parallel where the BSA was used as the internal reference.

4.4.4 D antigen detection

Five% RBCs solution 100 µL was injected over the surface at a flow rate of 10 µL/min. After the SPR signal reached a plateau, which indicated that the D

antigen on RBCs bound to an immobilized anti-D on the surface, the running buffer was passed over the surface for 25 min. The signal was determined from an increase of intensity from the region of interest (ROI). Regeneration of surfaces was done by using 5 mM NaOH and followed by the running buffer.

4.5 Results and discussion

4.5.1 Antibody immobilization

The optimum condition for anti-D immobilization was carried out by immobilizing 20% of anti-D in acetate buffer at varying pHs including 4, 4.5, 5.3 and 5.5. The pKa of the carboxydextran surface is about 3.5. When pH of the buffer is more than 3.5, the surface has a negative charge. The antibody, which has a positive charge, will attract to the surface via electrostatic attraction, figure 1.8. The suitable pH is the pH where the antibody is attracted to the surface the most. The suitable pH of the buffer is normally lower than pI of antibody.

When anti-D was immobilized on surface, some of BSA were able to link on the surface due to the presence of BSA along with the antibody. The immobilization in acetate buffer pH 4 gave the highest SPR signal. A pH of 4.5, 5.3 and 5.5 showed a lower and comparable SPR signal. The acetate buffer pH 4 had the highest signal; however, due to the presence of BSA, it cannot be ruled out there may be higher amounts of BSA bound onto the surface, as shown in figure 4.3. Based on the isoelectric point (pI) of BSA of about 4.7 is more than the acetate buffer pH 4. Thus BSA is having a positive charge which should prompt it to bind with the surface. A decrease of SPR signals were found when increasing the pH of the acetate buffer, eg. to more than the pI of BSA (at 5.3 and 5.5). At higher pH, BSA becomes negatively charged. Repulsive forces between the surface and BSA is dominant. As a result, BSA has less tendency to bind onto the surface and the signal should come from anti-D that binds on the surface instead of BSA. This is because anti-D consists of IgG mixed with IgM types that have a pI around 5.3-8.5 [122-124]. When the pH of the buffer is increased to be around 4.5, 5.3 and 5.5, the antibody will be bound on the surface more than BSA due to the charged property, but the immobilized signals

from the antibodies were much lower than BSA because of the lower concentration of antibodies compared with the BSA in the mixed anti-D solution.

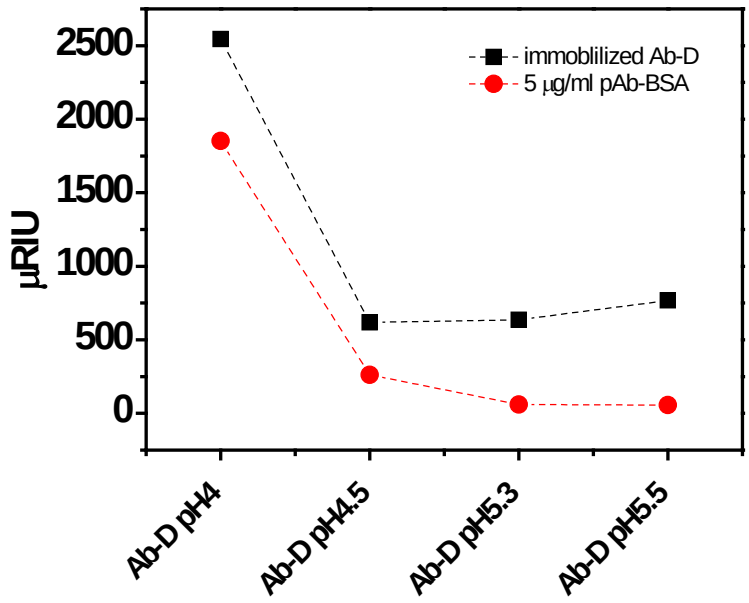


Figure 4.3 SPR immobilizing signals of the 20% mixed anti-D at various pH of acetate buffer (■) and SPR detecting signals from anti-BSA (●) after immobilization of anti-D.

Based on this assumption, a pH at 4 should have the highest amount of BSA bound to the surface. Polyclonal anti-BSA (pAb-BSA) was used to test the presence of the BSA on the surface. The immobilized mixed anti-D at pH 4 showed the highest SPR response among all immobilized conditions. This means that there were more BSA on the surface at the lower immobilized pH than at the higher pH. This supports the above assumption that at the lower immobilizing signals at pH 4.5, 5.3 and 5.5 there is less BSA bound to the surface. It is reasonable to assume that the anti-D might be immobilized more on the dextran surface at acetate buffer pH 4.5-5.5. Further validation was carried out in the next experiment by monitoring the binding with Rh+ RBCs.

4.5.2 Detection of D antigen on red blood cells

Four samples of RBCs (A10 Rh+, A17 Rh+, A62 Rh- and standard (std) cell Rh-) at 5%RBCs in PBS buffer pH 5, 6, 6.6, 7.4, 8, 8.5 and 9 were flowed over the mixed anti-D surface at various immobilizing pH. To find the suitable condition for detection of D antigen on RBCs, two factors were considered.

4.5.2.1 Effect of immobilizing pH on D antigen detection

Figure 4.4 shows the D antigen detection signal obtained from the surface which was immobilized with anti-D at different immobilization pHs. There were very low SPR responded from Rh- blood sample on all mixed anti-D surfaces due to the lack of D antigen on the RBCs. For the Rh+ blood sample, SPR signals were different on each anti-D surface. At an immobilized pH of 5.5, the surface showed the highest SPR response of around 400-700 μRIU followed by at the immobilized pH of 4.5 (around 300 μRIU) and at immobilized pH 4, respectively. This result could be related to the result from 4.5.1, that the immobilized pH at 4 showed the highest immobilization signals (from BSA) on the surface but showed lower signals for the Rh+ RBCs than at the immobilized pH of 5.5. It should be kept in mind that the optimal pH for anti-D immobilization is varied slightly from each lot of the antibody. The overall result could be proven that the BSA plays a major role in the anti D immobilization step.

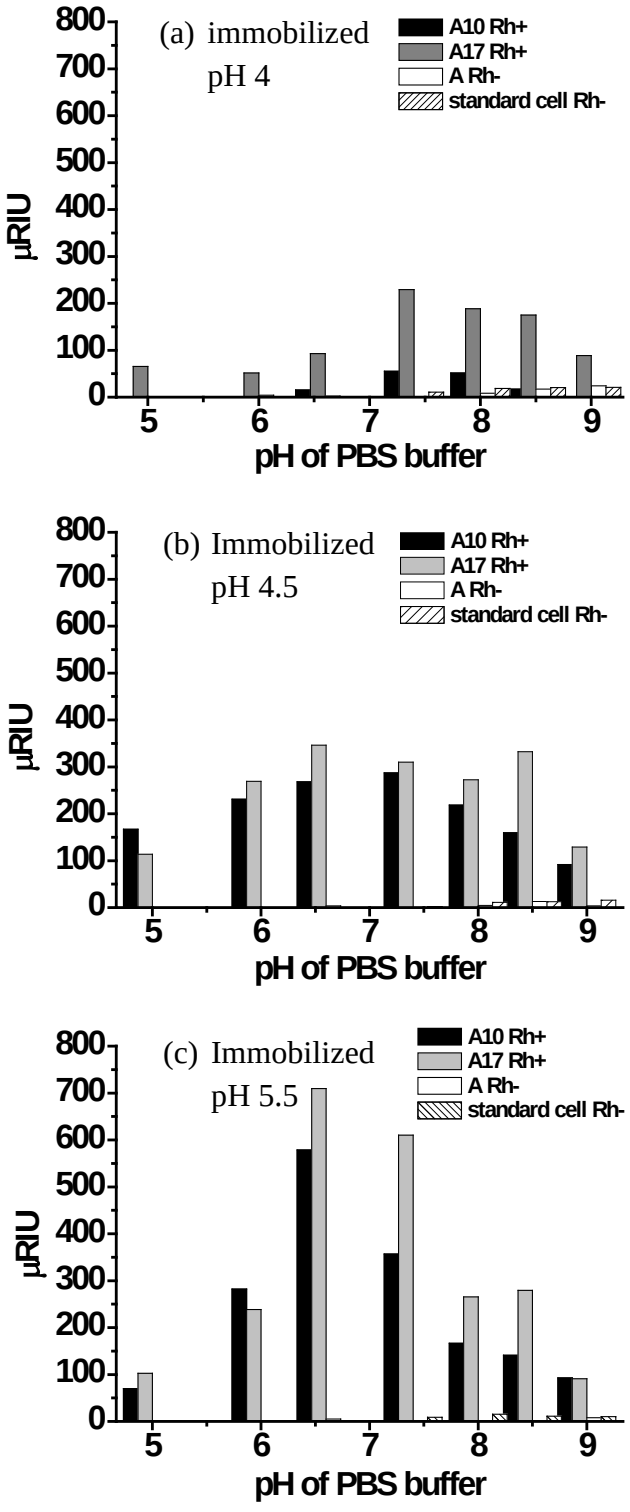


Figure 4.4 The SPR signals of 5% Rh- and Rh+ RBCs which diluted in different pH of PBS buffer (pH 5-9) on various pHs immobilization of anti-D mixed surface; a) pH 4.0, b) pH 4.5, and c) pH 5.5

4.5.2.2 Effect of pH buffer on D antigen detection

Five percent RBCs in PBS buffer pH 5, 6, 6.6, 7.4, 8, 8.5 and 9 was passed over the immobilized anti-D surface in order to observe the effect of pH buffer on the detection of D antigen on RBCs (figure 4.4). The SPR signals showed the same trend on all immobilized anti D surfaces. A PBS buffer pH of 6.6 and 7.4 was the optimal pH range for the D antigen detection because they showed the higher SPR signals compared to the lower and higher pH buffer. This may be due to many possibilities. At higher end or lower end of the pH, the pH can induce the denaturization of the immobilized anti-D which may interrupt the interaction between antigen and antibody due to the change of protein conformation. Another possibility may be due to the electrostatic charge in the solution. Normally carboxydextran has a negative charge at pH higher than 3.5 while the sialic acid presence on the red blood cell surface has a pKa around 4.7. This means that the RBCs have a net negative charge at physiological pH [125, 126]. At PBS buffer pH 5 and 6, the presence of hydrogen ions (H^+) in the solution may disturb the binding of D antigen on RBCs and anti-D antibody which is immobilized onto the surface. Hydrogen ions could bind to the negative charge on RBCs which can cause the hydrogen bonding between RBCs then the interaction of RBCs with the ion solution has more effect than the interaction between D antigen and anti-D. RBCs passed through the surface with less binding to the immobilized anti-D which relates to the lower of SPR signal. Unlike at the neutral buffer condition, pH 6.6 and 7.4, the D antigen on the RBCs could bind with the immobilized anti-D easier than at the lower pH. The crowding of RBCs occurred close to the sensor surface which showed in increase of SPR signal. At a pH 6.6 and 7.4, they seem like they were closer to the pI of the antibody. At this pH, the antibody had a positive charge which promoted the binding with RBCs surface. At a higher pH (pH 8-9) there were more OH^- ions which caused a repulsion force between OH^- ions and negative charge of RBCs and dextran surface rendering RBCs difficult to bind on the antibody surface which is represented in figure 4.5.

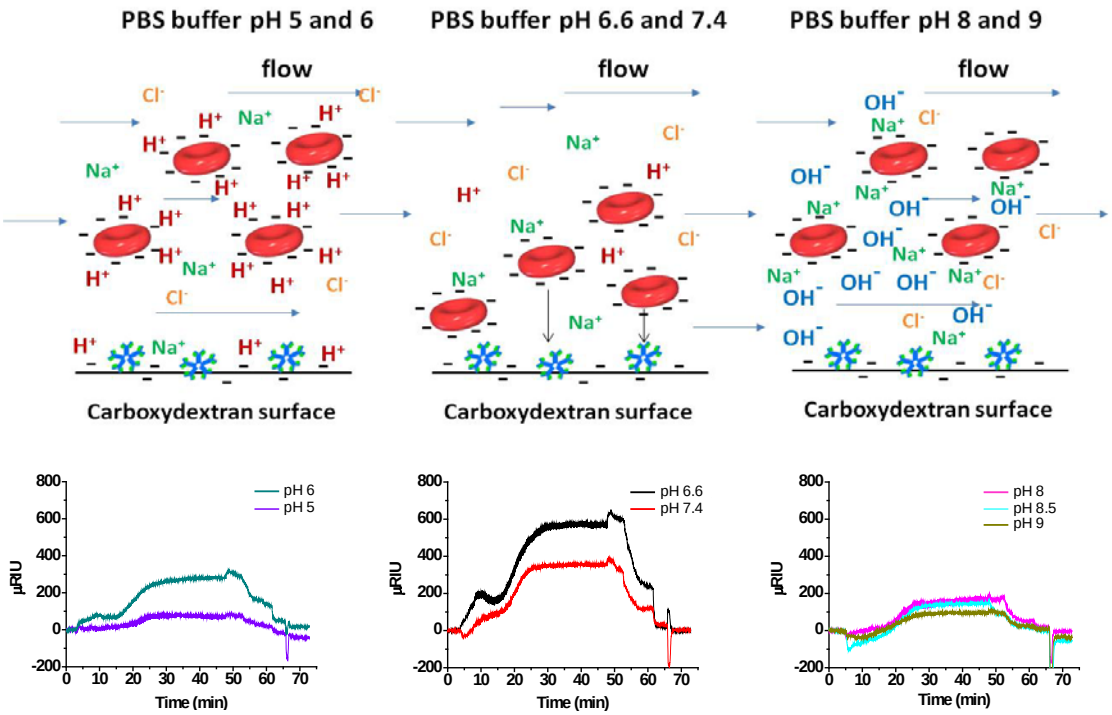


Figure 4.5 This schematic represents the effect of electrostatic charge for interaction of RBCs and carboxydextran surface in different pH solutions and their SPR response.

4.5.2.3 Detection of D antigen on RBCs samples

A total of 44 samples of RBCs including group A Rh+ n=10, B Rh+ n=10, AB Rh+ n=10, O Rh+ n=10 and RBCs Rh- n=4 were investigated for the consistency of the SPR technique. Five percent RBCs in PBS buffer pH 7.4 was flowed over immobilized anti-D surfaces. The results (figure 4.6) showed that the SPR technique could determine Rh D type in all RBCs correctly and agreed with the gel agglutination technique. The SPR signals were in good agreement with the results from 4.5.2.1 that at immobilized anti-D at pH 5.5 showed the highest SPR response. The Rh- RBCs showed a very low SPR signal (about 0-50 µRIU) which are indicated as a noise signals due to no D antigen on RBCs surface.

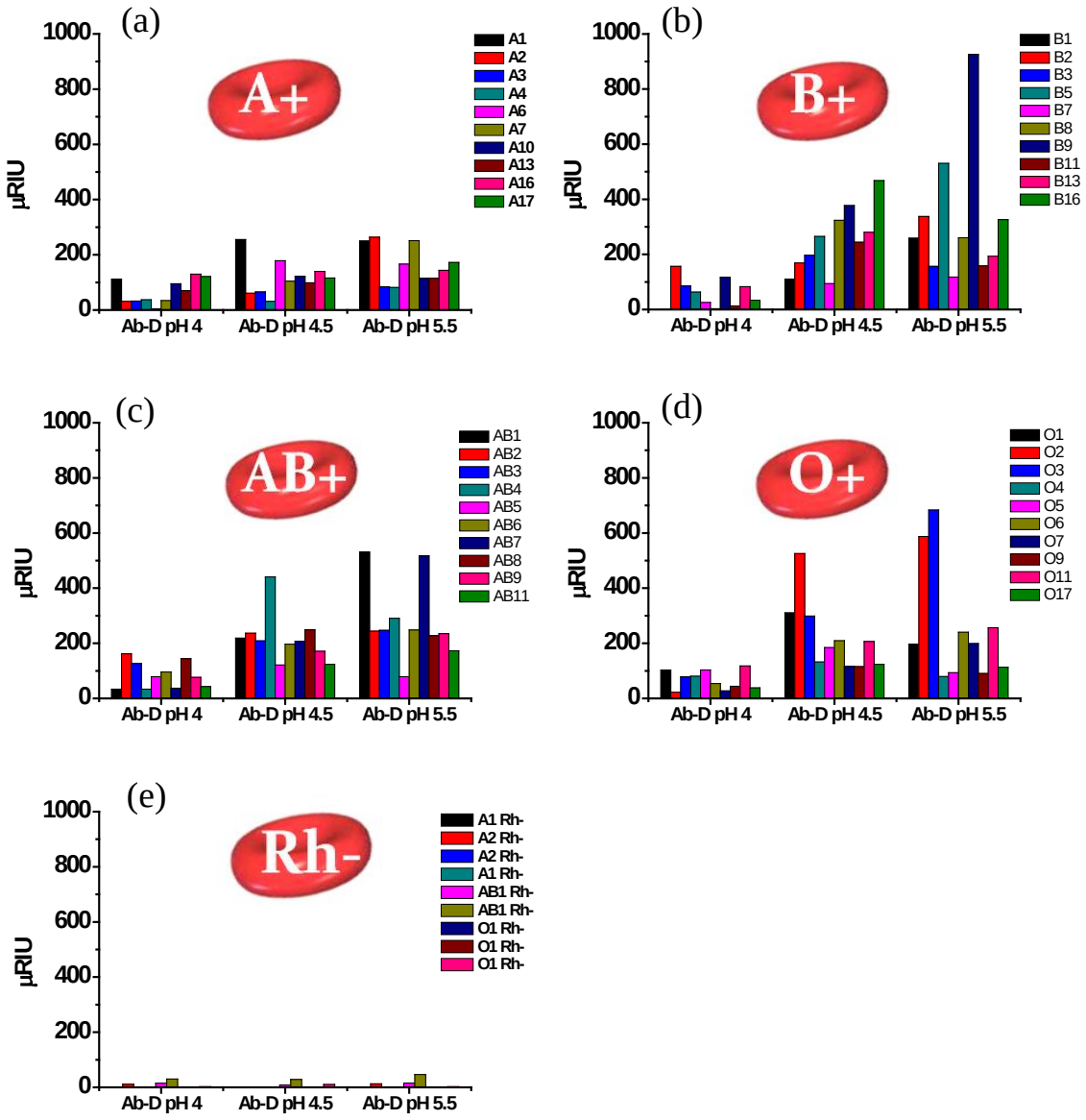


Figure 4.6 SPR signals of D antigen detection from 44 RBCs samples; (a) A Rh+,(b) B Rh+, (c) AB Rh+, (d) O Rh+ and (e) RBCs Rh- on immobilized anti-D at different pH of NaOAc buffer.

4.5.2.5 Effect of flow rate to D antigen detection

The flow rates had an effect on the binding of D antigens on mixed anti-D surfaces. From figure 4.7, B-RBCs (B1 Rh+, B2 Rh+ and B Rh-) at 5% concentration were flowed over the surface by varying the flow rate from 5 to 40

uL/min. The Rh- cells presented a very low signal of about 10-70 μRIU at all flow rates due to a lack of D antigen on the RBC surface. For Rh+ cells, the results showed that when the flow rate increases, the SPR signal decreases, which means that low amounts of RBCs can bind to the surface. The flow rate 5 uL/min showed a higher signal of Rh+ cells (about 800 μRIU) compared to the flow rate at 10 μL/min (about 300-500 μRIU). Both flow rates can distinguish the difference in the signal of Rh+ cells from Rh- cells. At a flow rate of 20 μL/min and higher, one cannot distinguish the signal of an Rh+ from Rh- cell. These results could be explained as follows: at high flow rate, the D antigen has short time for binding to anti-D then the lower signal is observed. When consider as RBCs is a biconcave shape and only some parts can attach on the surface, at high flow rate has more shear force which cause the RBCs to slip and pass by the surface. At a lower flow rate, there is a lower shear force. Thus RBCs have more time to bind with immobilized anti-D on the surface resulting in an increase of SPR signal.

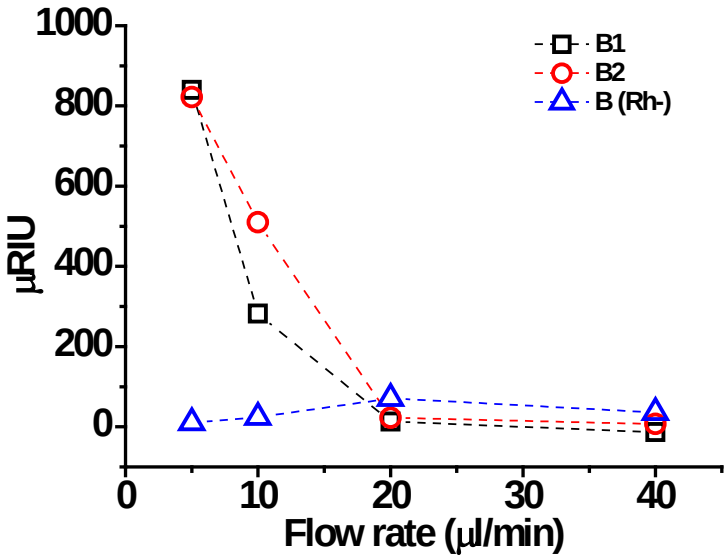


Figure 4.7 Effect of flow rate on the D antigen detection signal of 5% B RBCs (B1 Rh+, B2 Rh+ and B Rh-).

The suitable flow rate for D antigen detection (Rh D typing) is 10 μL/min due to shorter time measurement (about 20 mins) than that of 5 μL/min

(about 30 mins) and the high signal of Rh+ cells are observed and are clearly separate from an Rh- cell.

4.6 Conclusion

The SPR technique can be applied for the detection of D antigens on RBCs. On the optimization conditions, the suitable pH for immobilization of anti-D is in the range of 4.5-5.5 of NaOAc buffer. The immobilized pH can be varied from each sample of antibody. The optimum pH of PBS buffer for the RBC's sample is about 6.6-7.4 which presents a high interaction of D antigen and anti-D antibodies due to a proper charge property and suitable pH for antibody function. The optimum flow rate for D antigen detection is 10 μL/min because it takes a shorter experimental time and gives the higher signal of Rh+ cell.

CHAPTER V
INTEGRATED ABO-RH BLOOD TYPING ON DEXTRAN SENSOR SURFACE BY ARRAY SPR IMAGING TECHNIQUE

5.1 Introduction

The ABO blood system was the first blood-group system discovered by Landsteiner [97] and is one of the most important blood systems in blood transfusions. The ABO blood grouping consists of (a) forward cell grouping: the detection of presence of A, B, and D antigen on the red blood cells (RBCs) surface and (b) reverse serum grouping: the detection of anti-A or anti-B antibodies in serum. The presence of antigens and antibodies in humans are as follows:

(a) people who have blood group A have A antigens on the RBC's surface with anti-B in serum,

(b) people who have blood group B have B antigens on the RBC's surface with anti-A in serum,

(c) people who have blood group AB have both A and B antigens on the RBCs surface with no anti-A nor anti-B in serum,

(d) people who have blood group O have neither A nor B antigen on the RBC's surface with both anti-A and anti-B in serum.

An ABO incompatible blood transfusion is fatal due to complement activation along with disseminated intravascular coagulation (DIC). Compatible ABO blood grouping in both donor and recipient is indispensible to avoid deleterious complication.

The Rh blood system is the second important blood system after the ABO in blood transfusions because anti-D can cause a hemolytic transfusion reaction or hemolytic disease of the fetus and newborn. The people who are defined Rh positive (Rh+) will have D antigens on the RBC's surface. On the other hand, people who are defined as Rh negative (Rh-) will not have D antigens on RBCs. Both Rh+ and Rh-

people initially do not contain anti-D in serum but the Rh- people who were immunized by Rh+ blood transfusion, pregnancy or abortion may develop anti-D in their serum [113].

The conventional method for ABO and Rh blood typing is based on the agglutination technique, which includes a slide test, tube test, microbeads [127], strips [128] and microtiter plates [98, 129]. These techniques have some limitations they are time consuming, produce semi-quantitative results, require personal experience and have low sensitivity. To overcome these limitations, an in-house SPR technique is applied for ABO and Rh blood typing with the advantage of high sensitivity, rapid detection, label free, possible for adapting to automated systems and high throughput bioanalyzer.

In this chapter, the results of reverse serum grouping by SPR technique is discussed while the forward blood grouping and Rh typing can be seen in chapter III and IV. The integration of a ABO-Rh blood typing platform for the high-throughput blood typing device is shown in this chapter. Specific antibodies for A, B and D antigens were covalently immobilized on the CMD surface for ABO and Rh blood typing and synthetic blood group A and B antigens immobilized for serum grouping. The performance of SPR for ABO-Rh blood typing with whole blood was demonstrated. A single step of whole blood injection can classify ABO-Rh blood type at the same time. This technique allowed a convenient and high-throughput method for ABO-Rh blood typing.

5.2 Literature reviews

Quinn et.al, 1997 [99] applied the SPR (BIAcore ^{TM}) technique to detect A or B antigens on a RBC's surface by immobilized monoclonal IgM antibodies on a dextran surface. The investigation for clinical application has not been fully illustrated.

There are some alternative techniques to detect human blood group, anti-A and anti-B level such as an enzyme-linked immunosorbent assay (ELISA) [130, 131] and flow cytometry [132]. These techniques, however, are known to have a higher

cost, lower throughput, need for labeling and difficulty for automation. An easier and more rapid method for the detection of IgG anti-A/B antibodies by SPR was reported [133, 134]. IgG antibodies in plasma samples were detected by interaction with an immobilized blood group trisaccharide amine derivative and anti-IgG antibody by a sandwich assay. However, there are some limitations of using the SPR technique in measuring the IgM antibody which is possibly due to the high dissociation equilibrium constants (Kd) of the IgM antibody [135].

A novel microplate agglutination method (MAM) for blood grouping and reverse typing without the need for immobilization of neither antigen nor antibody and centrifugation was published by Spindler et al., 2001 [136]. Two types of MAM were used, with and without agarose for forward grouping and reverse grouping. For forward blood grouping, anti-A, anti-B, and anti-D antibodies were incubated with RBCs samples in a v-bottom plate for 40 minutes and the results were read by a photometer at 405 nm. For reverse blood grouping, RBCs were mixed with plasma and the suspension was transferred to the v-bottom agarose plate and incubated for 1 hour. The result was monitored by a photometer at 620 nm. Agarose was used as a separating medium which forms a layer between the sample and the surface of the wells. A positive result is indicated by the adherence of test RBCs on the ladder space, and a negative result is when RBCs fell in the center of the wells. The forward blood grouping showed a false-negative of around 0.37% that was probably caused by decreased antigen expression of donor RBCs and by the dilution factor of the antibody. In the case of reverse blood grouping, a low quantity of antibodies in sera seems to be the cause of a false-negative reaction. This method does not require the immobilization, centrifugation and washing steps, but it takes a long measurement time, only suitable microplates can be used and need to optimize for optimum dilution of each antibodies used.

A solid-phase microarray for blood grouping was reported by Robb et al., 2006 [137]. Antibodies or serum proteins were printed on nitrocellulose-coated slides. The highly specific blood grouping was done on a planar microarray by using a labeled erythrocyte for fluorescent data readout. The SPR technique showed the specific interaction of anti-A with blood group A erythrocyte and no reactivity detected for group B and O erythrocytes. Moreover, the study of interaction between

anti-A to the immobilized synthetic blood group A antigens were performed while varying the surface, immobilization technique and antibody probes for microarray-based blood group serology. A comparison among various techniques involved with the detection of antigens and antibodies for blood typing was tabulated in table 5.1.

Table 5.1 Comparison of methods involved with the detection of antigen and antibody for blood typing

Detection	Method	Substrate	Sample	Results	Advantage	Disadvantage	Ref
Antigen A and B on RBCs	SPR (direct detection)	Dextran surface immobilized with monoclonal anti-A and anti-B IgM (>95% pure)	Human blood group A, B, AB, O erythrocytes.	Detection limit of A erythrocyte $<0.33 \times 10^9$ RBC/mL.	- quantitative - high specific - labeled free - regenerate with 20mM NaOH	- the signal vary between different donors	[99] Quinn et al. (1997)
Anti-A and anti-B antibody (IgG and IgM)	ELISA (sandwich assay)	Polystyrene plate Coated with blood group substance A and B	Purified IgG and IgM type of blood group antibodies from human serum	Mean content of anti-A IgG from 15 serum samples is 3.98±8.74 μg/g total IgG.	- quantitative - specific - reproducible	- need labeling 2nd antibody - can not regenerate	[131] Bunchs and Nydegger (1989)
Anti-A and anti-B antibody (IgG and IgM)	Flow cytometric	-	Human serum incubate RBCs	Anti-A/B IgM was the main antibody to predict blood group. Anti-A/B IgG was found in blood group O sample. But anti-A or B IgG rarely found in blood group B or A sample.	- quantitative - more sensitive and more specific than ELISA (due to the binding of antibody to natural ABO antigen on RBCs)	- need labeling 2nd antibody - can not regenerate	[132] Stussi et al. (2005)
Anti-A and anti-B Antibody (IgG)	SPR (sandwich assay)	Dextran surface immobilized with blood group A/B trisaccharide amine derivative	Human serum	- Plasma AB (n=10) showed cut off value at 50 Resp. Diff. - Anti-A/B IgG in plasma O showed 50-300 Resp. Diff. - Anti-A/B IgG in plasma B or A show 50-300 Resp. Diff.	-rapid detection - regenerate with 50mM NaOH - no labeling of 2nd antibody	-poor in measuring IgM antibody	[133] Kimura et al. (2004)

Table 5.1 Comparison of methods involved with the detection of antigen and antibody for blood typing (cont.)

Detection	Method	Substrate	Sample	Results	Advantage	Disadvantage	Ref
Forward cell grouping and reverse serum groupin	A novel microplate agglutina-tion method (MAM)	Microplate with and without agarose for forward and reverse grouping	Forward; antibody incubate with RBCs. Reverse; plasma mixed with RBCs.	From 2225 sample blood donors, in case forward grouping showed 0.37% false negative. Reverse grouping gave 0.09% and 0.67% false positive and false negative.	- no immobilization step - no need for centrifugation - detect various sample - Forward and reverse grouping can be done at the same time - possible for adapting to fully automate system	-time consuming -limited type of microplate - need to do antibody titer for efficient results	[136] Spindler et al. (2002)
Antigen on RBCs	Solid phase microarray	Poly-L-lysine slide slides printed with antibody specific ABO blood group	Erythrocyte labeled with fluorescein isothiocyanate (FITC)	ABO blood group were achieved with cross react	- quantitative - highly specific - high throughput - multiple surface printing - various conditions can be monitored	- need labeling erythrocyte - high cost	[137] Robb et al. (2005)
Antigen A on RBCs and ranking anti-A antibodies	SPR	Dextran surface immobilized with anti-A and synthetic blood group A antigen	Unlabelled erythrocyte and various cell line anti-A	Can quantified the interactions of an immobilized anti-A with erythrocytes of various specificities Various Anti-A were ranked according to the strength of reaction with immobilized A antigen	- quantitative - highly specific - no labeling erythrocyte - real time measurement - regenerate with 1mM NaOH	- lower throughput when compare with microarray	

5.3 Objective

To combine the ABO and Rh blood typing of whole blood in the same SPR sensor chip in an array detection platform.

5.4 Experiment

5.4.1 Reagent

Monoclonal anti-A, anti-B and anti-D were used against A, B and D antigens. Anti-A and anti-B were IgM type, but the anti-D was IgG mixed with IgM. All antibodies were composed of bovine serum albumin (BSA) because they were designed for the agglutination test. The antibodies were obtained from the Thai Red Cross Society. Synthetic A and B antigens that were conjugated with BSA were purchased from Dextra Laboratories, UK. Blood and serum samples were left-over samples from the Ramathibodi Hospital Blood Bank (Bangkok, Thailand). This work was approved by the Ramathibodi Hospital Ethics Committee.

5.4.2 Sample preparation

EDTA blood samples were centrifuged at 4500 rpm to separate the plasma. RBCs were washed with PBS buffer and stored in Alsever preservative at 4ºC. The removed plasma was stored at -20ºC before use. For forward cell grouping and Rh D typing, 5% (v/v) RBCs was used and was prepared by taking 1:20 dilution of 100%RBC in PBS pH 7.4. The plasma sample for reverse serum grouping was prepared by diluting the plasma in PBST 0.005% at 1:10 dilution. The whole blood sample was prepared by taking 1:5 dilution of EDTA whole blood in PBST pH 7.4.

5.4.3 Ligand immobilization

The CMD surface was prepared using the method by Lofas [57]. The CMD was activated with 0.4M EDC and 0.1M NHS for 10 minutes to form reactive N-ester groups for ligand immobilization. The conditions for preparation of the ligands were different for each immobilized molecule; anti-A and anti-B antibodies were diluted at 1:10 in sodium acetate pH 5.3, anti-D antibodies were diluted at 1:5 in

sodium acetate pH 5.3 and 40 μg/ml of synthetic blood group A and B antigen in sodium acetate pH 4. BSA 10 μg/ml in sodium acetate pH 4 was used for the reference channel. The ligands flowed through the surface for 15 minutes which was followed with ethanolamine pH 8.5 for 10 minutes to deactivate the remaining reactive N-ester group.

5.4.4 Sample detection

The samples of 5% RBCs and 1:10 plasma diultion 100 μL was injected into the SPR machine at a flow rate 10 μL/min. After waiting 10 minutes, a running buffer was passed over the surface until the SPR signals were stable. The signals were monitored as a change in reflectivity at the region of interest (ROI). The ABO (forward cell grouping and reverse serum grouping) and Rh blood types were classified. The surface was regenerated by 5 mM NaOH and up to 20 mM NaOH for the plasma sample. Measurements were done in the seven parallel microchannels of flow cell, for each channel has its own control ROI using BSA. Whole blood at 1:5 dilution of group A, B, AB and O were trailed for ABO-Rh blood types in the same run.

5.5 Results and Discussion

5.5.1 Serum grouping

Serum grouping is an important method to confirm ABO blood types in addition to forward cell grouping. The synthetic blood group A and B antigens conjugated with BSA were immobilized on the CMD surface.

5.5.1.1 Pre-concentration of antigen A and B conjugated BSA

Finding the optimum pH for the immobilizing antigen was done by the pre-concentration method (see figure 1.9). Antigen A and antigen B which was conjugated with BSA at 10 μg/ml in various pHs of sodium acetate (NaOAc) buffer were prepared. Based on the assumption that BSA have a pI value around 4.7, the optimum pH buffer should be more than 3.5 (CMD surface has negative charge)

but lower than 4.7 (BSA have positive charge) the BSA will covalently immobilize onto the surface. Hence the pH range 5.5, 5, 4.5 and 4 of NaOAc buffer which covered a pI of BSA were used. The solutions flowed over the CMD surface without activating the carboxylic group. After the pH 5.5 and 5 solutions were injected, the SPR signals sharply decreased due to the change in the bulk refractive index and came back to baseline when the PBS filled up, as shown in figure 5.1. This means that the antigen was not attached to the surface. This may be due to the electrostatic repulsion between the negative charge of BSA and the dextran surface. At a pH 4.5, a slight change of SPR signal was observed after injecting the solution. The signal was deeply decreased and then gradually increased until reaching the original baseline. This may be due to an interaction of BSA with the surface. At a pH 4, lower than the pI of BSA, the positively charged BSA has more electrostatic attraction to the surface causing a steep increase of the signal.

A Sodium acetate buffer pH of 4 was chosen for immobilization of antigen A and B conjugated BSA onto the dextran surface. At this pH, positively charged BSA has greater possibility to link on the surface than the other pH.

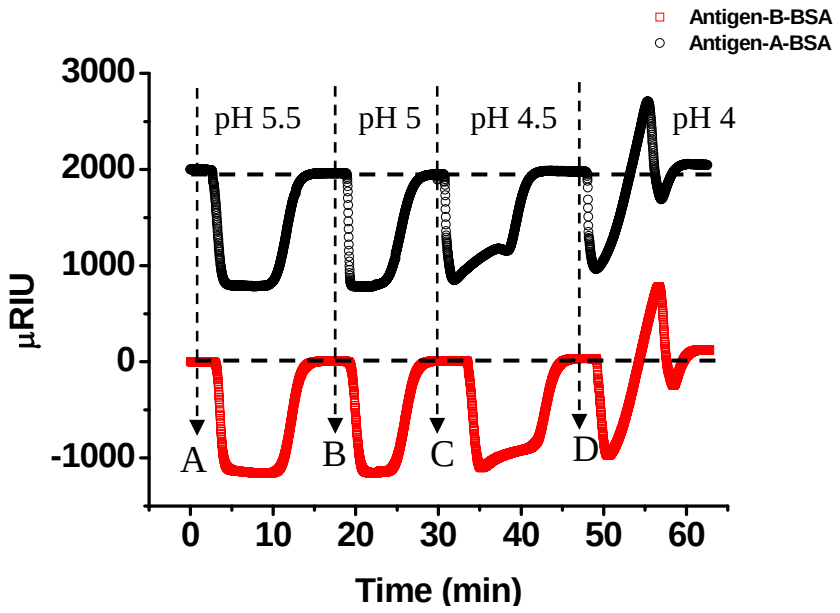


Figure 5.1 Pre-concentration of antigen A and antigen B conjugated with BSA at various pH; starting injection at point A pH 5.5, point B pH 5, point C pH 4.5 and point D pH 4.

5.5.1.2 Immobilization of antigen A and B conjugated BSA

The antigen A and antigen B conjugated BSA at concentrations of 1, 5, 20 and 40 μg/mL in NaOAc pH 4 were used to find the optimum concentration of antigen immobilization on the CMD surface. The SPR signals were plotted for all the antigen concentrations in figure 5.2 where an increased SPR signal depended on antigen concentration. The signal of both antigen A and antigen B conjugated BSA began to reach the saturation point at a concentration of 20 μg/mL and reached a plateau at 40 μg/mL. The antigen concentration of 40 μg/mL was chosen to ensure the greatest amount of the immobilized antigens on the surface.

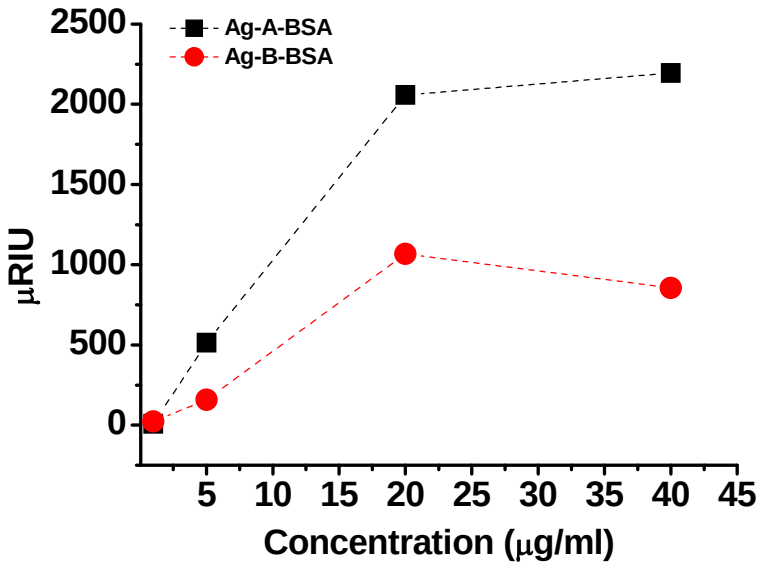
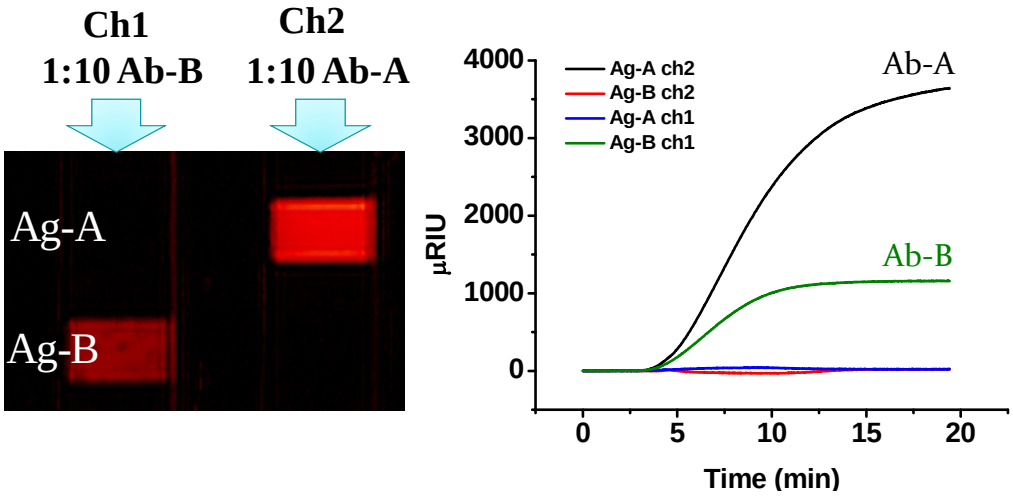


Figure 5.2 Immobilization signal against concentration of antigen A conjugated BSA (Ag-A-BSA) and antigen B conjugated BSA (Ag-B-BSA) on the CMD surface

5.5.1.3 Testing of immobilized antigen A and B conjugated BSA surface with anti A and anti B

In figure 5.3, antigen A and antigen B conjugated BSA were immobilized on the dextran surface along the horizontal line. Testing of the surface was carried out by injecting the monoclonal antibody B IgM through channel 1 and antibody A IgM through channel 2. The SPR image (figure 5.3 (a)) showed an increased color at the cross section area that has interactions between immobilized

antigens and their antibodies. Antibody B interacted with the antigen B surface but did not interact with antigen A surface (channel 1). Antibody A interacted with the antigen A surface but not with the antigen B surface (channel 2). These SPR image results of can be related to the SPR signal (figure 5.3 (b)). There was no non-specific binding or cross binding from the antibody. The next step is to test the surface with a plasma sample.



(a) (b)

Figure 5.3 Functional testing of the surface; (a) SPR imaging by immobilization of antigen A and antigen B conjugated BSA on horizontal line and injection of monoclonal antibody B and antibody A in channel 1 (ch1) and channel 2 (ch2), (b) SPR signal that is related to the signal from SPR imaging

5.5.1.4 Finding the suitable dilution of plasma sample

Plasma samples at various dilutions were tested in order to find a suitable dilution for SPR measurement (figure 5.4). An extra precaution must be taken for the plasma because many proteins are present in the plasma and may cause non-specific binding. For plasma blood group AB (figure 5.4 (a)), only antibodies specific to either A or B antigen should be present in the plasma. The signal should detect for both antigen A and antigen B. Any signal which shows up should originate from the other proteins present in the plasma, which is referred to as non-specific

binding. For plasma blood group A (figure 5.4 (b)), the specific signal will occur on the antigen B surface due to anti- B in the plasma. At a low plasma concentration, the difference between specific and non-specific signals couldn't be observed which may be due to the lower amount of antibodies in the plasma. At a lower dilution (1:10 and 1:5), there was less interference from the non-specific binding. For plasma blood group B (fig 5.4 (c)), the specific signal occurs on antigen A surface due to anti-A in the plasma. At a 1:10 and 1:5 dilutions, a highly specific signal occurred. For plasma blood group O (figure 5.4 (d)), both anti-A and anti-B were observed on both antigen A and antigen B surfaces. All these results show that the lower dilution (1:5) injected produced the higher signal but the problem was that more non-specific binding signals occurred. Therefore a dilution of 1:10 seems to be the suitable dilution of plasma to avoid non-specific binding and still be able to observe specific binding.

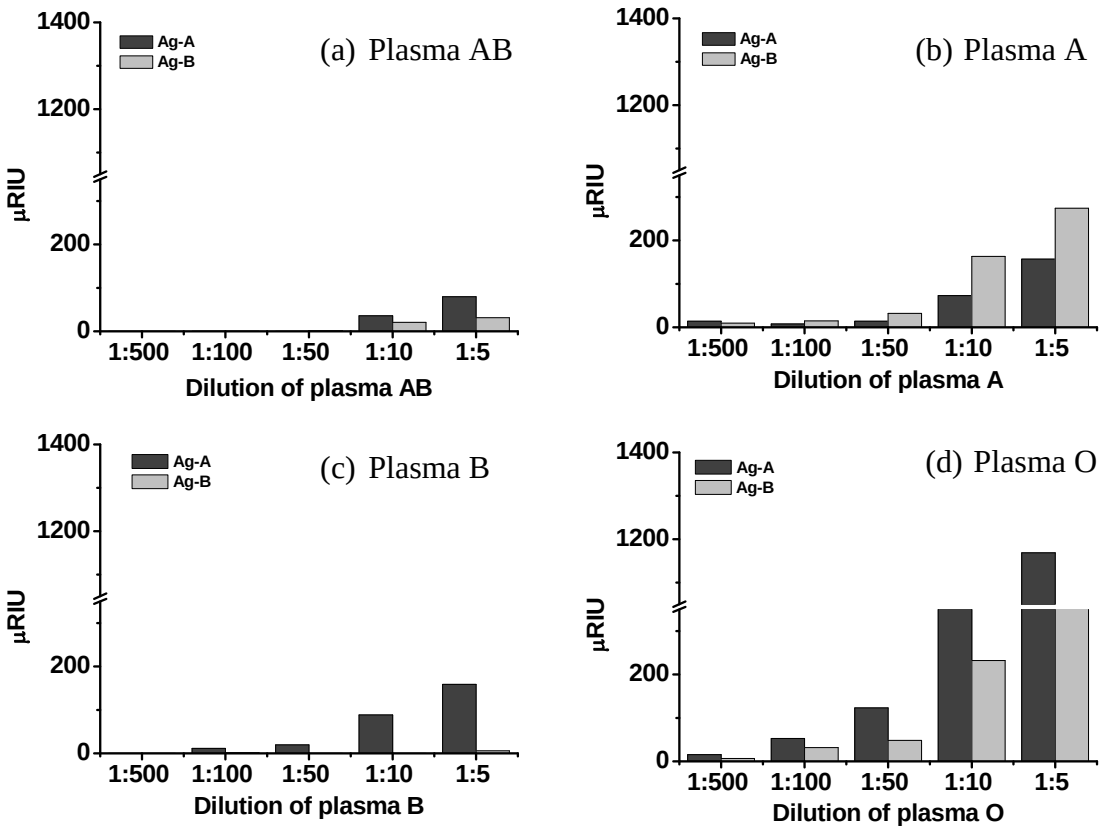


Figure 5.4 The SPR signal from various dilutions of plasma sample; (a) plasma blood group AB, (b) plasma blood group A, (c) plasma blood group B and (d) plasma blood group O.

5.5.1.5 Serum grouping by various plasma samples

One hundred and twelve plasma samples were tested for reverse ABO blood types by the SPR technique, including plasma blood group AB n=19, plasma blood group A n=29, plasma blood group B n=33 and plasma blood group O n=31. All plasma samples were diluted at a 1:10 dilution in PBST buffer and were flowed over the surface. For surface regeneration, 20 mM NaOH was used to wash off antibodies and proteins in the plasma and the surface could be used for the next cycle. SPR signals of each plasma group are shown in figure 5.5. From total 19 samples of plasma AB (figure 5.5 (a)) gave the average SPR signal on antigen A surface approximately 29.52 ± 31.48 μRIU and on antigen B surface approximately 25.32 ± 26.31 μRIU. These signals from plasma AB were used as a cut off value for each antigen A and antigen B surface which meant the highest signal of non-specific binding from the plasma samples. In other words, the signal, which is higher than this cut off value, is noted to be the positive signal.

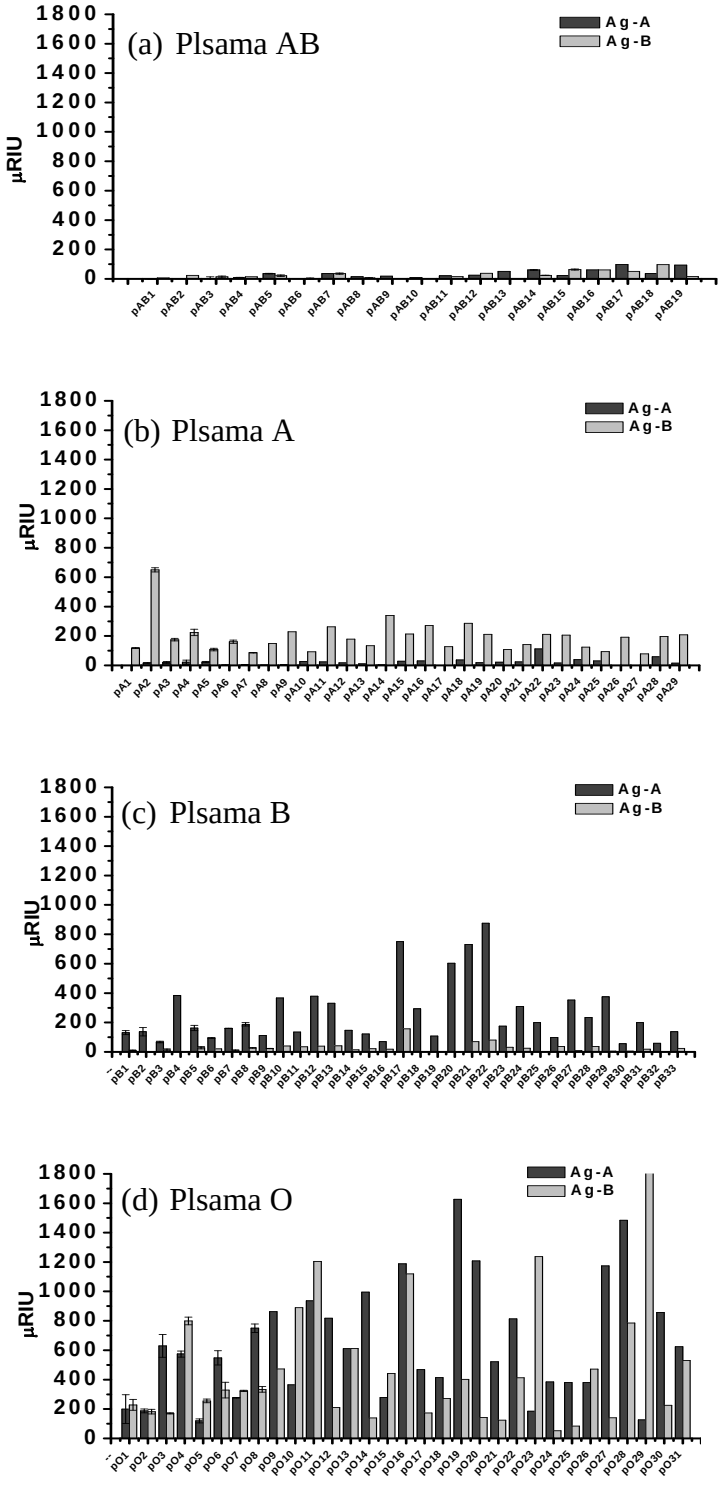


Figure 5.5 SPR signal of each plasma group on antigen A and antigen B conjugated BSA surface; (a) plasma blood group AB, (b) plasma blood group A, (c) plasma blood group B and (d) plasma blood group O.

For plasma blood group A (figure 5.5 (b)), almost all plasma blood group A samples were grouped correctly however they showed quite a low signal with antigen B surface of around 200 μRIU but much higher than the cut off value. One from 29 samples of plasma blood group A showed false-positive values (see table 5.2) on antigen A surface (3.45%) that is non-specific binding occurred more than the cut off value on antigen A surface. For plasma blood group B (figure 5.5 (c)), most of signals were around 200-400 μRIU, 3 of 33 samples showed false-positive (9.09%) signals on the antigen B surface and 2 of 33 samples showed false-negative (6.06%) signals on the antigen A surface that mean specific signal of plasma blood group B on antigen A surface lower than the cut off value (table 5.2). For plasma blood group O (figure 5.5 (d)), all plasma samples were grouped correctly, and the specific signal on antigen A and antigen B surfaces were higher than the cut off value. Specific signals taken from plasma blood group O were higher than both plasma blood group A and plasma blood group B; this may be explained by the ability of SPR for quantitative analysis of antibodies in plasma.

However there are some problems in serum grouping such as low specific signal, some non-specific signal, and false-positive or false-negative results. These problems might come from the quantity of antibodies in each individual sample [136] and by the interference from other proteins in the plasma. Further study into preventing non-specific binding or cross reaction signals needs to be investigated. In the case of low specific SPR signal (plasma A on antigen B surface and plasma B on antigen A surface), an increase of specific signal may be done by injection of anti-IgG antibodies after the plasma sample (sandwich assay) [133]. However, ABO blood typing must be classified under a combination of reverse serum grouping and forward cell grouping which can classify blood groups with 100% accuracy.

Table 5.2 False-positive and false-negative results from serum grouping by SPR technique

Plasma group.	Antigen tested	False-positive	False-negative
AB (n=19)	A	-	-
	B	-	-
A (n=29)	A	1 (3.45%)	0
	B	0	0
B (n=33)	A	0	2 (6.06%)
	B	3 (9.09%)	0
O (n=31)	A	0	0
	B	0	0
Total (n=112)		4 (3.57%)	2 (1.79%)

5.5.2 Cell Grouping

Anti-A and anti-B antibodies were immobilized by amine coupling onto the CMD surface. The optimum condition was a 1:10 dilution of antibodies in sodium acetate with pH 5.3 to avoid some BSA being linked onto the surface. Both antibodies showed comparable SPR immobilization signal. Dilution of the RBC's total 28 samples including A n=7, B n=7, AB n=7 and O n=7 at 5% in PBS buffer were flowed over the surface. A Specific signal of their antigen antibody couple was performed, as shown in figure 5.6. A-type RBCs responded with an anti-A probe but not with an anti-B probe, B-type RBCs reacted with an anti-B probe but not with an anti-A probe. Finally, AB-type RBCs reacted with both anti-A and anti-B probes, but O-type RBCs did not responded with either.

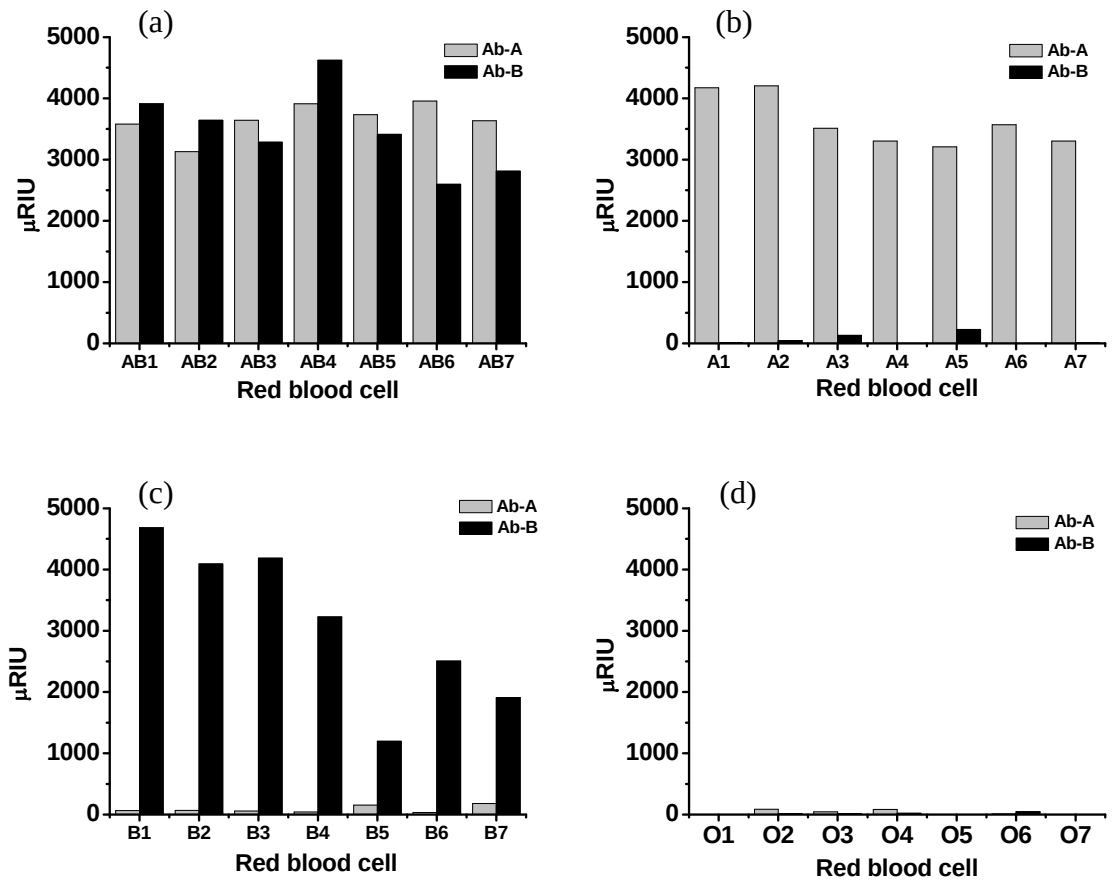


Figure 5.6 Bar chart representing the specificity of SPR technique for forward ABO blood grouping; (a) AB-RBCs, (b) A-RBCs, (c) B-RBCs and (d) O-RBCs

The detected SPR signal from all RBC samples, including anti-A and anti-B, are shown in table 5.3. The signals were averaged from each of the RBC's blood group samples. From table 5.3, specific signals for each antibody were more than 3000 μRIU (bold numeral) which were much higher than a non-specific signal. The non-specific signal for the cell grouping was approximately 0-140 μRIU that was obtained from the lowest and highest value from O-type RBCs to the anti-B surface (-4.61±51.35) and B-type RBCs to the anti-A surface (85.11±58.02) respectively. The non specific signal from RBCs to the surface may come from physical adsorption or hydrogen bonding between the RBC's surface and the CMD surface. Each measurement cycle was regenerated with 5 mM NaOH to wash RBCs from the surface while antibodies still remained on the surface for use in the next cycles. All the results

of cell grouping by SPR technique were consistent (100% accuracy) with the standard gel agglutination technique.

Table 5.3 SPR signal from cell grouping

Blood group	No. of test	Signal (μRIU)				Accuracy (%)
		Anti-A		Anti-B		
		Ave	SD	Ave	SD	
AB	7	**3655.49**	**272.27**	**3468.16**	**682.17**	100
O	7	10.94	71.39	-4.61	51.35	100
A	7	**3610.33**	**414.84**	55.14	90.85	100
B	7	85.11	58.02	**3114.26**	**1296.10**	100

5.5.3 Rh (D) typing

The second most important method in blood transfusion after ABO blood typing is Rh (D) typing. Rh positive (Rh+) and Rh negative (Rh-) blood types are found by the presence of D antigens on the RBC's surface. This work demonstrated that the SPR technique had the ability to detect D antigens on the RBC's surface. By the solid phase immobilization technique, anti-D (IgM mixed IgG) antibodies at dilution 1:5 in sodium acetate and pH 5.3 was the optimum condition for selection of anti-D immobilized onto the surface instead of BSA. This was due to the same reason as immobilization of anti-A and anti-B types for cell grouping.

A total of 50 samples of RBCs including group A Rh+ n=10, B Rh+ n=10, AB Rh+ n=10, O Rh+ n=10 and RBCs Rh- n=10 (A, B, AB and O) were used. Five % RBCs in PBS buffer pH 7.4 was flowed over mixed anti-D surfaces. The specificity of this technique for D antigen binding to immobilize anti-D on the surface is demonstrated in figure 5.7. The immobilized anti-D probe has interaction with Rh+ RBCs but not with Rh- RBCs.

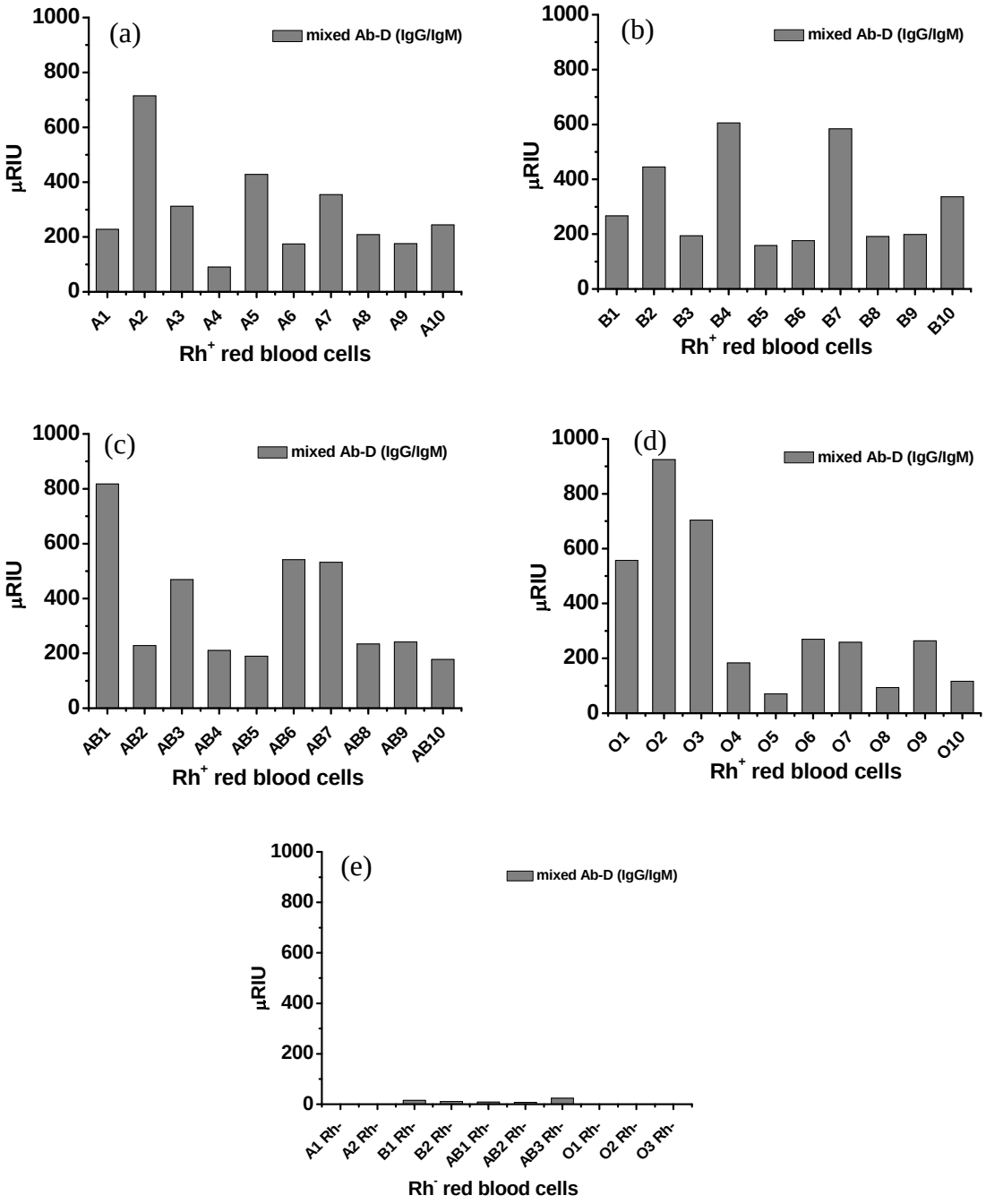


Figure 5.7 Bar chart represents the specificity of SPR technique for Rh (D) typing; (a) Rh+ A-RBCs, (b) Rh+ B-RBCs, (c) Rh+ AB-RBCs, (d) Rh+ O-RBCs and (e) Rh- RBCs

The signals of Rh (D) typing by SPR technique is shown in table 5.4, it shows that the SPR technique could detect all Rh+ RBCs correctly with the average signal of 329.28±211.11 μRIU which were much higher than Rh- RBCs which were 3.64±11.45 μRIU. All the results accorded with the gel agglutination technique with 100% accuracy. However, the signal from RBCs still varied which may be caused by the flow system, the antigenic site on the RBC's surface or the other factors such as salt concentration. An in depth investigation of the antibodies with D antigen on RBCs is still needed. The specific signal of D antigen to anti-D (table 5.4) were lower than A and B antigen to their specific antibodies from cell grouping (table 5.3). This may be caused by the position of the binding site for D antigen and amount of D antigen on the RBC's surface. D antigen is the polypeptide span on the red cell membrane which has a fewer amount of antigenic sites than A and B antigen, which is the highly branched chain of oligosaccharides extended from the RBC's surface that would have lower possibility to bind antibodies on the surface than A and B antigens (see figure 3.1). Moreover, the branch chain of oligosaccharides may be facilitating the binding of A and B antigens to their antibody but obstructs for the D antigen binding to its antibody.

Table 5.4 SPR signal from Rh typing

Rh type	No. of test	SPR	Signal (μRIU)		Accuracy (%)
			Ave	SD	
D positive	40	40	329.28	211.11	100
D negative	10	10	3.64	11.45	100

5.5.4 Array detection platform for integration of ABO-Rh blood-typing

The combination of ABO-Rh blood-typing including cell grouping, serum grouping and D antigen detection were performed by the SPR imaging technique as shown in figure 5.8. A convenient method with a single step of whole blood sample injection was proposed. The immobilization ligands were in a horizontal line from top to bottom; anti-A, anti-B, antigen-A, antigen-B and anti-D antibody. Immobilization for each ligand used the optimum conditions that are described above. For the sample

injection, whole blood was diluted 1:5 in PBST pH 7.4 buffer (around 1:10 dilution of serum with around 10% of RBCs) volume 100 μL. Channel 1 (ch1) was the whole blood group AB Rh+, channel 2 (ch2) was the whole blood group B Rh+, channel 3 (ch3) was the whole blood group A Rh- and channel 4 (ch4) was the whole blood group O Rh+. All blood samples flowed over the surface in parallel and ABO-Rh blood types were determined after 20 minutes.

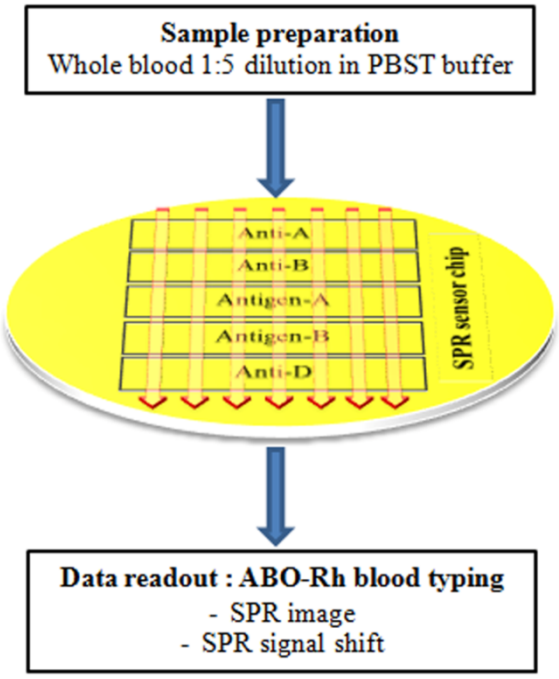


Figure 5.8 This schematic represents the method for ABO-Rh blood typing on an integrated ABO-Rh SPR sensor chip

The SPR data processing is shown as figure 5.9. SPR image showed the specific signals from antigen to their antibody in the brighter color of cross section area (figure 5.9 (a)) which relates to the SPR signal changed (figure 5.9 (b)). The whole blood group AB Rh+ had specific SPR responded with anti-A, anti-B, anti-D surfaces but very low signal with antigen A and B surfaces due to a lack of antibodies for ABO blood groups in the plasma. The whole blood group B Rh+ showed specific response with the anti-B, anti-D and antigen A surfaces but had very low signal on the anti-A and antigen B surfaces, which were defined as non-specific signals, would

come from other proteins in the plasma. The whole blood A Rh- had responded with anti-A and antigen B surfaces but had a lower signal with anti-B, anti-D and antigen A surfaces due to lack of antigen D on the RBC's surface and antibody A in the plasma. The whole blood group O Rh+ had a signal on anti-D, antigen A and B surfaces but had a very low non-specific signal on anti-A and anti-B surfaces. These results are an example of applying the SPR imaging technique for combination of ABO-Rh blood-typing in the same sensor chip as an array platform. This would be exposed the advantage of convenient and easy method by single step of whole blood sample injection. Cost savings can be realized because the surface can be regenerated. Test times can be reduced because various samples can be tested in parallel. Furthermore, this SPR imaging technique can be automated for routine work.

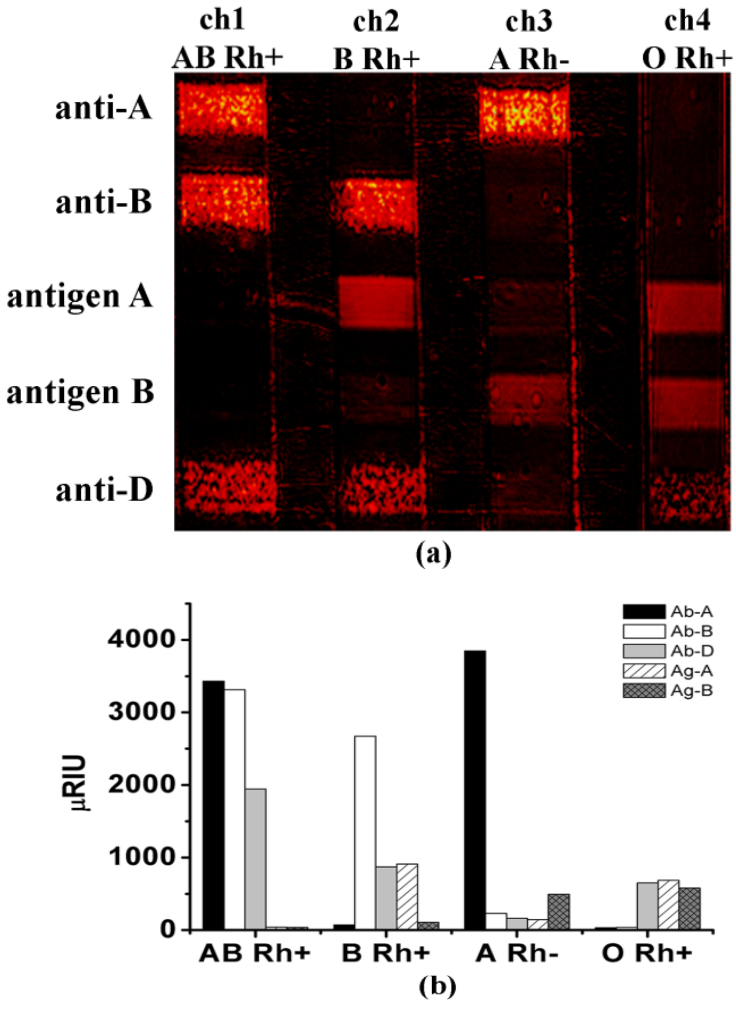


Figure 5.9 Integrated SPR sensor chip for ABO-Rh blood grouping array by injection 1:5 dilution of whole blood; (a) SPR image and (b) SPR signal

5.6 Conclusions

The SPR imaging technique showed possibility to detect A, B and D antigens on a RBC's surface and antibodies specific to ABO blood group in serum. All samples of RBCs were correctly grouped for cell grouping and Rh (D) detection produced a specific signal much higher than a non-specific signal. Serum grouping showed a lower signal than cell grouping, but the signal is still distinguishable for the specific signal and non specific signal from other proteins in the plasma. The surface can be regenerated at least 10 times with 5-20 mM NaOH. The SPR imaging technique has the potential for the combination of ABO-Rh blood-typing in the same sensor chip which is the convenient and easy method by single step of whole blood sample injection. This work is forming the prototype of SPR technology which is expected to be developed and applyed in blood bank applications.

REFERENCES

1. Wood, R.W., *On a remarkable case of uneven light in a diffraction grating*, in *Philos. Mag.*1902. p. 396-402.
2. Rayleigh, L., *On the dynamical theory of gratings.* Proc. R. Soc. London, Ser. A, 1907. **79**: p. 399.
3. Fano, U., *The theory of anomalous diffraction gratings and of quasi-stationary waves on metallic surfaces (Sommerfeld's waves).* J. Opt. Soc. Am., 1941. **31**: p. 213-222.
4. Otto, A., *Excitation of surface plasma waves in silver by the method of frustrated total reflection.* Z. Phys, 1968. **216**: p. 398-410.
5. Kretschmann, E. and H. Raether, *Radiative decay of non radiative surface plasmons excited by light(Surface plasma waves excitation by light and decay into photons applied to nonradiative modes).* Zeitschrift Fuer Naturforschung, Teil A, 1968. **23**: p. 2135.
6. Liedberg, B., C. Nylander, and I. Lunstrom, *Surface plasmon resonance for gas detection and biosensing.* Sensors and Actuators, 1983. **4**: p. 299-304.
7. Salamon, Z., H.A. Macleod, and G. Tollin, *Coupled plasmon-waveguide resonators: a new spectroscopic tool for probing proteolipid film structure and properties.* Biophysical journal, 1997. **73**(5): p. 2791-2797.
8. Chaiken, I., S. Rosé, and R. Karlsson, *Analysis of macromolecular interactions using immobilized ligands.* Analytical Biochemistry, 1992. **201**(2): p. 197-210.
9. Davies, J., *Surface plasmon resonance—the technique and its applications to biomaterial processes.* Nanobiology, 1994. **3**: p. 5-16.
10. Löfås, S., et al., *Bioanalysis with surface plasmon resonance.* Sensors and Actuators B: Chemical, 1991. **5**(1): p. 79-84.

11. Liedberg, B., I. Lundström, and E. Stenberg, *Principles of biosensing with an extended coupling matrix and surface plasmon resonance.* Sensors and Actuators B: Chemical, 1993. **11**(1): p. 63-72.
12. Wood, S., *DNA-DNA hybridization in real time using BIAcore.* Microchemical Journal, 1993. **47**(3): p. 330-337.
13. Säfsten, P., et al., *Screening antibody–antigen interactions in parallel using Biacore A100.* Analytical Biochemistry, 2006. **353**(2): p. 181-190.
14. Medina, M., et al., *Real-time analysis of antibody binding interactions with immobilized E. coli O157: H7 cells using the BIAcore.* Biotechnology Techniques, 1997. **11**(3): p. 173-176.
15. Lakowicz, J.R., *Radiative decay engineering 3. Surface plasmon-coupled directional emission.* Analytical Biochemistry, 2004. **324**: p. 153-169.
16. Beusink, J.B., et al., *Angle-scanning SPR imaging for detection of biomolecular interactions on microarrays.* Biosensors and Bioelectronics, 2008. **23**(6): p. 839-844.
17. Tudos, R.B.M.S.a.A.J., *Handbook of Surface Plasmon Resonance*2008: The Royal Society of Chemistry.
18. Zeng, Y.T.a.X., *Surface Plasmon Resonance: An Introduction to a Surface Spectroscopy Technique.* Journal of Chemical Education, 2010. **87**: p. 742-746.
19. Hutchinson, A.M., *Evanescent Wave Biosensors Real-Time Analysis of Biomolecular Interactions.* Molecular Biotechnology, 1995. **3**: p. 47-54.
20. Homola, J., S.S. Yee, and G. Gauglitz, *Surface plasmon resonance sensors: review.* Sensors and Actuators B: Chemical, 1999. **54**(1): p. 3-15.
21. Willets, K.A. and R.P. Van Duyne, *Localized surface plasmon resonance spectroscopy and sensing.* Annu. Rev. Phys. Chem., 2007. **58**: p. 267-297.
22. Patskovsky, S., et al., *Properties and sensing characteristics of surface-plasmon resonance in infrared light.* JOSA A, 2003. **20**(8): p. 1644-1650.
23. http://www.discoversensiq.com/technology/spr-principles/, *Overview of Surface Plasmon Resonance*.
24. http://www.proteomics.iastate.edu/seminars/BIACore/, *TechnologyNotes*.

25. Steiner, G., *Surface plasmon resonance imaging.* Analytical and bioanalytical chemistry, 2004. **379**(3): p. 328-331.

26. Puttharugsa, C., et al., *Development of surface plasmon resonance imaging for detection of Acidovorax avenae subsp. Citrulli (Aac) using specific monoclonal antibody.* Biosensors and Bioelectronics, 2011. **26**(5): p. 2341-2346.

27. Campbell, C.T. and G. Kim, *SPR microscopy and its applications to high-throughput analyses of biomolecular binding events and their kinetics.* Biomaterials, 2007. **28**(15): p. 2380-2392.

28. http://microgen.ouhsc.edu/biacore.htm.

29. Mouvet C., H.R., Maciag C., Luff B., Wilkinson J., Piehler J., Brecht A., Gauglitz G., Abuknesha R., Ismail G., Anal Chim Acta, 1997. **338**: p. 109.

30. Minunni, M. and M. Mascini, *Detection of pesticide in drinking water using real-time biospecific interaction analysis (BIA).* Analytical Letters, 1993. **26**(7): p. 1441-1460.

31. Gobi, K.V., et al., *Highly sensitive regenerable immunosensor for label-free detection of 2, 4-dichlorophenoxyacetic acid at ppb levels by using surface plasmon resonance imaging.* Sensors and Actuators B: Chemical, 2005. **111**: p. 562-571.

32. Koubova V, B.E., Karasova L, Skvor J, Homola J, Dostalek J, Tobiska P, Rosicky J, Sensor Actuator B Chem, 2001 **74**: p. 100.

33. OH, B.K., et al., *Detection of Escherichia coli O157: H7 using immunosensor based on surface plasmon resonance.* Journal of microbiology and biotechnology, 2002. **12**(5): p. 780-786.

34. Slavık, R., J. Homola, and E. Brynda, *A miniature fiber optic surface plasmon resonance sensor for fast detection of staphylococcal enterotoxin B.* Biosensors and Bioelectronics, 2002. **17**(6): p. 591-595.

35. Besselink, G.A.J., et al., *Signal amplification on planar and gel-type sensor surfaces in surface plasmon resonance-based detection of prostate-specific antigen.* Analytical Biochemistry, 2004. **333**(1): p. 165-173.

36. Masson, J.F., et al., *Sensitive and real-time fiber-optic-based surface plasmon resonance sensors for myoglobin and cardiac troponin I.* Talanta, 2004. **62**(5): p. 865-870.

37. Ladd, J., et al., *DNA-directed protein immobilization on mixed self-assembled monolayers via a streptavidin bridge.* Langmuir, 2004. **20**(19): p. 8090-8095.

38. Fitzpatrick, B. and R. O'Kennedy, *The development and application of a surface plasmon resonance-based inhibition immunoassay for the determination of warfarin in plasma ultrafiltrate.* Journal of immunological methods, 2004. **291**(1): p. 11-25.

39. Biacore, A., *Biacore sensor surface handbook*, 2003, BR-1005-71, Uppsala.

40. Shankaran, D.R. and N. Miura, *Trends in interfacial design for surface plasmon resonance based immunoassays.* Journal of Physics D: Applied Physics, 2007. **40**(23): p. 7187.

41. Kusnezow, W. and J.D. Hoheisel, *Solid supports for microarray immunoassays.* Journal of Molecular Recognition, 2003. **16**(4): p. 165-176.

42. Hendrix, M., et al., *Direct observation of aminoglycoside-RNA interactions by surface plasmon resonance.* Journal of the American Chemical Society, 1997. **119**(16): p. 3641-3648.

43. Gobi, K.V., C. Kataoka, and N. Miura, *Surface plasmon resonance detection of endocrine disruptors using immunoprobes based on self-assembled monolayers.* Sensors and Actuators B: Chemical, 2005. **108**(1): p. 784-790.

44. Lan, E., B. Dunn, and J. Zink, *Sol-gel encapsulated anti-trinitrotoluene antibodies in immunoassays for TNT.* Chemistry of materials, 2000. **12**(7): p. 1874-1878.

45. Davis, F. and S.P.J. Higson, *Structured thin films as functional components within biosensors.* Biosensors and Bioelectronics, 2005. **21**(1): p. 1-20.

46. Lundström, I., *Real-time biospecific interaction analysis.* Biosensors and Bioelectronics, 1994. **9**(9-10): p. 725-736.

47. Disley, D.M., et al., *Covalent coupling of immunoglobulin G to self-assembled monolayers as a method for immobilizing the interfacial-recognition layer*

of a surface plasmon resonance immunosensor. Biosensors and Bioelectronics, 1998. **13**(11): p. 1213-1225.

48. Tronin, A., et al., *Optimisation of IgG Langmuir film deposition for application as sensing elements.* Sensors and Actuators B: Chemical, 1996. **34**(1): p. 276-282.
49. Löfås, S. and B. Johnsson, *A novel hydrogel matrix on gold surfaces in surface plasmon resonance sensors for fast and efficient covalent immobilization of ligands.* Journal of the Chemical Society, Chemical Communications, 1990(21): p. 1526-1528.
50. Disley, D.M., et al., *Covalent coupling of immunoglobulin G to a poly (vinyl) alcohol-poly (acrylic acid) graft polymer as a method for fabricating the interfacial-recognition layer of a surface plasmon resonance immunosensor.* Biosensors and Bioelectronics, 1998. **13**(3): p. 383-396.
51. Ferretti, S., et al., *Self-assembled monolayers: a versatile tool for the formulation of bio-surfaces.* TrAC Trends in Analytical Chemistry, 2000. **19**(9): p. 530-540.
52. Ulman, A., *Formation and structure of self-assembled monolayers.* Chemical reviews, 1996. **96**(4): p. 1533.
53. Bain, C.D., et al., *Formation of monolayer films by the spontaneous assembly of organic thiols from solution onto gold.* Journal of the American Chemical Society, 1989. **111**(1): p. 321-335.
54. Patel, N., et al., *A scanning probe microscopy study of the physisorption and chemisorption of protein molecules onto carboxylate terminated self-assembled monolayers.* Applied Physics A: Materials Science & Processing, 1998. **66**: p. 569-574.
55. Lee, J.W., et al., *Characterization of a self-assembled monolayer of thiol on a gold surface and the fabrication of a biosensor chip based on surface plasmon resonance for detecting anti-GAD antibody.* Biosensors and Bioelectronics, 2005. **20**(7): p. 1422-1427.
56. Choi, S.H., J.W. Lee, and S.J. Sim, *Enhanced performance of a surface plasmon resonance immunosensor for detecting Ab–GAD antibody based on the*

modified self-assembled monolayers. Biosensors and Bioelectronics, 2005. **21**(2): p. 378-383.

57. Lofas, S., *Dextran modified self-assembled monolayer surfaces for use in biointeraction analysis with surface plasmon resonance.* Pure and applied chemistry, 1995. **67**(5): p. 829-834.
58. Handbook, B.S.S., *version AA*, 2003, BR-1005-71, Biacore, Uppsala, Sweden.
59. Handbook, B.C.A., *version AA*, December 2001, SE-754 50 Uppsala, Sweden.
60. Nieba, L., et al., *BIACORE analysis of histidine-tagged proteins using a chelating NTA sensor chip.* Analytical Biochemistry, 1997. **252**(2): p. 217-228.
61. Rich, R.L. and D.G. Myszka, *BIACORE J: a new platform for routine biomolecular interaction analysis†.* Journal of Molecular Recognition, 2001. **14**(4): p. 223-228.
62. Katsamba, P.S., et al., *Kinetic analysis of a high-affinity antibody/antigen interaction performed by multiple Biacore users.* Analytical Biochemistry, 2006. **352**(2): p. 208-221.
63. Frazier, R., et al., *Characterization of protein-resistant dextran monolayers.* Biomaterials, 2000. **21**(9): p. 957-966.
64. Dugdale, D.C., *Albumin - serum*, 2009, Medical Director, A.D.A.M., Inc.
65. *Microalbumin*, 2009, Chronolab A.G. Switzerland.
66. Busby, D.E. and R.C. Atkins, *Detection and measurement of microalbuminuria: a challenge for clinical chemistry.* MLO Med Lab Obs, 2005. **37**(2): p. 8-9.
67. Association, A.D., *Standards of Medical Care in Diabetes—2010.* Diabetes Care, 2010. **33**(Supplement 1): p. S11-S61.
68. Babazono, T., C. Takahashi, and Y. Iwamoto, *Definition of Microalbuminuria in First-Morning and Random Spot Urine in Diabetic Patients.* Diabetes Care, 2004. **27**(7): p. 1838-1839.
69. Hillege, H., et al., *Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population.* Circulation, 2002. **106**(14): p. 1777.
70. Gilbert, R., et al., *Microalbuminuria: prognostic and therapeutic implications in diabetes mellitus.* Diabetic Medicine, 1994. **11**(7): p. 636-645.

71. Contois, J., et al., *Analytical validation of an HPLC assay for urinary albumin.* Clinica Chimica Acta, 2006. **367**(1-2): p. 150-155.

72. Randolph L Pearson, M., Martin Goldberg, MD, FACP, Ankush Gulati, MD, *Diabetic nephropathy*, 2007, Elsevier Inc.

73. Liang, A.H., Y.J. Huang, and Z.L. Jiang, *A rapid and sensitive immunoresonance scattering spectral assay for microalbumin.* Clinica Chimica Acta, 2007. **383**(1): p. 73-77.

74. Giampietro, O., et al., *Which method for quantifying "microalbuminuria" in diabetics?* Acta diabetologica, 1992. **28**(3): p. 239-245.

75. Wu, T.L., et al., *Microplate ELISA for urine microalbumin: reference values and results in patients with type 2 diabetes and cardiovascular disease.* Annals of Clinical & Laboratory Science, 2005. **35**(2): p. 149-154.

76. Mogensen, C., *Microalbuminuria as a predictor of clinical diabetic nephropathy.* Kidney Int, 1987. **31**(2): p. 673-689.

77. Watts, G., et al., *Assessment of immunochemical methods for determining low concentrations of albumin in urine.* Clinical chemistry, 1986. **32**(8): p. 1544-1548.

78. Zhao, L., J.M. Lin, and Z. Li, *Comparison and development of two different solid phase chemiluminescence ELISA for the determination of albumin in urine.* Analytica chimica acta, 2005. **541**(1): p. 197-205.

79. Torffvit, O. and J.Ö.R. Wieslander, *A simplified enzyme-linked immunosorbent assay for urinary albumin.* Scandinavian journal of clinical & laboratory investigation, 1986. **46**(6): p. 545-548.

80. Comper, W.D., G. Jerums, and T.M. Osicka, *Differences in urinary albumin detected by four immunoassays and high-performance liquid chromatography.* Clinical biochemistry, 2004. **37**(2): p. 105-111.

81. Thakkar, H., et al., *Development and validation of a particle-enhanced turbidimetric inhibition assay for urine albumin on the Dade aca® analyzer.* Clinical chemistry, 1997. **43**(1): p. 109-113.

82. Marre, M., et al., *Laser immunonephelometry for routine quantification of urinary albumin excretion.* Clinical chemistry, 1987. **33**(2): p. 209-213.

83. Toto, R.D., *Microalbuminuria: definition, detection, and clinical significance.* The Journal of Clinical Hypertension, 2004. **6**: p. 2-7.

84. Sakai, G., et al., *Selective and repeatable detection of human serum albumin by using piezoelectric immunosensor.* Sensors and Actuators B: Chemical, 1995. **24**(1): p. 134-137.

85. Lee, W., et al., *Detection of human serum albumin on protein array using scanning tunneling microscopy.* Ultramicroscopy, 2010. **110**(6): p. 723-728.

86. Shumaker-Parry, J.S., R. Aebersold, and C.T. Campbell, *Parallel, quantitative measurement of protein binding to a 120-element double-stranded DNA array in real time using surface plasmon resonance microscopy.* Analytical chemistry, 2004. **76**(7): p. 2071-2082.

87. Johnsson, B., S. Löfås, and G. Lindquist, *Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors.* Analytical Biochemistry, 1991. **198**(2): p. 268-277.

88. Jung, Y., J. Jeong, and B. Chung, *Recent advances in immobilization methods of antibodies on solid supports.* The Analyst, 2008. **133**(6): p. 697-701.

89. Saphire, E.O., et al., *Contrasting IgG structures reveal extreme asymmetry and flexibility.* Journal of Molecular Biology, 2002. **319**(1): p. 9-18.

90. Lu, B., M.R. Smyth, and R. O'Kennedy, *Tutorial review. Oriented immobilization of antibodies and its applications in immunoassays and immunosensors.* Analyst, 1996. **121**(3): p. 29R-32R.

91. Norde, W., *Adsorption of proteins from solution at the solid-liquid interface.* Advances in colloid and interface science, 1986. **25**: p. 267-340.

92. Baud, M., *Data analysis, mathematical modeling.* Methods of immunological analysis, 1993. **1**: p. 656-671.

93. Rodbard, D., *Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays.* Clinical chemistry, 1974. **20**(10): p. 1255-1270.

94. Dudley, R., et al., *Guidelines for immunoassay data processing.* Clinical chemistry, 1985. **31**(8): p. 1264-1271.

95. Healy, M.J.R., *Statistical analysis of radioimmunoassay data.* Biochemical Journal, 1972. **130**(1): p. 207.

96. Vadgama, P., Crump, P.W., *Biosensors: Recent trends.* Analyst, 1992. **117**: p. 1657-1669.

97. Landsteiner K, *Zur Kenntnis der antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe.* Zentralblatt Bakteriologie, 1900. **27**: p. 357-62.

98. Malomgré, W. and B. Neumeister, *Recent and future trends in blood group typing.* Analytical and Bioanalytical Chemistry, 2009. **393**(5): p. 1443-1451.

99. Quinn, J.G., et al., *Detection of blood group antigens utilising immobilised antibodies and surface plasmon resonance.* Journal of immunological methods, 1997. **206**(1-2): p. 87-96.

100. Boozer, C., et al., *Looking towards label-free biomolecular interaction analysis in a high-throughput format: a review of new surface plasmon resonance technologies.* Current Opinion in Biotechnology, 2006. **17**(4): p. 400-405.

101. Daniels, G. and I. Bromilow, *Essential guide to blood groups*2007: Wiley Online Library.

102. http://www.vachiraphuket.go.th/www/public-health/index.php?name=knowledge&file=readknowledge&id=111.

103. http://www.cryst.bbk.ac.uk/pps97/assignments/projects/emilia/Adhesion.HTM.

104. Peungthum, P., *Implicated factors and optimal condition for antigenic ABO blood typing by surface plasmon resonance technique.* Unpublished Master's Thesis, Faculty of Science, Mahidol University.

105. Bayer, W.L., et al., *Testing for a blood group immunological reaction*, 1986, Google Patents.

106. Yves, L., et al., *Method for detecting antigens and/or antibodies*, 1995, Google Patents.

107. Yves, L., et al., *Method and test kit detecting antigens and/or antibodies*, 1999, Google Patents.

108. Chachowski, R.K., T.M. Setcavage, and K.J. Reis, *Column agglutination assay and device using biphasic centrifugation*, 1996, Google Patents.

109. Furukawa, K., et al., *Monoclonal antibody panel for blood group a antigen*, 1991, Google Patents.

110. American Association of Blood Banks, *AABB Guidelines and Standards for Blood Banks and Transfusion Services, 1-24th editions* 2006, KARGER.

111. Daniels G., ed. *Human Blood Groups*. Second ed. ed. 2002, Blackwell Science.

112. http://www.ncbi.nlm.nih.gov/books/NBK2269/.

113. Urbaniak, S.J. and M.A. Greiss, *RhD haemolytic disease of the fetus and the newborn.* Blood Reviews, 2000. **14**(1): p. 44-61.

114. Levine, P. and R.E. Stetson, *An unusual case of intra-group agglutination.* JAMA: The Journal of the American Medical Association, 1984. **251**(10): p. 1316-1317.

115. Landsteiner, K. and A.S. Wiener. *An agglutinable factor in human blood recognized by immune sera for rhesus blood*. 1940. Royal Society of Medicine.

116. Levine, P. and E.M. Katzin. *Isoimmunization in Pregnancy and the Varieties of Isoaǵǵlutinins Observed*. 1940. Royal Society of Medicine.

117. Avent, N.D. and M.E. Reid, *The Rh blood group system: a review.* Blood, 2000. **95**(2): p. 375-387.

118. Westhoff, C.M., *The Rh blood group system in review: a new face for the next decade.* Transfusion, 2004. **44**(11): p. 1663-1673.

119. Uthemann, H. and A. Poschmann, *Solid-phase antiglobulin test for screening and identification of red cell antibodies.* Transfusion, 2003. **30**(2): p. 114-116.

120. Sinor, L., et al., *Solid-phase ABO grouping and Rh typing.* Transfusion, 2003. **25**(1): p. 21-23.

121. Vyas, G.N. and K. Venkateswaran, *Simultaneous human ABO and RH (D) blood typing or antibody screening by flow cytometry*, 1998, Google Patents.

122. http://www.agrisera.com/en/info/igg.html.

123. Awdeh, Z., A. Williamson, and B.A. Askonas, *One cell–one immunoglobin. Origin of limited heterogeneity of myeloma proteins.* Biochemical Journal, 1970. **116**(2): p. 241.

124. Rosén, A., K. Ek, and P. Aman, *Agarose isoelectric focusing of native human immunoglobulin M and alpha 2-macroglobulin.* Journal of immunological methods, 1979. **28**(1-2): p. 1.

125. Eylar, E.H., et al., *The contribution of sialic acid to the surface charge of the erythrocyte.* Journal of Biological Chemistry, 1962. **237**(6): p. 1992-2000.

126. Coulter, C.B., *The isoelectric point of red blood cells and its relation to agglutination.* The Journal of general physiology, 1921. **3**(3): p. 309-323.

127. Reis, K., et al., *Column agglutination technology: the antiglobulin test.* Transfusion, 2003. **33**(8): p. 639-643.

128. Plapp, F.V., J.M. Rachel, and L.T. Sinor, *Dipsticks for determining ABO blood groups.* The Lancet, 1986. **327**(8496): p. 1465-1466.

129. Wegmann, T.G. and O. Smithies, *A simple hemagglutination system requiring small amounts of red cells and antibodies.* Transfusion, 1966. **6**(1): p. 67-73.

130. Buchs, J. and U. Nydegger, *Quantitation of anti-A and anti-B IgG antibodies in therapeutic iv immunoglobulin by indirect ELISA.* Transfusion Science, 1990. **11**(1): p. 113-121.

131. Buchs, J.P. and U.E. Nydegger, *Development of an ABO-ELISA for the quantitation of human blood group anti-A and anti-B IgM and IgG antibodies.* Journal of immunological methods, 1989. **118**(1): p. 37-46.

132. Stussi, G., et al., *Isotype-specific detection of ABO blood group antibodies using a novel flow cytometric method.* British journal of haematology, 2005. **130**(6): p. 954-963.

133. Kimura, S., et al., *Rapid quantitation of immunoglobulin G antibodies specific for blood group antigens A and B by surface plasmon resonance.* Transfusion, 2004. **45**(1): p. 56-62.

134. Yurugi, K., et al., *Rapid and accurate measurement of anti-A/B IgG antibody in ABO-unmatched living donor liver transplantation by surface plasmon resonance.* Transfusion Medicine, 2007. **17**(2): p. 97-106.

135. Lee, J., et al., *Demonstration of IgM antibodies of high affinity within the anti-Gal [alpha] 1-3 Gal antibody repertoire* Transplantation, 1998. **66**(8): p. 1117-1119.

136. Spindler, J.H., H. Klüter, and M. Kerowgan, *A novel microplate agglutination method for blood grouping and reverse typing without the need for centrifugation.* Transfusion, 2002. **41**(5): p. 627-632.

137. Robb, J., et al., *Development of non-agglutination microarray blood grouping.* Transfusion Medicine, 2005. **16**(2): p. 119-129.

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