Kinetic analysis of IgM monoclonal antibodies for determination of dengue sample concentration using SPR technique

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\section*{ABSTRACT}
Surface plasmon resonance (SPR) sensing is recently emerging as a valuable technique for measuring the binding constants, association and dissociation rate constants, and stoichiometry for a binding interaction kinetics in a number of emerging biological areas. This technique can be applied to the study of immune system diseases in order to contribute to improved understanding and evaluation of binding parameters for a variety of interactions between antigens and antibodies biochemically and clinically. Since the binding constants determination of an anti-protein dengue antibody (Ab) to a protein dengue antigen (Ag) is mostly complicated, the SPR technique aids a determination of binding parameters directly for a variety of particular dengue \textit{Ag-Ab} interactions in the real-time. The study highlights the doctrine of real-time dengue \textit{Ag-Ab} interaction kinetics as well as to determine the binding parameters that is performed with SPR technique. In addition, this article presents a precise prediction as a reference curve for determination of dengue sample concentration.

\section*{KEYWORDS}
biosensor; dengue virus; kinetic analysis; surface plasmon resonance

\section*{Introduction}
Existing biochemistry techniques for biomolecular detection, such as enzyme-linked immunosorbent assay (ELISA),\textsuperscript{1-3} polymer chain reaction (PCR),\textsuperscript{4-6} and fluorimmuno assay (FIA)\textsuperscript{7,8} are complicated, tedious and time-consuming. These methods require specific reagents and labels for biomolecular detection.\textsuperscript{9-11} Biosensors that can rapidly diagnose and have high-sensitivity detection capabilities for different types of biomolecules are thus very much desired in the field of life sciences.

Surface plasmon resonance (SPR) sensors\textsuperscript{12-14} have been extensively utilized to specifically detect certain biological molecules in liquid mediums for medical diagnosis.\textsuperscript{15,16} In describing the function of SPR biosensors, the binding of ligand and analyte leads to real-time refractive index changes in the reflected light, which directly represents the change in ligand-analyte binding interaction quantity.\textsuperscript{17-20} In this experimental technique, finding the affinity (binding constant) of an antibody is substantially important for optimization of such a work. These basic parameters are useful in different studies, for instance, thermodynamic study of the \textit{Ag-Ab} interaction in molecular basis or using antibodies as conformational probes. These binding information may lead to calculation of their topology and concentration measurement of antigens which are active biologically.\textsuperscript{21,22}

Different experimental studies have been done to ease the computation of binding parameters, such as, the kinetic parameter for the interaction between \textit{Ags} and \textit{Abs} or the dissociation constant. Among these approaches, separation of free reactants and bound reactants are more preferred.\textsuperscript{23,24}

There are some reports of using SPR method in binding parameters measurements in which both qualitative and quantitative applications were involved. Among them, active molecular concentration, association and disassociation rates ($k_a$, $k_d$), and binding constants can be named which ultimately
leads toward the finding of thermodynamic information of under experiment medium. In addition, after completion of first experiment, the SPR biosensors can be applied to finding the mechanism of binding interaction and its stoichiometry.22,25,26

**Biacore configuration and SPR chip construction**

Biacore 3000 from GE Healthcare is a real-time system for biomolecular interaction analysis using SPR technique.27-29 With this method it is possible to monitor the formation and dissociation of biomolecular complexes on chip surfaces. CM5 (carboxymethyl dextran matrix) is served as a biochip for this research.30 The CM5 is a particular chip of this system for numerous detections in microbiological areas.

According to this biosensor structure that is based on the Kretschmann configuration, a glass surface covered with a gold thin film provided the physical conditions for producing the surface plasmon resonance signal. Gold and silver are the most suited metals for surface plasmon resonance sensing. Silver has better surface plasmon resonance characteristics than gold, because of the larger real part of its dielectric constant.31 However, silver has poor long-term stability. Gold is more environmentally stable, is chemically more inert, has lower reactivity, does not react with commonly used fluids such as water and alcohols, and is compatible with a wide range of chemicals. Since gold was utilized in the CM5 chip as a metal layer, all assays are examined through this biosensor in the research.

According to the CM5 structure, there is a carboxymethyl-dextran matrix acting as a linker layer with 100 nm thickness. An inert hydrophilic environment which is suitable for the most biomolecular interactions is relatively provided by this matrix. Also, the immobilization is efficiently done from dilute solutions.32,33 N-ethyl-N-(dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) are intended to activate the biosensor surface before sample injection. Ethanolamine and 10 mM glycine-HCl with pH 2.0 are the washing solutions for removing particle that binds to the sensor surface and completing the immobilization procedure. For sample dilution, 10 mM buffer (pH 4.5 sodium acetate) is used in the samples that obtains an adequate concentration.34,35

A baseline of resonance signal was primarily determined by washing the surface with buffer having a fixed amount of bound ligand. The analyte was subsequently added to this buffer flow. Binding of analyte to the immobilized ligand caused a rise in refractive index of chip surface, thereby changing the SPR angle which is directly proportional to the number of ligand-analyte pairs. Changing SPR angle is named the resonance units (RU) as presented in Fig. 1A.

![Figure 1. Schematic diagram describing the interaction of Ab with Ag on the chip surface; curve (A) schematic response signal, showing association (I), equilibrium (II) and dissociation phases of each resonance signal, and curve (B) changing refractive index at the sensor surface, which are caused by the concentration’s change of sample medium when the antibodies (Abs) attach to the immobilized antigens.](image-url)
where 1000RU corresponds to a change of angle $\approx 0.1^\circ$ and 1RU corresponds to a change of refractive index $\approx 1 \times 10^{-6}$. It should be noted that if an analyte does not attach to the immobilized ligand, there is no variation in the SPR angle. Otherwise the bound analyte produces a positive SPR signal in the sensorgram (Fig. 1B).

Nowadays, the global prevalence of dengue fever are dramatically growing and the rapid dengue diagnostic tests have been developed beside of dengue vaccines.\(^{36,37}\) In present study, an antigen represents a molecule attached to the sensor chip’s surface, which is serotype 2 of the dengue virus (DENV2), and the dengue 2 specific monoclonal antibody is examined as a sample applied at various concentrations. Here, thorough description of the quantitative expression and evaluation of binding constants for $Ag\_Ab$ interaction kinetics are theoretically expressed and then employed to achieve the dengue $Ag\_Ab$ interaction kinetics. Subsequently, after calculation of all binding parameters, we obtain an approximate linear plot which aims to determine a concentration of each patient sample.

**Results and discussion**

The antigen immobilization site was characterized using atomic force microscopy (AFM); model VEECO DIMENSION 3000. The AFM machine imaged the chip surface in contact mode with 0.01–0.025 Ohm-cm antimony (n)-doped silicon probe. The presence of immobilized antigens was clarified through a 3-dimensional AFM image (Fig. 2). The AFM image shows a top view of the chip surface which presents 2 types of different peaks. Since the amine groups have been already adhered upon gold layer, the homogeneous and low peaks reveal the amine groups and second, higher sporadic peaks reveal the immobilized antigens (ligands) on the chip surface. The amine groups have a role of binding protein to antigen, which attached very well to the surface of chip.

**Presentation of output**

Evaluating both kinetic and equilibrium situations were studied to determine the sample concentration with the particular conditions. The assays were examined in similar environmental variables (e.g. same temperature and buffers) and used the dengue monoclonal antibodies as a sample, but, with different concentrations. The sample was diluted to concentrations of 1:400, 1:200, 1:100, 1:50, and 1:25 by adding 10 mM sodium acetate solvent with pH 4.5. Then 100 $\mu$l sample volume was injected on the chip surface with 30 $\mu$l/min flow rate.

The sensorgrams (resonance signal versus time) were collected at several different concentrations of injected samples. In subsequent concentrations (Fig. 3), a high quantity of dengue monoclonal antibodies at a 1:25 concentration caused sudden, rapid saturation in the binding phase between antibodies and antigens at the chip surface. This concentration was chosen to end the data collecting.

**Data analysis**

The representative sensorgrams were derived from injection of different concentrations of dengue monoclonal antibody samples. The plot $dR/dt$ vs. $R$ of each concentration was presented in Fig. 4. According to the association kinetics analysis, the slope of plot $dR/dt$ versus $R$ gives the value of $S$ (equation 5) for each concentration. Since the density of engaged monoclonal antibody is 0.85 nM, the concentration ($C$) of 1:400, 1:200, 1:100, 1:50, and 1:25 are 2.125 pM, 4.25 pM, 8.5 pM, 17 pM, and 34 pM respectively.

With regards to Fig. 4, the values for $S$ were found for each concentration and fitted with linear regression model in Fig. 5. According to equation 5, the association rate constant ($k_a$) and dissociation rate constant ($k_d$) of monoclonal dengue antibody were determined $13.2 \times 10^9 M^{-1}s^{-1}$ and $2.8 \times 10^{-2}s^{-1}$ respectively from the plot $S$ against $C$ (Fig. 5).
addition, the association constant $K_A$ and dissociation constant $K_D$ of monoclonal dengue antibody were obtained $4.71 \times 10^{-11} \text{ pM}^{-1}$ and $2.12 \times 10^{-12} \text{nM}$ respectively.

By considering the binding response in equilibrium condition against dengue sample concentration, equation (6) can be fitted according to monoclonal antibody concentration data which is shown in Fig. 6. This curve, as a reference, can determine the concentration of each sample and also the maximum binding response ($R_{\max}$) at the equilibrium condition. Based on Fig. 6, the $R_{\max}$ has been determined $20 \times 10^3 \text{ RU}$.

In addition, $\Delta$ represents the correction shift from the baseline.

**Case study**

Serums from infected patients to the dengue virus were diluted at different concentrations of dengue antibodies through laboratorial enzyme-linked-immune-sorbent assay (ELISA) method (low positive (LP); positive/negative (P/N) ratio of $< 3$, mid positive (MP); P/N ratio of $= 3$ and $< 5$, and highly positive (HP); P/N ratio of $= 5$ and $> 5$). It is obvious that the P/N ratio of $< 2$ is
a definitive negative case. According to the Table 1, P/N ratio, NS1, and IgM results of each patient serum were presented via the ELISA method. According to this method, the positive IgM results a proving the presence of the dengue virus, and the P/N ratio shows the IgM antibody quantity in each sample. In addition, the positive result of NS1 test indicates the presence of dengue virus in blood, but, its negative result indicates the late examination of the sample. In addition, each sample was examined using SPR Biacore machine for determination of binding signal variation (Table 1). Therefore, by having concentration reference curve (Fig. 6), simply the dengue antibody concentration ($C_{Ab}$) was interpolated based on the obtained binding signal variation of each patient serum.

**Conclusion**

In this research, the SPR technique was applied for biomolecular interaction analysis. The binding constant and stoichiometry data of dengue Ag-Ab interaction were calculated properly. Basically, SPR technique has been employed to evaluate the intrinsic affinities along with determination of rate constants for binding dengue antibody to its antigen. This study was demonstrated the SPR technique can remarkably detect and quantify the binding of dengue monoclonal antibodies to the chip surface. At the end, a reference plot for determination of sample concentration was obtained by different dengue monoclonal antibody concentrations.

**Methods and materials**

**Principle of Ag-Ab interaction kinetics in real-time**

In biosensors which are based on the SPR technique, one of reactants as a probe (Ag) is immobilized on the sensor surface and another reactant as target (Ab) is suspended into a liquid sample and flowed to the chip surface. The suspended target reacts with the immobilized probe and binds to each other if the target is its relevant probe (Fig. 7). Change in the SPR signal at resonance units (RU) graph due to the bound Ag-Ab, is plotted as a function of time (Fig. 7, curve (a)). Through this graph, the binding kinetics of Ag-Ab interaction is obtained. The formation of surface-bound Ag-Ab between target Ab and the immobilized Ag on the surface, can be represented as

\[
Ab_{bulk} \xrightarrow{k_m} Ab_{surface} + Ag \xrightarrow{k_a} Ag_{Ab} \xrightarrow{k_d} Ab_{surface} + Ag
\]

where $k_m$ is defined as a rate constant of mass transport to and from the chip surface. Since the rate constant is the same for mass transport, in both directions, the rate is equal. The association and dissociation rate constants are defined as $k_a$ and $k_d$ for formation of Ag-Ab complex respectively.\(^2\)

In an ideal situation, the Ab transport to the chip surface and its transport on the linker layer would not affect the binding kinetics. This occurs when the flow rate is quickly compared with binding. In this case, the sample (that includes Ab) concentration quickly becomes constant in time and uniform in space. It will equal to the

![Figure 5. Plot of slope value S vs. Ab concentration.](image)

![Figure 6. Plot of the binding response deviation versus Ab concentration.](image)

| Type of sample | P/N ratio | NS1 | IgM | $\Delta R(RU)$ | $C_{Ab}(pM)$ |
|---------------|-----------|-----|-----|----------------|--------------|
| LP            | 2.47      | CC  | 6,560| 0.84           | 6,560 0.84   |
| MP            | 3.75      | CC  | 9,380| 1.87           | 9,380 1.87   |
| HP            | 7.38      | ¡C  | 12,782| 3.76          | 12,782 3.76  |

**Table 1. The patient serum data with biosensor results.**

![Table 1](image)
concentration of injection \([B]_0\) in a bulk phase. In addition, the measured forward and backward rate constants approach to the constants of binding interaction kinetics. According to mentioned conditions, the complex formation rate can be depicted as

\[
d\frac{[Ag_{\cdot}Ab]}{dt} = k_a[Ab]([Ag]_0 - [Ag_{\cdot}Ab]) - k_d[Ag_{\cdot}Ab]
\]  

(2)

where \([Ag_{\cdot}Ab]\) is a quantity of bound target, \([Ab]\) is a quantity of unbound target, \([Ag]_0\) is the whole quantity of probe (Ag) on a chip surface. The rates of association/dissociation binding are clearly observable and the binding parameters will be obtained in the following.

If all number of probe is stated as a maximum target binding capacity on the surface, the concentration conditions can formerly be named as the SPR response signal \(R\). Under pseudo 1st-order conditions that the unbound target concentration, as a constant, is retained in the flow cell. The binding equation can be defined as

\[
d\frac{R}{dt} = k_aC(R_{\text{max}} - R) - k_dR
\]  

(3)

where \(dR/dt\) represents the changing rate in the response signal. In this equation, \(R\) and \(R_{\text{max}}\) come from measured and maximum response signals in each binding. The value \(C\) comes from the injected target concentration (M), constant \(k_a\) is an association rate \((M^{-1}s^{-1})\) and constant \(k_d\) is a dissociation rate \((s^{-1})\). Fig. 7A presents a scheme of response signal along with the defined parameters. According to equation 3, it can be rearranged for having an association kinetics analysis as:

\[
d\frac{R}{dt} = k_aCR_{\text{max}} - (k_aC + k_d)R
\]  

(4)

Therefore, the plot \(dR/dt\) vs. \(R\) is theoretically obtained as a straight line with slope \(- (k_aC + k_d)\) for kinetics of the Ag_Ab interaction. At \(R=0\) as initial binding rate is directly proportional to the sample concentration which can be used in concentration measurements. Through a single association response signal, the constants \(k_a\) and \(k_d\) can be specified if \(R_{\text{max}}\) is known. For determination of constant \(R_{\text{max}}\) experimentally, a high concentration of sample is examined to fully saturate the response signal. The desirable methodology is to acquire the sensorgram for different sample concentrations. To analyze the association and disassociation rates, the graph of changes in the total SPR response signal \(dR/dt\) versus \(R\) produces a value \(S\) as a slope of the response signal which relates the association and dissociation rates. It can be defined as:

\[
S = k_aC + k_d
\]  

(5)

According to the Fig. 7B, the association constant \(K_A\), can be calculated as \(K_A = k_a/k_d(M^{-1})\). At equilibrium
state, \( dR/dt = 0 \) and equation (3) can be reformulated as:

\[
\frac{R_{eq}}{C} = K_A R_{max} - K_A R_{eq}
\]

Therefore, the steady state association constant \( K_A \) can be found from a plot of \( R_{eq}/C \) vs. \( R_{eq} \) and the dissociation constant \( K_D \) can be calculated as \( 1/K_A \). On the other hand, a plot of \( S \) vs. \( C \) is a straight line with slope \( k_a \) (Fig. 7C). In theory, the intercept on the vertical axis (\( C = 0 \)) delivers \( k_d \), but in practice, this cannot be used as a reliable measure of a dissociation rate constant if there is \( k_a C >> k_d \). The dissociation is calculated by:

\[
\ln \frac{R_0}{R_t} = k_d (t - t_0)
\]

where \( R_0 \) is defined as an initial response level at \( t_0 \). The variable \( R \) and \( t \) represent the values acquired through the dissociation curve.\(^{22}\)

As a description of SPR method functionally, the incident laser light is reflected inside the prism which is usually coated with a thin gold layer from the outside. At a critical incident angle, an electron resonant oscillation appears on the surface of gold layer which results in sudden decrease of reflected light intensity. Since the critical angle varies in the different materials presented on the metal surface, causing the changes in refractive index of medium, this method has been utilized to measure biomolecular interactions at liquid-solid interface. In this method ligands were immobilized to the surface of sensor and analyte flows past this surface to interact and form a complex. A SPR response signal is generated just after interaction and can be observed in the real-time. Changing of refractive index in the immobilized ligands, which is related to the changing of adsorbed mass, affect the SPR biosensor. Hence, it can detect the targets interacting with the probe that is immobilized on the sensor chip.

Basically when samples containing the relevant antibody is injected on an immobilized antigen surface in a cycle of experiment, the resulting sensorgram can be divided into 3 essential phases: first is the association of \( Ab \) with \( Ag \) which takes place during the injection of sample; second is the steady-state phase or equilibrium at the end of injection, where the \( Ab \) binding rate is balanced; and third is the dissociation of \( Ab \) from the chip surface that takes place during buffer flow.

### Sample collection

The monoclonal antibody samples for this research were provided by the Center for Disease Control and Prevention (CDC). In addition, blood specimens were obtained from the University Malaya Medical Center. The approval study protocol was provided by the institutional review board of the University of Malaya Medical Center (Ethics no. 782.90). The patients’ consent and asymptomatic donors were obtained prior to blood collection, and the study was conducted in accordance with the “Declaration of Helsinki.”

### Ligand immobilization

In our experiment, 6 \( \mu l \) of dengue antigen (serotype 2) was diluted with 194 \( \mu l \) acetate buffer, which was immobilized on the surface of the Biacore chip. It should be noted that the titer of dengue virus serotype 2 (DENV2) used for this study, was obtained \( 2 \times 10^5 \) pfu/ml as ligand concentration. The EDC and NHS were utilized to activate the surface of chip via a mixture of 120 \( \mu l \) EDC and 120 \( \mu l \) NHS to produce reactive succinimide esters. The loose ligands were removed by specific buffers on the chip surface subsequently. Deactivation and taking off all loose associated ligands were carried out by 80 \( \mu l \) ethanolamine solution and 500 \( \mu l \) 10 mM glycine-HCl buffer with pH 2.0. In addition, the flow rate of immobilization process was adjusted on 10 \( \mu l/min \).

Upon sensor surface construction, the experiments were conducted by exposing the surface to each concentrated serum containing IgM antibody. The dengue IgM antibody began to bind once the serum was introduced to the sensor surface – a process that continued while an equilibrium position took place between the serum antibody concentration and immobilized dengue antigen concentration. At this point, the process of binding was studied by replacing the liquid phase with the buffer and recording the dissociation of attached antibodies until an equilibrium ratio between dengue IgM antibodies and immobilized dengue antigen concentration. This phase of association and dissociation can be performed at different serum concentrations to examine the concentration effect has on the time-dependent diagnostic process for the dengue fever.
Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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