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# Modelling of Displacement Method in Surface Plasmon Resonance Sensing

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Existing methods of detecting explosives have many problems in terms of stability, sensitivity, high selectivity, and rapid sensing for any given situation. In this study, we selected a displacement method using a surface plasmon resonance (SPR) immunosensor, which is one of the ways to overcome these problems. In an SPR displacement immunoassay, the methods to realize a high sensitivity were investigated by experimental findings. However, they were not investigated by theoretical approaches. Hence, we developed a method with a higher sensitivity based on the kinetic theory of this system. The strategy of an SPR displacement immunoassay to realize a high sensitivity was determined using the two-layer model simulation by a theoretical approach. As a result of the simulation, we found that a high sensitivity was realized using a combination of an antigen analogue and an antibody with a small association constant or a combination of an antigen and an antibody with a large association constant. This means that the equilibrium constants affect the sensitivity of an SPR displacement immunoassay, which is one of assays under nonequilibrium conditions. In addition, we investigated the relationship between association equilibrium constants and the sensitivity using conjugates and a hapten to detect 2,4,6-trinitrotoluene (TNT). The result of the experiment supported the theoretical inferences.

## 1. Introduction

Various explosive detection methods have been used to prevent damage from explosives. However, conventional methods have some problems in terms of stability, cost, sensitivity, high selectivity, and rapid sensing for any given situation. Accordingly, many additional detection methods have been developed to realize various requirements. A displacement method using a surface plasmon resonance (SPR) immunosensor is one of the ways to fulfill requirements on stability, high selectivity, and rapid sensing.

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SPR immunosensors, which have high stability based on SPR as the sensing principle and high selectivity based on antigen-antibody interaction, can be used for real-time sensing.[1,2] SPR sensors can measure changes in refractive index over a gold film (sensor chip) surface in real time and with high sensitivity without labeling using resonance between electrons on the gold film surface and light. In addition, SPR sensors have high reproducibility; hence, they are excellent for analysis under nonequilibrium conditions. However, since SPR sensors do not have selectivity, it is necessary to modify the surface of a sensor chip to supplement selectivity. SPR sensors can detect changes in refractive index over a sensor chip as changes in resonance angle. Changes in mass in the association or dissociation of proteins can be measured using SPR sensors, because the relationship between mass change and resonance angle shift in the association or dissociation of proteins is linear.[3] Antigen-antibody interactions take a long time to reach equilibrium, *e.g.*, 10–20 min. Therefore, SPR immunosensors are excellent for rapid sensing.

A displacement method has a slightly lower sensitivity than an indirect competitive method. For example, the indirect competitive method using SPR immunosensors can detect 10–100 ppt (pg/ml) 2,4,6-trinitrotoluene (TNT, $C_7H_5N_3O_6$); on the other hand, the displacement method using SPR immunosensors can detect 1–10 ppb (ng/ml) TNT.[1,2,4,5] However, the displacement method is capable of rapid sensing, because it requires no incubation before measurement; on the other hand, the indirect competitive method requires about 15 min incubation before measurement.[5,6] Generally, an SPR immunoassay requires two substances; i.e., an antibody as a ligand immobilized on the sensor surface and an antigen as an analyte to induce antigen-antibody interactions. However, it is difficult to detect low molecular weight (200–300) antigens by SPR sensing, because the mass change is insufficient.

Hence, in the case of using a low molecular weight antigen, the SPR immunoassay requires opposite composition; i.e., an antigen or an antigen analogue as a ligand immobilized on the sensor surface and an antibody as an analyte to induce antigen-antibody interactions.[1,2,4–9] As the molecular weights of antibodies are generally about 150,000, they cause a refractive index change sufficiently large to be detected by highly sensitive SPR sensors. Antigen analogues are substances that have a similar conformational epitope to a certain antigen; hence, antigen analogues interact with specific antibodies for the antigen.

The displacement method using SPR immunosensors is performed by the following steps.[1,2] Firstly, antigen analogue molecules were immobilized on the sensor surface, as shown in Fig. 1(a). Secondly, an antibody solution was introduced over the antigen analogue surface to induce antibody-antigen analogue interactions, as shown in Fig. 1(b). When antibody molecules were bound to the antigen analogue surface, an antigen (sample) solution was introduced over the surface-bound antibody to enhance dissociation of the antibody from the sensor surface owing to the antigen-antibody interaction in the solution, as shown in Figs. 1(c) and 1(d). We could estimate the concentration of the antigen in the sample solution by measuring the loss of antibody mass.

In the displacement method using SPR immunosensors, guides for combinations of antigens, antigen analogues, and antibodies, which enable a more sensitive detection of

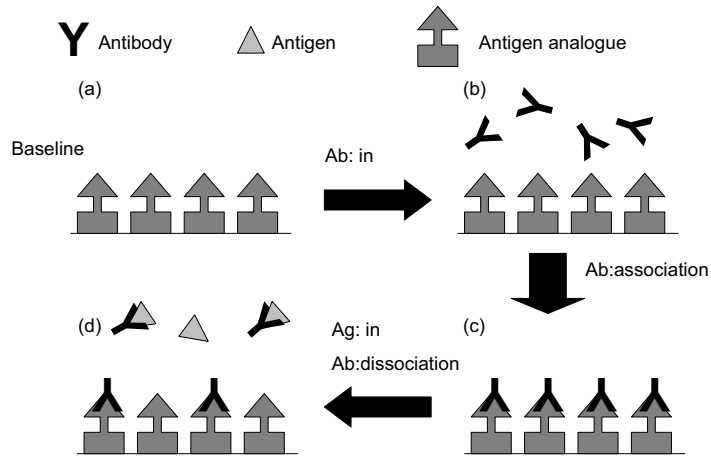


Fig. 1. Schematic diagram of surface conditions at each phase in SPR displacement immunoassay.

explosives, were suggested by the experimental results of 10 ppb TNT.[1] However, no experiment was performed for a wide range of TNT concentrations, and furthermore, the guides for combinations were not investigated theoretically. In this study, we constructed a theoretical model of the method, and performed some simulations in order to investigate the guides for combinations of antigens, antigen analogues, and antibodies. The purpose of this study is to theoretically find strategies for combinations of antigens, antigen analogues, and antibodies, which enable a more sensitive detection of explosives (preferably, 100 ppt sample detectable).

## 2. Theory

### 2.1 *Definition of model*

To realize highly sensitive sensing, we should select conditions that are optimum for antigens, antigen analogues, and antibodies. In this study, we performed a simulation of the displacement method based on a two-layer theoretical model to determine the optimum conditions.

We set some measurement conditions for a model of the displacement method. We used Biacore X (GE Healthcare Bioscience) as a representative sensing system. A gold film on the sensor chip was modified with antigen analogues, hereafter called hapten (Hap), as ligands. Antibodies (Ab) as analytes were associated with and dissociated from the sensor chip. We assumed that the antigen (Ag) or Ab solution can be introduced into a flow cell at a constant concentration and flow speed. A shift of resonance angle, which is the basis of sensing, indicates a change in refractive index over the sensor chip surface. In the association or dissociation of Ab, however, a 1,000 RU resonance angle shift corresponds to a shift of mass of about 1 ng/mm$^2$.[3] Hence, we can estimate the shift of mass over the sensor chip surface from the shift of the resonance angle, and can estimate the concentration of hapten-antibody complexes (Hap-Ab).

An Ab solution of constant concentration was introduced into the flow cell and over the sensor chip surface. Ab is expected to associate with Hap. The system over the sensor chip surface composed of Ab, Hap and Hap-Ab is considered to achieve the equilibrium state. Then, an Ag solution was introduced over the sensor chip surface into the flow cell to induce the dissociation of Hap-Ab and the association of antibody-antigen complexes (Ab-Ag). We construct a theoretical model such that the system in the flow cell has two layers,[10] the solution layer and the equilibration layer. The solution layer occupies most of the system and is a layer where solution flows. On the other hand, the equilibration layer is a thin layer over the sensor chip surface. This layer is generally called the unstirred layer,[11] which is not affected by solution flow. For convenience, we call the interface between these two layers the 'interface' hereafter. In each layer, solute molecules move freely; however, we assume that only Ab move into and out of each layer by diffusion at the interface. To distinguish Ab in the solution layer from Ab in the equilibration layer, we call the former 'Ab' and the latter 'Abs.'

We define each layer in terms of the dissociation of Ab as follows. In the solution layer, a Ag solution of constant concentration is introduced, and Ag binds to Ab, which comes from the equilibration layer by diffusion. In the equilibration layer, Abs dissociate from Hap-Ab and then the remainder is Hap. Some Abs move into the solution layer by diffusion. Figure 2 shows the two-layer model illustrated by a simple diagram. Hence, we suppose that only one binding site of each Ab molecule can bind to Ag or Hap, despite each Ab molecule having two binding sites.

## 2.2 *Definition of response*

Using the displacement method, we immobilized antigen analogue molecules (Hap) on the sensor chip surface as ligands. We take the resonance angle under this condition as the baseline. Next, an Ab solution of constant concentration was introduced into the flow cell for a certain time, and Ab molecules associated with Hap. The signals

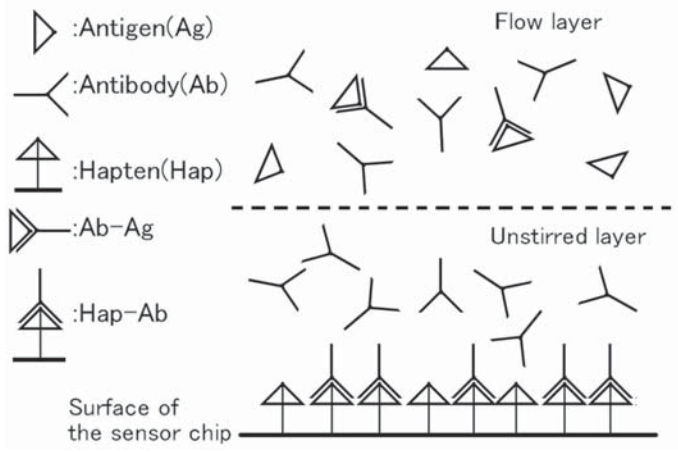


Fig. 2. Simple diagram of two-phase model.

of the sensorgram were recorded as the basic amount of Ab that associated with Hap, $\Delta\theta_{A0}$, at the end of introducing the Ab solution. Then, a buffer solution was introduced for a certain time to dissociate Hap-Ab spontaneously. The signals of the sensorgram were recorded as the amount of Ab that associated with Hap, $\Delta\theta_{A1}$, at the end of the dissociation of Hap-Ab without Ag. A regeneration solution was introduced to wash out all Ab molecules that associated with Hap.

Similarly, we took the baseline, the basic amount of Ab that associated with Hap, $\Delta\theta_{B0}$, and the amount of Ab that associated with Hap, $\Delta\theta_{B1}$, when an Ag solution was introduced instead of a buffer solution without Ag for a certain time for the dissociation of Hap-Ab. In ideal conditions, $\Delta\theta_{B0}$ is equal to $\Delta\theta_{A0}$. In real experiments, however, $\Delta\theta_{B0}$ differs slightly from $\Delta\theta_{A0}$. If Ag at a high concentration were present in the Ag solution, a greater amount of Ab is considered to dissociate from Hap-Ab and the dissociated Ab would associate with Ag. We calculated the ratio of remaining Ab from these results. The remaining Ab ratio was defined as $\dfrac{\Delta\theta_{B1}/\Delta\theta_{B0}}{\Delta\theta_{A1}/\Delta\theta_{A0}}$. In this study, the response of the displacement method was defined as $1 - \dfrac{\Delta\theta_{B1}/\Delta\theta_{B0}}{\Delta\theta_{A1}/\Delta\theta_{A0}}$.

Figure 3 shows a sensorgram for the displacement method. A larger response is obtained in the case of using a higher concentration of Ag solution. Therefore, we can estimate the amount of Ag in the sample solution and measure Ag concentration. However, the following three conditions must be constant: the concentration of an Ab solution, time of introducing an Ab solution ($T_a$), and time of introducing a buffer or Ag solution ($T_d$).

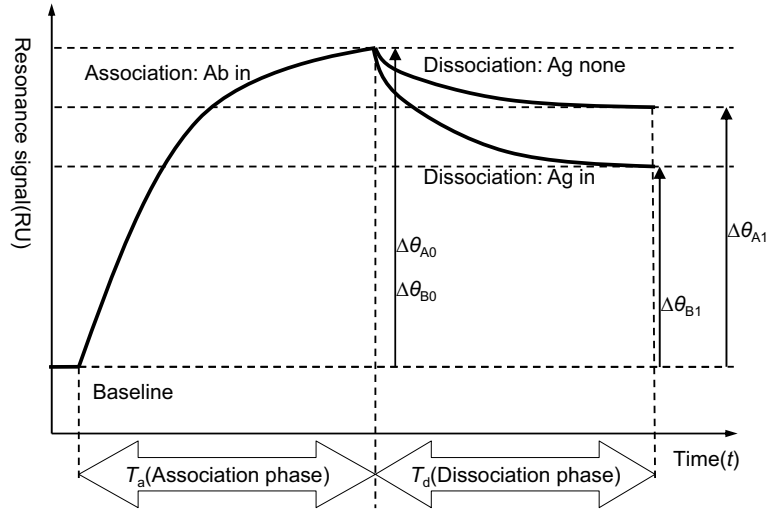


Fig. 3. Schematic of sensorgram for displacement method.

### 2.3 *Simplification of model*

We simplified the model by making assumptions for the simulation of the two-layer model. We assumed chemical equations in the model as follows, where the brackets [ ] indicate the concentration of the substance within the brackets:

$$[Hap\text{-}Ab] \underset{k_2}{\overset{k_2'}{\rightleftharpoons}} [Hap]+[Abs], \tag{1}$$

$$[Abs] \underset{k_D'}{\overset{k_D}{\rightleftharpoons}} [Ab], \tag{2}$$

$$[Ab]+[Ag] \underset{k_1}{\overset{k_1'}{\rightleftharpoons}} [Ab\text{-}Ag]. \tag{3}$$

We introduced the association (dissociation, or diffusion) rate constant '$k$' to the chemical equations. Equation (1) is the chemical reaction in the equilibration layer, where $k_2$ is the association rate constant, and $k'_2$ is the dissociation rate constant. Equation (2) expresses the chemical reaction in the interface, where $k_D$ is the diffusion rate from the equilibration layer to the solution layer, and $k_{-D}$ is the diffusion rate constant in the opposite direction. Equation (3) is the chemical reaction in the solution layer, where $k_1$ is the association rate constant, and $k'_1$ is the dissociation rate constant.

Based on these kinetic reactions, we can formulate corresponding equations, and then, discuss the dependence of detection sensitivity for the antigen on binding constants such as $K_1$ (= $k_1/k'_1$) and $K_2$ (= $k_2/k'_2$). The procedure to obtain the resulting equations is tedious, and hence, is shown in the appendix.

### 2.4 *Theoretical results*

We simulated the numerical model using scilab-4.1.2, which is an open-source numerical computing system. We defined the step size of the simulations as 0.5 s. Four quantities, namely, $[Abs]_0$, $[Hap\text{-}Ab]_0$, $k_D$ (= $k_{-D}$) and $\alpha$ were set constant in all the following simulations. $K_1$, $K_2$, and $[Ag]$ were defined in each simulation.

We investigated the responses at $t$ = 60 s for different values of the association equilibrium constants $K_1$ and $K_2$. We defined $t$ = 60 s on the basis of $T_d$ in a real experiment. Figure 4 shows results of the simulation.

As shown in Fig. 4, the responses did not reach 1 despite increasing $[Ag]$ and approached asymptotically certain different limits. Each limit can be considered to appear owing to a short dissociation time ($T_d$). In such a short-time detection, $T_d$ is not sufficient for the responses to reach 1. Hence, it is expected that the responses can reach 1 in a long-time detection; for example, the case of $T_d$ = 6,000 s. As shown in Fig. 4, which indicates $K_1$ dependence, the rising of responses occurs under low $[Ag]$ conditions with a large $K_1$. Hence, the responses under low $[Ag]$ conditions and thus the sensitivity became high with a large $K_1$. Whether $K_1$ is large or small, the limits of each response were the same.

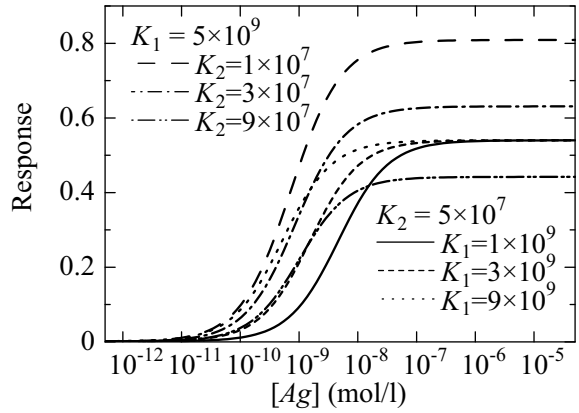


Fig. 4. Simulated responses for different values of $K_1$ and $K_2$ in 60 [s].

As shown in Fig. 4, which indicates $K_2$ dependence, the rising of responses occurs under low [*Ag*] conditions with a small $K_2$; hence, the sensitivity became high. The limit of each response rose with increasing $K_2$. Therefore, $K_2$ affects the responses from the low [*Ag*] region to the high [*Ag*] region. It is difficult to distinguish whether the large response under low [*Ag*] conditions is due to the height of the upper limit of the response or a change in sensitivity unrelated to the upper limit of the response, because the limit of each response is affected by $K_2$. Comparison with the results of simulation under sufficiently long $T_d$ conditions is required for this consideration. Therefore, we do not conclude this consideration in this section. Hence, we propose three ways to increase response at certain [*Ag*] as follows.

One way is to make $K_2$ small. However, it is necessary to investigate whether a small $K_2$ makes the sensitivity high or not. Another way is to make $K_1$ large. However, the influence of $K_1$ does not appear at a high [*Ag*]. We assume that this way is effective for improving the limit of detection (LOD). The last one is to take a long dissociation time, but this is not good for rapid sensing.

We investigated the responses at $t$ = 6,000 s using each association equilibrium constant. $t$ = 6,000 s was estimated to be sufficiently long to reach equilibrium conditions in the entire system. Hence, we defined the response at $t$ = 6,000 s as a quasi-unlimited-time response in the model against a limited-time response at $t$ = 60 s. Figures 5 and 6 show results of the simulation.

As shown in Figs. 5 and 6, the responses approach 1 asymptotically under high [*Ag*] conditions. As shown in Fig. 5, which indicates $K_1$ dependence, the rising of responses occurs under low [*Ag*] conditions with a large $K_1$. This trend is similar to that in $t$ = 60 s simulation; hence, the sensitivity was higher. As shown in Fig. 6, which indicates $K_2$ dependence, the responses approach 1 asymptotically, which is different from those in $t$ = 60 s simulation. However, the responses rose under low [*Ag*] conditions when $K_2$ was small. This trend is similar to that in $t$ = 60 s simulation; hence, the sensitivity was

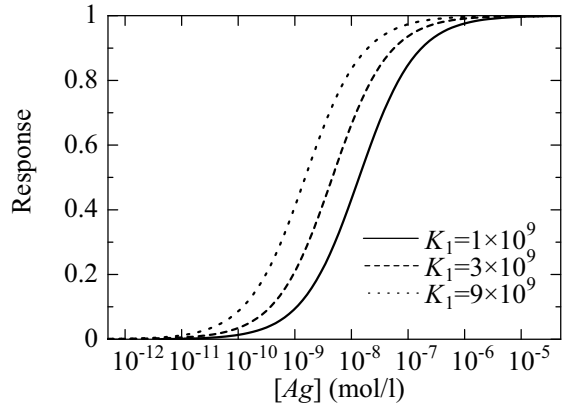


Fig. 5. Simulated responses for different $K_1$ values in 6,000 [s].

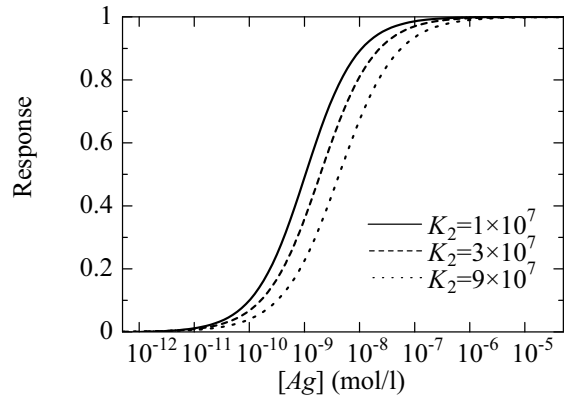


Fig. 6. Simulated responses for different $K_2$ values in 6,000 [s].

higher. We can conclude that a small $K_2$ makes the sensitivity high. Hence, we propose two ways to raise the sensitivity as follows.

One way is to make $K_2$ small, and the other is to make $K_1$ large. These two ways are the same as those suggested for $t$ = 60 s simulation.

## 2.5 *Guide from the model*

Regarding the displacement-sensing model, a more sensitive response can be obtained at a smaller $K_2$ or a larger $K_1$. A small $K_2$ means a weak Hap-Ab association. A large $K_1$ means a strong Ab-Ag association. In the case of the limited time simulation, the upper response limits depend on $K_2$ and $T_d$.

Hence, we should select optimum $K_1$, $K_2$, and $T_d$ for each condition. Regarding SPR immunoassay, however, it is difficult to change the combination of Ab and Ag, because

of the need to use a specific Ab against a sample Ag. Because there is no latitude for selecting the combination of Ab and Ag, it is not necessary to focus on $K_1$. To realize rapid detection, we should not prolong $T_d$; hence, it is unnecessary to focus on $T_d$. On the other hand, there is some latitude for choosing the combination of Ab and Ag analogue. Therefore, we concluded that $K_2$ should be small for the optimum setting of each condition in SPR displacement immunoassay.

## 3. Experiment

We proposed that a small $K_2$ provides a large response and a high sensitivity as determined from the simulation of SPR displacement immunoassay based on the two-layer model. Because $K_2$ means the strength of Hap-Ab association, we can investigate our proposal using some antigen analogues (haptens or conjugates) against the same Ab and Ag combination. In this study, we used three different antigen analogues (1 hapten and 2 conjugates), and investigated their effects on $K_2$ and responses.

### 3.1 *SPR immunosensor*

In this study, we used Biacore X (GE Healthcare Bioscience) as an SPR sensor. Temperature was constant at 25°C. HBS buffer (10 mM HEPES-buffered saline, 150 mM NaCl, 0.05% [w/v] Tween 20, pH 7.4) was used as the running buffer. HEPES means 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid. HBS buffer solution was sterilized and degassed in advance. HBS buffer, the same as the running buffer, was used for the dilution of Ag or Ab solution. NaOH (50 mM) was used as the regeneration solution. The flow rate of each solution was basically 10 µl/min.

We tried to detect TNT (Chugokukayaku Co., Ltd.), which is a common nitroaromatic explosive compound. A mouse anti-TNT monoclonal antibody (Strategic Diagnostics Inc.), which realized specific binding to TNT, was used as an analyte.

We used three types of antigen analogue having different $K_2$ values for the anti-TNT antibody (hereafter, Ab or antibody) as ligands; 2,4-dinitrophenylated chicken egg albumin (DNP-ovalbumin, LSL; hereafter, DNP-OVA), DNP-hemocyanin keyhole limpet (KLH, LSL), and DNP-glycine (formula weight [FW]: 241.16, Tokyo Chemical Industry Co., Ltd.). Both DNP-OVA and DNP-KLH were conjugates. DNP-glycine was a hapten. The amount of the DNP group was 4.0±1.0 per molecule on both conjugates. The sensor chip surface was modified using self-assembled monolayers (SAMs) in immobilizing antigen analogue molecules over the surface. We used 16-mercaptohexadecanoic acid (MHA, FW: 288.5, Sigma-Aldrich) and polyethylene glycol (PEG6)-COOH aromatic dialkanethiol (FW: 694.95, Senso Path Technologies) as SAMs. 16-MHA was used for binding conjugates. PEG6-COOH aromatic dialkanethiol was used for immobilizing the hapten.

### 3.2 *Sensor chips*

In this study, we used 'Sensor Chip Au' (SIA Kit Au, GE Healthcare Bioscience) as sensor chips. Binding conjugates to the 16-MHA surface and immobilizing hapten to the PEG6-COOH aromatic dialkanethiol surface were performed in accordance with

the previous method.[4,9] The conjugates, two types of the antigen analogues, had free amino groups, whereas the hapten, another antigen analogue, had the carboxyl group. To immobilize conjugates over the sensor chip surface by amino coupling, we formed a SAM, which had the carboxyl group at one end of its carbon chain, on the gold film surface. On the other hand, binding the hapten over the sensor chip surface could not be carried out similarly, because both the SAM and the hapten had the carboxyl group. Therefore, we used ethylendiamine ($NH_2CH_2CH_2NH_2$, FW: 60.10), which has two amino groups at both ends. We immobilized ethylendiamine to the SAM by amine coupling. The hapten was immobilized with ethylendiamine similarly.

### 3.3 *Ab association kinetics*

We analyzed sensorgrams from the kinetic point of view using BIAevaluation 3.2 RC1, which is an attached application of the Biacore series. The model used for kinetic analysis was 'bivalent analyte with mass transfer.' $K_2$ analyzed using the application and the model, in which two binding sites of the analyte are used for the association with the antigen analogues, is not completely equal to $K_2$ defined in the two-layer model. However, the meanings of magnitude of both $K_2$ are the same; that is, both $K_2$ mean the strength of binding between the antibody and the antigen analogue. Hence, we determined that $K_2$ calculated using this application can be used for estimating our inference.

We investigated Ab association kinetics as follows. Ab solutions were prepared at seven different concentrations; 0.1, 0.5, 1, 5, 10, 50, and 100 μg/ml (ppm).

First, the baseline was taken. Next, an Ab solution was introduced into the flow cell for 120 s. After waiting for 30 s, the regeneration solution was introduced for 30 s. These steps were carried out in the order from the lowest to the highest Ab concentration. Some of the Ab solutions can reach saturation of Hap-Ab association by 60 s. We used the lowest [*Ab*] solution among them for the detection of TNT.

### 3.4 *Detection of TNT*

We calculated the responses using the result sensorgrams of TNT detections. We examined responses and estimated the LOD. The LOD was three times as large as the standard deviation (SD). However, the responses under the same antigen analogue condition had different SDs by each [*Ag*] detection; hence, we used the SD of the lowest [*Ag*] (1 ppt) detection.

TNT detection using the displacement method was performed as follows. We used the Ab solution selected in previous 'Ab association kinetics.' Ag solutions at seven different concentrations, 0 (HBS without TNT), 1, 10, 100 pg/ml (ppt), 1, 10, and 100 ng/ml (ppb), were prepared.

First, the baseline was taken. Next, the Ab solution was introduced for 60 s. After waiting for 30 s, a Ag solution was introduced for 60 s, and then, the regeneration solution was introduced for 30 s. Such steps were carried out using Ag solutions in the order from 1 ppt to 10 ppb. We performed these detections three times, and 100 ppb Ag solution was measured once and last. Detection of 100 ppb Ag solution was carried out once, because TNT solution at this high concentration adsorbs and remains on the

flow channel, and residual TNT markedly affects the measurement. Each response was calculated using each RU value. The signal at 10 s before the flow of Ag solution was defined as $\Delta\theta_0$. The signal at 10 s after the flow of Ag solution was determined as $\Delta\theta_1$. Figure 1 shows the conditions of the sensor chip surface at each step.

## 4. Results and Discussion

Figure 7 shows three responses obtained by TNT detection using each antigen analogue. The $K_2$ and LOD of the responses are shown. We mainly investigated three points: the relationship between $K_2$ and response, correlation between $K_2$ and LOD, and the limit of response. The inferences for the three points from the above simulation were as follows.

- A small $K_2$ causes a large response.
- A small $K_2$ causes a low LOD.
- Responses approach not 1 but certain different limits asymptotically under high [*Ag*] conditions.

  LOD is often high despite the large response under low [*Ag*] conditions because of a large SD. Hence, we distinguished a large response from a low LOD.

  Figure 7 shows the relationship for $K_2$:

$$K_2(\text{DNP-glycine}) < K_2(\text{DNP-OVA}) < K_2(\text{DNP-KLH}). \quad (4)$$

We focused on the responses in this relation. Responses to the same Ag solution concentration beyond 10 ppb showed the following relationship:

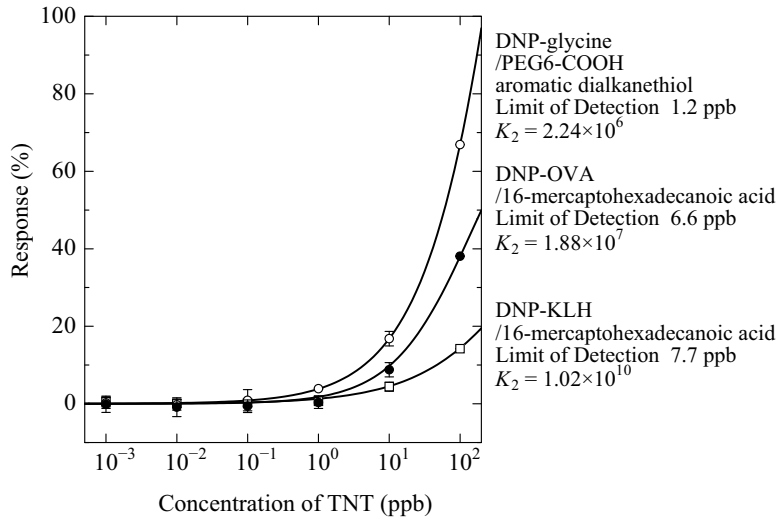


Fig. 7. Responses to TNT in the case of using three antigen analogues.

$$R(\text{DNP-glycine}) < R(\text{DNP-OVA}) < R(\text{DNP-KLH}) . \ (R\text{: response}) \quad (5)$$

From 1 ppt to 1 ppb, the responses were lower than LOD; hence, we disregarded such responses. Therefore, Fig. 7 shows that a small $K_2$ causes a large response to the same Ag solution concentration. This result was the same as the expectation of the simulation.

Next, the LODs obtained using each antigen analogue were 6.6 ppb for DNP-OVA, 7.7 ppb for DNP-KLH, and 1.2 ppb for DNP-glycine. Their relationship was as follows:

$$\text{LOD(DNP-glycine)} < \text{LOD(DNP-OVA)} < \text{LOD(DNP-KLH)} . \quad (6)$$

This relationship was the same as the above relationship for $K_2$. Hence, Fig. 7 implies that a small $K_2$ causes a low LOD. This result was the same as the expectation of the simulation.

Besides, we focused on the upper limits of responses. Figure 7 shows that there are no limits of responses in this experiment. It is different from the expectation of the simulation. Various factors are considered as the causes; for example, the size of $K_1$ and ignored losses of Ab and Ab-Ag. It is possible that the upper limits of responses can be observed by measuring Ag solution concentration beyond 100 ppb. However, the detection of high-concentration TNT is difficult, because adsorbed and remaining TNT on the flow channel markedly affects the measurement. Hence, it is difficult to confirm this influence from the experiment.

We obtained the responses and the LODs, which support the expectations of the simulation. Although the upper limits of responses were not found, there are no practical problems. Hence, we propose the use of a hapten and an antibody with a weak interaction (i.e., a small $K_2$) for optimum setting of the displacement method, and can conclude that the two-layer model simulation in this study showed a sufficient validity. Hence, in this study, we concluded that we obtained the following results.

- The strategy of an SPR displacement immunoassay to realize a high sensitivity was determined using the two-layer model simulation by a theoretical approach.
- The equilibrium constants ($K_1$ and $K_2$) affect the sensitivity of an SPR displacement immunoassay, which is one of assays under nonequilibrium conditions.

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## Appendix

### A. *Details of simplification*

Using eqs. (1)–(3), the following chemical kinetics hold:[12]

$$\frac{\mathrm{d}}{\mathrm{d}t}[Hap\text{-}Ab] = -\frac{\mathrm{d}}{\mathrm{d}t}[Hap] = k_2[Hap][Abs] - k'_2[Hap\text{-}Ab], \tag{A1}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}[Abs] = k'_2[Hap\text{-}Ab] - k_2[Hap][Abs] + k_{-\mathrm{D}}[Ab] - k_{\mathrm{D}}[Abs], \tag{A2}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}[Ab] = k'_1[Ab\text{-}Ag] - k_1[Ab][Ag] + k_{\mathrm{D}}[Abs] - k_{-\mathrm{D}}[Ab] - f_\alpha, \tag{A3}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}[Ag] = k'_1[Ab\text{-}Ag] - k_1[Ab][Ag] + f_\beta, \tag{A4}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}[Ab\text{-}Ag] = k_1[Ab][Ag] - k'_1[Ab\text{-}Ag] - f_\gamma. \tag{A5}$$

Equations (A1)–(A5) are simultaneous differential equations, and instantaneous kinetics depend on the concentration of substances. The term '$f$' means loss or gain of substances by flow in the solution layer.

We simplified the simultaneous differential equations using some presumptions. Because of this simplification, the simulation of the model became a qualitative (not quantitative) simulation. However, there is no problem, because the purpose of this study is not to make a "quantitative simulation" but to find "strategies" realizing high sensitivity. We used some assumptions as follows.

- Ag concentration does not change with time ($[Ag] = [Ag]_0$).
- The loss of Ab and Ab-Ag from the solution layer is zero ($f_\alpha = f_\gamma = 0$).
- The total amount of Ab molecules, which are stand-alone or compose complexes, in the flow cell is conserved during the dissociation of Ab.
- Diffusion is slower than the other chemical reactions (eqs. (1) and (3) are under equilibrium conditions).
- When there is no difference in Ab concentration between the solution layer and the equilibration layer, the diffusion of Ab is apparently completed ($k_D = k_{-D}$; hereafter $k_D$).

On the basis of the assumptions, the following equations are realized:

$$K_2[Hap][Abs] = [Hap\text{-}Ab], \tag{A6}$$

$$\frac{d}{dt}[Ab] = -\frac{d}{dt}[Abs] = k_D([Abs] - [Ab]). \tag{A7}$$

$$K_1[Ab][Ag] = [Ab\text{-}Ag]. \tag{A8}$$

Equation (A6) is the equilibrium equation of the equilibration layer, and eq. (A8) is the equilibrium equation of the solution layer. Each '$K$' has the following relation, which means the association equilibrium constant: $K_2 = k_2/k'_2$ , $K_1 = k_1/k'_1$.

One differential equation containing a variable is integrated by setting default conditions using eqs. (A6) and (A7).

## B. *Calculation of the model*

To investigate the numerical simulation of the model we have to select default values and orders of constants appearing in eqs. (A6) and (A7), and represent the concentrations of substances as variable expressions. We apply these parameters to eqs. (A6) and (A7), and obtain a differential equation, which can be calculated.

We define the concentration of substances at the time '$t$' as follows:

| Substance | Concentration at $t$ | Default value ($t = 0$) |
|---|---|---|
| $[Hap\text{-}Ab]$ | $[H]_0 - x$ | $[H]_0 - x\ (t = 0)$ |
| $[Hap]$ | $x + x_0$ | $x\ (t = 0) + x_0$ |
| $[Abs]$ | $x - \alpha(y + z)$ | $x\ (t = 0)$ |
| $[Ab]$ | $y$ | 0 |
| $[Ag]$ | $[Ag]$(constant) | $[Ag]$ |
| $[Ab\text{-}Ag]$ | $z$ | 0 |

- $[H]_0 = [Hap\text{-}Ab]_0 + x\ (t = 0) = [Hap\text{-}Ab]_0 + [Abs]_0$
- $[Hap\text{-}Ab]_0$: maximum association concentration at the end of introduction of Ab solution into the flow cell ([*Hap-Ab*] at the start of the dissociation phase in Fig. 3)
- $[Abs]_0$: concentration of Ab solution ([*Abs*] at the start of the dissociation phase in Fig. 3)
- $[H]_0$: [*Hap-Ab*] when all Ab molecules in the system are assumed to be bound to Hap
- $x_0 = [Hap]_0 - x\ (t = 0) = [Hap]_0 - [Abs]_0$
- $[Hap]_0$: concentration of Hap not bound to Ab at the end of introduction of Ab solution ([*Hap*] at the start of the dissociation phase in Fig. 3)
- $x_0$: [*Hap*] when all Ab molecules in the system are assumed to be bound to Hap
- $\alpha$: volume ratio between the equilibration layer and the solution layer

We introduced a volume ratio between the equilibration layer and the solution layer into the model. Because we defined that the total amount of Ab molecules in the entire system is constant, we can describe the movement of Ab molecules as an add-subtract calculation of the amount of substances, which is the product of concentration and volume. However, we have to correct the add-subtract calculation of concentration using the volume ratio, because concentration is defined as the quotient of the amount of a substance using the volume ratio. The volume ratio '$\alpha$' is a coefficient to correct calculations. Using the concentrations of substances at $t$, eqs. (A6) and (A7) become as follows:

$$K_2(x + x_0)[x - \alpha(y + z)] = x + x_0, \tag{A9}$$

$$K_1 y[Ag] = z, \tag{A10}$$

$$\frac{dy}{dt} = k_D[x - \alpha(y + z) - y]. \tag{A11}$$

Equation (A9) is regarded as a quadratic equation of $x$. Hence, we obtain

$$x = \frac{\alpha(y + z) - x_0 - \frac{1}{K_2} + \sqrt{\left[\alpha(y + z) - x_0 - \frac{1}{K_2}\right]^2 + 4\left[x_0\alpha(y + z) + \frac{[H]_0}{K_2}\right]}}{2} \geq 0. \tag{A12}$$

Equations (A10) and (A12) are substituted to eq. (A11), and then we can calculate the model as a differential equation of $y$. The mathematization of the model was finished.

We have to define various numerical constants such as $K_1$ and $K_2$, and the order of default values of [*Abs*] and [*Hap-Ab*] to calculate the model. We estimate the constants and the orders from some references as follows.[8,13–15]

- $K_1$: approximately $10^9$ $M^{-1}$
- $K_2$: approximately $10^7$ $M^{-1}$
- $[Ag]$: $5\times10^{-5}$ – $5\times10^{-13}$ M
- $[Abs]_0$: $2\times10^{-7}$ M

- $[Hap\text{-}Ab]_0$: $1\times10^{-6}$ M
- $k_D$: approximately 0.1 $s^{-1}$
- $\alpha$: approximately 4

We assumed that $[Ag]$ ranges from 0.1 ppt to 10 ppm with approximately 200 molecular weight. Four quantities, $[Abs]_0$, $[Hap\text{-}Ab]_0$, $k_D$, and $\alpha$, are related to the volumes of the equilibration and solution layers. The thickness of the equilibration layer depends on not only flow rate but also a solute's diffusion constant, '$D$.' Mass transport coefficient, '$k_M$,' which represents the speed of molecules moving in and out between the unstirred layer and the flow layer, increases with flow rate. The thickness of the equilibration layer '$d$' is in inverse proportion to $k_M$. In the case of SPR immunosensors, $D$, $k_M$ and $d$ generally have the following values:[13,14] $D \approx 10^{-11}$ $m^2/s$, $k_M \approx 10^{-5}$ m/s, $d \approx 10^{-5}$ m.

Fundamentally, these three approximations are used in the case of $[Abs] \leq [Ab]$ situations (adversely, $[Abs] \geq [Ab]$, in this model). In this study, however, we considered that the approximation can be introduced into our model. The size of the flow cell (Biacore X) can be estimated.

$$\text{length} \times \text{width} \times \text{height} = 2.4 \times 0.5 \times 0.05 \text{ mm}^3$$

The maximum Ab association is defined to be about 1,500 RU (approximately 1.5 ng/$mm^2$). The molecular weight of Ab is estimated approximately 150,000. $[Hap\text{-}Ab]_0$ is calculated using the size of the flow cell and the thickness of the equilibration layer. The order of $k_D$ is estimated by dividing $k_M$ by the height (0.05 mm) of the flow cell. $\alpha$ was defined as $\alpha = (\text{height} - d)/d$, which is approximately 4.

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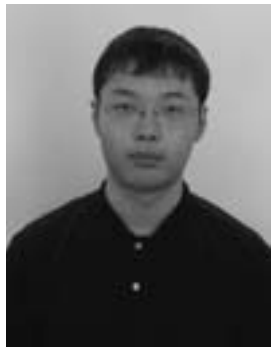

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