Surface Plasmon Resonance Immunosensor for IgE Analysis Using Two Types of Anti-IgE Antibodies with Different Active Recognition Sites

Li, Yan
Department of Applied Chemistry, Graduate School of Engineering, Kyushu University

Ren, Jujie
Department of Applied Chemistry, Graduate School of Engineering, Kyushu University

Nakajima, Hizuru
Department of Applied Chemistry, Graduate School of Engineering, Kyushu University

Soh, Nobuaki
Department of Applied Chemistry, Graduate School of Engineering, Kyushu University

他

出版情報：Analytical Sciences. 23 (1), pp.31-38, 2007-01-10. 公益社団法人日本分析化学会
バージョン：

ANALYTICAL SCIENCES JANUARY 2007, VOL. 23

Original Papers

Surface Plasmon Resonance Immunosensor for IgE Analysis Using Two Types of Anti-IgE Antibodies with Different Active Recognition Sites

Yan LI, Jujie REN, Hizuru NAKAJIMA, Nobuaki SOH, Koji NAKANO, and Toshihiko IMATO†

Department of Applied Chemistry, Graduate School of Engineering, Kyushu University, 744 Motooka, Nishi, Fukuoka 819-0395, Japan

A simple and novel method for the determination of an IgE antibody based on a surface plasmon resonance immunosensor for the diagnosis of an allergy is described. The method involves the use of an anti-IgE(D) antibody and an anti-IgE(H) antibody, which reacts with the Ce2 domain and the Ce3 domain of the IgE antibody. The anti-IgE(D) antibody was immobilized on the gold surface of a sensor chip by physical adsorption. An IgE antibody sample was incubated by adding it to an anti-IgE(H) antibody solution to form an anti-IgE(H) immunocomplex through a reaction of the Ce3 domain of the IgE antibody. The incubated solution was introduced onto the sensor chip and the immunocomplex of the IgE-anti-IgE(H) then reacted with the anti-IgE(D) antibody immobilized on the sensor chip through the Ce2 domain of the IgE antibody part of the IgE-anti-IgE(H) immunocomplex. The detection limit of the present method for the determination of the IgE antibody was about 10 ppb. The affinity constants for the anti-IgE(H) antibody immunocomplex with the IgE antibody in solution and that of the anti-IgE(H) antibody immunocomplex with the IgE antibody immobilized on the sensor chip by a biotin-streptavidin interaction were estimated to be 4.1×10^7 M^{-1} and 5.8×10^6 M^{-1}, respectively. The affinity constant for the immunocomplex of the anti-IgE(H) antibody with the IgE antibody with the anti-IgE(D) immobilized on the sensor chip was estimated to be 4.9×10^7 M^{-1}, 20-times larger than the affinity constant for the IgE antibody immunocomplex with the anti-IgE(D) antibody immobilized on the sensor chip, based on a direct immunoassay method of the IgE antibody under the same experimental conditions.

(Received September 4, 2006; Accepted November 6, 2006; Published January 10, 2007)

Introduction

An immunoglobulin E (IgE) antibody, recently discovered by Ishizaka *et al*. in 1967,[1] has proven to play a very important role in the development of allergies in humans. The IgE antibody binds to mast cells, basophils, monocytes, and langerhans *via* receptors for the IgE antibody, when the IgE antibody on the surface of those cells is cross-linked with allergens, and these cells then degranulate and release chemical mediators, leading to allergic symptoms.[2] The IgE levels are *ca*. 300 ppb (ng/mL) in healthy humans.[3,4] However, a number of factors in humans, including age, gender, smoking habits, and atopic status, have been reported to influence the serum levels of the IgE level.[5-7] Especially, the level of the IgE concentration in serum provides important information for the diagnosis of allergy, since the level of the IgE antibody is dramatically changed by allergens.

A number of methods for the determination of the IgE antibody, such as radioallergosorbent test (RAST) and enzyme linked immunosorbent assay (ELISA), have been developed to date.[8,9] However, these methods have some disadvantages. For example, although the RAST method provides high sensitivity, the separation of an antibody or an antigen conjugate labeled with a radioactive species is required, and an operator must be highly trained because of the radiological safety issues involved. The ELISA method provides a high analytical throughput, but tedious washing and separation procedures are involved, thus constituting major disadvantages.[10]

An instrument based on surface plasmon resonance phenomena (SPR), which occurs on the surface of a gold film, has been recently used for the determination of toxic compounds and plant viruses as well as for studies of DNA hybridization.[11-14] The SPR instrument has some advantages with respect to versatility and capability of monitoring molecular interactions in real time without the need for isotopic labeling.[15]

In this paper, we wish to report on a simple and novel method for the determination of an IgE antibody for allergy diagnosis based on an SPR immunosensor. The method uses two types of mouse anti-IgE antibodies, *i.e*., an anti-IgE(D) antibody and an anti-IgE(H) antibody, which react with two different domains, Ce2 and Ce3, in the same IgE moiety, which permits the sensitivity to the IgE antibody to be enhanced. Namely, we utilized the fact that the anti-IgE(H) antibody reacts with the Ce3 domain and the anti-IgE(D) antibody reacts with the Ce2 domain in the IgE antibody. In the present method, we employed two kinds of immunoreactions: one involved a reaction of the IgE antibody to be determined with the anti-IgE(H) antibody in an incubation solution to form an immunocomplex of the anti-IgE(H) antibody with the IgE antibody by a specific reaction with the Ce3 domain in the IgE antibody. The second was a reaction of the anti-IgE(D) antibody immobilized on the surface of a gold film of a sensor chip with the immunocomplex of the anti-IgE(H) antibody with

† To whom correspondence should be addressed.
E-mail: imato@cstf.kyushu-u.ac.jp

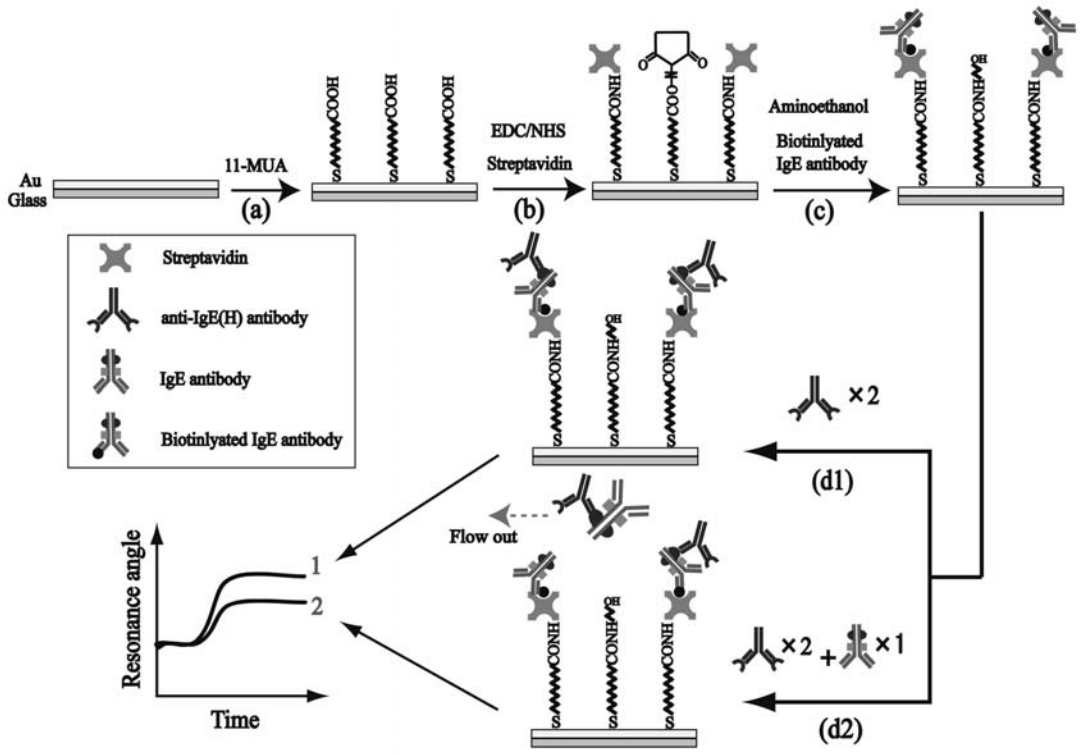

Fig. 1 Diagram for the determination of the affinity constant of anti-IgE(H) complex with an IgE antibody in solution by an indirect competitive assay using a senor chip immobilized with a b-IgE antibody.

the IgE antibody in the incubation solution *via* the Ce2 domain of the IgE antibody in the immunocomplex. The IgE antibody was determined by the SPR response due to binding the immunocomplex of the anti-IgE(H) antibody with the IgE antibody to the anti-IgE(D) antibody immobilized on the sensor chip. Compared with the IgE antibody, since the immunocomplex of the anti-IgE(H) antibody with the IgE antibdy has a larger molecule weight than the IgE antibody itself, a larger dielectric constant change is induced in the vicinity of the gold film, which would be expected to lead to a larger SPR response. Owing to this enhancement of the sensor response, a detection limit for the determination of the IgE antibody as low as 10 ppb could be achieved.

Experimental

Chemicals and equipments

The mouse anti-IgE(H) antibody and the mouse anti-IgE(D) antibody were purchased from Funakoshi Co. (Japan). 11-Mercaptoundecanoic acid (11-MUA) was purchased from Sigma Co. Streptavidin and a biotinlyation kit, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), were purchased from Dojindo Laboratories Co. (Japan). The human IgE antibody was purchased from Calbiochem Co. (USA). All other reagents were of analytical grade and were used without further purification. Deionized water (resistance higher than 18 MΩ) was used to prepare all solutions. Phosphate-buffered saline (PBS, pH 7.2) containing 0.05 wt% Tween 20 was used as a carrier solution. The surface plasmon resonance (SPR) sensor system used in this work was constructed from an SPR sensor with a flow cell (SPR 20, Denki Kagaku Keiki Co., Japan), a sample injector with a 100 μL sample loop (Rheodyne, 7125) and a syringe pump (Carvo 3000X). The SPR sensor was interfaced with a personal computer (Macintosh G4, USA) and the SPR signals were stored on the hard drive. A bare sensor chip was purchased from Eliotech Co. (Japan). The sensor chip comprised a gold thin film (thickness of 45 nm) deposited on a cover glass (18 × 18 × 0.15 mm) with the assistance of a thin chromium film (thickness 5 nm) between them to improve the adhesion of the gold thin film to the cover glass. The sensor chip was attached to the flow cell using a silicon sheet (0.5 mm thick) with a groove (3 × 14 mm). The flow cell, attached to the sensor chip, was set on a prism of the SPR sensor by a coating of matching oil (refraction index of 1.516, from Margil, USA) on the prism.

Evaluation of the affinity constant of an anti-IgE(H) immunocomplex with an IgE antibody in solution and that with the IgE antibody immobilized on a sensor chip

The procedures for the immobilization of a biotinlyated IgE antibody and evaluation of the affinity constant for an anti-IgE(H) immunocomplex with an IgE antibody in solution are shown in Fig. 1. The gold thin film on the sensor chip was cleaned by soaking in a Piranha solution (mixture of H_2SO_4 and H_2O_2 at a volume ratio of 3:1) for 10 min. After rinsing with pure water, the sensor chip was rinsed additional 3 times with ethanol. The sensor chip cleaned with the Piranha solution was then immersed in a 50 mM 11-MUA ethanol solution for 24 h to form a self-assembled monolayer of 11-MUA as an anchor membrane on the sensor chip (step (a) in Fig. 1). The carboxylic groups of the 11-MUA layer on the sensor chip were activated by immersing the sensor chip in a mixed EDC/NHS solution (molar ratio, 2:1) for 50 min.[16] After placing the resulting sensor chip in a flow cell of the sensor chip, a 100 μL portion of a 500 ppm streptavidin solution was introduced into the carrier solution from an injector for immobilization on the activated sensor chip, followed by the injection of a 1 M aminoethanol

solution for deactivation of the unreacted 11-MUA on the sensor chip (step (b) in Fig. 1). The IgE antibody was biotinlyated using a biotinlyation kit according to manufacture's instructions, in which an amino group reactive biotin reagent in the kit reacted with the IgE antibody. The resulting biotinlyated IgE (b-IgE) antibody was immobilized onto the gold surface of the sensor chip *via* a biotin–streptavidin interaction. A 100-μL aliquot of a 9.5 ppm biotinlyated IgE (b-IgE) antibody solution was introduced on the sensor chip for immobilization of the b-IgE antibody on the sensor chip, on which streptavidin had been previously immobilized (step (c) in Fig. 1).

An anti-IgE(H) antibody solution at various concentrations from 3 to 26 ppm was introduced to the sensor chip immobilized with the b-IgE antibody in order to evaluate the affinity constant of the immunocomplex of the b-IgE antibody with the anti-IgE(H) antibody (step (d1) in Fig. 1). In this case, the anti-IgE(H) antibody reacted with the Ce3 domain of the b-IgE immobilized on the sensor chip. Samples were prepared by incubating a 7.8 ppm anti-IgE(H) solution with an IgE antibody from 0.7 to 9.8 ppm for 30 min. The incubated mixture was introduced on the sensor chip and the sensor response was measured. In this case, the anti-IgE(H) antibody competitively reacted with the IgE antibody in the incubation solution and with the b-IgE antibody, which was immobilized on the sensor chip. The immunoreaction of the anti-IgE(H) antibody with the IgE antibody in the incubation solution and that with the b-IgE antibody immobilized on the sensor chip are shown in step (d2) of Fig. 1.

Method for the determination of an IgE antibody using anti-IgE(D) and anti-IgE(H) antibodies and estimating the affinity constant of the immunocomplex of an IgE-anti-IgE(H) antibody with an anti-IgE(D) antibody immobilized on the sensor chip

A diagram of the analytical procedures involved in the present method is shown in Fig. 2. An anti-IgE(D) antibody was immobilized on the gold surface of a sensor chip by physical adsorption for a preparation of the sensor chip (step (a) in Fig. 2). The physical adsorption of the anti-IgE(D) antibody was investigated by introducing a 100-μL aliquot of an anti-IgE(D) solution at several concentrations from 40 to 200 ppm onto a freshly cleaned sensor chip, where a carrier PBS solution was pumped at a flow rate of 0.02 mL/min. An anti-IgE(D) antibody solution at a concentration of 100 ppm was finally selected for immobilization on the sensor chip for determining the IgE antibody in all subsequent experiments. After immobilization of the sensor chip with the anti-IgE(D) antibody, a 100-μL aliquot of a 1000 ppm BSA solution was then introduced twice for blocking nonspecific adsorption on the bare gold surface of the sensor chip (step (b) in Fig. 2). A 100-μL aliquot of a 50 ppm anti-IgE(H) antibody solution was mixed with the IgE antibody solution from 13 ppb to 1 ppm, and the mixture was incubated for 30 min, to prepare sample solutions. In the incubation solution, the IgE antibody reacted with the anti-IgE(H) antibody *via* the Ce3 domain of the IgE antibody to form an immunocomplex of the anti-IgE(H) antibody with the IgE antibody. A 100-μL aliquot of the incubation solution was then introduced on the sensor chip, where the anti-IgE(D) antibody had been immobilized (step (c2) in Fig. 2). The immunocomplex of the anti-IgE(H) antibody with the IgE antibody reacted with the anti-IgE(D) antibody on the sensor chip to form an anti-IgE(D)-IgE-anti-IgE(H) immunocomplex on the sensor chip *via* the Ce2 domain of the IgE antibody. The excess of free anti-IgE(H) antibody in the incubation solution flowed out from the sensor chip without any interaction on the sensor chip. The angle shift of the SPR sensor was monitored during the above procedures. A freshly prepared sensor chip was used for only one determination, and the possible regeneration of the sensor chip was not investigated in this work.

Direct analysis of the IgE antibody with the anti-IgE(D) antibody immobilized sensor chip

In order to compare the sensitivity of the present method to the IgE antibody in a direct immunoassay, where the anti-IgE(D) antibody is immobilized on the sensor chip under the same conditions and a sample IgE antibody solution from 2.5 to 20 ppm was introduced onto the sensor chip under the same experimental conditions as described in the previous section (step (c1) in Fig. 2).

Theoretical Considerations for Evaluating Affinity Constants for the Immunocomplex Used in This Work

Evaluation of the affinity constant for the anti-IgE(H) antibody complex with an IgE antibody on the sensor chip

The affinity constant of the anti-IgE(H) antibody complex with a b-IgE antibody immobilized on the sensor chip can be evaluated by assuming Langmuir-type adsorption, as follows.[10] When an anti-IgE(H) antibody solution is introduced on the sensor chip, the surface on which the b-IgE antibody is immobilized by a biotin–streptavidin interaction, an immunoreaction *via* the Ce3 domain of the b-IgE antibody that occurs on the sensor chip is expressed as followed (cf. step (1) in Fig. 1):

$$\underline{\text{b-IgE}} + \text{anti-IgE(H)} \longrightarrow \underline{\text{b-IgE-anti-IgE(H)}}, \quad (1)$$

where $\underline{\text{b-IgE}}$ denotes the b-IgE antibody immobilized on the sensor chip and anti-IgE(H) denotes the anti-IgE(H) antibody in the sample solution. $\underline{\text{b-IgE-anti-IgE}}$ is an anti-IgE(H) antibody immunocomplex with the b-IgE antibody on the surface of the sensor chip.

The affinity constant, K_1, of the immunoreation Eq. (1) can be expressed by

$$K_1 = \underline{[\text{b-IgE-anti-IgE(H)}]}/(\underline{[\text{b-IgE}]} \times [\text{anti-IgE(H)}]), \quad (2)$$

where the parentheses without any underline denotes the concentration of the chemical species in the solution, expressed in mol/dm^3, and that with an underline denotes the surface concentration of the chemical species immobilized on the sensor chip, expressed in nmol/mm^2. If a Langmuire-type adsorption is assumed to hold for binding of the anti-IgE(H) antibody to the b-IgE antibody on the sensor chip, and the total surface concentration of the b-IgE antibody is assumed to be $\underline{[\text{b-IgE}]}^{\text{T}}$, the following equation can be derived:

$$\underline{[\text{anti-IgE(H)-b-IgE}]}/\underline{[\text{b-IgE}]}^{\text{T}} = [\text{anti-IgE(H)}]/(1 + K_1 \times [\text{anti-IgE(H)}]). \quad (3)$$

In this case, since the change in the SPR sensor signal (angle shift), $\Delta\theta_1$, is proportional to the surface concentration of the anti-IgE(H) antibody bound to the sensor chip, $\Delta\theta_1$ can be rewritten in following form:

$$\Delta\theta_1/\Delta\theta_{1,\max} = [\text{anti-IgE(H)}]/(1 + K_1 \times [\text{anti-IgE(H)}]), \quad (4)$$

where $\Delta\theta_{1,\max}$ denotes the maximum of the angle shift of the SPR sensor, where all of the b-IgE antibody immobilized on the sensor chip is completely bound to the anti-IgE(H) antibody.

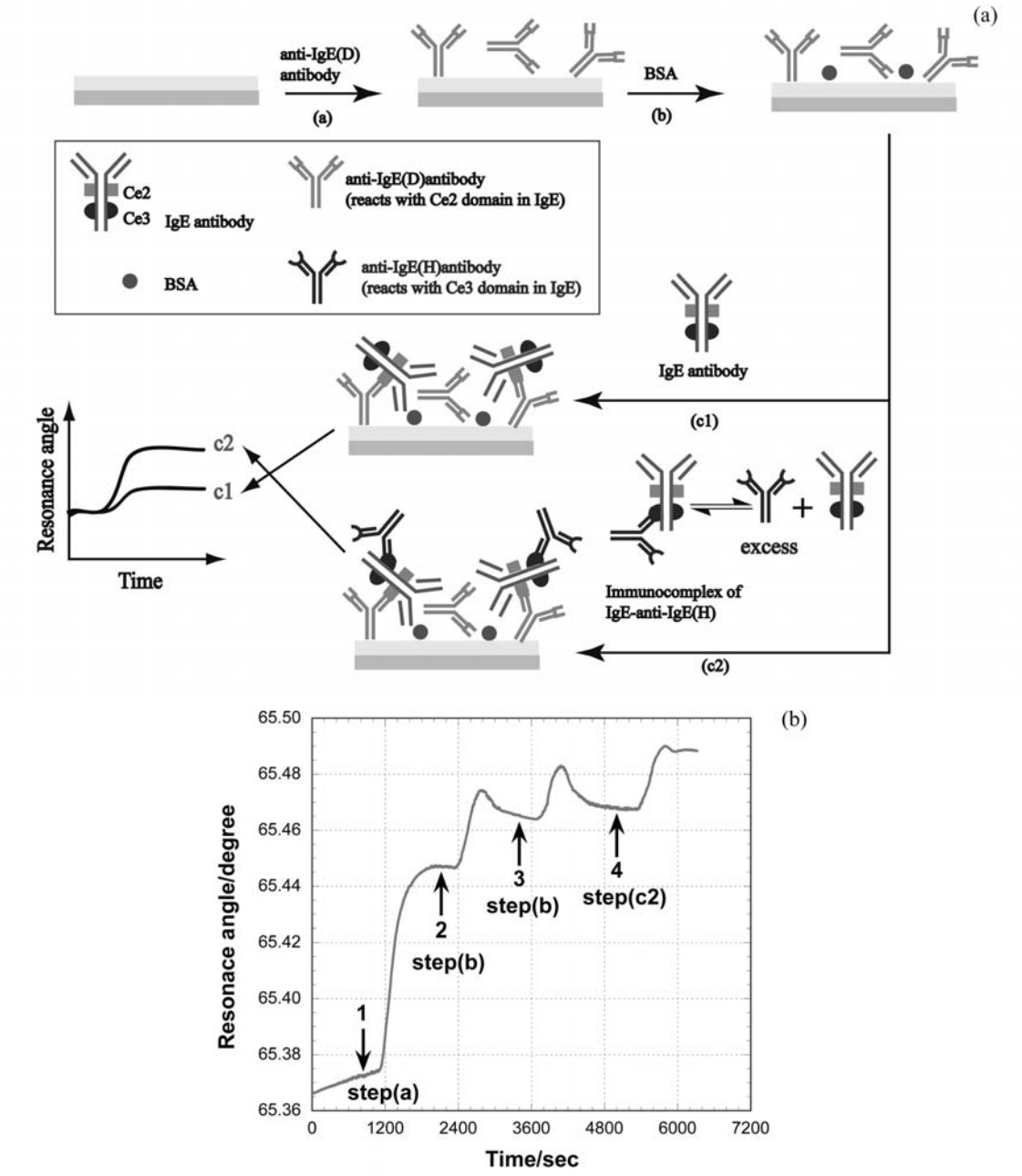

Fig. 2 (a) Protocol for the determination of IgE based on a surface plasmon resonance immunosensor using two types of anti-IgE antibodies and the direct immunoassay for the determination of the IgE antibody. (b) Typical sensorgram obtained using two types of anti-IgE antibodies. The arrow indicates the following procedures: 1, injection of a 100 ppm anti-IgE(D) antibody solution; 2, injection of a 1000 ppm BSA solution for blocking the bare gold surface; 3, injection of a 1000 ppm BSA solution for blocking the bare gold surface; 4, injection of an incubation solution containing 50 ppm of anti-IgE(H) antibody and 650 ppb of IgE antibody.

The following equation can be derived, from Eqs. (3) and (4):

$$1/\Delta\theta_1 = 1/\Delta\theta_{1,max} + 1/([\text{anti-IgE(H)}]\times K_1 \times \Delta\theta_{1,max}). \quad (5)$$

When $1/\Delta\theta_1$ is plotted against 1/[anti-IgE(H)], a linear plot can be obtained, and $\Delta\theta_{1,max}$ and K_1 can be evaluated from the values of the slope and the intercept of the straight line.

Evaluation of the affinity constant of the anti-IgE(H) antibody complex with an IgE in solution

The affinity constant of the anti-IgE(H) antibody complex with the IgE antibody in solution can be determined in the same manner as is assumed to be a Langmuir-type adsorption for competitive immunoreaction of the anti-IgE(H) antibody to the IgE antibody in the solution and to the b-IgE antibody immobilized on the sensor chip.

The immunoreaction of the anti-IgE(H) antibody with the IgE antibody *via* the Ce3 domain in the IgE antibody in the solution is expressed as follows (cf. step (d2) in Fig. 1):

$$\text{IgE} + \text{anti-IgE(H)} \longrightarrow \text{IgE-anti-IgE(H)}, \quad (6)$$

where IgE and IgE-anti-IgE(H) are the IgE antibody and an immunocomplex of the anti-IgE(H) antibody with the IgE antibody in the incubation solution. The affinity constant of the immunocomplex, K_2, of Eq. (6) is expressed by

$$K_2 = [\text{IgE-anti-IgE(H)}]/([\text{IgE}]\times[\text{anti-IgE(H)}]). \quad (7)$$

From the mass balance in terms of the anti-IgE(H) antibody in the incubation solution, the following equation holds:

$$[\text{anti-IgE(H)}]^T = [\text{anti-IgE(H)}]+[\text{anti-IgE(H)-IgE}], \quad (8)$$

where $[\text{anti-IgE(H)}]^T$ is the initial total concentration of the anti-IgE(H) antibody in the incubation solution. In this study, $[\text{anti-IgE(H)}]^T$ was 7.8 ppm, and [anti-IgE(H)] and [anti-IgE(H)-IgE] were the free anti-IgE(H) antibody and an immunocomplex of the anti-IgE(H) antibody with the IgE antibody, respectively, in the incubation solution. When the incubation solution containing the free anti-IgE(H) antibody and the immunocomplex of the anti-IgE(H) with the IgE antibody was introduced on the sensor chip, on which the b-IgE antibody was immobilized on the surface, only the free anti-IgE(H) antibody was bound to the b-IgE antibody immobilized on the sensor chip. The immunocomplex of the anti-IgE(H)-IgE flowed out from the sensor chip because the active site for the Ce3 domain in the IgE antibody was already occupied by the anti-IgE(H) antibody. The concentration of the free anti-IgE(H) antibody in the incubation solution is derived using Eqs. (7) and (8) as follows:

$$[\text{anti-IgE(H)}] = [\text{anti-IgE(H)}]^T/(1 + K_2\times[\text{IgE}]). \quad (9)$$

By inserting Eq. (9) into Eq. (5) and rearranging the terms, the following equation is obtained:

$$1/\Delta\theta_2 = 1/\Delta\theta_1 + K_2\times[\text{IgE}]/(\Delta\theta_{1,\max}\times K_1\times[\text{anti-IgE(H)}]^T), \quad (10)$$

where $\Delta\theta_2$ is the SPR angle shift observed when the incubation solution is introduced on the sensor chip. When $1/\Delta\theta_2$ is plotted against [IgE], a linear plot would be expected between the two parameters, and K_2 can be obtained from the slope of the line.[17-20]

Evaluation of affinity constants of the complex of the IgE antibody and an immunocomplex of IgE-anti-IgE(H) with an anti-IgE(D) antibody immobilized on sensor chip

The affinity constant for the IgE antibody complex with the anti-IgE(D) antibody immobilized on the sensor chip (step (c1) in Fig. 2), K_3, can be evaluated in the same manner that Eq. (5) was derived, as follows:

$$1/\Delta\theta_3 = 1/\Delta\theta_{3,\max} + 1/([\text{IgE}]\times K_3\times\Delta\theta_{3,\max}), \quad (11)$$

where the value of $\Delta\theta_3$ is the SPR angle shift produced when an IgE antibody solution is introduced onto the sensor chip; $\Delta\theta_{3,\max}$ is the maximum of the SPR angle shift, where all of the anti-IgE(D) immobilized on the sensor chip is completely bound to the IgE antibody. When the value for $1/\Delta\theta_3$ is plotted against 1/[IgE], a linear relationship between the two parameters can be obtained. K_3 and $\Delta\theta_{3,\max}$ can be evaluated from the value of the slope and the intercept of the straight line.

The affinity constant for an immunocomplex of anti-IgE(H)-IgE with the anti-IgE(D) antibody on the sensor chip, K_4 (step (c2) in Fig. 2), can be evaluated by the following equation, which is derived from the same manner as Eq. (11):

$$1/\Delta\theta_4 = 1/\Delta\theta_{4,\max} + 1/([\text{IgE-anti-IgE(H)}]\times K_4\times\Delta\theta_{4,\max}), \quad (12)$$

where $1/\Delta\theta_4$ is the SPR angle shift derived from an IgE antibody solution containing an excess of the anti-IgE(H) antibody introduced onto the sensor chip, and $\Delta\theta_{4,\max}$ is the maximum SPR angle shift, where all of the anti-IgE(D) on the sensor chip is completely bound to the IgE-anti-IgE(H) immunocomplex. In this case, the [IgE] term in Eq. (11) is replaced by the [IgE-anti-IgE(H)] term in Eq. (12), because the IgE antibody in the incubation solution can be considered to have been converted to the form of the immunocomplex of [IgE-anti-IgE(H)] under the conditions of an excess of the anti-IgE(H) antibody in the incubation solution. When the value of $1/\Delta\theta_4$ is plotted against the value of 1/[IgE-anti-IgE(H)], a linear relationship between two parameters is obtained. K_4 and $\Delta\theta_{4,\max}$ can be estimated from the value of the slope and intercept of the straight line. The concentration of the immunocomplex of IgE-anti-IgE(H) in the incubation solution can be regarded as the same concentration as that of the IgE antibody. The concentration of the anti-IgE(H) antibody was kept constant at 50 ppm and concentration of the IgE antibody in the incubation solution was varied from 13 ppb to 1 ppm.

Results and Discussion

Evaluation of the affinity constant of the anti-IgE(H) antibody immunocomplex with the IgE antibody on the sensor chip

After introducing a 500 ppm streptavidin solution on an 11-MUA activated sensor chip, an angle shift of 0.08° was observed, caused by the immobilization of streptavidin on the sensor chip. This indicates that the surface concentration of streptavidin immobilized on the sensor chip was 1.5×10^{-5} nmol/mm^2, by taking into account BIAcore's specification that an angle shift of 0.1° corresponds to a mass change of 1 ng/mm^2,[21,22] and that the molecular weight of streptavidin is 53 kDa. The injection of a 100 μL aliquot of a 9.6 ppm b-IgE antibody solution onto the sensor chip resulted in an angle shift of 0.1°, indicating that the surface concentration of the b-IgE antibody was *ca.* 5.6×10^{-7} nmol/mm^2. Judging from the surface concentration of streptavidin and the b-IgE antibody, about 27 moles of streptavidin were estimated to be immobilized for each mole of the b-IgE antibody on the surface of the sensor chip. When an anti-IgE(H) antibody solution at concentrations from 3 to 26 ppm was introduced on the sensor chip, on which the b-IgE antibody was immobilized, the angle shift increased with an increase in the concentration of the anti-IgE(H) antibody up to 13 ppm, reaching at a constant value, as shown in Fig. 3. The maximum in the angle shift, which was due to the fact that all of the b-IgE antibody on the sensor chip was bound to the anti-IgE(H) antibody, was obtained at a concentration of the anti-IgE(H) antibody of 13 ppm. The value of the angle shift from Fig. 3 is 0.035°. The affinity constant of K_1 for the immunocomplex of the anti-IgE(H) antibody with the b-IgE antibody immobilized on the sensor chip can be evaluated from Eq. (5) using the data shown in Fig. 3. In Fig. 4, the reciprocal of the angle shift is plotted against the reciprocal value of the concentration of the anti-IgE(H) antibody, expressed in units of the molar concentration. A good linear relationship between the two parameters was obtained, indicating that the Langmuir-type adsorption holds. The affinity constant, K_1, and $\Delta\theta_{1,\max}$ were estimated to be 5.8×10^6 M^{-1} and 0.034°, respectively, from the intercept and slope of the straight line in Fig. 4.

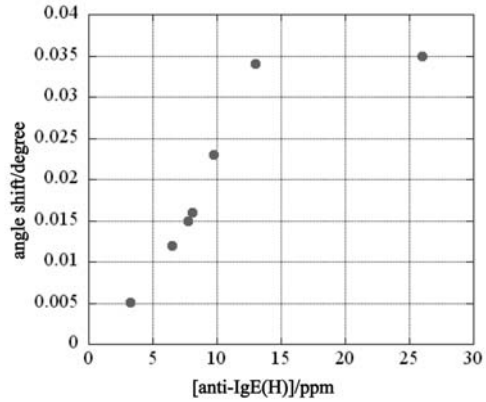

Fig. 3 Relationship between angle shift and the concentration of anti-IgE(H) antibody for the sensor chip immobilized with a biotinlyated IgE antibody.

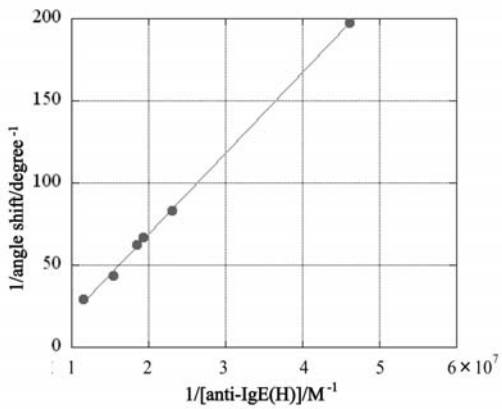

Fig. 4 Relationship between the 1/angle shift and 1/[anti-IgE(H)] for verification of the Langmuir adsorption isotherm.

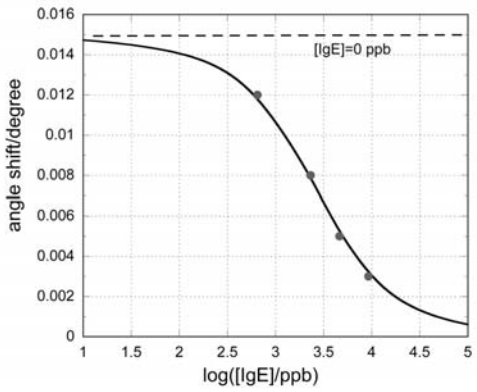

Fig. 5 Relationship between the angle shift and log([IgE]/ppb).

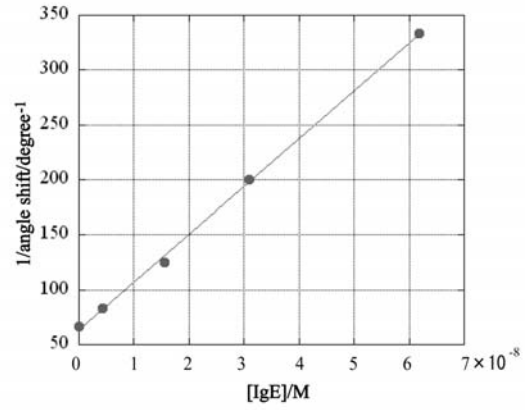

Fig. 6 Relationship between the 1/angle shift and the concentration of the IgE antibody for calculating the affinity constant of the anti-IgE(H) antibody complex with the IgE antibody. Data are taken from Fig. 5.

Evaluation of the affinity constant of the anti-IgE(H) immunocomplex with the IgE antibody in solution

The affinity constant of the anti-IgE(H) antibody immunocomplex with the IgE antibody in solution was determined by an indirect competitive assay of the IgE antibody using an incubation solution containing 7.8 ppm of the anti-IgE(H) antibody and the IgE antibody in a concentration range of 0.65 to 9.3 ppm (Fig. 1, step (d2)). A concentration of 7.8 ppm for the anti-IgE(H) antibody was chosen for the concentration of the anti-IgE(H) antibody in the incubation solution. At this concentration, the anti-IgE(H) antibody would be expected to bind to about half of the total b-IgE antibody molecules immobilized on the sensor chip, judging from Fig. 3. When an incubation solution containing the anti-IgE(H) antibody at a concentration of 7.8 ppm and IgE antibody in the concentration range from 0.65 to 9.3 ppm was introduced on the sensor chip, the relationship between the angle shift of the sensor and the concentration of the IgE antibody in the incubation solution was obtained. The results are shown in Fig. 5. As can be seen, the angle shift decreases with increasing the concentrations of the IgE antibody in the incubation solution, indicating that the IgE antibody in the incubation solution inhibits the binding of anti-IgE(H) antibody to the b-IgE antibody immobilized on the sensor chip. Namely, the active site of the anti-IgE(H) antibody for recognition of the Ce3 domain is occupied with the IgE antibody in the incubation solution *via* the formation of an immunocomplex of IgE-anti-IgE(H); also, concentration of the free anti-IgE(H) antibody, which can react with the b-IgE on the sensor chip, decreases with an increase in the IgE antibody in the incubation solution. The affinity constant for the anti-IgE(H) antibody complex with the IgE antibody in the incubation solution can be estimated from Eq. (10) using the data in Fig. 5 in the same manner as described above. According to Eq. (10), the reciprocal values for the angle shift were plotted against the concentration of the IgE antibody in the incubation solution, as shown in Fig. 6. A good linear relationship between both parameters was obtained, indicating that the Langmuir-type adsorption also holds in this case. The affinity constant, K_2, for the immunocomplex of the anti-IgE(H) antibody with the IgE antibody in solution was estimated to be 4.1×10^7 M^{-1} from the slope of the straight line in Fig. 6. The value of the affinity constant for the anti-IgE(H) antibody complex with the b-IgE antibody on the sensor chip, K_1, is 8-times smaller than that for the IgE antibody in solution, K_2, which indicates that the reactivity of the IgE antibody with the anti-IgE(H) antibody may be reduced after the biotinylation reaction or immobilization on the sensor chip. Namely, this may be due to the fact that the Ce2 domain of the IgE antibody that reacts with the anti-IgE(H) antibody is hindered after the biotinylation reaction or immobilization on the surface of the sensor chip. The detection limit for the determination of the IgE antibody by this indirect competitive assay method read from 80

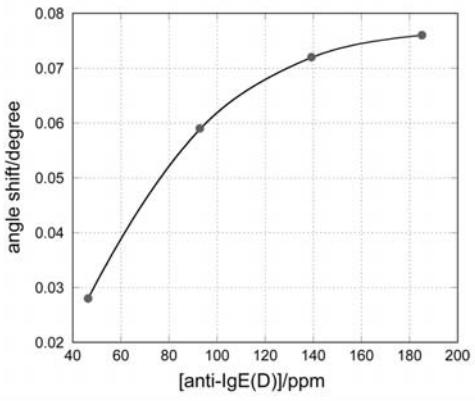

Fig. 7 Relationship between the angle shift and the concentration of the anti-IgE(D) antibody for immobilization on the sensor chip by physical adsorption.

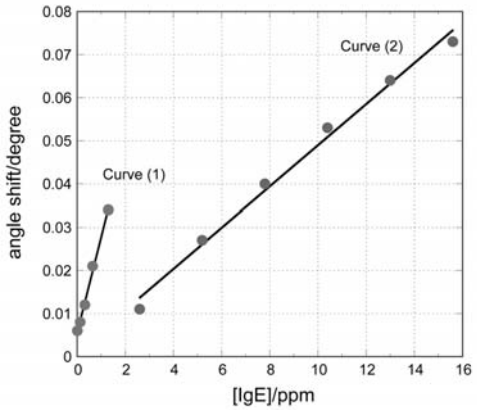

Fig. 8 Calibration curves for determining the IgE antibody. Curve (1) was obtained by introducing an incubation solution containing the IgE antibody in the range from 13 ppb to 1 ppm and 50 ppm of anti-IgE(H) antibody. Curve (2) was obtained by direct immunoassay of the IgE antibodies. Conditions: Carrier solution, PBS, 0.02 mL/min; sample volume, 100 μL; sensor chip, anti-IgE(D) antibodies immobilized onto gold film; protocol shown in Fig. 2.

to 85% inhibition of the immunoreaction between the anti-IgE(H) antibody with the IgE antibody in Fig. 5, was about 500 ppb. The method described in the following section is much more highly sensitive.

Preparation of a sensor chip by immobilization of an anti-IgE(D) antibody

The sensitivity of the SPR sensor is principally governed by the extent of change in mass on the surface of the sensor chip. Therefore, if a larger change in the mass on the sensor chip can be induced, the sensitivity for determining the IgE antibody would be enhanced. In the present SPR immunosensor for the IgE antibody, to enhance the mass change in the IgE antibody, the IgE antibody was converted to a larger molecular weight species by a reaction with an anti-IgE(H) antibody, which has an active site for recognition of the Ce3 domain in the IgE antibody. Meanwhile, the IgE antibody contains an additional Ce2 domain and an anti-IgE(D) antibody, which allows selective binding to the Ce2 domain of the IgE antibody. In this experiment, we prepared a sensor chip, on which the anti-IgE(D) antibody was immobilized for binding an immunocomplex of the IgE antibody that was converted to a larger molecular-weight species. The preparation of the sensor chip was based on physical adsorption, as described in the experimental section. As shown in Fig. 7, the angle shift, which is due to physical adsorption of the anti-IgE(D) antibody on the sensor chip, increases with increasing the concentrations of the anti-IgE(D) antibody in the solution introduced on the sensor chip. When the concentration of anti-IgE(D) antibody solution was larger than 140 ppm, the angle shift approached nearly a constant value, indicating that the surface of the sensor chip became saturated with the anti-IgE(D) antibody. In a subsequent experiment for preparing a sensor chip, an anti-IgE(D) antibody solution at a concentration of 100 ppm was selected. Assuming the BIAcore's specification that an angle shift of 0.1° corresponds to 1 ng/mm^2 holds, the present surface concentration of the anti-IgE(D) antibody was estimated to be 0.6 ng/mm^2 when a 100 ppm solution was introduced on the sensor surface.

Calibration of the IgE antibody using two types of antibodies of anti-IgE(D) and anti-IgE(H) antibodies

The protocol for the present immunoassay and a typical sensorgram for the determination of the IgE antibody are shown in Figs. 2(a) and (b), respectively. When a 100 μL aliquot of a 100 ppm anti-IgE(D) antibody solution was introduced on the sensor chip for immobilization of the anti-IgE(D) antibody, the resonance angle increased by about 0.06°, and then reached a constant value. A 100-μL aliquot of a 1000 ppm BSA solution was then introduced on the sensor chip twice to block any nonspecific adsorption to the bare gold surface. An angle shift of 0.02° was observed for the blocking. In the case of introducing a 650 ppb IgE antibody solution containing 50 ppm of an antibody solution in the incubation solution, an angle shift of 0.02° was obtained, as shown in Fig. 2(b). This angle shift is due to the fact that the immunocomplex of IgE-anti-IgE(H) in the incubation solution was bound to the anti-IgE(D) antibody immobilized on the sensor chip. When an incubation solution containing 50 ppm of anti-IgE(H) antibody and the IgE antibody in a concentration range from 13 ppb to 1 ppm was introduced on the sensor chip, a relationship between the angle shift and the concentration of the IgE antibody in the incubation solution was obtained, as shown in Fig. 8 (curve (1)). The detection limit for the determination of the IgE antibody, defined as the concentration at an angle shift 3-times larger than the noise level, is about 10 ppb. Since this detection limit for the proposed method for the IgE antibody is much lower than the levels of the IgE antibody present in healthy people (300 ppb), the proposed method has potential for use in the diagnosis of allergies. On the other hand, a relationship between the angle shift and the concentration of the IgE antibody, obtained by a direct immunoassay, in which the IgE antibody solution at several concentrations from 2.5 to 20 ppm was directly introduced on the sensor chip immobilized with the anti-IgE(D) antibody, is shown in Fig. 8 (curve (2)). The detection limit for the direct immunoassay method for the determination of the IgE antibody is about 1 ppm. The slopes of curves (1) and (2) in Fig. 8 were calculated to be 0.022 and 0.0048 degree/ppm, respectively. Therefore, the sensitivity of the proposed method using two types of anti-IgE antibodies is about 4.5-times higher than that of the direct immunoassay method.

Evaluation of the affinity constants for the IgE antibody complex and IgE-anti-IgE(H) with the anti-IgE(D) complex with the antibody immobilized on the sensor chip

The affinity constant of the IgE antibody complexed with the

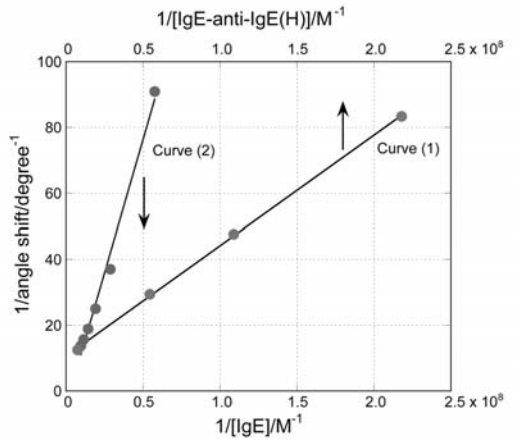

Fig. 9 Relationship between the 1/angle shift and 1/[IgE] or 1/[IgE-anti-IgE(H)] for calculating the affinity constants for the IgE antibody complex or IgE-anti-IgE immunocomplexed with anti-IgE(D) immobilized on sensor chip. Curve (1) was obtained from data curve (1) in Fig. 8. Curve (2) was obtained from data curve (2) in Fig. 8.

anti-IgE(D) antibody immobilized on the sensor chip, K_3, was estimated to be 2.3×10^6 M^{-1} from the intercept and slope of curve (2) in Fig. 9, using Eq. (11) and the data in Fig. 8 (curve (2)). On the other hand, the affinity constant for the IgE-anti-IgE(H) antibody complex with the anti-IgE(D) antibody immobilized on the sensor chip, K_4, was estimated to be 4.9×10^7 M^{-1} from the intercept and slope of curve (1) in Fig. 9, using Eq. (12) and the data in Fig. 8 (curve (1)). The high sensitivity of the proposed method compared with the direct immunoassay method may be due to the fact that the affinity constant for the IgE-anti-IgE(H) complex with the anti-IgE(D) antibody on the sensor chip is about 20-times larger than that for the IgE antibody complex with the anti-IgE(D) on the sensor chip. The detection limit of the proposed method is about 100-times lower than that for the direct immunoassay method for determining the IgE antibody. This improvement in sensitivity and detection limit may be due to an effective increase in IgE antibody by a reaction with the anti-IgE(H) antibody as well as the larger affinity constant of the immunocomplex of IgE-anti-IgE(H) with the anti-IgE(D) antibody immobilized on the senor chip than that of the IgE antibody complex with the anti-IgE(D) antibody.

Conclusions

A sensitivity enhanced immunosensor method based on surface plasmon resonance, using two types of anti-IgE antibodies for the determination of the IgE antibody, is described. The detection limit is *ca*. 10 ppb. The affinity constants for the anti-IgE(H) antibody complex with the IgE antibody in solution and with the b-IgE antibody immobilized on the sensor surface were estimated be to 4.1×10^7 and 5.8×10^6 M^{-1}, respectively. The sensitivity of the proposed method for the IgE antibody was enhanced by about 4.5-times compared with that of the direct immunoassay method. This enhanced sensitivity of the proposed method is due to a large change in the dielectric constant induced by conversion of the IgE antibody to the IgE-anti-IgE(H) immunocomplex. Use of the SPR immunosensor method in the diagnosis of allergies is currently underway.

References

1. K. Ishizaka, T. Ishizaka, and M. M. Hornbrook, *J. Immunol*., **1966**, *97*, 75.
2. M. Ban and D. Hettich, *Toxicol. Lett*., **2001**, *118*, 129.
3. V. S. Chowdary, E. C. Vinaykumar, J. J. Rao, R. Rao, K. R. Babu, and V. Rangamani, *J. Allergy Asthma Immunol., Indian*, **2003**, *17*, 21.
4. M. P. Kreuzer, C. K. O' Sullivan, M. Pravda, and G. G. Guilbault, *Anal. Chim. Acta*, **2001**, *442*, 45.
5. R. A. Barbee, M. H. Halonen, W. Kaltenborn, M. Lebowitz, and B. Burrows, *J. Allergy Clin. Immunol*., **1987**, *79*, 919.
6. F. Cetinkaya and M. Cakir, *Pediatric Otorhinolaryngology*, **2005**, *69*, 493.
7. A. L. Wright, D. Sherrill, C. J. Holberg, M. Halonen, and F. D. Martinez, *J. Allergy Clin. Immunol*., **1999**, *104*, 585.
8. P. L. B. Bruynzeel and L. A. M. J. Houbern, *Clini. Chim. Acta*, **1981**, *112*, 315.
9. I. P. Lewkowich, J. D. Rempel, and K. T. Hayglass, *J. Immunol. Methods*, **2004**, *286*, 123.
10. N. Matsubashi and H. Asagawa, "*Meneki Kese Gaku* (in Japanese)", **1979**, Kodansha, Tokyo, 25.
11. J. W. Choi, K. W. Park, D. B. Lee, W. Lee, and W. H. Lee, *Biosens. Bioelectron*., **2005**, *20*, 2300.
12. P. M. Boltovets, B. Snopok, V. R. Boyko, T. P. Shevchenko, N. S. Dyachenko, and Y. M. Shirshov, *J. Biol. Methods*, **2004**, *121*, 101.
13. M. Gotoh, M. Hasebe, T. Ohia, Y. Hasegawa, Y. Shinohara, H. Sota, J. Nakano, and M. Tosu, *Gene Anal. Biomol. Eng*., **1997**, *14*, 17.
14. Y. Sato, K. Sato, K. Hosokawa, and M. Maeda, *Anal. Biochem*., **2006**, *355*, 125.
15. C. Boozer, G. Kim, S. Cong, H. Guan, and T. Londergan, *Curr. Opin. Biotechnol*., in press.
16. S. H. Choi, J. W. Lee, and S. J. Sim, *Enzyme Microb. Technol*., **2004**, *35*, 683.
17. G. Sakai, K. Ogata, T. Uda, N. Miura, and N. Yamazoe, *Sens. Actuators, B*, **1998**, *49*, 5.
18. K. V. Gobi, M. Sasaki, Y. Shoyama, and N. Miura, *Sens. Actuators, B*, **2003**, *89*, 137.
19. D. R. Shankaran, K. V. Gobi, T. Sakai, K. Matsumoto, K. Toko, and N. Miura, *Sens. Actuators, B*, **2004**, *100*, 450.
20. G. Sakai, S. Nakata, T. Uda, N. Miura, and N. Yamazoe, *Electrochim. Acta*, **1999**, *44*, 3849.
21. E. Laplantine, L. Vallar, K. Mann, N. Kieffer, and M. Aumailley, *J. Cell Sci*., **2000**, *113*, 1167.
22. P. Sriramarao, P. Steffner, and K. R. Gehlsen, *J. Biol. Chem*., **1993**, *268*, 22036.