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Laboratory Exercises

Surface Plasmon Resonance Label-free Monitoring of Antibody Antigen Interactions in Real Time*

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Detection of biologically active compounds is one of the most important topics in molecular biology and biochemistry. One of the most promising detection methods is based on the application of surface plasmon resonance for label-free detection of biologically active compounds. This method allows one to monitor binding events in real time without labeling. The system can therefore be used to determine both affinity and rate constants for interactions between various types of molecules. Here, we describe the application of a surface plasmon resonance biosensor for label-free investigation of the interaction between an immobilized antigen bovine serum albumin (BSA) and antibody rabbit anti-cow albumin IgG1 (anti-BSA). The formation of a self-assembled monolayer (SAM) over a gold surface is introduced into this laboratory training protocol as an effective immobilization method, which is very promising in biosensing systems based on detection of affinity interactions. In the next step, covalent attachment via artificially formed amide bonds is applied for the immobilization of proteins on the formed SAM surface. These experiments provide suitable experience for postgraduate students to help them understand immobilization of biologically active materials via SAMs, fundamentals of surface plasmon resonance biosensor applications, and determination of non-covalent biomolecular interactions. The experiment is designed for master and/or Ph.D. students. In some particular cases, this protocol might be adoptable for bachelor students that already have completed an extended biochemistry program that included a background in immunology.

Keywords: Biosensor, bioanalytical chemistry, surface plasmon resonance, SPR, antibody, antigen, education.

Various biologically active materials ranging from small organics [1] to huge multiprotein complexes [2] play an incredible role in the physiological functions of living species. Fast and efficient analysis of such compounds is required in molecular biology and biochemistry [3, 4]. Here, biosensors are the most promising tools since they do not require sophisticated sample preparation, are simple to use, and can be successfully applied in biological and biochemical education [5].

Biosensors are divided into two major classes: catalytic biosensors [6] and affinity interaction-based biosensors [7]. Some bioanalytical protocols based on the application of biosensors have been presented: potentiometric detection of urea, amperometric detection of glucose based on the application of redox mediators [8] and depletion of dissolved oxygen [9], and detection of hydrogen peroxide concentration by an oxygen electrode [5]. The application of soluble redox mediators and/or detection of reaction substrates/products were applied in each case. However, direct (label-free) detection of analytes by bioelectronic devices [10] is the most promising and challenging opportunity in biosensorics. One of the most promising label-free detection methods in biosensorics is based on detection of surface plasmon resonance since this method allows one to monitor binding events in real time without labeling. Therefore, the system can be used to determine both affinity and rate constants for interactions between all types of biomolecules. SPR[1] is often applied for biomedical purposes, especially for investigation of some drugs affecting cell proliferation [11, 12].

The laboratory protocol described here will demonstrate the application of SPR for monitoring of the binding interaction between immobilized protein BSA and dissolved antibody rabbit anti-cow albumin IgG1. Formation of a

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[1] The abbreviations used are: SPR, surface plasmon resonance; SAM, self-assembled monolayer; PBS, phosphate-buffered saline; MUA, 11-mercaptoundecanoic acid; EDC, *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide; NHS, *N*-hydroxy succinimide.

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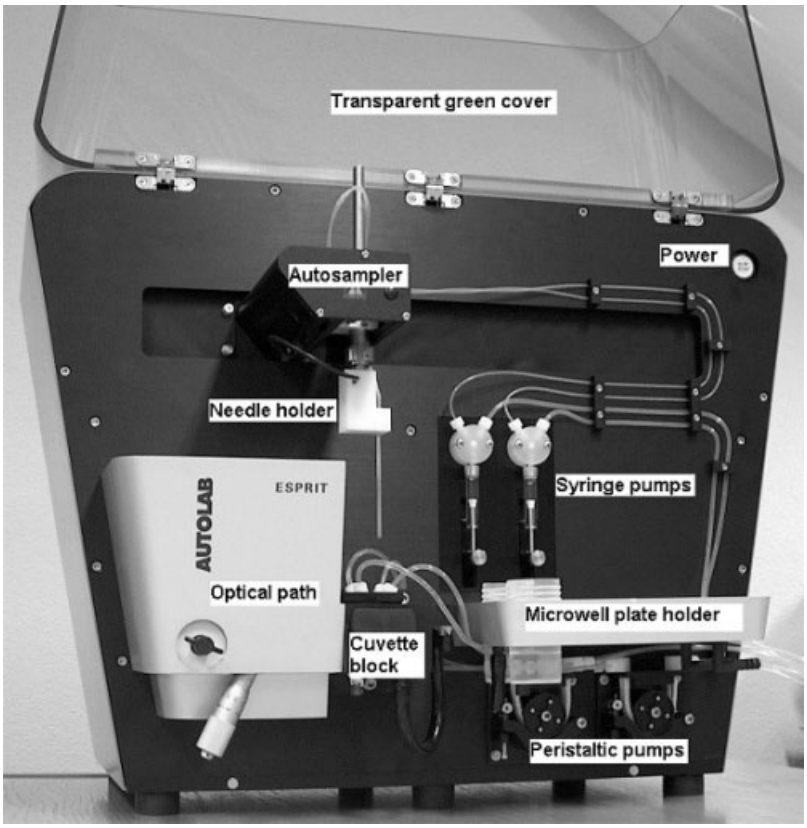

FIG. 1. **The Autolab ESPRIT.**

SAM over a gold surface is introduced as a promising immobilization method for biosensing systems.

EXPERIMENTAL PROCEDURES

Planning and Preparation for the Experiment—The number of students performing experiments should not exceed five for one stand, and an optimal amount is two students per stand. The teacher, depending on practical experience with the application of SPR, should reserve from 8 to 20 h for preparation of the experiments described below.

Method for Monitoring of Antibody-Antigen Interactions—An SPR analyzer Autolab ESPRIT (Fig. 1) developed by ECO Chemie was applied for educational detection of antibodies in solution. If this instrument is not available, any other SPR analyzer (*e.g.* any types of SPR analyzers from Biacore AB (Uppsala, Sweden) or other manufacturers) might be easily applied for the proposed training experiment. Principally, any commercially available SPR analyzer should not require any special adaptation to perform this experiment. The experimental procedure is designed for two 4-h laboratory periods. Solution preparation and formation of a self-assembled monolayer is performed in the first period. SAM stabilization and activation, BSA immobilization, remaining reactive ester deactivation, and interaction of anti-Cow albumin with BSA are accomplished in the second period.

Chemicals—All chemicals should be of analytical grade and used as received (if not otherwise stated). The solutions should be prepared by using an HPLC grade water purification system. All of the chemicals for this experiment were purchased from Fluka (Fluka Chemie AG, Buchs, Switzerland). The chemicals used are listed in Table I.

Formation of SAM—The bare sensor disk is coated by a SAM consisting of substituted thiols since gold has a strong interaction with sulfur. To achieve this goal, the sensor disk is incubated in 1 mM 11-mercaptoundecanoic acid (MUA) in ethanol for 4 h. A repetitive sequence of coupling buffer solution, 10 mM sodium acetate, pH 4.5, and 100 mM HCl, both in 1-min intervals lasting for 1 h, is used for stabilization of the monolayer and for measuring the baseline. The buffer pH is adjusted with acetic acid.

TABLE I
Chemicals

Name	CAS No.[a]
11-Mercaptoundecanoic acid	71310-21-9
PBS buffer	57-30-7
Hydrochloric acid	7647-01-0
Albumin from bovine serum	9048-46-8
Rabbit anti-cow albumin	
Ethanol	64-17-5
Immersion oil	
N-Hydroxy succinimide	6066-82-6
N-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride	25952-53-8
Ethanolamine	141-43-5
NaOH	1310-73-2
Sodium acetate trihydrate	6131-90-4

[a] CAS No., Chemical Abstracts Service number.

Surface Activation—A sensor disk coated with MUA is deposited on top of a slider with a glass prism (Fig. 2). Then, the slider is positioned inside the Autolab ESPRIT using a cuvette holder (Fig. 1). A mixture of 0.1 M *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC) and *N*-hydroxy succinimide (NHS) in water is used for immobilized MUA activation. This solution can be supplied automatically by pumping it from a corresponding vessel or manually by pipetting. During the activation routine, solutions without any air bubbles should be used. The activation step lasts for 5 min. The mixture of EDC/NHS is then washed away with a coupling buffer. The EDC/NHS activation can be repeated at least three times on the same chip to increase the activation yield; EDC is very unstable and should be stored in a freezer at −20 °C. Diluted EDC aliquots can be frozen at −20 °C and stored before use. After defrosting, an EDC aliquot should be mixed with NHS and immediately used for experiments.

Immobilization of the BSA—After activation of the MUA immobilized on the sensor surface, the BSA solution should be added

FIG. 2. **Sensor disk on top of a slider with a glass prism.**

either manually by pipetting or via a preprogrammed automatic immobilization sequence routine. Immobilization of BSA is performed in both channels of the SPR cuvette (Fig. 3). During the immobilization routine, solutions without any air bubbles should be used since they may lower the efficiency of coupling. The ligand is coupled covalently either by amine or by thiol functional groups on a modified gold sensor disk. Immobilization of 2.5 μg of BSA protein dissolved in a coupling buffer lasts for 15 min. If no decay in the minimal refraction angle is detected after washing with 10 mM phosphate buffer solution (PBS) containing 0.05% Tween 20, it means that the surface is sufficiently loaded by coupled BSA.

To increase BSA coupling yield, it is recommended that students increase the ligand concentration close to the sensor surface during the reaction. Therefore, the ligand should be dissolved in buffer with a low salt concentration, *e.g.* 10 mM sodium acetate. This provides an environment where the ligand can be electrostatically preconcentrated at the surface. The negatively charged carboxylated hydrogel can interact with positively charged residues of ligand, thus attracting the ligand toward the chip. By choosing a pH that is lower than the isoelectric point (pI) of the ligand by 0.5–1 pH units, the ligand remains positively charged. During the application of this protocol, be aware that under these conditions, the concentration of the ligand is 10–1,000-fold lower when compared with the salt concentration. The coupling efficiency is dependent on pH, and the pH in the experiments proposed herein should be between pH 3.5 and pH 8.5. Otherwise, the dextran carboxylic groups become protonated below a pH of 3.5. The ligand concentration is reversibly dependent on pH, meaning that if immobilization is performed at a lower pH, a higher ligand concentration is required to achieve the same immobilization efficiency. Also, the duration of the reaction is important for efficiency of ligand binding. Some ligands react very fast, and maximal immobilization efficiency is achieved within 1 min, whereas other ligands are less active. It is not recommended that students perform the ligand immobilization reaction for longer than 30 min due to the relatively short half-life of the activated NHS-ester.

Deactivation of Active Surface—After immobilization of the BSA, it is essential to neutralize unreacted reactive esters remaining on the formed SAM surface. Reactive esters are deactivated with 1 M ethanolamine, pH 8.0. If the Autolab ESPRIT is used in this laboratory experiment, then ethanolamine can be supplied automatically by pumping it from a corresponding vessel or manually by pipetting. During the deactivation routine, solutions without any air bubbles should be used. The deactivation step should last for 5 min. After this, ethanolamine is washed away with a 10 mM PBS buffer solution. Please note that ethanolamine is very unstable and should be stored in a freezer at −20 °C.

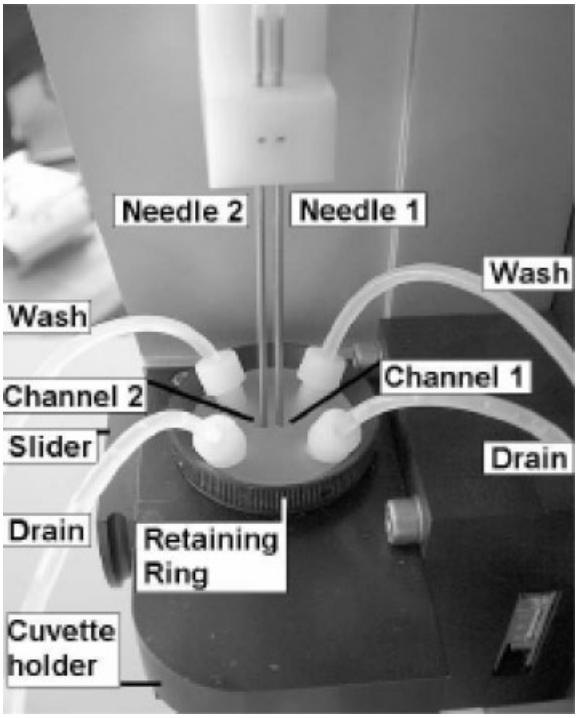

FIG. 3. **Sample in the injection system.**

Stabilization Conditions Prior to Final Interaction Analysis—When the BSA (ligand) is finally covalently coupled to the sensor disc surface, its interaction with anti-BSA (analyte) can be tested. A repetitive sequence of 10 mM PBS buffer solution, pH 7.4, and 100 mM HCl, both in 1-min intervals lasting for 1 h, is used for stabilization of the surface and for measuring the baseline. If BSA is properly immobilized on the disk surface, then stable baselines are registered. In some cases, an unstable baseline may be obtained. This effect may result from some unstable SAM formations, from non-specifically bound ligands still not fully removed after coupling that now slowly diffuse from the surface, or from bound analyte that is not completely removed by the regeneration procedure.

After each BSA binding experiment, the sensor disc surface

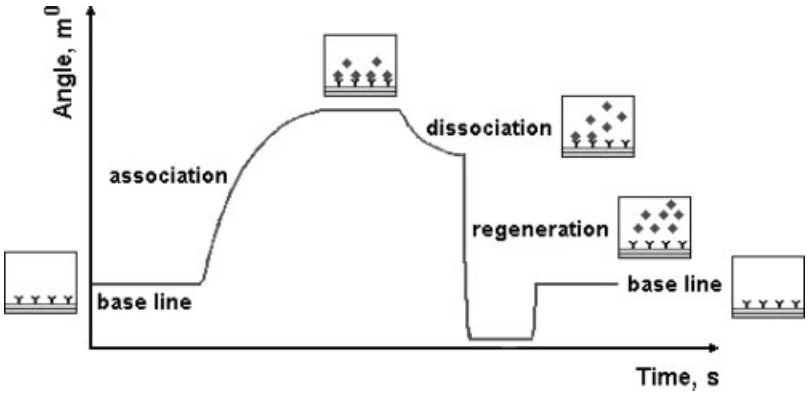

FIG. 4. **Interpretation of SPR signal *versus* time.**

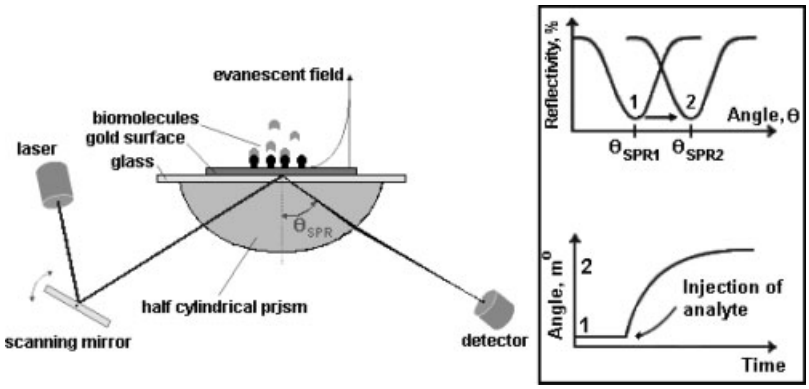

FIG. 5. **Illustration of the surface plasmon resonance principle in real-time kinetic analysis.**

should be completely regenerated prior to the next measurement. Anti-BSA antigen that is tightly associated with immobilized BSA, but non-covalently bound, can be removed with 100 mM HCl. For other analyte-ligand complexes, the best regeneration conditions depend on the stability of the formed protein complex.

Interaction of Anti-BSA Antibody—The prepared surface is stabilized before registering a baseline with PBS buffer at pH 7.4 for 5 min. After the baseline becomes stable, the first concentration (the lowest detectable concentration) of anti-Cow albumin is added. The BSA and anti-BSA interaction is monitored for 15 min followed by dissociation in PBS buffer (Fig. 4). The interaction complex is then regenerated with 100 mM HCl for 5 min (Fig. 4). After restoring the PBS baseline, the interaction cycle is repeated for the other antibody concentrations. Reference measurements are performed with the rabbit anti-human α-2-macroglobuline antibody. The concentration of the reference antibody is equivalent to the anti-BSA antibody in every individual experiment.

Important—Because of high affinity and fast interactions, the bulk solution can become depleted of free analyte in a diffusion layer just above the surface. This mass transport-limited effect can be significantly decreased by lowering the ligand capacity on the surface. More reliable binding data and faster kinetics are then obtained. Therefore, it is suggested to begin experiments with low ligand capacity-based disks and low analyte concentrations.

RESULTS AND DISCUSSION

Surface plasmon resonance is a physical process that can occur when plane-polarized light hits a metal film under total internal reflection conditions. Usually, the SPR-generating surface is composed of a ~50-nm-thick layer of metal deposited on glass. Because the SPR effect can only take place on thin metal layers in which the electrons behave as a free electron gas, meaning that their motions are independent of the charge that they leave behind when moving, the choice of metal is limited to copper, silver, aluminum, and gold. Gold is by far the most effective substrate for SPR measurements and immobilization of biologically active molecules via self-assembled monolayers. SPR is detected by measurement of the intensity of the reflected light. At the total internal reflection angle, a sharp decrease of reflected light intensity is measured. The position of the SPR angle depends on the refractive index in the substance close to the sensing surface. The refractive index near the sensor surface changes because of the binding of macromolecules to the surface. As a result, the total internal reflection angle will change according to the amount of bound macromolecules. There is a linear relationship between the amount of bound material and the shift of the total internal reflection angle [13]. One of the advantages of SPR is that the method allows one to study biomolecular interactions in real time. There is also no need for any labeling (*e.g.* with radioactivity or fluorescence), which, in addition to saving time, helps in preserving biomolecular nativity. SPR biosensors have been used to study a wide range of biomolecular interactions, providing both qualitative (identification, site specificity, epitope mapping) and quantitative (kinetics, affinity, and concentration analysis) information [11, 14, 15]. In a typical SPR biosensing experiment, a ligand is immobilized on an SPR-active gold-coated glass slide, which forms one wall of a thin flow cell. An analyte in an aqueous buffer solution is induced to flow across the sensor surface by injecting it into the flow cell. The interaction of analyte with the ligand attached to the surface causes a change on the metal surface and within the surface plasmon field. The resulting shift in the resonant wavelength of the incident light is measured. The light passes through the prism and slide, reflects from the gold, and passes back through the prism to a detector (Fig. 5). The high sensitivity of the optical

response is due to the fact that there is a very efficient, collective excitation of conduction electrons near the gold surface. Since SPR detection is independent of the chemical nature of the sample being analyzed, in principle, all types of molecules such as proteins, lipids, nucleic acids, and small molecules such as drugs, substrates, and cofactors can be used to monitor biomolecular interactions [16].

The Autolab ESPRIT is a unique double channel SPR biosensor incorporating an auto-sampler. This instrument is equipped with universal control and data acquisition software. The revolutionary design of the Autolab ESPRIT allows the application of this device for simultaneous optical-electrochemical investigations currently of special interest to the biochemistry community [17].

By using the Autolab ESPRIT, a linear relationship between the amount of bounded material and shift in SPR angle is exploited for estimation of analyte concentration. The SPR angle shift in millidegrees is used as a biosensor response unit to quantify the binding of macromolecules to the sensor surface. The SPR biosensor response also depends on the refractive index of the bulk solution. A change of 120 millidegrees represents a change of $\sim$1 ng/mm^2 in surface concentration of protein or a change in bulk refractive index of $\sim 10^{-3}$.

In an SPR biosensing experiment, the entire gold surface containing immobilized ligand is thoroughly washed with buffer. After the appearance of a stable baseline (Fig. 4), a solution containing the analyte is introduced (association phase). The interaction of analyte with the ligand attached to the surface results in an increase of the signal. At the end of the analyte injection, the sample is replaced by a continuous flow of buffer, and a decrease in signal now reflects dissociation of analyte from the surface-bound complex. The surface is then regenerated with a particular regeneration reagent, and the signal returns to a stable baseline in the presence of the buffer.

Because of the very specific interaction between the immobilized biologically active material and analyte, no other biomaterials are able to form complexes with the immobilized biologically active molecules and significantly influence the analytical signal of the SPR biosensor. A shift in the reflection angle of the reflected light is interpreted as the analytical signal. The signal is proportional to the concentration of analyte involved in affinity complex formation. Therefore, the extent of formation of the affinity complex between the analyte (in this experiment, anti-BSA) present in the solution phase and the immobilized ligand (BSA) is easily observed and quantified by monitoring the shift of the minimal reflection angle.

A basic antigen-antibody interaction model is exploited to describe the function of this analytical system,

$$\text{BSA} + \text{anti-BSA} \underset{K_{\text{dissociation}}}{\overset{K_{\text{association}}}{\rightleftharpoons}} \text{Complex}$$

REACTION 1

which is simplified to

$$[A] + [B] \rightleftarrows [AB]$$

REACTION 2

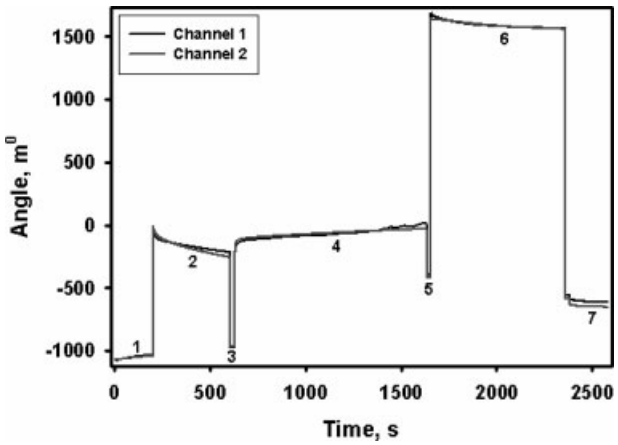

FIG. 6. **SPR signal versus time.** *Line 1*, baseline (10 mM PBS); *line 2*, activation of the MUA with a mixture of EDC/NHS; *line 3*, washing with 10 mM PBS; *line 4*, immobilization of BSA; *line 5*, washing with 10 mM PBS; *line 6*, deactivation of remaining reactive esters with ethanolamine; *line 7*, washing with 10 mM PBS.

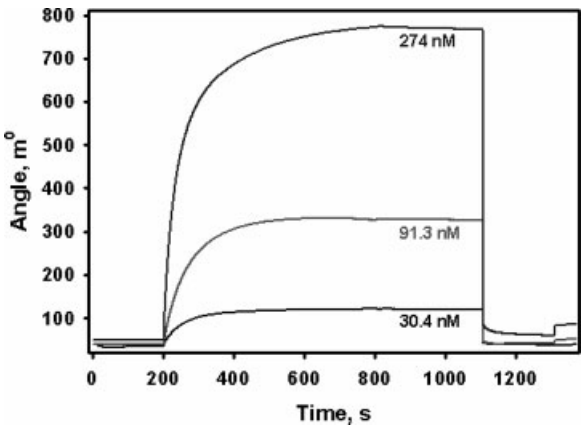

FIG. 7. **Real-time monitoring of immobilized BSA interaction with anti-BSA present in the sample at three different analyte (anti-BSA) concentrations: 30.4, 91.3, and 274 nM.**

where [A] is the concentration of BSA, [B] is the concentration of anti-BSA, and [AB] is the concentration of BSA and anti-BSA complex.

The complex formation rate can be described as

$$\frac{d[AB]}{dt} = K_a[A][B] - K_d[AB] \qquad \text{(Eq. 1)}$$

where K_a is the association rate constant and K_d is the dissociation rate constant.

The integrated rate equation for the determination of SPR signal during the association phase is

$$R_t = \frac{K_a \cdot [C] \cdot R_{max}}{K_a \cdot [C] + K_d}\left(1 - e^{-K_a[C]+K_d)t}\right) + R_0 \qquad \text{(Eq. 2)}$$

where t is time, R_0 is the signal at the start of the phase, [C] is the concentration of the analyte, rabbit anti-BSA, R_t is the measured signal at any time (t) during evaluation of the SPR signal, and R_{max} is the maximal response at saturating concentrations of analyte [18].

Equation 2 can be adapted to

$$R_t = E(1 - e^{-Kst}) + R_0 \qquad \text{(Eq. 3)}$$

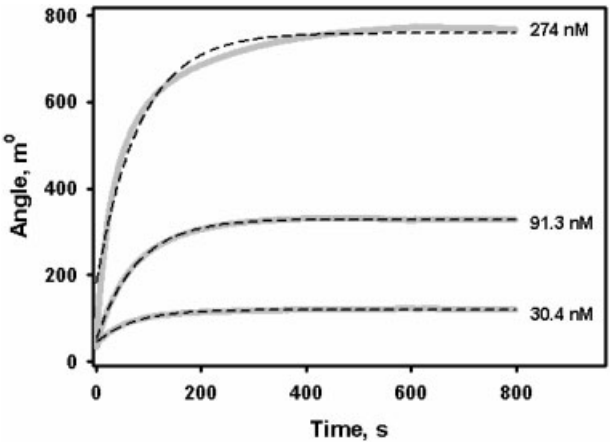

FIG. 8. **Data processing by Autolab ESPIRIT kinetic software and calculation of equilibrium angle.** *Dashed lines* represent real-time kinetic measurement data obtained during measurements of antigen-antibody interactions; *solid lines* are generated by Autolab ESPIRIT kinetic software.

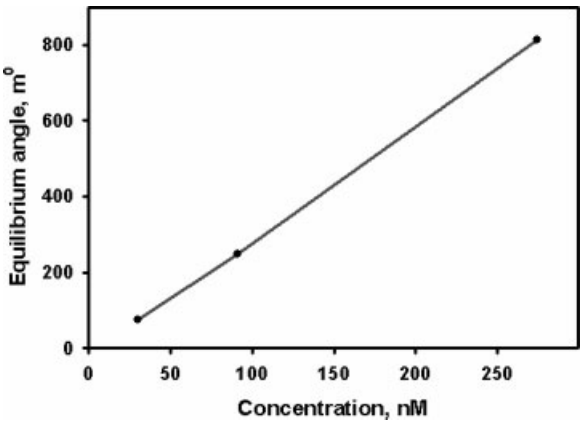

FIG. 9. **Concentration of anti-BSA *versus* equilibrium angle.**

where E is the SPR signal at equilibrium corresponding to the association of BSA and anti-BSA,

$$K_s = K_a[C] + K_d \quad \text{(Eq. 4)}$$

The association rate constant (K_a) is obtained from the slope of a plot of K_s *versus* the concentration of anti-BSA. Theoretically, the dissociation rate constant (K_d) can be obtained from the intercept of this plot; however, K_d values obtained in this way should be treated with care [19].

Activation of the MUA immobilized on the sensor surface with a mixture of EDC/NHS, immobilization of BSA, and deactivation of the remaining reactive esters with ethanolamine are shown in Fig. 6. The immobilization of BSA was performed in both channels of the SPR cuvette. After washing, no decay in the refraction angle representing minimal reflected light intensity was detected, thus indicating that the surface was sufficiently loaded by coupled BSA.

Sensorgrams representing anti-BSA interaction with immobilized BSA are shown in Fig. 7. From each sensorgram (Fig. 8), the SPR signal at steady-state conditions (equilibrium angle) is determined, and the resulting equilibrium angles are plotted *versus* anti-BSA concentration (Fig. 9). This can easily be done using Autolab ESPRIT kinetic software. SPR signals representing analyte binding were obtained at several concentrations of IgG1 (30.4–274 nM) to determine the association and dissociation rate constants, and thus, the overall affinity constant for the interaction.

For the interaction of protein BSA and anti-BSA, K_a was $9.36 \times 10^3\ \text{M}^{-1}\text{s}^{-1}$, and the intercept value, k_d, was $7.85 \times 10^{-3}\ \text{s}^{-1}$. The association rate constant K_a and the dissociation rate constant K_d are then obtained from the linearization of data corresponding to the steady-state phase of the association/dissociation. The value of K_d can also be obtained from the dissociation phase [12]. It has become apparent that there are more ways to evaluate the data as, for example, the mass transport limitations or conformational change after binding have not been taken into account in this simplified evaluation. All SPR data are stored in IBO format and can be used later by any data acquisition program (Excel, Sigma Plot, Origin, etc.) for data analysis and display.

Several questions about the accuracy, precision, and reproducibility of the SPR sensors may arise from the data obtained. Results obtained by the teacher and individual students in a laboratory can be compared to determine variations between individual sensors.

Presently, there are laboratory experiments that permit students to generate meaningful data for qualitative and quantitative analysis. Although this experiment is designed for a qualitative analysis course, it is also applicable to other courses where quantitative assessment of data is performed. The students not only analyze the data that they generate but also compare their data with the rest of the class. A compilation of all student results is given to other students for analysis. Additionally, identification of instrumental problems and development of extended experiments can be an important part of the laboratory work as well. The students will then be introduced to the SPR method and will gain experience in working with the SPR equipment.

Prior to the experiments, students are introduced to safety precautions and questioned about their SPR biosensor application backgrounds. After an introduction, they should scrutinize the whole amplified laboratory protocol. Then, each student should be given one sensor disk to perform all experiments described herein. The students should start the experiments according to the directions in the prepared handout, which can be modified based on traditional laboratory practices. The descriptive handout should be given to students prior to entering the laboratory. A short report form should be completed after the first week of the laboratory. Students are also required to hand in a formal laboratory report with all of their calculations and an explanation of their individual projects.

Conclusions and Pedagogical Implications—Students who perform this laboratory-based experiment will gain understanding of modern bioanalytical systems devoted to the determination of large molecules. In addition, the basic training on protein-protein interaction kinetics presented here will help students better understand some biochemical regulation processes.

SAFETY ISSUES

There are hazards associated with hydrochloric acid and sodium hydroxide. Both combine exothermically with wa-

ter. Solutions must be prepared in a fume hood. To avoid skin contact with this and other reagents, latex gloves must be used. The solutions should be disposed of as inorganic waste.

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