Anal. Chem. **2002,** *74,* 6106−6110

Application of an Absorption-Based Surface Plasmon Resonance Principle to the Development of SPR Ammonium Ion and Enzyme Sensors

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Two new types of surface plasmon resonance (SPR) sensors that can determine the concentration of ammonium cations and urea were realized based on the previously reported theory of the absorption-based SPR measurement method. The change of the dielectric constant caused by the change of the light absorption characteristics of dyes incorporated in a sensing membrane phase is utilized in these SPR sensors. The determination of ions using the SPR sensor was realized by detecting the SPR signals of the minimum reflectance related to the change of absorption spectra of the dye in the ion optode membrane consisting of an ammonium-selective ionophore (TD19C6) and a lipophilic cationic dye (KD-M11) that shows absorption spectral changes due to protonation and deprotonation. A SPR enzyme sensor that can determine the concentration of urea was prepared by the combination of this ion optode membrane and an enzyme membrane based on urease. With the newly developed SPR sensors, the intensity changes of the reflectance at the fixed SPR resonance angle are monitored, which is different from conventional SPR sensors where usually the change of the SPR resonance angles is detected. In a continuous-flow experiment using the SPR ion sensor for NH_4^+ ion determination, a dynamic measurement range from 10^{-5} to 10^{-2} M was achieved. In the case of the enzyme-based SPR urea sensor, a dynamic range from 10^{-4} to 10^{-1} M was observed in a stopped-flow batch arrangement. It is expected that this sensing technique can be applied for the SPR-based detection of a wide range of low molecular weight analytes.

A surface plasmon resonance (SPR) sensor is a sensing device that measures changes of the refractive index (RI) or the dielectric constant near the surface of a thin metal layer using the surface plasmon resonance phenomenon.[1,2] The sensor has attracted much attention as a biosensor, because it requires no labeling procedures and, therefore, is useful for affinity analysis.[3−6] Biomolecular processes such as antigen−antibody reactions[7−11] or DNA−DNA interactions[12−14] can be monitored using this kind of sensor system. For this purpose, the measurement is carried out by analyzing substantial changes of the refractive index on the gold surface of the sensor chip caused by specific adsorption of the analytes. A conventional SPR sensor measures the shift of the reflection minimum angle (θ_{SPR}); however, low molecular weight analytes do not change the refractive index sufficiently.[15,16] In this context, we focused on the imaginary part of the refractive index and proposed an absorption-based SPR sensor and its theoretical background.[17] To demonstrate some examples of a practical application of this SPR sensor theory, we here report on a SPR ammonium ion sensor and a SPR enzyme sensor for the detection of urea. Other combinations of enzymes and ionophores can be applied in the same method, enabling the detection of a variety of ionic species and low molecular weight analytes by

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10.1021/ac0258655 CCC: $22.00
Published on Web 10/23/2002

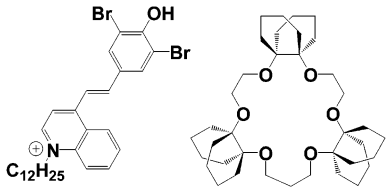

Figure 1. Chemical structures of the neutral ammonium ionophore TD19C6 and the lipophilic cationic dye KD-M11.

monitoring the imaginary part of the refractive index in contrast to conventional SPR techniques.

The sensing phase on the sensor chip is based on a plasticized poly(vinyl chloride) (PVC) membrane containing a neutral ammonium ionophore (TD19C6, Figure 1),[18] a lipophilic anionic additive (KTCPB), and a lipophilic cationic dye (KD-M11, Figure 1),[19] which shows maximum light absorption ($\lambda_{max} = 630$ nm) at the wavelength of the incident light coupling with the surface plasmon wave. When the target cations in an aqueous sample solution are extracted into the organic PVC membrane by complex formation with the neutral ionophore, deprotonation of the dye is induced in order to maintain the electrical neutrality of the membrane phase.[20−23] This cation exchange process results in an absorption spectral change of the sensing membrane. As shown by the Kramers−Kronig equation,[24,25] the light absorption characteristics of the sensing phase are related to the dielectric constant. The change of the dielectric constant of the membrane causes a change in the phase adjustment terms of the surface plasmon resonance and, as a consequence, results in a modification of the incident angle and the reflectance intensity.

On the basis of this principle, it is expected that the ammonium concentration can be determined by following the intensity changes of the reflectance instead of the changes of the resonance angle. Additionally, a SPR enzyme sensor is demonstrated in which an enzyme membrane containing urease is pasted on the ammonium-selective ion optode membrane. The substrate urea is hydrolyzed into ammonia and carbon dioxide by the enzymatic catalysis of urease.[26] The resulting ammonia is converted into ammonium cations in the pH-buffered aqueous sample system, followed by the extraction into the ion optode membrane. Thus, the concentration of urea can be determined by using an absorption-based SPR sensor, where the change of the dielectric constant is monitored. This study demonstrates that the newly proposed theoretical background for an absorption-based SPR sensor can be actually applied to an ion sensor and an enzyme sensor.

EXPERIMENTAL SECTION

Reagents. The highest grade commercially available reagents were used for the preparation of the test buffer solutions. The distilled and deionized water used had a resistivity of greater than 1.5×10^7 Ω cm at 25 °C. The ammonium-selective ionophore (TD19C6), potassium tetrakis(*p*-chlorophenyl)borate (KTCPB), and *o*-nitrophenyl octyl ether (NPOE) were purchased from Dojindo Laboratories (Kumamoto, Japan). Cellulose acetate and cellulose acetate hydrogen phthalate were purchased from Kanto Chemical Co. (Tokyo, Japan). Dodecylbenzenesulfonic acid was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Poly(vinyl chloride) (high molecular weight type) was obtained from Sigma Chemical Co. (St. Louis, MO). Carboxylated PVC (carboxyl content 1.8%) was purchased from Aldrich Chemical Co. (Milwaukee, WI), and urease was purchased from Funakoshi Co. (Tokyo, Japan). The lipophilic cationic dye (KD-M11) was prepared according to a previously reported procedure.[17] THF was dried and distilled over sodium benzophenone ketyl prior to use.

SPR Apparatus. A SPR sensor (SPR-20, DKK Co., Ltd., Tokyo, Japan) equipped with a LED as a light source (630 nm) and a CCD camera (XC-77, Sony Co., Tokyo, Japan) was used in this experiment. The prepared sensor chips (vide infra) were placed on the high-RI glass prism ($n = 1.79$) with a hemisphere shape. The backside of the sensor chip was adhered to the prism using a matching oil ($n = 1.79$, Cargille Laboratories Inc.), while the membrane on the front side of the sensor chip faced toward a flow cell via a spacer made from silicone rubber. SPR signals were recorded and analyzed on a computer through an imaging board connected to the CCD camera. The reflectivity was calculated by dividing the intensity of the p-polarized light by that of the s-polarized light in order to reduce the effect of intensity fluctuations arising from the light source.

Preparation of Sensor Chips and Ion Optode Membranes. The sensor chips were prepared by evaporating a 5-nm layer of chromium followed by a 45-nm layer of gold on a glass plate (SFL-6, $n = 1.79$). A total of 40 mg of PVC, 80 mg of NPOE, 2.48 mg (4.1 μmol) of TD19C6, 2.33 mg (4.1 μmol) of KD-M11, and 2.01 mg (4.1 μmol) of KTCPB were dissolved in THF, and the solvent was slowly evaporated until the mixture reached a total weight of 0.77 g. A 0.2-mL aliquot of this cocktail solution were dropped on the gold film of the sensor chip, and it was rotated at 4000 rpm for 5 min using a spin-coater (1H−D3, Mikasa Co. Ltd.) to give an ion optode membrane of ∼1-μm thickness. After being dried for 1 h under vaccum, the chip was stored in a refrigerator. The glass-supported ion optode membrane on the gold film was immersed into 0.1 M aqueous HCl for complete protonation of the dye and then conditioned in 0.05 M HEPES buffer solution adjusted to pH 7.0 for 5 h. In parallel, the same cocktail solution was spin-coated on an OHP film for the measurement of conventional absorption spectra.

Preparation of Enzyme Membranes. A total of 300 mg of cellulose acetate and 300 mg of cellulose acetate hydrogen phthalate were dissolved in 5 mL of acetone. The solution was poured onto the surface of 1 L of water containg 8 g of dodecylbenzenesulfonic acid. The obtained membrane was immersed into 0.2 M phosphate buffer solution adjusted to pH 5.0

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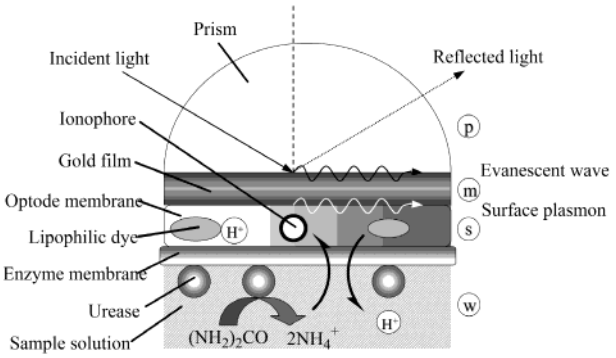

Figure 2. Schematic representation of the sensing system for the SPR enzyme sensor. In this Kretschmann configuration, the glass prism, the metallic film, the sensing layer, and the sample solution phase are labeled p, m, s, and w, respectively.

for 1 h and then washed with water. The enzyme solution (200 mg/mL) prepared in 0.1 M phosphate buffer adjusted to pH 7.0 was dropped on the cellulose membrane and was kept at 0 °C for 3 days in a refrigerator. The enzyme membrane was washed thoroughly with the same buffer solution and adhered to an ion optode membrane (vide supra), prepared from carboxylated PVC instead of PVC because the former is more effective for adhesion to hydrophilic cellulose membrane.

Measurement Conditions. HEPES buffer, 0.05 M, adjusted to pH 7.0 with $Ca(OH)_2$ was used as the running buffer, and the sample solutions were introduced into the flow cell at a rate of 0.2 mL/min using a HPLC pump. The intensity of the reflectance of the p- and s-polarized light was measured after the SPR signals reached an equilibrium after 8-min contact with the sample solutions, and then the sensing membrane was regenerated by washing for 2 min with running buffer before the injection of the next sample. Since the response time is dependent on the sample concentration, an equilibration time of 8 min was used in all cases, which is sufficient to guarantee a stable optical signal also at low concentrations. It is assumed that the time-limiting step for the sensor response is the diffusion of the produced ammonia through the enzyme layer.[27]

On the other hand, a batch system experiment was also evaluated. In this case, the injected sample solution was kept on the sensing membrane for 8 min without flow, the intensity was recorded, and then the cell was washed with running buffer for 2 min.

The conventional absorption spectra were recorded on a Hitachi U-2000 spectrometer. The ion optode membrane supported by an OHP film was cut and placed in a quartz cell and filled with the sample solution, and its spectra were measured.

THEORY

A schematic representation of the prepared SPR ion/enzyme sensor is shown in Figure 2. A simulation of the SPR response was performed in order to estimate the theoretical SPR signals for the case of the ion optode membrane containing dyes. The Fresnel equation was applied to a three-layer model in which the sensing system consists of a prism, a metal film, and the PVC sensing layer. The reflectance R of the light is given by the Fresnel equation for p-polarized light as follows,

$$R = \left|\frac{\gamma_{pm} + \gamma_{ms}\exp(i2k_{mz}d)}{1 + \gamma_{pm}\gamma_{ms}\exp(i2k_{mz}d)}\right|^2 \quad (1)$$

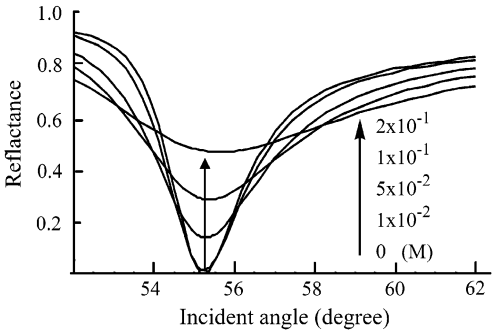

Figure 3. SPR curves simulated for the ion optode membrane containing increasing concentrations of deprotonated KD-M11. The wavelength of the incident light used for the calculation is 630 nm.

Based on eq 1, the SPR curves as shown in Figure 3 are expected for the absorption-based SPR ion sensor using the dye KD-M11 with an absorption maximum at 630 nm and a wavelength of the incident light of 630 nm. SPR curves were calculated for increasing concentrations of the deprotonated dye. According to the working principle of cation exchange optodes described above, this corresponds to an increase in the ammonia or urea concentration, respectively. The detailed calculation parameters are given as Supporting Information.

The degree of changes of the absorption and the refractive index are generally dependent on the wavelength and are related to each other as mathematically described by the Kramers−Kronig equation:

$$\Delta n(E) = \frac{ch}{2\pi^2}\int_0^\infty \frac{\Delta\alpha(E_0)}{(E_0)^2 - E^2}\,dE_0 \quad (2)$$

where $\Delta n(E)$ is the change of the refractive index and $\Delta\alpha(E)$ is the change of the absorption coefficient. Since $E = hc/\lambda$ and $\alpha(E) = A(E)\ \ln 10/l$ in which $A(E)$ is the absorbance and l is the light path length, eq 2 can be transformed to

$$\Delta n(\lambda) = \frac{\ln 10}{2\pi^2 l}\int_0^\infty \frac{\Delta A(\lambda_0)}{(1/\lambda_0)^2 - (1/\lambda)^2}\frac{d\lambda_0}{\lambda^2} \quad (3)$$

as a function of the wavelength. According to eq 3, the inflection point of the wavelength-dependent refractive index curve is coincident with the absorption maximum as shown in Figure 4. When the wavelength of the incident light is coincident with the absorption maximum of the dye, no angle shift of the SPR signal is induced because no change of the real part of the refractive index due to light absorption does occur. The signal broadening is only due to the changes of the dielectric constant arising from the effect of light absorption.

RESULTS AND DISCUSSION

In this work, our developed TD19C6 having three decalino groups in a 19-membered crown ether was selected as the

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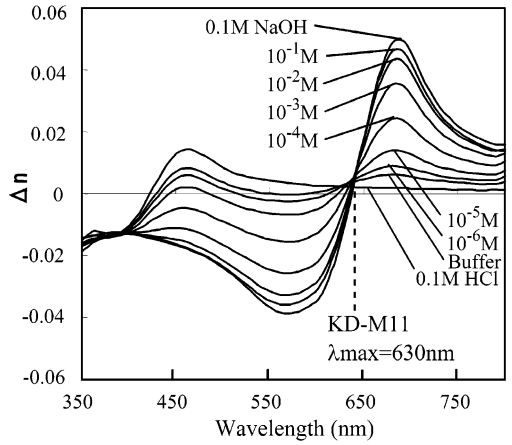

Figure 4. Relationship between the wavelength and change of refractive index of the ion optode membrane for varying NH_4^+ concentrations calculated by the Kramers−Kronig equation using experimental data shown in Figure 5.

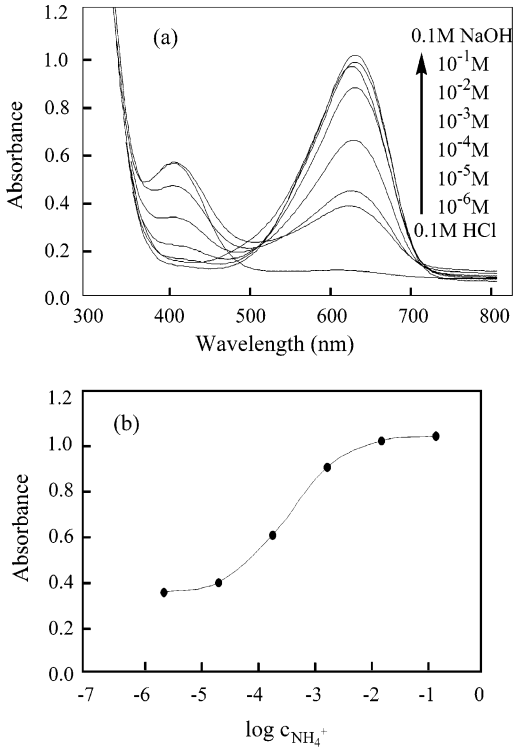

Figure 5. (a) Absorption spectra of the ion optode membrane based on TD19C6 and KD-M11 at various concentrations of NH_4^+ in HEPES-buffered (0.05 M) solution at pH 7.0 and (b) the response curve of the membrane at 630 nm.

ammonium ionophore. When this ionophore was applied to an ion-selective electrode (ISE), excellent selectivity for NH_4^+ was realized (log K, $NH_4^+/K^+ = -1.0$ and $NH_4^+/Na^+ = -3.5$). The lipophilic cationic dye KD-M11 has an absorption maximum at 420 nm in the protonated form and at 630 nm in the deprotonated form.[17]

Figure 5 shows the absorption spectra of the ion optode membrane based on TD19C6 and KD-M11 at various concentrations of NH_4^+ as obtained with the sensing film cast onto a glass plate. As can be seen in Figure 5b, the optode membrane responds to variations in the NH_4^+ concentration in the range from 10^{-5} to 10^{-2} M. For the determination of NH_4^+ using the SPR ion sensor,

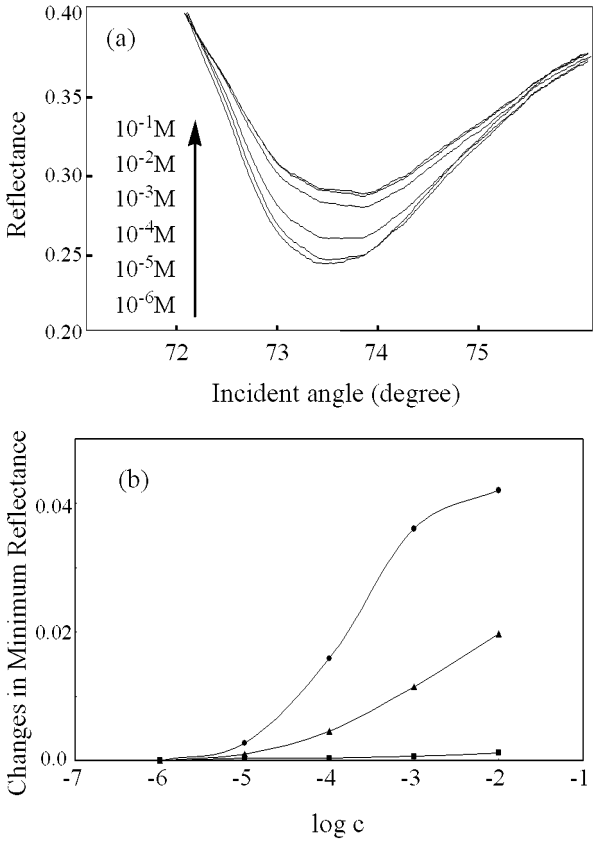

Figure 6. (a) SPR signals of the ion optode membrane based on TD19C6 and KD-M11 at various concentrations of NH_4^+ in HEPES-buffered (0.05 M) solutions at pH 7.0 and (b) the response curve of the membrane at 73.3° for (●) NH_4^+, (▲) K^+, and (■) Na^+.

a LED emitting at a wavelength of 630 nm was used as a light source. The NH_4^+ concentration could be measured from the concentration of 10^{-5} M as shown in Figure 6a. By monitoring the reflectance at the fixed incident angle of the lowest intensity (73.3°), a response curve for NH_4^+ was obtained. As shown in Figure 6b, the response curve obtained from the SPR ion sensor is similar in shape to the one that was observed with the absorption-based ion optode membrane in Figure 5b. Furthermore, the SPR ion sensor showed high NH_4^+ selectivity over K^+ and Na^+, comparable to the selectivity pattern as described for ISEs. The short-term repeatability of the sensor was examined. When the same sensing membrane was alternately exposed to an ammonia sample and running buffer for several times, a standard deviation of 5.9% ($n = 5$) was found for consecutive measurements of 10^{-3} M NH_4^+ samples.

As an example for further extending the range of application, the absorption-based SPR ion sensor for the determination of NH_4^+ was applied to the development of a SPR enzyme sensor that was designed to be able to measure the concentration of urea in aqueous solutions. Urease is known to catalyze the hydrolysis of urea according to the following reaction scheme.

$$(NH_2)_2CO + 2H_2O + H^+ \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^-$$

A cellulose membrane containing urease was adhered to the ammonium ion optode membrane, and the SPR signals were

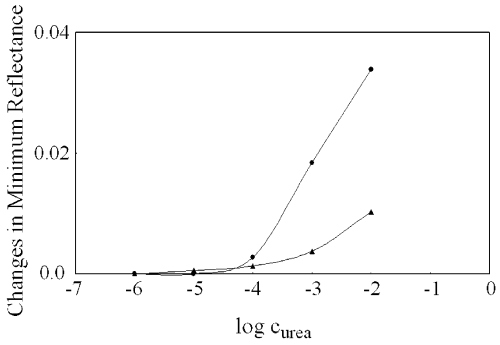

Figure 7. Response curves of the SPR enzyme sensor for urea solutions in (●) flow and (▲) batch systems (73.3°).

measured in both flow and batch systems. The response signals obtained in the flow system were similar to those observed with the ion optode membrane alone (Figure 6a). A signal broadening and an increase of the reflectance were observed as the concentration of urea increases.

As shown in Figure 7, the sensitivity at low sample concentrations and the detection limit in the batch process were better than that observed for the flow system. In contrast to the S-shaped response curve found for the batch system, the curve obtained with the flow system shows only a gentle slope, which probably corresponds to the beginning of a S-shaped curve. These differences were attributed to the fact that the NH_4^+ ions resulting from the urea hydrolysis are washed away from the sensing membrane in the flow system. Therefore, the experimentally observed better performance in the batch system is explained by the longer residing time of NH_4^+ in the proximity of the sensing membrane leading to a more efficient extraction into the ion sensing membrane. The standard deviation observed for consecutive sample measurements and regeneration with buffer using the same sensing membrane in a batch system was 11.7% ($n = 5$) at an urea concentration of 10^{-3} M. Freshly prepared PVC membranes were stable over a period of one month or longer when kept at −20 ×b0°C in the dark, and the enzyme membrane is stable for at least one week at a temperature of 4 °C.

CONCLUSIONS

A light absorption-based SPR sensor was shown to be useful for the indirect determination of ions or low molecular weight biological components using dye-incorporated membranes by following the changes of the imaginary part of the refractive index (the change of the reflectance minimum). As demonstrated in this work, a SPR ammonium ion sensor showed NH_4^+ concentration-dependent changes of SPR signals and gave a response curve, sensitivity, and a dynamic measuring range similar to that of an ion optode membrane based on absorption spectral changes. Especially in the case where the wavelength of the incident light is matched with the absorption maximum of the dye, the response curves are easily obtained and are useful for quantitative analysis because only a change of the reflectance occurs without any change of the refractive index. This type of sensing contributes to simple SPR instrumentation. Furthermore, the determination of a biological component was shown to be possible by combining an enzyme reaction with this SPR ion sensing system. Although the detection of low molecular weight compounds using SPR sensors has been regarded to be difficult so far, we have succeeded in the development of a SPR ion sensor by applying the absorption-based surface plasmon resonance theory. It is expected that the methods demonstrated in this study can be applied to the determination of a large variety of chemical species. While SPR will not replace ion-selective electrodes or optodes as the first method of choice for the detection of ionic species and low molecular analytes, the extension of available analytical parameters is an important contribution to the development of multianalyte detection systems. Ongoing developments in SPR techniques leading to multichannel and two-dimensional SPR systems will allow the simultaneous real-time and label-free quantification of a wide variety of analytes ranging from high molecular weight biomolecules to low molecular weight ions, including species not detectable by conventional SPR instruments.

ACKNOWLEDGMENT

Partial support of this investigation by the Kanagawa Academy of Science and Technology and the Ministry of Science and Technology is acknowledged. This study was also partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture. D.C. gratefully acknowledges a research fellowship granted by the Science and Technology Agency of Japan (STA).

SUPPORTING INFORMATION AVAILABLE

More detailed description of the theoretical simulations and the used parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review June 18, 2002. Accepted September 23, 2002.

AC0258655