Development of high-sensitivity molecular adsorption detection sensors

— Biomolecular detection for highly-developed diagnosis, medication, and medical treatments —

Makoto Fujimaki * **and Koichi Awazu**

High sensitivity sensors for detection of molecules are aspired in various fields of application, particularly in medical technology, pharmacy and environmental sciences. In the present research, we have developed a high-performance molecular detection sensor for diagnosis, medication, and medical treatments that utilizes waveguide modes as basic technology. We successfully obtained sensors with high sensitivity and stability by applying nano-perforation technology, optical simulation and experiments, and an originally developed sensing-plate fabrication process. In this report, the scenario of the present research, elemental technologies and their synthesis for achieving the scenario and the properties of the realized sensors are described. In addition, a rapid progress in sensing performance obtained by a breakthrough is reported.

1 Introduction

One of the important functions expected of medical sensors is the early detection of diseases. With the recent advances in medicine, many substances associated with various diseases have been identified, and the presence and progress of the disease can be determined by detecting these substances. The possibility of curing a disease increases dramatically by early discovery. To find a disease in its early stage, it is necessary to detect these substances even at low concentration. This means that there is a demand for higher sensitivity of the sensor.

Other functions of the sensor include the identification of the cause of the disease and observation of the patient's progress. For example, in case of a cold or food poisoning, it is necessary to immediately identify the virus or bacterium that is causing the symptoms. When the patient is admitted to a hospital and administered some drug, it is important to conduct an on-spot observation to see whether the drug is taking effect. In such situations, the sensors are expected to quickly detect the target substance. In addition to sensitivity, the sensor must also be compact so it can be portable, and be capable of performing without being influenced by the environment in which the measurements are made.

If a high-performance sensor allows early discovery of diseases, early identification, and accurate progress observation with minimal stress, the patient can be expected to recover early. This will reduce physical strain on the patient, as well as reduce the time and cost of medical care.

Of course, there are many methods for detecting minute substances, but considering use in a medical care situation, it must be of low cost and the device must not require extensive training to operate. For certain diseases and conditions, sensors that fulfill such requirements have been designed and put to actual use. They include the influenza diagnosis using the immunochromatography method (Tamitest Influenza AB of Roche Diagnostics K.K., QuickVue Rabid SP Influ of DS Pharma Biomedical Co., Ltd.) and pregnancy test using gold colloid chormato-immunosorbent assay (Dotest of Rhoto Pharmaceutical Co., Ltd., Check One Fast of Arax Co., Ltd.).

However, in many cases, effective sensors that can surmount the issues of very low concentration of target substance produced by the disease or difficulty in separation from other substances have not been developed. Much is expected in future developments. With these demands and with objectives of establishing a biosensing technology that enables quick discovery of a disease at its early stage, even at small hospitals, we developed a biological substance sensor that is highly sensitive to certain minute substances, compact so it is portable, and that enables stable measurements not dependent on the measurement environment. Also, one of the major issues was the mixing of various substances that may interfere with the target substances when using human samples such as blood, urine, or saliva. Therefore, we developed a method to prevent the interference caused by these foreign substances.

2 Development of molecular adsorption detection sensor

Center for Applied Near-Field Optics Research, AIST   Tsukuba Central 4, Higashi 1-1-1, Tsukuba, 305-8562, Japan   * E-mail : m-fujimaki@aist.go.jp

Figure 1 shows the issues that must be solved in sensor development, performance that can be expected by solving the issues, and merit that can be gained through the performance gained. The sensor must detect the presence of a certain substance (sample) at high sensitivity, stably, and with low noise. For the development of this sensor, we applied the technology of detecting the change in dielectric environment resulting from specific adsorption of an analyte through the change in waveguide mode[Term 1] [1][2]. The characteristics of this technology and the scenario for achieving higher performance through this development are explained below.

2.1 Principles of the EFC-WM sensor and expected performance

The surface plasmon[Term 2] resonance (SPR) sensor is known as a biosensor that is available on the market mainly for R&D and has extremely similar mechanism to the evanescent-field[Term 3] -coupled waveguide-mode (EFC-WM) sensor that we developed[1]. To help understand the mechanism of the EFC-WM sensor, we shall briefly explain the measurement principle of the SPR sensor that is already available for practical application.

In general, in the SPR sensor, a metal film is deposited onto the glass substrate surface with high refractive index, and a prism made from the same glass is placed on the opposite side of the metal film surface. Usually, Au and Ag are used for the metal film since surface plasmon resonance can be excited in them with visible light. Figure 2(a) shows the optical system frequently used in the SPR sensor. This optical configuration is called the Kretschmann configuration[3]. In the Kretschmann configuration, when the light is incoming from the prism under total reflection condition, the surface plasmon is excited on the metal film surface at a specific incidence angle. This angle of incidence is called the 'resonance angle'. When the light is incoming at the resonance angle, the incident light combines with the surface plasmon and the intensity of reflected light decreases significantly. Since the surface plasmon is sensitive to the change of permittivity at the metal surface, this resonance angle changes when biomolecules are adsorbed on the surface and the intensity of reflected light changes greatly. The adsorption of biomolecules can be detected by detecting the change of the intensity of the reflected light. To detect only specific molecules, the metal surface is modified with a substance that specifically adsorbs the target molecule.

The sensing plate used in the EFC-WM sensor has a reflective film and a transparent dielectric waveguide on the substrate glass[4][5]. Same optical system as the SPR sensor can be used for measurement. Figure 2(b) shows the optical configuration of the EFC-WM sensor using the Kretschmann configuration. When light is incoming via the prism as in the SPR sensor, coupling occurs with waveguide mode that propagates the waveguide and the incident light through the evanescent field generated at the reflected film at a certain angle of incidence (resonance angle). When light is incoming around this specific angle, the intensity of reflected light decreases significantly. Since the waveguide mode is sensitive to the surface condition as in the SPR mode, when biomolecules adsorb onto the waveguide surface, the aforementioned resonance angle changes and the intensity of reflected light changes. The EFC-WM sensor uses this intensity change of reflected light to detect the molecule adsorption.

SPR sensor is a label-free detection method where the sample is not tagged with a labeling substance, and its greatest characteristic is having high sensitivity that enables sample detection without labeling. Therefore, the complex procedure of labeling is unnecessary, and detection can be carried out easily. Also, since the sample is not labeled, the properties and features of the original sample are not lost. Therefore, the behavior of the target molecule, for example, in what condition the specific molecules are adsorbed can be observed accurately. However, in terms of sensitivity, it is said to be double to triple-digit inferior to the highly sensitive detection method using labeling substance, such as the enzyme-linked immunosorbent assay (ELISA)[6] (however, direct comparison is difficult since sensitively is greatly influenced by various factors such as the type and size of the target molecule, capture method of the target molecule, measurement environment such as whether it is in blood or in buffer solution, or presence of foreign substance). The EFC-WM sensor is a label-free detection method as in the SPR sensor. Until now, the EFC-WM sensor fell behind the SPR sensor as a molecule adsorption sensor even with

Issues	Expected performance	Merits gained	Application
High sensitivity	Detection of small molecules Detection of small quantity or low concentration substance	Identification of various diseases, pathogens, bacteria Identification of disease in its early stage	Medicine
Stability	Improved physical stability Improved chemical stability Stabilization of sensitivity against temperature	Prevention of misdiagnosis by damage Does not break when handling Increased lifespan of sensor Stable manufacturing; reduced manufacturing error Stable against sample (acid, alkali, corrosiveness, reactivity) Air conditioning not necessary; can be used outdoors	Drug discovery Check-up Health Environmental measurement
Noise reduction	Reduction of effects by foreign substance and nonspecific adsorption	Accurate diagnosis; highly sensitive diagnosis	Antiterrorism

Fig. 1 Issues, expected performances, merits gained, and fields of application of the sensor.
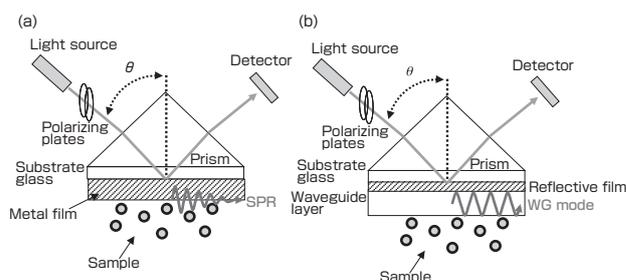

Fig. 2 (a) Optical configuration used in the SPR sensor. (b) Optical configuration used in the EFC-WM sensor.

its advantages of having no limitations to the reflective substance and of being able to use both s and p waves. The greatest reason is because the absolute value of change of resonance angle at the time of molecule adsorption is small compared to the SPR sensor. However, the EFC-WM sensor has a sharp band of resonance angle, and therefore can sense large changes in reflectance property at a small angle change. Also, compared to the SPR sensor, the preparation of the EFC-WM sensor is complex since the waveguide layer must be created. However, we thought the key to increasing sensitivity was devising this waveguide layer. If the sensitivity of the EFC-WM sensor can be raised double to triple digits by using these features, it will have sufficient sensitivity as a molecule adsorption sensor.

Other than sensitivity, stability in the environment in which the sensor is used, particularly in room temperature, is required. Highly sensitive detection method is affected readily by the environment because of its high sensitivity. In general, biomolecules are dissolved in water in some form such as in blood, urine, or buffer solution. Since the permittivity of water changes according to temperature, the sensor that detects the change in permittivity as in the SPR or EFC-WM sensor is extremely unstable against temperature. In developing a highly sensitive sensor, solving the problem of temperature stability is a major issue.

When developing the EFC-WM sensor as an effective sensing method usable in the medical field, there are various requirements in its performance. However, unlike the SPR sensor which can be used only with material that produces SPR, the EFC-WM sensor has higher degree of freedom where any material, as long as it reflects light, can be used as the reflective film, and any material can be used as the waveguide layer, as long as it is a transparent film. There are several areas that can be devised to increase sensitivity and performance. Therefore, we drew the following scenario to increase the sensor performance.

2.2 Scenario to increase the performance of the EFC-WM sensor

First we looked at the structure of the waveguide. Figure 3 shows the simulation result of the electric field profile in the waveguide when a waveguide mode is excited therein. Here the incident light was s-polarized light with wavelength of 632.8 nm, the refractive index of substrate glass was 1.769, the reflective film was Au with thickness of 40 nm, and the waveguide layer was silica glass with thickness of 500 nm. The wavelength surface was immersed in water. The incident light was incoming from the left side of the figure. As shown in the figure, the electric field was strong inside the waveguide, while it became weak at the surface. If the molecules could be guided to the area with a strong electric field, they could be detected sensitively. Therefore, we considered forming pores in the waveguide layer and guiding

the sample into the waveguide[7][8]. Pore formation will increase the surface area of the waveguide and will increase the number of adsorbed samples. This is also expected to contribute to higher sensitivity. The size of the pore should be sufficiently smaller than the wavelength of the incident light to prevent scattering. Therefore, when visible light is used, the diameter of the pore should be several tens to 100 nm. Since greater increase in surface area can be obtained by deepening the pores, the pores should be deep so they penetrate the waveguide layer. As a method for forming nanopores with such small diameters and high aspect ratio, we used selective etching by hydrofluoric acid (HF) vapor of latent tracks formed by irradiation with swift heavy ions[9]. Using this technology, it was possible to form nanopores with diameters of several 10 nm and aspect ratio of 40 or more[8].

Next we reviewed the materials for the sensing plate, particularly the reflective film[5]. In the conventional EFC-WM sensor, there are many reports of using noble metals such as Au and Ag for the reflective film[10][11]. High sensitivity has been obtained by using such materials. However, these metals have extremely poor adhesion with the glass substrate and plastic substrate used in the EFC-WM sensor or the dielectric layer used as waveguide layer, and have problems of easily peeling away. Therefore, it is necessary to introduce an adhesive layer to maintain high reliability for practical use, but addition of an adhesive layer produces problems of reduced sensor sensitivity, increased cost, and increased manufacturing error. Using optical simulation, we investigated what kind of optical properties a material should have for the EFC-WM sensor, and conducted a comparison of sensor performance by fabricating various sensing plates.

Based on the results obtained by the above-mentioned development, we found the silica glass created by thermal oxidation of Si was appropriate as waveguide for nanopore formation, and Si was appropriate as a reflective film. Based
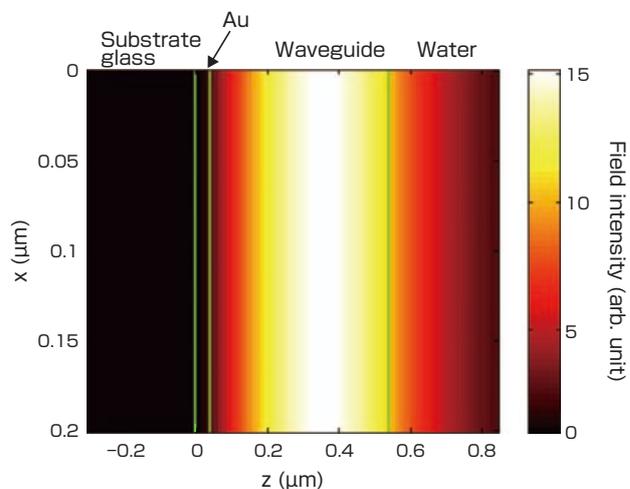

Fig. 3 Simulation result of the electric field profile in a waveguide in which a waveguide mode is excited.

on these results, we used a substrate called silicon-on-quarts (SOQ) which comprises of single-crystalline Si layer on silica glass substrate[12] to fabricate the sensing plate, and devised a method for fabricating the waveguide by oxidizing the single-crystalline Si layer[13]. We called the plate fabricated with this method monolithic sensing plate. In addition, we were able to achieve dramatic high sensitivity using the property in which the monolithic sensing plate sensitively detects the optical absorption of an adsorbent substance. This will be explained in chapter 4. Figure 4 shows the series of R&D.

3 Results of the development

The results obtained in this research are presented as follows.

3.1 Achievement of high sensitivity through nanopore formation technology

As mentioned above, we used the selective etching by HF vapor of latent tracks formed by irradiation with swift heavy ions for formation of nanopores in the waveguide. For ion irradiation, the 12 UD Pelletron tandem accelerator at the University of Tsukuba was used. The ion irradiation method is shown in Fig. 5. The Au ions accelerated at 150 MeV were irradiated onto a Al foil with thickness of 0.8 μm. The ions were scattered by the foil, and a uniform ion beam with low current density was formed. The current density of the ion beam was set to be 100 pA/cm^2 on the sensing plate. The reason for using low current density was to accurately control ion fluence, since the ion fluence was extremely low at the order of 10^9 to 10^{10} per 1 cm^2. For the vapor etching, a 20 % HF solution was used. An irradiated sensing plate was placed in a container of HF solution so it would not become immersed in HF solution, yet the sample would be exposed to HF vapor.

Figure 6 shows the SEM photographs of the surface and cross section of a thermally grown SiO$_2$ film with thickness of 2.0 μm which was irradiated with Au ion and etched by
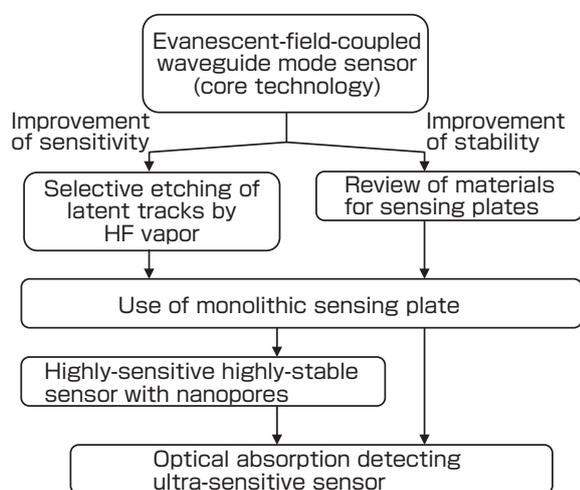
HF vapor for 60 min. The temperature of HF solution during etching was 21.5 ºC. The figure shows that the formed pores penetrate the SiO$_2$ film. The thickness of the SiO$_2$ film after etching was 1.9 μm. That is, the aspect ratio of the pore was 42. Using this method, nanopores with diameters in several 10 nm order could be formed accurately.

Sensitivity improvement was attempted using this method, by forming nanopores in the waveguide layer of the sensing plate with silica glass waveguide. A glass (OHARA, S-LAH66, refractive index of 1.76924 at 632.8 nm) was used as a substrate. The glass was cut and polished into a 20 mm × 20 mm piece with thickness of 1 mm. Au was used as the reflective film. Cr layers were used as adhesive layers between the Au film and the glass substrate and between the Au film and the waveguide layer. These films were formed by the vacuum deposition method. The thickness of the Au film was 53 nm, and that of the Cr layer was 0.8 nm. The waveguide layer was formed by RF magnetron sputtering using a silica glass plate as a sputtering target. The thickness of the waveguide was 550 nm. After the sputtering, a thermal annealing at 600 °C for 24 hrs was applied to the substrate in order to densify the formed waveguide layer. The nanopores were formed on the waveguide surface using the aforementioned method. The Au ion fluence was 5.0 × 10^9 cm^{-2}, and HF vapor etching was done for 30 min. The temperature of HF solution was 19.0 ºC. Figure 7 shows the SEM photographs of the surface and cross section of the plate after etching. As shown in the figure, nanopores of diameters of about 30 nm can be observed. It can also be seen that the pores penetrated to the Au layer. The thickness of the waveguide layer after the etching was 400 nm.

A right triangle prism made of S-LAH66 was optically attached to the fabricated plate using matching oil, and the measurement of incident angle dependence of reflectance was conducted using the Kretschmann configuration. The light source was s-polarized He-Ne laser (632.8 nm). A cuvette was placed on the waveguide to support liquid samples. The detection sensitivity was assessed by modifying the waveguide surface with biotynil group and by observing the change in reflectance by specific adsorption of streptavidin[Term 4] on biotin[Term 5]. Streptavidin was dissolved
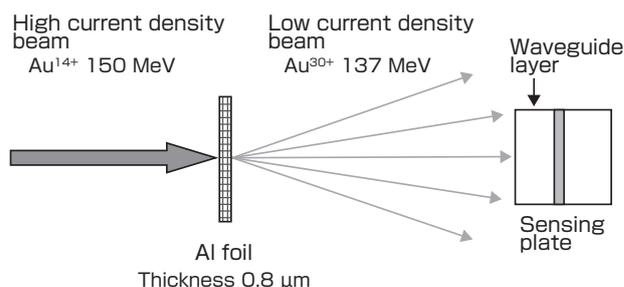
Fig. 4 Synthesis of R&D.

Fig. 5 Ion irradiation method used in nanopore formation.

in PBS buffer, and the concentration was set to 100 nM. Figure 8 shows the measurement results. Figures 8(a) and 8(b) show the results of measurement using the sensing plates with nanopores and without nanopores, respectively. White dots show the reflectance property measured with filling the cuvette with PBS buffer without streptavidin, and black dots show the reflectance property after the adsorption of streptavidin on biotin when the cuvette was filled with PBS buffer containing streptavidin. In both cases, the dip caused by excitation of waveguide mode was observed, and it could be seen that the peak position shifted by adsorption of streptavidin. The amount of shift of the peak by the adsorption of streptavidin in the substrate with nanopores was 0.38°. On the other hand, the peak shift was 0.06° in the substrate without pores. It was shown that dramatic improvement in sensitivity could be obtained by forming pores. However, as shown in Fig. 8(a), the width of the peak widened and the depth decreased by the formation of nanopores. This is thought to be due to the roughening of the
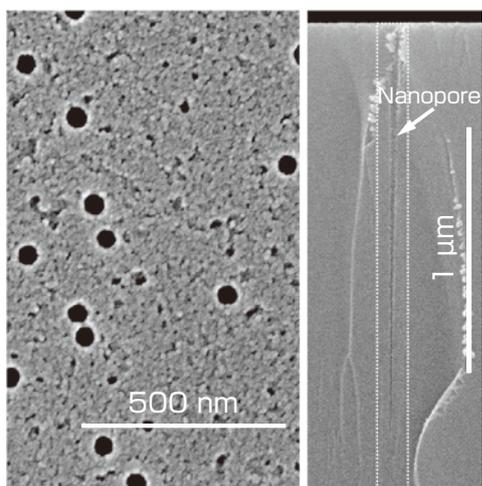
waveguide surface by the etching. In fact, as shown in Fig. 7, particle-like roughness could be observed on the waveguide surface after the etching. This problem was greatly improved by the development of monolithic sensing plate as explained in section 3.3.

3.2 Material of the reflective film

The sensitivity of EFC-WM sensor is greatly dependent on the optical property of the reflective film. Therefore, we conducted simulations to project sensor sensitivity for various reflective film materials, and also actually fabricated several types of sensors for demonstration.

Figures 9(a), 9(b), and 9(c) show the results of the calculation of reflectance property when Au, W, and Si are used as reflective films, respectively. The refractive index of the substrate used for calculation was 1.769; the thickness of the reflective films were 40, 20, and 30 nm, respectively; and the refractive index and thickness of the waveguide layer were 1.485 and 500 nm. The incident light was s-polarized monochromatic light with wavelength of 632.8 nm. The waveguide surface was assumed to be immersed in water. Although the shape of the reflectance property differs by reflective film materials, peaks are observed in the reflectance properties in all cases. The shape of the reflectance property, that is, whether the waveform peaks upwards or downwards, is determined by the intensity of the background reflected light and the condition of resonance. The positions of these peaks shift due to substance adsorption to the waveguide surface.

To learn what kind of optical property a material should have

Fig. 6 SEM photographs of the surface (left) and cross section (right) of nanopores formed on a thermally-grown SiO$_2$ film.
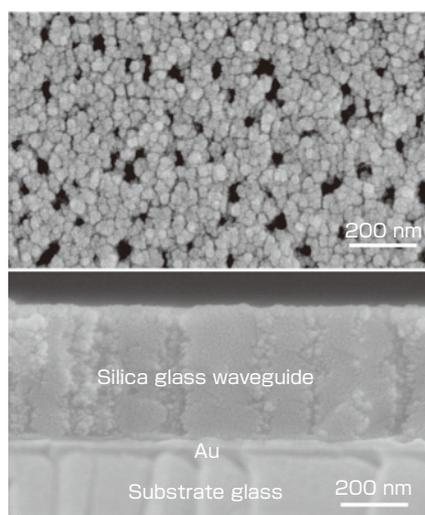

Fig. 7 SEM photographs of the surface (top) and cross section (bottom) of the sensing plate with nanopores.
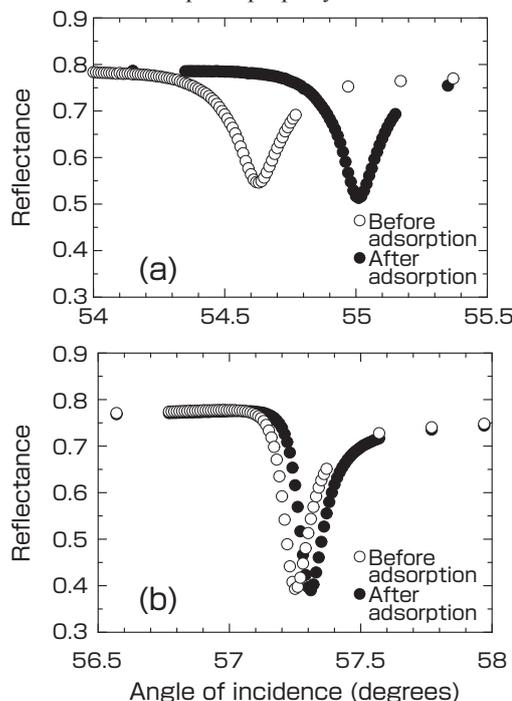

Fig. 8 Reflectance property before and after adsorption of streptavidin on biotin observed using the sensing plate with nanopores (a) and without nanopores (b).

as a reflective film, we calculated the relationship between the complex refractive index $n + k$i of the reflective film material and the detection sensitivity. The calculation results are shown in Fig. 10. The figure shows the relationships between the maximum change in reflectance obtained when substance with thickness of 5 nm and refractive index of 1.45 was adsorbed to the waveguide surface, and the n and k of the reflective film. Here, the wavelength of incident light was 632.8 nm, and the refractive index of the substrate glass and prism was 1.769. Also, the thickness and refractive index of the waveguide was 350 nm and 1.485 respectively. The optimal values for incident angle of light, direction of polarization, and thickness of the reflective film were calculated by simulation. The material with a complex refractive index with a large change in reflectance can be the considered material suitable as the reflective film material. In the figure, the complex refractive indices of some materials are shown as black dots. From the calculation result, Au, Ag, and Cu, which are materials that have been conventionally used, showed high sensitivity. It could also be seen that Si and Ge, which are materials with large n and small k, also showed good sensitivity.

Sensing plates were fabricated using some of the materials listed in Fig. 10, and observation was done using biotin-streptavidin adsorption as in the previous experiment. Streptavidin had a diameter of about 5 nm and a refractive index of 1.45, so similar results could be expected as previous calculations. Table 1 shows the reflective film materials used in the experiment, the maximum reflectance change $|\Delta R_{ex}|$ due to the streptavidin adsorption for each reflective film material, and the maximum reflectance change $|\Delta R_{cal}|$ obtained by the above calculation. When Au was used as the reflective film, the Au layer peeled off after forming the sensing plate without the adhesive layer, and no experimental values could be obtained. The highest value of $|\Delta R_{ex}|$ obtained in the experiment was 0.505 when Cu was used. However, Cu, although not as bad as Au, had poor adhesiveness, and some peeling occurred. When using a Au reflective film with Cr adhesive layers with a thickness of 0.8 nm, $|\Delta R_{ex}|$ was 0.263. This was a relatively high value among the experimental values obtained, but the sensitivity was way lower than the calculated value $|\Delta R_{cal}| = 0.719$ when Au only was used as the reflective film. The next highest value

Table 1 Relationship among the reflective materials used for the sensing plate fabrication, the values of |ΔR_{ex}|, and the values of |ΔR_{cal}|.

Reflective film material	|Δ R_{ex}|	|Δ R_{cal}|
Au	NA	0.719
Cr/Au/Cr	0.263	0.380
Cu	0.505	0.683
Cr	0.064	0.101
W	0.070	0.098
a–Si	0.234	0.271
Ge	0.139	0.405

was 0.234 when a-Si was used. Large reflectance change was expected in the calculation for Ge, but the actual sensitivity was about one-third of the calculated value. This is thought to be because the calculated value was obtained using the complex permittivity of single-crystalline Ge, while in the experiment, Ge layer was deposited using the sputtering method and the formed Ge layer was amorphous.

In this research, it was found that high sensitivity could be obtained in conventionally used materials such as Au, Ag, and Cu, but there was a problem in their stability. Although stability issue could be solved to some degree by introducing the adhesive layers, the sensitivity with the adhesive layers was the same as when Si was used as the reflective film. Also since Si has extremely high adhesiveness with glass materials, Si was suitable as the reflective film material for securing both stability and sensitivity.

3.3 Monolithic sensing plate

From the above approach, it was found that our nanopore formation technology was effective in increasing sensitivity, and the Si reflective film was effective in both sensitivity and durability. However, as shown in the SEM photographs of Fig. 7, the surface of the waveguide layer formed by the sputtering method became roughened by the nanopore formation and the reflectance property deteriorated, and as a result, sufficient improvement of sensitivity could not be

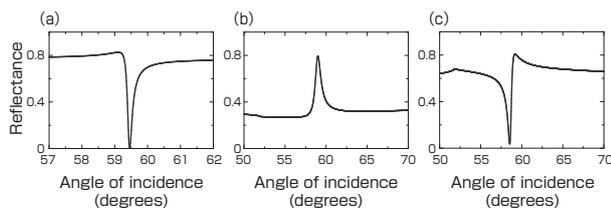(a) (b) (c)

Reflectance

Angle of incidence (degrees)

Fig. 9 Calculation results of incident angle dependency of reflectance when Au (a), W (b), and Si (c) are used as reflective films. The thicknesses of reflective films are 40, 20, and 30 nm, respectively.

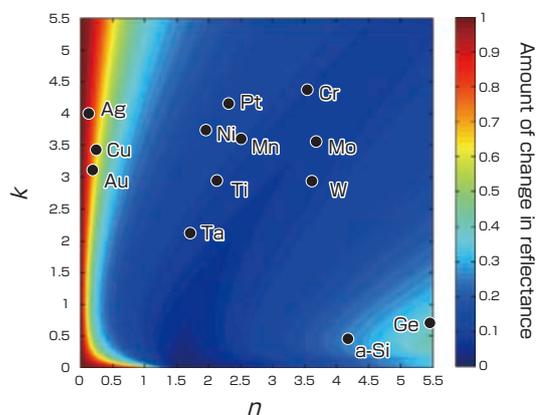Amount of change in reflectance

k

n

Fig.10 Calculation result of relationship between the complex refractive index of the reflective film material and detection sensitivity. The black dots in the graph show the complex refractive index of some materials that may be used as the reflective film.

obtained. Since almost no surface roughening occurred in the thermally grown SiO_2 film as shown in Fig. 6, we considered the thermal oxidation process of Si in the waveguide layer formation. When Si is used as the reflective film, and if the Si layer is formed thick and the waveguide is formed by thermal oxidation of the surface, we can fabricate a sensing plate with a thermally grown SiO_2 film as a waveguide. In this case, it is necessary to use a glass substrate that can endure high-temperature treatment.

To realize this idea, we considered using the SOQ substrate for sensing plate formation. Since thick oxidized layer was necessary for waveguide formation, we incorporated the water vapor oxidation method[14] where the speed of oxidizing the Si layer of the SOQ substrate was fast, and we fabricated a plate having a single-crystalline Si reflective layer and a SiO_2 waveguide layer on a silica glass substrate. The fabrication process of the sensing plate is shown in Fig. 11. The thickness of the single-crystalline Si layer before the thermal oxidation was 440 nm. When this layer was oxidized for 1 hr in oxygen atmosphere containing water vapor at 1000 ºC, the Si layer surface was oxidized and a waveguide layer with thickness of 482 nm was formed. The remaining Si layer of a thickness of 220 nm would function as the reflective film. We named this sensing plate 'monolithic sensing plate'.

The fabricated sensing plate was set in the optical setup as shown in Fig. 12 to conduct molecule detection tests. Figures 13(a) and 13(b) show the changes in reflectance properties
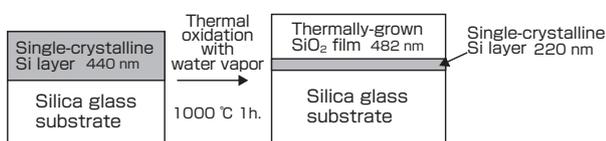
when biotin-streptavidin adsorption was detected using the monolithic sensing plates with nanopores and without nanopores, respectively. The incident light was s-polarized light of He-Ne laser (632.8 nm wavelength). The diameter of the nanopores was about 50 nm, and there were 5×10^9 pores/cm^2. The white dots in the figures show the incident angle dependency of the reflected light intensity before adsorption, while the black dots show the intensity after adsorption. It can be seen that the amount of shift of peak position increased about 10 times by the nanopore formation. By the nanopore formation, the width of the dip increased slightly while the depth hardly changed. This is due to the reduction of damage during etching.

To ensure the improvement of sensitivity by nanopore formation theoretically, we conducted a simulation using the Fresnel equation. Figures 14(a), 14(b), and 14(c) are conceptual diagrams used in the simulation. Figure 14(a) shows a conventional SPR sensor, 14(b) shows an EFC-WM sensor using a monolithic plate without nanopores, and 14(c) shows an EFC-WM sensor using monolithic plate with nanopores. The prism of the SPR sensor was a right triangle prism with a refractive index of 1.769 and the sensing plate was assumed to have a gold film with a thickness of 51 nm formed on a substrate with a refractive index of 1.769. The prism of the EFC-WM was an isosceles triangle prism having a vertex angle of 30° with refractive index of 1.456. The substrate of the sensing plate was silica glass (n = 1.456), the thickness of the Si reflective layer was 220 nm, and the thickness of the waveguide layer was 450 nm. As in the experiment condition, the diameter and the number of nanopores were set to be 50 nm and 5×10^9 pores/cm^2, respectively. As an imitation of the adsorption of streptavidin,

Fig.11 Explanation of the process for fabricating the sensing plate by thermal oxidization of the single-crystalline Si layer of the SOQ substrate.
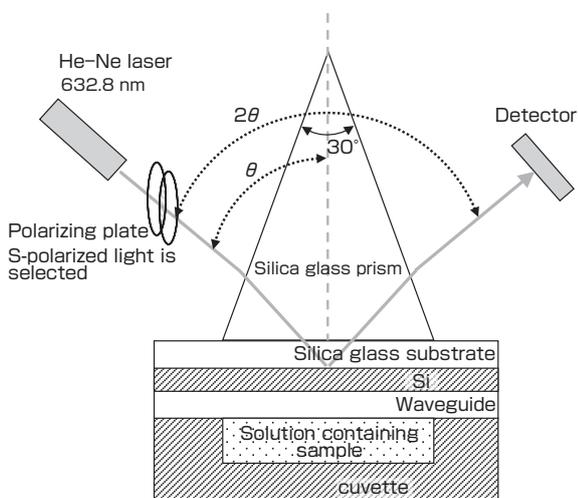
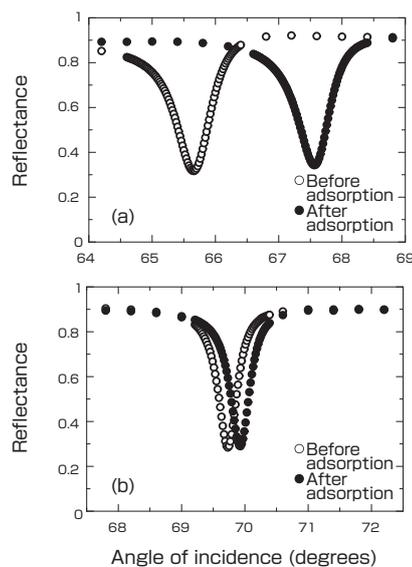**Fig.12 Optical setup used in molecular detection using the monolithic sensing plate.**

Fig.13 Reflectance property before and after adsorption of streptavidin on biotin observed using the monolithic sensing plate with nanopores (a) and without nanopores (b). The concentration of streptavidin was 1.5 μM.

it was assumed that a layer (light pink layers in Fig. 14) with a thickness of 5 nm and a refractive index of 1.45 was formed on the detection surface. Figures 14(d), 14(e), and 14(f) show the calculation results of the reflectance property before and after the molecule adsorption shown in Figs. 14(a), 14(b), and 14(c), respectively. In these sensors, the sensitivity is higher if the full-width at half maximum (W) of the dip is small and the shift of the peak position (S) is large. Namely, the sensitivity is higher if S/W values is higher. Table 2 shows the values of change in reflectance (ΔR), S, W, and S/W caused by the molecule adsorption in the experiment and in the simulation. In case of the EFC-WM sensor without nanopores, almost all the values obtained in the experiment agree well with the values obtained in the simulation. In case of the EFC-WM sensor with nanopores, the S values were greater in the experiment value. To obtain the shift of S = 1.91 obtained in the experiment for calculation, the diameter and the number of the nanopores had to be set at 65 nm and 6×10^9 pores/cm^2, respectively. This means that the diameter and the number of the nanopores formed in the experiment were slightly larger than those in the fabrication setting. As shown in Table 2, the value of S/W obtained in the experiment was 2.98 with nanopores, and 0.514 without nanopores. These values were both larger than the theoretical S/W value of the SPR sensor, and it was about 4 times greater without nanopores and about 25 times greater with nanopores.

Monolithic sensing plate is excellent in stability. The monolithic plate is physically stable because the substrate, the reflective film, and the waveguide layer are atomically

Table 2 Comparison of sensitivity of SPR and EFC-WM sensors. Sim. is calculated values, and Ex. is experimental values.

	ΔR	S	W	S/W
SPR sensor Sim.	0.15	1.00°	8.4°	0.12
EFC-WM sensor without nanopores Ex.	0.38	0.19°	0.37°	0.51
EFC-WM sensor without nanopores Sim.	0.40	0.17°	0.34°	0.49
EFC-WM sensor with nanopores Ex.	0.60	1.91°	0.64°	2.98
EFC-WM sensor with nanopores Sim.	0.63	0.72°	0.34°	2.12

bonded to each other. Also, since it is composed only of Si and SiO$_2$, it is chemically stable.

4 Breakthrough

To apply this sensor to the actual detection of substance originating from various diseases, we conducted a hospital survey to see which substances should be targeted. In this survey, we realized that many sensors used at hospitals for similar purposes detect disease substance using color density. The monolithic sensing plate is sensitive to changes in refractive index as mentioned above, and it is even more sensitive to changes in optical absorption, or density of "color." Therefore, we redesigned the sensor so it can detect changes in "color" more sensitively. The dip seen in the reflectance property of the EFC-WM sensor changes in the angle direction or along the horizontal axis direction against the change in refractive index, while for the change in optical absorption, it changes in reflectance intensity or vertical axis direction. Therefore, we changed the structure of the
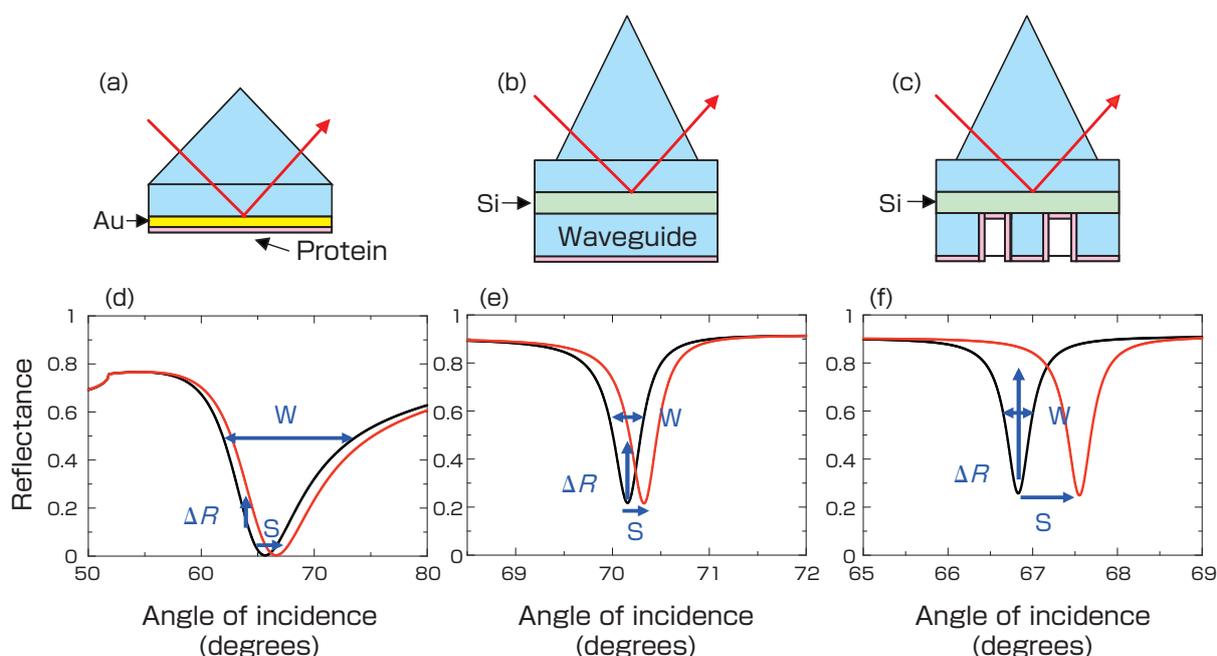

Fig.14 Conceptual diagram of molecule adsorption in the SPR sensor (a), the EFC-WM sensor without nanopores (b), and the EFC-WM sensor with nanopores (c). (d), (e), and (f) show the calculation results of reflectance property before and after the molecule adsorption in (a), (b), and (c), respectively.

sensing plate so the change in vertical direction would appear more clearly. Specifically, we reduced the thickness of the Si reflective layer.

The fabricated sensing plate had a single-crystalline Si layer of a thickness of about 35 nm and a thermally grown SiO_2 waveguide layer of a thickness of about 520 nm on a silica glass substrate. After modifying the waveguide surface with biotin, this plate was set in the optical setup shown in Fig. 12 to conduct the detection test using immunogold conjugate as a sample. The immunogold conjugate consists of 4 to 5 streptoavidins attached to a Au nanoparticle with diameters of 20 nm. The Au nanoparticle absorbs the incident light. We used Tris-buffered saline containing 10 pM of this sample. Figure 15 shows the reflectance property before and after the introduction of the sample. White dots show the reflectance property before introduction and black dots show the reflectance property 20 hrs after introduction. Reduction in reflectance of 0.046 was observed, indicating that the sensor successfully detected the sample at such low concentration.

Next, to examine improvement in sensitivity by using dyes, we detected the capture of streptavidin by biotin after dying the streptavidin with a blue dye, Coomassie Brilliant Blue G-250. This dye has an optical absorption band at around 600 nm. The sensing plate and the optical setup used were the same as the previous experiment. The detection test was done using a PBS buffer containing 100 pM of the dyed streptavidin. Figure 16 shows the reflectance property before and after introduction of the sample. White dots show the reflectance property before introduction, and black dots show the reflectance 1 hr after introduction. In this case also, sufficiently large change in reflectance was observed. This detection sensitivity was about three orders of magnitude higher than the sensitivity of a conventional EFC-WM sensor.

In the above two examples, change in the dip position accompanying the change in refractive index was thought to have occurred by the adsorption of the samples, but the amount of molecule adsorption was small in both cases, and the change in dip position could not be confirmed clearly. In this type of sensor, further improvement of sensitivity could be expected by nanopore formation. This shall be our future subject of study.

In this method, when settings are done to measure only the change in depth of the dip, the detection sensitivity will not be affected by temperature change. This is because the change in refractive index of water by temperature difference causes a change only in the position of the dip, and does not accompany any change in the depth of the dip. Also, since detection is achieved by capturing the optical absorption of substances, there is almost no effect on reflectance property even if a substance without optical absorption is adsorbed. That is, even when some foreign substance adheres to the detection surface, as long as the substance does not absorb the incident light, it will not be detected. Therefore, this method is scarcely affected by adhesion of foreign substances. As described, the present method has several advantages over the conventional method.

5 Composition of the research

The flow of the development is summarized in Fig. 17. In the research for increasing the performance of the sensor, we used a synthesis method called strategic selection[15]. First, to increase the sensitivity of the EFC-WM sensor, which is the core technology, based on simulation, we selected nanoprocessing to the waveguide layer. While we were able to improve the sensitivity by conducting the nanoprocessing, new problems arose in the physical stability of the sensor and the evenness of the processed surface. To solve these problems, we returned to material selection, and selected reflective film materials from a different perspective, that is, we set adhesiveness and processing capabilities as well as sensitivity as new standards of selection. We found that
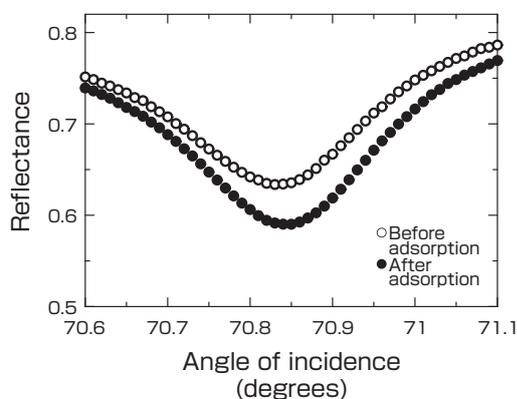

Fig.15 Reflectance property before and after adsorption of streptavidin with gold nanoparticles (immunogold conjugate, concentration 10 pM) on biotin, observed using optical absorption detecting monolithic sensing plate.
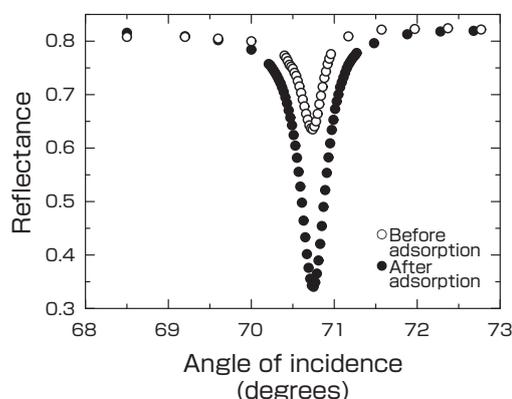

Fig.16 Reflectance property before and after adsorption of streptavidin dyed with Coomassie Brilliant Blue G-250 (concentration 100 pM) on biotin, observed using optical absorption detecting monolithic sensing plate.

Si was suitable as a reflective film, and devised a method for forming the waveguide layer by thermal oxidation. Single-crystalline Si is suitable to obtain a uniform thermally grown SiO_2 layer, which is scarcely damaged by the nanoprocessing. Therefore, we decided to use the SOQ substrate with a single-crystalline Si layer on a silica glass substrate. By forming the waveguide by thermal oxidation of the Si layer, we were able to develop a high-performance sensor as described above. By strategically combining several elemental technologies, we were able to obtain an integrated technology in the form of a high-performance sensor.

6 Future issues

There are two major issues in realizing the sensor. One is to demonstrate its function by detecting a target substance in actual measurement environment, that is, using samples of human origin such as blood and saliva that contain various foreign substances. Here, "selectivity" is important. The primary issue is to obtain high selectivity where other substances are separated from the target substance accurately. Therefore, to realize the actual outcome, it is important to develop a substance that can specifically capture a target substance originating from a particular disease. Also, we would like to aim for high selectivity by using the detection method using color based on what we learned through this study and by developing dyes that can specifically colorize target samples. The second issue is production costs. Since it is for medical use, it is desirable to keep the cost of the sensing plate to about 100 yen at most. We hope this can be achieved by incorporating the integration technology and mass-producing the product.
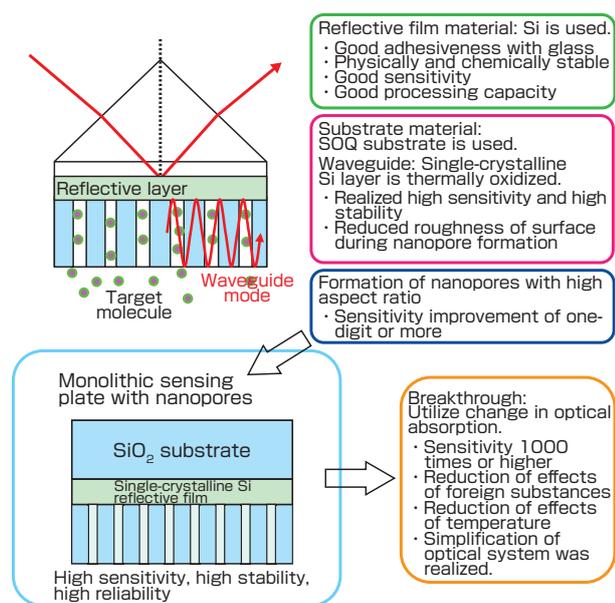
7 Conclusion

Fig.17 Flow of the R&D.

Through this sensor development, we were able to obtain detection sensitivity that can sufficiently meet the demand of practical use. We were also able to show the possibility of reducing the effects of foreign substances and temperature change that were major issues in the conventional sensors. The developed sensing plate has high physical and chemical stability. Moreover, though it was not mentioned in the paper, by using the SiO_2 waveguide, it is possible to use silane coupling for surface modification in molecule detection. Silane coupling is a very strong, simple, and low-cost surface modification method. Also, our device is suitable for downsizing. Currently, we are working on a desktop device about the size of a college dictionary. We obtained good results for the factors assumed at the start of the development. In the future, we would like to cooperate with the researchers of fields that require such sensors, and promote collaboration with medical institutions and researchers of different fields such as chemistry and biosciences. We would also like to continue our research to realize a sensor that can serve the medical field.

Acknowledgement

Terminology

Term 1. Waveguide mode: When light is transmitted by total reflection within a limited medium, the angles of reflection are limited and become intermittent. The distribution of light intensity within the medium forms "nodes" where the light intensity is mutually strengthened and weakened by resonance. The state where the light is propagating while maintaining such light intensity distribution is called the waveguide mode. One example is the propagation mode in an optical fiber.

Term 2. Surface plasmon: Plasmon is a phenomenon in which the free electrons in metal vibrate as a group. In general, light does not couple with plasmon, but an

evanescent wave can couple with surface plasmon. That means the surface plasmon can be excited by the evanescent field.

Term 3. Evanescent field: When light is reflected, it penetrates into the medium that is reflecting the light. The electromagnetic field that penetrates the medium is called the evanescent field. Under total reflection condition, light penetrates into the medium with low refractive index to about one wavelength.

Term 4. Streptavidin: A glycoprotein with molecular weight of about 60,000. It has extremely high affinity to biotin, and the biotin-streptavidin adsorption reaction is used to bind various biomolecules and nanoparticles.

Term 5. Biotin: Molecular weight 244.31, molecular equation $C_{10}H_{16}N_2O_3S$. It is also called vitamin B7 or vitamin H.

References

[1] W. Knoll: Optical characterization of organic thin films and interfaces with evanescent waves, *MRS Bulletin* 16, 29-39 (1991).

[2] W. Knoll: Interfaces and thin films as seen by bound electromagnetic waves, *Annu. Rev. Phys. Chem.* 49, 569-638 (1998).

[3] E. Kretschmann: Die bestimmung optischer konstanten von metallen durch anregung von oberflächenplasmaschwingungen, *Z. Physik* 241, 313-324 (1971).

[4] M. Fujimaki, C. Rockstuhl, X. Wang, K. Awazu, J. Tominaga, T. Ikeda, Y. Koganezawa and Y. Ohki: Biomolecular sensors utilizing waveguide modes excited by evanescent fields, *J. Microscopy* 229, 320-326 (2008).

[5] M. Fujimaki, C. Rockstuhl, X. Wang, K. Awazu, J. Tominaga, N. Fukuda, Y. Koganezawa and Y. Ohki: The design of evanescent-field-coupled waveguide-mode sensors, *Nanotechnology* 19, 095503-1-7 (2008).

[6] J. Y. Douillard and T. Hoffman: Enzyme-linked immunosorbent-assay for screening monoclonal-antibody production using enzyme-labeled second antibody, *Methods in Enzymology* 92, 168-174 (1983).

[7] K. Awazu, C. Rockstuhl, M. Fujimaki, N. Fukuda, J. Tominaga, T. Komatsubara, T. Ikeda and Y. Ohki: High sensitivity sensors made of perforated waveguides, *Opt. Express* 15, 2592-2597 (2007).

[8] M. Fujimaki, C. Rockstuhl, X. Wang, K. Awazu, J. Tominaga, T. Ikeda, Y. Ohki and T. Komatsubara: Nanoscale pore fabrication for high sensitivity waveguide-mode biosensors, *Microelectron. Eng.* 84, 1685-1689 (2007).

[9] R. G. Musket, J. M. Yoshiyama, R. J. Contolini and J. D. Porter: Vapor etching of ion tracks in fused silica, *J. Appl. Phys.* 91, 5760-5764 (2002).

[10] M. Osterfeld, H. Franke and C. Feger: Optical gas detection using metal film enhanced leaky mode spectroscopy, *Appl. Phys. Lett.* 62, 2310-2312 (1993).

[11] K. H. A. Lau, L. S. Tan, K. Tamada, M. S. Sander and W. Knoll: Highly sensitive detection of processes occurring inside nanoporous anodic alumina templates: A waveguide optical study, *J. Phys. Chem. B* 108, 10812-10818 (2004).

[12] T. Abe, K. Sunagawa, A. Uchiyama, K. Yoshizawa and Y. Nakazato: Fabrication and bonding strength of bonded silicon-quartz wafers, *Jpn. J. Appl. Phys.* 32, 334-337 (1993).

[13] M. Fujimaki, C. Rockstuhl, X. Wang, K. Awazu, J. Tominaga, Y. Koganezawa, Y. Ohki and T. Komatsubara: Silica-based monolithic sensing plates for waveguide-mode sensors, *Opt. Express* 16, 6408-6416 (2008).

[14] B. E. Deal and A. S. Grove: General relationship for the thermal oxidation of silicon, *J. Appl. Phys.* 36, 3770-3778 (1965).

[15] R. K. Lester and N. Kobayashi: Hope for *Synthesiology* – Discussion with Professor Lester, *Synthesiology* 1, 139-143 (2008) (in Japanese).

Author

Makoto Fujimaki
Completed the doctoral course at Waseda University in 1998. Doctor (Engineering). Engaged in research of optical communication devices at Waseda University and Montreal University in the Research Fellowships of the Japan Society for the Promotion of Science for Young Scientist. Engaged in development of power electronics devises and optical communication elements at the Electrotechnical Laboratory as Domestic Research Fellow, Japan Science and Technology Corpration. After serving as an associate professor of Waseda University, joined AIST in 2004. Works on the development of biosensing technology using near-field optics. Appointed director of AIST Technology Transfer Venture, and works to commercialize the technology developed at AIST. In this paper, mainly worked on the optical design and integrated the overall concept.

Koichi Awazu
Completed the doctoral course at the Tokyo Institute of Technology in 1991. Doctor (Engineering). Joined the Electrotechnical Laboratory in 1991, and engaged in research of accelerator application engineering. Visiting researcher at the Montreal University from 1996 to 1998. Chief researcher at the New Energy and Industrial Technology Development Organization from 2001 to 2002. Visiting professor at the Institute for Molecular Science from 2002 to 2004. Team leader of the Center for Applied Near-Field Optics Research from 2003. Visiting professor at the Faculty of Engineering, The University of Tokyo from 2005. Engages in research of nanophotonics, as well as in fused discipline of medicine and beam application. In this paper, investigated nanopore formation technology and waveguide formation technology.

Discussion with Reviewers

1 R&D scenario

Question (Naoto Kobayashi, AIST Special Advisor)

It is clear that we need the development of ultra-sensitive molecule sensors. What effects that were unseen before can we expect when the results are achieved in this R&D? Is it merely that we get higher sensitivity, more stability, and easier to use sensors compared to conventional ones? If those are the results, what differences do increased sensitivity and stability make? Please explain those points and also describe the R&D scenario leading up to this.

Answer (Makoto Fujimaki)

In sensor development, improvement of sensitivity and stability is number one. Also, other important points include accurate identification of the sample or low-noise detection.

Figure 1 is a summary by functions, "high sensitivity," "high stability," and "low noise." Specific issues, merits obtained by solving each issue, and final target of application are summarized. This figure was inserted at the beginning of chapter 2. Also, the research scenario was shown as a diagram in Fig. 4, and added to the end of section 2.2.

2 Summary of elemental technologies
Question (Naoto Kobayashi)

I understood that the main focus of the development of this paper was to improve sensitivity of the "evanescent-field-coupled waveguide-mode (EFC-WM) sensor" that has been already developed by forming pores with high aspect ratio in the waveguide so the number of molecules adsorbed to the waveguide surface would be increased and by measuring the change in reflectance due to the change in the waveguide mode by the adsorption, to improve the stability by the development of the monolithic sensing plate, and to further improve the sensitivity by detecting optical absorption of samples.

Even though an EFC-WM sensor has more advantages than a SPR sensor in that there is no limitation on reflective substance and that both s and p waves can be used, why wasn't it put to practical use?
Answer (Makoto Fujimaki)

The principle of the EFC-WM sensor has been known for a long time. The greatest reason it fell behind the SPR sensor for measuring molecular adsorption is because the absolute value of changes in resonance angle caused by molecular adsorption is smaller compared to the SPR sensor. Also, it has the disadvantage of requiring complex maneuvers to form the waveguide layer. I added these points in the latter half of section 2.1.
Question (Naoto Kobayashi)

I understood that one of the major contributions of the authors is to improve the sensitivity by making nano-sized pores with high aspect ratio. In this paper, it is described that the sensitivity is improved because the nanopores with high aspect ratio greatly increases the surface area to which the sample substance can be adsorbed, and because the sample substance is distributed within the waveguide in which the electric field is strongly confined. However, theoretical discussion using calculation and simulation is lacking. I recommend you include some quantitative explanation including the data for dependence of sensitivity on the density of pores.
Answer (Makoto Fujimaki)

To theoretically check the improvement of sensitivity by the nanopore formation, we conducted simulation using the Fresnel equation, and compared the results against the experimental values. In this sensor, sensitivity is higher when the full width at half maximum (W) of the dip is small and amount of shift (S) of the peak position is large. Namely, the sensitivity is higher when the S/W value is large. In the case of an EFC-WM sensor using a monolithic plate with nanopores, the increase in the S/W value by the nanopore formation was very large, about 4 times in the simulation and 6 times in the experimental value. We added this point in section 3.3 as a result showing the improvement of sensitivity.
Question (Naoto Kobayashi)

In the Breakthrough chapter, you mentioned that you realized an ultra–sensitive sensor (about 1000 times) using the reflectance change by optical absorption. In this case, what effects are there by the change of dielectric environment such as the change in refractive index and the change of resonance angle? Or, is there very little effect on the angle change due to extremely small amounts of adsorption? Even if that is so, do you think there is sufficient effect of the nanopores? If so, please explain. Also in this case, is the Kretschmann configuration optimal?

Answer (Makoto Fujimaki)

Angle change does occur in this case, but the adsorption is very small and hardly any angle change occurs. I added this in chapter 4. Although we haven't conducted experiments yet, nanopores do have effect, and sensitivity will increase with pores. I added this also to chapter 4. For configuration, regardless of having or not having nanopores, it does not have to be always a Kretschmann configuration. However, this configuration is easy to use because it can be built readily and the optical system is simple.

3 Synthetical approach
Question (Naoto Kobayashi)

In this study, I think the build up of synthesiology is still insufficient. In the paper, the content, significance, and effects of the individual elemental technologies are well described. However, I recommend you describe in detail the uniqueness and originality of synthesis in which the technologies were combined to create an ultra-sensitive, stable sensor.
Answer (Makoto Fujimaki)

In our research to increase the performance of the sensor, we used a synthesis method called strategic selection. First, to increase the sensitivity of EFC-WM sensor, which is our core technology, we selected nanoprocessing of the waveguide layer based on the simulation. To solve the problems of physical stability and evenness of the processed surface of the sensor, we returned to material selection, and selected the reflective film material suitable for the sensor. We found that Si was suitable as a reflective film, and decided to use the SOQ substrate consisting of a single-crystalline Si layer on a silica glass substrate. By strategically combining several elemental technologies, we were able to obtain an integrated technology in the form of a high-performance sensor. We added this description in chapter 5, and provided detailed explanation of Fig. 17.

4 Future developments
Question (Naoto Kobayashi)

I think this research produced great results in ultra-sensitivity and stability of molecule adsorption sensor, but I think there are still many issues to be considered before it can be put to practical use in the medical scene. I hope you add the prospect for realization and the issues that must be solved.
Answer (Makoto Fujimaki)

In chapter 6, I added the prospect for realization and issues, which are mainly the reduction of noise or removal of foreign substance, the reduction of non-specific adsorption, and more accurate identification of samples to be detected. I also added comments about the cost. If these issues are solved, we expect this technology will be realized.

5 Measures against pandemic influenza
Question (Naoto Kobayashi)

Will this technology help in the early detection of the new pandemic influenza virus that is currently spreading globally?
Answer (Makoto Fujimaki)

One of the themes on which our group is currently focusing our efforts is "ultra-sensitive detection and quick identification of new pandemic influenza virus." We have so far succeeded in detecting the virus fragment (called the HA). Using the high-sensitive molecule detecting technology that we developed, in the near future, we wish to develop a device that can be used to prevent the spread of influenza pandemic.