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Highly Sensitive SPR Biosensor Based on Nanoimprinting Technology

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1. Introduction

Detection of biomolecular interactions is becoming more important as a technique to achieve rapid diagnoses of incipient diseases and preventive medical care. Among various detection techniques currently available (e.g., fluorometry, quartz crystal microbalance, etc.), surface plasmon resonance (SPR)-based biosensing has received much attention since it does not require any labelling of the analytes and enables high-throughput real-time sensing. The SPR technique allows very fast measurements of the order of several minutes, whereas conventional enzyme-linked immunosorbent assay (ELISA) methods are often lengthy processes. Recently, SPR-based biosensors have been extensively applied to analyses in biomedical (Vaisocherová et al., 2006), environmental (Dostálek et al., 2006), and food sciences (Ladd et al., 2006).

In conventional SPR, the evanescent field penetrates into the metal surface by as much as ~300 nm (Stenberg et al., 1991, Homola, 2003). When the target analyte binds to the metal surface, changes in the local refractive index occur, which in turn causes the SPR angle to shift. However, the sensing of target molecules suffers due to unwanted noise factors such as the instability of the temperature and the change in the refractive index of the mobile phase. Thus, the "sensing depth" of conventional SPR is significantly larger than the range required for practical use such as in clinical diagnoses. In this paper, we demonstrate that the sensing depth of SPR can be controlled by producing a pattern of periodic metal nanogrooves on the sensor surface.

2. Advantages of SPR biosensor based on nanoimprinting technology

2.1 Surface plasmon resonance (SPR)

Surface plasmon resonance (SPR) is an interactive coupling phenomenon between light (electric field) and free electrons in metal. When the wave number and the frequency of propagating light match those of the eigenmode of the free electrons, the energy of the propagating light is transferred to the oscillation of free electrons. The coupling that occurs near the surface of the metal is called surface plasmon resonance (SPR) (Homola, 2003). It is known that SPR can be roughly classified in two types; the first type is propagating SPR and the second type is localized SPR.

To generate propagating SPR, light must be translated in the evanescent field for the matching of the wavenumber and frequency of the propagating light. In general, propagating SPR is generated using a prism with a Kretschmann configuration (Kretschmann & Raether, 1968). A thin gold layer (thickness, about 50 nm) is prepared on a glass substrate, which is then attached on the prism surface with matching oil. When light enters into the prism, total internal reflection of light occurs on the glass surface as a result of the thin gold layer. By changing the incident angle or the wavelength of the incident light, propagating SPR can be generated when the coupling condition is satisfied. The generated SPR propagates along the gold surface as the collective oscillation of the free electrons near the gold surface. At that time, the reflection of the incident light is almost absorbed for SPR generation.

Localized SPR occurs on nano-metal structures such as metal nanocolloids (diameter of several tens of nanometres) (Nath & Chilkoti, 2004) and metal nanorods (Huang et al., 2011) and so on. Localized SPR does not require an evanescent field. And the propagating light can couple with the eigenmode of the free electrons in the metal nanostructure. One main difference between localized SPR and propagating SPR is that the localized SPR does not propagate along the metal surface, and that the electric field generated by the localized SPR is much smaller than that of the propagating SPR. The depth of the electric field generated by the localized SPR is several tens of nanometres in size, which means it is smaller than the diffraction limit of light.

These two types of SPR have been extensively studied in physics, and are now demonstrated for the application of practical biosensors.

2.2 SPR biosensor

One important characteristic of SPR is that its coupling condition sensitively depends on the refractive index of a dielectric material located in close proximity to the metal substrate. Therefore, we can find the binding of biomolecules whose refractive index is larger than that of water on the metal surface by detecting the change of coupling angle or wavelength of incident light. To realize a practical biosensor, we immobilize probe molecules, such as antibodies that can capture specific target molecules, on the metal surface. And after that, the sample reagent is applied on its surface. When the target molecules are included, the signal change, which can be expressed as a shift of the resonant angle or wavelength, can be observed according to the concentration of the target molecules. The strong points of the SPR biosensor are that detection can be achieved without any labelling of fluorescent molecules on the target biomolecules and that it realizes quantitative and real-time sensing. As a result, it can also provide the dissociation/association coefficients that cannot be obtained by conventional detection methods.

The biosensor based on the propagating SPR principle was first commercialized by Pharmacia Biosensor AB in 1990 (Homola et al., 1999). And the SPR biosensors are widely used in the pharmaceutical field and research field now. However, the commercialized SPR biosensors are generally very expensive. Thus, low-cost and high sensitivity SPR biosensors have been demanded for a long time.

Some researchers have already started to use localized SPR in biosensors. As mentioned above, localized SPR has very unique physical characteristics that rely on the coupling between light and free electrons that occurs without the need for an optical prism. Also, the resonant electric field, "sensing depth" is much smaller than the diffraction limit of light, which means that areas further than several tens of nanometres from the metal surface are not detected by this sensor. This provides a unique advantage for biosensors as the size of

the biomolecules is generally only about ten nanometres and the background noise can be all but eliminated by localized SPR rather than propagating SPR (Fig. 1). As a result, the detection system can be much simpler and the signal-to-noise ratio can be high as a result of using localized SPR.

In recent studies, biosensors using localized SPR have been keenly studied and some groups have reported that they could detect disease related biomolecules by using localized SPR.
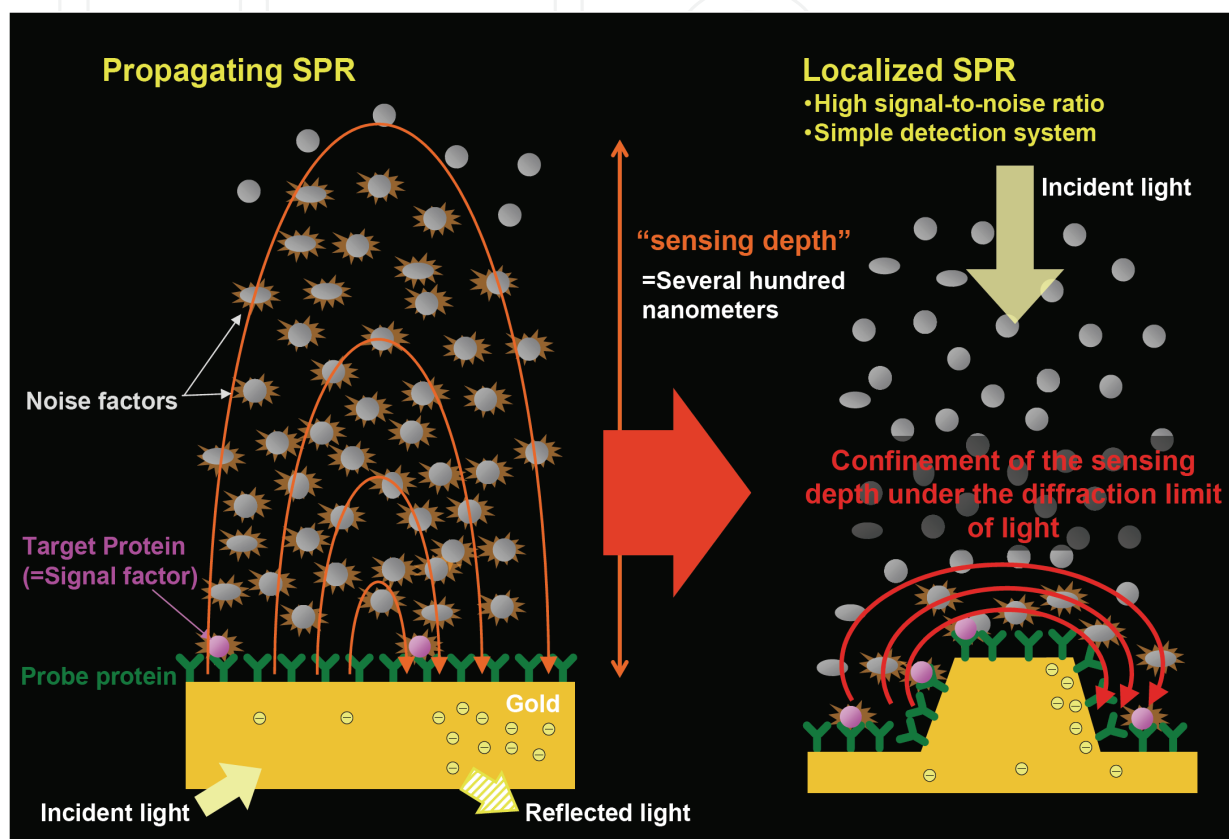

Fig. 1. Reduction of the background noise by localizing the "sensing depth" close to the surface.

2.3 SPR biosensor based on nanoimprinting technique

Localized SPR has a great potential to realize small-sized, easy operation, low-cost and high sensitivity biosensors. However, it is still challenging to fabricate uniform nanopatterns on a wide area of the metal surface. For instance, metal colloids immobilized on a substrate are commonly used as a sensor substrate. To realize a uniform quality in colloid diameter and shape, high process control in deoxidization of metal ions is necessary. In addition, the uniform immobilization of colloids on the sensor surface while avoiding aggregation and density fluctuations are still challenging in mass production. As a stable nanofabrication method, electron beam lithography is a viable candidate. However, the patterns are produced by scanning a single electron beam across a wafer, which is a time consuming and costly process. Other methods such as nano-sphere lithography etc. also have low pattern reproducibility and process throughput. To overcome these conventional problems, our group has proposed a unique way to prepare the metal nanostructures for localized SPR by using nanoimprinting technology (Table 1).
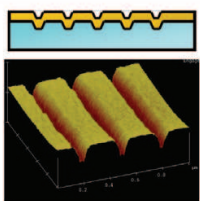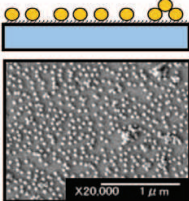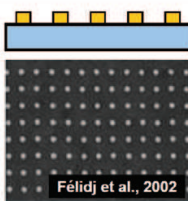
	Nanoimprint method	Metal colloidal method	Electron beam method
Structure Image			
Pattern Reproducibility	Good	Not good (aggregations)	Good
Cost (Process Throughput)	Good (~several minutes)	Not good (~several hours)	Bad (~several days)
Tuning	Easy	Possible	Possible

Table 1. Advantages of Nanoimprint method compared with conventional methods.

3. Fabrication procedure of nanoimprinting SPR biosensor device

3.1 Nanoimprint method

Nanoimprinting technology was first proposed by S. Y. Chou et al. in 1995 (Chou et al., 1995). Prior to this, nanoscale patterns were fabricated using time consuming nanopatterning techniques such as X-ray lithography, electron beam lithography etc. Nanoimprinting technology basically uses the pattern transfer principle and it can foreshorten the process time. Its general process is below.

1. Prepare the master substrate with nanoscale patterns on its surface by using electron beam lithography etc.
2. Make a metal mould from the master substrate by an electroforming process.
3. Press the metal mould onto a polymer surface with heating produced during ultraviolet (UV) irradiation.
4. Peel off the metal mould from the solidified polymer.
5. Repeat steps 3) and 4) for each new polymer surface.

The preparation of the master substrate involves a conventional nano-fabrication technique, which is time consuming. However, the fabricated master substrate can be used to produce the metal mould, which can be used repeatedly. The replication process time with the metal mould is much shorter than the master fabrication process, and generally takes only several tens of seconds. It is demonstrated that nanoscale patterns as small as 5 nm can be successfully transferred by this method. By using this fabrication technology, devices with nanoscale patterns can be fabricated with very high process throughput and in low-cost.

This process is keenly focused and has been demonstrated to have wide application in various electrical (CMOS, FET, patterned media etc.), optical (anti-reflection structure etc.) and energy devices (organic solar cells, fuel cells etc.) and so on.

3.2 Nanoimprinting process for SPR biosensor device

To generate localized SPR, nanosized metal colloids and metal nano rods have been used and studied. High process reproducibility and stability are, however, demanded for the

biosensor products. Furthermore, low-cost sensing devices are necessary to realise disposable usage to avoid contamination resulting from repeated use of a sensing device. These demands are not satisfied by the conventional methods as the chemical fabrication process is still unstable and of high-cost.

To overcome these conventional problems, we have proposed to make a localized SPR biosensor by using the nanoimprinting technique. The main process flow is shown in Fig. 2. As a first step, nanopatterns were created in a photoresist, ZEP520A (Nippon Zeon, Japan) coated on an 8 inch silicon wafer. The nanopatterning step typically takes around 9 hours to pattern a 45 mm² area. After that, the nanopatterned area was sputtered with Ni (CS-200S, ULVAC, Japan) and then electroformed with Ni (SA1m, Digital Matrix, USA) to produce a metal mould having a thickness of 250-300 µm. This metal mould is used to replicate the nanoscale patterns onto a polymer surface. Polymer resin was first deposited onto a glass substrate and then the metal mould was pressed onto the polymer surface with heating or UV irradiation. After solidification of the polymer resin, the metal mould was peeled off from the replicated polymer surface. In general, this process takes only several tens of nanometres. As a last step, a thin gold layer was sputtered onto the surface of the polymer replica. The gold nanoscale patterns generate localized SPR when exposed to incident light.

By this process, the single metal mould can be used repeatedly. As a result, nanopatterns having substantially the same dimensions can be fabricated on the surface of the replica, which is difficult to achieve by using the conventional colloid base method. And the process cost can be also very low.
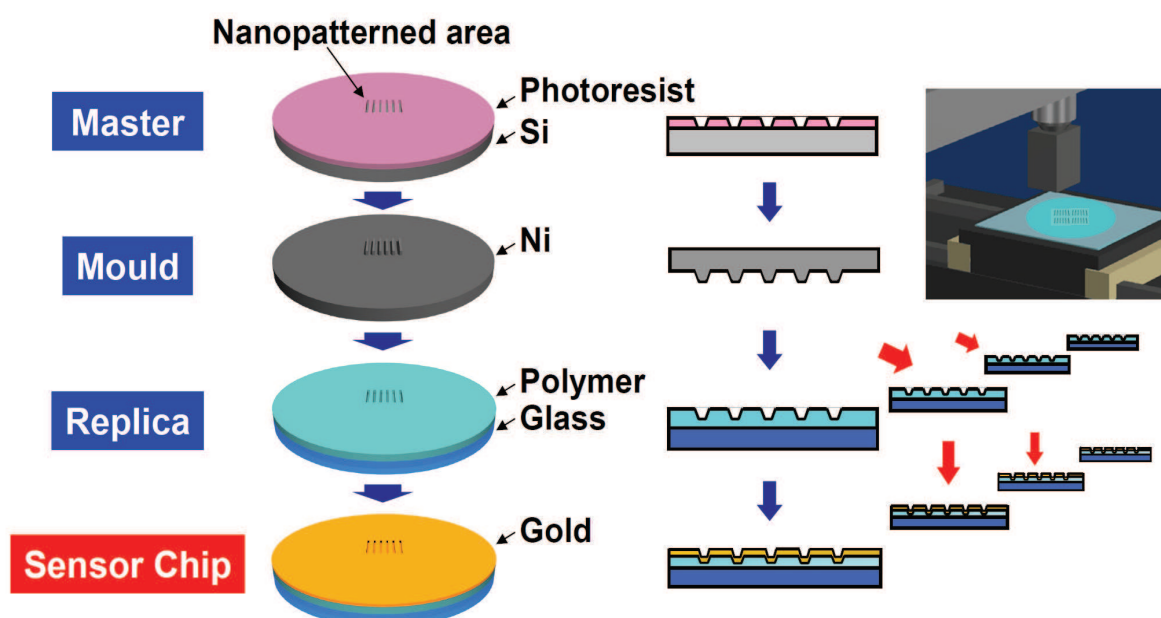

Fig. 2. Fabrication process diagram for the nanoimprint method.

Fig. 3 shows the sensor chip fabricated by the nanoimprinting technique. The nanopatterned areas are slightly red in colour, which means that green light is absorbed by the localized SPR (Fig. 3a). The nanostructures on the sensor chip surface are produced by the nanoimprint injection moulding method. The replication process takes 15 seconds. The period (300 nm) of the nano patterns and the gap size (100-140 nm) of the nanogrooves was confirmed by atomic force microscopy (AFM) image (Fig. 3b).
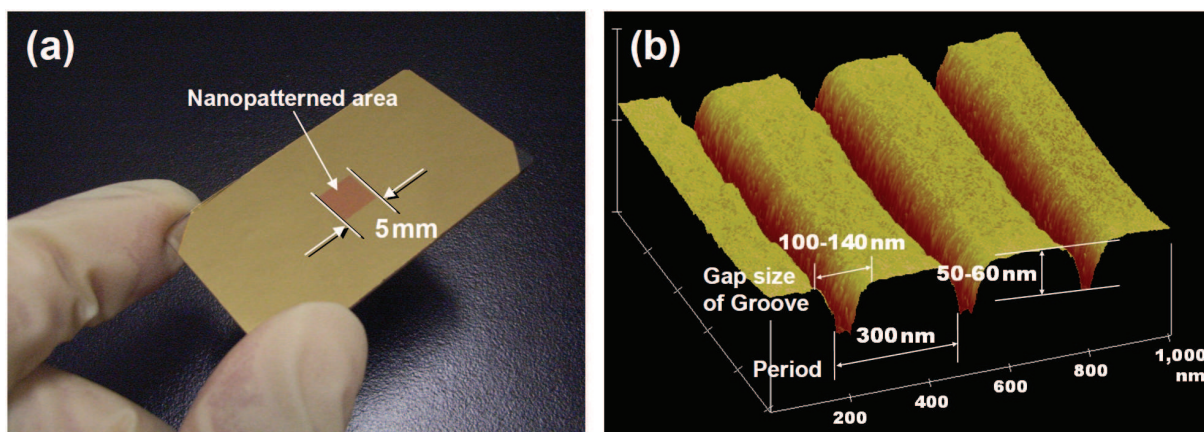
Fig. 3. The sensor chip fabricated by the nanoimprinting technique (a) and an AFM image of the nanopatterned area (b).

4. Design of nanogroove structure for SPR biosensor

4.1 Simulation methods

An analysis of the physical interaction between the metal nanostructures and the incident light (electric field) is necessary to design the shape, size, and period of the metal nanostructures. Here, we have used two simulation methods, finite-difference time-domain (FDTD) and rigorous coupled-wave analysis (RCWA) in this study.

FDTD is a major photonic analysis tool in which the space is divided into a small mesh, the so-called "Yee mesh". The electric and magnetic fields in each mesh are solved according to Maxwell's equation step by step. The dynamic behaviour of the electric field can be calculated for an arbitrary material environment by this method. However, the simulation time and memory space required for solving such complex structures are considerable. We, therefore, used the RCWA method for a static analysis. In the RCWA method, the space is transformed using the Fourier transfer method and solved. Though only the periodic structure can be analysed, the simulation time and memory required for the RCWA method are much smaller than that required for the FDTD method. We used these two methods for each purpose complementarily and optimized the metal nano structures for application in a high sensitivity biosensor.

4.2 Basic design of periodic nanogroove structure

While conducting the FDTD simulations, we found that the resonance occurs inside the metal nano-gap when the periodic nanogrooves are prepared on the metal surface. This resonance is a kind of SPR and its resonant electric field depends on the size of the nanogroove. As shown in Fig. 4, when the gap size of the nanogroove is several tens of nanometres in size, the depth of the resonant electric field is smaller than 100 nm, which overcomes the diffraction limit of light. In this simulation, the light (wavelength, 670 nm) is focused on the sensor substrate from the front side. This result means that localized SPR can occur when periodic nanogroove structures are prepared on the metal surface.

It is also proved that the resonant wavelength can be tuned by changing the structural parameters of the nanogroove. The relationship between the structural parameter and the resonant wavelength is shown in Table 2. The depth of the resonant electric field, the so-called "sensing depth", is very important for a biosensor because when the sensing depth is
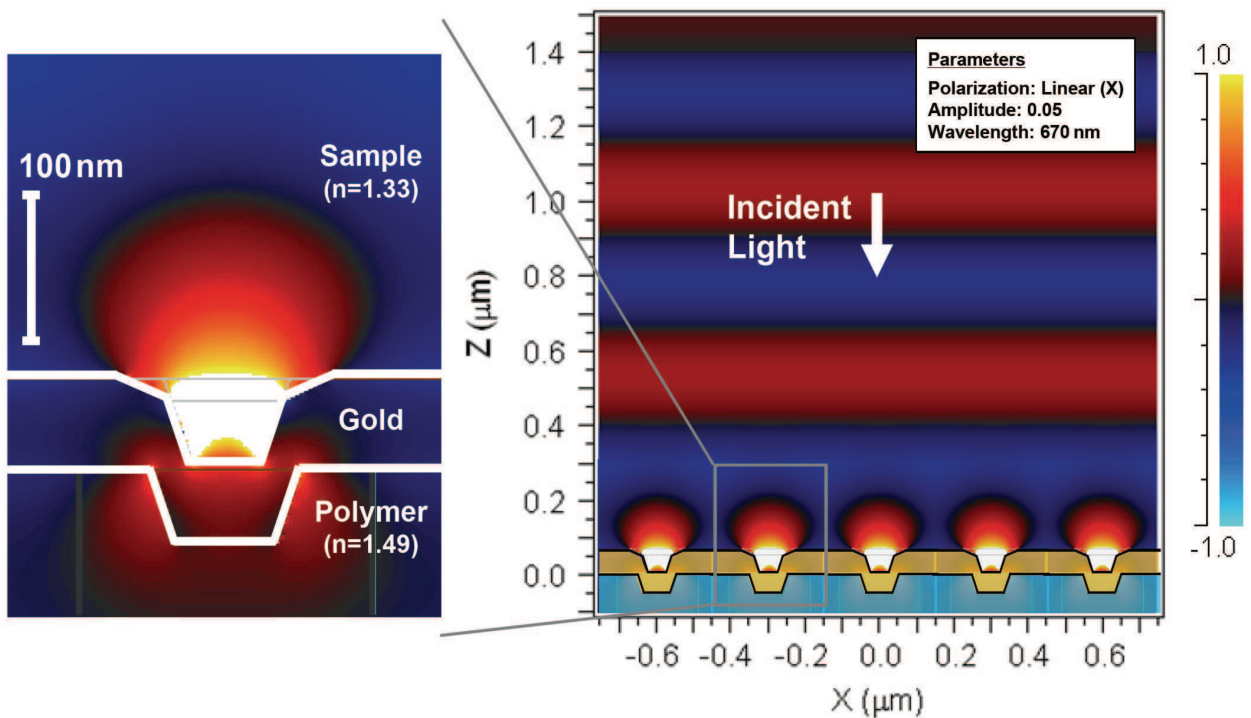
Fig. 4. FDTD simulation of the electric field generated by nanogap structures.

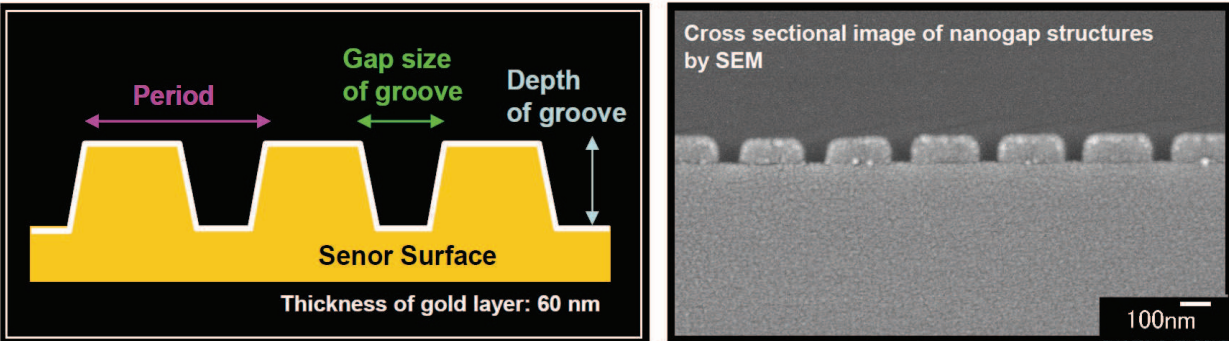| Structural parameter	Effects on the resonance condition
Period	The larger the period, the longer the resonant wavelength and the larger the depth of the resonant electric field.
Gap size of groove	The larger the gap size, the shorter the resonant wavelength.
Depth of groove	The deeper the groove, the longer the resonant wavelength.

Table 2. The relationship between the structural parameter and the resonant wavelength.

too small, the target molecules are not detected. Moreover, when the sensing depth is too large, the background noise is included in the signal. The unique point of this nanogroove SPR is that the sensing depth can be easily selected by changing the structural parameters. The optimal sensing depth can be tuned according to the sizes of the probe molecule and target molecule. Furthermore, the resonant wavelength can be tuned by adjusting the gap size and the depth of the nanogroove. The wide-range tuning of the sensing depth and the resonant wavelength are not easy to accomplish in conventional localized SPR.

4.3 Experimental evaluation of periodic nano-groove structure

As a next step, we evaluated the optical characteristics of the metal nanogroove structures experimentally. Metal nanogroove structures were fabricated by the nanoimprinting process to yield structures with different period and gap sizes. The depth of the nanogrooves was found to be 50 nm, as determined by the thickness of the polymer photo-resist on the master substrate. And the gap size of the groove is varied by changing the dose energy of the electron beam on the master substrate. After making the nickel mould, the replica substrate is produced by the replication process using UV irradiation. A thin gold layer (thickness 80-100 nm) was then deposited on the replica's surface. Fig. 5 shows the optical image of the fabricated device. We can observe a reflection colour change by changing the period and gap size of the nanogrooves. When the period gets shorter, the colour changes from green to red. This result means that the absorption wavelength decreases (red to green). Also, when the gap size gets smaller (dose energy gets smaller), the pattern colour changes from red to green. This means that the resonant wavelength gets longer when the gap size gets smaller. These results are well identical to the simulation results.
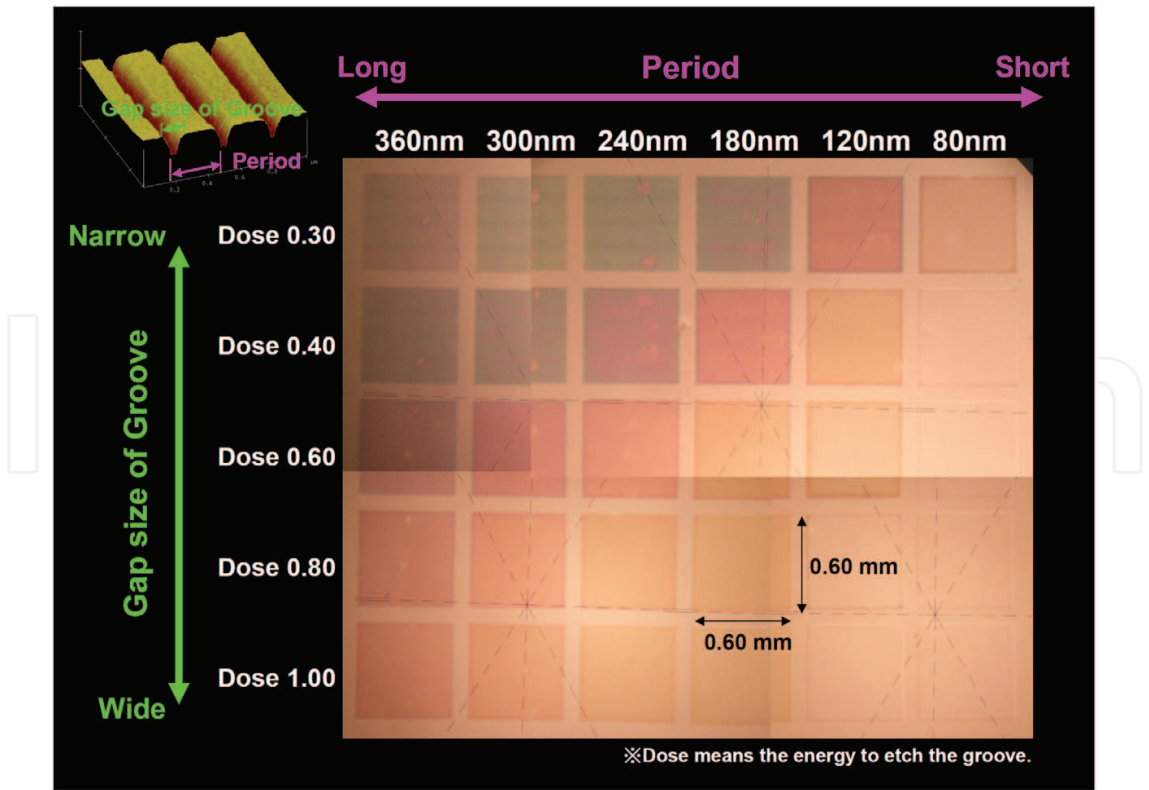

Fig. 5. The metal nanogroove structures changed their period and gap sizes by the nanoimprinting process.

5. Design of immobilization layer for an SPR biosensor

5.1 Three key factors for surface preparation

For SPR biosensing in solution, it is necessary that one interaction partner (probe molecules) is immobilized on the sensor surface to capture the target molecules. Conventional immunochemical methods such as ELISA are based on the simple physical adsorption of probe molecules onto a plastic plate. However, it is thought that a more sophisticated approach is required for surface preparation of a sensor surface for SPR biosensing. This is because the sensitivity of SPR biosensors is highly dependent on the binding capacity of the immobilized probe molecules on the sensor surface and on the resistance of the surface to nonspecific protein adsorption. The performance of the sensor surface is supported by three crucial factors (the capture agent, the surface chemistry, and the surface matrix). Fig. 6 shows a schematic diagram of an immobilization layer for SPR biosensing and the desired characteristics of the three key factors.
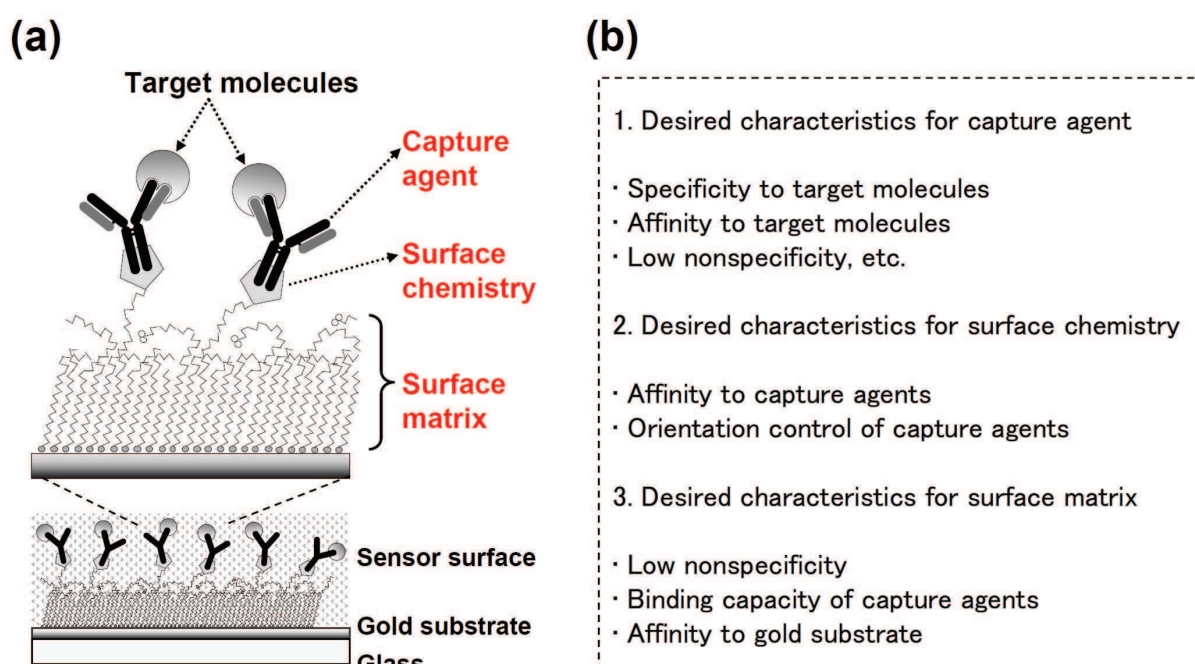

Fig. 6. Schematic diagram of the immobilization layer (a) and desired characteristics for the three key factors (b).

5.2 Basic design of the immobilization layer
5.2.1 Capture agent

In principle, it is difficult for SPR sensors to clearly distinguish the signal component of target molecules from the background noise factors associated with non-specific absorption. First, the capture agent must have the capability for specific recognition of the target molecules. Proteins such as immunoglobulin (IgG), which are known as immune antibodies, are frequently used as capture agents on account of their high specificity towards their target antigens (Table 3, Besselink et al., 2004, Yang et al., 2005). Second, the selection of capture agents with high affinity (equilibrium dissociation constant, in units of molar concentration; $K_D < 10^{-9}$) is necessary to achieve high sensitivity. Recently, nucleic acid aptamers and synthetic peptides have been developed as artificial antibodies with high specificity, high

Capture agent

General tendency for Affinity — Low affinity → High affinity

Native molecules → Specificity (high)
- Polyclonal antibody (not purified) = antisera
- Monoclonal antibody (not purified)
- Monoclonal antibody (1)(2) (purified)
- Polyclonal antibody (affinity purified)

Artificial molecules → Specificity (high-low)
- Small molecule (3)
- DNA/RNA aptamer (4)
- Peptide/Cyclic peptide (5)
- Antibody fragment (variable region) ScFv (6) VHH (7)
- Antibody fragment (Fab region, etc.) Fab, Fab'2 (8)

Surface chemistry

Orientation — Random → Site specific

Covalent → Stability (high)
- Succinimidyl ester-Amines (9)(10) Aldehyde-Amines Epoxide-Amines, Thiol group
- Cutinase- (11) Phosphonate
- Maleimide-Thiol group (8) Bromoacetyl-Thiol group Thioester-Cysteine at N terminus

Non-covalent → Stability (Low)
- Avidin-Biotin (12) → Stability (Middle)
- Protein A/G (13) Anti-IgG antibody
- Glutathione-GST (14) Maltose-MBP
- Ni-NTA-His tag (15) Antibody-Peptide tag (FLAG,HA,myc, etc.)

Surface matrix

Examples of matrix and expected effects

	Simpleness	High density	High orientation	Low nonspecificity	Hybrid type
	Untreated (physical adsorption (3) Thiol group, etc.) (13)	Polymer (Poly-lysine etc.) (12)(16)	self-assembled monolayer (SAM) (7)(17)	phospholipid layer	SAM and EG fused layer (18)(19)
	Protein layer (BSA, etc.) (4)	Polysaccharide (2)(9) (dextran, etc.)	Protein layer (13) (Protein A/G, etc.)	Ethylene glycol (EG)	Protein and phospholipid fused layer (20)(21)
				Polysaccharide (2)(9) (dextran, etc.)	SAM and phospholipid fused layer (22)

(1) Besselink et al., 2004; (2) Yang et al., 2005; (3) Kim et al., 2007; (4) Polonschii et al., 2010; (5) Katz et al., 1995; (6) Torrance et al., 2006; (7) Huang et al., 2005; (8) Bonroy et al., 2006; (9) Johnsson et al., 1991; (10) Lahiri et al, 1999; (11) Kwon et al., 2004; (12) Huang et al., 2002; (13) Lee et al., 2007; (14) Ha et al., 2007; (15) Sigal et al., 1996; (16) Wazawa et al, 2006; (17) Masson et al., 2007; (18) Prime & Whitesides, 1993; (19) Sigal et al., 1998; (20) Athey et al., 2005; (21) Shah et al., 2007; (22) Ishizuka-Katsura et al., 2008

Table 3. Examples of three factors (capture agent, surface chemistry, and surface matrix) that are important in the formation of immobilization layers.

affinity, and ease of size control, using the molecular evolutionary systematic evolution of ligands by exponential enrichment (SELEX) process and phage display method. The obtained artificial antibodies have been often used as capture agents in SPR (Katz et al., 1995, Polonschii et al., 2010).

5.2.2 Surface chemistry

A coupling method involving activated N-hydroxysuccinimide esters is one of the most commonly used surface chemistry techniques for anchoring capture molecules to a sensor surface (Table 3, Johnsson et al., 1991, Lahiri et al, 1999). Since the target of this activated ester is any amino group that is present on the protein molecule with high probability, this coupling method is applicable to various capture agents used for SPR biosensing. To obtain highly sensitive SPR signals, the orientation of capture agents should be considered. The percentage of biochemically active capture agents that can interact with the target molecules would be higher if the orientation of the capture agents on the sensor surface can be improved. As a result of this improvement, the SPR response would be increased several times. As a typical example, the surface chemistry to immobilize proteins via hexahistidine tags (His-tag) has been used (Sigal et al., 1996). In recent years, mutated proteins, such as functional fusion proteins, have been used for achieving oriented immobilization of capture agents and simplification of the immobilization process (Terrettaz et al., 2002, Ha et al., 2007, Park et al., 2009, Le Brun et al., 2011). Some mutated proteins are already on the market as commercial layers (Athey et al., 2005).

5.2.3 Surface matrix

Polymers, polysaccharides, self-assembled monolayers, phospholipid and protein layers, among other, have all been reported as surface matrices (Table 3). One of the most important functions of the surface matrix in SPR biosensors is the suppression of non-specific adsorption of contaminants to the sensor surface. For this purpose, the introduction of oligo(ethylene glycol) molecules is highly effective (Prime & Whitesides, 1993, Sigal et al., 1998). Moreover, it is also important to increase the binding capacity of the capture agents. This factor, which determines the maximum signal variation of the SPR sensor, can control the dynamic range of the biosensing. One example of a surface matrix that has been successful in increasing the binding capacity of capture agents is the carboxymethylated dextran matrix provided by GE Healthcare (Sweden). The carboxymethylated dextran matrix provides a three-dimensional space with a thickness of 100 nm for target molecule binding (Yang et al., 2005, Johnsson et al., 1991).

5.3 Preparation of an immobilization layer on the sensor surface

To accomplish highly sensitive SPR biosensing with a nanoimprinted sensor device, the thickness of the probe layer using such as antibodies should be ~20 nm, as the sensing depth is about 40–80 nm. For this purpose, we attempted to introduce self-assembling layers of ORLA18 proteins (Orla Protein Technologies, UK) onto the sensor surface. The scaffold structure of ORLA proteins is based on the stable structure of the beta-sheet and beta-barrel mutated porin outer membrane protein (Omp) of *Escherichia coli*. It was firstly reported by the research group of J.H. Lakey in 2002 that OmpF proteins can be immobilized directly on a gold surface via thiol-gold bonds formed between the gold and the cysteine residues of the Omp protein (Terrettaz et al., 2002). Moreover, the surface loops of the monomeric porin OmpA can be replaced by anything from short peptides to larger protein domains. The

advanced ORLA protein (ORLA18) is designed to present precisely oriented antibody (IgG)-binding domain structures (two Z-domains of protein A) as single layers with a thickness of ~10 nm on surfaces (Athey et al., 2005).

The surface preparation process of the ORLA18 protein layer is described below and shown in Fig. 7.

1. Treatment of the gold surface on the nanoimprinted sensor device by injecting an aqueous solution containing 1% (v/v), beta-mercaptoethanol.
2. Self assembly of the scaffold protein on the gold surface by injecting a 5 µM ORLA18 dissolved in ROG-8 buffer (Orla Protein Technologies).
3. Stabilization of the scaffold proteins and masking of the spaces between the proteins in the monolayer using 1x filler solution (Orla Protein Technologies).
4. Antibody binding on the ORLA18 protein layers by injecting a 100 µg/mL antibody dissolved in Tris-buffered saline (TBS; 10 mM Tris, pH 7.5, 150 mM NaCl) solution.
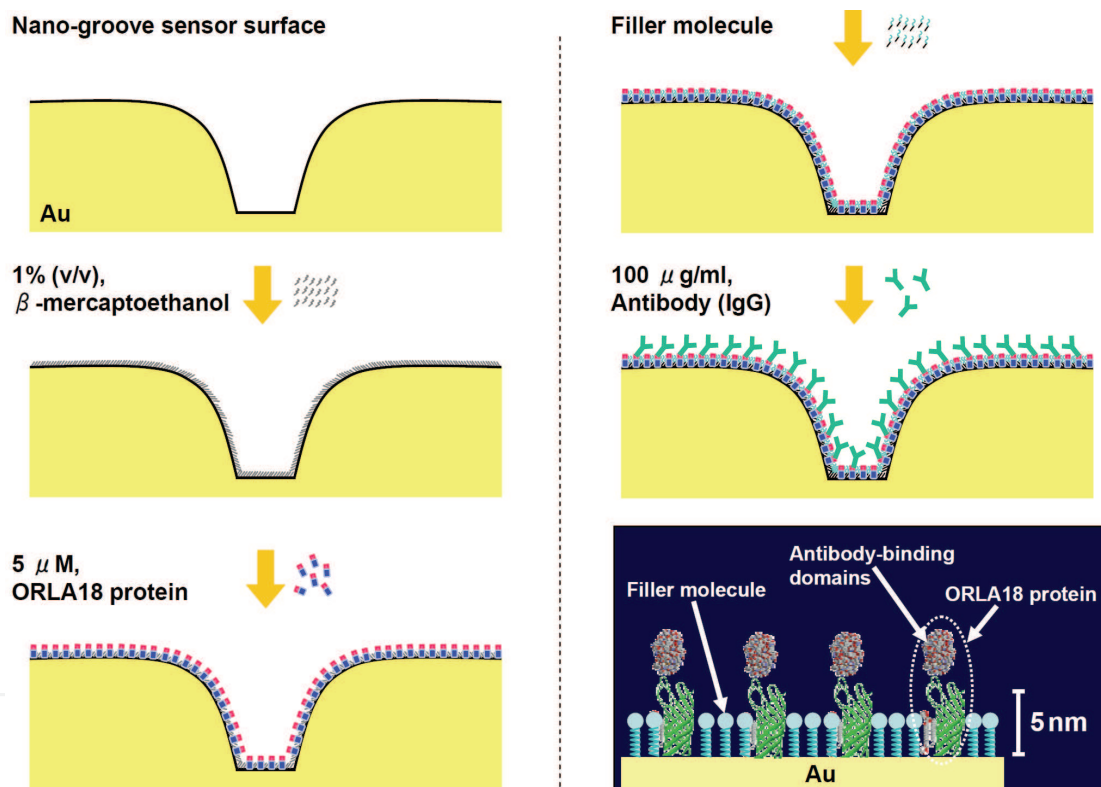
Fig. 7. Preparation process of an antibody-immobilization layer on the nanogroove sensor surface.

6. SPR biosensing using the nanoimprinted sensor device

6.1 Development of the SPR biosensor system

As a prototype of the nanoimprinted SPR biosensor system, we developed a desktop model for use in a laboratory (see Fig. 8). The dimensions of this model are W250mm × D250mm × H150mm, which is 10 times smaller than the commercialized SPR systems (Biacore-X, GE Healthcare, US).

Fig. 9 shows the optical system employed inside the prototype. The white light from the halogen lamp (LS1-LL, Ocean Optics Inc, US) is collimated and irradiated onto the sensor
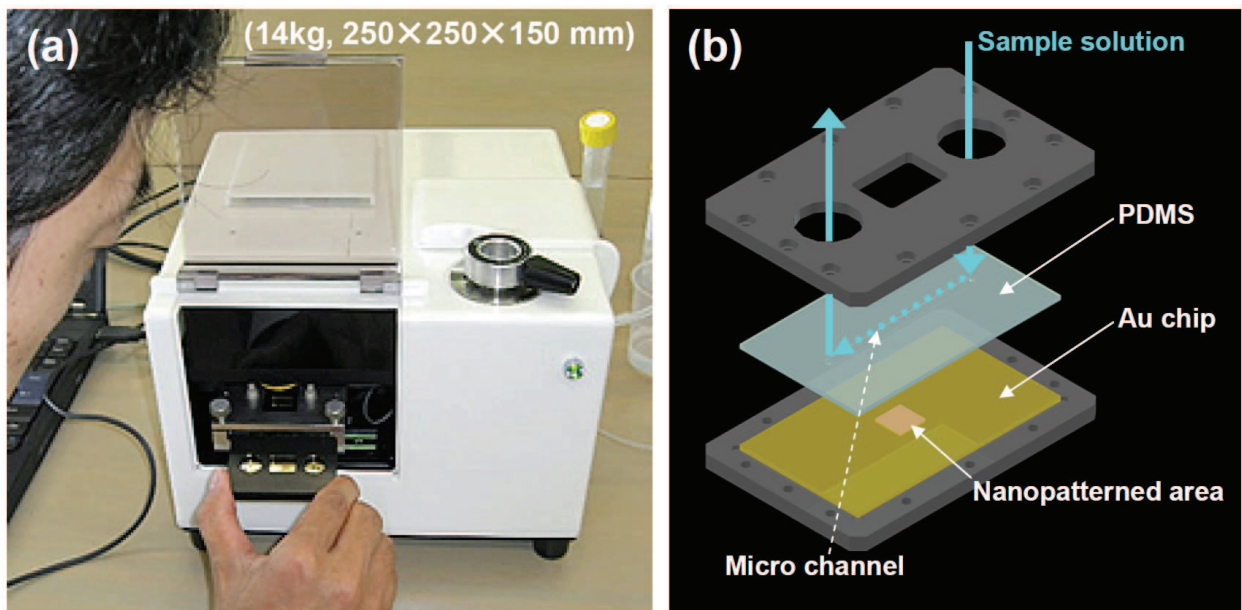
Fig. 8. Prototype of the nanoimprinted SPR biosensor system (a) and a schematic representation of the sensor chip including a microchannel (b).
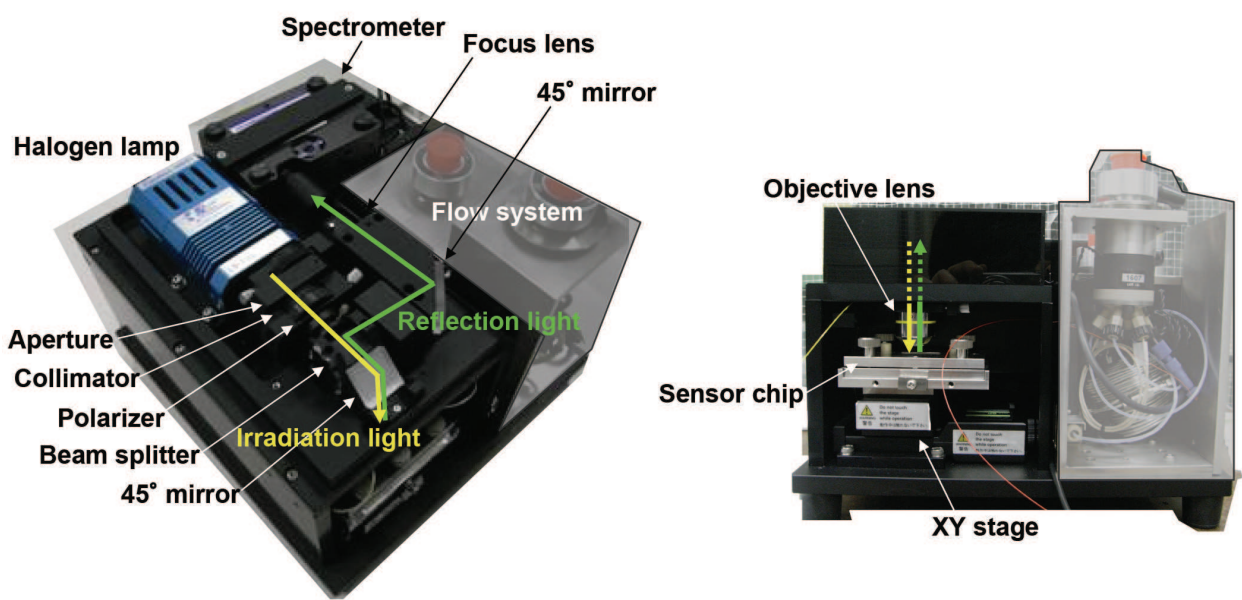

Fig. 9. Optical system inside the nanoimprinted SPR biosensor system.

surface through the objective lens (Plan N ×10, Olympus Co., Japan). The reflected light is gathered by the object lens and split by the half prism (BS CUBE NON-POL VIS 47121, Edmund Optics Inc., US) before reaching the spectrometer (USB4000, Ocean Optics Inc., USA). The resonant wavelength is analyzed by measuring the reflection spectrum data in real time. The motorized linear XY stage (SGSP15-10, Sigma Koki Co., Ltd, Japan) is located under the sensor device holder and is configured to enable multiple points on the sensor chip to be monitored by programming the detection points in advance. A correct and stable liquid flow control system is also very important. Several pumps, such as Veritas and plunger pumps, were considered for use as a flow control system in the prototype. In the

end, an electro osmotic flow (EOF) pump was selected for use. The EOF pump (RP7SP, Nano fusion Technology, Japan) is a very small-sized low-cost pump that provides a non-pulsating flow.

We attached a flow sensor (ASL1430-24, Sensirion, US) downstream of the EOF pump. The applied voltage to the EOF pump was determined under the feedback control of the flow sensor to realize a small and stable flow.

6.2 Two-dimensional monitoring of antibody-binding on the ORLA18 surface

The ORLA18 protein was immobilized on the nanogroove sensor surface according to the immobilization process shown in Fig. 7. To suppress non-specific adsorption, the remaining area was blocked using an oligo(ethylene glycol) (OEG) self-assembled monolayer composed of hydroxyl-terminated thiol $(HS(CH_2)_{11}(OCH_2CH_2)_3OH)$ (Dojindo, Japan) molecules. After the docking of the ORLA18-immobilization gold substrate onto the sensor chip cassette (Fig. 8b), the SPR measurements were started. The two-dimensional variation of the SPR peak wavelength shift on the nanogroove sensor surface was monitored by the nanoimprinted SPR biosensor system of Fig. 8a. After the injection of 100 µg/ml rabbit polyclonal antibodies and IgG (Monosan, Netherlands) dissolved in TBS (pH 7.5) buffer, the specific signal corresponding to antibody-binding was confirmed in the ORLA18-immobilized area (Fig. 10, top left-hand corner of the immobilized area).
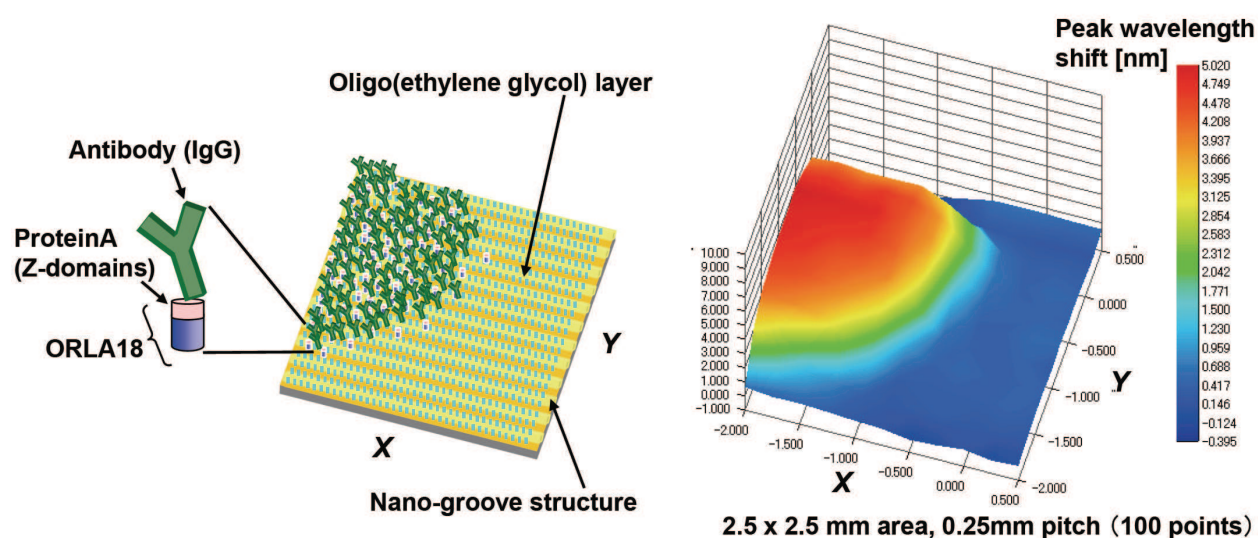

Fig. 10. 2D monitoring of the antibody-binding process on the ORLA18 surface using the nanoimprinted SPR biosensor system.

6.3 Evaluation of the sensing depth on the nanoimprinted sensor device

To demonstrate that the sensing depth is confined to a much smaller region in this sensor, we have attempted to compare the "bulk effect" between this sensor and the propagating SPR. Undiluted fetal bovine serum (FBS) purchased from Japan Bioserum (Japan) was used as a model of blood serum, which contains various proteins as background noise factors (Fig. 11).

In the propagating SPR (Biacore-X, GE Healthcare Co.), whose sensing depth is several hundred nanometers, the signal change caused by the undiluted FBS injection was about 8,000 RU, while the signal change from the binding of the antibody (IgG) dissolved in TBS

(pH 7.5) buffer was 2,100 RU. In the nanoimprinted SPR sensor, the peak shift caused by the undiluted FBS injection was 1.2 nm, while the peak shift caused by the binding of IgG dissolved in TBS (pH 7.5) buffer was 3.5 nm. The signal-to-noise ratio (IgG binding/FBS signal before washing) was 0.26 and 2.92 in the propagating SPR and the nanoimprinted SPR, respectively (Fig. 11). This shows that the nanoimprinted SPR sensor is more than 10 times less subject to bulk effects, due, for example, to the other constituents of blood and temperature fluctuations. This indicates that the sensing depth of the SPR is much smaller than that of a conventional propagating SPR sensor. This will help fabricate small-sized equipment in which temperature control is not necessary. Also, the washing protocol to separate the signal caused by the binding of the target molecules from the signal caused by the mixture of other floating biomolecules can be omitted. These advantages promise to lead to the development of protein and point-of-care chips, which are expected to become prevalent in diagnostic and healthcare applications.
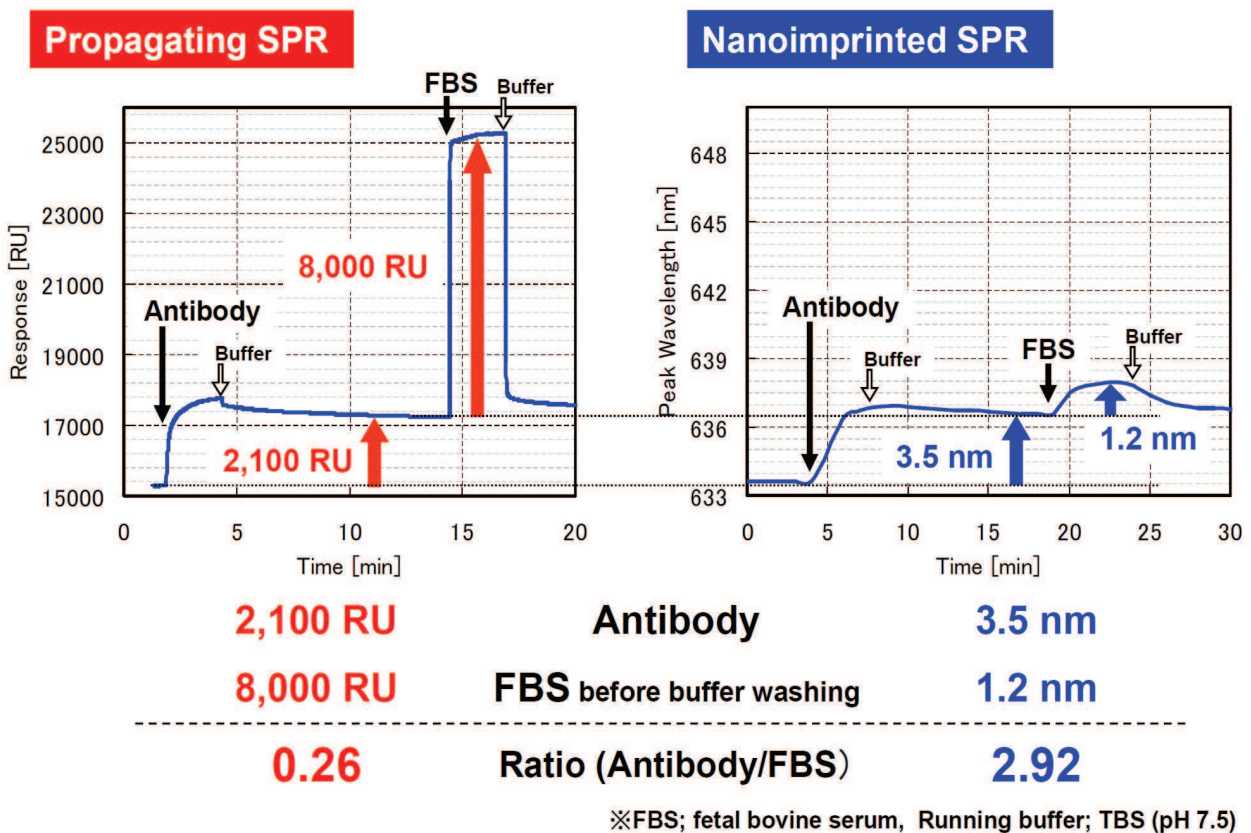

Fig. 11. Comparison of the signal-to-noise ratios of the nanoimprinted SPR and propagating SPR.

6.4 Detection of alpha-fetoprotein using the nanoimprinted SPR biosensor

Using the nanoimprinted SPR biosensor, we performed the quantitative detection of alpha-fetoprotein (AFP), a tumor marker. The AFP concentration in healthy human serum is approximately ~20 ng/ml, but its level increases markedly to over several hundred ng/ml in patients with liver cancer (Teramura & Iwata, 2007). Currently, the cut-off-value of AFP for clinical diagnosis is 200 ng/ml. Hence, the sensitive detection of the AFP using the nanoimprinted SPR system must be useful in cancer diagnosis. For the highly sensitive

detection of AFP, an affinity purified rabbit polyclonal antibody (95% IgG) against human AFP was purchased from Monosan (Netherlands). Pure human AFP (a single band on SDS-PAGE) was obtained from Morinaga Institute of Biological Science (Japan). These AFP and Anti-AFP were diluted in Hepes-buffered saline (10 mM HEPES, pH 7.4, 150 mM NaCl) solution containing 0.005% (v/v) Surfactant P20 and 3 mM EDTA, which was used as a running buffer in the flow cell of the SPR system.

At first, we attempted to detect the AFP molecules on the anti-AFP-immobilized ORLA18 surface under condition 1 in Fig. 12. However, degradation of the signal was observed in low concentration. Then the detection limit of the nanoimprinted SPR biosensor was estimated to be more than several hundred ng/mL (Fig. 13). To obtain a higher variation of the SPR signal, we attempted to increase the efficiency of the antigen-antibody reaction by five times higher flow rate (100 µl/min) and two times larger than the contact time (6 min) of this reaction under condition 2. Fig. 14 shows the results of AFP detection at condition 2. The error bar indicates three standard deviations of the base line signal after the injection of a zero-concentration AFP solution. After several experiments, finally we realized that reducing the diffusion time of AFP is essential. Under condition 1 (Fig. 12), the mass-transport effect (Karlsson et al., 1991) can be larger because of the size of its flow cell. Therefore, under condition 3, we decreased the flow cell size. In this condition, the signal degradation at low concentration was observed under condition 1 to be significantly improved (Fig. 13). Then we estimated that the detection limit of AFP by the nanoimprinted SPR biosensor is ~20 ng/mL. This value already overcomes the cut-off value of 200 ng/mL in a clinical diagnosis.
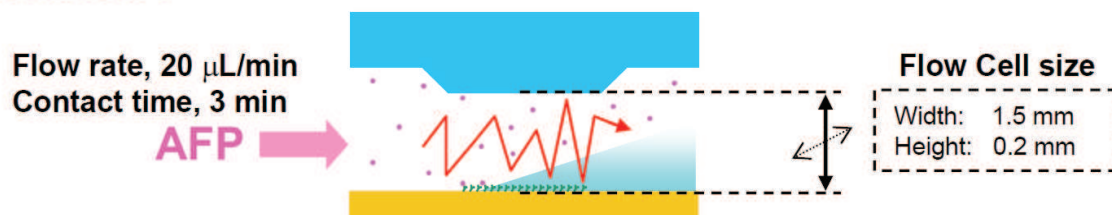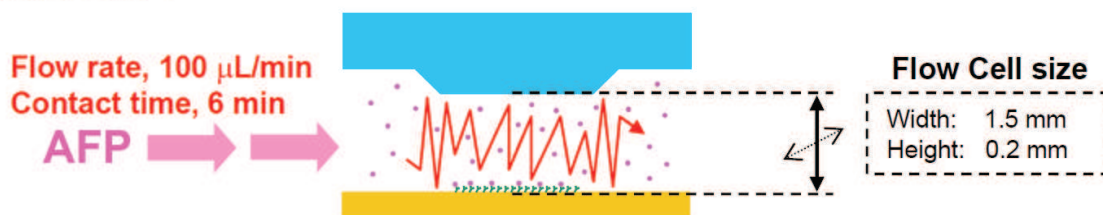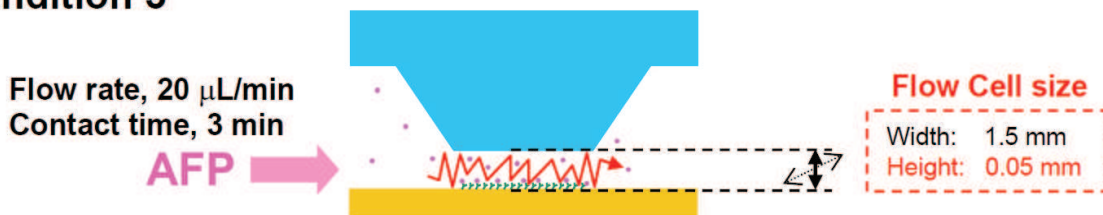

Fig. 12. Experimental conditions for AFP detection using the nanoimprinted SPR biosensor.
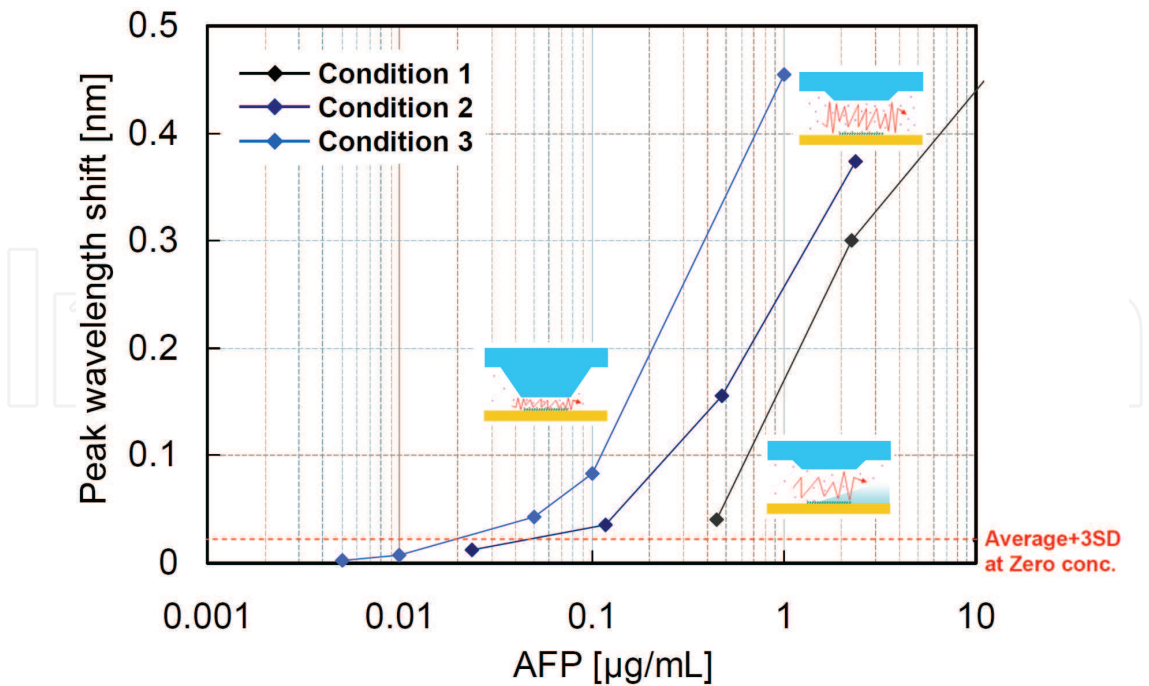
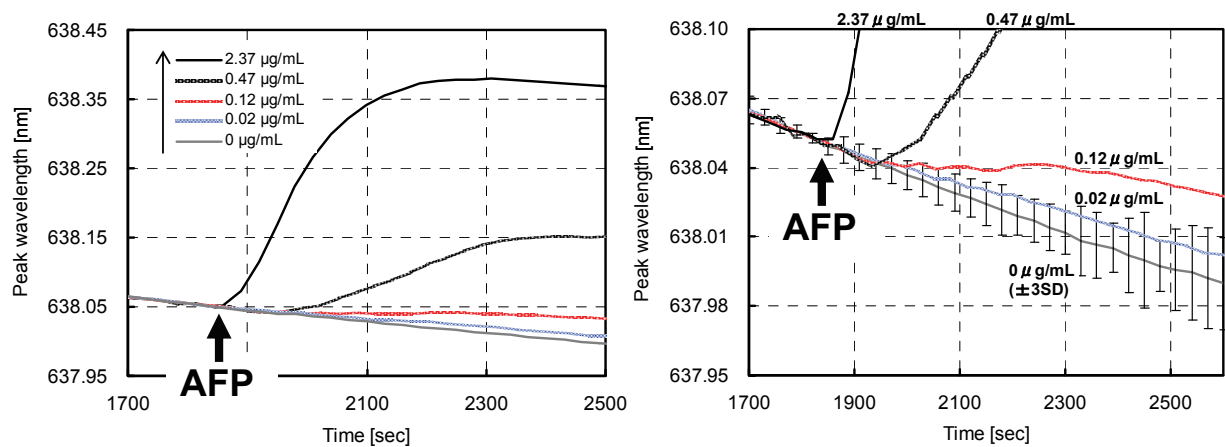Fig. 13. Standard curves for AFP detection at different experimental conditions.

Fig. 14. Results of AFP detection using the nanoimprinted SPR biosensor. The right graphs show the expansion of the left graphs.

7. Present research - Development of palm-sized model -

Localized SPR has good potential to be used more widely as it can provide real-time, quantitative and easy operation sensing without any labeling on the target molecules. For various usages such as point-of-care testing, food analysis, and environmental tests, we have developed a palm-sized prototype based on the nanoimprinted SPR biosensor.

Fig. 15 shows a picture of the prototype. The dimensions are W77mm × D52mm × H56mm and its weight is only 240 g. The electric power used in this model is all supplied by the USB cable connected to the PC. The sensor chip is inserted from the front side into the equipment. The injection of the liquid sample through the micro channel is conducted by the
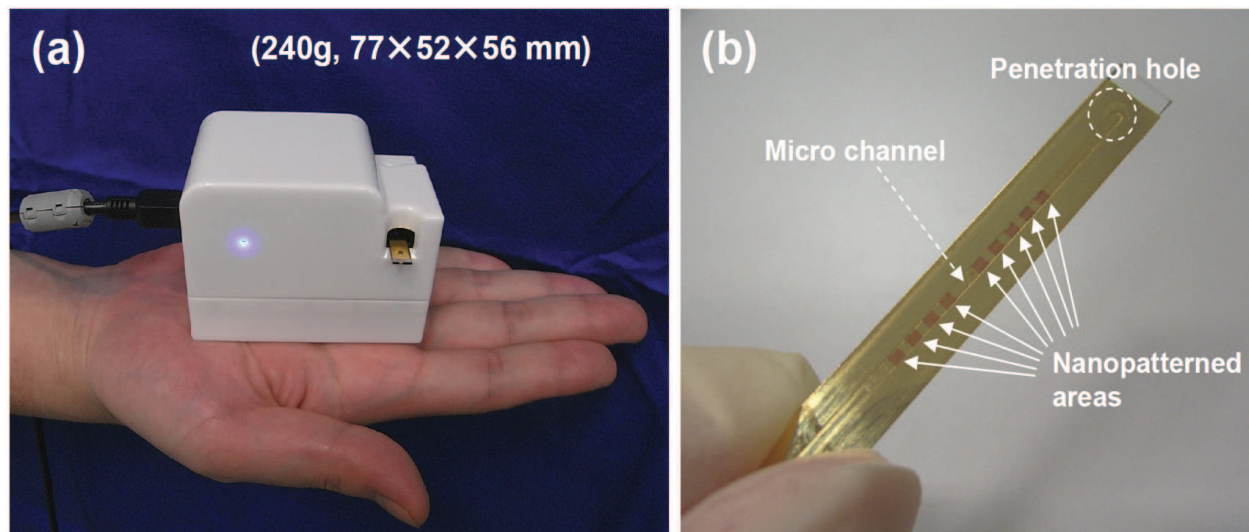
Fig. 15. Palm-sized model of the nanoimprinted SPR biosensor system (a) and the sensor chip including a micro channel (b).

syringe pressure. Fig. 16 shows the optical system of this model. A red laser (LM10-650, Kyoritsu-Electric Corporation, Japan) is used as a light source and the change of the reflection power is detected in this model. The light from the light source is split into four beams and four spot areas, which are detected at once. And the reflected light is detected by the photo-diode (S8745-01, Hamamatsu Photonics, Japan).
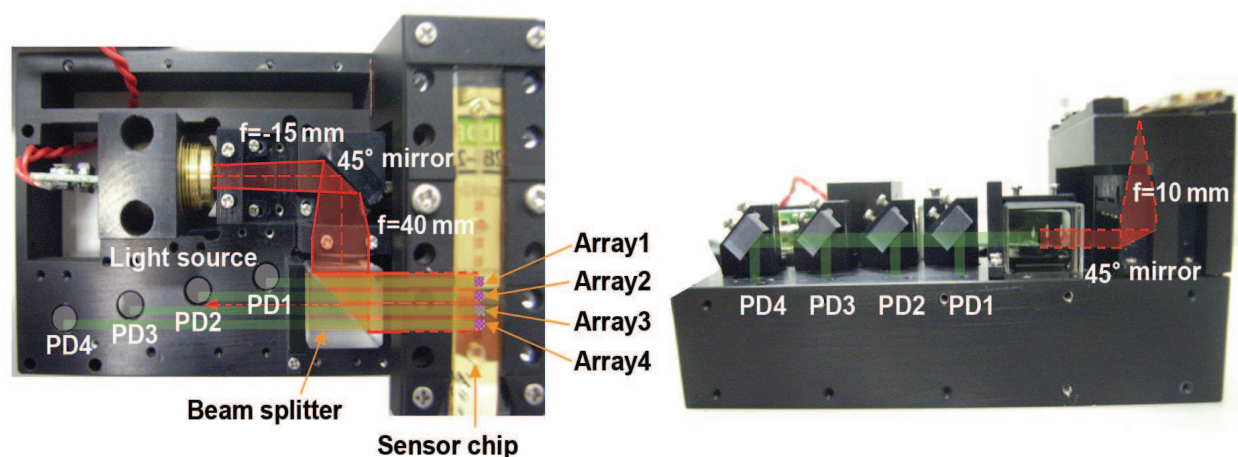

Fig. 16. Optical system inside the palm-sized nanoimprinted SPR biosensor.

To demonstrate the detection of biomolecules by using this model, we have attempted to detect avidin protein. The sensor surface is previously modified by the biotinylated alkyl thiol (BAT) layer according to the protocol below.

1.   Biotin-terminated tri(ethylene glycol)hexadecanethiol (OEG-BAT) (Assemblon, USA) and 11-hydroxy-1-undecanethiol (HUT) (Dojindo, Japan) are solved in 99.5% ethanol.
2.   The sensor chip was immediately immersed in the ethanol solution containing 0.95 mM OEG-BAT and 0.05 mM HUT.
3.   The sensor chip is rinsed with an ethanol solution after 10 minutes of immersion.
4.   The sensor chip is dried under a stream of nitrogen.

5. Inject 0.1 mg/mL NeutrAvidin (Thermo Scientific, USA) dissolved in Hepes-buffered saline (10 mM HEPES, pH 7.4, 150 mM NaCl) solution containing 0.005% (v/v) Surfactant P20 and 3 mM EDTA.

The result is shown in Fig. 17. NeutrAvidin was injected at about t=50 sec. As a result, the reflection change of 6% was observed in this system. This result indicates that the detection of biomolecules can be realized by this small and simple model.
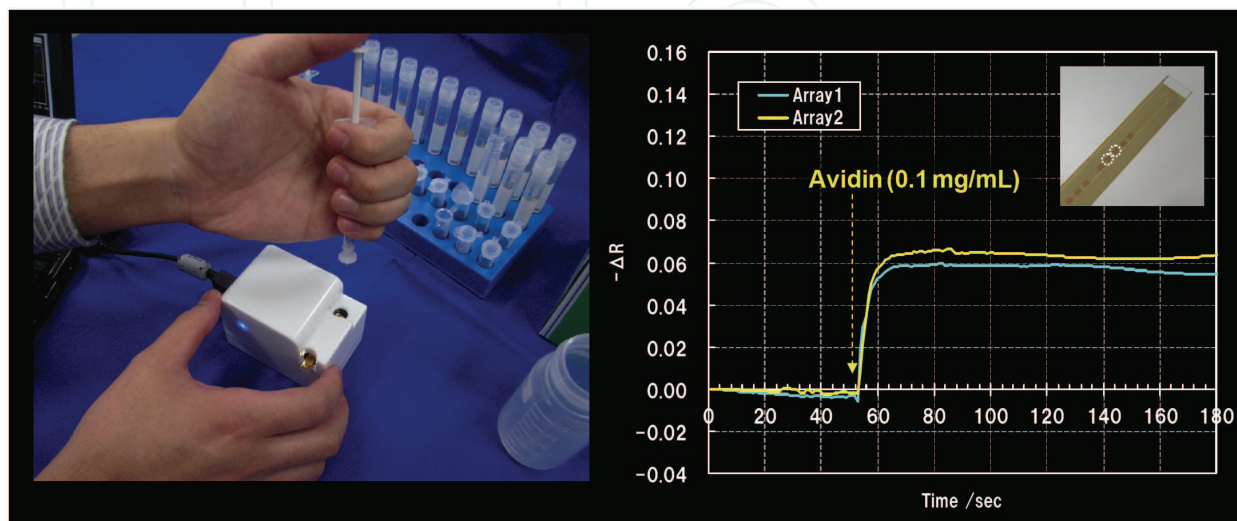

Fig. 17. Avidin protein detection using the palm-sized nanoimprinted SPR biosensor.

8. Conclusions

In this study, we accomplished a 10-times-higher signal-to-noise ratio measurement than conventional SPR using an SPR biosensor based on a gold nanogroove surface. Notably, using nanoimprinting technology, we have developed metal nanopatterns on a sensor surface with a process that has high reproducibility and throughput. Furthermore, the size of the prototype based on this detection principle was 10 times smaller than in commercialized SPR systems. Using the SPR prototype, we accomplished quantitative AFP detection as low as ~20 ng/mL. For this, 1) the smaller thickness of the probe layer and 2) the immobilization of antibodies on the sensor surface in a well-oriented manner were essential. A self-assembled fusion protein, the ORLA18 layer, was useful for accomplishing this purpose. Decreasing the size of the flow cell to reduce the diffusion time of the AFP was also very important. To the best of our knowledge, the detection limit of 20 ng/mL is the highest sensitivity achieved for direct detection of AFP using an SPR biosensor.

Recently, we were also successful in detecting AFP of about 100 pg/ml with an enhanced assay method by stepwise application of a biotinylated secondary antibody and streptavidin-bound colloidal gold. This suffices as a practical way to measure other important biomarkers such as prostate-specific antigen and carcinoembryonic antigen. Thus, the effectiveness of our SPR sensor has been demonstrated by the achievement of a high signal-to-noise ratio and highly sensitive detection of tumor marker protein. Our nanoimprinting technology-based SPR biosensor technology will have various useful applications, such as for medical diagnoses, environmental monitoring, and in the food industry.

9. Acknowledgments

10. References

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