

Monitoring surface-assisted biomolecular assembly by means of evanescent-field-coupled waveguide-mode nanobiosensors

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Abstract Biological self-assembly is a natural process that involves various biomolecules, and finding the missing partner in these interactions is crucial for a specific biological function. Previously, we showed that evanescent-field-coupled waveguide-mode sensor in conjunction with a SiO₂ waveguide, the surfaces which contain cylindrical nanometric holes produced by atomic bombardment, allowed us to detect efficiently the biomolecular interactions. In the present studies, we showed that the assembly of biomolecules can be monitored using the evanescent-field-coupled waveguide-mode biosensor and thus provide a methodology in monitoring assembly process in macromolecular machines while they are assembling.

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Introduction

All molecular interactions that take place in the cells are classified as interactome that includes interactions between protein and protein, protein and small ligand, DNA and protein, RNA and protein, and others. Since these interactions are very important, a wide range of methods are available for their detection. Berggård et al. [1] have reviewed the analysis of protein–protein interactions by the use of affinity-tagged proteins, the two-hybrid system, and some quantitative proteomic techniques. Previously, we have analyzed antigen–antibody, protein–lipid, and protein–RNA interactions, and interactions of proteins involved in human blood coagulation by a system that is based on the surface plasmon resonance (SPR) technique [2–4]. To characterize protein–protein interactions in vitro, Miernyk and Thelen [5] examined five biochemical approaches, namely, co-immunoprecipitation, blue native gel electrophoresis, in vitro binding assays, protein crosslinking, and rate-zonal centrifugation. Each of these approaches has its own strengths and weaknesses, particularly with regard to the sensitivity and specificity of the method. A high sensitivity means that many of the interactions that occur in reality can be detected by the screen. The main requirements for a biosensor approach to be valuable in research and commercial applications in preference to laboratory-based techniques are that the target molecule can be readily recognized and that suitable biological-recognition elements are readily available and can be easily handled.

Among several analytical devices that can be used to analyze biomolecular interactions, waveguide sensors are a good choice and have several advantages. With a nanopore-fabricated surface, a waveguide-based sensor can have a large surface area that confers sensitivity [6]. The evanescent-field-coupled (EFC) waveguide-mode sensor that we used in this study has a multilayer structure of SiO₂ waveguide, a thin single crystalline Si layer, and a SiO₂ glass substrate. The sensor design is that of the Kretschmann configuration, in which modifications in the dielectric environment near the waveguide surfaces are detected with a high sensitivity by measuring change in reflectivity [7, 8]. Reflected spectra are measured by rotating the resonator with respect to the laser and recording the reflected signal intensity as a function of the angle of incidence. Shifts in this resonance resulting from changes in the local refractive index as a result of the adsorption of biomaterials can be used as biosensor for nucleic acids or proteins. The total sensing system with a Kretschmann geometry is shown in Fig. 1.

Previously, we have analyzed the influence of nanometric holes on the waveguide surfaces, and it was shown that increase in the hole sizes was proportional to the increase in the sensitivities. The surfaces with larger than 50 nm holes was found to be more suitable for biomolecular interactions as determined by the Evanescent-field-coupled waveguide mode sensor. However, hole sizes on the surfaces exceeding 100 nm causes scattering, and interference with visible light leads to

weakening and broadening of the resonances [9]. On these optimized bombarded surfaces and using amine-coupling reactions, in the present investigation, streptavidin was attached to the glutaraldehyde (Glu)-functionalized surface, and then subsequent assembly of various biomolecules on the streptavidin moiety was evaluated by using the EFC-waveguide as a SiO₂-based sensor chip. The use of this surface modification for protein attachment and the creation of an environment for various biomolecular interactions can give an ideal platform for the analysis of broad range of biological entities, especially in identifying unknown interacting partners and inhibitor library screening for drug discovery.

Materials and methods

Reagents and biomolecules

We purchased 3-(triethoxysilyl)propan-1-amine (3APTS) from Sigma-Aldrich (Tokyo, Japan) and Glu from Wako Chemicals (Osaka, Japan). ImmunoPure streptavidin was obtained from Pierce Biotechnology (Woburn, MA, USA). Chemically synthesized biotin-dT₂₀ was purchased from Sigma Genosys (Tokyo, Japan). Factor IX was purchased from American Diagnostica (Stamford, CT, USA), and Factor-IX binding protein (Factor IX-bp) was purified from the venom of the habu snake (*Trimeresurus flavoviridis*), as discussed before [4]. The hemagglutinin (HA) protein of the influenza A virus was kindly provided by Dr. Kawasaki (National Institute of Advanced Industrial Science and Technology, Japan). Anti-HA influenza A monoclonal antibody was purchased from Chemicon, USA.

A 33-mer stable RNA-aptamer, previously selected against factor IXa [10], was enzymatically synthesized by in vitro transcription using T7 RNA polymerase on a synthetic DNA template. A template with the T7 promoter region (in italics) 5'-AGTAATACGACTCACTATAGG-GATGGGGACTATACCGCGTAATGCTG-3' was synthesized to generate the double-stranded DNA. Using this template DNA and the primers 5'-AGTAATACGACTCACTATAGG-3' (forward) and 5'-(T)₂₄ATGGGGAGGCAG-CATTACGCGGTATA-3' (reverse), a polymerase chain reaction (PCR) reaction was performed with a commercial PCR kit (Ex Taq kit, Takara Bio, Shiga, Japan). The reaction mixture was cycled at 94 °C for 70 s, 55 °C for 50 s, and 72 °C for 70 s for 15 cycles. The PCR product was precipitated and used for RNA preparation by in vitro T7 transcription. Transcription was performed at 37 °C overnight, with a DuraScribe transcription kit (Epicentre Biotechnologies, Madison, WI, USA). Afterwards, the products were treated with 2 U of DNase I (RNase free) for 10 min at 37 °C to remove the template DNA. They

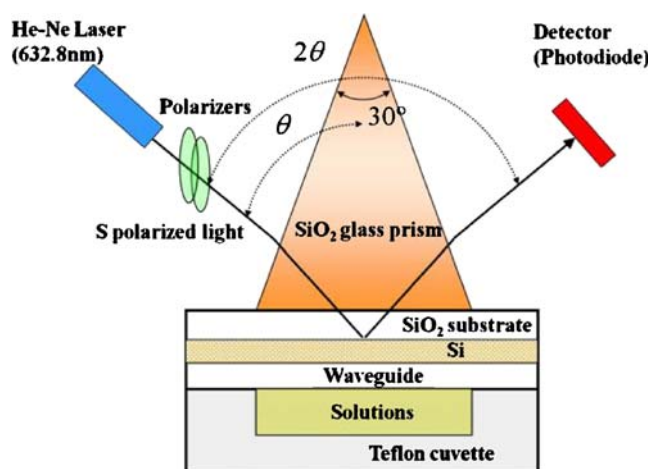


Fig. 1 Schematic representation of the experimental setup for the analysis of biomolecular interactions. The samples are optically matched to the base of a silica glass prism and mounted on a goniometer. A Teflon cuvette containing the sample is connected to the waveguide side of the samples. A He-Ne laser operating at 632.8 nm irradiates the single crystalline Si layer at angles higher than the angle necessary for total internal reflection. The reflected light is detected by a photodiode. The photodiode is also mounted on another goniometer and moved simultaneously with the samples. Appropriate samples are injected into the Teflon cuvette and analyzed

were then mixed with an equal volume of 2× urea buffer (7 M urea, 50 mM ethylenediamine tetraacetic acid, 90 mM Tris borate containing 0.05% bromophenol blue). The reaction mixtures were denatured at 90 °C for 2 min and fractionated on a 15% polyacrylamide gel containing 7 M urea. The RNA band was excised, and the RNA was eluted from the gel. The RNAs were vacuum-concentrated, re-dissolved in water, and then assayed by means of their absorbance at 260 nm. The resulting transcribed RNA (57-mer) had a 33-nucleotide moiety that specifically recognizes human factor IXa at its 5' end and 24 nucleotides of A residues at its 3' end. For the negative reaction, a single-stranded DNA molecule with 20 bases (dT₂₀) was synthesized chemically.

Preparation of the waveguide sensor surfaces

We used the same method for preparing the waveguide surface that we have used previously [11]. The substrate that we used was a silicon-on-quartz substrate (Shin-Etsu Chemical) comprising a 440-nm-thick single crystalline Si (100) layer on a 1.2-mm-thick SiO₂ glass substrate. The substrate was cut into plates measuring 25×25 mm. These plates were thermally oxidized in an electric furnace in an atmosphere of O₂ containing water vapor at 1,000 °C at ambient pressure for 1 h. The resulting oxidized layer forms the waveguide layer. The waveguide was exposed to 137-MeV Au ions at room temperature and a residual pressure below 1×10⁻⁴ Pa by using the 12 UD Pelletron tandem accelerators at the University of Tsukuba. Then, hydrofluoric acid (HF) vapor etching was applied to the exposed waveguide using 20% aqueous HF solution.

Functionalization of the SiO₂ surface

To functionalize the SiO₂ waveguide with amine groups, the chip was first immersed in a hydroxyl solution for 24 h at room temperature to attach OH groups to the surface of the silica. Free hydroxide ions were removed by washing several times with (Hepes)–KOH (pH 7.4). The modified surface of the waveguide substrate was further modified by immersion in a 0.5% (v/v) ethanolic solution of 3APT for 24 h. The 3APT reacted with surface hydroxyl groups of the SiO₂ waveguide to give an amine group-functionalized substrate that was rinsed with ethanol and dried in a stream of nitrogen gas.

Preparation of streptavidin-derivatized SiO₂ surface and analysis of assembled biomolecular interactions

The amine-functionalized SiO₂ surface, produced as described above, was treated with a 2.5% solution of Glu for 3 h at room temperature to form an aldehyde-activated

surface. After incubation, the surface was washed thoroughly with Hepes–KOH (pH 7.4). A 100-nM solution of streptavidin was injected, and the surface was incubated until it reached saturation. After incubation, the injection chamber was thoroughly washed with Hepes–KOH buffer to remove any free molecules, and measurements were taken. Unreacted free aldehyde groups were blocked by treatment with a 3% solution of bovine serum albumin (BSA). By using these surfaces, the interactions of streptavidin with biotin-dT₂₀ were monitored at a concentration of 100 nM; other steps were similar to those previously described. By using this streptavidin–biotin-dT₂₀ layer as a base, interactions with various biomolecules (anti-factor IX aptamer, factor IX, and factor IX-bp) were analyzed at a 100-nM concentration. Each analysis was performed at a saturation level of attachment with thorough washing to remove free molecules. Specific interactions between factor IX-bp and factor IX were determined as described before using surface plasmon resonance [4]. Interactions of antibody–antigen on the present system were monitored with anti-HA monoclonal antibody and HA of influenza A with the concentrations of 25 and 100 nM, respectively.

Measurement with the Kretschmann geometry

The system for the waveguide-mode sensor with Kretschmann geometry is shown in Fig. 1. All measurements were performed as described previously [11]. Briefly, the sensor chip is mounted on the base of a SiO₂ glass prism to form an optically contiguous medium. An S-polarized He–Ne laser operating at 632.8 nm was used as the light source for the measurements. The beam was directed onto the base of the prism at an angle that gave total internal reflection. We also clamped a Teflon flow cell onto the waveguide to permit the injection and extraction of various dielectric media and reagents. To observe the optical waveguide modes, the reflectivity was measured for various angles of incidence at a constant temperature of 20.8 °C.

Results and discussion

Biologists are interested in biomolecular assembly events, particularly those that occur in cells, so it is essential to be able to identify missing partners in these events by using real-time monitoring systems. In developing chemical and biological sensors using functionalized SiO₂ surfaces, we have previously demonstrated the feasibility of using the EFC-waveguide sensor to detect biomolecules and have shown that the presence of nanometer-sized holes on the SiO₂ sensor chips improves the sensitivity of detection [6, 12] as a result of an increase in the number of adsorbed

molecules on the surfaces. We have also shown that these types of chips are mechanically and environmentally stable [12]. The presence of a single crystalline Si layer for reflection has been shown to provide a good sensitivity and stability [11], so this sensor system combines good optical and mechanical properties to permit the detection of biomolecular interactions. Moreover, waveguide sensors can detect small amounts of adsorbed materials on their surface and are capable of a higher sensitivity sensor [7, 12, 13]. Since, s-polarization shows higher sensitivity rather than p-polarization [8], in the present study we used s-polarization. To get good waveguide propagation, SiO₂ layer (~480 nm) was thermally grown on Si layer (~220 nm) and shown in Fig. 1. With this setup, we modified the SiO₂ surface to permit attachment of proteins, and we demonstrated the system to be suitable for analysis of various events involving the assembly of interacting biomolecules.

To prepare the surface for such analysis, we modified the SiO₂ surface by stepwise chemical treatments and finally functionalized the surface with Glu to permit coupling to amine groups (Supplementary Fig. 1). First, hydroxyl groups are introduced onto the surface of the SiO₂ by immersion in an aqueous hydroxide solution. After this modification, amine groups are introduced by coupling with a bifunctional silane reagent, as reported previously [12]: this kind of silane coupling reaction has been shown to be suitable for the immobilization of biomolecules [14, 15]. On the amine group-functionalized surface, we formed a link with the aldehyde group at one end of a Glu molecule and left other terminal aldehyde group of Glu free to react with a protein to form a Schiff's base [16]. As reported in the literature, the Glu coupling reaction on chip was performed at a pH of 7.4, a slightly alkaline environment, as a lower pH than this would reduce the efficiency of the coupling reaction [16]. In a similar manner, Montisci et al. [17] covalently coupled lectins through Glu to poly(lactide) microspheres: in this case, Glu reacts with hydroxyl or amino groups. In the present study, with the aid of the EFC-waveguide sensor, we used SiO₂ modified with an amine-coupling reagent and Glu (2.5%) as a homobifunctional reagent to study assembly processes involving proteins and nucleic acids, and we commonly used the same concentration (100 nM) which is higher than the sensitive range used before.

Streptavidin is tetrameric protein with a molecular mass of about 60,000 Da that is widely used in molecular biology because of its extraordinarily strong affinity to the vitamin biotin; this interaction ranks among one of the strongest known noncovalent interactions. To analyze these interactions and other biomolecular assembly processes using a SiO₂ surface that is suitable for use as an EFC-waveguide sensor, the following steps were carried out, as summarized

in Fig. 2. The streptavidin was immobilized by covalent bonding to the free aldehyde groups of the silica-attached Glu molecules, and any residual free unreacted Glu aldehyde groups were subsequently blocked by reaction with BSA. After thorough washing of the assembly with Hepes–KOH (pH 7.4), excitation of a waveguide mode was experimentally observed as a sharp dip in reflectivity as a function of the incident angle. In this optical system, changes in the angular positions ($\Delta\theta$) were measured as 0.15° and 0.12° for Glu and streptavidin attachments, respectively; similarly, the maximum changes in reflectivity (ΔR) these molecules were 0.09 and 0.07 (Fig. 3a, b). To evaluate the suitability of the streptavidin-modified surface for studies on streptavidin–biotin interactions and for the hybridization of Watson–Crick base pairs, we injected a solution of the biotin-attached poly(T) bases until saturation was reached. When the streptavidin was completely saturated with biotin-dT₂₀ molecules, measurements were taken, and shifts in the resonance showed values of $\Delta\theta=0.06^\circ$ and $\Delta R=0.04$ (Fig. 3c). This shift also includes signal from the interactions between the unreacted Glu and BSA. This signal could be at its minimal as we used excess of streptavidin to react with the Glu surface. With biotin-dT₂₀ molecules as the base layer, we then went on to study various other biomolecular interactions to demonstrate the suitability of the system for studies on a broad range of biomolecules. To check the specificity of this surface, we first injected a DNA molecule with similar bases (dT₂₀) as a noncomplementary negative reaction. As expected, we did not detect any angular shift upon injection of the noncomplementary DNA, indicating that no further attachment occurred with this molecule (Fig. 3d). For hybridization analysis using the biotin-dT₂₀ surface, we injected the RNA-aptamer molecule containing a poly(A₂₄) moiety onto the biotin-dT₂₀-modified surface, and we found a shift in the spectrum with values of $\Delta\theta=0.11^\circ$ and $\Delta R=0.07$ (Fig. 3e). Here, we used an important aptamer against factor IXa from the human coagulation system that has previously been shown to be a potential anticoagulant in clinical tests [10, 18]. The changes in the resonance shift during this molecular assembly indicated the specificity and suitability of the linker system in further analyses with the EFC-waveguide sensor. Hybridization of RNA with poly(A₂₄) on biotin-dT₂₀ was performed with four additional bases to create a spacer during duplex formation, and greater spectral changes were observed. This surface is suitable for attaching any aptamer and for their analysis with a target ligand as a diagnostic tool: this is an alternative to the use of antibodies [2]. To prove the utility of the present sensor system, we attached the anti-factor IXa aptamer, which can bind to factor IX or factor IXa with equal affinity, and analyzed the interaction with factor IX in the presence of calcium ions, as reported before [3, 10].

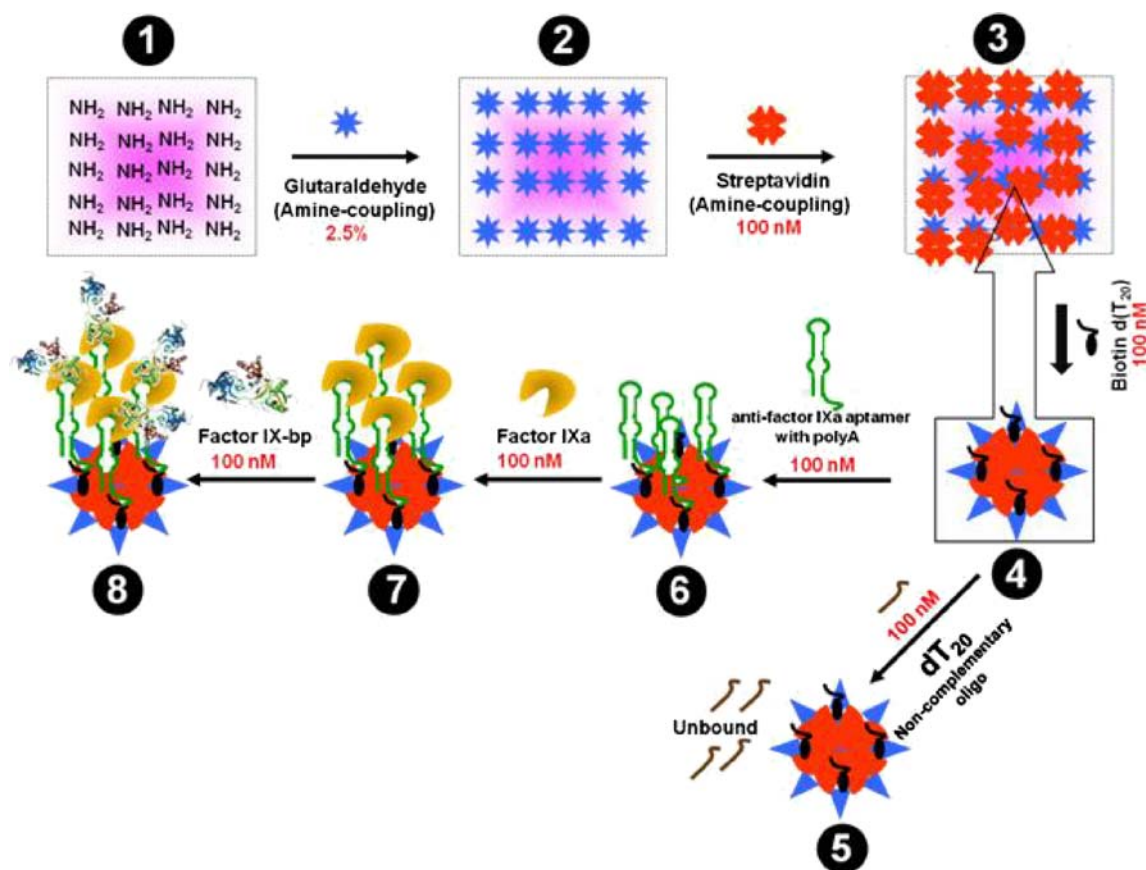


Fig. 2 Schematic representation of the assembly of biomolecules on the SiO₂ surface through the glutaraldehyde linker. Glu (2.5%) was first linked to amino groups on a functionalized SiO₂ waveguide, and other end of the Glu molecule was then reacted with amine groups present on the streptavidin (100 nM). Biotin-dT₂₀ (100 nM) was

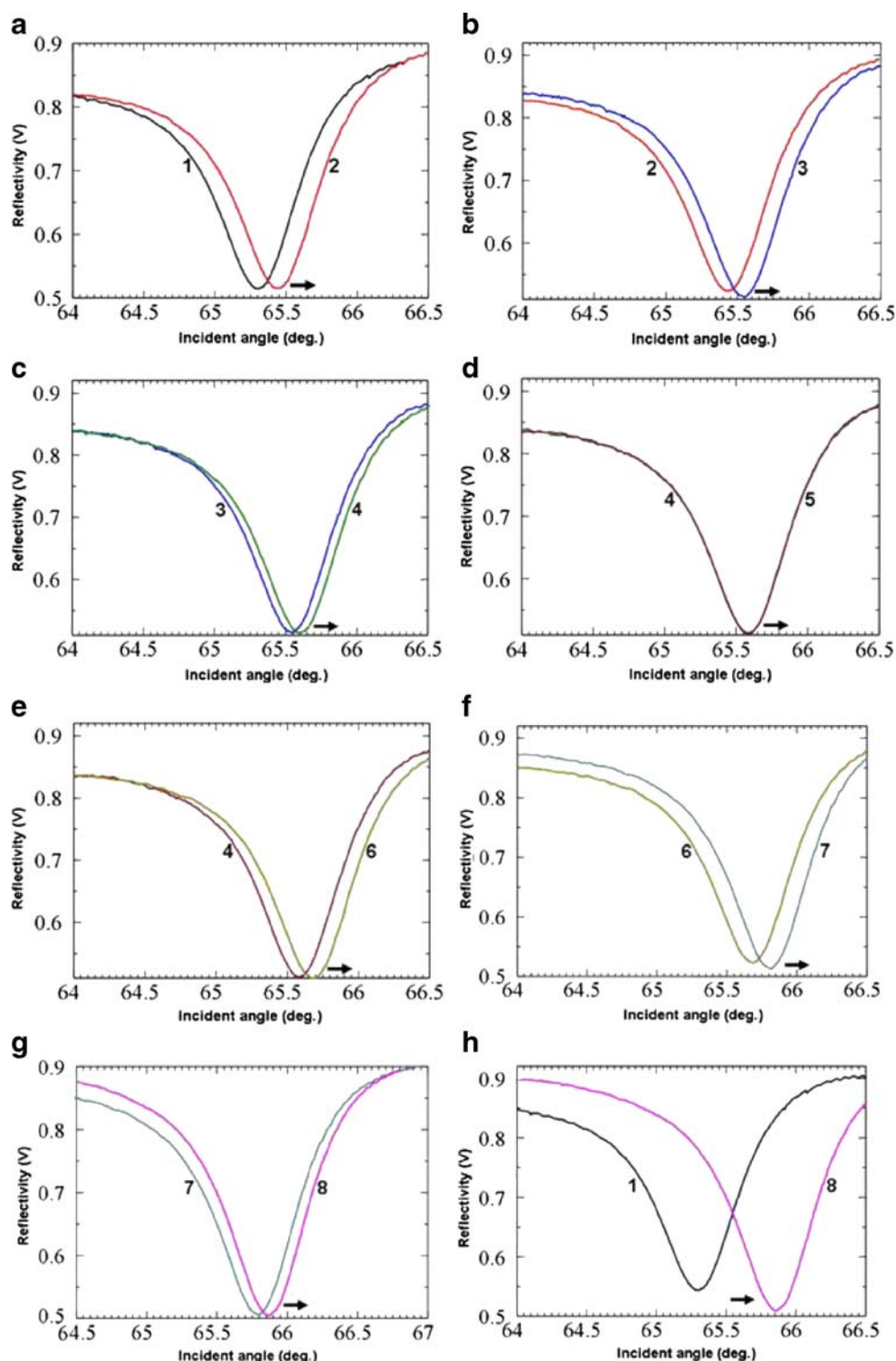
passed through streptavidin layer in order to attach anti-factor IXa aptamer containing polyA tail (100 nM). Further, human factor IXa, followed by factor IX binding protein, was assembled with similar concentrations, and all binding events were evaluated by using the EFC-waveguide-based sensor

When we injected the factor IX onto the anti-factor IXa RNA-aptamer-attached surface under suitable buffering conditions, we observed a clear angular shift, with values of $\Delta\theta=0.12^\circ$ and $\Delta R=0.09$ (Fig. 3f). To prove the specificity of attachment of these molecules, we injected factor IX-bp onto a surface coated with factor IX in the presence of calcium and magnesium ions, which are important for proper molecular folding and interactions [4, 19]. We found a specific angular shift with values of $\Delta\theta=0.07^\circ$ and $\Delta R=0.04$ (Fig. 3g). Figure 3h shows the shift in resonance associated with the overall molecular attachments until factor IX-bp binding: this corresponds to a $\Delta\theta$ of 0.55. These results not only correlated with our SPR analysis (Supplementary Fig. 2) but were also in agreement with our previous analysis that the calcium and magnesium ions are essential for the interactions between the factor IX-bp and factor IX [4].

Similarly, to prove the reliability of present sensor system, we attempted to replicate a sandwich assay routinely used in diagnosis of important proteins. Specifically, we attempted a sandwich assay using hemagglutinin

of influenza A virus and their monoclonal antibody as shown in Fig. 4. For this, initially, as mentioned above, Glu surface on the chip was made to attach anti-HA monoclonal antibody. Stepwise attachment of anti-HA antibody on Glu layer followed by HA of influenza A was performed and clear shifts were observed as described in above case. Further, when we make the sandwich of HA molecules between anti-HA, the changes in the resonance was found to shift further (Fig. 5a–d). These results indicate the suitability of waveguide-mode sensor for antibody–antigen interactions. In general, two kinds of antibodies that recognize two regions of antigens are used in a sandwich assay, but we used only one kind of monoclonal antibody against the HA in our sandwich assay for demonstration purpose. Our results suggest that the HA antigen that we used has the ability to bind two antibodies, as HA known to form trimer. The results obtained from these investigations proved that the modified surface of the SiO₂-waveguide sensor can be used to monitor interactions of various biomolecules during assembly events. As reported before, this EFC

Fig. 3 The angular shifts obtained upon binding of various molecules onto the SiO₂ surface: **a** attachment of Glu; **b** immobilization of streptavidin on Glu; **c** attachment of biotin-dT₂₀; **d** negative reaction with dT₂₀; **e** attachment of anti-factor IX aptamer with poly(A₂₄); **f** immobilization of human factor IX on anti-factor IX-attached surface; **g** attachment of factor IX-binding protein onto factor IX-immobilized surface; **h** angular shift obtained between amino-surface (*I*) and factor IX-binding protein (8). Arrows indicate the direction of the angular shift. Glu (2.5%) was used; all other molecules were used at a 100-nM concentration. Numbering (1 to 8) represents the various steps (see Fig. 2)



sensor has been successfully used in studies of biomolecular interactions to detect low concentrations (of the low-nanomolar order) of adsorbed molecules; in this system, an altered dispersion relation of waveguide modes by molecular adsorption causes a strong perturbation of the mode, resulting in a significant shift in the incident angle at which the mode is excited [6, 7, 12].

To improve our understanding of biological interactions, we need sensors that are capable of recognizing biomolecules, so the interaction of surface and interfacial chemistry with the biological response of materials is a fundamental area of study in the field of biomaterial surface science [20, 21]. On the basis of our present results with the reagents 3APT and Glu, which are common coupling agents for

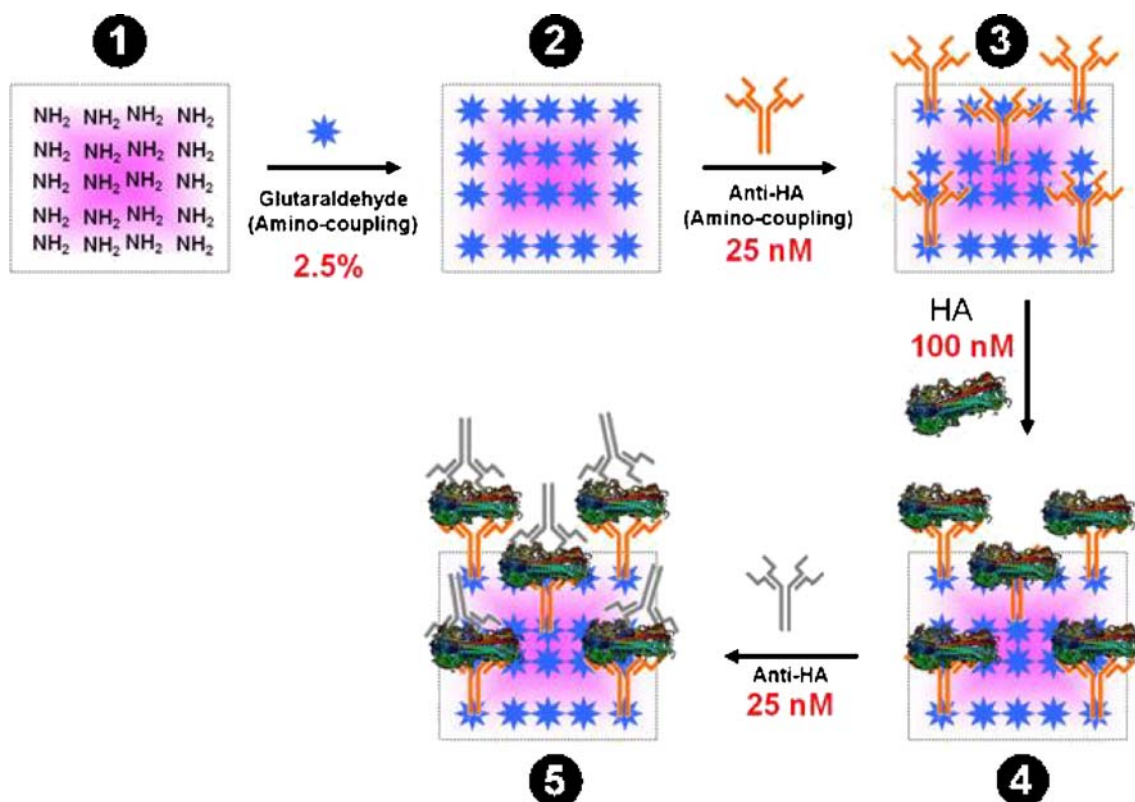
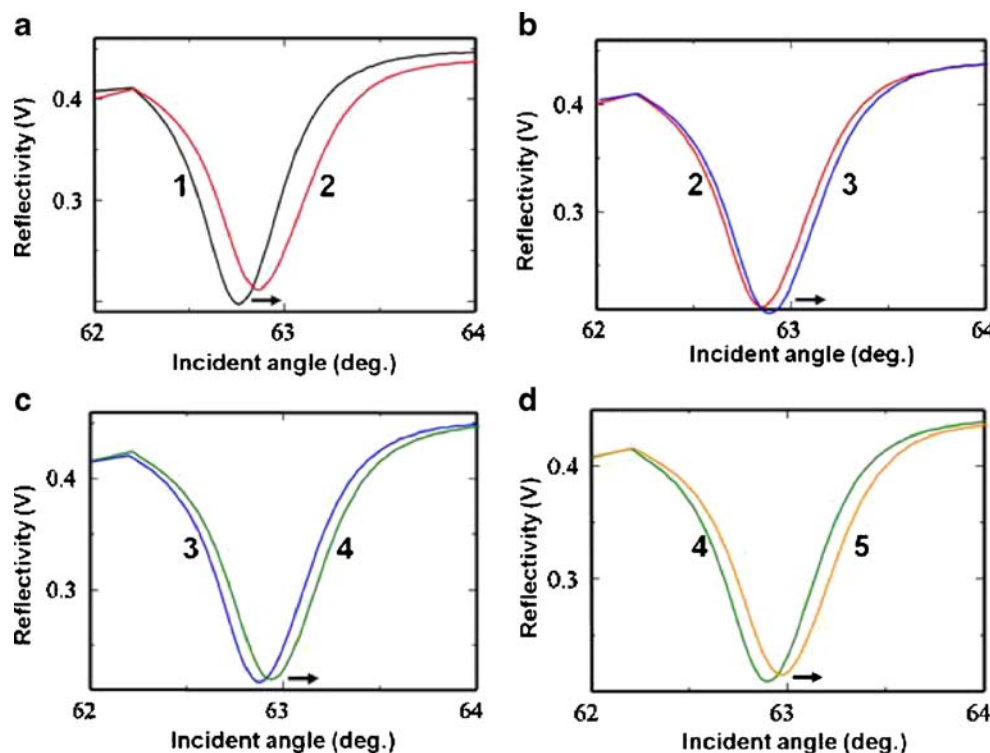


Fig. 4 Schematic representation of the sandwich model on the SiO₂ surface through the glutaraldehyde linker. Glu (2.5%) was first linked to amino groups on a functionalized SiO₂ waveguide, and other end of the Glu molecule was then reacted with amine groups present on the anti-hemagglutinin monoclonal antibody (25 nM). One hundred

nanomolars of HA from human influenza A was passed through anti-HA. Further, anti-HA was again allowed to bind with the similar concentration (25 nM), and all binding events were evaluated by using the EFC-waveguide-based sensor

Fig. 5 The angular shifts obtained upon binding of HA of influenza A virus and their monoclonal antibody onto the SiO₂ surface. The molecules are attached as follows: **a** attachment of Glu; **b** immobilization of anti-HA monoclonal antibody on Glu; **c** attachment of HA on anti-HA antibody; **d** attachment of anti-HA on HA. Arrows indicate the direction of the angular shift. Glu (2.5%) was used. Anti-HA and HA are used as 25 nM and 100 nM, respectively. Numbering (1 to 5) represents the various steps (see Fig. 4)



silicon-based materials [22], it is clear that surface modifications of SiO₂ chips and a detection system with an EFC waveguide mode form a suitable immunosensor for studies on antigen–antibody interactions and for the detection of protein–ligand assembly events. In addition, the possibility of the attachment of chains of a molecular assembly, as demonstrated here, will open a pathway to the analysis of any biomolecule of interest. The approach with antibody–antigen or interactions of proteins and other ligands on SiO₂ is amenable for studies to be carried out by several detection systems. For example, by using *Schistosoma japonicum* antibodies, Wang et al. [15] have performed an immunoagglutination assay by using SiO₂ particles as the sensitive system. Moreover, the surface modification of SiO₂ in a manner suitable for use in an EFC device, as presented here, permits the use of real-time, label-free evanescent wave methods for studying protein–ligand interactions, and it is possible to discriminate between changes in reflectivity originating from specific interactions from those associated with nonspecific surface interactions. The present studies may also result in the development of suitable platforms for identifying inhibitors of biomolecular assembly processes.

These kinds of EFC waveguide-mode sensors are capable of detecting lower concentrations of molecules similar to SPR sensors [8, 23, 24] and also carries advantages over SPR. It has been known that dips seen in reflectivity due to induction of waveguide modes are much sharper than those due to SPR, and the sharp resonance brings about a higher sensitivity. In addition, it has also been shown that perforation of a waveguide in an EFC waveguide sensor effectively enhances its sensitivity. The EFC waveguide sensor applied in the present research consists of a SiO₂ glass substrate, a single crystalline Si layer, and a thermally grown SiO₂, and the layers are atomically bonded with each other, whereas a typical SPR sensor uses a high refractive index glass plate coated with Au thin layer. According to the difference in the structures, the EFC waveguide sensor is more stable than SPR sensors, and high reproducibility is expected by the EFC waveguide sensor. The methodology described in the present study will not only be useful in identifying unknown partners of biomolecular receptors during biomolecular self-assembly events but also be useful in identifying bound molecules, as these can be recovered from the sensor surface. Additionally, this system is suitable in drug discovery for finding appropriate drug leads from chemical libraries.

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