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Development of label-free optical diagnosis for sensitive detection of influenza virus with genetically engineered fusion protein

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ABSTRACT

An active immobilization method utilizing the metal-binding property was developed and examined for its ability to facilitate the biosensing of avian influenza virus. The special biosensing performance with optical plasmonic analysis, including surface plasmon resonance (SPR) was evaluated on gold substrate and also by SPR imaging (SPRi) and localized SPR (LSPR) system where antigen–antibody interaction occurs. A complete optical analytical system was developed by integrating microarray and fabricating nanoparticles onto a single glass chip, thus allowing specific and sensitive diagnosis with subsequent binding. Reaction condition for the maximum reactivity was optimized by SPR analysis and more sensitive interaction was performed by SPRi analysis. Furthermore, ultra-sensitive detection was successfully developed up to the target molecules of 1 pg mL^{-1} by LSPR analysis. The advanced phase-in of enhanced plasmonic sensing system allows more efficient and sensitive detection by switching fabrication processes, which were prepared on the gold surface using the nanoparticles. This inflow contains the gold binding polypeptide (GBP)–fusion protein, which was expressed in recombinant *Escherichia coli* cells, was bound onto the gold substrates by means of specific interaction. The GBP–fusion method allows immobilization of proteins in bioactive forms onto the gold surface without surface modification suitable for studying antigen–antibody interaction. It was used for the detection of influenza virus, an infectious viral disease, as an example case.

1. Introduction

Various valuable real-time tools, *in situ* analysis of dynamic surface events, have been developed for studying biomolecular interactions on the ultra-thin films [1–3]. Aside from the design of label and probes, active areas of research include an advanced method such as time-resolved or spatially resolved, evanescent wave, laser-assisted spectroscopy, surface plasmon resonance (SPR), and multidimensional data-acquisition [4–8]. Among them, SPR sensor system offers the advantages of label-free, rapid response time, highly sensitive and real-time detection of binding events between biomolecules [1–5]. Unfortunately, conventional SPR reflectometry sensor requires sophisticated optical instrumentation associated with the detection system. This limitation is significant, because biosensors are urgently in demand for high-throughput and cost-effective monitoring. For the enhancing the detection sensitivity, fiber bundle also has been employed for purposes of imaging or for biosensor array in recent years [3–8]. Fiber optic sensors are based on either direct or indirect (indicator-based) sensing schemes. In the first, the intrinsic optical properties of the analysis must be measured, for example its refractive index, absorption, or emission. In the second, the color or fluorescence of an immobilized indicator, label, or any other optically detectable bioprobe have to be monitored. The success of SPR in general and in the form of fiber-optic sensors, in particular, is impressive.

To overcome the limit of detections, we evaluated a SPR, SPR imaging (SPRi)- and localized SPR (LSPR)-based optical biosensor for label-free monitoring of biorecognition events. As to gold nanoparticles, SPR is localized at the particle surface, and therefore the use of these LSPR for sensing as in the case of non-planar metal surface is possible. The Mie theory describes that there is a restriction for the movement of electrons through the internal metal framework, when the size of the metal particle is scaled down to nano-level [9]. The collective charge density oscillations of nanoparticles are defined as LSPR. LSPR absorption bands are characteristic of the type of the nanomaterial, the diameter

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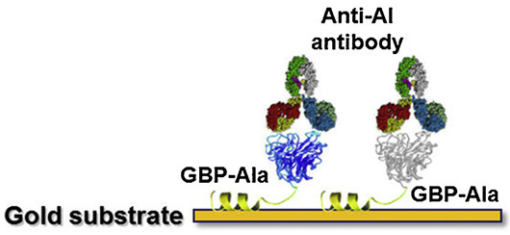

Fig. 1. Schematic diagram of bio-recognition element onto the gold substrate by GBP–fusion proteins. GBP–AIa immobilized on the bare gold surface is sequentially bound by specific interaction between AIa and its anti-AI antibody.

of nanoparticles and their distribution [10]. LSPR-based optical biosensor can be set up without using the specific configurations, for example, the attenuated total reflection optical setup or waveguide coupling. It is possible to fabricate very small devices based on the LSPR technique with a simple optical setup.

With the advances in biosensor technology, many kinds of biosensors based on LSPR have been developed. Using a gold nanoparticle monolayer immobilized on the glass substrate, Okamoto and Yamaguchi has observed the increase of both wavelength and absorbance of the LSPR band as the reflective indices of the sample solutions or the thickness of the films on the surface increased [11]. Developing the biosensor from this technique, Nath and Chilkoti reported an efficient assay for quantify biomolecular interactions in real time on the functionalized surface by measuring the transmission absorption spectra [12]. Moreover, they optimized their chips by investigating the influence of nanoparticle sizes on the sensitivity of the sensors [13]. Another type of LSPR-based biosensor based on triangular silver nanoparticles was developed [14–16]. These silver nanostructures were measured by UV–vis extinction spectroscopy. The determination of organophosphorous pesticides using LSPR has recently been reported by Lin et al. [17]. Chau et al. reported the development of fiber-optic chemical and biochemical probes based on LSPR [18]. Fujiwara et al. measured the binding between the antibodies and proteins using LSPR [19]. Liu et al. recently developed a nanoplasmonic molecular ruler for measuring the nuclease activity and DNA foot printing [20].

However, the device fabrication has been time-consuming and controlling the uniformity in the size of nanometals has been difficult, and resulted in poor reproducibility. For the improvement of these issues, a simple way to excite the LSPR phenomenon was archived by construct a gold-capped nanoparticle array chip. Himmelhaus and Takei have first reported this type of biosensor [21,22]. It depends on the monolayer formation of surface-adsorbed polystyrene spheres, following by the deposition of a thin gold layer on their surface. This LSPR biosensor has demonstrated its linear dependence on the refractive index of the surrounding environment. The shift to the longer wavelength as well as the change in absorbance strength was also observed in the devices as the biomolecules adsorb on the gold surface.

In this work, SPR-based optical biosensor using multi-spot gold-capped nanoparticle array chip for label-free detection of avian influenza (AI) virus as a rapid and user-friendly alternative to conventional techniques is demonstrated. The gold binding polypeptide (GBP)–fusion protein for the AI surface antigen immobilization was immobilized on the gold surface as shown in Fig. 1. Firstly, a bare gold chip was used for immobilization of the GBP–fusion protein, and its specific antibody can be bound subsequently. For enhancing the sensing signals, SPR imaging and LSPR analysis were developed in this study. The multi-spot gold-capped nanoparticle array chip, a LSPR exciting structure, was fabricated by using silica nanoparticles. The optimization as well as characteristics of the LSPR-based optical biosensor was then performed, which brings several advantages such as sensitivity, low-cost and ease to fabrication, and alternatively promises the recent complex optical system in the analysis applications for the dynamic biological interaction.

2. Experimental

2.1. Chemicals and reagents

Restriction enzymes were purchased from New England Biolabs (Berverly, MA, USA). Agarose was from Cambrex BioScience Rockland (Rockland, ME, USA). Unless otherwise stated, all chemical reagents were purchased from Sigma. Ni-NTA spin kit was from Qiagen (Hilden, Germany). All oligonucleotides were synthesized at Genotech (Daejeon, Korea).

2.2. Plasmid construction and protein preparation

Escherichia coli BL21(DE3) ([F$^-$ *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (DE3)], Novagen, Darmstadt, Germany) was used as host strains for general cloning works and gene expression studies. Polymerase chain reaction (PCR) experiments were performed with a PCR Thermal Cycler Mini (Bio-Rad) using High Fidelity PCR System (Takara). Restriction enzymes were purchased from New England Biolabs. DNA sequences of all clones were confirmed by automatic DNA sequencer (ABI Prism model 377, Perkin Elmer).

GBP is for the convenient monitoring of protein immobilization. As described in the previous report, the DNA fragments encoding GBP-fused genes were obtained by overlapping PCR amplification using the plasmid pET-6HGBP containing the 6 histidine and GBP coding gene [23]. The DNA fragments encoding the GBP and the AI viral surface antigen (AIa) were amplified by PCR amplification using the primers P1 (5′-GAAACAGCATATGCACCATCACCATCACCACCACGGCAAAACCCAGGCGACCAG-3′) and P2 (5′-GTACTCGAGGATCGGACGGTTGCTGCCTTTCCAGTTATCACGACAAGACTGAATGGTACCGCT-3′) and the plasmid pET-6HGBP as a template for the construction of 6His-GBP-AIa fusion gene. And then, the PCR product was digested with *Nde*I and *Xho*I, and then cloned into the pET-22b(+) to make pET-6HGBP-AIa.

Recombinant *E. coli* BL21(DE3) strain harboring pET-6HGBP-AIa was cultivated in Luria–Bertani (LB) medium (10 g L^{-1} bactotryptone, 5 g L^{-1} yeast extract and 5 g L^{-1} NaCl) supplemented with 100 μg mL^{-1} of ampicillin at 37 °C and 250 rpm. At the OD_{600} (DU 600 Spectrophotometer, Beckman) of 0.4, cells were induced with 0.1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) for the production of the GBP–fusion protein. After induction, cells were further cultured for 4 h, and harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C. The fusion protein was purified by affinity chromatography using Ni column. Protein concentration was determined by Bradford's method using bovine serum albumin (Sigma) as a standard. The purified proteins were characterized by high-performance liquid chromatography (Agilent) (see Supplementary Information Fig. S1).

2.3. Prediction of putative antigenic regions of H5N1 and H9N2 AI neuraminidase protein

By analyzing the primary structure of H5N1 and H9N2 AI neuraminidase protein obtained from a "chicken and H5N1 neuraminidase structure database" (http://protein.gsc.riken.go.jp/Research/Na/), the putative antigenic regions of envelope protein were predicted. The hydrophilicity, flexible region, antigenicity, and surface probability of H5N1 and H9N2 AI neuraminidase protein were calculated by Kyte–Doolittle plots, Karplus–Schulz prediction, Jameson–Wolf prediction, and Emini prediction, respectively (see Supplementary Information Fig. S2) [24–27].