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Highly sensitive localized surface plasmon resonance immunosensor for label-free detection of HIV-1

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Abstract

A highly sensitive label-free immunosensor for the detection of HIV-1 is newly developed based on localized surface plasmon resonance (LSPR) method. Uniform nanopattern of circular Au-dots (10-20 nm) was fabricated on indium tin oxide (ITO) coated glass substrate by simple electrochemical deposition method. The surface of Au nanopattern was modified with HIV-1 neutralizing gp120 monoclonal antibody fragments. The modified substrate was employed to measure various concentrations of HIV-1 particles quantitatively based on the shift of longitudinal wavelength in the UV–Vis spectrum which results from the changes of local refractive index induced by specific antigen-antibody recognition events. The detection limit of the HIV-1 particles was estimated to be 200 fg/mL, which is 10 fold higher than that of previously reported virus detection method based on LSPR. Since fabricated LSPR immunosensor has high sensitivity and selectivity, it is a promising approach for biological/medical sample analysis and various kinds of virus detection.

From the Clinical Editor: A localized surface plasmon resonance-based virus detection method is shown to have an order of magnitude improvement in detectable concentration of HIV-1 particles. Similar approaches may be used for screening other viral particles as well.

Key words: AIDS; HIV-1 virus; Localized surface plasmon resonance; Biosensor; Immunosensor

The detection and quantization of viruses are basis for various fields of applications from food production/sanitation to diagnostics and therapeutics.[1] Viruses are diverse in their mechanism but usually virus structures are comprised of DNA or RNA genome surrounded by capsid proteins, which are associated with an entry to the host cell.[2] Normally, all of the existing viruses require a host to propagate their lives and many are related to the human pathogens.[3] Generally, diseases such as flu or common cold are self-examined by the host immune response, but most serious disease caused by viruses are avoiding this response and threatening host survivals.[4] HIV is a well known variant to cause acquired immunodeficiency syndrome (AIDS) which not only leads to a life-threatening infections but also contains the generic symptoms that are difficult to diagnose. Therefore, rapid detection of viruses has emerged as important aspect for human lives for virus infection. But, conventionally used virus determination and quantification methods are not sensitive enough and they are tedious and time consuming.

To achieve this goal, various techniques have been developed for sensitive and selective virus biosensors based on QCM,[5–7] electrochemical,[8–11] and optical methods.[12,13] Among these methods, optical detection methods are particularly more promising compared with other methods. Unlike other methods, optical biosensors allow the signal transduction of the biomolecular binding reaction without any physical connection between the excitation source and the detection channel.[14] Moreover, optical biosensors are well-suited for the physiological solutions and do not change in the ionic strength of the sample solutions.[15] However, there are some drawbacks still persistent on the currently used optical biosensors, which requires precise alignment of light onto the sample surface.[16] As a result, the developed methods are not particularly suitable for medical applications.

Conflict of interest: Authors declared no conflict of interest.

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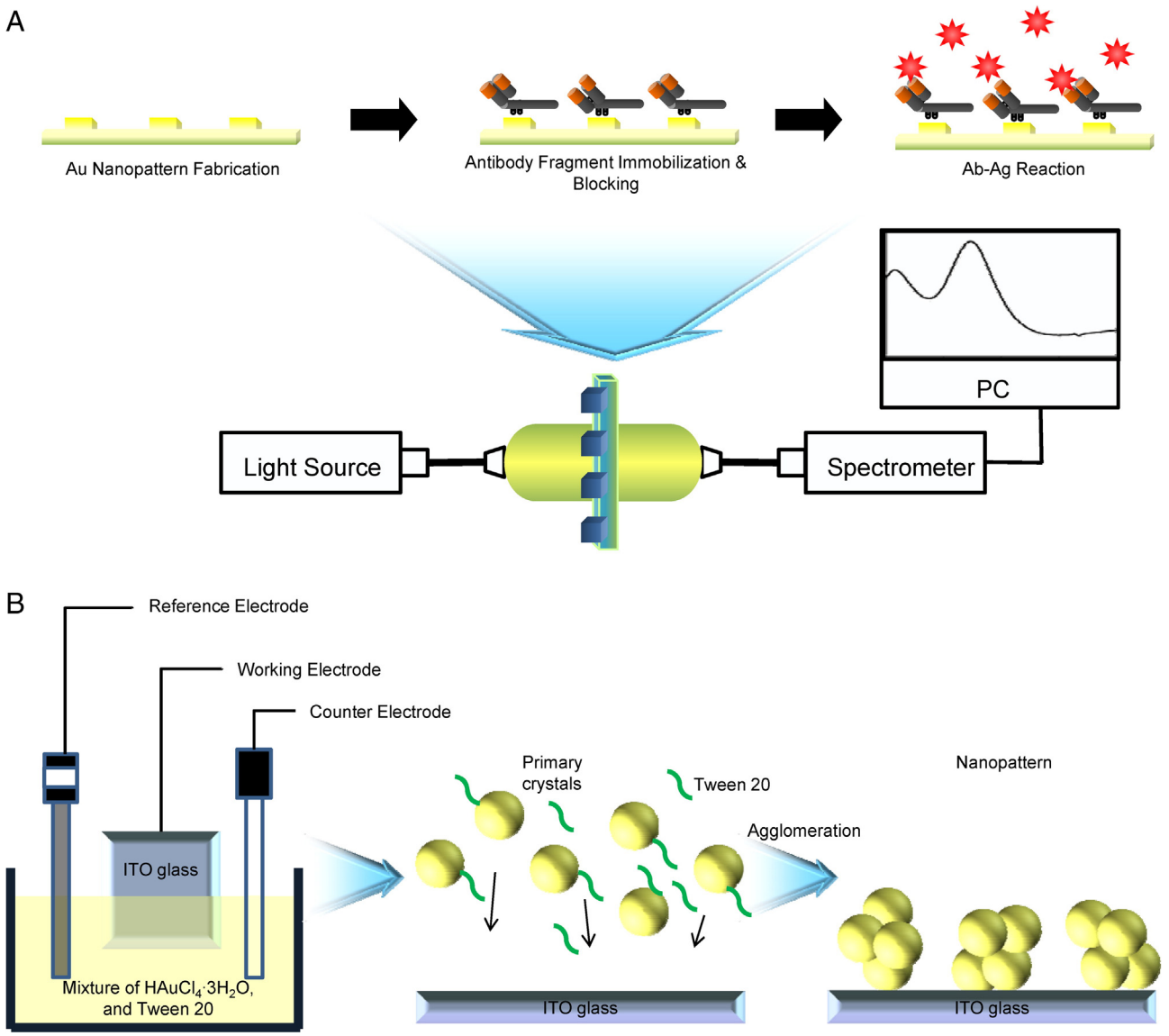

Figure 1. Schematic representation of (**A**) the immunoassay configuration with LSPR method for the quantitative detection of HIV-1 particles and of (**B**) Au nanopattern fabrication method based on electrochemical deposition in the presence of surfactant.

In contrast, recent advances in nanofabrication methodologies led the development of nano-plasmonic biosensors due to its potential applicability for developing highly sensitive miniaturized optical devices, sensors, as well as in medical diagnostics and therapeutics.[17,18] With nanofabrication methodologies, metal nanostructures can be developed with controlled shape, size, and space such as nanoparticles (NPs), nanoarray structures.[19] Moreover, with the emergence of quantitative electromagnetic modeling tools better understanding of the optical properties of isolated and electromagnetically coupled nanostructures of various sizes and shapes was analyzed. With the ability in controlling the geometry and optical properties of nanostructures,[20] various strategies for modifying the surfaces of these materials made it possible to develop highly sensitive and selective chemical/biological sensors.[21] Few studies have been performed to develop a biosensor based on LSPR method to detect virus such as hepatitis B (HBV) and swine origin influenza A (H1N1). But, still these methods use complex fabrication steps and require labeling probes for sensitive detection. [22–24] Hence, with a simple low-cost fabrication technology for nanostructures for formation of nanodot associated with a label free virus immunosensor based on LSPR method for the detection of HIV-1 has not been reported yet.

In this study, a highly sensitive, selective and label-free LSPR immunosensor to detect HIV-1 particles based on gold nanodot was proposed for the first time. 10-20 nm sized circular shaped highly ordered Au nanopattern was fabricated by simple electrochemical deposition method as a sensing layer on the ITO substrate. Au nanopattern with an oriented antibody layer was developed by gold and thiol reaction. The fabricated biosurface has been employed to detect the HIV-1 virus like particles (VLPs) based on absorbance changes, which resulted

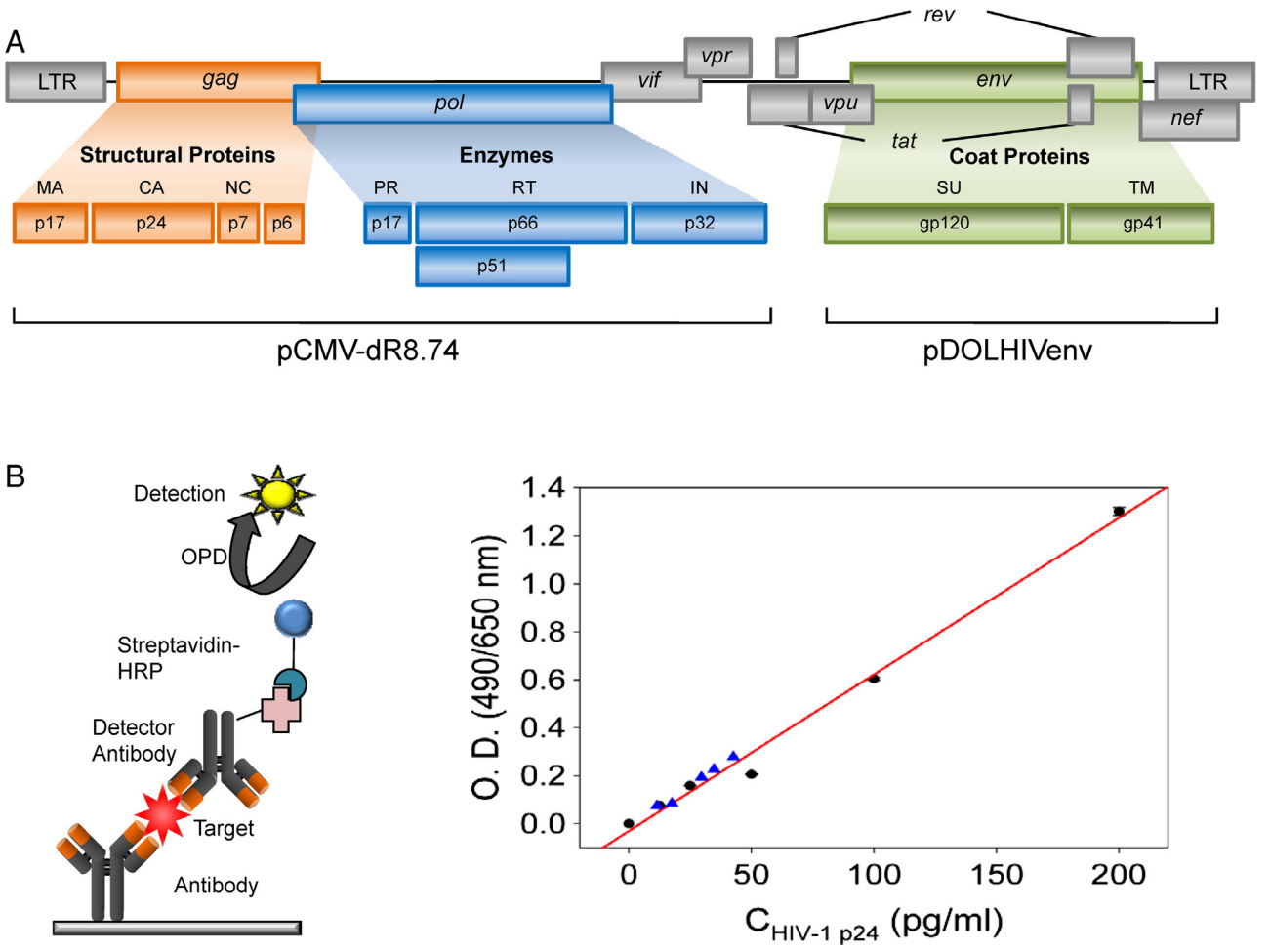

Figure 2. (**A**) Representative HIV-1 RNA genome structure and two different plasmids, pCMV-dR8.74 and pDOLHIVenv consist of gag, pol, and env components. (**B**) The linear relationship between the O.D. value (490/650 nm) of (●) standard HIV-1 p-24 antigen over the range 5-200 pg/mL with correlation coefficient (R^2) is 0.9905 and the measured concentration of (▲) produced HIV-1 VLPs.

from the change in refractive index on Au surface with immunoreactions. Developed HIV-1 sensor with the ability of rapid preparation and high sensitivity provides an approach for biological sample analysis and investigating interactions between biological molecules in medical field.

Methods

Materials

All solutions were prepared with distilled and deionized by Millipore [(Milli-Q) water (DDW > 18 MΩ)]. For the fabrication of Au nanopattern, Hydrogen tetrachloroaurate trihydrate (III) (99.9+%), polyoxyethylene sorbitan monolaurate (Tween 20) were purchased from Sigma-aldrich (St. Louis, MO, USA). The broadly HIV-1 neutralizing gp120 monoclonal antibody (gp120MAb), 2G12, was purchased from Polymun Scientific (Vienna, Austria). 2-mercaptoethlamine (2-MEA) was purchased from Sigma-Aldrich (St. Louis, MO, USA) to produce fragmented antibody. Casein was purchased from Sigma (St Louis, MO, USA) and applied after the reaction gold-thiol reaction. The reduction of disulfide bond in the antibody (IgG) heavy chain was carried out in the phosphate-EDTA buffer (pH 6.0, 1 L base: sodium phosphate 100 mM, EDTA 5 mM) purchased from Sigma (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) purchased from Gibco (Carlsbad, CA, USA) and used for HEK-293 cell. Two different plasmids, pCMV-dR8.74 and pDOLHIVenv were kindly provided from Dr. Byung-Chan Kim from KIST (Korea). Fetal bovine serum (FBS), antibiotics (penicillin-streptomycin, 10,000 U/ml of penicillin sodium, and 10,000 μg/ml of streptomycin sulfate in 0.83% saline), and trypsin (0.05% trypsin, 0.53 mM EDTA-4Na) were obtained from Gibco (Invitrogen, Grand Island, USA). Phosphate buffered saline (PBS) (pH 7.4, 10 mM) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used in this study were of reagent grade and obtained commercially.

Fabrication of Au nano pattern

Au nano pattern was electrochemically fabricated by using a potentiostat (CHI-660, CH Instruments, USA) as shown in Figure 1, *B*. A three-electrode system composed of ITO-coated

glass substrate as a working electrode, a platinum wire as the auxiliary electrode and Ag/AgCl as the reference electrode.[25] ITO-coated glass substrates were cleaned by sonication for 15 min using 1% Triton X-100 solution, DIW and ethanol sequentially, and then by basic piranha solution (1:1:5, H_2O_2:NH_3:H_2O) for 30 min at 80 °C. Finally, the substrates were cleaned by DIW and then dried under N_2 stream to obtain a clean ITO surface. Au nanopattern was electrochemically deposited on ITO substrates (20 mm × 10 mm) using a 0.5 mM $HAuCl_4$ aqueous solution containing in 30 s, Tween 20 as a surfactant. The potential was maintained at −0.9 V (vs. Ag/AgCl) and the deposition temperature was controlled to maintain at 25 °C in with electric-heated thermostatic water bath. In order to remove any surfactant traces that may have adsorbed on the developed pattern, the substrates were rinsed with DIW and then boiled with isopropyl alcohol for 5 min. Finally the substrates are washed with DIW and dried under N_2 stream. The active area for electrochemical deposition of Au nanopattern was 20 mm × 10 mm.

HIV-1 virus like particle production

Human embryonic kidney cells (HEK-293 T) was obtained from American type culture collection (ATCC). The cells were stored in deep frozen portion at −70 °C and cultivated in Dulbecco's modified Eagle's medium (DMEM) glutaMax supplemented with 10% (FBS) and 1% penicillin-streptomycin which were obtained from Gibco BRL (Rockville, MD, USA). The HEK 293 T cells were grown in T25 culture flasks (Corning, USA) with atmosphere of 5% CO_2 at 37 °C and 70% humidity. At 80% confluence, the cell line was maintained through the subculture at a density of 1×10^5 cells/ml on culture plates at a period of 2 or 3 days. On the grown HEK293 cells were co-transfected by $CaCl_2$ transfection with two different plasmids, pCMV-dR8.74 and pDOLHIVenv,[26] which contain Gag and Pol, and Env components of HIV-1, respectively (Figure 2, *A*). Co-transfected HEK293 cells were again cultured in DMEM medium supplemented with 10% FBS and 1% streptomycin/penicillin at 37 °C in 5% CO_2. After 2 days, supernatant was collected and HIV-1 particles were concentrated by ultracentrifugation (26,000 rpm for 2 h at room temperature) and re-suspended in DMEM medium and kept at −80 °C until use. The concentration of produced virus-like particles (VLPs) was determined by p24 Enzyme Linked Immuno Sorbent Assay (ELISA) assay kit (Perkin-Elmer, Boston, MA).

Immobilization of antibody fragment by self-assembly technique

The antibody fragments were prepared on the basis of chemical reduction using 2-MEA. 10 mg of 2-MEA was added to the 1 mL antibody solution (up to 10 mg/mL) for the fragmentation of immunoglobulin G (IgG) molecules. The reaction was carried out for 90 min under 37 °C. After incubation, residual 2-MEA was removed through dialysis against phosphate buffered saline (PBS, pH 7.4) – 5 mM ethylenediaminetetra acetic acid (EDTA) buffer. The fragmentation of antibody enables orientated immobilization on the surface, which leads to the enhancement of sensitivity, selectivity. Fragmented antibody solution, which is containing

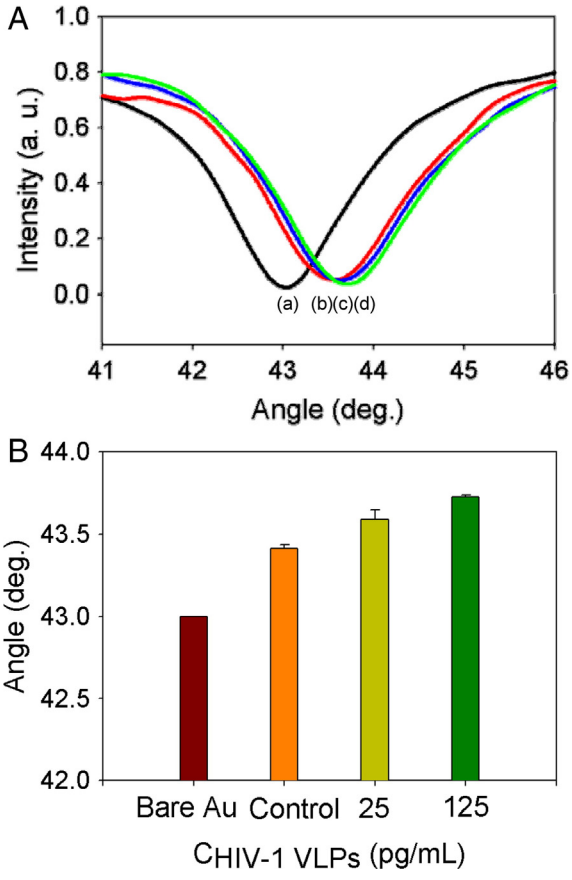

Figure 3. (**A**) Representative SPR spectra of the (a) Bare Au substrate, (b) antibody layer immobilized with antibody fragment and blocking material, (c) antigen (5 pg/mL of HIV-1 VLPs), (d) antigen (25 pg/mL of HIV-1 VLPs). (**B**) Plot of SPR angle shift versus different layer.

native thiol (−SH) group was applied to the Au surface and incubated overnight at 4 °C to form biosurface by self-assembly method. Also, various concentrations of HIV-1 VLPs were introduced to the surface for 1 h at 37 °C for further measurements in a humid chamber. The binding mechanism involved in the process is HIV-1 virus neutralizing monoclonal antibody, 2G12, binds to N-glycans with α(1,2)-linked mannose terminal at N295, N332, and N392 positions near to the V3 loop in the region of gp120.[27]

Determination of HIV-1 VLPs by surface plasmon resonance method

A glass plate (SF10 n = 1.723, 18 mm × 18 mm, Korea Electro-Optics Co., Ltd., Korea) was used as the solid support and gold (Au) was sputtered to the glass substrate with a thickness of 430 Å after chromium (Cr) was sputtered as an adhesion material with a thickness of about 20 Å for further experiments. The immunoassay was conducted using SPR spectroscopy with a built-in He-Ne laser beam that had a wavelength of 632.8 nm (MultiskopTM, Optrel Gbr, Germany). The p-polarized light beam was measured by a photo multiplier tube (PMT) sensor. A glass prism (BK 7) having a 90° angle was used with Kreschmann ATP coupler. The plane face of the 90°

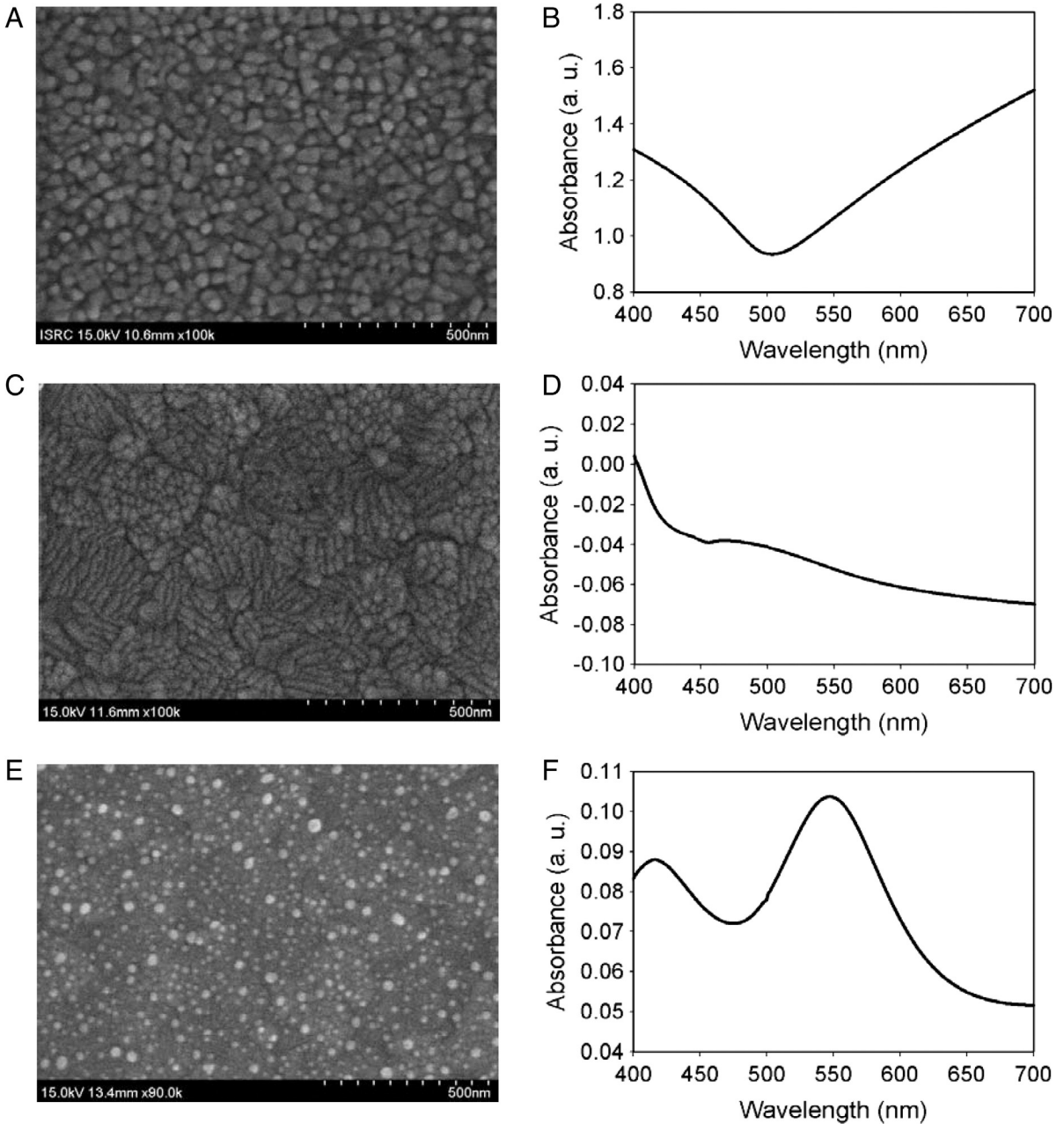

Figure 4. SEM image of (**A**) thin Au film, (**C**) bare ITO substrate, (**E**) Au nanopattern on ITO substrate, and measured absorption spectrum of (**B**) thin Au film, (**D**) bare ITO substrate, (**F**) Au nanopattern on ITO.

glass prism was coupled to a cover glass via index matching fluid (Benzyl benzoate, Merck, Germany). The resolution of the angle reading of the goniometer was 0.001°. All samples were monitored at room temperature and the variation of the incidence angle was between 38° and 50°.[28]

Determination of HIV-1 VLPs by localized surface plasmon resonance method

The binding of analytes to the molecular probe was monitored by the LSPR peak shift in the UV–Vis spectrum resulting from the changes of local refractive index induced by the immunological reaction. As the reaction occurs on the surface the local refractive index changes at a given wavelength which is due to the extension of light absorption of molecular film. All absorption spectra were obtained by using monitoring UV–Vis spectral changes in transmission mode of JASCO V-530 UV-spectrometer.

Results

Validation experiment for the production of HIV-1 VLPs based on conventional ELISA

To classify the production and concentration of HIV-1 VLPs from cell culture medium, commercially allowed Alliance HIV-1 P24 ANTIGEN ELISA Kit was used. Since, the enzyme-linked

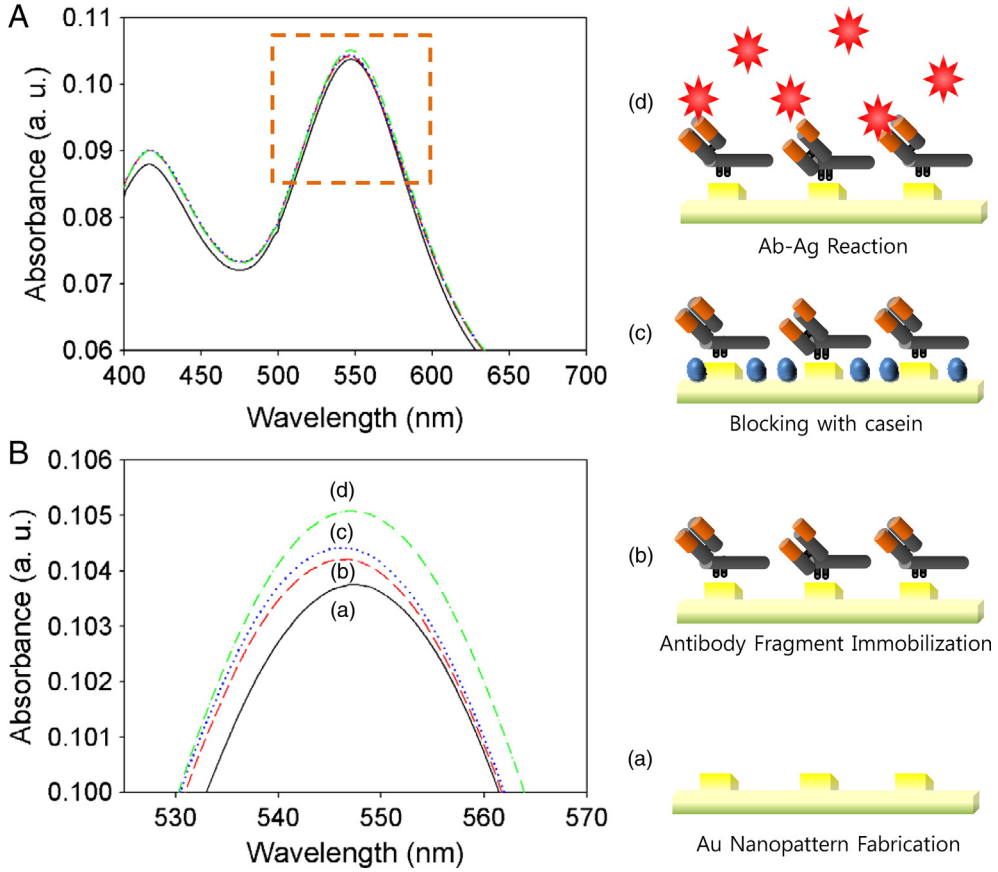

Figure 5. (**A**) Optical characteristics of the (a) bare Au nanopattern on ITO substrate, (b) fragmented antibody immobilized layer, (c) blocking with casein, (d) HIV-1 VLPs immuno reacted layer, respectively. (**B**) Optical characteristics of zoomed part of wavelength between 520 to 570 nm.

immunosorbent assay (ELISA) is a common serological test for the determination of particular antigens or antibodies. The produced sample solution was diluted for the concentration determination and calibrated against obtained standard curve with correlation efficiency ($R^2 = 0.9905$).

$$F = -0.0301 + 0.0065 * C$$

Where F is O.D. (optical density) value and C is concentration of analytes (pg/mL). From these results, the produced HIV-1 VLPs (▲) with absorbance values which are equal or greater than the cutoff factor of ~0.054, are considered as initially reactive and used for further experiments (Figure 2, *B*).

Analysis of HIV-1 VLPs from cell culture supernatant using SPR

The interaction between the metal and antibody fragment, antibody fragment with HIV-1 VLPs was monitored with the surface plasmon resonance spectroscopy. Figure 3 shows the SPR spectroscopy results of bare Au surface, antibody fragment layer and different concentration of HIV-1 particles on Au surface respectively. Based on the SPR angle of bare Au surface, the SPR angle of the fragmented antibody layer was shifted approximately 0.412°. The nonspecific binding reaction was prevented by blocking procedure with 5% (w/w) of casein, which did not bring any significant change in SPR angle (data not shown).[29] Moreover, the addition of 25 pg/mL and 125 pg/mL of HIV-1 VLPs to the bio-surface modified with fragmented antibody and casein induced a small shift of 0.178° and 0.313° successfully, but below 25 pg/mL significant SPR angle change could not be distinguished. This result indicates that simple SPR method for the detection of HIV-1 VLPs is not sensitive enough to determine successfully the presence of HIV-1 below the 25 pg/mL range.

Characterization of plasmon effect on Au nanopattern

After fabrication procedures of Au nano pattern, the LSPR band was monitored in the visible range. As a result, the Au nanopatterned layer substrate for LSPR excitation could be obtained. Figure 4 shows clear difference between SEM image and the measured absorption spectra of a (A, B) thin Au film, (C, D) pure ITO substrate and (E, F) Au nanopatterned ITO substrate. The thin Au film absorbs light throughout the visible regions due to free electron absorption, for the nanoparticles free electron oscillator strength for absorption is strongly pulled into a dipolar absorption peak at around 550 nm. Moreover, the resonant electromagnetic behavior of metal nanoparticles is based on the confinement of the conduction electrons which is related to the small particle size. The conduction electrons inside the particle that moves upon plane-wave excitation relate to the radiation of wavelength, leading the polarization on the particle surface.[30] These polarized charges act as an effective restoring force, allowing for a resonance to occur at a specific frequency. Consequently, a resonant enhanced field inside the particle produces

a dipolar field around the particle, which leads enhancement of absorption and scattering.

Quantitative analysis of HIV-1 VLPs from cell culture supernatant using LSPR

LSPR measurements were performed on the fabricated Au nanopatterned ITO substrate, after immobilization of fragmented antibody, which resulted in an increment of LSPR signal. After the blocking procedure with 5% (w/w) of casein on the surface of fragmented antibody immobilized Au nanopattern, the specific binding reaction of HIV-1 VLPs could be also observed by the specific absorbance intensity increments (Figure 5). To prove the reusability and selectivity, the immune-reaction and its elution were confirmed by changes in absorbance difference as shown in Figure 6, *A*. On the fragmented antibody immobilized biosurface, 1 pg/mL concentrated HIV-1 VLP solution was introduced for the measurement, which results in an increase in absorbance based on specific immuno-reaction. On contrary, when 3 M KCl solutions were applied to break antibody-antigen binding interactions, the absorbance returns back to its position where it originally was. To prove 3 M KCl solution breaks the antibody-antigen binding interactions effectively without damaging the protein structure, higher concentration of HIV-1 VLPs (25 pg/mL) was introduced. Since higher concentrated HIV-1 VLPs were applied more immunoreaction occurs on the Au nanopatterned substrate due to their specific binding affinity to the immobilized antibody fragments which results in the increment on the absorption intensity at wavelength around 550 nm. From the results, it can be concluded that fabricated Au nanopattern ITO substrate is appropriable to develop virus sensor to detect immunoreactions with high selectivity and reusability.

Based on the successful immunological recognition reactions, pure DMEM solution was also introduced for the negative control experiment, where no change in the absorbance peak was observed (Figure 6, *B*). On the fragmented antibody modified surface, the various concentrations of HIV-1 VLPs were performed by monitoring the absorbance changes as refractive index (RI) changes on an Au nanopattern surface. Increment in absorbance intensity in LSPR spectroscopy was observed at various HIV-1 VLPs concentrations of 200 fg/mL to 125 pg/mL which were independently introduced. The absorbance slightly increases and shows red-shift as concentration of HIV-1 VLPs increases, which is caused due to the number of antigen-antibody recognition events, occurred on the surface. The LSPR measurement appears to be highly sensitive for the detection of HIV-1 VLPs concentrations at low levels. Even at 200 fg/mL of HIV-1, a distinct response in the absorbance intensity increase could be obtained. The dynamic range obtained in the concentration range of 200 fg/mL to 125 pg/mL shows the linearity with increasing HIV-1 VLPs concentration having a correlation coefficient of 0.990 (Figure 6, *C*).

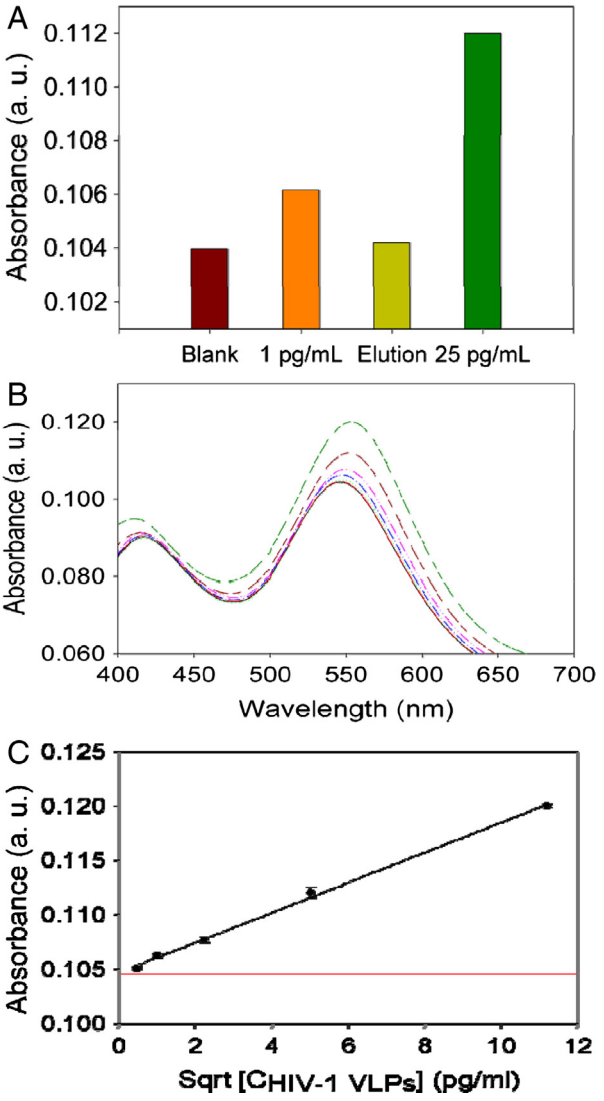

Figure 6. (**A**) Change in absorbance peak based on immunoreactions and its elution (a) blank electrode, (b) 1 pg/mL of HIV-1 VLPs, (c) elution with KCl and (d) 25 pg/mL of HIV-1 VLPs respectively. (**B**) Absorbance peak change at wavelength around 550 nm LSPR absorbance strength changes with different HIV-1 VLPs concentrations (—) antibody fragments with blocking, (— —) negative control experiment with pure DMEM, (●●●) 200 fg/mL, (—●—●) 1 pg/mL, (— ●● —) 5 pg/mL, (— -) 25 pg/mL, and (— –) 125 pg/mL respectively. (**C**) Calibration characteristics of the different concentration of HIV-1 VLPs range from 200 fg/mL to 125 pg/mL with correlation coefficient (R^2) of 0.9990. The detection limit of LSPR based biosensor for HIV-1 particle was 200 fg/ml.

Discussion

To understand the phenomena and for diagnosis of AIDS, the HIV was intensively studied over the years. Several studies on the molecular structures and main base of HIV virus particles are previously identified. Moreover, the biochemical specificity, the replication factors, cycle and the immune-suppression affect on a human immune system were also obtained.[31] From the RNA genome of HIV, *gag*, *pol*, and *env* include the information to produce the structural proteins such as capsid proteins, viral enzymes, and precursor (gp160) (Figure 2, *A*) and remaining genes are regulatory genes for the infection and replication.[32] In this study, to determine HIV-1 particles quantitatively, HIV-1 VLPs were produced by co-transfecting pCMVdR8.74 and pDOLHIVenv plasmids to the HEK293 cells. Thus, the

produced HIV-1 VLPs represent noninfectious genuine viruses relying on the absence of genomic components inside the produced virus like particles.

Traditional virus detection methods such as enzyme-linked immunosorbent assays (ELISA), polymerase chain reaction (PCR), and cell culturing are not compatible for the point of care use, because ELISA technique requires multiple steps and several agents which have a potential to create quenching reactions.[33] PCR requires considerable sample preparation steps and can be easily interrupted by inhibitors.[34] In addition, it is quite difficult to detect newly emerged strains of infectious agents.[35] Also, cell culturing is a time-consuming method and it needs highly skilled and labor intensive process where some viruses cannot be cultured at all.[36]

Therefore, even several methods have been analyzed to develop sensitive virus sensor such as (i) QCM sensor based hybridization, which has major limitations to overcome (a) its low sensitivity, (b) uncontrollable response, (c) lack of integration capability and (c) expensive instruments;[5–7] (ii) electrochemical sensor based on voltammetry and impedance technique, which is inexpensive, robust and easy to operate, however, (a) it still requires labeling mediator for sensitive detection and (b) stability and reproducibility problem remains;[8–11] (iii) optical detection methods such as optical fibers and surface plasmon resonance (SPR), which are fast, reliable and sensitive but still have some drawbacks such as (a) requires precise alignment of light to the samples and (b) expensive instruments are needed for sensitive measurement[12,13] (Table 1). Apart from these detection methods, few studies have been performed to develop highly sensitive biosensor by LSPR method to detect virus more efficiently. Label free detection of hepatitis B (HBV) and influenza virus has been reported, in which the fabrication of nanostructures such as nanorod was complicated and time consuming.[22,23] Also, the detection of a swine origin influenza A (H1N1) by LSPR method was reported, in which the labeling probes for sensitive detection were used.[24]

Comparing these techniques, in the present study, circular shaped highly ordered Au nanopattern was fabricated by simple and fast electrochemical deposition method within 30 s which acts as a sensing layer and fragmented antibody was immobilized in an oriented manner based on gold-thiol interaction, to enhance the sensitivity and selectivity. Having these advantages, HIV-1 VLPs were successfully determined based on absorbance changes, which resulted from refractive index change on the Au pattern surface with immunoreactions without any labeling materials. The detection limit of the developed LSPR sensor was estimated at 200 fg/mL with correlated coefficiency ($R^2 = 0.9990$), which is 10 fold higher than that of previously reported virus detection method based on LSPR.[23,24]

Since the newly developed LSPR for HIV-1 particle has the advantages of rapid preparation, high sensitivity and selectivity, it might become a promising method in the field of disease comprehension, treatment, and monitoring. High throughput screening for virus detection will uncover the vital information and help in understanding the disease much better, which ultimately leads to the development of more efficient drugs for the treatment or prevention. But, this method still requires large improvement to distinguish different binding events regarding multiple analytes. Since LSPR signal is mainly dependent on the refractive index around the metal structure and the specificity can be achieved through biomolecular interactions. Combination of LSPR with analytical method such as Raman spectroscopy can allow the identification and improvement of the detection limits. Even though, proposed LSPR sensor promises various potential applications, interdisciplinary research effort is still required to develop highly sensitive and reliable system, which could replace currently available commercialized biosensor devices and applications.

Table 1
Summarizes the detection limit of the virus detection methods along with those reported in literature.

Detection method	Virus	Detection Limit	Reference
QCM	Hepatitis B	8.6 pgL^{-1}	Yao et al
	Vaccinia	25 ng	Kleo et al
	Influenza A (H5N1)	0.0128 HAU	Wang et al
Voltammetry	Hepatitis B	1.94×10^{-8} M	Ding et al
	Herpes	2 nM	Tam et al
EIS	Rabies	0.5 μgmL^{-1}	Hnaien et al
	Dengue	1 pfu mL^{-1}	Nguyen et al
Optical	Hepatitis C	60 pM	Griffin et al
SPR	Influenza	0.5 μgmL^{-1}	Nilsson et al
LSPR	Influenza A (H1N1)	13.9 pg mL^{-1}	Chang et al
	Influenza	1 pg mL^{-1}	Park et al
	Hepatitis B	0.01 IU mL^{-1}	Wang et al
	HIV-1	200 fg mL^{-1}	Present work

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