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Detection of influenza virus by electrochemical surface plasmon resonance under potential modulation

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In this study we report the development of a novel viral pathogen immunosensor technology based on the electrochemical modulation of the optical signal from a surface plasmon wave interacting with a redox dye reporter. The device is formed by incorporating a sandwich immunoassay onto the surface of a plasmonic device mounted in a micro-electrochemical flow cell, where it is functionalized with a monoclonal antibody aimed to a specific target pathogen antigen. Once the target antigen is bound to the surface, it promotes the capturing of a secondary polyclonal antibody that has been conjugated with a redox-active methylene blue dye. The methylene blue displays a reversible change in the complex refractive index throughout a reduction-oxidation transition, which generates an optical signal that can be electrochemically modulated and detected at high sensitivity. For proof-of-principle measurements, we have targeted the hemagglutinin protein from the H5N1 avian influenza A virus to demonstrate the capabilities of our device for detection and quantification of a critical influenza antigen. Our experimental results of the EC-SPR-based immunosensor under potential modulation showed a 300 pM limit of detection for the H5N1 antigen.

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1. INTRODUCTION

Recently, a novel electrochemical surface plasmon resonance (EC-SPR) methodology that merges important features of the surface plasmon resonance (SPR) technique and electrochemical impedance spectroscopy (EIS) [1,2] has been proposed and demonstrated. The EC-SPR technique combines the electric potential modulation of EIS and the optical detection of SPR to reach superior performance for detecting and quantifying molecular binding events at the device active interface. For biosensing applications, a main advantage of the new technique resides in its immunity to unwanted signals from bulk refractive index changes, which therefore increases detection selectivity and sensitivity [3]. In recent years, Tao and co-workers have successfully used the new technique in the imaging mode by applying an AC potential modulation to the sensor surface and then collecting the amplitude and phase components of the AC-SPR signal [4–6]. They were able to study small molecules (60–120 Da) including their binding kinetics and affinity, which typically cannot be detected by conventional SPR [7,8]. A biosensor based on the electrochemical surface plasmon resonance technique has been developed by the Meunier group [9] by immobilizing onto the SPR surface a thiolated-DNA strand that has been conjugated to a redox methylene blue (MB) probe. The MB probe displays a strong and robust change in its optical absorption associated with its redox transition. The DNA strand features a stem-loop that initially constrains the MB label to stay close to the electrode surface (the ON configuration). In the absence of the target DNA, an applied potential modulation at the electrode interface that oscillates around the formal potential of the redox probe will modulate the optical SPR signal, which can then be used to provide information on the redox activity of the MB probe. As a result, a strong amplitude in the optical signal reports the absence of the target analyte. When the target DNA is present in the sample under scrutiny, it will then bind to the immobilized DNA strand and such an event will open the original stem loop displacing the MB farther away from the electrode surface (the OFF configuration). This configuration will then reduce the number and probability of MB molecules that undergo a redox transition under an applied potential modulation, and therefore an optical signal of lower amplitude is detected indicating the presence of the target analyte. Although the overall concept was successfully demonstrated, under this sensing strategy the OFF configuration (when the

redox probe has been displaced further away from the electric interface but yet resides close to the surface) still provides a small but non-negligible chance for the electron transfer process to occur, which can generate an undesirable optical signal. Therefore, for this embodiment the optical background signal (under a high concentration of the target DNA) was relatively high with a negative impact in the detection sensitivity, which was reported in the nanomolar range.

Previous work by Ghithan *et al.* [10] has demonstrated a novel strategy for pathogen analysis by combining the high sensitivity of a single-mode, electro-active, integrated optical waveguide (SM-EA-IOW) platform with a biologically specific sandwich immunoassay, where the redox probe is forced to bind to the device interface exclusively when the target analyte is present in the test sample. This sensor strategy has been demonstrated experimentally [10] to have very high sensitivity (in the pico-molar range) due to the long path length of the propagating light wave inside the SM-EA-IOW device. However, for developing an inexpensive and easy-to-use transduction platform that offers a simpler path towards an arrayed-detection technology with multiplexed interrogation confined to a small device footprint, the single-bounce reflection of an electrochemically modulated SPR configuration can be advantageous.

In order to advance a biosensing technology that is compatible with multiplex detection and displays high sensitivity, we have developed and demonstrated here a novel immunosensor-based strategy for direct detection of viral pathogens by incorporating a sandwich bioassay on the electrochemically modulated SPR platform. The detection principle is based on the interaction between the monoclonal anti-H5 (H5N1) capture antibody, the hemagglutinin (HA) virus antigen, and the MB-labeled secondary antibody. HA is a ubiquitous glycoprotein component of the influenza virus capsid responsible for binding the virus to host cells and is thus a useful antigen for our assay. The particular isoform of HA present on a flu virus is used to classify the virus, e.g., virus H5N1 contains type 5 HA. Once and only if the target antigen is bound to the device surface, it promotes the capturing of a secondary polyclonal antibody that has been conjugated with the redox-active MB dye. Then by applying a modulated electric potential to force the oscillation of the MB across one of its redox transitions, a modulated optical signal in reflection is created and detected. Such methodology exclusively detects an optical signal that is electrically modulated and constrained to the analytical interface, which provides a means to significantly reduce the effects of background signal that comes from the substrate or fluid media. Additional selectivity in the analytical signal is obtained by modulating the applied potential around the formal potential of the redox probe and by choosing the laser wavelength to be centered at the optical transition of the dye.

2. MATERIALS AND EQUIPMENT

A. Reagents

A monoclonal anti-H5 (H5N1) capture antibody (250 μg, 2 mg/ml), polyclonal anti-H5 (H5N1) (20 μg/ml, 1 ml, molecular weight = 144 kDa, goat polyclonal IgG), and HA protein of the H5N1 influenza virus (200 μg/ml, 0.5 ml) were purchased from Santa Cruz Biotechnologies, Inc (Dallas, Texas). Phosphate buffered saline (PBS) 50 mM (pH = 7.4), 3-Mercaptopropionic acid (MPA), 1-ethyl-3-(3-dimethylaminopropyl) Carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (St. Louis, Missouri). MB ester (5 mg, molecular weight = 598.12 g/mol), was purchased from Biosearch Technologies (Novato, California). In the last step, the conjugated polyclonal anti-H5 (H5N1) was purified by using a resin column (Thermo Fisher Scientific, Waltham, Massachusetts) and a 50 mM PBS solution as elution buffer.

B. Experimental Setup

In order to demonstrate the EC-SPR immunosensor, chromium (5 nm) and gold films (50 nm) were deposited on soda lime glass slides (VWR, 48300-048) using a DC magnetron sputtering technique (Nano-Master). The gold-coated glass slide was then mounted in a homemade micro-electrochemical flow cell with a three-electrode configuration and connected to an equilateral BK-7 glass prism (Edmund Optics) using index matching gel ($n_D = 1.52$, Cargille) The gold film served as the working electrode (WE), while two gold-plated pins (surface area = 2.85 mm^2, Mouser Electronics, 8451-0-00-34-00-00-33-0) were used as reference (RE) and counter electrodes (CE). A potentiostat (CHI 660D, CH Instruments, Inc.) was connected to the three electrodes to apply and control the potential at the working electrode. The optical setup is based on the widely used Kretschmann configuration. A CW laser (Obis from Coherent) at the wavelength of 685 nm and operated at 40 mW was deployed in combination with a half-wave plate and a linear polarizer to establish linearly polarized light at transverse-magnetic (TM) polarization; the laser beam was directed towards the entrance facet of the coupling prism to excite the surface plasmon wave as illustrated in Fig. 1.

First, measurements were taken to optimize the sensor platform. For these measurements, the electrochemical flow cell was mounted on a rotation stage to precisely control the incident angle, while the reflected optical intensity at different angles of incidence was monitored by a power meter (Newport, model 1930C) at a particular applied potential. Second, measurements were taken under cyclic voltammetry (CV) modulation. For these measurements, the electrically modulated reflected optical signal was monitored at a fixed angle by the power meter and transferred to an oscilloscope (Agilent, DSO8104A Infiniium). Finally, measurements were taken under an AC potential modulation. For these measurements, a function generator (DS345, Stanford Research Systems) was connected to the potentiostat to apply a sinusoidal potential modulation to drive the oscillation of the MB across its redox state. The modulated optical signal of the reflected light was monitored at a fixed incident angle by the power meter and then delivered to a lock-in amplifier (SR830 DSP, Stanford Research Systems), where the magnitude and the phase of the analytical signal was collected and recorded by the oscilloscope.

C. Sensor Preparation

The sensor interface of the gold-coated glass slide was sequentially cleaned in an ultrasonic bath with deionized (DI) water, acetone, and ethanol for 10 min in each solution, and then

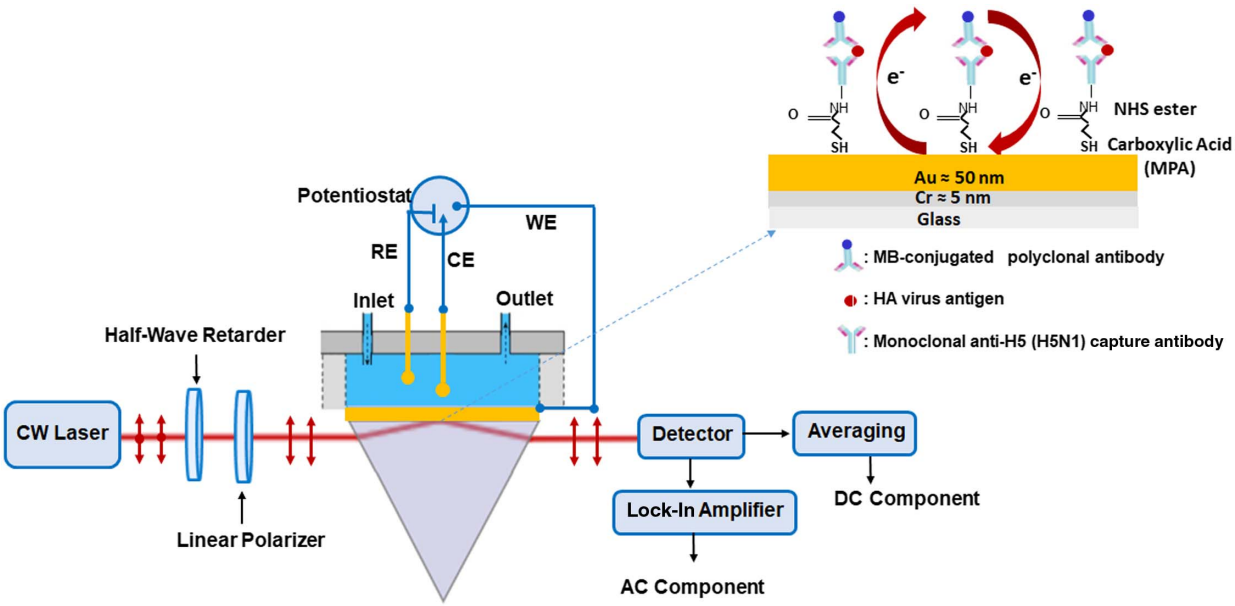

Fig. 1. Schematic representation of the EC-SPR setup, which includes a potentiostat for electrical control, a laser light source, a retarder, and a polarizer for transverse-magnetic polarized light. A detector, a lock-in amplifier, and an oscilloscope were used for data collection. Inset, a glass slide coated with a 5 nm chromium adhesion layer followed by a 50 nm layer of gold to create a biosensor surface. A molecular assembly formed by layers of MPA, EDC, and NHS were used to functionalize the biosensor interface.

gently dried under N_2 gas. The device was then immediately immersed into a 10 mM solution of 3-mercaptopropionic acid (MPA) in ethanol for 24 h to establish a self-assembled monolayer (SAM) on the device interface. After rinsing the sensor surface gently with ethanol and DI water and drying it with N_2 gas, the device was placed in a PBS buffer solution (pH = 7.4) of 2 mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 5 mM N-hydroxy succinimide (NHS) for 1 h to convert the terminal carboxylic groups to active NHS esters [11,12]. Subsequently, a covalent bond between a monoclonal anti-H5 (H5N1) capture antibody and the active NHS ester was formed by immersing the sensor surface in a solution of 0.5 μg/ml capture antibody in the PBS buffer at 4°C overnight (see the inset of Fig. 1). Finally, the sensor surface with the immobilized capture antibody was rinsed with the PBS buffer to remove any unbounded antibodies and then stored at 4°C. All liquid injection into the electrochemical cell was handled by a manually activated syringe pump.

3. RESULTS AND DISCUSSIONS

A. Optimization of the Analytical Signal

The principle of the EC-SPR sensor relies on a strong difference ΔI of the optical response between the reduced and oxidized states of the conjugated MB ester in the full immunoassay assembly. However, because the conjugated MB ester has similar spectroscopic properties as the pristine MB molecule under the redox process, we have searched for the ideal SPR configuration to optimize the EC-SPR response with the highest optical signal ΔI using the original MB molecule.

Figure 2 shows typical SPR reflectance curves against the effective refractive index inside the prism (also known as Snell's invariant) for a PBS buffer solution and a 2.6 μM MB solution, where both samples were measured at two electrical potentials applied to the working electrode: −0.35 V (which corresponds to a reduced state of MB) and 0.05 V (an oxidized state of MB).

The black and blue curves in Fig. 2 show the SPR reflectance for the buffer solution at −0.35 V and 0.05 V, respectively. After injecting the MB solution at −0.35 V potential (red curve), the position of minimum reflectance has shifted to a higher value of effective refractive index; however, the value of minimum reflectance is about the same as obtained for the buffer solution at this applied potential (black curve). Such behavior confirms that the change is mainly due to a modification in the real part of the refractive index near the surface plasmon wave, as MB molecules replace water molecules. The almost constant value in the minimum of reflectance indicates the high transparency of the MB molecule at −0.35 V (MB is highly transparent in its reduced state). On the other hand, at

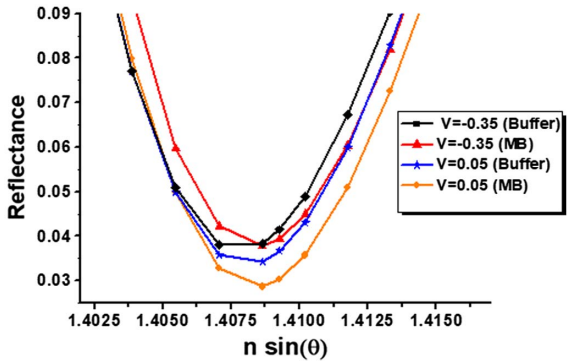

Fig. 2. SPR reflectance for the PBS buffer and MB solutions at −0.35 V and 0.05 V.

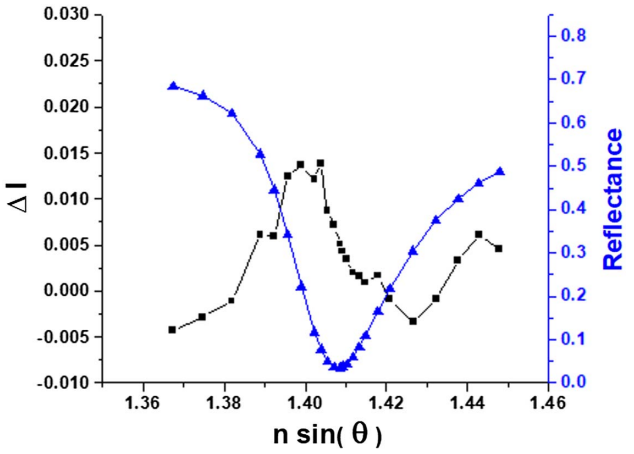

Fig. 3. Right y axis shows the SPR reflectance for the buffer solution, while the left y axis shows the difference in the optical signal ΔI between the oxidized state and the reduced state of the MB molecule.

the 0.05 V potential (orange curve) a significant change in the value of the reflectance minimum of the MB solution was observed (when compared to the buffer solution data at that potential, blue curve), which is consistent with significant changes in the imaginary part of the refractive index of the MB molecule. These results confirm that the MB absorption change under the redox process is present in our experiment and can potentially be deployed as a useful probe.

Next, an optical signal I was determined by subtracting the reflectance of the MB solution by the reflectance of the PBS buffer solution; such operation was performed at each effective refractive index and at two applied potentials (−0.35 and 0.05 V) corresponding to the reduced and oxidized states of the MB molecule, respectively. The optical signal difference I between the two oxidation states of the MB sample was then determined against the effective refractive index (Fig. 3, black trace), while the blue curve (y axis on the right side of the plot) provides the conventional SPR reflectance of the platform submersed in buffer solution.

These results show that the highest magnitude of the optical signal difference ΔI is observed in the linear region on the left side of the resonant angle of the conventional SPR curve. These experimental results come from changes in both the real and imaginary parts of the refractive index of the MB molecules when undergoing a redox process, and they define the best fixed angle (i.e., ideal effective refractive index) to implement EC-SPR measurements that will maximize the amplitude of the optical modulation when an electrochemical modulation is applied.

B. Cyclic Voltammetry (CV) Technique

For a proof-of-principle demonstration on the capabilities of the EC-SPR sensor for detection and quantification of a critical influenza antigen, a monoclonal anti-HA (H5N1) capture antibody was immobilized on the sensor surface by covalently binding it to a 3-mercaptopropionic acid (MPA) self-assembled monolayer (SAM). The sensor surface was mounted on an electrochemical flow cell and stabilized with a PBS buffer solution under CV potential modulation from (−0.4 to 0.2 V) at a scan rate of 0.02 V/s. Two measurements are highlighted below: in the first measurement, a 50 μL solution of MB-labeled polyclonal antibody at a concentration of 10 μg/ml was incubated in the flow cell for 30 min before rinsing with a PBS buffer. In the second measurement, a 50 μL solution of 200 ng/ml HA protein of the H5N1 influenza virus was prepared in 0.2 M PBS buffer and incubated in the flow cell for 30 min before rinsing with the PBS buffer; this step was followed by injecting a 50 μL solution of MB-labeled polyclonal antibody solution at a concentration of 10 μg/ml and incubating for 30 min before the flow cell was rinsed with PBS buffer to remove any unbound MB-labeled polyclonal antibodies. Figure 4 shows the difference in the optical signal I under cyclic voltammetry for the two measurements described above: in the presence of the 200 ng/ml HA virus antigen (black trace) and in the absence of the HA virus antigen (red trace). Please note that each trace has several overlapping cycles of the applied electric potential.

The results in the presence of antigen show a reversible and sharp transition of the optical signal I as the modulation potential crosses the formal potential of the redox probe (approximately at −0.17 V) and indicates the presence of the HA virus antigen, which is responsible for capturing and retaining the active redox probe (MB) at the device interface. In contrast, the absence of HA virus antigen (red trace) translates into an almost constant optical signal. This control experiment also confirms that the non-specific adsorption of the MB-labeled polyclonal antibody on the device interface was successfully kept to negligible levels. Above all, these results unambiguously confirm the ability of the EC-SPR platform to detect the presence of the HA virus antigen through the modulated optical signal under a CV scan.

C. AC Voltammetric Technique

The data above shows that, at least for a concentration of 200 ng/ml of the HA virus antigen solution, the EC-SPR platform under CV potential modulation can be used to detect the

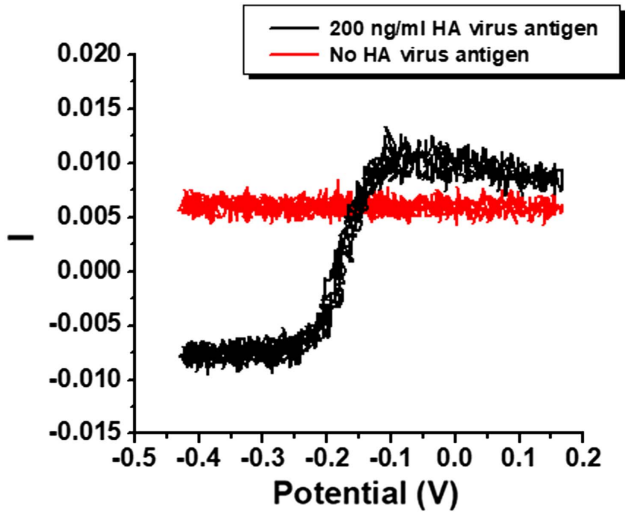

Fig. 4. Optical signal I under a CV scan for the full immunoassay sandwich at scan rate of 0.02 V/s. The red trace shows the data in the absence of HA virus antigen, while the black trace represents the data in the presence of HA virus antigen.

target analyte. However, to reach detectability at much lower analyte concentrations, better interrogation schemes should be implemented. For this purpose, an AC voltammetric modulation was deployed to drive the redox process and consequently the optical signal with a much-improved signal-to-noise ratio while delivering a faster transduction time [13].

In order to apply the AC voltammetric modulation, the modulation frequency needs to be experimentally determined; the resonance frequency is essentially connected to the electron transfer kinetics of the redox process occurring on the electrode surface. To do so, a sinusoidal potential modulation was applied on the EC-SPR sensor with the full immunoassay sandwich at a fixed DC bias potential (−0.17 V), and several frequencies ranging from 50 to 500 Hz were measured. The SPR optical signal ΔI_{SPR} was determined by subtracting the baseline SPR optical signal, which was taken after the capture of the HA virus antigen from the SPR optical signal collected after the incubation (and rinsing) of the MB-labeled polyclonal antibody. The SPR optical signal ΔI_{SPR} versus the frequency of the sinusoidal potential modulation is shown in Fig. 5. A clear peak centered at about 250 Hz corresponding to the highest electron transfer rate of the relevant redox process was determined. Driving our device at this modulation frequency will help eliminate spurious effects coming from thermal noise, mechanical vibration, and other detrimental sources.

Once the modulation frequency that maximizes the SPR optical signal ΔI_{SPR} was determined, a sinusoidal potential modulation with an amplitude of 0.04 V and a frequency of 250 Hz was employed at several different DC bias potentials ranging from −0.4 to 0 V; such DC potential range was chosen to encompass the formal potential of the MB redox probe. Figure 6 shows the SPR optical signal obtained by the lock-in amplifier at each bias potential in the absence of HA virus antigen and for three different concentrations of HA virus antigen (20, 50, 100 ng/ml).

As shown in Fig. 6, the plot of SPR optical signal against the DC bias potential displays a maximum intensity at approximately −0.17 V, which confirms the redox activity of the MB probe. Away from the formal potential of the redox probe, the optical signal decreases substantially and reaches a non-zero value due to changes in the real part of the refractive index as

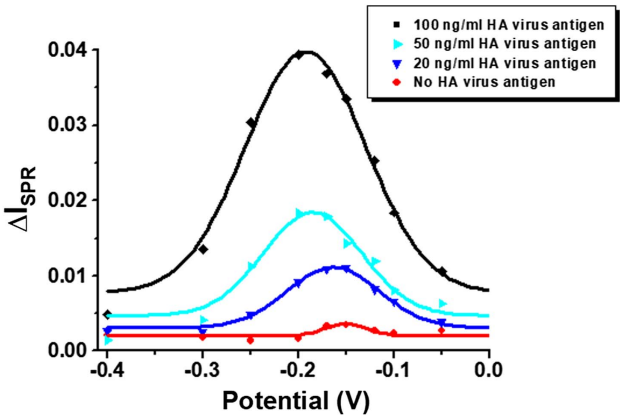

Fig. 6. SPR optical signal ΔI_{SPR} versus the DC bias potential in the absence of HA virus antigen and for different concentrations of the HA virus antigen measured under a sinusoidal potential modulation at the frequency of 250 Hz and an amplitude modulation of 0.04 V.

the MB-labeled polyclonal antibody adsorbs onto the sensor surface. The peak intensity of the SPR optical signal as reported by the redox probe is proportional to the surface concentration of the HA virus antigen and provides a direct route for the quantification of the HA virus antigen in the solution. A small peak of the SPR signal in the absence of the HA virus antigen (red line in Fig. 6) did not go unnoticed, and it most likely indicates the presence of a small number of non-specific adsorption events; such artifacts are expected to be reduced by optimizing the surface density of the monoclonal anti-H5 (H5N1) capture antibody to fully cover the active device interface. The corresponding peak intensity of the SPR optical signal for the different bulk concentrations of the HA virus antigen were determined, and the results are plotted in Fig. 7.

The calibration curve for detection shows a linear response, and the limit of detection was determined using the 3-sigma standard, which corresponds to an analyte concentration that would generate a signal that is three-fold the signal measured when no virus analyte was present in the system. Such procedure resulted in a value of 16 ng/ml or 300 pM for the limit of detection. The detection sensitivity obtained here for the influenza virus based on an immunoassay on the EC-SPR platform did not reach the high levels of the SM-EA-IOW device

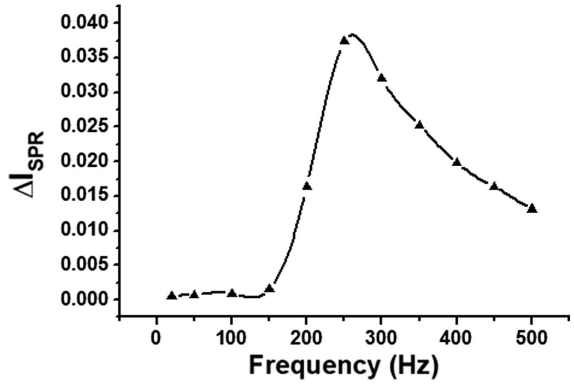

Fig. 5. SPR optical signal ΔI_{SPR} measured under AC potential modulation at the DC bias potential of −0.17 V and a HA virus antigen concentration of 200 ng/ml.

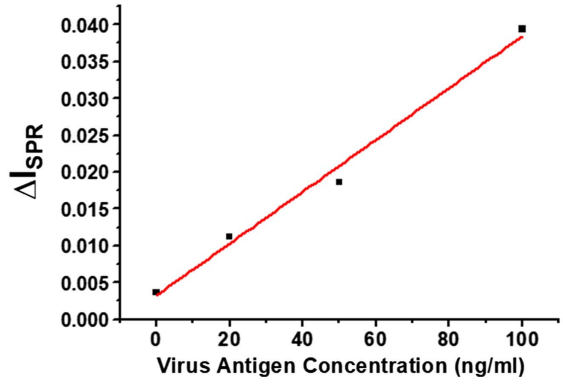

Fig. 7. Calibration curve based on AC measurements for detection of different concentrations of the HA virus antigen.

(4 ng/ml or 77 pM) already reported in the literature by us [10], although it does show improved performance when compared to previous work based on a redox nanoswitch probe coupled to a DNA strand (5 nM) [9]. Most importantly, the EC-SPR sensor, which features a single-bounce reflection interrogation scheme, offers a high compatibility for multiple-array detection in a small footprint that can make it a very attractive sensing technology.

4. CONCLUSIONS

In this study we have developed an electrochemical surface plasmon resonance (EC-SPR) strategy for immunosensing detection based on a sandwich assay that deploys an electrically modulated optical signal featured by a redox probe where no chemical labeling of the analyte is required. The sensor principle takes advantage of the highly specific interaction between a monoclonal capture antibody, a virus antigen, and a redox-labeled antibody. The potential modulation of the redox probe creates a robust and highly selective analytical signal for specific detection of events of interest. We have demonstrated here the ability for true detection of a virus antigen where the modulated optical signal of the redox-active probe provided a direct and fast quantification of the virus antigen with a 300 pM limit of detection. Overall, the EC-SPR sensor strategy has the potential for high sensitivity detection of multiplexed targets with a small footprint that only requires a small amount of sample material at low concentrations of a target antigen. The sensor can be further functionalized and used in clinical settings as a fast way to detect a variety of pathogens.

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