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Differential-Phase Surface Plasmon Resonance Biosensor

Ying-Chang Li,[†] Ying-Feng Chang,[‡] Li-Chen Su,[†] and Chien Chou*[,†,‡,§]

Department of Optics and Photonics, National Central University, Jhongli, Taiwan, 320, and Institute of Biophotonics, and Department of Biomedical Imaging and Radiological Science, National Yang-Ming University, Taipei, Taiwan, 112

In this paper, a novel differential-phase-sensitive surface plasmon resonance biosensor (DP-SPRB) is proposed and developed, in which a two-frequency laser is integrated with a differential amplifier in order to analytically convert the phase modulation into amplitude modulation. With the use of the conventional envelope detection technique, the differential phase is precisely decoded in real time in terms of the demodulated amplitude. In order to verify high detection sensitivity of the DP-SPRB, a sucrose−water solution and glycerin−water solution at low concentrations were both tested, and the experimental results confirm that the detection sensitivity on wt % concentration of the sucrose solution is 0.00001%. Moreover, the real-time monitoring mouse IgG/antimouse IgG interaction shows the minimum concentration of mouse IgG to be at 10 fg/mL. To our knowledge, this is the highest sensitivity ever measured by a surface plasmon resonance biosensor. However, because of the limited dynamic range of DP-SPRB, it can only apply to biomolecule interactions at extremely low concentration.

Surface plasmons, the internal collective oscillation of free electrons with a dielectric on noble metal surfaces such as gold or silver, can be excited by the evanescent wave generated by the incident laser beam undergoing attenuated total reflection (ATR). As the evanescent wave satisfies the phase-matching condition with the surface plasma wave (SPW) excited on the metal/dielectric interface, the maximum coupling between these two waves takes place while SPW is on resonance.[1] Theoretically, SPW excitation is sensitive to the variation of the refractive index or thickness of the test medium in the vicinity of a metal surface.[2] Thus, the surface plasmon resonance biosensor (SPRB) was proposed and widely used as a biosensor in biomolecule interactions in areas such as pharmaceutical development and life sciences.[3,4] Conventional SPRB is based on the construction of exciting a single surface plasma wave on a metal surface, with the minimum intensity of the reflected laser beam measured either in terms of its incident angle or wavelength on resonance. Therefore, real-time tracking of the resonance angle or wavelength of SPRB is able to monitor the biomolecular interactions in real time in terms of the variation of effective refractive index (Δn_{eff}) of the medium. It relates to the thickness (d) and refraction index (n) simultaneously in the interacted region of SPW.[5] Two categories used in order to dynamically sense the biomolecule interactions include (a) noninterferometric[6] and (b) interferometric[7] SPRB. In noninterferometric SPRB, Δn_{eff} is determined by means of precisely tracking the wavelength or incident angle on resonance. However, the limited spatial resolution of a charge-coupled device (CCD) and the slow variation of reflected intensity near the resonance angle result in the detection sensitivity of a commercial instrument such as the Biacore T100 (Biacore Inc., Uppsala, Sweden) at the detection limit of $\Delta n_{\text{eff}} \sim 3 \times 10^{-7}$ RIU (refractive index unit), or 0.3 RU, whose baseline noise is quoted at 1×10^{-7} RIU (0.1 RU).[6,8,9] In contrast, the interferometric SPRB combines SPW and a polarized interferometer in the temporal[7,10] or spatial domain[8,11] through the detection method either by amplitude-sensitive or by phase-sensitive on Δn_{eff} measurement. Although sensing the amplitude or phase of the interference signal in the temporal domain (heterodyne) or in spatial domain (spatial fringes) provides higher sensitivity of detection, the order of $\Delta n_{\text{eff}} = 10^{-8}$ RIU on detection sensitivity had been demonstrated successfully. However, the high sensitivity to environmental disturbance and the slow response of the phase measurement based on a phase-lock loop also limit the applicability of interferometric SPRB on real-time monitoring of biomolecular interactions. Recently, Chou and co-workers[5,10] proposed an amplitude-sensitive paired SPRB which integrates a two-frequency paired linear polarized P and S laser beam with a slight difference on temporal frequency and with a polarized heterodyne interferometer able to monitor the biomolecule interactions in real time. Their method is based on amplitude demodulation by use of a

* To whom correspondence should be addressed. Phone: 886-2-28267061. Fax: 886-2-28251310. E-mail: cchou@ym.edu.tw.

† National Central University.

‡ Institute of Biophotonics, National Yang-Ming University.

§ Department of Biomedical Imaging and Radiological Science, National Yang-Ming University.

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conventional envelope detection technique, which is characterized by high sensitivity and fast response simultaneously. In addition, a pair of SPWs is excited simultaneously. Consequently, the detection sensitivity is improved up to 10^{-9} RIU on IgG/anti-IgG interaction and was verified experimentally.[10] In the meantime, a wide dynamic range of 10^5 was performed as well. Such improvement can be attributed to a common-path noise rejection mode provided in PSPRB, which immunes environmental disturbances such as temperature variations within the reaction chamber. Moreover, the ratio of the amplitudes of P- and S-polarized heterodyne signals is also measured to effectively reduce the effect of laser intensity instability of the measurement. In order to further enhance the detection sensitivity of SPRB and allow it to be detected at extremely low concentration or small molecule interaction detection in solution, the phase-sensitive detection[7] is suggested, because a large phase change is generated near the SPR angle compared with the amplitude variation of the reflected laser beam theoretically. However, a limited dynamic range is also produced.[7] Thus, as the conventional phase lock-in technique is used for phase detection, a slow response is produced, too. For numerous applications, such as the single-molecule detection (SMD) at extremely low concentration,[12] a method to serve fast time response and high detection sensitivity based on phase measurement is urgently needed for real-time monitoring of biomolecule interactions. In response to this need, the differential-phase SPRB (DP-SPRB) is proposed and set up in this research, in which a single SPW, rather than a pair of SPWs, is excited on a metal/dielectric interface of an SPR device by using the P wave (TM wave), while the S wave (TE wave) is totally reflected by the metal thin film to serve as the reference signal. This setup not only reduces the effect of laser intensity fluctuation by ratio of the amplitudes of reflected P and S waves but also converts the phase modulation to amplitude modulation for high-sensitivity phase measurement in real time.

The working principle of the DP-SPRB is derived in the Principle of the DP-SPRB section, while the experimental verification is shown in the Experimental Results section. Meanwhile, the minimum detectable concentration of the proposed DP-SPRB on small molecules such as sucrose−water solution and glycerin−water solution was tested. Experimentally, the minimum detection sensitivity to large biomolecules, such as mouse IgG/antimouse IgG interaction, was measured and analyzed. Finally, the performance of the DP-SPRB is discussed in the Conclusion and Discussion section.

PRINCIPLE OF THE DP-SPRB

The optical setup of the DP-SPRB is shown in Figure 1, in which a two-frequency laser of paired highly correlated linear polarized waves, P wave and S wave, is adopted (where ω_p and ω_s are the temporal frequencies of the P and S waves, respectively). The formula, $\Delta\omega = \omega_P - \omega_S$, represents the beat frequency of the laser beam. In this experiment, the polarized two-frequency laser beam is produced using a Zeeman splitting He−Ne laser with a 632.8 nm wavelength. The signal beam ($P_1 + S_1$ waves) and the reference beam ($P_2 + S_2$ waves) are generated through a beam splitter (BS). A_1 and A_2 are two analyzers located in the

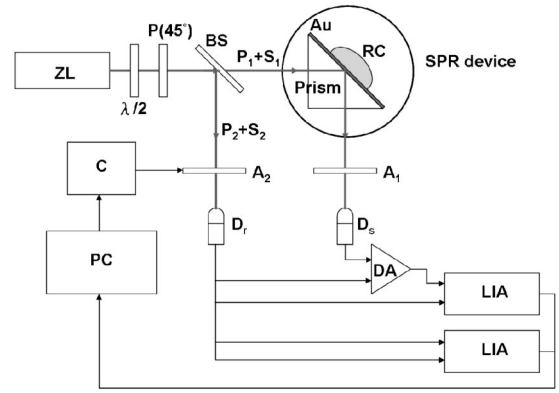

Figure 1. Schematic of the DP-SPRB: ZL, Zeeman laser; $\lambda/2$, half-wave plate; P, polarizer; BS, beam splitter; RC, reaction chamber; D_{ref}, D_{sig}, photodetectors; A_1, A_2, analyzers; DA, differential amplifier; LIA, lock-in amplifier; C, controller; PC, personal computer.

signal and reference beams, respectively, where A_1 is adjusted on its the azimuth angles at 45° to the *x*-axis, while A_2 is adjusted at an angle θ_r to the *x*-axis simultaneously. Thus, two heterodyne signals, $I_{sig}(\Delta\omega t)$ and $I_{ref}(\Delta\omega t)$, from the signal and reference channels are produced by photodetectors, D_{sig} and D_{ref}, accordingly.

$$I_{sig}(\Delta\omega t) = A'_{P1}A_{S1}\cos[\Delta\omega t + \Delta\phi'_{sig}] \quad (1)$$

$$I_{ref}(\Delta\omega t) = A_{P2}A_{S2}\sin 2\theta_r \cos(\Delta\omega t + \Delta\phi_{ref}) \quad (2)$$

where (A_{P1}, A_{S1}) and (A_{P2}, A_{S2}) represent the amplitudes of the P_1, S_1, and P_2, S_2 light waves. Meanwhile, A'_{P1} and $\Delta\phi'_{sig}$ are the attenuated amplitude and phase shifts, respectively, of the reflected P_1 wave due to surface plasma wave (SPW) excitation on the gold/dielectric interface in the SPR device shown in Figure 1. In the meantime, A_{S1} is the amplitude of S_1 wave, which is independent of the SPW excitation in this arrangement, whereas θ_r is adjusted until the amplitudes of eqs 1 and 2 are equal, allowing us to arrive at

$$A_{P2}A_{S2}\sin 2\theta_r = A'_{P1}A_{S1} = K \quad (3)$$

The output intensity from differential amplifier (DA) becomes

$$\Delta I(\Delta\omega t) = I_{sig}(\Delta\omega t) - I_{ref}(\Delta\omega t) = \left|2K\sin\left(\frac{\Delta\phi'}{2}\right)\right|\sin(\Delta\omega t) \quad (4)$$

where $\Delta I(\Delta\omega t)$ is the amplitude-modulated (AM) signal.[13] The differential phases $\Delta\phi' = \Delta\phi'_{sig} - \Delta\phi_{ref}$ and $\Delta\phi'_{sig} = \Delta\phi'_{p1} + \Delta\phi_{sig}$ are defined due to $\Delta\phi_{sig} \cong \Delta\phi_{ref} = n(\Delta\omega/c)l \cong 0$, under the condition of $\Delta\omega = 1.67$ MHz, $l \cong 20$ cm, and $c = 3 \times 10^{10}$ cm/s in this setup. With $\Delta\phi' \cong \Delta\phi'_{p1}$, we now arrive at

$$\Delta\phi'_{P1} = 2\sin^{-1}\left(\frac{|\Delta I|}{2K}\right) \quad (5)$$

It is obvious that the DP-SPRB is able to measure the differential phase, $\Delta\phi'_{P1}$, in terms of the demodulated amplitude of the AM heterodyne signal from the DA in real time. Due to

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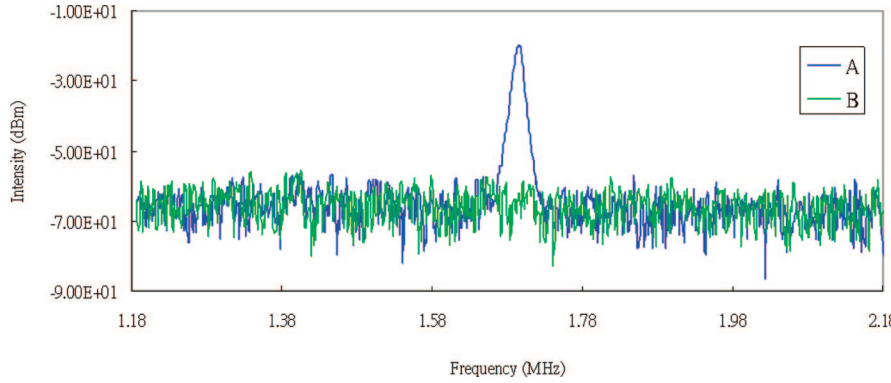

Figure 2. Power spectrum of the output signal from the DA. Curve A is the noise floor of the detected heterodyne signal at tridistilled water, and curve B is the electronic noise level when the laser beam was totally blocked in the measurement.

the fact that the differential phase is dependent on both the variations of refractive index and thickness simultaneously, the phase shift in the experiment therefore follows the relation of

$$\Delta\phi = \frac{\omega}{c} d\Delta n_c + \frac{n_c \omega}{c}\Delta d = \Delta n_{eff}\frac{\omega}{c} d \qquad (6)$$

In Figure 1, two lock-in amplifiers are used in the DP-SPRB for simultaneous monitoring and measuring K in order to satisfy the condition of eq 3 during the measurement. As the intensity ratio is shown in eq 5, this results in DP-SPRB being insensitive to the laser intensity fluctuation in the measurement. Thus, the DP-SPRB is able to measure Δn_{eff} in the vicinity near the gold surface of the SPR device at high sensitivity. Since the amplitude of eq 4 is demodulated by using the envelope detection technique, then the differential phase, $\Delta\phi'_{P1}$, of the signal beam is obtained via the demodulated amplitude in real time. Additionally, the DP-SPRB converts the phase modulation into amplitude modulation by eq 4. Then the differential-phase measurement can reach high detection sensitivity and in real time by means of amplitude detection rather than direct phase measurement by using the phase lock-in technique.[13] At the same time, the common-path configuration of the DP-SPRB decreases environmental disturbance as well. Therefore, the effect of temperature variation in the reaction chamber of the SPR device is canceled out efficiently. This is critical to the high detection sensitivity on Δn_{eff} by DP-SPRB experimentally. In addition, the near field of the SPW can enhance the ability of the DP-SPRB regarding to its specificity and selectivity of biomolecule binding affinity. Moreover, the novel balanced detector detection via the differential amplifier in DP-SPRB is able to perform shot-noise-limited detection.[13] The experimental verification of the shot-noise-limited detection will be demonstrated and discussed in the next section.

EXPERIMENTAL RESULTS

Since the DP-SPRB integrates a novel balanced detector scheme with a common-path polarized heterodyne interferometer, a shot-noise-limited detection is theoretically applicable. Therefore, in order to experimentally verify shot-noise-limited detection of the DP-SPRB, tridistilled water was measured when the incident angle of the laser beam is set near the SPW resonance angle, with the power spectrum of the output signal from the DA measured through a spectrum analyzer (Advantest R3132, Japan). Figure 2 shows the noise floor of −6.5 dBm on testing tridistilled water, with a peak value of −2 dBm at the beat frequency $\Delta\omega$ = 1.67 MHz observed due to the residual phase retardation of the P and S waves produced by the optical components in the DP-SPRB. It is obviously seen that the overlap of the electronic noise level which was under the condition of the laser beam was totally blocked with the noise floor of the power spectrum by testing tridistilled water as shown in Figure 2. This result verifies the shot-noise-limited detection of the DP-SPRB. Moreover, to quantitatively verify the detection sensitivity of the DP-SPRB, a sucrose (Sigma, St. Louis, MO) solution of different wt % concentration, defined by (weight of solute/weight of solution) × 100%, was tested. In this experiment, a bare gold chip (BR-1005-42, Biacore AB, Sweden) of which the reaction area is 7 × 3.5 mm^2 and the thickness is 45 nm is adopted for measuring only the change of refractive index (Δn) of the sucrose solution while the thickness is not involved in this experiment. The detection sensitivity of the DP-SPRB can then be calculated precisely. The refractive index is calibrated by quantitatively checking the concentration of sucrose solution according to the calibration table list in ref 14. In this experiment, the sucrose solution was injected into the reaction chamber of the stagnant designed for measurement, in which the temperature variation in the reaction chamber was ignored because the common-phase rejection mode was provided by the DP-SPRB definitely. In the mean time, the ratio of the amplitudes of the output heterodyne signal from the DA and the reference signal in Figure 1 was measured using a lock-in amplitude (LIA), in which the effect of laser intensity fluctuation is reduced significantly. Thus, the high sensitivity and real-time measurement of differential-phase detection in terms of the demodulated amplitude is verified.

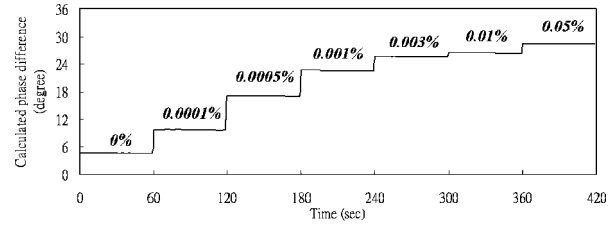

Figure 3. Phase shift obtained from DP-SPRB at different wt % of sucrose−water solutions, which is in a range of 0.0001−0.05%.

Figure 3 shows the varying time response of different concentrations of wt % of the sucrose water solutions from 0.0001% to 0.05%. It demonstrates a limited dynamic range of the DP-SPRB when the concentration is higher than 0.003% on the differential-

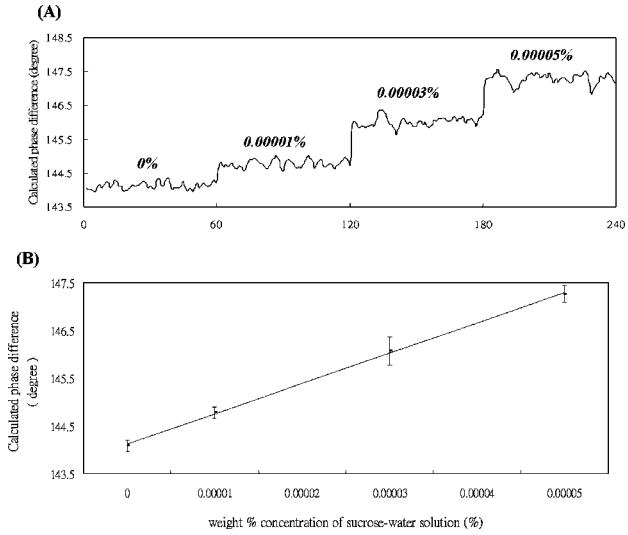

Figure 4. Measurements of sucrose−water solutions: (A) the time response of different weight concentrations from 0% to 0.00005%; (B) linear dependence of phase shift vs concentration of sucrose−water solutions.

phase measurement. Separately, different wt % concentrations from 0.00001% to 0.00005% of sucrose−water solution were tested as well. The detection sensitivity of the DP-SPRB at 0.00001% of the sucrose−water solution is shown in Figure 4. According to the table of refractive index related to wt % concentration of the sucrose−water solution,[14] the concentration at 0.00001% is equivalent to $\Delta n = 1.4 \times 10^{-8}$ RIU relative to pure water. The thickness variation is not involved either in this experiment. Theoretically, the detection sensitivity of the DP-SPRB can be calculated according to $(S/N)^{-1} = \delta(\Delta V)/(\Delta V)$, where S/N refers to the signal-to-noise ratio of the detected heterodyne signal, and $\delta(\Delta V)$ is the standard deviation of the signal as noise and ΔV acting as signals accordingly. In Figure 4, S/N ≅ 5 is calculated at 0.00001% of the sucrose−water solution. This result indicates that the detection sensitivity of the DP-SPRB is $\delta(\Delta n) \cong 2.8 \times 10^{-9}$ RIU. In Figures 3 and 4, the dynamic range of the DP-SPRB is 100 (from 0.00001% to 0.001%), as determined by a linear relationship of output voltage versus wt % concentration of the sucrose−water solution. To extend this method into protein−protein interaction, such as mouse IgG/antimouse IgG interaction in real time, the sensitivity on Δn_{eff} is calculated theoretically at $\delta(\Delta n_{\text{eff}}) \cong 1.7 \times 10^{-11}$ RIU, because of the large molecular weight of the IgG molecule (150 kDa) compared with a small molecular weight of the sucrose (342 Da).[10] The signal level is enlarged by the proportion of their dependence on the molecular weight to refractive index. The sensitivity of the glycerin−water solution on wt % concentration in a range from 0.001% to 0.05% was tested as well. Figure 5 shows the experimental results in which the detection sensitivity becomes $\Delta n \cong 3.0 \times 10^{-7}$ RIU at 0.001% concentration relative to pure water (where S/N ≈ 43). Therefore, the detection sensitivity at 0.001% of the glycerin−water solution is $\delta(\Delta n) \cong 7 \times 10^{-9}$ RIU. By considering the ratio of the molecular weight of glycerin (92 Da) to sucrose (342 Da), the detection sensitivity on the sucrose−water solution at $\delta(\Delta n) \cong 1.9 \times 10^{-9}$ RIU is estimated. This is consistent with the previous result obtained by measuring

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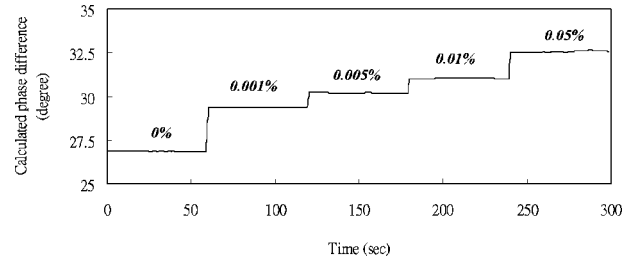

Figure 5. Time response of different concentrations of glycerin−water solution. The range of glycerin−water solutions of weight concentration is from 0% to 0.05%.

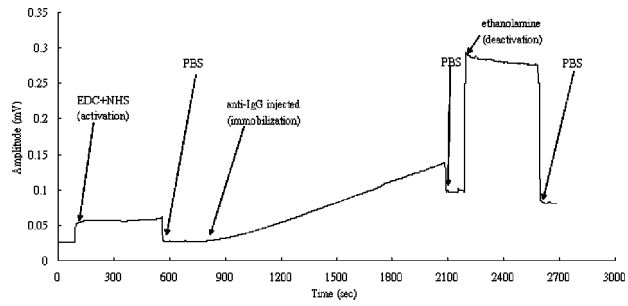

Figure 6. Sensorgram of antimouse IgG immobilized on the CM5 biochip.

sucrose−water solution in Figure 4. Afterward, the CM5 sensor chip (Biacore AB, Sweden) was used in order to measure detection sensitivity of the DP-SPRB by directly measuring IgG/antimouse IgG interaction in real time. Initially, the sensor chip was immobilized by rabbit antimouse IgG at 40 μg/mL concentration following the standard amine coupling method provided by Biacore, Inc.[15] The protocol for this method is as follows:

(1) The sensor chip was activated by immersing a solution of 0.2 M *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.05 M *N*-hydroxysuccinimide (NHS) in tridistilled water,

(2) the rabbit antimouse IgG in 10 mM sodium acetate, pH 5.0, was immobilized via reaction of its nucleophilic groups, and

(3) the excess esters were deactivated using 1 M ethanolamine hydrochloride adjusted to pH 8.5 with sodium hydroxide, which also obviated loosely bound protein.

Figure 6 illustrates the sensorgram of the immobilization of antimouse IgG at 40 μg/mL on the CM5 sensor chip in which a covalent binding of antimouse IgG onto the sensor surface was performed properly. The sensorgram of Figure 6 was obtained using our own designed amplitude-sensitive PSPWB[10] as a large dynamic range of detection is available. Afterward, the immobilized antimouse IgG interacting with IgG at extreme low concentration in PBS (pH = 7.4) buffer solution was measured in real time. The binding kinetics at 10 fg/mL (or 67 aM) of mouse IgG interacting with immobilized antimouse IgG was measured successfully via the DP-SPRB, while S/N ≅ 78 was calculated as shown in Figure 7. Since the protocol of the standard amine coupling method provided by Biacore Inc. can block the nonspecific binding with high efficiency during the deactivation step of the biochip,[15] the measured

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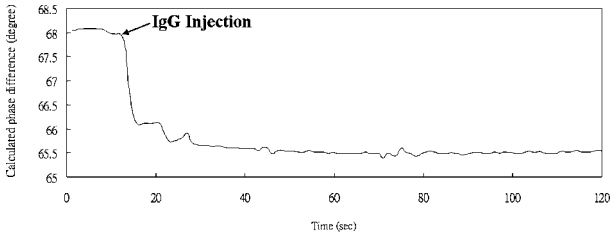

Figure 7. Time response of IgG/anti-IgG interaction of the concentration of IgG at 10 fg/mL in PBS buffer solution.

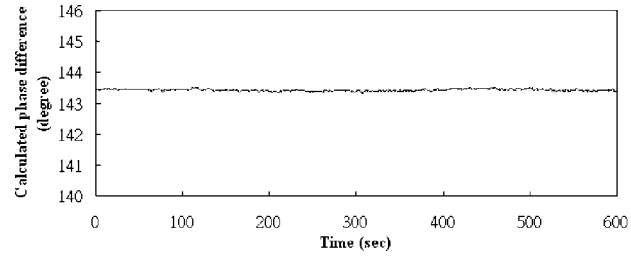

Figure 8. Phase stability of DP-SPRB on testing pure water using a lock-in amplifier.

result shown in Figure 7, with the specific binding between IgG and anti-IgG, is verified in this experiment, with the volume of the reaction chamber identified at 600 μL. Meanwhile, the diffusion equation of the injected mouse IgG in the reaction chamber is ignored because of the extreme low concentration of mouse IgG in the reaction chamber in which the equilibrium state can be reached quickly. Aside from this, the certainty of high-specificity binding between IgG and anti-IgG interaction was also ensured in this experiment.[15] If the binding constants such as dissociation or the association rate constants of biomolecule interactions are of interest, different concentrations of mouse IgG are necessarily detected so that the association and dissociation rate constants can be properly obtained via the DP-SPRB.[16] From the time response of the binding kinetics of mouse IgG and antimouse IgG in Figure 7, higher detection sensitivity of the DP-SPRB is anticipated as well. Simultaneously, the amplitude instability caused by excess noise of the laser is reduced significantly with the intensity normalization by the reference beam. Meanwhile, the environmental disturbance and laser frequency noise of common-phase noises are canceled out due to the common-path configuration of DP-SPRB. Moreover, the limited dynamic range of the DP-SPRB only allows small molecule detection or extreme low concentration in order to preserve the output signal in the linear region. Large biomolecules can easily result in saturation in the output signal where a nonlinear response of binding kinetics of the biomolecule interactions is produced. In other words, high detection sensitivity at extreme low concentrations of biomolecule interactions, for example, the SMD, becomes one of many possible research directions aimed toward meeting the requirement by using the DP-SPRB. In this experiment, identifying the differential-phase stability by testing tridistilled water near the SPW resonance angle was recorded using LIA (Standard Research 844). The phase stability at 0.036° per 10 min in this experiment was achieved as shown in Figure 8. Theoretically, the dynamic range on the differential phase by the DP-SPRB is $0° < \Delta\varphi \leq 180°$. However, the true dynamic range on the differential-phase measurement by the DP-SPRB is limited only by the dynamic response of the rapid phase change on SPW excitation.[2,7] In this research, the DP-SPRB is proposed and experimentally verified to perform the highest sensitivity ever on effective refractive index in real time. Furthermore, the conversion ability of phase modulation into amplitude modulation of the DP-SPRB in a common-path interferometer by using a two-frequency paired linear polarized laser beam not only enhances sensitivity but also initiates a quicker response. These are able to monitor the biomolecule interactions at extremely low concentration where the shot-noise-limited detection is performed as well.

CONCLUSION AND DISCUSSION

Recently, the phase-sensitive interferometric SPR biosensor was proved able to perform on the detection sensitivity of up to $\Delta n_{\text{eff}} \cong 10^{-8}$ RIU.[7] However, it results to the slow response on phase decoding using the phase lock-in technique, and as such a real-time measurement on biomolecule interaction detection becomes impossible under this limitation. In addition, the excess noise from laser intensity fluctuation and the environmental disturbance further degrade the detection sensitivity. In this research, we propose the use of the DP-SPRB which can effectively overcome such deficiencies and significantly improve detection sensitivity. Additionally, the synchronized detection and coherence detection of heterodyne signals, which are dependent on the spatial and temporal coherence between paired polarized waves, are critical to the sensitivity of the DP-SPRB. In other words, the scattering effect of the surface plasma wave through the roughness of gold film lowers the correlation between paired polarized waves in the DP-SPRB. This results in a lower heterodyne efficiency and S/N of the heterodyne signal from DA. Therefore, a high-quality gold film and high degree of coherence of the two-frequency laser are both integral toward the effective performance of the DP-SPRB.

In conclusion, the features of this novel DP-SPRB method include a highly spatially and temporally correlated two-frequency paired linear polarized laser beam integrated with a novel balanced detector which can successfully detect 0.00001% of weight concentration in sucrose−water solution. This is equivalent to the sensitivity of $\delta(\Delta n) \cong 2.8 \times 10^{-9}$ RIU on sucrose−water solution measured experimentally. Meanwhile, a sensitivity on 10 fg/mL (67 aM) of mouse IgG interaction with antimouse IgG was demonstrated by the DP-SPRB as well. To our knowledge, this is the highest sensitivity ever measured by SPRB. However, a narrow dynamic measurement range is resulted because of phase detection of SPW excitation. Thus, only extreme low concentrations or small molecules can be measured in the linear response region by the DP-SPRB for biomolecule interactions. Potentially, the DP-SPRB can be suggested for single-molecular detection at extremely high detection sensitivity in real time.

In summary, the features of the DP-SPRB able to measure the binding kinetics of biomolecule interactions rely on the following properties: (1) a two-frequency paired linear polarized laser beam, (2) a novel balanced detector to result in shot-

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noise-limited detection, (3) a phase-to-amplitude modulation conversion resulting in differential-phase decoding by a conventional envelope detection technique, (4) a real-time detection via demodulated amplitude detection, (5) a synchronized detection resulting in high S/N of the heterodyne signal, (6) a common-phase noise-rejected mode to immune the background phase noises, and (7) an insensitivity to the scattering effect of the tested medium.

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