Sergiy Patskovsky, Renaud Jacquemart, Michel Meunier, Gregory De Crescenzo, Andrei V. Kabashin

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A B S T R A C T
We here describe a scheme of spatially modulated surface plasmon resonance (SPR) polarimetry that enables to combine ultra-high phase sensitivity with good signal-to-noise background. The proposed approach uses spatial modulation of s-polarized component by birefringent elements and the extraction of phase-polarization information by Fourier-transform methods. This scheme was tested for monitoring the interactions between an antibody and its biological partner. Our experimental data, collected by amplitude-sensitive and phase-sensitive polarimetry demonstrate that the latter scheme provides at least one order of magnitude improvement in terms of detection limit.

1. Introduction

Within the last two decades, surface plasmon resonance (SPR) has emerged as a novel powerful tool for biomolecular interaction analysis, drugs discovery and, more generally, to life science research [1–3]. SPR sensing technology is based on the detection of refractive index changes occurring within a thin layer of liquid (10–200 nm) near a metallic surface. Providing that a biological species is covalently bound or captured to a surface, its interaction with its biological partner being present in the fluid above the surface will lead to biological complex formation according to the law of mass action. These interactions thus imply a net mass increase at the biosensor surface, the latter being proportional to the changes to be attributed to non-specific interactions (i.e. binding of the biological partner to the surface itself), it is then possible to derive both kinetic and thermodynamic parameters related to the interactions. This approach has been routinely applied to the study of macromolecular interactions such as protein-protein, protein-DNA or protein-polysaccharide interactions using various optical biosensors now commercially available (for a recent review, the authors refer the reader to Rich and Myszka, 2006 and references therein). Recent studies also reported the use of SPR devices for the detection and characterization of small molecular weight compounds (~200 Da) binding to macromolecules [4–6] (Myszka, 2004). These recent breakthroughs highlighted the limits of detection of current biosensors since appropriate signal levels required immobilization of high amounts of biological partners to biosensor surface. Indeed, as common SPR biosensor detection principle is based on mass accumulation, the lower the molecular weight of the species to be detected is, the higher the surfacic density of bound partner must be. Hence, detection of very low molecular weight compounds is still an issue. That is, commercially available SPR biosensors are generally implemented in the Kretschmann configuration to direct p-polarized light through a glass prism and reflect it from a gold film. When the tangential x-component of the incident optical wave vector, \( k_x \), is matched with \( k_{sp} \), the pumping light energy is transferred to surface plasmons according to:

\[
k_x = k_0 n_{glass} \sin \theta_{inc} = k_{sp},
\]

where \( n_{glass} \) corresponds to the refractive index of the prism and \( \theta_{inc} \) to the angle of incidence. In this case, SPR is observed as a dip in the angular (spectral) dependence of the reflected intensity. In fact, the presence of such a strict condition of wave vector matching implies that any change of the refractive index of the neighbor medium that contacts gold will drastically affect it. SPR sensors employ this property and monitor changes of the biological film thickness due to binding events in the close vicinity of the gold layer responsible for SPR to occur. In most SPR biosensor instruments,
the information about the biomolecular interactions is obtained from measurements of angular [7–9] or spectral [10] characteristics of light reflected under SPR. In this case, the detection limit of such instruments is about \((5–10) \times 10^{-6}\) at the best in terms of the refractive index change (expressed in RIU), which corresponds to \(1 \text{ pg mm}^{-2}\) of biomaterial accumulating at the biosensor surface.

It has been shown in several studies [11–16] that the sensitivity of SPR measurements could be improved by at least two orders of magnitude by monitoring the phase characteristics of light instead of its intensity. Such a gain in sensitivity was attributed to the phase jump across SPR, which was demonstrated to be much sharper than dips observed when monitoring intensity changes [11,12].

In this paper, we propose a phase-sensitive SPR scheme for biosensing purposes. As instrumental implementation, we used a scheme of common path SPR polarimetry, which provides high sensitivity under a relatively simple experimental arrangement and the absence of inertial noises. Even though the detection limit of this experimental scheme (better than \(10^{-6}\) RIU) was limited by external temperature and intensity drifts, our results clearly indicated that phase-sensitive measurements provided better detection limit than that obtained by classical intensity monitoring.

2. Methodology

2.1. SPR system setup

A schematic diagram of the instrument design used in this manuscript is shown in Fig. 1, panel A. The SPR effect was produced in conventional Kretschmann-Raether prism arrangement. A 30 mW laser diode operating at a wavelength of 660 nm was used as the light source. The wavelength was chosen in order to optimize the phase response for the SPR sensor utilizing 50 nm gold film. A polarizer and a half-wavelength plate enabled to set linear polarization with a variable contribution of s- and p-components. The beam was then filtered by an optical filter, consisting of a lens and a pinhole, and was directed onto the SPR prism coupling system (i.e. the sensor head). The sensor head used a custom-made glass prism (BK7 or SF11 glass) with a proper base angle to provide near-normal incident angle. A commercial biosensor chip (CMS, BIACORE Inc., Piscataway, NJ) corresponding to a glass slide covered with a thin layer of gold to which a three-dimensional, non-reticulated carboxy-methylated dextran matrix is anchored for macromolecule coupling purposes (see section below for sensor chip preparation), was then placed in immersion contact with the prism. The SPR coupling at a specific angle of light incidence on the sensor head (SPR angle) made the excitation of surface plasmons possible over gold/adjacent medium interface. The SPR effect was accompanied by a drastic decrease of intensity of p-polarized component at the SPR angle and a sharp jump of its phase. The spatial distribution of the reflected light intensity was then recorded by a CCD camera and examined by proper software image treatment.

The proposed design involved two additional birefringent elements, which enabled to spatially modulate the beam and then extract phase information. The modulation was produced by a birefringent wedge (spatial phase retarder), which was placed after the filter. The axis of the retarder was oriented perpendicular to the plane of SPR production. After passing the wedge, the beam became spatially modulated along the wedge axis with periodic changes of phase relations between the s- and the p-polarized components, hence giving rise to periodically elliptical polarization. An exit polarizer (analyzer) composed the second birefringent element, which was placed just after the SPR coupling block. The analyzer was oriented at 45° to enable interference between projections of s- and p-polarized components to its axis. This gave rise to the appearance of dark and bright fringes that were associated with the above-stated periodic wedge-caused modulation of phase difference between the s- and the p- components. Fig. 1, panel B shows a typical image of interference patterns as observed with the CCD camera. Here, horizontal fringes corresponded to the wedge-caused modulation of the beam. The phase information was then extracted by the Fourier transform method from the interference pattern. Here, horizontal line-to-line or pixel-to-pixel algorithms for data treatment can be applied. This approach enabled to fully restore the phase and intensity information over the laser spot size area.

2.2. Biological materials and methods

2.2.1. Materials

Chicken egg-white lysozyme (Cat# L3790) was purchased from Sigma–Aldrich (Canada). Rabbit polyclonal anti-lysozyme antibody (Cat# AB391-1, lot# 216035, 10 mg/mL) was purchased from ABCam Inc (Cambridge, MA). All the chemicals and buffers used for SPR experiments were purchased from Biacore Inc. (Piscataway, NJ).
2.2. Methods

2.2.2.1. Biological system. An antibody/protein biological system consisting of a rabbit polyclonal antibody (154 kDa) directed against egg-white lysozyme was selected to test our experimental system. This biological system was selected based on (i) the well-documented high stability of lysozyme (i.e., conservation of its enzymatic activity even after drastic cleaning conditions and after long term storage) and (ii) since we previously showed, by conventional SPR, that the selected polyclonal anti-lysozyme antibody is able to bind to lysozyme when immobilized on biosensor surface.

2.2.2.2. Sensorchip surface generation. For this study, the sensorchip surface corresponded to a CM5 chip (Biacore Inc.) to which the antibody was covalently coupled using a BiacoreTM 3000 instrument and its Surface Prep Unit SP 2 module provided by the manufacturer. This approach allowed us to prepare a single, large area surface being 3.3 times larger than those generated when using standard BiacoreTM surface preparation procedure. Covalent coupling of lysozyme was achieved by standard amine coupling procedure adapting the protocol described in [17]. During immobilization, flow rate was set at 10 μL/min. Injections of a 0.05 M N-hydroxysuccinimide (NHS) and 0.2 M N-ethyl-N-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC) mixture (70 μL) were followed by lysozyme immobilization (70 μL at 1 μM in sodium acetate, pH 5.5). A solution of 0.1 M ethanolamine-HCl (70 μL, pH 8.5) was used to block the remaining activated carboxyl groups.

2.2.2.3. Detection of lysozyme/antibody interactions by phase-sensitive SPR. Maximum phase sensitivity of SPR is achieved at the angles close to the optimal SPR angle given by Eq. (1). Therefore, preliminary system calibrations were performed by measuring the reflected light intensity as a function of angle of incidence. Angle corresponding to minimal reflectivity was used for the following real-time measurements.

An in-house-designed open flow cell allowing for injection of soluble sample or still measurements was used for the detection of lysozyme-antibody interactions. Buffer used to fill the flow cell was HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20). Upon addition of buffer to the flow cell, baseline stabilization was established prior to several additions of buffer (for stability confirmation) or concentrated antibody solution (10 mg/mL). The concentration of antibody in the cell was thus increased in a stepwise fashion. The SPR signal corresponding to the interaction of the soluble antibody with immobilized egg-white lysozyme was followed in real time. Fourier transform method was applied to test both phase and intensity of the reflected light at the same time. Phase response was found to be more stable than intensity response to light source intensity fluctuations.

3. Results and discussions

In a first series of experiments, calibration of the system was carried out by flowing different refractive index solutions on the sensorchip. Glycerol/water solutions with different refractive indices, i.e. different glycerol/water ratios ranging from 0.008 to 2.58% (w/v) were used. Known refractive index values associated to these solutions allowed us to calibrate the instrument as well as to determine the detection limit of our system. Fig. 2, panel A shows the phase responses we measured when increasing glycerol content. One can see that, by measuring the phase, we were able to easily detect even relatively small variations of glycerol concentration (0.008%) corresponding to a refractive index change of 10⁻⁵. Taking into account the level of noises (0.03°), we can estimate that the detection limit in our system is better than 10⁻⁶ refractive index units. Such detection limit level is lower by almost an order of magnitude than in conventional SPR devices. It should be noted that measured value of the detection limit does not represent the physical limit given by phase characteristics (as it was shown in [11,12], the phase sensitivity can be lower than 10⁻⁸ if SPR production conditions are well optimized), but is related to a relative predominance of the signal over noises in our experimental setup. We reason that in our case these noises were mainly related to laser power and temperature drifts. It is clear that the mentioned drifts can be drastically reduced by optimizing the design of the system and introducing an active thermo stabilization system. The calibration curve corresponding to phase variation as a function of refractive index is presented in Fig. 2, panel B. One can see that the phase response was almost linear until more than Δn = 10⁻³ RIU, suggesting that the phase presents a good sensing parameter under small refractive index changes. At larger RI changes the phase signal started to saturate. This phenomenon is explained by a limited dynamic range of phase measurements in the case of fixed angle of light incidence. The dynamic range of phase measurements can be improved by using either schemes with converging beam or applying feedback loops. The development of these configurations is now in progress and will be published elsewhere.

The system was then applied to real biosensing experiments, i.e. monitoring the specific interaction between a polyclonal antibody and lysozyme that had been previously immobilized on a commercially available sensorchip coated with carboxymethylated dextran (see Section 2.2). Typical real-time phase and intensity responses are shown in Fig. 3. Stable baselines for both phase and intensity.
signals were obtained when the measurement cell was filled with 0.5 mL buffer. Baseline stability was further tested by manually adding buffer in the measurement cell (10 μL added twice). As can be seen in Fig. 3, no major variations of the signal were observed upon addition. In contrast, subsequent additions of concentrated antibody samples (5 or 10 μL additions) led to significant phase-sensitive signal changes to be correlated to antibody-lysozyme interaction resulting from antibody accumulation at the sensorchip surface. After each antibody addition, transient profiles were consistent with those expected from a biological interaction occurring under total mass transport limitation. That is, stepwise additions of antibody resulted in thermodynamic equilibria (i.e. signal stabilization) being reached within approximately 60–100 min. This time scale is consistent with our biological interaction being kinetically limited by the diffusion of soluble antibody to the vicinity of the derivatized surface since no stirring was performed after sample addition.

As shown in Fig. 3, phase provided a higher noise-to-signal ratio. In fact, for the same noise level, the signal corresponding to phase sensitivity measurement was at least one order of magnitude higher than that corresponding to intensity measurement. It should be noted that this comparative test was performed only in optimal phase measurement conditions, corresponding to the minimum of reflected intensity.

4. Conclusion

Thanks to a simple scheme allowing for the simultaneous measurements of phase sensitivity and intensity, we have experimentally demonstrated that phase-sensitive spatially-modulated SPR polarimetry provides substantial gain of at least one order of magnitude when applied to biomolecular interactions. Further gain is expected to be achievable in a close future by decreasing temperature and laser intensity drifts in our current system. Furthermore, the sensing approach that is presented here relies on a detection scheme that is compatible with a spatial resolution over the SPR-supporting gold surface, hence rendering it amenable to multi-sensing tasks that are required in array configurations.

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