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Multiplexing of radiative-surface plasmon resonance for the detection of gastric cancer biomarkers in a single optical fiber

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ABSTRACT

The simultaneous detection of multiple biological analytes is achieved for the first time using a single optical fiber based surface plasmon resonance (SPR) biosensor. This is achieved by collecting the plasmonic wave re-scattered by a rough metallic coating deposited onto two separate sections (sensing regions) of a single multimode optical fiber. The results obtained showed that two gastric cancer biomarkers (apolipoprotein E and clusterin) are detected in clinically relevant concentrations each on a separate sensing region, simultaneously, something that cannot be done in traditional fiber based SPR biosensors that read the transmitted or reflected light to monitor the position of the resonance. While this multiplexing demonstration has been performed with two different biomarkers, it paves the way for the multiplexed detection of a larger number of biomarkers using a simple fiber optic based SPR sensor for point of decision diagnostics.

1. Introduction

In medical diagnostics there is a growing need for biosensing technologies capable of screening a large amount of samples in a relatively short time and at a low cost, toward the point of decision diagnosis [1–3]. The detection of a single biomarker has proven insufficient for the diagnosis of most pathologies [4,5]. It has been shown that the regulation of multiple biomarkers needs to be considered for an accurate diagnosis of pathologies including cancer [6], while the results obtained for a single biomarker can often be influenced by other environmental and/or physiological conditions, leading to a false positive results. In particular, a number of biomarkers have been identified as having anomalous regulation in patients with gastric cancer [7], which is the second leading cause of cancer death worldwide and is one of the most common malignancies experienced in Asia [8,9]. Identification of early-stages disease may be the most promising approach for reducing gastric cancer mortality [9]. In particular, studies on patients affected by gastric cancer have shown a 2.5-fold over expression of apolipoprotein E (apoE) and a 2-fold under expression of clusterin (CLU), compared to the physiological regulation range of 23–49 μg/ml and of 250–450 μg/ml respectively [9–12]. However, other studies demonstrate that tumor progression and malignancy correlate to increased expression of CLU [13], and the apparent discrepancy of data may be related to the fact that CLU exists in various isoforms [9]. Obviously one may ask about accuracy of the diagnostic, especially considering that the normal regulation ranges of the two mentioned proteins are fairly large and that there may be an overlap between the healthy and unhealthy patients. The answer to this problem is therefore to increase the number of biomarkers tested simultaneously, reducing the uncertainty of the diagnostic.

Surface plasmons are collective oscillations by free electrons on a nanometric metallic film at the interface with a dielectric. They are sensitive to change in refractive index [14,15], and can be probed via illumination with a polarized laser through a prism, on top of which a metal such as gold is deposited. Observation of the reflected light at different incidence angles reveals a dip in the reflectivity is observed when the SPR conditions are fulfilled. Commercial available SPR biosensors systems [16] have proven effective in detecting the presence of multiple analytes and in determining their concentration [17]. However the price of such systems is typically too high to enable their use as a point of decision screening tool. In addition at present tests that are tailored to be specific to a certain pathology are limited [1].

Optical fiber based SPR biosensors have been demonstrated in different configurations, and although to date they have not reached the sensitivities and detection limits achieved by SPR devices that use the standard Kretschmann configuration, they offers some advantages including the possibility of realizing dip sensors, the degree of miniaturization achievable [14] and

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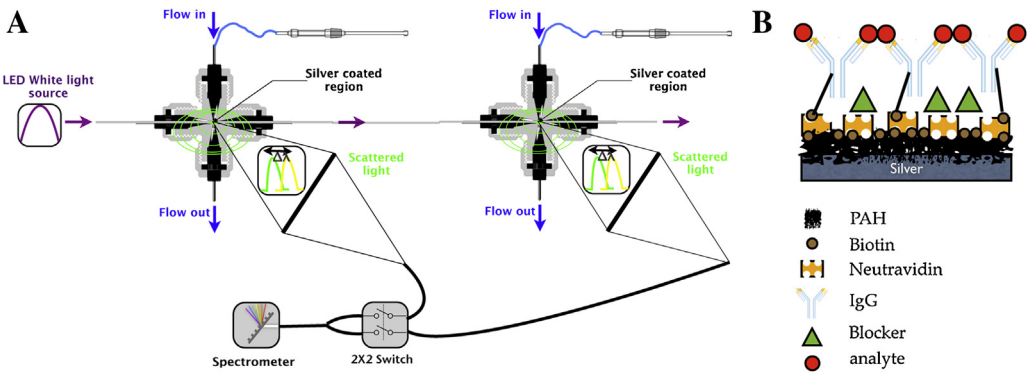

Fig. 1. (A) Representation of the radiative SPR multiplexed architecture. The plasmonic wave scattered from the sensing regions (SRs) is collected and analyzed by a spectrometer. (B) Surface functionalization strategy. Neutravidin is attached to the biotinylated surface in order to have biotinylated antibodies to bind in a oriented fashion.

the convenience of the industrial production of optical fibers [18]. Typically, a section of the fiber core is coated with a metal (e.g. gold) and white light is coupled into the fiber; the transmitted light is monitored by a spectrometer and a dip is observed at the wavelengths satisfying the SPR conditions [14,19].

The principle drawback of optical fiber based SPR biosensing devices is their limited versatility for the realization of biosensors that can detect multiple analytes simultaneously. In fact, because of the nature of the measurement performed to assess the SPR signal (transmitted or reflected light), only one sensing region can be engineered per optical fiber, and consequently only one analyte can be detected per SPR fiber sensor [20].

We have recently shown [21] that the intrinsically non radiative nature of the SPR process can be overcome using a rough metallic coating, enabling the re-scattering of the plasmonic wave and turning SPR into a radiative process. A spectral analysis of the emitted light enables the characterization of the SPR signal in a different way, which allows a radically different sensor architecture with additional capabilities. In this paper we demonstrate for the first time that with this architecture the detection of multiple gastric cancer biomarkers is possible within a single unstructured optical fiber using a simple optical configuration.

2. Materials and methods

2.1. Sensor preparation

The optical fiber based SPR biosensor was prepared coating a in-house produced bare (unstructured) optical fiber with a rough silver film, as reported in Francois et al. [21]. Briefly, an optical fiber made of F2 Schott lead silicate glass with a refractive index of 1.62 and a diameter of 130 μm was extruded from a bulk glass sample into a rod and drawn into a fiber using a fiber drawing tower. The fiber was subsequently coated with a polymer with a refractive index of 1.52. A 1 m long portion of the fiber was used to prepare the sensors. Two short (≈2.5 mm) sections separated by 40 cm were mechanically stripped of the polymeric coating and coated with a nanometric silver film using the Tollens reaction [22] to obtain two sensing regions (SR) in series. A solution of 20 ml of $AgNO_3$ 0.24 mol/l and 40 μl of KOH 0.25 mol/l was reacted with a 20 ml mixture of methanol and glucose (1.9 mol/l) 1:2, in a Petri dish in presence of the two stripped sections of the fiber for 8 min at room temperature. After deposition, the sensing regions were thoroughly rinsed in Millipore water and dried in air. The sensors were then immersed for 60 min in a 2 mg/ml solution of Poly(allylamine hydrochloride) (PAH) in 1 M NaCl, rinsed with Millipore water and dried under a stream of nitrogen. PAH is a positively charged polyelectrolyte that adsorbs onto the negatively charged silver surface, introducing amine groups for further bioconjugation.

2.2. Optical setup and data processing

Light emitted by a white LED (6500K, Thorlabs) was focused and coupled into the optical fiber. For each SR the scattered plasmonic wave was collected by a microscope objective (20×), focused into a patch cable (200 μm core), and fed into a cooled compact CCD spectrometer (Ocean Optics, QE65000, with a SNR of 1000:1) through a 2 × 2 switch to select either one of the two SRs to analyze (see Fig. 1A). The switch was programmed to periodically switch between the two channels with a lag of 5 s. For biosensing experiments, each recorded spectrum was typically averaged over 3 s (5–10 averages, depending on the acquisition time) to further reduce the SPR fluctuations. A cross-correlation function was applied to the recorded experimental spectra to calculate the SPR shift overtime. The top inset in Fig. 2A shows a typical recorded spectrum for the re-scattered SPR signal from a sensor immersed in Millipore water. The inset in Fig. 2B shows the fluctuation of the SPR position over time in phosphate buffered saline (PBS, pH = 7.4). The standard deviation of this sample data is below 0.01 nm.

2.3. Surface functionalization

In order to ensure the correct orientation of the antibodies (see Fig. 1B), which is crucial especially for the detection of small proteins [23], both the SRs were functionalized with biotin (0.6 mg/ml), a vitamin containing a carboxylic group, that was covalently bound to the amine-terminated surface of PAH in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.2 M) and N-hydroxysuccinimide (0.2 M) for about 30 min under flow rate of 20 μl/min, and the SPR signal was monitored over time. After a thorough PBS wash, the surface of both SRs were exposed for about 30 min to a 20 μl/min flow of 400 nM Neutravidin, a tetrameric protein that binds specifically biotin, previously immobilized, forming a strong non-covalent bond ($K_D \sim 10^{-15}$ M) [24]. After a thorough PBS wash, SR1 was further functionalized with a solution of biotinylated anti-CLU IgG (330 nM, R&D System) flowed at 20 μl/min for about 30 min, allowing the biotin function of antibodies to bind to free groups on Neutravidin (see Fig. 1B). SR2 was functionalized with biotinylated anti-apoE IgG (330 nM, MabTech) instead, for about 30 min.

After the immobilization of antibodies, the sensor was exposed to a blocking reagent (The Blocking Solution, Candor) for 45 min (flow rate of 10 μl/min) in order to block any unreacted binding sites, preventing non-specific binding. This approach has proven effective to block unspecific binding [23]. The exposure time of the

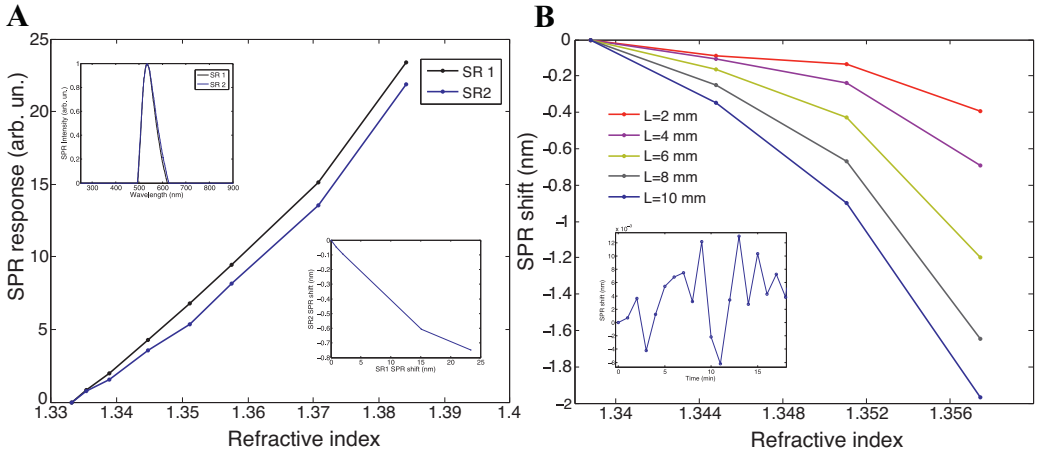

Fig. 2. (A) SPR shift response vs. bulk refractive index of water/glycerol solutions for the two SRs. Top inset shows a typical SPR signal scattered from the two SRs. Bottom inset shows the SPR response observed in SR2 as a function of the SPR shift induced in SR1. (B) SPR shift response of SR2 as a function of refractive index changes induced in SR1 for different SR1 lengths. Smaller SR1 lengths caused small cross-talk. The inset shows the typical fluctuation over time of the SPR response in PBS.

species in the flow cell was varied until no further shift in the SPR position was observed as a means of ensuring a complete saturation of the available functional groups.

3. Results and discussion

3.1. Characterization of the double sensing region SPR biosensor

The top inset in Fig. 2A shows a typical radiated SPR signal (which occurs at 520 nm) from the two SRs when exposed to water. The response of the system was characterized by exposing the two SRs to glycerol/water solutions at various concentrations to obtain a range of bulk refractive indexes. This was done to demonstrate that changes in refractive index could be detected separately in the two SRs. Fig. 2A shows the shift of the SPR signal of the two SRs as a function of the bulk refractive index, and demonstrates that both SRs are sensitive to changes in refractive index and therefore can be used to detect two different analytes within the same optical fiber, a first step for realizing a multiplexed fiber based SPR platform. The sensitivity is in the order of 740 nm/RIU, smaller than the best reported values for fiber optics based SPR sensors operating in this range [14,25]. However, the limit of detection (LOD) is often reported as a mean to measure the performance of a sensor, although it is not an intrinsic characteristic of the sensor because it depends on the equipment used to measure it. The LOD is often defined as the ratio between the sensitivity (S) and the resolution of the sensor (R) of the sensor [27]. As far as our system is concerned, we can discriminate a SPR shift of 0.03 nm, calculated as 3 times the spectral noise (see inset in Fig. 2B) [26,27] which is in our case the main limiting factor compare to other source of noise such as the amplitude noise and thermal noise as defined by White and Fan [27], and resulting in a LOD of 4×10^{-5} RIU. Such a LOD is not as good as the best reported fiber based SPR sensors with LOD down to 10^{-5} RIU [14,28], but it is still comparable to other recently reported values for fiber based SPR [29,30].

Note that the overall sensitivity, in terms of nm/RIU shift, differs slightly between the first and second SR. This is due to cross-talk between the SRs; in fact, the light probing the second SR has a spectral content slightly different from the input light, that is partially dissipated through the metal and re-scattered after interacting with the first SR. Furthermore, the spectral density of light after the first SR depends on the refractive index surrounding SR1, and therefore we expect it also to have an effect on the plasmonic wave emitted by the second SR. Indeed, if we vary the bulk refractive index of SR1 and we observe the SPR response of SR2, we observe that a small SPR shift occurs on SR2 (see bottom inset in Fig. 2A), which is only caused by a change of refractive index in SR1. This cross-talk, defined as the SPR shift observed on SR2 as a result of a change in refractive index in SR1, and the effect of this artifact on our system is explored below. As evident in the bottom inset in Fig. 2A, this cross-talk is estimated to be below 5% (calculated as the ratio between the shift observed in SR2 and that induced in SR1) for this experimental conditions, which is, in absolute terms, in the same order of magnitude of the noise level measured in SR2 when a small protein binds to SR1.

3.2. Determination of sensing regions cross-talk length

The impact of cross-talk depends on two factors: the coupling efficiency of light to the SPR and the length of the first sensing region. The former is dependent on the details of metallic layer and has also an effect on the amount of light scattered by the SR and therefore on the signal to noise ratio (SNR). The trade-off between SNR, which is improved with higher light coupling efficiency to SPR, and cross-talk, which is decreased with lower light coupling efficiency into the plasmonic wave, reduces the space for further improvements by just varying the chemical conditions of the metal deposition process. The latter factor can be easily controlled during the fabrication process of the sensors, and can be used to optimize the system and limit the cross-talk between the SRs to value that suit the application. The cross-talk was systematically studied as a function of the SR1 length and of the refractive index surrounding SR1, and the results are presented in Fig. 2B. As the refractive index of SR1 increases, the SPR response observed in SR2 is blue-shifted. The absolute amount of blue-shift observed in SR2 strongly depends on SR1 length. Unsurprisingly, a longer SR1 causes a larger blue-shift on SR2 for a certain refractive index change in SR1, thus increasing the cross-talk artifact.

However, assuming a 2.5 mm length for SR1, the artifact on SR2 is below 5% in terms of SPR response (see bottom inset in Fig. 2A), and for typical SPR responses due to the binding of biomolecules such as IgG, a typical absolute value of the artifact is as small as hundredths of a nanometer, which still lies within the fluctuations of the SPR signal.

Fig. 3A represents the SPR response of the two SRs during the functionalization process. Note that the solutions were injected with a delay between the two SRs to demonstrate that the cross-talk is negligible in these circumstances. Indeed, we observe that

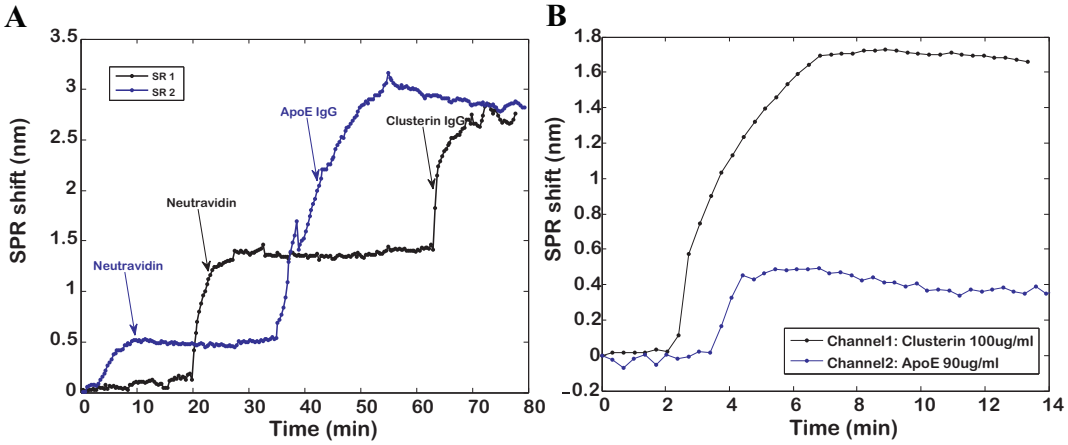

Fig. 3. (A) Real-time SPR response during the binding of neutravidin and of anti-clusterin IgG and anti-apoE IgG for SR1 and SR2 respectively. (B) Real-time SPR response over binding of key gastric cancer biomarkers. Human clusterin (SR1) and human apoE (SR2) are simultaneously detected in less than 15 min.

the baseline of SR2 is stationary while SR1 sees binding events and vice versa, which proves that the multiplexed platform we propose is suitable for biomarkers detection.

3.3. Detection of gastric cancer biomarkers

CLU and apoE were chosen as test proteins because of their importance as gastric cancer biomarkers [7,9,11]. As mentioned in the introduction, studies on patients affected by gastric cancer have shown a 2.5-fold over expression of apoE and a 2-fold under expression of CLU, compared to the physiological regulation range of 23–49 μg/ml and of 250–450 μg/ml, respectively [9–12]. Therefore we have deliberately chosen to use concentrations for these two proteins in the clinically relevant range which would allow to formulate a clinical diagnostic, 90 μg/ml solution for human apoE and 100 μg/ml solution of human CLU. As previously mentioned, biotinylated anti-CLU IgG were immobilized on SR1 and biotinylated anti-apoE IgG on SR2, in order to impart different functionalities to the SRs, and to enable selectivity to either to human CLU or to human apoE. In Fig. 3B the SPR response of the two SRs is presented when SR1 is exposed to a 100 μg/ml solution of human CLU under a flow rate of 10 μl/min (black line), and SR2 is exposed to a 90 μg/ml solution of human apoE under a flow rate of 10 μl/min (blue line). Quick (sub 15 min) simultaneous detection of both CLU and apoE with an optical fiber based multiplexed SPR biosensor is demonstrated, showing the potentialities of the architecture we propose, based on the analysis of the scattered plasmonic wave, with a simple fabrication process and high versatility.

4. Conclusion

In summary, we have demonstrated that our radiative SPR platforms allows for the implementation of multiple sensing regions, which are sensitive to change of the surrounding refractive index. A degree of cross-talk between the two sensing regions has been observed when changes of refractive index were induced on the first one. This artifact was extensively characterized to fully understand its effect as a function of the length of the sensing regions and of the refractive index, with smaller SRs lengths causing less cross-talk. We have also demonstrated the simultaneous detection of two gastric cancer biomarkers CLU and apoE in clinically relevant concentrations, down-regulated and up-regulated, respectively. It was found that in our experimental conditions the cross-talk between the SRs does not impact the detection of the particular set of gastric cancer biomarkers that we have tested. In addition, based on our results we can project the number of sensing regions that could be employed in a situation where higher number of biomarkers are to be detected. In fact, assuming to employ an architecture where only one long SR is present, functionalized along its length with different capture IgGs, we can estimate from our results that the length of the SR, that gives a cross-talk below 10%, would be 5 mm. Setting up a 500 μm spacing between the different IgG sections, achievable with commercial spotting systems, up to 10 different biomarkers could simultaneously be detected.

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Alexandre François is a Research Associate at the Institute for Photonics & Advanced Sensing (IPAS) at the University of Adelaide. After receiving his PhD in Electrical Engineering from the University of Sherbrooke (Canada) and a post-doctoral experience working in the pharmaceutical company (Fujirebio Inc.), Dr François specialized himself in optical biosensing technologies and especially label free technologies such as surface plasmon resonance and cavity mode spectroscopy with a strong emphasis on medical diagnosis applications.

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Tanya Monro is Professor at the University of Adelaide. She was awarded the Bragg Gold Medal for the best Physics PhD in Australia in 1998 for her PhD work at The University of Sydney. Her research focuses on optical materials and micro/nanostructured optical fibers and devices for use in next generation lasers, sensors and nonlinear devices. She is Director of the Institute for Photonics & Advanced Sensing (IPAS) at The University of Adelaide. IPAS pursues a transdisciplinary agenda, bringing together physics, chemistry and biology to create disruptive technologies. She has published over 450 papers.