How the surrounding environment affects the biosensing performance of resonant nanopillars arrays: Under dry conditions or immersed in fluid

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ABSTRACT

In this work we demonstrate the advantage of performing the biosensing process of a refractive index optical biosensor under dry conditions, in comparison with the biosensing structure immersed in fluid. We developed a biosensing experiment over a specific transducer based on resonant nanopillars (R-NPs) arrays. The optical interrogation to monitor the recognition events was firstly performed with the R-NPs in dry, only in contact with the air, and secondly with the R-NPs immersed in water. We observed a significant enhance in the sensitivity of the biosensing curve response for the R-NPs in dry conditions, leading an improvement of the Limit of Detection (LoD) in more than one order of magnitude. These results are also in good correlation with 3D-Finite difference time domain simulations carried out for both fluid conditions. According to this result, any interferometric optical bio-transducer for in-situ diagnosis will improve the sensitivity in case it can operate in dry conditions. Moreover, measuring in simple drops of biological samples, in dry conditions, will be a relevant issue for Point of Care Devices.

Keywords:

Biosensing enhance between dry/wet conditions

Optical biosensor

Resonant nanopillars

In-situ diagnosis

Vertical optical interrogation

Label-free

1. Introduction

High sensitivity optical detection methods are a continuous matter of study, in which scientific community is working persistently to achieve the development of novel biosensing systems with improved performance [1]. This work is specially focused on the field of micro and nano technology, where many optical integrated sensors and biosensors [2,3] have proven to be effective and strong candidates to become the technology on which the diagnosis systems lab-on-a-chip [4] and Point of Care detection devices (PoC) must be based [5–7].

Presumably the best known label-free optical biosensor is the SPR (Surface Plasmon Resonator) [8], which is based on the refrac-

tive index change (RI) and is being widely used commercially. Moreover, last trends in research are addressing miniaturization and portability [9]. In addition, optical biosensors based on interferometric transducers can measure biological moieties by measuring the change in refractive index (RI) caused by the bioconjugates interaction [10]. Some examples of interferometric transducers are Mach Zehnder [11], BICELLS [12], Young interferometer [13] or photonic crystals [14], among others. Also optical waveguides and ring resonators are used in biosensors based on optical interferometry [15,16].

Interferometric transducers based on R-NPs are an efficient technology for emerging diagnosis systems. The optical properties of the materials of which the R-NPs are formed and the specific distribution of these materials along the pillars, produce a sharp resonance mode formation when R-NPs are vertically interrogated, due to the central cavity between the Bragg Reflectors forming the R-NPs.

In previous works we demonstrated the performance of R-NPs made of $\text{Si}_3\text{N}_4/\text{SiO}_2$ [17] and their feasibility to be used as biochem-

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ical sensors [18,19]. For it, we immersed the pillars in fluids with different refractive index (RI), and we interrogated them vertically from the backside. R-NPs showed high throughput in detecting small changes in the RI of the media in which they were immersed. The biological events produce shifts in resonance modes suitable to be monitored along the immobilization of bioreceptors or during the specific recognition of target biomolecules. Thereby, in a different former study we confirmed the possibility of binding biomolecules to R-NPs surface and therefore their feasibility to work as multiplexed label-free optical biosensors, where the response of identical R-NPs arrays transducers exhibited a good reproducibility [20]. In that work, a standard immunoassay was developed over the pillars for the biosensing demonstration.

In the present work we study the comparison of the biosensing performance under dry conditions or immersed in fluid of a transducer based on R-NPs arrays. We demonstrate through a biosensing experiment, the increased performance of R-NPs when they are vertically interrogated in dry conditions. The obtained results can be extended to other interferometric optical biosensors reported in the literature.

2. Materials and methods

In this work we compare the biosensing response of the same R-NPs arrays by using two different characterization modes. One of them is with the R-NPs immersed in the dilution sample; in this case water, but depending of the assay could also be Phosphate Buffer Solution (PBS) or other biological sample (Fig. 1-A). The other is with the pillars in dry conditions (Fig. 1-B). The drying process effect was previously reported as an efficient method to accurately carry out immunoassays where biosensing signals fit properly with the theoretical calculations [7,12,20,21].

We developed an IgG/anti-IgG standard immunoassay purchased from Sigma Aldrich (I4231/R3756) for the comparison of the biosensing performance of the two different interrogation procedures. All the experiments were carried out under controlled room temperature at 20 °C plus/minus 2 °C. Humidity was also stable during the experiments.

The chip developed is composed by eight different R-NPs arrays of $1 \times 1 \text{ mm}^2$. We call BICELLS (Biophotonic Sensing Cells) to these arrays when they are biofunctionalized with the bio-receptors [21]. The chip's layout has distributed 2 rows and 4 columns of BICELLS over a quartz substrate. This distribution of BICELLS provides multiplexing features to the sensor chip. R-NPs are formed by 10 pairs of Bragg Reflectors ($\text{Si}_3\text{N}_4/\text{SiO}_2$) with a central cavity of 200 nm of SiO_2 . The lattice parameter among the pillars forming the array is 500 nm and the pillars diameter is around 200 nm. The fabrication of the R-NPs involves several steps, from thin film layer deposition, laser interference lithography, an intermediate lift-off process and a final etching of the pillars; this fabrication process has been reported in detail elsewhere [17].

The chip was located in a holder-fabricated *ad-hoc* with the pillars facing down, as described in previous works [20]. Surface modification, biofunctionalization and biological recognition process for the immunoassay purpose affected to all the R-NPs arrays from the sample chip. However, according to the aforementioned previous work, we assumed that the biosensing response would be similar for every BICELLS, thus in this work the presented comparison data correspond to the characterization of one single BICELL (R-NPs array) from the multiplexed chip. After every sensing step, the arrays were independently, vertically interrogated with a bundle of fiber with a beam spot size of 500 μm diameter, with a spectrometer (with a resolution of 0.1 nm), covering the visible range in which the resonance mode can be observed. (Fig. 1-C). Every step of the immunoassay was characterized within the two

comparing methodologies: on one side, by immersing the pillars in water and on the other hand, with the pillars in dry conditions.

For the immunoassay over the R-NPs, they were firstly biofunctionalized with the IgG. For that reason, we developed a silanization protocol based on 3-aminopropyl trimethoxysilane (APTMS), and glutaraldehyde (GA) as linker to the amine groups of the antibody, which is further described in our previous work [20]. The immobilization of the IgG over R-NPs was carried out through the incubation of a drop of 50 $\mu\text{g}/\text{mL}$ of IgG, over night and in humid conditions in order to avoid the evaporation of the sample drop. Subsequently the chip with the R-NPs was rinsed with PBS-tween and water and properly dried with clean air. Then the chip was placed on the empty holder (with the R-NPs facing down) for the interrogation of the R-NPs in dry conditions and later on, the holder was filled with water for the interrogation of the R-NPs in humid conditions. Secondly, this process of incubation, rinsing, drying and both methods of interrogation, (in dry conditions and with the R-NPs immersed in water), was repeated for the recognition of increasing concentrations of anti-IgG with no regeneration phase between them. The incubation time of the increasing concentrations of anti-IgG was 30 min (Fig. 2).

Finally, we have performed theoretical calculations for the R-NPs spectra using the commercial software RSoft [22], and in particular its feature for Finite Difference Time Domain calculations Fullwave [23]. We have obtained reflectivity referred to silicon of the photonic structure for both dry and immersed in water conditions, considering a variable thickness biofilm layer with a refractive index of 1.41 surrounding the pillars, with a thickness ranging from 0 to 20 nm. For the calculations we have considered three-dimensional space domain, with a grid resolution varying from 20 nm for the bulk pillars, to 5 nm for the biofilm layer.

3. Results and discussion

We vertically interrogated the BICELLS without any previous treatment in order to have a reference signal of the spectral mode for both characterization procedures: with pillars immersed in water and with pillars in contact with air (Fig. 3).

In Fig. 3, the pink line represents the reflectance signal as a function of the wavelength when the R-NPs array is untreated and in dry conditions. The Blue spectrum corresponds to the untreated R-NPs array immersed in water. It can be observed a smaller intensity of the transducing signal in liquid infiltrated R-NPs, which barely reaches 1.5 relative units of the reflectance referred to silicon. Besides, the resonance mode is centered at 582 nm, in comparison, when the pillars are in dry conditions; it is centered at 485 nm.

Two other parameters may be mention although, in this case they are not decisive to determine biosensor performance: the signal-to-noise ratio (SNR) and the width of the resonance dip, expressed as the Full Wave at Half Maximum (FWHM). Both parameters provide information about the biosensor yield [24]. For the same transducer, a higher SNR ratio and a smaller FWHM are observed for the R-NPs in dry conditions.

The relative shift of the resonant deep during the silanization process and during biofunctionalization (IgG immobilization onto the sensing surface), for both measuring typologies, is represented in Fig. 4. The pink bars show the shift of the R-NPs resonance position after every biofunctionalization step, when the R-NPs are dry. The shift is around 2.2 nm after APTMS silanization and 6.14 nm for the IgG immobilization over the silane layer. For the same R-NPs immersed in water (blue bars) the shift is 0.4 nm for the APTMS and 1.23 nm for the IgG attachment. These results do not imply that the amount of antibody attached on the surface of the pillars is lower for pillars immersed, because it is the same R-NPs sample. However, they reveal that the changes occurring over biosensing structures,

Fig. 1. A. Scheme of pillars immersed in water. B. Scheme of pillars in dry conditions. C. Layout of the measurement system.

Fig. 2. Process carried out for the interrogation of the immunoassay steps with both methods, (A. with R-NPs dry and B. with R-NPs immersed).

are more easily observed when R-NPs are not immersed. The shift corresponding to the IgG immobilization is approximately 3 times the shift of the silane layer formation.

As summary, we can state that higher wavelength displacement results in greater sensitivity (m). Also, due to the lower FWHM and higher SNR, the uncertainty (u) on the calculation of the minimum

position of the resonance, is smaller for R-NPs interrogated in dry conditions.

Thus, due to these figures, we expect to achieve a better performance in terms of limit of detection (LoD) under dry conditions. In order to demonstrate and check these abovementioned assumptions, we carried out a recognition experiment to obtain the biosensing curve response (Fig. 5-A). For this recognition phase,

Fig. 3. Transducing signal for different characterization modes before any chemical or biological treatment: Pink spectra: The R-NPs array response in dry conditions and blue spectra the same R-NPs array response immersed in water. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Biofunctionalization steps (silane surface modification and IgG immobilization) as a function of relative shift of the resonant mode for arrays immersed (blue bars) and not immersed (pink bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

different concentrations of anti-IgG were incubated in the R-NPs BICELLS. The point in 0 represents the stable biolayer of IgG and the subsequent points correspond to the relative shift for the increasing concentrations of anti-IgG (2.5, 5, 10, 15, 20 and 50 $\mu\text{g/mL}$). Fig. 5-B and C corresponds to the transducing signal of the R-NPs for the IgG is immobilization and for the recognition of anti-IgG at a concentration of 50 $\mu\text{g/mL}$, assuming that surface is saturated at that concentration. It can be seen the higher resonant mode shift for interrogation of R-NPs in dry conditions.

The relative shift of the highest concentration of anti-IgG recognition for the dry pillars, is 4.36 nm and for the immersed pillars is 0.68 nm. Knowing the IgG and anti-IgG both biomolecules have the same size (around 12 nm), it would be expected a similar shift for the recognition of the anti-IgG, than for the immobilization of IgG

(6.14 and 1.23 for R-NPs dry and in humid condition respectively) if the yield of the recognition was 100%. Therefore we assume that probably the orientation of the IgG was not the ideal, and consequently there were some fragment antigen areas (Fab), which were not available for the recognition process.

Fig. 6 shows the reflectivity spectra calculated using 3D-FDTD algorithm, without considering an attached biofilm layer. The effect of adding this layer is a shift in the resonant mode; in the case of dry conditions this mode is centered around 484 nm, whereas for water conditions the dip is around 570 nm (Fig. 6-A). We have calculated the shift of the resonance mode when adding a biofilm layer of 5, 10, 15 and 20 nm (Fig. 6-B), for both fluid and dry conditions. The total shift, considering 20 nm of biofilm is of 2.84 nm (immersed in water) and 16.32 nm (dry conditions); more than 5

Fig. 5. Recognition curve of anti-IgG of one of the BICELLS. R-NPs in dry conditions (pink). R-NPs immersed in water (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. A. Calculated spectra for the RNPs for dry conditions and immersed in water, without considering any biofilm (reference calculation). B. Resonant mode position for calculated spectra as a function of biofilm thickness considered, from 0 to 20 nm, for both fluid conditions.

times (5.75) higher for dry conditions. The maximum shift reached experimentally is of 2.31 nm (water) and 12.7 nm (dry), considering the biofunctionalization of IgG plus the anti-IgG recognition shift; 5.5 times higher for dry conditions in this case. If we compare experimental and theoretical data, we observe that the saturation shift for both conditions seems to correspond in the two cases with a biofilm thickness around 17–18 nm (Fig. 7-B). This is in accordance with the relatively low shift for the biorecognition process,

and thus a bit lower than expected (a biofilm layer of 20–25 nm was expected for this calculations).

After every recognition step, 7 repeated measurements were taken in order to obtain the standard deviation of the resonance position. For the standard uncertainty (u) calculation, it was considered the resolution of the measurement system (R), the number of repeated measurements (n) and its standard deviation (s) as expressed in Eq. (1) [25]. This Eq. (1) is the law of propagation of uncertainty, where other factors, such as the temperature are

Table 1
Quality parameters of both interrogation techniques.

	FWHM nm	Uncertainty (U) nm	Sensitivity (m) nm/($\mu\text{g mL}^{-1}$)	Limit of Detection (LoD) $\mu\text{g/mL}$
R-NPs immersed in water	31.41	0.11	0.01	8.08
R-NPs in dry conditions.	25.20	0.10	0.17	0.60

considered negligible. The expanded uncertainty (U) was obtained with a coverage factor of 3 (Eq. (2)).

$$u^2 = \frac{R^2}{12} + \frac{s^2}{n} \quad (1)$$

$$U = 3 \cdot u \quad (2)$$

For a further comparison of the different techniques we calculated the LoD of the sensor under the two media conditions, air and water.

$$\text{LoD} = \frac{U}{m} \quad (3)$$

Where m is the sensitivity, and is calculated as the slope of the linear range between the first point of recognition and the saturation concentration point. The aforementioned quality parameters comparing both measuring techniques are summarized in Table 1.

As it could be inferred from the resonant modes in Fig. 2, the standard uncertainty for the minimums identification, is higher when R-NPs are measured in wet conditions, due to a lower SNR and a higher FWHM. Therefore the expanded uncertainty U is also higher for the immersed R-NPs.

Considering 20 $\mu\text{g/mL}$ as the saturation point we observed that the sensitivity (m), when the R-NPs are dry, is one order of magnitude higher regarding wet pillars (0.014 and 0.1716 $\text{nm}/\mu\text{g mL}^{-1}$ respectively). Thus, sensitivity, which is the resonance shift as a function of the analyte concentration, has a greater influence on the LoD determining compared with the uncertainty, which is very similar for both cases of study. In accordance with uncertainty and sensitivity values, the LoD, expressed in Eq. (3), is improved 13.2 times if the experiment is performed with the pillars in dry conditions.

This improvement is mainly due to the higher shift of the resonance mode induced by the higher Refractive Index Contrast (Δn) between R-NPs and air. The Δn between R-NPs and water is lower than the Δn existing between R-NPs and air. In wet conditions, the index of the surrounding media is 1.33, and 1.41 for the biolayer, whereas in dry conditions the index of the surrounding media is 1. This higher contrast results in higher values of the resonance shift, as seen experimentally.

4. Conclusions

In this work we evidence the result of measuring the same sample, with the same measuring system but in two different media: water and air. There have been demonstrated the benefits of measuring the biosensing response of R-NPs in dry conditions, where an enhancement in the limit of detection, in more than one order of magnitude is reached. Theoretical results are also in good correlation with experimental data. Moreover, the comparison results of this scientific report could be applied to other label-free optical transducers reported in the literature.

Finally, the R-NPs are susceptible to be fabricated at industrial level; moreover, their better performance in dry conditions makes them potentially ideal optical transducers to be implemented in Point of Care devices for in-situ diagnosis (portable and fast diagnosis systems), avoiding complex fluidic systems which are heavier, harder to handle and probably more expensive. This also could be applied to other interferometric transducers.

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