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Silicon oxynitride waveguides as evanescent-field-based fluorescent biosensors

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Abstract

Channel waveguide-based evanescent-field optical sensors are developed to make a fully integrated chip biosensor. The optical system senses fluorescent analytes immobilized within a micrometric sized bioreactor well realized within an optical waveguide. The main novelty of this work is related to the fact that, within the bioreactor well, the excitation of the fluorescent signal is achieved by means of the evanescent field propagating through a silicon oxynitride waveguide. The immobilization of the emitting molecules is realized by functionalization of the waveguide surface by a wet chemical method. These photonic biosensors are successfully applied to detect low surface concentration (10^{-11} mol cm^{-2}) of a green emitting organic dye. This approach could permit the selective detection of a wide range of chemical and biological species in complex matrices and can be exploited to set-up array-based screening devices. In this regard, the preferential excitation of the dye molecules in the close vicinity of the exposed waveguide core is also analysed.

Keywords: optical bionsensors, fluorescent detection, evanescent-field excitation, SiON/SiO_2 waveguides, biophotonics

(Some figures may appear in colour only in the online journal)

1. Introduction

Compact and portable chip-biosensor devices able to perform laboratory operations at the end-user premise are very attractive in a wide range of fields such as medicine, biology, defence and food industry [1–3]. The most diffused approach for such lab-on-a-chip biosensors is based on disposable optical micro-transducers functionalized with a biological layer (e.g. antibodies, enzymes, DNA, aptamers and scaffolds [4, 5]) for the selective immobilization of the target analyte [1, 6, 7]. In addition to the high sensitivity, reliability, immunity to electromagnetic interference, and fast response of the optical techniques, one of the main properties of these approaches is the high selectivity of the biorecognition event [4]. A higher degree of selectivity is reached in the case of the fluorescent-based 'sandwich' approach. This is a two-step recognition method whereby the target biomolecules are selectively marked with a fluorescent tag in addition to the selective immobilization on the bioreceptor layer [1]. This approach eliminates the need to purify the analyte from complicated mixtures before the measurement, simplifies the assay, and increases the sensitivity by the use of fluorescent markers. To fully exploit this selectivity, it is needed to confine the interrogation volume (i.e. the interaction volume of the optical probe) to the biorecognition layer where the target biomolecules are immobilized. In this way only the analytes selectively bound to the structure are labelled and detected, while the excess of fluorescent markers floating in the sample solution does not contribute to the signal. This is successfully accomplished by making use of the evanescent

field of the optical mode propagating through a waveguide structure. When a light wave is coupled into a waveguide, the associated electromagnetic field is not completely confined to the waveguide core; but it exponentially decays along the surrounding medium [8, 9]. Let us call n_{eff} the effective index of the waveguide mode and n_{sample} the refractive index of the medium in the region where the so-called evanescent field decays; then the characteristic penetration depth (i.e. the distance, d, at which the evanescent field amplitude decreases by a factor of $1/e$) is determined by [1, 10, 11]

$$d = \frac{\lambda}{2\pi} \frac{1}{\sqrt{n_{\mathrm{eff}}^2 - n_{\mathrm{sample}}^2}}. \quad (1)$$

From equation (1) and considering that $n_{\mathrm{eff}} > n_{\mathrm{sample}}$, it is clear that in the visible range the evanescent field of a waveguide provides a sensing region which is confined to a thickness <200 nm [6, 12]. The evanescent-field approach has been used during the last years for the development of a large variety of both label free and marked biosensors [13, 14]. Among others, the fabrication of glass [11], polymer [10, 15] and semiconductor [16, 17] planar waveguides for total internal reflection fluorescence (TIRF) microscopy has been reported. The commercially available 'zeptosense' system [17] is worth noting, which by combining the evanescent-field and fluorescent methods can reach a detection limit of 0.8 zeptomoles for a spot diameter of 150 μm (i.e. a surface concentration of 10^{-18} mol cm^{-2}), and a wide dynamical range. Other commercially available biosensors are based on label-free methods where the transduction process involves the modification of the effective refractive index induced by the immobilization of the analyte onto the core. Some examples of these system are the resonant-wavelength-grating-based biosensor 'OWLS$^{\mathrm{TM}}$ 210' [18] (sensitivity $\sim$1 ng cm^{-2}) and the single beam grating coupled interferometer developed by Creoptix [19] (surface sensitivity $\sim$0.01 ng cm^{-2}). Note that a surface sensitivity of 1 ng cm^{-2} entails a surface concentration of $\sim 10^{-12}$ mol cm^{-2} for a molecular weight of 397.8 Da corresponding to the fluorescent analyte used here. Some excellent reviews [13, 14] provide comparative information about these and other optical biosensors. They show the higher potential sensitivity of the fluorescent and evanescent-filed combined solutions in comparison with the label-free systems. However, most of the TIRF biosensors are based on planar waveguides in which the excitation beam is coupled into the structure by prisms or by objective lens. Therefore, in the case of multiarray biosensors the fluorescent signal is excited at the same wavelength [17], which limits the choice of the fluorophore probes. In this work, we prove the concept of an evanescent-wave biosensors based on silicon oxynitride channel waveguides. This design allows for the development of compact multichannel chips where each emission signal might be excited at different wavelengths. The structures are produced by CMOS fabrication techniques that enable the mass scale production of inexpensive disposable photonic layers. As another novelty, during the fabrication process the topmost layer of the photonic layer is adapted for the immobilization of biomolecules containing free amino (NH_2) groups by a further wet chemical process.

Photonic biosensors based on Si compatible materials offer the advantage of exploiting the well-established CMOS micro-fabrication technologies for the development of densely packed multichannel arrays within a single chip. In addition, these techniques enable the development of monolithic systems in which the optical transducer is integrated together with the optoelectronic components of the sensor chip. Within CMOS compatible materials, silicon oxynitride (SiON) has proved to be a good candidate for the development of both marked and label-free biosensors, including Mach–Zehnder interferometers, Fabry–Pérot and disc resonators, as well as absorption-based biosensors [20–23]. One of the main characteristics of this material lies in the flexibility to adjust the refractive index within a wide range as a function of the nitride content, from 1.45 (SiO_2) to 2.00 (Si_3N_4) [24, 25]. Furthermore, SiON/SiO_2 waveguides are particularly appealing for dye-labelled biosensors, thanks to their low optical losses in the UV–visible region. For instance propagation losses as low as 1 dB cm^{-1} at 633 nm were reported for waveguides grown by plasma-enhanced chemical vapour deposition (PECVD) [23, 26].

This paper reports about an evanescent-wave and fluorescent-based optical transducer consisting of a SiON/SiO_2 channel waveguide where a reactor well has been formed for the delivery of biological sample solutions. This system has been conceived as the photonic layer of a fully integrated chip biosensor incorporating the microfluidic and optoelectronic systems required for the detection process. As a main novelty the SiO_2 cladding was removed along the sensing area while preserving a thinned SiON core. According to our FDTD simulation, the optical mode still propagates through the thinned waveguide core while its evanescent tail spills out into the bioreactor region [27]. These simulations also indicate that the illumination of the sensing volume is homogeneous and confined to a few tens of nanometres from the surface of the channel waveguide. In agreement with equation (1) the thickness of the sensing volume can be easily adjusted to different analyte dimensions either by controlling the refractive index of the solution filling the bioreactor well [27] or by changing the excitation beam wavelength.

In our previous work the fluorescence emission collected by the SION core was theoretically analysed. In contrast, in this work we experimentally test the performance of the structure when collecting the fluorescence emission from the top of the bioreactor well. We use this vertical detection configuration in order to reproduce the design of the whole biosensor device where the photonic layer will be implemented. In this final device the photonic structure is interfaced with an ad hoc SPAD photodiodes matrix, which can be much more easily aligned on the top of the bioreactor. The present experimental work deals with the surface detection of dye molecules immobilized on the bioreactor bottom surface. This requires to adapt the composition of the bottom surface of the bioreactor for the immobilization of the fluorescent analyte. For these experiments we used 5-(aminomethyl) fluorescein—AMF—dye molecules as the fluorescent analyte: a green emitting xanthene derivative, which is commonly used as an immunodiagnostic fluorescent probe [28].

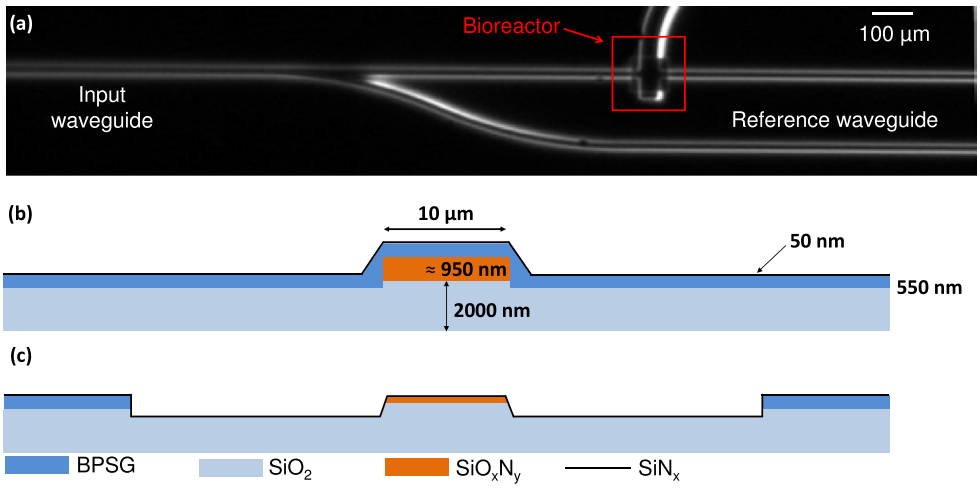

Figure 1. Device description. (*a*) Optical image of the top view of the photonic layer. (*b*), (*c*) Schematic cross-sections of the waveguide structure before (*b*) and at the bioreactor well (*c*). Note that in (*b*) and (*c*) there is a scale factor of 2 between the vertical and horizontal dimensions.

2. Device description and detection set-up

The overall layout of the prototype device is presented in figure 1(*a*) (more details in [27]). Main components of the optical layer are a set of channel waveguides and the micrometric reactor site for the analyte immobilization. For the detection experiments a laser beam is butt-coupled into the input waveguide and transmitted towards the bioreactor where the fluorescent emission is excited. The structure also includes a Y splitter before the bioreactor. Thus, the reference branch allows monitoring the stability of the excitation beam and the reproducibility of the alignment independently of the absorption losses caused by the bioreactor content.

The structures were fabricated in a standard silicon fabrication line by PECVD and low-pressure chemical vapour (LPCVD) deposition processes in combination with 1100 °C annealing treatments to remove light-absorbing hydrogen bonds; and optical lithography for pattern definition and dry reactive ion etching (RIE) for the pattern transfer process. Figure 1 shows a schematic representation of the transversal cross section of the waveguide before and at the bioreactor well. As illustrated in figure 1(*b*) the waveguide structure consists of a 2 μm thick SiO_2 buffer layer ($n = 1.45$) and a 1 μm thick SiO_xN_y core layer ($n = 1.85$) both deposited by PECVD; a ~0.5 μm thick borophosphosilicate glass (BPSG) layer ($n = 1.45$ nm) grown by LPCVD in order to optimize the surface planarity after RIE plays the role of the top cladding [29]. The waveguide was 10 μm wide to better match the dimension of the bioreactor. Fabrication of bioreactor well (figure 1(*c*)) was also carried out by lithography/RIE. In this process the etching time was adjusted to completely remove the BPSG top cladding, as well as to thin the core of the waveguide section that crosses the bioreactor. This is carried out in order to decreases the confinement of the propagating modes and therefore to increase the interaction between the evanescent field and the bioreactor content. Finally, the whole system was coated with a thin SiN_x film (50 nm of thickness) intended to accommodate the surface chemical composition of the structure for the immobilization of amino containing biomolecules by the chemical process described below; but without disturbing the optical properties of the photonic layer. It is important to remark that both, the design of the channel waveguide and the fabrication protocol, have been optimized for the production of low losses waveguides in the visible range where organic dyes are excited and emit, with propagation losses below 3 dB cm^{-1} at $\lambda = 473$ nm. To illustrate this point, figure 2(*b*) shows the propagation of a 473 nm excitation beam up to the micro-bioreactor as well as the beam transmitted after the micro-bioreactor. The bioreactor and the waveguide dimensions are also controlled by the requirement of a sensing area large enough to be potentially functionalized by a spotter with the trapping agent (i.e. to deliver the recognition element onto the active sensor surface). Therefore, a $50 \times 50\,\mu m^2$ bioreactor site was selected, and a waveguide width of 10 μm was chosen to interact with a representative area of the bottom surface of the bioreactor.

For the detection experiments a homemade modular experimental set-up was used (figure 2(*a*)). The excitation section consists of a DPSS laser source at 473 nm (CIEL from Laser Quantum) coupled to a tapered optical fibre which is mounted on a piezoelectric micropositioning system for the alignment process. The optical transducer herein analysed has been devised for its further implementation into a portable biosensor device in which the fluorescence signal is detected by a SPAD photodetector array installed over the photonic layer. In order to emulate this architecture, in our experimental system the fluorescence emission is collected from the top of the structure. The detection system consists of an objective lens (20×) coupled with an ultra-zoom (12× maximum magnification) to achieve a field of view of the same order of magnitude of the bioreactor area. The collected emission is coupled by a set of lens and optical fibres into a spectrometer (SOPRA DMS2) interfaced with a cooled photomultiplier tube.

3. Results

To check the suitability of the photonic layer for the fluorescent detection of fluorescent analytes two different kinds of studies

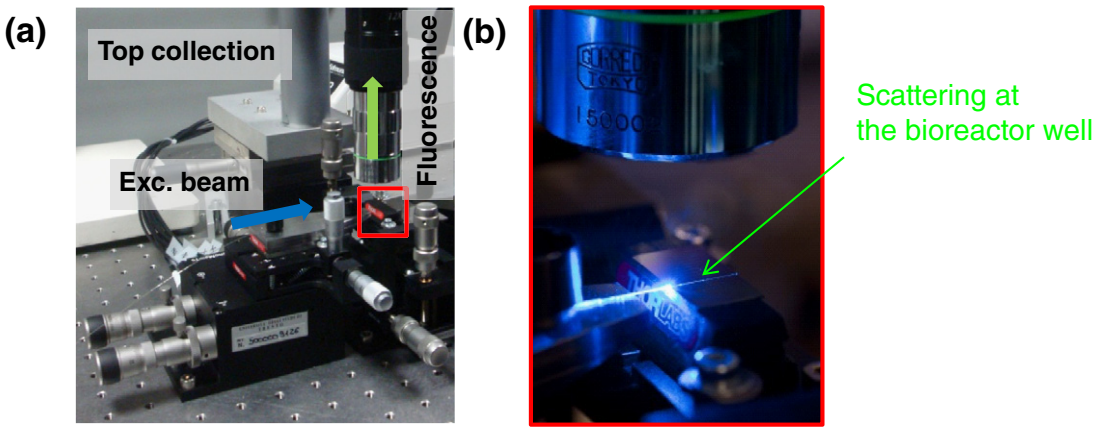

Figure 2. (*a*) Photograph of the experimental set-up used for the detection test. The photonic chip is located on the sample holder within the red square. (*b*) Enlarged view of the photonic chip when a 473 nm blue laser beam is propagating through the structure. Note that the light signal is propagating all over the chip from the input facet to the output facet. The bright point at midway is the location of the reactor and the spot is due to the scattering of the light at this location.

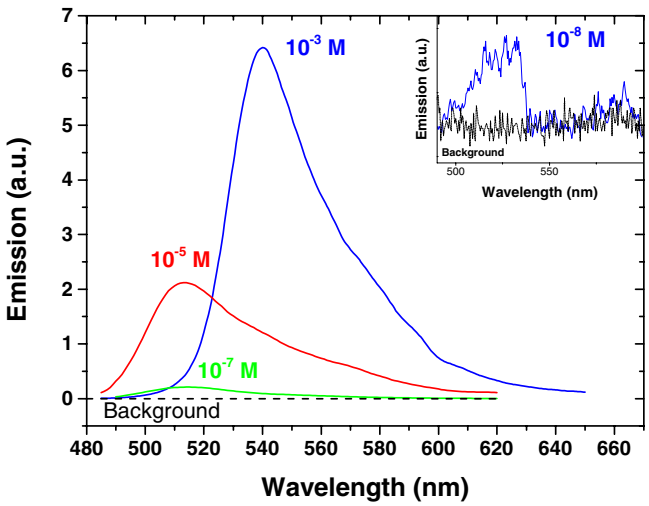

Figure 3. Fluorescence spectra acquired for AMF–water solutions delivered in the bioreactor. The insert shows the emission for a 10^{-8}M solution. The background line is measured with an empty bioreactor.

have been carried out by employing AMF as fluorescent marker. In the first series of experiments volumetric sensing has been performed. In this case, the bioreactor was filled with different AMF–water solutions; therefore, the detection of the dye molecules in water is analysed. Figure 3 shows the various emission spectra acquired for different dye concentrations. Emission is excited here by the laser beam propagating in the waveguide and collected from the top of the structure. As shown in figure 3, the emission maxima are located in the 500–550 nm spectral region which corresponds to the emission range of AMF molecules in water solution. This confirms the suitable excitation of the fluorescent dye by the laser beam transmitted through the bioreactor. For a high dye concentration of 10^{-3}M the green emission from the dye solution is strong enough to be detected by visual inspection through the microscope; whereas it progressively decreases with the dye concentration. No linear relationship between the dye concentration and the emission intensity was observed in our experiments. This result may be due to two different factors: first reabsorption of the fluorescent emission as a consequence of the overlapping of the emission and absorption bands and due to the localized excitation of the emission on the bottom of the reactor. Such behaviour is typically observed for high concentrations of organic dyes with a short Stokes shift, which is the case for AMF. The second factor is the formation of aggregated species with a lower quantum yield that leads to the reduction in the emission efficiency as the dye concentration increases. This is a common effect in xanthene derivative molecules that is accompanied by a red-shift of the emission band [30, 31]. In fact, this phenomenon also explains the red-shift of the emission maximum observed in figure 3 as the dye concentration increases. It is worth noting that the minimum concentration that we were able to detect was 10^{-8} M (see inset in figure 3), a value which is close to the limit of detection of other photonic biosensors [32].

The second class of experiments was aimed at the surface sensing. Point of care and biosensing applications demand for a selective response towards the target molecule. This can be addressed by functionalizing the optical transducer with a biological layer for the specific recognition of the target analyte. Binding of biological molecules (which should form the recognition layer) on an inorganic surface requires an organic interlayer bearing reactive groups able to immobilize the desired bioreceptor. We set up a simple functionalization protocol to introduce an organic interface onto the microwells which exposes carboxylic (COOH) groups able to react with the amino group and form an amide bond. These groups can be used to immobilize biomolecules containing free amino groups. To test the performances of the functionalized surface integrated in the sensor chip, we anchored a fluorescent marker bearing an amino group (AMF) to the functionalized sensor surface.

Wet chemical functionalization of the thin SiN_x final layer of the photonic structure was carried out by the process summarized in figure 4. Before functionalization the samples were cleaned by sonication in trichloroethylene, acetone and 2-propanol. Afterwards, they were immersed in a 2% HF

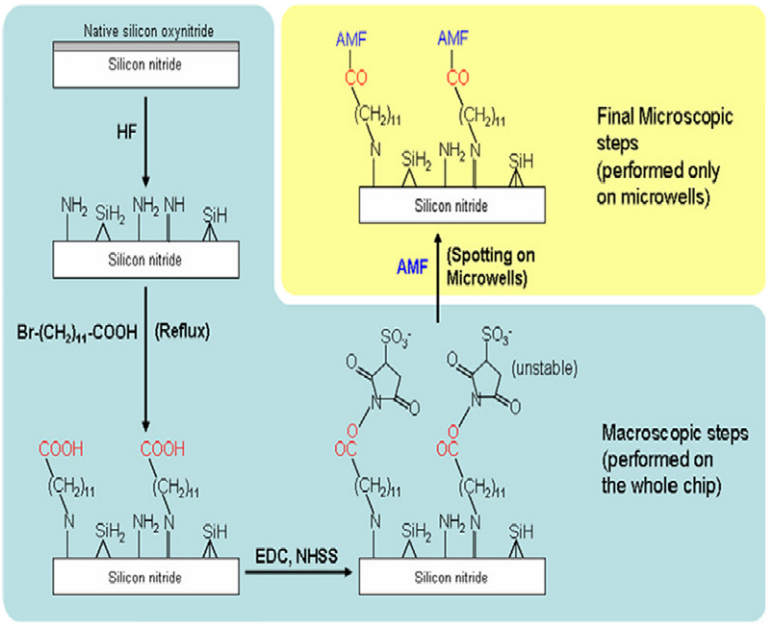

Figure 4. Scheme of the chemical reactions used for the immobilization of the AMF molecules on the bioreactor.

solution (1 min) to remove the native oxide, rinsed with water and dried under a gentle nitrogen flux. Thereupon, in order to introduce the carboxylic groups, the samples were immersed into a 0.08 M solution of Br-dodecanoic acid (Br-$(CH_2)_{11}$-COOH) in toluene and kept under reflux (110 °C) in argon atmosphere for 120 min [33]. The samples were then rinsed by sonication in dichloromethane, acetonitrile, water and methanol and dried with nitrogen. Carboxyl moieties immobilized inside the microreactors were then marked using AMF. First the carboxylic groups were activated by dipping the photonic chips into a solution of 75mM N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDC) and 15mM N-hydroxysulfosuccinimide sodium salt (NHSS) for 30 min [34]. Then the samples were rinsed with water, dried with nitrogen and the microwells were immediately spotted with a 100 μM AMF solution. The dye was dissolved into DMSO : 0.1M phosphate buffer, pH 8 (1 : 9), which was proved to provide a good reaction medium. For the spotting process a BioOdyssey Calligrapher MiniArrayer, BIO-RAD, with a 100 μm capillary tip was used. After spotting, the samples were stored in the dark for an incubation time of 120 min. Afterwards, the samples were thoroughly rinsed in the DMSO–phosphate buffer solution and in water in order to remove those dye molecules that are not chemically bond to the bioreactor surface. Finally they were dried under a nitrogen flow. The same functionalization process was carried out on a set of reference silicon nitride layers that were analysed in a commercial spectrofluorometer. These analyses revealed that after the final cleaning process a surface concentration of 10^{-11} mol cm^{-2} of fluorescent AMF molecules remains immobilized on the SiN_x layer. The good performance of the spotting process was also tested by fluorescence microscopy (see figure 6(*a*)).

As a representative example, figure 5 shows the emission spectrum recorded for a functionalized bioreactor with a dye

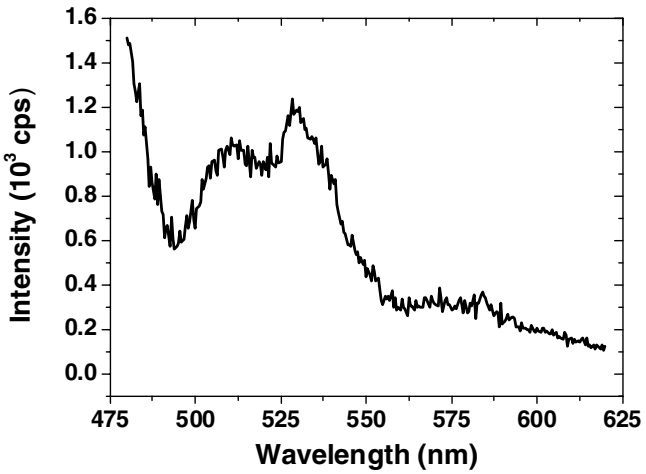

Figure 5. Emission spectrum recorded for a functionalized bioreactor with an AMF surface concentration of 10^{-11} mol cm^{-2}.

surface concentration of 10^{-11} mol cm^{-2}. This spectrum depicts a weak broad emission band extending from 500 to 550 nm, which is the emission spectral region of AMF. This result proves the excitation of the immobilized AMF molecules by means of the evanescent field of the waveguide, as well as the capability of the system to detect low surface concentrations of this dye (10^{-11} mol cm^{-2}). Indeed considering the bioreactor area (2500 μm^2) this surface concentration entails an amount of 10^{-16} moles of AMF in the bioreactor bottom surface. Nevertheless, this surface concentration is higher than the sensitivity of other biosensors described in the introduction. At this point it is important to stress that this is not an optimized device, and that this study just aims to experimentally demonstrate the concept of CMOS-fabricated SiON channel waveguides for the surface detection of immobilized fluorescent molecules. Some issues to be solved are related to the photobleaching of the dye and

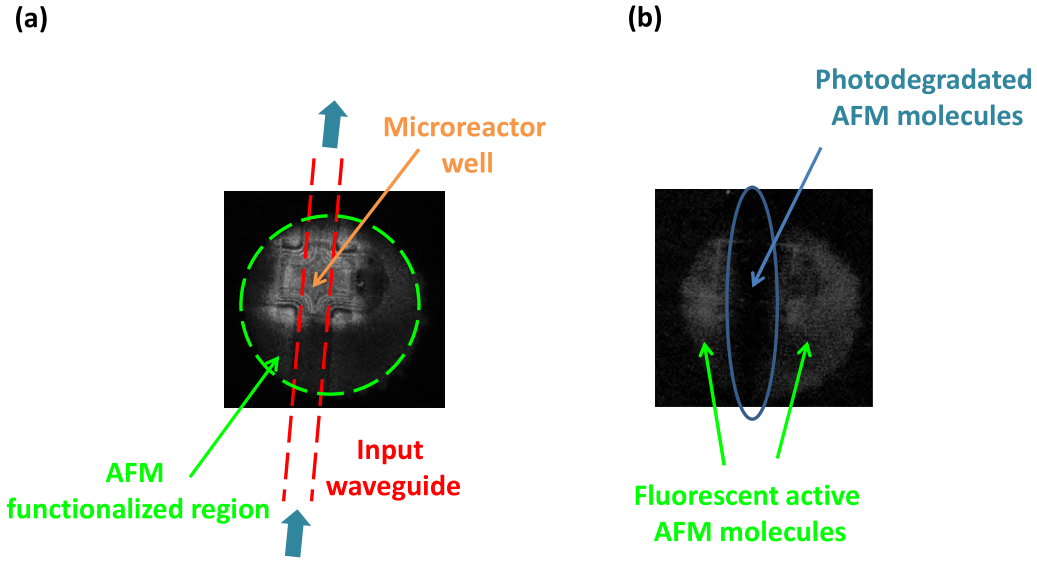

Figure 6. Fluorescence microscopy image of a micro-bioreactor (*a*) after the functionalization process and (*b*) after a prolonged detection experiment.

the anisotropy of the fluorescence emission. In our preliminary experiments, we noticed that the AMF molecules undergo a fast photodegradation. The AFM bleaching was demonstrated by analysing some reference samples by fluorescence microscopy, before and after exposing them to the laser beam (see later). For this reason we suppose that in the absence of bleaching the signal shown in figure 5 could be much more intense. In fact the sensor detection test in figure 5 requires the alignment of the optical set-up, a process during which the immobilized dye molecules is exposed to the excitation laser beam for at least two min. In addition, it is also important to take into account that the Fermi golden rule states that the spontaneous radiation rate is affected by the photon mode density which in turn is determined by the dielectric environment. Thus, as was experimentally reported and theoretically analysed [35, 36], the emission from the dye molecules at the waveguide core interface is not isotropic; and most of the energy is radiated towards the high refractive index material (i.e. the waveguide core). Nevertheless our experimental results prove the suitability of the photonic layer to detect low surface concentration of a fluorescent analyte even under such adverse conditions (i.e. dye photobleaching and detection from the top of the structure).

Further proofs of the interaction between the evanescent field and the trapped dye molecules are provided in figure 6. Figure 6(*a*) shows the fluorescence image recorded on the bioreactor just after the functionalization and figure 6(*b*) shows the fluorescence image of the same bioreactor after the surface sensing experiment. In figure 6(*b*) the photobleaching of the AMF molecules caused by the propagation of the laser in the waveguide is apparent. Photodegradation affects only those AMF molecules immobilized on the surface of the waveguide. This reveals that the excitation of the dye is due to the evanescent field of the propagating mode. Hence, the excitation is very localized (note that the other AMF molecules preserve their luminescence activity) in agreement with our previous theoretical FDTD simulations [27]. This opens the possibility of using the functionalized structures for the specific detection of a target molecule without the need of the final cleaning procedure, even when analysing complex samples containing different fluorescent biomolecules, as discussed in the introduction.

4. Conclusions and summary

A SiON channel waveguide-based optical transducer has been developed and tested. This simple photonic layer has been conceived for its implementation in a modular-stack lab-on-chip biosensor. The working concept is based on the fluorescent detection of the fluorophore markers excited by means of the evanescent field propagating through the waveguide. In this way, a very localized excitation of the fluorescence is possible. In order to selectively detect a given biomolecule, the structure can be functionalized with a biorecognition layer for the specific immobilization of the target molecules. As a proof of concept the photonic layer has been successfully applied in the fluorescent detection of AMF molecules bound to the bottom of a bioreactor, for a low dye surface concentration of 10^{-11} mol cm^{-2}. In these experiments the photobleaching pattern observed after illumination confirms that the dye excitation is mainly mediated by the evanescent field of the optical modes propagating in the SiON waveguide. In addition, the penetration depth of the evanescent-wave (i.e. the thickness of the interrogation volume) can be easily adjusted as a function of the refractive index of the bioreactor content (see equation (1)). Thus, by filling the reactor well with the suitable solvent the system might allow for the detection of larger analytes [37]. These characteristics together with the possibilities of the employed fabrication techniques (CMOS fabrication techniques) allow one to extend the design to the development of a single-system sensors array with multianalyte capabilities, by functionalizing each single sensing structure with a specific biorecognition layer.

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