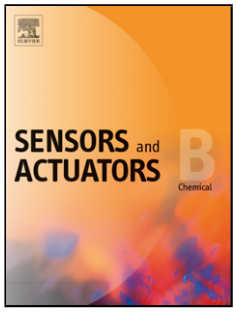

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Organic distributed feedback laser for label-free biosensing of ErbB2 protein biomarker

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ABSTRACT

The human epidermal growth factor receptor 2 (ErbB2) protein plays an important role in human malignancies. Its overexpression has been recognized as a feature of a malignant cancerous phenotype in breast cancer cell lines, and has become one of the most widely investigated clinical indicators of breast, ovarian, gastrointestinal and lung cancers. In this work a vertically emitting organic distributed feedback (DFB) laser has been used to detect the ErbB2 protein. This DFB laser consists of a polystyrene (PS) film containing a perylenediimide laser dye, deposited over a second-order one dimensional grating fabricated on fused silica by thermal-nanoimprint lithography and subsequent reactive ion etching processes. Specificity of the system to ErbB2 protein biomarker, achieved by functionalizing the PS with anti-ErbB2 monoclonal antibodies, is demonstrated. A concentration limit of detection for ErbB2 protein of 14 ng/mL has been obtained, and no cross-reactivity has been observed with bovine serum albumin (BSA) and tumor necrosis factor alpha (TNFα) proteins. These findings open the possibility of using this type of biosensors in clinical applications.

Keywords

Label-free biosensors, organic lasers, distributed feedback, perylenediimides, ErbB2 biomarker

1.-Introduction

Label-free biosensors must simultaneously provide high sensitivity, large dynamic range and sufficient resolution for detection of mass density changes less than < 1 pg/mm^2 in order to have an impact on the most challenging detection applications [1]. Label-free resonant optical sensors generally detect shifts in the resonant wavelength caused by the interaction between the target molecule and the evanescent portion of resonant modes, and the amount of wavelength shift is proportional to the density of captured biomolecule on the sensor surface. The narrow spectral linewidth achieved by using quality factor (Q-factor) (>10^5) passive optical resonators enables sensor systems to resolve smaller wavelength shifts associated with the detection of analytes at low concentration, or biomolecules with low molecular weight, such as drug compounds [2,3]. While detection resolution can be substantially improved through the use of high

Q-factor passive resonators, it is generally at a cost of a decrease of the sensitivity and the dynamic range of the system. Only a few examples of passive resonators have achieved high Q-factor and high sensitivity simultaneously [4]. In addition, the implementation of high Q-factor optical resonators typically requires high precision alignment for evanescent light in/out coupling, providing potential limits to their practical application. One way to solve this problem is to use organic distributed feedback (DFB) lasers. These laser biosensors are simultaneously capable of a high sensitivity and a high degree of resolution, since they operate with single mode and narrow linewidth emission [5-7]. A recently reported strategy to work with good figures of merit for both, resolution and sensitivity, consists in using a photonic crystal resonant reflection technique. It works with a simple optical setup and has showed to be useful for studying cell dynamics or the presence of single metallic or dielectric nanoparticles conjugated with antibodies for biosensing [8,9].

DFB laser biosensors show significant advantages for label-free biosensing applications including: (i) simple implementation -these sensors do not require high precision for positioning of optical fibers or waveguides to the resonator perimeters-; (ii) the chip can be fabricated following low-cost replication techniques and active materials can be applied using spin-coating or dip-coating, extensible to roll-to-roll manufacturability [10]; (iii) they can be fabricated on flexible plastic substrates which can be incorporated into standard well microplates [10] for multiplex detection. In addition, the recent demonstration of a DFB laser optically pumped by a Light Emitting Diode (LED) [11] can lead to compact biosensing devices and promising setups for their commercialization. A thorough review of the literature only shows a few reports of organic DFB lasers used as biosensing devices [6,10,12,13]. Lu et al. [6] demonstrated the capability of a DFB device to detect 3.4 nM (60 ng/mL) of human immunoglobulin G (IgG) protein. That laser consisted on a Rhodamine 590 doped SU-8 active film spin-coated over a grating engraved over UV-curable polymer and a 30 nm-thick top layer of titanium dioxide (TiO_2). Tan et al. [10] fabricated similar DFB devices, with the organic active film prepared by horizontal dipping and demonstrated their use to detect tumor necrosis factor alpha (TNFα) down to 0.625 µg/mL. In a later work [12], with the same kind of DFB devices they detected 34 nM (600 ng/mL) rabbit IgG. Haughey et al. [13] fabricated DFB lasers based on an oligofluorene truxene active film deposited by spin-coating on top of the grating engraved on an epoxy resist and no TiO_2 layers. They studied the avidin-biotin interaction and demonstrated detection down to 1 µg/mL of avidin using a biotin-functionalized DFB.

Sensitivities (S_b) in the biological range (at n = 1.33) of around 20 nm/RIU have been achieved with DFBs used for the detection of proteins [5,14]. Better sensitivities, of the order of 70-150 nm/RIU have been obtained by experiments and simulations [6,15] with DFB laser sensors coated with a thin layer of high refractive index of TiO_2 (n= 2.1). However, M. Lu et al. [6] fitted the wavelength emission versus refractive index curve over a 14 nm tuning range, while C. Vannahme et al. [15] calculated the sensitivity at refractive indexes close to n = 1.5, thus far from the biological range. DFB sensor

resolutions of 0.72 pm [15] have been obtained by fitting the spectral peak to a center of mass model over many laser pulses. Lower resolutions, below 0.5 pm, have been reported [12] using spectrometers with higher optical resolution and triggering the spectrometer to detect the laser pulse only, although this may limit the compactness on the optical setup. Considering that the limit of detection (LOD) of the DFB laser sensor is given by the ratio resolution/sensitivity [2], state-of-the art figure-of-merit for DFB laser sensors are around 7×10^{-6} RIU [15]. In a previous work [16], we have fabricated a DFB laser based on a polystyrene (PS) film doped with a highly photostable perylenediimide derivative (perylene orange, PDI-O) and demonstrated its use as a bulk refractive index sensor with a LOD of 2.5×10^{-5} RIU. In the present paper a vertically emitting organic second-order 1D DFB laser composed of PS/PDI-O active film, similar to that recently reported, is demonstrated for label-free biosensing of human epidermal growth factor receptor 2 (ErbB2) protein biomarker. Specific biofunctionalization of the DFB laser has been successfully carried out.

2.-Materials and methods

2.1 Materials, reagents and solutions

The thermal-NIL resist mr-I7010R was purchased from Microresist Technology GmbH (Germany). PS (Mw = 35000 g/mol) and toluene were purchased from Sigma-Aldrich (Spain). PDI-O (purity higher than 99.5%) was purchased from Phiton (Germany). Recombinant human ErbB2 protein, rabbit anti-ErbB2 monoclonal antibody (anti-ErbB2) and rabbit anti-ErbB2 monoclonal antibody conjugated to fluorescein isothiocyanate (FITC) were purchased from Sino Biological Inc. (China). BSA was from Sigma-Aldrich (Spain) and TNFα from Peprotech (United Kingdom). Phosphate buffer saline and carbonate-bicarbonate buffers were prepared by dissolving readily prepared tablets from Sigma-Aldrich. All other chemicals were of analytical grade and ultrapure water (Millipore Milli-Q) was used throughout.

2.2 Device structure

The structure of the device used as a biosensor (see Fig. 1a) is a waveguide-based organic semiconductor laser. It has been built by a relief grating patterned on a transparent fused silica substrate (refractive index $n_s = 1.46$, at $\lambda = 580$ nm) on which the active material, PDI-doped PS ($n_f = 1.59$, at $\lambda = 580$ nm), is coated as a thin film with a thickness of $h_f = 160$ nm. Active films were prepared by spin-coating a toluene containing PS, as inert polymer, and 1.0 wt% (with respect to PS) of PDI-O. Film thickness was determined by the fringe pattern of the absorption spectrum, measured by a Jasco V-650 UV-VIS spectrophotometer. The resonator is a second-order 1D DFB grating with a period of $\Lambda = 376$ nm and a grating depth of $d = 60$ nm, and has been fabricated by thermal-NIL and subsequent reactive ion etching processes [16]. An Atomic Force Microscope micrograph of one of the obtained gratings is shown in Fig.1b.

INSERT FIGURE 1a

INSERT FIGURE 1b

2.3 Biosensor surface functionalization.

After thorough water rinsing and N_2 drying of the fabricated DFB devices, the specific biofunctionalization of the surface with anti-ErbB2 monoclonal antibodies was carried out by means of an overnight incubation at 4ºC of a 20 µg/mL antibody solution in pH 9.6 carbonate-bicarbonate buffer. After another cleaning step, a 2 hours long incubation of BSA (5 mg/mL) in PBS buffer (pH 7.3) ensured perfect surface coverage for minimizing potential non-specific binding. Incubations with ErbB2 protein biomarker were carried out during 2 hours at different concentrations ranging from 0 up to 10000 ng/mL also in PBS buffer. All incubations were performed with a carefully pipetted 10 µL drop covering all the DFB array structure in a humid atmosphere to avoid evaporation.

2.4 Optical characterization

Optical characterization of the DFB sensors, before and after functionalization, was performed by pumping and collecting the emitted light through the substrate (see Fig. 1a), with the sample placed horizontally with respect to the optical table. This geometry, which requires the use of a transparent substrate, avoids disturbing the analyte with the pump beam. The excitation beam shape is elliptical (minor axis of 1.1 mm), and it is incident at an angle of around 20° with respect to the normal to the film plane (green arrow). The emitted light is collected (red arrow) perpendicularly to the sample surface. A pulsed Nd:YAG (YAG-yttrium aluminum garnet, 532 nm, 5.5 ns, 10 Hz) laser was used to excite the sample and the light was collected by an Ocean Optics MAYA 2000 fiber spectrometer, with a nominal resolution in determining the spectral peak wavelength and linewidth of 0.07 nm and 0.13 nm, respectively. The measured spectrum is formed by discrete points separated by steps of 0.035 nm.

3.-Results and discussions

The DFB sensor shows one single peak, which before functionalization appears at λ_{DFB} = 555.2 nm (see Fig. 2). Its lasing threshold and the photostability half-life are 200 kW/cm^2 and around 20 min (1.2×10^4 pump pulses), respectively [16]. The photostability half-life is defined as the time or the number of pump pulses at which the emitted laser intensity decays to half of its maximum value. Its sensitivity in the biological range (n = 1.33) is S_b = 32 nm/RIU [16], which is comparable to those reported for other single-layer waveguide DFB biosensors [6,10,12,13]. The sensor resolution was calculated by fitting the central wavelength emission by a center of mass calculation [15], obtaining a resolution of 0.8 pm and a LOD of 2.5 x 10^{-5} RIU [16].

INSERT FIGURE 2

With the aim of proving the biosensing capabilities of the developed DFB lasers, an immunoassay for the detection of ErbB2 protein (185 kDa) was developed. This protein

is an important oncogene relevant to certain aggressive types of breast cancer and its overexpression has been proved to serve as biomarker of the status and progression of the illness [17]. Given the label-free properties of the DFB sensing, a direct immunoassay was designed (see scheme in Fig. 1a). The label-free format greatly simplifies other immunoassay formats based on molecular labels such as fluorescence, light absorption, radioactivity, etc.

For the specific detection of protein biomarkers in a surface-based biosensor one of the most important factors to obtain a good performance is the correct biofunctionalization of the surface with specific capture agents such as high-quality antibodies. In this case, for the PS functionalization with anti-ErbB2 antibodies (155 kDa), an optimization of the immobilization protocol was carried out. Physisorption of antibodies to a PS surface in various buffers and with different incubation times and temperatures was carried out. PBS buffer and carbonate-bicarbonate buffers with pHs ranging from 7 up to 9.6 and NaCl concentrations varying from 0 to 500 mM were employed in this optimization. A 10 µL drop of 10 µg/mL of anti-ErbB2 fluorescently-labeled with FITC in the mentioned buffers was incubated. After washing with water and N_2 drying, the fluorescence intensity and surface emission homogeneity were observed in a Zeiss fluorescence microscope. Best results were obtained with an overnight incubation at 4ºC in a pH 9.6 carbonate-bicarbonate buffer.

The optimized protocol was employed in DFB devices and the emission wavelength was measured before and after the anti-ErbB2 functionalization (see Fig. 2). An average shift of 0.51 nm was obtained, which compares well with other protein immobilization methods employed for DFB biosensors (see, *e.g.*, [10]).

When developing an immunoassay, it is always of the upmost importance to evaluate the specificity and cross-reactivity of the functionalized sensor surface with proteins other than the target. In this case, incubation of the anti-ErbB2 DFB surface with BSA at very high concentration (5 mg/mL), a process that was effectively used as surface blocking in all measurements, did not alter the emission wavelength of the DFB laser beyond the measurement uncertainty (see Fig. 3). This proved the correct formation of a specific anti-ErbB2 antibody monolayer on the surface. The specificity and cross-reactivity of the developed immunoassay was tested using a solution of TNFα, a related biomarker at a higher concentration (1 µg/mL) than those employed for the ErbB2 protein. As it can be seen in Fig. 3, where a positive sample of ErbB2 at 250 ng/mL is presented for comparison, the signal obtained was null which proved the high specificity of the developed DFB biosensor.

INSERT FIGURE 3

It can be seen that non-specific interactions due to large concentration of proteins are negligible compared to the specific signal of low concentration ErbB2 positive samples.

Several DFB chips were prepared to run the direct binding ErbB2 immunoassay on their surface with the objective of obtaining a calibration curve and a deeper characterization

of the biosensor. Solutions of ErbB2 prepared on PBS buffer with 0, 2, 10, 250 and 10000 ng/mL concentrations were incubated during 2 hours and the laser emission shift quantified. The DFB spectrum for the case of 10 ng/mL has been included in Fig. 2 to illustrate the displacements with respect to spectra obtained in previous stages of the immunoassay. With the displacements obtained for the different concentrations, a calibration curve is obtained (see Fig. 4). It is remarkable that even the lowest concentration of 2 ng/mL gave rise to a detectable wavelength shift. A LOD of 14 ng/mL was calculated as the concentration corresponding to a signal three times the standard deviation of the negative samples (which was considered as the measurement error). To the best of our knowledge (see related literature presented in Section 1, i.e., [6,10,12,13]), this is the lowest concentration LOD reported for a DFB-based protein biosensor. Since the refractive index sensitivity of the fabricated DFB laser is comparable to other published DFB biosensors, this better sensitivity could be because of an improved surface functionalization, a better molecular recognition (due to high affinity antibodies) or to a larger size of the ErbB2 protein analyte (185 kDa) which implies a larger refractive index change upon binding. It is worth mentioning that the established clinical cut-off for the ErbB2 biomarker is 15 ng/mL [18], which proves the potential usability of this biosensor. This LOD value is higher than that achievable with other types of sensors. For example, a LOD as low as 26 pg/mL have been obtained for the ErbB2 biomarker in diluted human serum samples using a highly sensitive amperometric magnetoimmunosensor [19]. However, DFB laser biosensing represents a cost-effective way to detect biomolecules and the DFB laser can be made entirely in polymer [20] or even be completely biocompatible [21]. It can lead to its use not only as an *in-vitro* diagnostic tool if not as an *in-vivo* biosensor for in-situ detection of biomolecules.

INSERT FIGURE 4

Conclusions

In this work a vertically emitting organic second-order 1D DFB laser has been used for the label-free detection of ErbB2 cancer biomarker. The DFB was composed of PS and a perylenediimide derivative and has been fabricated by thermal-NIL and subsequent plasma etching processes. A concentration LOD of 14 ng/mL ErbB2 has been obtained, with no cross-reactivity with BSA and TNFα proteins. This concentration LOD obtained for ErbB2 detection opens the possibility to use DFBs in clinical applications. The specificity demonstrated for ErbB2 detection shows how this technology can be extended for low-cost multiplex detection of protein biomarkers. These findings along with the low-thresholds that can be obtained for DFB laser emission and its compatibility with LED pumping could pave the way towards its implementation in medical devices.

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Biographies

Aritz Retolaza received the Chemistry Science degree in 1999 and the Ph.D. degree in thermal and mechanical properties of polymer blends, in 2004, both from the University of the Basque Country (Spain). Since 2005 he has worked in the Micro and Nanofabrication Unit of IK4-Tekniker, developing UV-lithography and NIL technologies. He has studied basic aspects of NIL, such as bending, demoulding and lift-off after imprinting, and he has applied this technology in different fields such as organic lasers, DNA manipulation in nanofluidic chips, effect of micropatterned surfaces on cell morphology and localized surface Plasmon resonance.

Josu Martinez-Perdiguero received his BS and PhD in physics with honors from the University of the Basque Country in 2005 and 2009 respectively. In 2010, he joined the Micro and Nanofabrication Unit of IK4-Tekniker and has since been researching on new highly sensitive biosensors with a special focus on novel concepts and practical

aspects such as lower limits of detection, performance with real samples and system integration. He is author of 20 indexed articles.

Santos Merino received the PhD degree in Solid State Physics from the Basque Country University, Spain, in 1997. Since then, he has been a Researcher at IK4-Tekniker. He is currently the Head of Micro and Nanofabrication Unit at IK4-Tekniker, developing most of the activity in biosensors and tissue engineering in Health, as well as nanofabrication-based processes for photonic applications.

Marta Morales-Vidal received the Optics and Optometry degree in 2007, the Optometry and Vision Sciences Master's degree in 2009, and the Molecular Nanoscience and Nanotechnology Master's degree in 2012, all of them from Alicante University (UA). She is currently working towards a PhD in Nanoscience and Nanotechnology in the UA in the "Organic electronics and photonics" group, with particular emphasis in the study of novel organic materials for lasing and the application of organic distributed feedback lasers for biosensing applications.

Pedro G. Boj obtained the PhD in physics in 1986 in the University of Valencia, in the area of optics in holographic recording materials and holographic optical elements. In 1982 he joined the Holographic Centre of the University of Alicante (UA). In 1985 he became Associate Professor of Optics at the UA, position that holds at present. His research activity evolved from the field of holography to the area of organic solid-state lasers, since 2002, when he joined the group of "Organic Electronics and Photonics" at the UA.

José A. Quintana received the Ph.D. degree in Optics from the University of Alicante (UA), Spain, in 1975. As Associate Professor he taught Solid-state Physics at the University of Valencia, Spain, from 1968 until 1972, and Physics and Optics at the UA, from 1973 until 2008. He conducted research in the area of holography from 1968 until 2000. He joined the "Organic Electronics and Photonics" group at the UA in 2004 and since then, he works in the area of Organic Electronics.

José M. Villalvilla graduated in Chemistry in 1985 in the Autonomous University of Madrid and received the PhD in physics in 1992 from the University of Alicante (UA), working in the area of dry recording in III-V materials with ionic beams**.** After postdoctoral work in Cambridge University, UK, during 1996, he became Associate Professor of Applied Physics at the UA, position that holds at present. In 2002 he joined the group of "Organic Electronics and Photonics". Since then, his research activity has

focussed on photoconductive polymers and on the fabrication of organic distributed feedback lasers by holographic lithography.

María A. Díaz-García received the Ph.D. in Physics in 1995 at the Autonomus University of Madrid, Spain. She was part of the pioneer group of Prof. Heeger (Nobel Prize in Chemistry **Author Biographies** 2000), at the Univ. of California in Santa Barbara, USA, which discovered stimulated emission in semiconducting polymers in 1996. She joined the faculty of the University of Alicante in 2001, where she founded the "Organic Electronics and Photonics" group, which leads since then. She was appointed full professor in 2010. Her latest research focuses on organic optoelectronic materials and devices, with major emphasis on organic lasers.

FIGURE CAPTIONS

Figure 1. a) Scheme of the DFB laser sensor, including excitation (green arrow) and collection (red arrow) geometry. Scheme of the direct capture immunoassay employed for ErbB2 biomarker detection is also shown. b) 3D image of one of the gratings, obtained by atomic force microscopy, before deposition of active film.

Figure 2. DFB spectra of the sensor device in each step of the immunoassay. From left to right: (A) before functionalization; (B) After functionalization with antiErbB2; (C) After BSA blocking; (D) After analyte addition at a concentration of 10 ng/mL. The symbols represent the collected experimental points.

Figure 3. Selectivity tests for the ErbB2 immunoassay.

Figure 4. Calibration curve constructed for the detection of ErbB2 standards, prepared in PBST pH 7.3, with a PDI-doped PS DFB sensor. The concentrations used are 2, 10, 250 and 10000 ng/mL. The concentration LOD was estimated as 14 ng/mL (Signal/Noise =3).

HIGHLIGHTS

- An organic distributed feedback laser is used to detect ErbB2 protein biomarker.
- An immunoassay for the specific detection of ErbB2 protein biomarker has been carried out.
- A concentration limit of detection of 14 ng/mL ErbB2 has been achieved.