



Long period fiber grating working in reflection mode as valuable biosensing platform for the detection of drug resistant bacteria

G. Quero^{a,1}, S. Zuppolini^{b,1}, M. Consales^a, L. Diodato^b, P. Vaiano^a, A. Venturelli^c,
M. Santucci^d, F. Spyraakis^d, M.P. Costi^d, M. Giordano^e, A. Borriello^{b,*}, A. Cutolo^a,
A. Cusano^{a,*}

^a Optoelectronics Group, Dept. of Engineering, University of Sannio, Benevento, Italy

^b Institute for Polymers, Composites and Biomaterials (IPCB)—CNR, Portici, Italy

^c Tydock Pharma S.r.l., Modena, Italy

^d Dept. of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy

^e Optosmart S.r.l., Portici, Italy

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ABSTRACT

We report here on a reflection-type long period fiber grating (RT-LPG) biosensor for the fast detection of class C (AmpC) β -lactamases (BLs), actually considered as one of the most important source of resistance to β -lactam antibiotics expressed by resistant bacteria. A standard LPG working in transmission configuration is first transformed in a more practical probe working in reflection mode and successively coated with a primary high refractive index (HRI) overlay of atactic polystyrene (aPS) in order to increase its surrounding refractive index sensitivity (SRI) in biological solutions. The aPS-coated RT-LPG is then coated by a secondary layer of poly(methylmethacrylate)-*co*-methacrylic acid (PMMA-*co*-MA) in order to provide the necessary surface functionalities to promote a stable covalent bioreceptors immobilization. The BLs detection has been performed by using the 3-aminophenylboronic acid (3-APBA) as biorecognition element, due to its excellent inhibition properties against class C BLs and specificity. Results here provided demonstrate that the proposed label free biosensor is capable of reliable detection of purified AmpC BLs in phosphate buffer solutions (PBS) with concentrations as low as one hundred nM, with a lowest limit of detection (LOD) of the order of a few tens of nM. The real effectiveness of the proposed biosensor has been also confirmed in lysate samples, which contain *Escherichia coli* bacteria overexpressing AmpC BLs.

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1. Introduction

The rapid spread of bacterial strains resistant to third and fourth generation of β -lactam antibiotics represents one of the most critical and pressing issues in clinical therapy [1–3]. One the main responsible for the proliferation of multidrug resistant bacteria can be ascribed to the high consumption of carbapenems antibiotics. Multidrug resistance forced, in fact, the administration of last generation drugs to treat a number of nosocomial infections, increasing the risk of developing new resistances [4,5]. Bacterial resistance is essentially based on four different mechanisms: (i) production of β -lactamases; (ii) decreasing of the affinity of Penicillin-Binding

Proteins (PBPs) for β -lactam antibiotics through mutations in the binding site; (iii) decrease expression of Outer Membrane Proteins (OMPs); (iv) overexpression of efflux pumps that export a wide range of substrates from the periplasmic space to the surrounding environment [6]. In particular, β -lactamases production represents the major mechanism of resistance exploited by Gram-negative bacteria. These proteins are able to inhibit β -lactam antibiotics by breaking the β -lactam ring through the nucleophilic attack of a catalytic serine (serine β -lactamases, classes A, C, D according to the Ambler classification [7]) or of a hydroxyl group coordinated by two catalytic zinc ions (metallo β -lactamases, class B) [1]. β -Lactamases able to inactivate third-generation cephalosporins and aztreonam are known as Extended Spectrum β -Lactamases (ESBLs) [8], while those even able of hydrolyzing carbapenems have been classified as carbapenemases [9]. It has been presaged that carbapenemase-producing *Enterobacteriaceae*, the most common pathogens responsible for both community- and hospital-acquired infections, will spread as well as ESBLs producers. The only

* Corresponding authors.

E-mail addresses: borriell@unina.it (A. Borriello), a.cusano@unisannio.it (A. Cusano).

¹ These authors contributed equally to this work.

possibility to fight bacteria diffusion is to adopt an efficient infection control strategy. This can be attained by developing rapid sensitive methodologies for the detection of β -lactamase producers [10]. Thus, besides trying to design new potential β -lactamase inhibitors or discover new antibiotics not hydrolyzed by ESBLs and carbapenemases, the early detection of β -lactamases produced by the infecting bacteria could represent a valuable strategy for reducing long-term hospitalization and prolonged antibiotic treatment [11]. For this reason, innovative diagnostic tools for rapid β -lactamases detection and identification in biological samples of infected patients are urgently needed. The potential benefits of having an easy working β -lactamase detection system would include reduced hospitalization times, lower illness severity, higher probability of administering the appropriate therapy, lower treatment failures, and reduction in the escalation of antibiotic resistance in clinical settings and the community. All these aspects would have a significant economic impact on the community life quality and on the health care system.

Potential multi-drug resistant bacteria (MDR) are currently screened by susceptibility testing using breakpoint concentration of antibiotics hydrolyzed by specific β -lactamases [12,13]. Even if these techniques represent the first and most common detection strategy, they are time-consuming, since they require bacteria growing on plates, and not always reliable, since many ESBL/carbapenemase producers do not confer obvious resistance levels to antibiotics and sometimes show lower minimum inhibitory concentration (MIC) values with respect to the clinical breakpoints [14]. To solve these limitations, different phenotypic assays for the specific detection of β -lactamase producing strains were developed. Chromogenic, iodometric and acidimetric tests are quite fast but less specific since they do not give specific indications about which β -lactamase is produced [15,16]. Metallo β -lactamase producers are often detected by chelating agents, with the obvious risk of having non-specific binding [17]. Molecular-genetic techniques still remain the reference standard for the identification of ESBL/carbapenemase genes. These real time PCR-based assays are more rapid and sensitive with respect to phenotypic assays, but they are also more expensive and they require the availability of trained microbiologists. Also they cannot detect new β -lactamases since the range of resistance genes to be identified has to be predefined.

More recently, direct methods for known and novel β -lactamases identification to be used in routine diagnostic laboratories have been reported including the Carba NP test [18] and MALDI-TOF mass spectrometry [19].

Unfortunately, both phenotype- and genetic-based methodologies are poorly adapted to the clinical need of rapidly isolating patients and prevent nosocomial outbreaks [5]. It seems that the only possibility to speed up and improve ESBLs/carbapenemases diagnosis rely on a better integration between expert reference laboratories, on the education of the laboratory personnel or on the development of innovative approaches for the direct detection on selective cultivation media [10]. In this perspective more sensitive and easy-to-use devices would be extremely useful for a rapid and precise detection of β -lactamase producing bacteria in an early infection stage.

In the last years, many efforts have been focused on the development of highly sensitive optical probes able to detect unlabeled biomolecules, as they would enable a true real time (and in situ) screening with minimal sample consumption and treatment. Several approaches, based on label-free methods have been proposed [20,21], including surface plasmon resonance (SPR) based biosensors [22,23], interferometric based biosensors [24,25], optical waveguide based biosensors [26], optical ring resonator based biosensor [27–29], photonic crystal based biosensor [30,31], and optical fiber based biosensor [32–34]. Among all, Long Period Fiber

Gratings (LPGs) have received great attention, in the last years, as reliable and high sensitivity label free biosensors [35–41].

One of the main limitations of this kind of fiber gratings in the bare configuration is the rather low sensitivity in aqueous solutions, especially for biochemical detection [42]. In order to overcome this limitation, in literature two different strategies have been developed. The first approach relies on coupling the propagating core mode with a high-order cladding mode near its turn-around point (TAP) [37,40,41,43]. TAP LPGs have demonstrated to attain values of volume RI sensitivity of the order of thousands of nm RIU⁻¹ [44,45]. The second approach is based on the exploitation of the modal transition phenomenon, that takes place with the integration of nano-scale polymer overlays onto the LPG surface [34,36]. Indeed, it is by now very well known that the SRI sensitivity of LPGs can be optimized at the desired working point through the deposition of a high RI (HRI) layer by acting on its thickness (ranging in hundreds of nanometers) [34,46]. However, essential condition to fully exploit the benefits of transition mode LPGs is that the attenuation bands would not lose visibility while working in transition mode. This is not verified for some materials and related deposition techniques [47,48] and it is achieved only when high optical quality overlays (low absorption and roughness) are used. Up to now the best results have been obtained with thin polymer layers deposited by dip-coating (DC), in particular with ordinary atactic polystyrene (aPS) [46].

One of the peculiarity of LPGs platforms is their operation in transmission mode which makes the device sensitive to the bending, therefore requiring the development of appropriate strain-free packages to host the LPG device. In particular, the bending applied on the LPG can introduce unexpected variations in the spectrum of the transmitted optical signal, thus complicating the sensor signal interpretation. In addition the capability to work in reflection mode addresses the mentioned issues and allows the easy integration of the reflective optrode in the vials containing the biological solution and represents a more practical and robust solution to be employed for concrete biological applications [49–52].

In this work, we report, for the first time to our best knowledge, on an innovative, robust and highly sensitive technological platform based on double-coated RT-LPG biosensors for the label-free detection of β -lactamases produced by resistant bacteria. In particular, in the model system we set up, the optical probes were functionalized with the 3-amino-phenylboronic acid (3-APBA) ligand, able to bind and detect the presence of a specific BL, i.e. AmpC, in PBS solutions. Once verified the potentiality of the proposed approach with purified AmpC contained in PBS buffer solutions, we also investigated its real effectiveness in biological solutions much more similar to samples extracted by infected humans. In particular, several detection experiments were carried out with lysate samples containing *Escherichia coli* bacteria overexpressing AmpC BL.

2. Materials and methods

2.1. Chemicals

Ethanol (EtOH), isopropyl alcohol (IPA, analytical grade 99.7), double distilled water (ddH₂O), chloroform (CHCl₃, analytical grade 99.9), atactic polystyrene (aPS, MW=280.000), poly(methylmethacrylate)-co-methacrylic acid (PMMA-co-MA, MW=34.000), aqueous ammonia (NH₃ 30% w/w), dextrose, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimidehydro-chloride (EDC), N-hydroxysuccinimide (NHS) and 3-amino-phenylboronic acid (3-APBA) were purchased from Sigma-Aldrich (Milan, Italy). Potassium hydroxide (KOH) were purchased by J.T. Baker. AmpC

β -Lactamase was provided by University of Modena and Reggio Emilia (Modena, Italy).

2.2. Design of transition mode HRI-coated LPG

A custom virtual environment for the design and simulation of multilayer-coated LPGs, realized with MATLAB® code and based on the transfer matrix method with the Linearly Polarized (LP) mode approximation [47], was used to identify the optimal design parameters of transition-mode HRI-coated LPG, including the grating period, the cladding mode order and the overlay thickness. The parameters of the optical fiber used for the simulations are contained in the specifications of the Fiber-core PS1250/1500: core and cladding radius of 3.5 μm and 63 μm , respectively and numerical aperture of 0.13. As described in details in Supplementary material, once fixed the mode order (LP_{07}), the operating wavelength (1550 nm, in order to benefit of the low cost optoelectronic equipments) and the refractive index (1.34, to optimize the operation in watery environment), the optimal design parameters turned out to be: grating period $\Lambda = 370 \mu\text{m}$, aPS layer thickness $t_{\text{aPS}} = 280 \text{ nm}$ and PMMA-co-MA overlay thickness $t_{\text{PMMA}} = 30 \text{ nm}$. With the considered parameters, SRI sensitivities of the double-coated RT-LPG transducer as high as $\sim 1400 \text{ nm/RIU}$ are expected.

2.3. RT-LPG fabrication and characterization

2.3.1. LPG inscription

The LPGs used in this experiment were inscribed in a boron doped photosensitive single-mode optical fiber (PS1250/1500, Fibercore Ltd.) by a point to point technique. The fiber was mounted on an automatic rotation stage able to continuously rotate the optical fiber during the LPG writing procedure, that was performed via a KrF pulsed excimer laser (LightBench 1000, Optec, Belgium) operating at a wavelength of 248 nm. Both the rotation stage and the laser action were controlled and synchronized by a personal computer in order to select the grating period (translation stage step and slit dimension), the grating length (number of irradiated points) and the induced RI change (number of laser pulses per point and laser fluence).

2.3.2. RT-LPGs fabrication

The main fabrication steps to realize the RT-LPGs are (see Fig. 1a): (i) the fiber cutting just after the grating, (ii) the Ag reflecting layer (i.e. the mirror) integration on the fiber end-face and (iii) the double layer (aPS and PMMA-co-MA) deposition on the RT-LPG surface. The first step (i) is of fundamental importance for the development of more practical and robust LPG working in reflection configuration, and was dictated by our biomedical applications, which required the probe to be immersed into laboratory vials containing the biological samples under tests. Crucial aspect in this step is the identification of the precise grating location along the optical fiber, in order to cut it just after the grating end in order to avoid the formation of interference fringes within the attenuation bands, typical of self-interfering LPGs [53]. For the Ag mirror integration on the fiber end-face, a freshly prepared silver nitrate solution (AgNO_3 0.1 M, 640 μL) and a potassium hydroxide solution (KOH 0.8 M, 440 μL) were mixed. The brown precipitate formed was dissolved by the addition of aqueous ammonia (NH_3 30% w/w, 2 drops) and stirring, and successively a dextrose solution (0.25 M, 64 μL) was added. After stirring, the LPG tip was dipped into the ready Tollen's reagent [54]. The fiber was removed after $\sim 30 \text{ min}$ (though the silver film formed on the wall of vial in 5 min) and dried in air. Fig. 1b reports the transmission spectrum of the attenuation band associated to the sixth order mode (LP_{07}) acquired before the optical fiber cut (blue line) as well as the reflected spectra after the optical fiber cut (red line) and after the mirror formation at the fiber

facet (green line). A significant baseline reduction of $\sim 15 \text{ dB}$ occurs after cutting, mainly due to the fact that light passing through the LPG is mostly transmitted at the fiber/air interface, and only a small portion ($\sim 3\text{--}4\%$) is reflected back into the fiber. Nevertheless, as the Ag mirror layer is formed at the fiber tip, almost all the initial power is recovered. We point out that the use of reflection-type LPGs not only is of fundamental importance to transform an LPG-based device in a more practical probe for concrete biomedical applications, but also enables to improve the resonance visibility (see Fig. 1b) because of the light double passing through the grating.

The aPS and PMMA-co-MA overlay depositions were performed via the dip-coating process by means of an automated system (NIMA Technology Micro-Processor Interface IU 4) at an immersion/extraction speed of 100 mm/min [35,46]. Deposition solutions were: 9.5% (w/w) of atactic polystyrene (aPS) in chloroform (analytical grade 99.9), and 10% (w/w) of poly(methylmethacrylate)-co-methacrylic acid (PMMA-co-MA) in chloroform:isopropanol (1:3, v/v). Fig. 1c reports the spectral position in air of the attenuation band related to the sixth order cladding mode in the bare reflection type LPG (green curve), after the aPS overlay deposition (violet curve) and after the second PMMA-co-MA layer deposition (yellow curve). The deposition of the 280 nm-thick aPS overlay caused a blue shift of the RT-LPG spectrum of about 22 nm, while the one occurred after the 30 nm-thick PMMA-co-MA deposition was of about 5 nm. The aPS and PMMA-co-MA layer thicknesses were retrieved by means of a reverse engineering approach carried out with the help of the virtual environment for the design and simulation of nano-scale coated LPGs (Section 1, Supplementary material).

2.3.3. Surrounding refractive index (SRI) sensitivity characterization

The SRI sensitivity of the fabricated RT-LPGs (see Fig. 2a) was carried out by submerging the probes into aqueous glycerol solutions characterized by different RI in the range 1.335–1.46. Fig. 2b reports the typical SRI sensitivity ($|\partial\lambda_c/\partial\text{SRI}|$) vs. SRI curve of the fabricated RT-LPGs which exhibits the typical resonance-shaped behavior of transition mode LPGs, thus confirming the fabrication process success [55,56]. In particular, it reveals that the SRI sensitivities of fabricated probes are $\sim 1500 \text{ nm/RIU}$ in correspondence of an SRI = 1.34. This experimental value resulted slightly higher than those obtained from the design process ($\sim 1400 \text{ nm/RIU}$), probably due to the slight differences between numerical and experimental values of the refractive indexes of the aPS and PMMA-co-MA layer, as well as of the glycerol solutions used for the SRI sensitivity characteristic (that were measured at 589 nm and for which dispersion was not taken into consideration).

2.4. Biochemical functionalization and biomolecular tests

Once fabricated the double-coated RT-LPG transducer, the attention was focused on the biochemical functionalization strategy in order to promote a stable covalent immobilization of the bioreceptor on the sensor surface. This represents a crucial step since, in many cases, the reliability of a biosensor strongly depends on the correct immobilization of the bioreceptor on the transducer surface [57]. As described in detail in the Supplementary material, the 3-aminophenylboronic acid (3-APBA), chosen as biorecognition element, is a well known AmpC BLS inhibitor, able to bind the protein with micro molar affinity ($K_i = 7.3 \mu\text{M}$ [58]), forming a covalent adduct and behaving as a transition state analogue [59]. Crystallographic evidenced showed that the ligand presents a favorable orientation within the protein binding site [60], likely allowing the anchoring reaction and the probe functionalization. The biochemical functionalization strategy here adopted consists in the sensor surface modification via the activation of carboxylic groups, and following immobilization of the bioreceptors-ligand complex

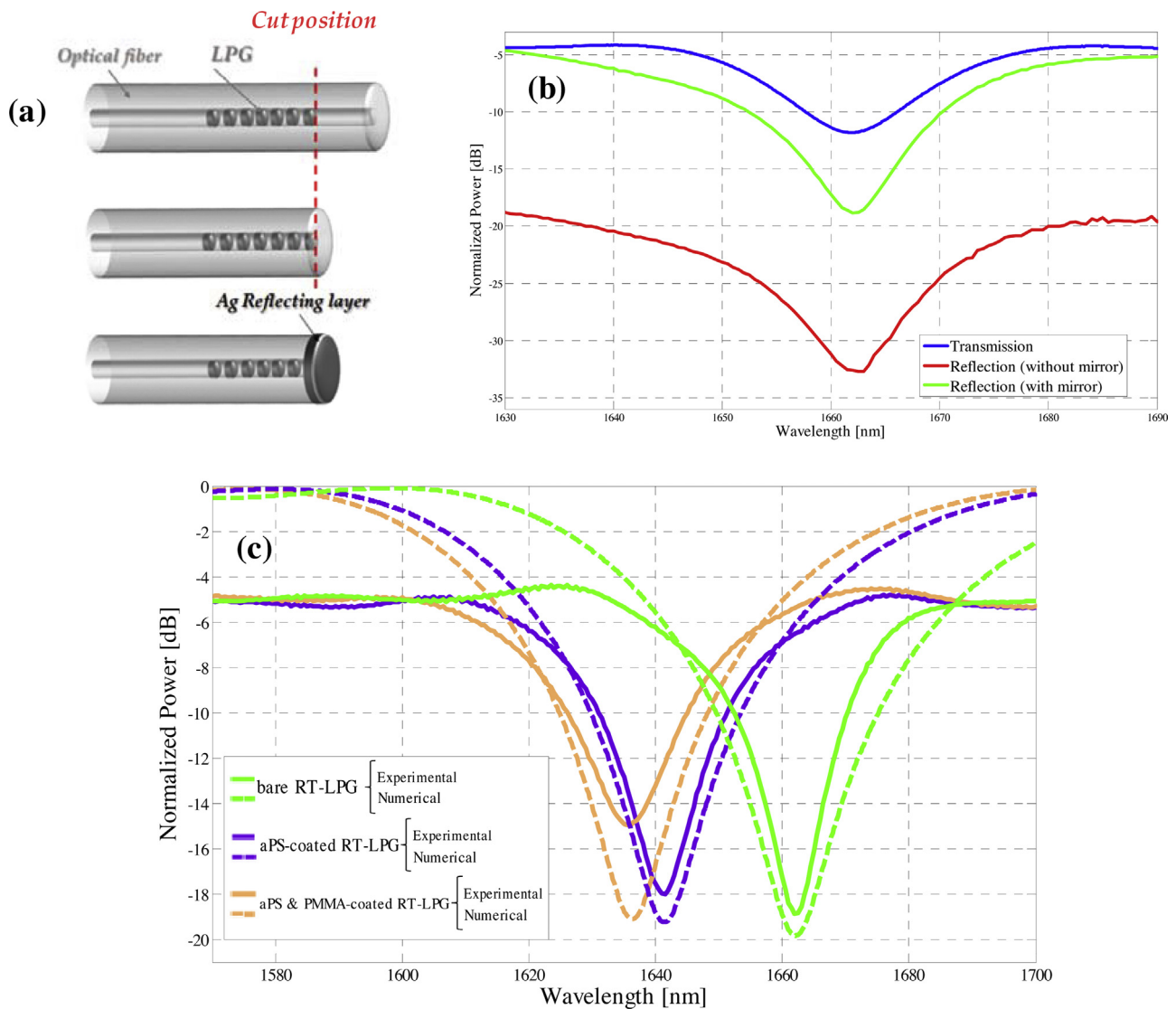


Fig. 1. (a) Schematic view of the main fabrication steps for the RT-LPG fabrication; (b) LPG spectra acquired in air before the fiber cut (blue line), just after the fiber cut (red line) and after the mirror integration (green line); (c) Reflection spectra of the bare (green line), aPS (violet line) and PMMA-co-MA (yellow line) coated RT-LPG. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

on the RT-LPG surface through the formation of a covalent bond. This strategy can confer reliability, selectivity and sustainability to the optical fiber device. Specifically, with the carboxylic functions provided by the PMMA-co-MA surface as anchoring points and the primary amino groups present on the ligand, the linkage chemistry consisted in the initial activation of the carboxylic groups through the reaction with *N*-ethyl-*N'*-(3-(dimethylamino) propyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), leading to an amine-reactive ester, and suitable for a successive reaction with the primary amino group present on the ligand. A final washing with ddH₂O was performed to remove the un-reacted ligand molecules. At this step, the biofunctionalized LPG probe was used for the AmpC binding test following this procedure: first immersion in ddH₂O, immersion in AmpC β -lactamase solution (in PBS buffer), washing with ddH₂O to remove the un-reacted protein. In order to evaluate the effectiveness and feasibility of the RT-LPG biosensor, the same procedure was performed using a complex matrix (lysate) solution with increasing AmpC concentrations from 100 nM to 300 nM.

2.5. Protein purification

AmpC β -lactamase from *Pseudomonas Aeuruginosa* was obtained by transfecting *E. coli* BL21(DE3) cells with the plasmid pET-9aAmpC in 1 L of LB-medium, in presence of kanamycin 50 μ g/mL, at 150 RPM, 37 °C, for 24 h. Protein expression was induced by adding IPTG (isopropyl- β -D-galactopyranoside, 1.0 mM) and incubating the culture at 150 RPM, 37 °C, for 6 h. After 6 h the cell broth was centrifuged at 4500 RPM, 4 °C, for 20 min. The supernatant was recovered, concentrated by ultrafiltration (Centricon Plus-70, Millipore), and desalted using an Hi-Prep desalting 26/10 column (GE Healthcare) with load-buffer (0.02 M Tris, 0.5 M NaCl, pH 7.0). The sample was then loaded on a XK 16/20 column packed with 25 mL of Affigel-10 (Biorad) functionalized with 3-MAPB (3-methylaminophenylboronic acid). The column was previously equilibrated with the load-buffer. The protein was eluted (F.R. 1 mL/min) with 5 column-volumes of elution-buffer (0.5 M boric acid, 0.5 M NaCl, pH 7.0). β -Lactamase activity was checked in each eluted fraction using cephalothin as substrate (100 μ M). The active fractions were merged, concentrated by ultrafiltration

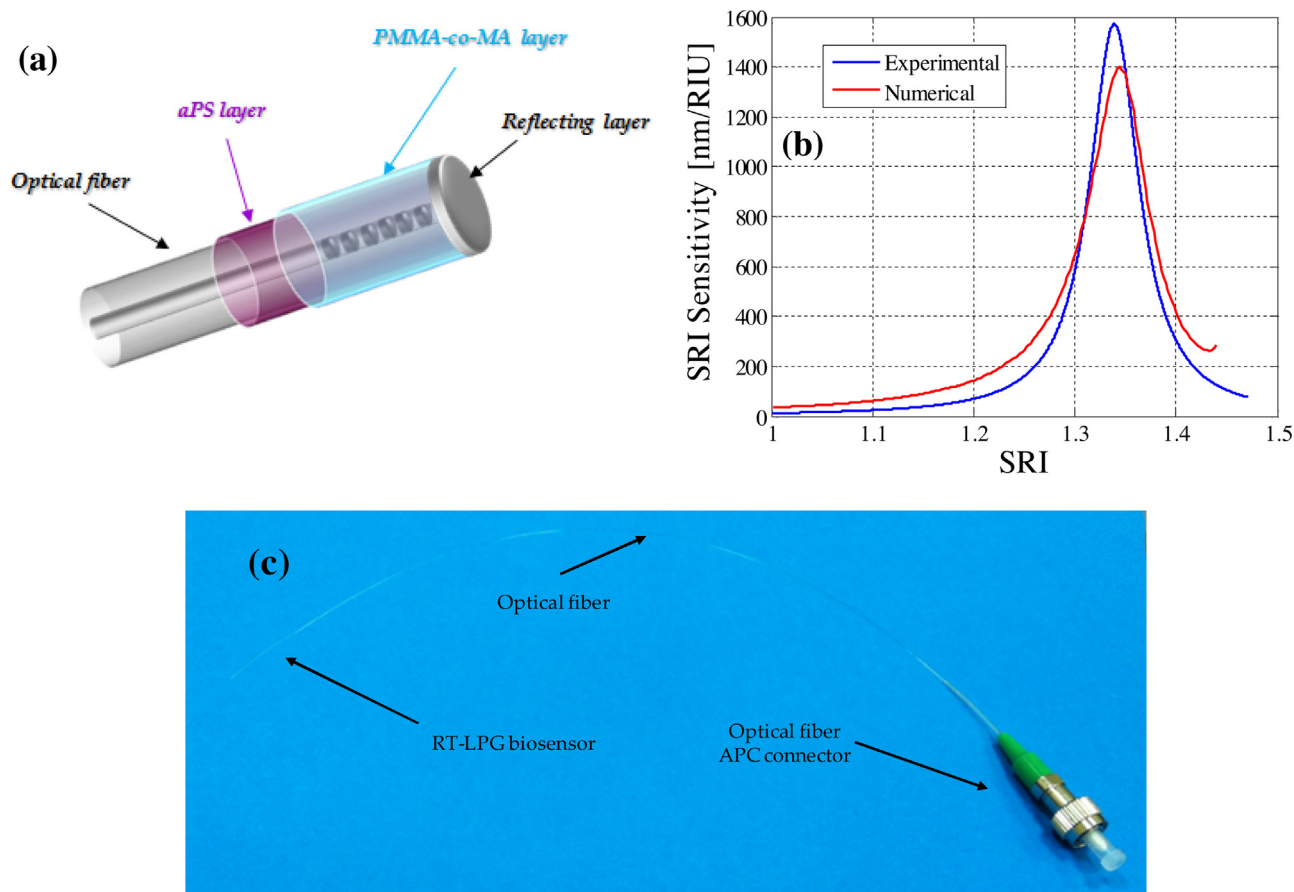


Fig. 2. (a) Schematic view of the final RT-LPG transducer and (b) experimental SRI sensitivity ($|\partial\lambda_c/\partial\text{SRI}|$) vs. SRI curve (blue line) for the cladding mode LP_{07} ; the numerical sensitivity curve is also reported for comparison (red line) and (c) a photograph showing the RT-LPG biosensor probe developed in this work. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Ultracel-10 K; Millipore) and checked by SDS-Page. The purified AmpC β -lactamase (1 mg/mL) was stored at -80°C .

2.6. Lysate preparation

E. coli BL21 (DE3) bacterial strain was grown on an agar plate. A small liquid culture (5 mL) of Luria-bertani (LB) broth was inoculated with bacterial colonies from agar plate and incubated at 120 RPM, 37°C for 4 h. The entire 5 mL liquid culture was poured into 0.25 L of LB-broth and incubated at 150 RPM, 37°C , for 6 h. The cell-broth was then centrifuged at 4500 RPM, 4°C , for 20 min; the cellular-pellet was recovered and suspended in Buffer-A (20 mM NaH_2PO_4 , 30 mM NaCl, pH 7.5). Cells were then broken by sonication and the cell-suspension centrifuged at 12000 RPM, 4°C , for 30 min. The supernatant was recovered, filtered by using 0.80/0.45 μm filters for a total final volume of 5 mL. Purified AmpC β -lactamase was added in filtered lysate sample in order to obtain a final protein concentration equal to 3.65 μM . It was thus possible to obtain experimental samples characterized by an increased biological complexity with respect to the purified protein sample.

2.7. Interrogation setup

An optoelectronic set-up comprising a broadband light source (with bandwidth 1200–1700 nm), a 2×1 directional coupler and an optical spectrum analyzer (OSA, ANDO AQ6317C, wavelength resolution 10 pm, dynamic range 60 dB) was used for the acquisition of the LPG reflection spectra at the different stages of the device

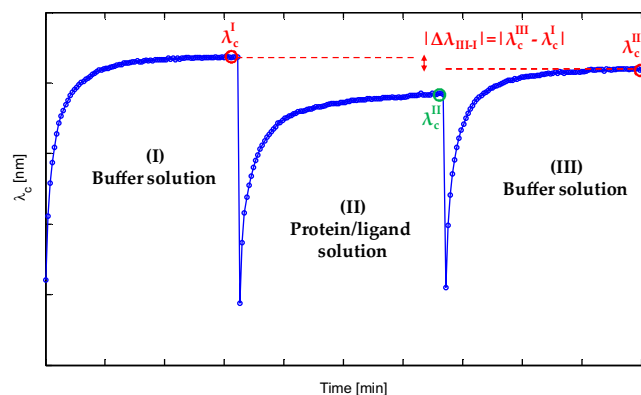


Fig. 3. Sensorgram during a generic biomolecular experiment involving the AmpC BLs (or ligand) binding to the RT-LPG surface.

fabrication and characterization. The OSA is connected to a personal computer and is controlled by a LabView plug-in, enabling the automatic acquisition of the RT-LPG spectra. Acquired spectra are then automatically filtered and elaborated by a MATLAB script that provides the central resonance wavelengths (λ_c) of each spectrum. During the biological experiments the RT-LPG spectra is automatically acquired every 45 s, therefore providing a continuous and real time monitoring of the interaction kinetics of the biological molecules on the RT-LPG surface.

2.8. Analysis methodology

With reference to the RT-LPG sensorgram reported in Fig. 3, a generic biological experiment involving the AmpC BLs (or ligand) binding test essentially consists of three main steps: the first step (I) relies on the RT-LPG probe immersion in a vial containing the buffer solution (in our case ddH₂O). It is worthy to observe that each time the optical probe is submerged into the aqueous environment there is a sort of acclimation of the polymeric layers expressed through a quite fast red wavelength shift [35] (lasting ~30 min) before the RT-LPG biosensor response reaches a steady state value (in this case λ_c^I). The probe is then immersed in the target biological sample (II) containing the protein (or ligand) and the whole binding kinetic (λ_{res} vs. time) is monitored until the LPG response reaches a plateau value (λ_c^{II}). Finally, the probe is extracted from the vial and is immersed in clean ddH₂O (III) in order to remove the protein (or ligand) molecules not permanently bound to the probe surface. Once completed the washing procedure the RT-LPG signal reaches a new plateau value (λ_c^{III}). Step III can be sometimes repeated to ensure that most of the target biomolecule a-specifically bound to the surface is completely removed. To evaluate the RT-LPG signal variation associated only to the protein (or ligand) attachment and not to the different RI of the used solutions, it is possible to consider the resonance wavelength variation between steps III and I ($\Delta\lambda_{III-I} = \lambda_c^{III} - \lambda_c^I$), where the probe is submerged in the same solution (i.e. ddH₂O) and no RI-induced wavelength variations can occur.

3. Results and discussions

3.1. RT-LPG transducer biofunctionalization

The typical real time sensorgram, reporting the time variation of the probe resonance wavelength obtained during the biofunctionalization of the RT-LPG transducer is reported in Fig. 4a. The optical probe was first immersed in 1.0 mL of ddH₂O (RI = 1.333) to acclimate the sensor surface and left inside it until a steady state level was reached (I). Successively, 500 μ L of freshly prepared EDC/NHS solution (0.05/0.03 M in ddH₂O, RI = 1.342) was added dropwise (II). The RT-LPG probe was left in the final solution (1.5 mL) for about 50 min (until the output signal stabilization) and then rinsed in 1.5 mL of clean ddH₂O (III). The RT-LPG resonance wavelength shift recorded between steps I and III ($\Delta\lambda_{III-I}$) and due to the activation of the carboxylic groups on the PMMA-co-MA surface, was about –4 nm. After the EDC/NHS coupling chemistry, in order to anchor the 3-APBA to the activated PMMA-co-MA surface, the device was immersed (IV) in a vial containing 1.5 mL of 3-APBA solution (3.38 mM in ddH₂O) for a reasonable time of 60 min, after which it was rinsed in clean ddH₂O (V) for about 30 min. Also in this case, a blue shift was observed for the optical signal $\Delta\lambda_{V-III} = -1.5$ nm between steps V and III, which is coherent with an attachment of 3-APBA molecules on the PMMA-co-MA surface. This step was repeated one more time (steps VI and VII) in order to guarantee the coverage of all available functionalized groups on the probe surface, and a further blue shift of –1.0 nm was obtained. Overall, results obtained from this phase confirm that the chemical functionalization allows the PMMA-co-MA surface carboxylic groups activation, which in turn facilitate the 3-APBA bioanchoring.

We also performed some tests to verify the durability of the functionalized probe: to this aim, the probe was kept immersed in buffer solution for a time longer than those typically used in our biological experiments. The preserved functionality of the device was confirmed by the continued stability of optical signal measured for overall immersion time (24 h, data not shown).

3.2. Purified AmpC β -lactamase detection tests

Once performed the RT-LPG transducer biofunctionalization, the attention was focused on the purified AmpC detection tests. Fig. 4b reports the real time sensorgram obtained with two increasing AmpC concentrations of 300 nM and 500 nM, respectively. Also in this case, the biofunctionalized RT-LPG probe was first acclimated in clean ddH₂O (VIII), and successively immersed in a PBS AmpC β -lactamase solution (IX) with concentration 300 nM for about 60 min, which is a reasonable time to allow the completion of the binding kinetic and permit all active sites on the probe surface to fish the AmpC molecules out. This is confirmed by the fact that a very stable steady-state value is reached at the end of the binding process by our RT-LPG device. The probe is then washed in clean ddH₂O (X). The wavelength shift recorded by our device between steps X and VIII ($\Delta\lambda_{X-VIII}$) resulted to be as high as –0.80 nm, therefore confirming the high sensitivity of the proposed optical fiber probe. After step X, the RT-LPG probe was submerged in a new PBS AmpC solution (XI) with a slightly higher concentration (500 nM), followed by its rinsing in ddH₂O (XII) for 60 min. A further (cumulative) blue shift of 0.90 nm was recorded for the resonance wavelength ($\Delta\lambda_{XII-X}$). The total (cumulative) shift measured by the RT-LPG device between steps XII and VIII ($\Delta\lambda_{XII-VIII}$) turned out to be –1.70 nm for a cumulative AmpC concentration of 800 nM.

Following the same protocol, several experiments have been carried out at high concentration levels in order to investigate the saturation grade of the PMMA-co-MA functionalized surface. To this aim, RT-LPG biosensors were fabricated by following the same procedure described in Section 2, and used for the biological experiments involving much higher β -lactamase concentrations (i.e. 1.5 μ M and 5.0 μ M). Measured resonance wavelength shifts of –4.15 nm occurred for the first concentration (1.5 μ M) and a further (cumulative) blue shift of 1.35 nm was observed for the second one (5.0 μ M).

It is worth considering that, even if the fabrication process is robust and repeatable, a normalization procedure (able to take into account the slight and unavoidable differences in the SRI sensitivities of different RT-LPG probes) has to be exploited in order to improve the device performances, reduce its limit of detection and permit its exploitation for concrete applications. Such differences may occur due to the LPG fabrication tolerances, or to slightly different aPS or PMMA-co-MA overlay thicknesses obtained from a device to another. To take into account this aspect, we performed a normalization of the optical device signal (in terms of resonance wavelength shift) to the wavelength shift occurred upon PMMA-co-MA layer deposition ($\Delta\lambda_{PMMA}$), which provides an indirect measure of the surface sensitivity of each RT-LPG probe. Thus, henceforth, we will consider the following observable (O):

$$O = \frac{\Delta\lambda_c}{\Delta\lambda_{PMMA}}$$

in order to compare results obtained with different RT-LPG probes and build up a unique calibration curve.

In Fig. 5 it is shown the optical biosensor calibration curve (blue curve) obtained using 5 different bioprobes for each tested protein concentration demonstrating the effectiveness of the proposed methodology. It reports the values of the observable O obtained in correspondence of the different AmpC β -lactamase concentrations (C). Cumulative values have been considered when a probe previously tested with a lower concentration is reused for an higher concentration experiments (e.g. step XI–XII). Fig. 5 reveals that the normalized wavelength shift (i.e. the observable O) initially increases in a quasi-linear manner with the increasing AmpC concentration until saturation is reached in correspondence of a protein concentration of ~1.0 μ M. This can be explained by the fact

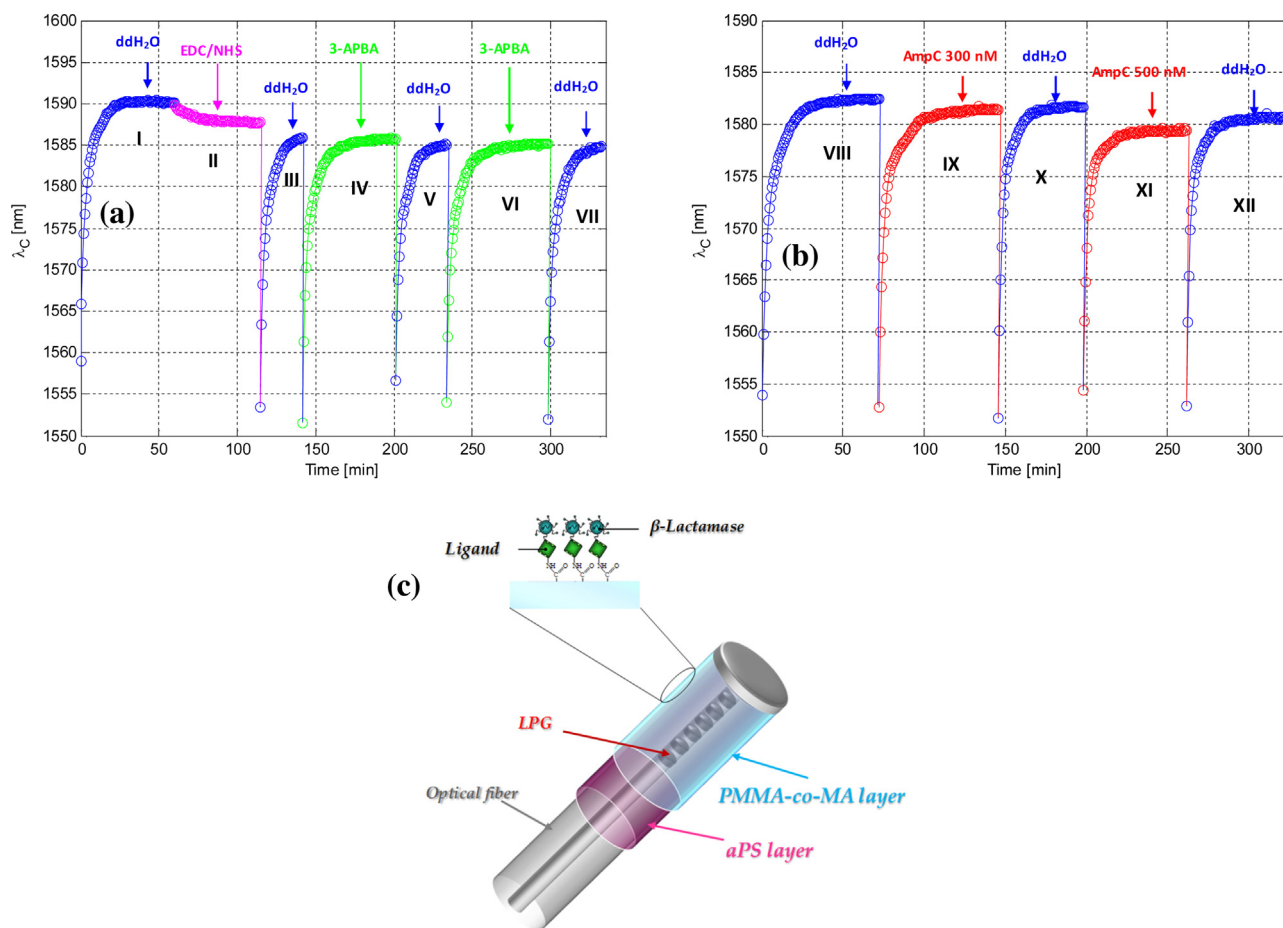


Fig. 4. (a) RT-LPG sensorgram during the biofunctionalization phase, (b) RT-LPG sensorgram during the AmpC β -lactamase binding experiments and (c) schematic of the RT-LPG probe coated by the different biological layers.

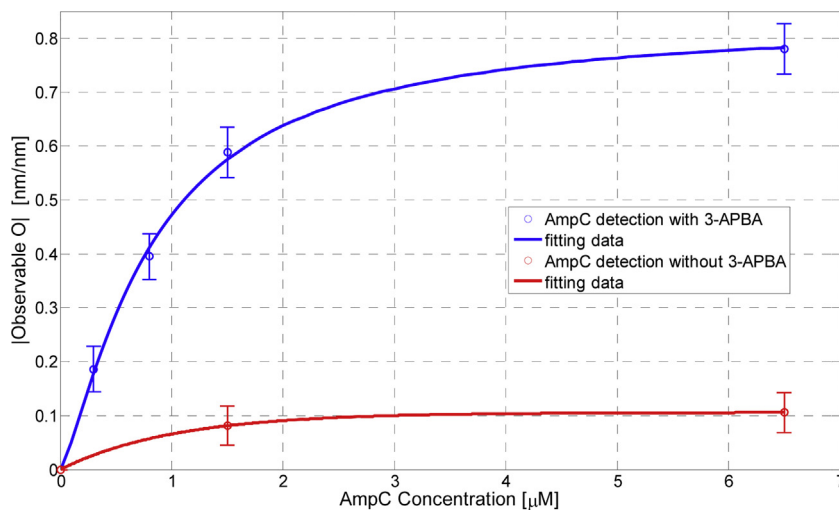


Fig. 5. Characteristic curves ($|O|$ vs. AmpC concentration) of the RT-LPG device obtained by the negative control tests using not-functionalized probes (red curve), the binding experiments with purified protein sample (blue curve). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that an increased β -lactamase concentration enhanced the surface coverage of the biofunctionalized RT-LPG surface. At high concentration ($>1.5 \mu\text{M}$) the sensor surface is almost completely covered with the target protein and therefore the resonance wavelength shift starts saturating.

A rough estimation of the RT-LPG biosensor limit of detection can also be performed by considering its sensitivity against AmpC

BLs, calculated as the slope of the characteristic curve (O vs. AmpC concentration) in the linear region, ranging from 0.3 to $\sim 1.0 \mu\text{M}$:

$$S_{\text{AmpC}} = \left| \frac{\partial O}{\partial C} \right| = 0.486 \mu\text{m}^{-1}$$

Therefore, by considering the minimum detectable value of the normalized observable (O_{\min}), expressed as:

$$O_{\min} = \frac{\Delta\lambda_{\min}}{\Delta\lambda_{\text{PMMA}}}$$

where, $\Delta\lambda_{\min}$ is the minimum detectable wavelength variation (set by the interrogation unit and equals to 35 pm), the LOD for the optical probe functionalized with 3-APBA is:

$$\text{LOD} = \frac{O_{\min}}{S_{\text{AmpC}}} \approx 20 \text{ nM}$$

In order to verify the goodness and effectiveness of our biological system, we also performed negative control (blank) tests allowing to assess the β -lactamase α -specific binding. To this aim we tested the response of RT-LPG probes (which underwent only the EDC/HNS coupling chemistry but without the immobilization of the 3-APBA bioreceptor) against biological solutions with AmpC concentrations of 1.5 μM and 5.0 μM . The biomarker concentrations were intentionally chosen so high (i.e. in the saturation level of the RT-LPG biosensor) in order to provide a “worst case” response against the α -specific protein binding onto the activated PMMA-co-MA surface of the device. The obtained values for the observable O (respectively -0.081 and -0.105) are reported in Fig. 5 (red curve) as a function of the tested concentrations. They reveal a response of the RT-LPG in the negative control case more than seven times lower in respect to the responses obtained with the biofunctionalized device, therefore confirming the high affinity of the chosen bioreceptor and, overall, the potentiality of the proposed bioprobe.

3.3. RT-LPG biosensor validation in lysate solutions

Once verified the correctness of the RT-LPG biosensor operation with purified AmpC contained in PBS buffer solutions, we also performed some preliminary experiments to test its real effectiveness and feasibility to detect the presence of β -lactamases in matrixes characterized by an increased biological complexity. In particular, several binding experiments with AmpC contained in lysate samples were carried out, exploring the capability of the optical device to reveal very low AmpC concentrations (i.e. 100 nM and 300 nM).

A representative sensorgram obtained during the “lysate” tests is reported in Fig. 6a. Similar to what made during the test with PBS buffer solutions, the biofunctionalized RT-LPG probe was first acclimated in clean ddH₂O (I), and successively immersed for about 60 min in the lysate sample (II) containing an AmpC BL concentration of 100 nM. In this step, differently from the previous cases, the resonant wavelength increased with time, reaching a final value higher than the plateau value obtained in ddH₂O. This can be attributed to the different refractive index of the lysate solution with respect to the ddH₂O one. When the probe is washed in clean ddH₂O (III), the device signal reaches a baseline value lower than that measured in step I, coherently with the binding of AmpC molecules on the functionalized RT-LPG surface. Although the very low BLs concentration used in this test, the wavelength shift recorded by our device between step III and I ($\Delta\lambda_{\text{lys,III-I}}$) resulted to be as high as -0.6 nm , thus demonstrating the capability to detect very low protein concentrations even in complex biological solutions. After step III, the RT-LPG probe was submerged in a new lysate sample (IV) containing a slightly higher AmpC concentration of 300 nM, followed by its rinsing in ddH₂O (V) for 60 min. A further (cumulative) blue shift of about 0.6 nm was measured by the optical probe ($\Delta\lambda_{\text{lys,V-III}}$). The total (cumulative) shift measured

by the RT-LPG biosensor in ddH₂O between steps V and I ($\Delta\lambda_{\text{lys,V-I}}$) turned out to be about -1.5 nm for a cumulative AmpC concentration of 400 nM. By comparing the preliminary characteristic curve (O vs. AmpC concentration) obtained during the “lysate” tests (black circles in Fig. 6b) with the one obtained during the experiments with the purified protein in PBS solutions (blue circles in Fig. 6b), a quite good agreement can be observed.

We also carried out some control experiments aimed to verify the output signal variation of the RT-LPG probe when inserted in a lysate solution without AmpC BL. Obtained results (see Fig. 6b, c) showed a very small response both in terms of resonance wavelength variation ($\Delta\lambda_{\text{lys,blank}} = -0.3 \text{ nm}$) and Observable ($|O| = 0.06$). This value for the observable resulted to be almost half the value obtained with the lysate solution containing 100 nM of AmpC BL, thus confirming once more the capability of our device to correctly detect the AmpC BL in complex media (i.e. in lysate solution), at the very low concentration of 100 nM.

Even if further experiments are needed to assess the capability of our approach to correctly operate in clinical samples, including in-depth specificity tests and also further detection experiments involving more complex biological solutions (among which true clinical samples), however preliminary results here shown demonstrate that the proposed optical device, with the help of the exploited biological system, is able to correctly operate also in complex biological samples, allowing the detection of AmpC BL at the very low concentration of 100 nM.

As a final comparison with other currently available methodologies for the detection of β -lactamases, we wish to emphasize that, as well described in Livermore and Brown [15], colorimetric, acidimetric and iodometric tests are rapid indicators of resistance to penicillin and ampicillin, but in Gram-negative bacteria present specificity issues. More accurate methodologies as susceptibility tests and genetic-based approaches are more time consuming. In this scenario, the presented methodology could allow a rapid and precise detection of β -lactamase producing bacteria in an early infection stage. To the best of our knowledge no similar device has been proposed so far. On the other hand, the main limitation of our RT-LPG biosensor actually relies on its response time which, in reported experiments, is mainly limited by batch operation. However, it is worth pointing out that the integration of the RT-LPG platform in a suitable optofluidic system enabling its operation in flow condition, would drastically reduce the biosensor response time, therefore completely overcome the above mentioned limitation.

4. Conclusions

A comprehensive study and a detailed analysis of a robust and high-sensitivity reflection-type long period biosensor capable of label-free detection of drug resistance infections biomarkers is discussed here for the first time. A standard LPG working in transmission mode was first transformed in a more practical probe able to work in reflection configuration; successively it was made sensitive in aqueous environment by the deposition of an HRI thin overlay of atactic polystyrene. The surface of the aPS-coated RT-LPG transducer was then coated with a further ultra-thin layer of PMMA-co-MA in order to provide the necessary conditions for a correct bio-functionalization. AmpC β -lactamases produced by resistant bacteria were used as infection biomarkers and a known β -lactamase inhibitor (i.e. 3-amino-phenylboronic acid) as the ligand and able to fish them out. 3-APBA is indeed able to bind to AmpC BL with micro molar affinity and presents a favorable orientation within protein binding site, likely allowing the anchoring reaction and the probe functionalization. A deep experimental campaign was carried out which demonstrated the capability of the pro-

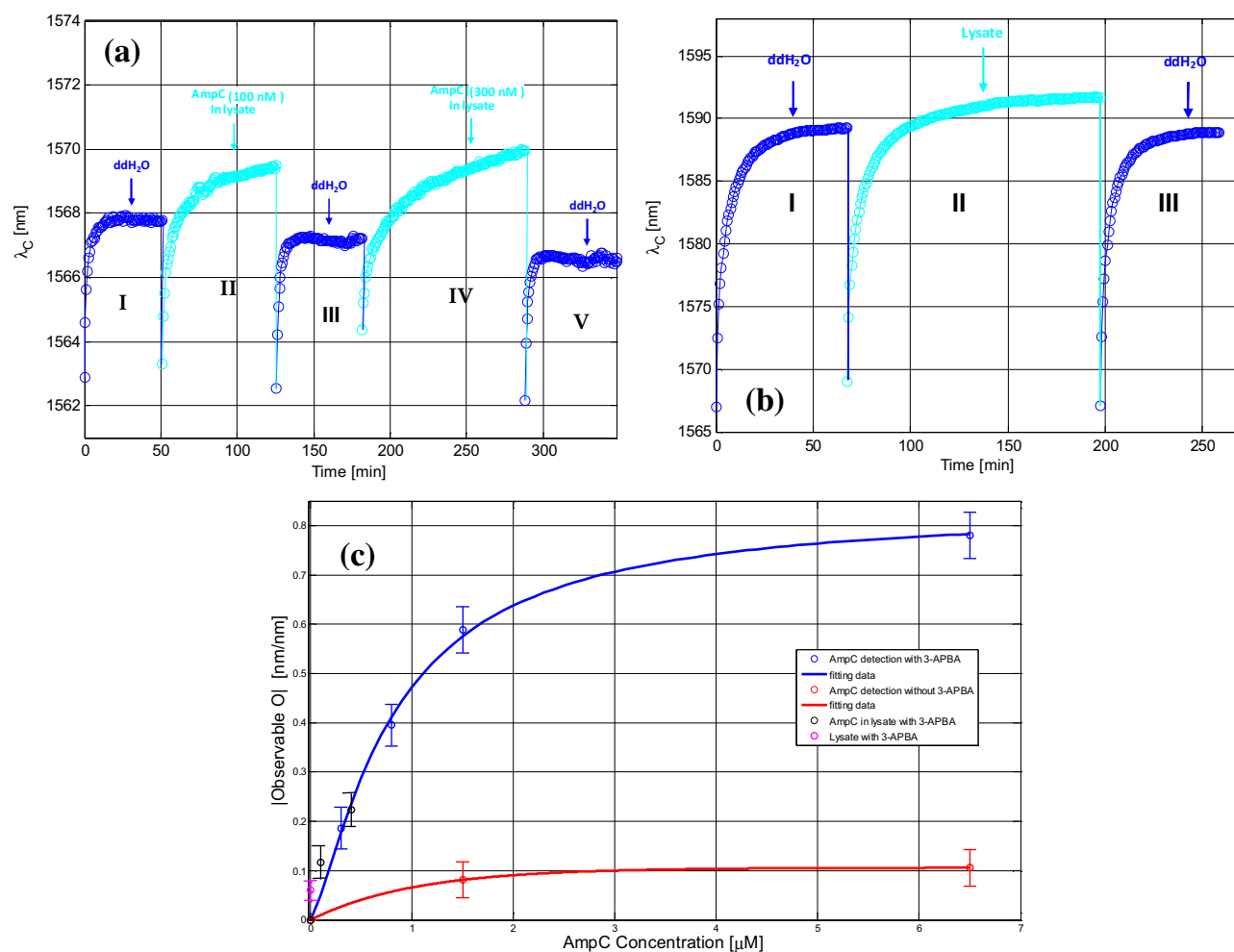


Fig. 6. RT-LPG sensorgram obtained during (a) the binding experiments in lysate solution sample and (b) a control test in lysate solution without the AmpC BL; (c) characteristic curves ($[O]$ vs. AmpC concentration) of the RT-LPG device obtained by the negative control tests using not-functionalized probes (red curve), the binding experiments with purified protein sample (blue curve) and with more complex lysate samples (black circles). The observable value obtained with a lysate solution without AmpC BL has also been inserted (magenta circle). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

posed optical biosensor to detect the presence of purified AmpC BL within phosphate buffer solutions, with concentration in the range 300 nM–5.0 μ M. In order to verify the efficacy of our biological system, we also explored the β -lactamase a-specific binding occurring onto the not functionalized sensor surface (i.e. without 3-APBA overlay) during some negative control (blank) tests. Obtained results evidence a response of the (not-biofunctionalized) RT-LPG in the negative control case more than seven time lower in respect to that provided by the biofunctionalized device, therefore confirming the high affinity of the chosen bioreceptors and, overall, the efficacy of the entire biological system. Finally, the proposed platform was also tested to detect the presence of β -lactamases in lysate complex matrixes. Some preliminary binding experiments with AmpC contained in lysate samples (with concentration of 100 nM and 300 nM) confirmed the correct operation of the optical device. Overall, results here provided clearly demonstrate the capability of the proposed optical biosensor to correctly perform the label-free detection of AmpC BLs (both in PBS or lysate sample) with a limit of detection of a few tens of nM opening new ways to study and investigate antibiotics resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.snb.2016.02.086>.

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Biographies

Giuseppe Quero was born on August 25, 1981 in Taranto, Italy. He is a postdoctoral researcher at the Optoelectronic Division of University of Sannio. He received the Ph.D. Degree in "Information Engineering" with a thesis with title "Lab-on-Fiber Technology for Sensing Applications" from the University of Sannio, in July 2012. In October 2010 and February 2015, he is the winner of the "EWOF'S 2010 Student Paper Award" in the Fourth European Workshop in Optical Fibre Sensors and of the "Best poster award" in the XVIII AISEM conference. His research interest is in the area of the chemical and biological sensing applications.

Simona Zuppolini received her degree (2009) and Ph.D. (2013) in Chemical Sciences (Organic Chemistry branch) at the University of Naples "Federico II". She is currently a post-doctoral research collaborator at the Institute of Polymers, Composites and Biomaterials (IPCB) of National Research Center (CNR) in Portici (Naples). Her studies

are mainly focused on the research and development of biosensors and sensors surface materials. Her scientific activity is attested by 6 publications.

Marco Consales is Assistant Professor at the Engineering Department of the University of Sannio, in Benevento. Since 2004 he has been carrying out an intensive research activity in the area of optoelectronics and photonics for chemical and biological sensing applications, with particular interest in the field of Lab-on-fiber devices, as demonstrated by 2 national patents, 10 invited book chapters, more than 35 publications on scientific journals and more than 60 publications on conference proceedings.

He is Editor-in-Chief of the “Nanomaterials and Nanotechnology” Journal (“Nanophotonics” topic), and member of the Editorial Board of Journal of Sensors, Journal of Nanotechnology in Diagnosis and Treatment and Advances in Optics.

Laura Diodato received her graduation in Chemistry in 2013 at the university of Federico II in Naples. She is working at the Institute IPCB of CNR in Naples. Her studies are mainly focused on the biofunctionalization of polymers on a fiber optic.

Patrizio Vaiano was born in Castellammare di Stabia, Naples, Italy, in 1988. He received his Master's Degree in Electronic Engineering from the University of Naples “Federico II” in December 2013. Since June 2014 he is a Ph.D. Student in Information Technologies for Engineering at the University of Sannio, where he collaborates with the Optoelectronic Group of Engineering Department. His research activity is focused on the development of optical fibers based devices for chemical and biochemical sensing applications.

Alberto Venturelli received her Ph.D. in Medicinal Chemistry from the University of Modena and Reggio Emilia (2004). More than 15 years of pharmaceutical research experience comprising specific background in Organic Synthetic Chemistry (*University of Modena and Reggio Emilia* and *School of Chemistry of the University of Exeter, UK*), Medicinal Chemistry (*University of Modena and Reggio Emilia* and *Imperial College of London, UK*) and administration of research projects committed to results (he is coordinator of the 7FP EU project “OPTObacteria”). He is currently CEO at TYDOCK PHARMA.

Matteo Santucci received his specialization degree in Pharmaceutical Biotechnology in 2012 at the University of Modena and Reggio Emilia. He is a Ph.D. student in Drug Science at the third year at the University of Modena and Reggio Emilia. He is working in the field of protein purification and biophysical methods for protein-ligand interaction characterization. He has published 3 papers

Francesca Spyraakis received her Ph.D. in Biochemistry and Molecular Biology in 2007 at the University of Parma. She is currently a post-doctoral researcher at the University of Modena and Reggio Emilia. Her studies are mainly focused on the development and application of *in silico* methodologies for the identification of protein ligands, and on the computational analysis of protein structural and dynamical properties. Her scientific activity is attested by more than 60 publications.

Maria Paola Costi has completed her Ph.D. Medicinal chemistry at the age of 29 years from the University of Modena and Reggio Emilia and postdoctoral studies from University of California San Francisco (UCSF). She is professor of Medicinal Chemistry at the Department of Life Science at Unimore. She has published more than 85 papers in international journals, many patents and serving as an editorial board member of a few journals. Board member of translational science in oncology group of the MITO network and WG leader of drug discovery and development of EUTROC. Coordinator of a number of collaborative European projects.

Michele Giordano, chemical engineer, received his Ph.D. in Materials Engineering in 1995 at the University of Napoli “Federico II”. Senior researcher at Institute for Polymers Composites and Biomaterials CNR in Portici since 2005. He is currently Visiting Professor at the University of Strasbourg and Associate Researcher at CERN in Geneva. His research activities are within the area of engineering and materials science. Research focuses are nano and macro composite materials, mainly polymer based, including multiscale design and processing of multifunctional composite materials, and materials engineering for sensing and optoelectronic applications. He is author of more than 130 peer reviewed (ISI indexed) scientific papers. H index 38 on Dec 2015.

Anna Borriello has a Ph.D. in Chemistry at University of Naples “Federico II” (1996) and is currently a research scientist at the Institute for Polymers, Composites and Biomaterials (IPCB) of the Italian National Research Council (CNR). Her research interests are Design, synthesis, testing, and engineering of smart systems. Current emphases include: (Bio) Sensors, Electrically Conductive Polymers, Nanofillers in polymer composite, polymer electrolyte membranes, Bio-Inspired Materials. He is author of more than 60 between peer reviewed scientific papers and industrial patent.

Antonello Cutolo was born on November 7, 1955. He received the Laurea degree in Electronic Engineering in 1978 from University of Naples “Federico II”, Italy. After serving the Italian Air Force, he was with the Applied Mathematics and Physics Laboratory, Technical University of Denmark (during 1980–1981). From 1981 to 1983, he was with Adone Storage Ring of Frascati to build a free electron laser (FEL). From 1983 to 1986, he was with the High Energy Physics Laboratory, Stanford, CA, where he was in charge of the broadband optical resonators, diagnostic equipment, and harmonic generator for the infrared FEL. In 1987, he was with Duke University. In 1986, he was a Professor of quantum electronics at the University of Naples, Naples, where he became a Professor of optoelectronics in 1993. Since 1999 he has been a Full Professor at University of Sannio in Benevento, Italy. His research interests include fiber-optic sensors, optoelectronic modulators and switching, laser beam diagnostic, and nonlinear optical devices. He has been scientific responsible of numerous research projects. Since 2002 he has been leading the Optoelectronic Pole of the research consortium “Centro Regionale Information Communication Technology–CeRICT”. He has authored several books, more than 20 patents and more than 300 publications on national and international scientific journals and conference proceedings. He has co-founded two high tech companies, Optosmart srl and OptoAdvance srl (spin off companies of the University of Sannio) and with core business on the development of fiber optic sensor arrays for chemical and structural monitoring. Furthermore, he has founded the research Consortium TOP-IN, whose main aim is the exploitation of advanced optoelectronics technologies for industrial applications.

Andrea Cusano received Laurea degree cum Laude in Electronic and Telecommunication Engineering and the Ph.D. in Optoelectronics from University of Naples (Federico II, Italy). He is currently Associate Professor at the Engineering Department of University of Sannio where he and Prof. Cutolo co-founded the Optoelectronic Group since 2002. He has published over 120 journal articles and 150 refereed conference communications related to the development of new fiber optic and photonic sensors for physical, chemical and biological sensing applications. In this context, he has also co-authored more than 10 chapters published in international books and invited papers in many prestigious international journals. He has been co-editor of several Special Issues related to photonics technologies for sensing applications published in prestigious International journals as the case of IEEE Sensors, IEEE Photonics, IEEE/OSA Journal of Lightwave Technology. He is Co-Editor of 7 international books: “Fiber Bragg Grating Sensors: Recent Advancements, Industrial Applications and Market Exploitation” Bentham e-book 2010, “Photonic Bandgap Structures. Novel Technological Platforms for Physical, Chemical and Biological Sensing” Bentham 2012, “Selected Topics on Metamaterials and Photonic Crystals”, World Scientific Pub. 2011, “Optochemical Nanosensors”, Taylor & Francis 2013, “Lab on Fiber Technology industrial companies (Ansaldo STS, Alenia WASS, Optosmart and MdTEch) and more than 10 national patents. He actually serves as Editor-in-Chief of the Journal of Optics and Laser Technology (Elsevier) and as Associate Editor for the Journal of Photonic Sensors (Springer). In 2015m, he was selected as Associate Editor of IEEE/OSA Journal of Lightwave Technology. He is a member of the technical committee of several international conferences such as IEEE Sensors, ICST, EWSHM, EWOFS. Prof. Cusano is cofounder of two spin-off companies ‘OptoSmart S.r.l.’ (2005) and “Optoadvance” (2011) and has been consultant for major companies of the Finmeccanica group such as Ansaldo STS and Alenia WASS. He has also collaborations with CERN in Geneva where he is working on the development of innovative sensors for high energy physics applications. He received many international recognitions and awards for efforts in the development of innovative optical sensing systems and has been Principal Investigator of many national and international projects. Finally, in 2015, he was nominated Technical director of a public-private aggregation (Top-in Optoelectronic Technologies for Industry) including several national research centers, 3 big companies from Finmeccanica and 11 SMEs.