Bacteriophage Adhesin-Coated Long-Period Grating-Based Sensor: Bacteria Detection Specificity

Marcin Koba, Mateusz Śmietana, Ewa Brzozowska, Sabina Górska, Monika Janik,
Predrag Mikulic, Andrea Cusano, and Wojtek J. Bock, *Fellow, IEEE*

Abstract— **In this work we study a label-free detection specificity of an optical fiber long-period grating (LPG) biosensor working near the dispersion turning point of higher order cladding modes. The LPG sensor is functionalized with bacteriophage adhesin and tested with specific and non-specific bacteria dry weights. It is shown that that such a biosensor is able to selectively bind to specific bacterial strains. In the experiments bacteria dry weights of *E. coli* B were used as a positive test and *E. coli* K12 and *Salmonella enterica* as negative tests. The resonance wavelength shift induced by *E. coli* B reaches over 90 nm, while for *E. coli* K12 and *Salmonella enterica* approximately 40 and 20 nm, respectively. Additionally, whole-cell-ELISA tests were made to confirm LPG-based biosensor outcomes, and numerical simulations were conducted to estimate bio-layer impact on sensor's performance.**

Index Terms— **optical fiber sensors, long-period gratings, label-free sensing, surface functionalization, biosensors, bacteriophage adhesin, bacteria detection.**

I. INTRODUCTION

IN recent years optical fiber sensing community has become more and more interested in bio-sensing [1-4]. One of the main trends is to apply label-free methods, where kinetics of reactions taking place on the sensor's surface can be monitored using devices sensitive to small changes of external refractive index (RI) or an increase of thickness of the aggregating bio-film [4]. Among many other label-free optical fiber sensors, an interesting platform is the one based on the long-period gratings (LPG). The LPG is a periodic modulation of refractive index within a core of an optical fiber [5]. The modulation induces coupling of the core mode with a series of cladding modes and results in resonance peaks appearing in the LPG transmission spectrum that correspond to the coupling effect. Thanks to interactions between the cladding modes and the external medium, the LPGs are sensitive to variations in the surrounding RI or formation of a thin, high-RI overlay on its surface. The sensitivity of an LPG is typically defined as a resonance wavelength shift per RI unit (RIU) of the external medium. It has been further shown that the highest sensitivity is at the dispersion turning point (DTP), where two resonances separate from each other with an increase of RI or a formation of the high-RI overlay [6]. There has been a number of biosensors shown, where the LPGs were applied for nucleic acids [2,7], bacteria [3] or bacteria parts [8], protein [4,9], or antigen [10] detection. Each of them requires functionalization of the LPG surface with the bio-molecules showing affinity to the measured molecules. In our previous work we have shown a highly sensitive sensor based on the LPG working near the DTP coated with bacteriophages [3] or their adhesins [8,11] for bacteria or bacterial endotoxin detection, respectively. The binding of bacteria to bacteriophages or their adhesins can be very specific, thus it is possible to implement both sensitive and selective sensing.

This work is devoted toward studies on specificity of the highly sensitive label-free biosensor based on LPGs. An analysis considers bacteriophage T4 adhesin, which is specific to the *E. coli* B bacteria strain. The advantage of biosensor functionalization with adhesins is the possibility for low-cost biosensitive molecule exchange and for sensor's surface regeneration [11]. The LPG-based sensing structure allows for monitoring of the binding phenomenon in real time and with high accuracy. In the presented study we investigate *Salmonella enterica*, *E. coli* K12 as non-specific, and *E. coli* B as highly specific strains [13]. The outcomes are crosschecked using a standard enzyme-linked immunosorbent assay (ELISA) test, and supported by numerical simulations, where bio-overlay was assumed to be a high-RI film on the fiber cladding which increased its thickness.
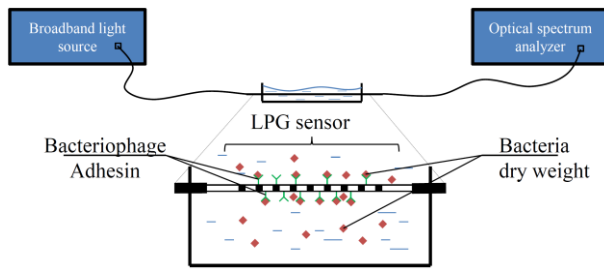
Fig. 1. A schematic representation of the measurement setup.

II. Experimental Details

The LPGs were written in hydrogen-loaded standard Corning SMF28 fiber using Pulse Master 840 high-power KrF excimer laser (λ = 248 nm). During the writing process of the grating the pulse repetition rate was set to 100 Hz, pulse duration to 12 ns, and peak pulse energy to approx. 10 mJ. The fiber was exposed to UV light through an amplitude chromium mask (Λ = 226.8 µm) for about 7 minutes. In order to stabilize the optical properties of the LPGs after the exposure, the gratings were annealed at 150 °C for approx. 4 hours to release excess hydrogen. Furthermore, to enhance the RI sensitivity towards DTP, the gratings were etched in HF acid for about 3 hours. The etching procedure resulted in the sensor's operation in the vicinity of the dual resonance regime. For the presented experiment three sensors were prepared, each of them being 4 cm long, and before functionalization the measured sensitivity of the structures in the considered spectral range was approx. 3000 nm/RIU [11].

After preparation of the LPG-based structures, their surface was bio-functionalized using N-(5-amino-1-carboxypentyl) iminodiacetic acid (AB-NTA), $NiCl_2$ reagents and recombinant phage protein (2.5 µg/ml) with a histidine-tag to obtain a well-defined orientation of the adhesin on the fiber's surface. This procedure comprised of chemical preparation of the fiber's surface and then of adhesin binding. At first, the fiber was immersed in the 0.01 % acetic acid and 2 % (3-Glycidyloxypropyl) trimethoxysilane solution at temperature T = 90 °C for about 3 hours, then it was left for about 16 hours at T = 20 °C in the 0.01M $NaHCO_3$ buffer (pH = 10) with 20 mM N-(5-amino-1-carboxypentyl) iminodiacetic acid solution. Next, the fiber was bathed in 10 mM $NiCl_2$ and 5 mM Glycine solution for about 2 hours at room temperature. Finally, after washing in $NaHCO_3$ buffer the fiber sensor was immersed in adhesin solution for about 1 hour [12]. *E. coli* B bacteriophage g37 adhesin was used as a bacteria specific layer.

The optical transmission of the LPG in the range of λ = 1550-1750 nm was monitored using a NKT Photonics SuperK COMPACT supercontinuum white light laser source and Yokogawa AQ6375 optical spectrum analyzer. A schematic drawing of the setup is shown in Figure 1. The ambient temperature (T) during the measurements was set to 22 °C and monitored with a HP 34970A Data Acquisition Unit equipped with a thermocouple. The tension of the LPG was held constant throughout the experiment.

Numerical analysis of the LPG response to growth of bio-layer on its surface was performed using OptiGrating by Optiwave. The implemented model of the fiber and LPG assumes core and cladding radii of 3.98 and 52.685 µm, respectively. The numerically obtained spectrum was tuned to results of the measurements for a LPG with no overlay. The RI of the outer medium (PBS) was set to n = 1.334 RIU at λ = 1550 nm, and RI of the bio-overlays was set to n = 1.45 [14,15] and 1.50 RIU [16] at λ=1550 nm. For PBS as well as for bio-solution the permittivity curves were assumed to be dispersive. Simulations were made for different bio-layer thickness up to d=100 nm.

An ELISA test was performed using a 96-well plate (Maxisorp, Nunc) that was covered with bacteria suspended in PBS (5 µg per well) and centrifuged at 600 x g (Heraeus megafuge 16R) at 4 °C for 20 minutes. 0.1% glutaraldehyde was added (250 µl/well) and incubated at room temperature (RT) for 30 minutes. Next, the blocking step was performed using 0.1% BSA-PBS solution supplemented with 0.1 M glycine (2 hours at RT). The plate was washed with PBS containing 0.05% Tween (PBS-T) and again, BSA-PBS solution was added (250 µl/well) and incubated at 4°C overnight. After washing, the adhesin solution was added (0.25 µg/well) and incubated at 37°C for 1 hour. Next, the plate was washed and rabbit polyclonal anti-adhesin antibodies (1:4000) were added and incubated at 37 °C for 1 hour. Finally, anti-rabbit Immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) was added (1:10000) and incubated at 37 °C for 45 minutes. 3, 3', 5.5'-tetramethylbenzidine (TMB) substrate was added to yield a color reaction measured at λ = 490 nm using the Biotec microplate reader.

III. Results and Discussion

A number of factors can shift the resonance wavelengths ($\lambda_{res}{}^m$) of the LPG [6,17]. The main relation describing wavelength-dependent coupling from the guided core mode (LP_{01}) to the mth cladding mode (LP_{0m}) is shown in (1), where ($n_{eff}{}^{01}$) is the effective refractive index of the propagating core mode, ($n_{eff}{}^{0m}$) is the effective refractive index of the mth cladding mode and (Λ) is the period of the LPG.

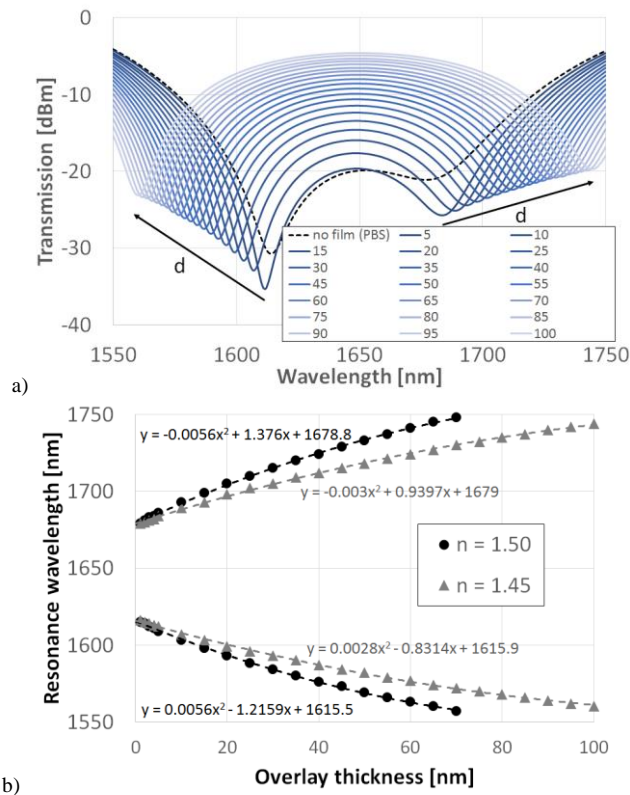$$\lambda_{res}^m = \left(n_{eff}^{01} - n_{eff}^{0m} \right)\Lambda \quad (1)$$

Fig. 2. Simulation of the LPG response to overlay growth up to d = 100 nm assuming overlay n = 1.45 and 1.50 RIU, where (a) shows evolution of transmission spectrum for n = 1.45 RIU and (b) corresponding resonance wavelength shifts.
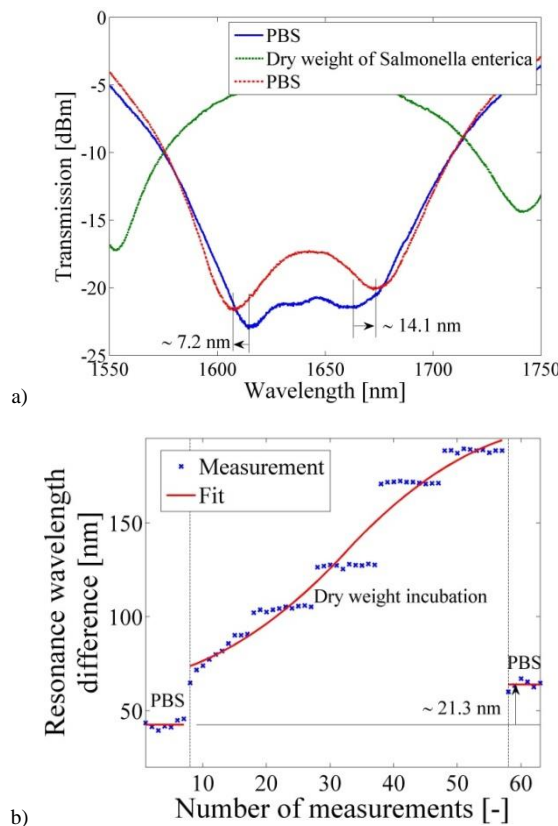

Fig. 3. a) An example of the measurement spectra, and b) measured resonance wavelength difference before, during and after *Salmonella enterica* application.
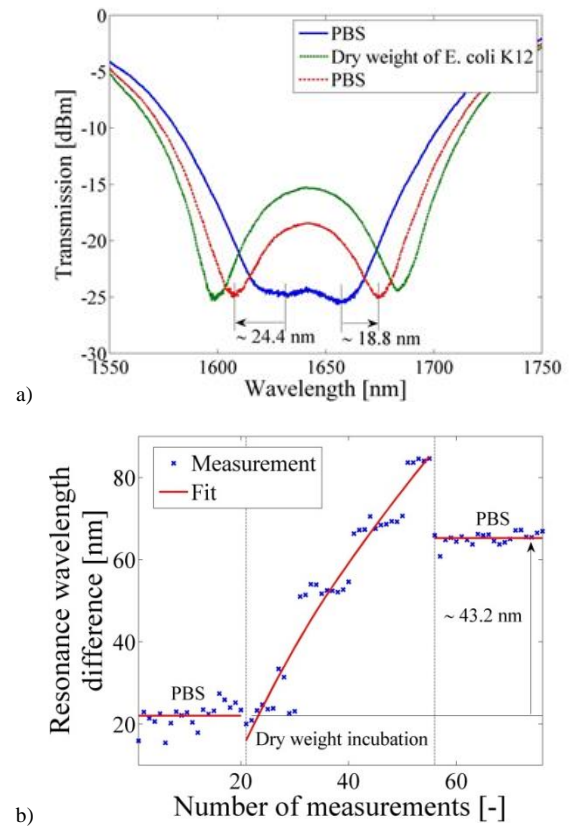

Fig. 4. a) An example of the measurement spectra, and b) measured resonance wavelength difference before, during and after *E. coli* K12 application.

A resonance wavelength shift can be alternated by variation of either the period of the grating or the effective indices of the modes. As far as the influence of ambient RI is concerned, only n_{eff}^{0m} can be influenced by the external medium, which in the investigated case can be a bio-overlay. According to (1) when the external RI increases or when the overlay is formed on the LPG surface, the λ_{res}^{m} shifts towards shorter wavelengths (experiencing a blue shift). When the LPG works close to the DTP the resonances increase their spectral separation with an increase of the external RI or formation of the bio-overlay. In Fig. 2, results of numerical simulations are shown to depict the effect of the increase in thickness of the overlay for two different values of its refractive index (*n*). Values typically assumed in the literature for a bio-overlay's *n* range from 1.45 to 1.5 RIU. It can be seen that the resonance wavelength values depend strongly on both thickness (*d*) and *n* of the overlay. Increase of *d* or *n* is followed by increase of separation of the resonances. Depending on *d*, the sensitivity to bio-overlay formation changes from approx. 1.25 and 1.75 nm/nm in the range 0-5 nm of bio-overlay thickness away from the LPG surface to 0.6 nm/nm at 70 nm away from the LPG surface for *n* = 1.45 and 1.5 RIU, respectively. The result suggests that the LPG in the label-free sensing configuration is more sensitive to small bio-material adhesion and in this case the optical properties of the bio-overlay are more profound than for larger bio-molecules or bio-compounds such as bacteriophage-bacteria, where the sensitivity is almost
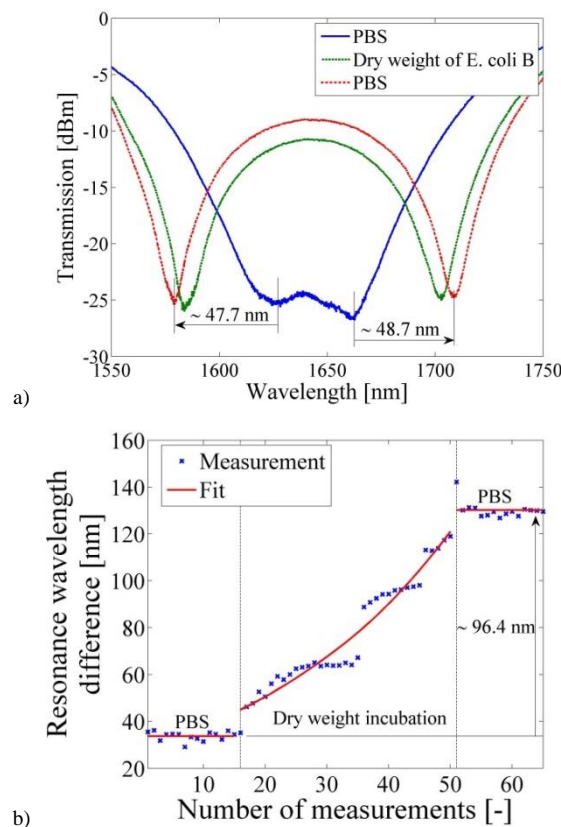
Fig. 5. a) An example of the measurement spectra, and b) measured resonance wavelength difference before, during and after *E. coli* B application.

Fig. 6. The results of the whole-cells-ELISA showing the adhesin gp37 binding to the bacterial cells.

constant. However, when *n* of bio-overlay is higher, smaller bio-molecules induce a higher spectral shift than for the binding effect of those with lower *n*. This dependence may be important in the case of non-specific binding of smaller bio-material. It must be also emphasized here that the simulated bio-overlay is assumed to be constant in *n* and uniform in thickness. These conditions are difficult to meet in reality, where bio-molecules usually form a randomly distributed structure. Their higher density at the surface may correspond to higher *n* of the overlay, thus from the numerical point of view the bio-overlay is treated in terms of averaged quantities therefore giving a qualitative insight into the experiment.

For the purpose of the experiment we used three LPG-based structures, each showing similar and high RI sensitivity of approx. 3000 nm/RIU. The LPG-based sensors, i.e., LPGs with a functionalized sensing region, after attachment of bacteriophage adhesin were washed several times in phosphate buffered saline (PBS). Then, the sensors were immersed in bacterial dry weights for about 1.5 h, and finally washed again in PBS. The concentrations of all bacteria solutions were 10^7 cfu/mL. Examples of the spectra obtained at each stage of the experiment are shown in Figures 3a, 4a, and 5a for *Salmonella enterica*, *E. coli* K12, and *E. coli* B, respectively.

The measured resonance wavelength change for these bacterial dry weights are depicted in Figures 3b, 4b, and 5b, respectively. The resonance wavelength change is observed in terms of the split of two double resonance minima. In the
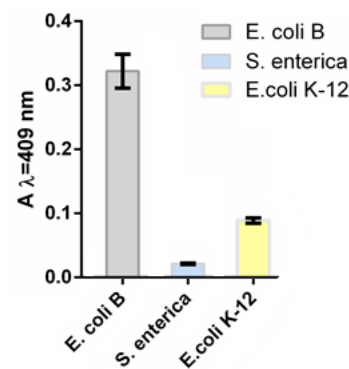
figures, blue crosses represent the values of resonance wavelength from the spectrum measurement, while the straight lines show the mean value between the points (in terms of mean square error). In Figures 3 to 5 we compare the shift of the resonance wavelength for PBS solution before and after introduction of the specific bacteria dry weights. The differences of resonant wavelengths for consecutive samples are summarized in Table I.

The results in Figures 3 and 4 show resonance shifts caused by the non-specific bacterial dry weights. These shifts are 21.3 and 43.2 nm for *Salmonella enterica* and *E. coli* K12, respectively. During the bacteria incubation process the wavelength shifts grow significantly, which is in general caused by bacteria aggregation on the fiber surface and to a relatively smaller extent by increase of their averaged *n*. The final PBS washing process greatly reduces the shift just by washing out the physically attached bacteria, especially in the case of *Salmonella enterica*. However, the overall results show changed values of resonance wavelengths; this indicates some non-specific bonding, which most probably is induced by accumulation of bacteria dry weight on the fiber surface and mixing with adhesin. The observed greater shift for *E. coli* K12 indicates some specificity in terms of biological affinity within the same species with *E. coli* B. This hypothesis was further confirmed in the whole-cell-ELISA test, Figure 6, [18]. During this test the bacteria were immobilized on a plate surface and then incubated with the adhesin. To detect the adhesin-bacteria cell interaction, we used rabbit polyclonal anti-adhesin antibodies first, and then anti rabbit IgG-HRP antibodies. The absorbance at λ = 409 nm (A at 409 nm) of a blank well (reagents without bacteria) was subtracted from the reading of each test.

In Figure 6, it is shown that the highest reactivity is observed for the adhesin with *E. coli* B bacteria – the positive control, and some reactivity is observed for *E. coli* K12, but almost none for *Salmonella enterica*.

TABLE I
RESONANCE WAVELENGTH SHIFT OBTAINED AS A RESULT OF EACH TEST.

Test No.	Bio-molecule dry weight	Wavelength shift [nm]
1	*Salmonella enterica*	21.3
2	*E. coli* K12	43.2
3	*E. coli* B	96.4

The specificity of the bacteria binding by the phage adhesin depends on the composition and structural peculiarities of receptors on the bacterial surface [19]. *E coli* B appears to contain glucose, heptose and 3-deoxy-D-mannooctulosonic acid (KDO) in molar ratio 2:3:3. Probably the two glucoses and spatial configuration around them are necessary for the adhesin and bacteria specific interaction [20]. In many cases, also bacterial membrane proteins are required for adhesin binding [19].

Finally, although the results for *E. coli* K12 and *Salmonella enterica* show some binding, the LPG-sensor as well as ELISA test indicate that the outcome for specific *E. coli* B is significantly greater (Table I, Figure 6). In this last case, after dry weight attachment the PBS washing only removes residual bacteria, which were not able to attach to the fiber surface functionalized with adhesin. The absence of any signal change after final PBS washing indicates the sensor's saturation – i.e. a certain thickness of bio-overlay is attained and no new molecules are being attached nor detached to/from surface of the fiber.

IV. CONCLUSIONS

In this work, we have shown the study of LPG-based structure as a promising platform for specific bio-sensing of bacteria dry weight. We demonstrated that the LPG-based biosensor is capable of specific bacteria strain detection (here *E. coli* B), giving strong and well distinguishable signal. We used phage adhesin, which seems to be a good recognition element for biosensors, and even better than a whole bacteriophage. The LPG-based sensor with adhesin coating is stable, free of bacterial contamination, and easy to use. The presented study of the biosensor was confirmed using a well-established biological test (ELISA), which also indicated the same non-specificities. We also preformed numerical simulations to give additional qualitative insight into sensor's behavior with a bio-overlay, showing the high importance of bio-overlay parameters.

REFERENCES

[1] Tian, Y., Wang, W., Wu, N., Zou, X. and Wang, X., "Tapered optical fiber sensor for label-free detection of biomolecules," *Sensors*, vol. 11, no. 4, pp. 3780-3790, Mar. 2011.

[2] Chen, X., Zhang, L., Zhou, K., Davies, E., Sugden, K., Bennion, I., Hughes, M. and Hine, A., "Real-time detection of DNA interactions with long-period fiber-grating-based biosensor," *Opt. Lett.*, vol. 32, no. 17, pp. 2541-2543, 2007.

[3] Smietana, M., Bock, W.J., Mikulic, P., Ng, A., Chinnappan, R. and Zourob M., "Detection of bacteria using bacteriophages as recognition elements immobilized on long-period fiber gratings," *Opt. Express*, vol. 19, no. 9, pp. 7971-7978, 2011.

[4] Pilla, P., Foglia Manzillo, P., Malachovska, V., Buosciolo, A., Campopiano, S., Cutolo, A., Ambrosio, L., Giordano, M. and Cusano, A., "Long period grating working in transition mode as promising technological platform for label-free biosensing," *Opt. Express*, vol. 17, no. 22, pp. 20039-20050, 2009.

[5] Vengsarkar, A. M., Lemaire, P. J., Judkins, J. B., Bhatia, V., Erdogan, T. and Sipe, J. E., "Long-period fiber gratings as band-rejection filters," *J. Lightwave Technol.*, vol. 14, no. 1, pp. 58-65, Jan. 1996.

[6] Shu, X., Zhang, L. and Bennion, I., "Sensitivity characteristics of long-period fiber gratings," *J. Lightwave Technol.*, vol. 20, no. 2, pp. 255-266, Feb. 2002.

[7] Jang, H. S., Park, K. N., Kim, J. P., Sim, S. J., Kwon, O. J., Han, Y.-G. and Lee, K. S., "Sensitive DNA biosensor based on a long-period grating formed on the side-polished fiber surface," *Opt. Express*, vol. 17, no. 5, pp. 3855-3860, Feb. 2009.

[8] Brzozowska, E, Śmietana, M., Koba, M., Górska, S., Pawlik, K., Gamian, A., and Bock, W.J., "Recognition of bacterial lipopolysaccharide using bacteriophage-adhesin-coated long-period gratings," *Biosens. Bioelectron.*, vol. 67, pp. 93-99, May 2015.

[9] Wang, Z., Heflin, J. R., Van Cott, K., Stolen, R. H., Ramachandran, S. and Ghalmi, S., "Biosensors employing ionic self-assembled multilayers adsorbed on long-period fiber gratings," *Sens. Actuators B Chem.*, vol. 139, no. 2, pp. 618-623, 2009.

[10] Kim, D. W., Zhang, Y., Cooper, K. L. and Wang, A., "Fibre-optic interferometric immuno-sensor using long period grating," *Electron. Lett.*, vol. 42, no. 6, pp. 324-325, Mar. 2006.

[11] Koba, M., Śmietana, M., Brzozowska, E., Górska, S., Mikulic, P., and Bock, W.J., "Reusable Bacteriophage Adhesin-coated Long-period Grating Sensor for Bacterial Lipopolysaccharide Recognition," *J. Lightwave Technol.*, vol. 33, no. 12, pp. 2518-2523, Jun. 2015.

[12] Smietana, M., Koba, M., Brzozowska, E., Krogulski, K, Nakonieczny, J., Wachnicki, L., Mikulic, P., Godlewski, M., and Bock, W.J. "Label-free sensitivity of long-period gratings enhanced by atomic layer deposited TiO2 nano-overlays," *Opt. Express*, vol. 23, no. 7, pp. 8441-8453, 2015.

[13] M. Koba, M. Śmietana, E. Brzozowska, S. Górska, P. Mikulica, A. Cusano, and W. J. Bock, "Detection specificity studies of bacteriophage adhesin-coated long-period grating-based biosensor," in Proc. SPIE, Vol. 9634, 963426, 24rd International Conference on Optical Fiber Sensors, Curitiba, 2015.

[14] A. Tsargorodskaya, A. V. Nabok, and V. Ray, "Ellipsometric study of the adsorption of bovine serum albumin into porous silicon," *Nanotechnology*, vol. 15, no. 5, pp. 703−709, Mar. 2004.

[15] A. Ricciardi, M. Consales, G. Quero, A. Crescitelli, E. Esposito, and A. Cusano, "Versatile Optical Fiber Nanoprobes: From Plasmonic Biosensors to Polarization-Sensitive Devices," *ACS Photonics*, vol. 1, no. 1, pp. 69-78, 2014.

[16] A.J. Steckl, H. Spaeth, H. You, E. Gomez, and J. Grote, "DNA as an Optical Material," Optics and Photonics News 22(7), 34-39 (2011).

[17] M. Smietana, W.J. Bock, P. Mikulic, and J. Chen, "Tuned Pressure Sensitivity of Dual Resonant Long-Period Gratings Written in Boron Co-doped Optical Fiber,", *J. Lightwave. Technol.*, vol. 30, no. 8, pp. 1080-1084, Apr. 2012.

[18] E. Brzozowska, M. Koba, M. Śmietana, S. Górska, M. Janik, A. Gamian, and W. J. Bock, "Label-free Gram-negative bacteria detection using bacteriophage-adhesin-coated long-period gratings," submitted for publication.

[19] D. V. Rakhuba, E. I. Kolomiets, E. Szwajcer Dey, and G. I. Novik, "Bacteriophage receptors, mechanisms of phage adsorption and penetration into a host cell," *Pol. J. Microbiol.*, vol. 59, no. 3, pp. 145-155, 2010.

[20] P. Prehm, B. Jann, K. Jann, G. Schmidt, and S. Stirm, "Cell-wall lipopolysaccharides of ampicillin-resistant mutants of Escherichia coli K-12," *Eur. J. Biochem.*, vol. 66, no. 2, pp. 369-377, 1976.

Marcin Koba received the M.Sc. degree in optoelectronics in 2006 and Ph.D. degree in electronics in 2011, both from the Warsaw University of Technology, Warsaw, Poland.

His current interests include laser physics, solid state physics, photonics, fiber optic sensing structures, and metrology.

Mateusz Śmietana received the B.Sc., M.Sc., Ph.D. (with distinction), and D.Sc. degrees in electronics from the Warsaw University of Technology (WUT), Poland, in 2000, 2002, 2007, and 2014, respectively.

Since December 2015, he has been an Associate Professor in the Institute of Microelectronics and Optoelectronics, WUT. He was a postdoctoral fellow at Virginia Tech and Université du Québec en Outaouais (UQO), Canada. He received

scholarships from the European Social Fund through the WUT Development Programme (Poland) and from the FQRNT by the Ministère de l'Éducation, du Loisir et du Sports du Québec (Canada). He has authored and co-authored more than 80 scientific papers. His fields of interest are fiber-optic sensors and thin films.

Ewa Brzozowska received a M.Sc. degree in biotechnology in 2002 from the University of Wroclaw, Poland and Ph.D. degree in biology in 2009 from the Institute of Immunology and Experimental Therapy Polish Academy of Science (IIET, PAS), Wroclaw, Poland. Since 2014, she has been an Assistant Professor in the IIET.
Her research interest include bacteriophages and bacteriophage proteins as antibacterial agents and as tools for bacteria detection, bacteriophage- bacteria interaction mechanisms and antibacterial proteins structure determination.

Sabina Górska received a M.Sc. degree in biotechnology in 2006 from the Wroclaw University of Technology, Wroclaw, Poland and Ph.D. degree in biology in 2011 from the Institute of Immunology and Experimental Therapy Polish Academy of Science (IIET, PAS), Wroclaw, Poland. Since 2013, she has been an Assistant Professor in the IIET.
Her research interest include polysaccharide antigens structure determination**,** understanding their biological function as well as the understanding of the bacteria-host and bacteria-bacteria cross-talk.

Monika Janik received her M.Sc. degree in biotechnology in 2015 from the Institute of Immunology and Experimental Therapy Polish Academy of Science (IIET, PAS), Wroclaw, Poland.
She is currently working toward her PhD degree at the Université du Québec en Outaouais (UQO), Canada.

Predrag Mikulic received an Associate of Science Degree as a Telecommunication Engineering Technologist from the University of Sarajevo, Yugoslavia in 1989.
He has more than 20 years of professional high-tech experience in the industry. His major interests are thin-film deposition, excimer lasers, photonic devices, fiber sensors, and procedures for splicing dissimilar fibers including photonic crystal fibers.

Andrea Cusano …

Wojtek J. Bock (M'85-SM'90-F'03) received the M.Sc. degree in electrical engineering and the Ph.D. degree in solid state physics from Warsaw University of Technology, Poland, in 1971 and 1980, respectively.
Since 1989, he has been a Full Professor of Electrical Engineering at the Université du Québec en Outaouais (UQO), Canada. Since 2003, he has held a Canada Research Chair Tier-I in Photonics and has been Director of the Photonics Research Center at UQO. His research interests include fiber-optic sensors and devices, multisensor systems, and systems for precise measurement of non-electric quantities. He has authored and co-authored more than 370 scientific papers, patents and conference papers in the fields of fiber optics and metrology which have been cited about 3000 times.