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Label-free Gram-negative bacteria detection using bacteriophage-adhesin-coated long-period gratings

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Abstract: This paper presents a novel application of a highly sensitive sensor based on long-period gratings (LPGs) coated with T4 bacteriophage adhesin for Gram-negative bacteria detection. We show here, that the sensor evidently recognizes *Escherichia coli* K-12 (PCM2560), whereas in the reference tests – ELISA and BIAcore – the results are questionable. For LPGs sensor the resonant wavelength shift observed for *E. coli* K-12 was approximately half of that measured for *E.coli* B (positive control). The BIAcore readings (RU) for *E. coli* K-12 were at 10% level of the signal obtained for *E .coli* B. These results confirm the improved sensitivity of the LPGs sensor. Moreover, we also show that application of adhesin may allow for efficient detection of *E. coli* O111 (PCM418), *Klebsiella pneumoniae* O1 (PCM1) and *Yersinia enterocolitica* O1 (PCM1879). The specificity of binding bacteria by the adhesin is discussed and it is determined by a distinct region of lipopolysaccharide receptors and/or by the presence of outer-membrane protein C in an outer membrane of Gram-negative bacteria.

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1. Introduction

Nowadays, detection of pathogenic bacteria is critical in the food industry and in monitoring of water reservoirs. The current detection methods are still typically based on conventional cultivation of bacterial pathogens following standardized and generally accepted protocols. These methods are time-consuming and fail to provide rapid information about contaminating microorganisms [1]. In recent years, much attention has been given to the application of label-free detection mechanisms based on optical fiber sensors. These sensors can detect changes in thickness or in the optical properties of the bio-layer formed on the surface of target microorganisms. The advantages of this type of sensor include real-time detection, immunity to undesired electromagnetic fields and high resistance to harsh environmental conditions, as well as the possibility of multiplexing, and the capability of remote sensing [2–4]. Over the years, several highly sensitive sensors based on surface plasmon resonance (SPR), reverse symmetry waveguides (RSW), metal clad waveguides and long-period gratings (LPG) have been reported [3]. Although these sensors offer an extremely high sensitivity (~2000 nm/RIU; RIU: refractive index unit) the requirement of metallic film, bulky prism, and/or optical

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circulator, for the SPR based sensors, and special waveguide geometry for the RSW waveguides often increases the size and overall cost of the sensor. LPG-based sensors are compact, easy to fabricate and the major advantage of the sensors is the possibility to tune the resonance wavelength anywhere in the electromagnetic spectrum including the highly sensitive infrared region [3]. Optical fiber sensors based on LPGs are of wide interest. They offer high sensitivity and robustness, and can be mass-produced. The LPG is a periodic modulation of the optical fiber core, which induces coupling between the core mode and a series of cladding modes. This coupling in turn results in the appearance of a series of resonances in the LPG transmission spectrum. Since cladding modes are employed in the sensing mechanism, the spectral response of the grating strongly depends on a number of environmental influences. LPG-based devices that can be used to measure temperature, strain, hydrostatic pressure, and bending have been reported [5,6]. However, the ability to detect changes in the external refractive index (RI) is the main advantage of the LPG sensor. Thanks to this RI sensitivity, an LPG can be used to detect variations in thickness and in the optical properties of the surrounding material, and can thus be applied for label-free biosensing [2,7]. The LPG-based platform is capable of real-time monitoring of interactions between bio-molecules at the grating surface. LPG sensors can be fabricated using several available techniques, and their resonant wavelength response can be easily tuned up to the operational range of standard optical interrogation systems [8–10]. It must be noted that a number of investigated liquids, including the buffer and bacteria solutions used in biosensing experiments, can accelerate corrosion of metal-containing devices. For this reason, the fused silica surface of the LPG is very promising as a corrosion-resistant and biocompatible sensor interface. Due to the LPG's simplicity and absence of fragile or corrosive overlays, the device can be even regenerated and reused [11,12]. In our previous work, we have shown a highly sensitive label-free LPG-based sensor for detection of bacterial lipopolysaccharide (LPS), which is a major component of the outer surface membrane in almost all Gram-negative bacteria [13]. LPS is an extremely strong stimulator of innate or specific immunity in diverse eukaryotic species, ranging from insects to humans. In higher doses it can also lead to pathological reactions such as septic shock [14]**.** The sensor mentioned above was functionalized with T4 phage adhesin. In this paper, we report our recent studies on the ability of an LPG-based sensor to detect and to specifically recognize the whole bacteria. The sensing effects are compared to responses obtained using methods based on whole-cell enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR). The biological aspect of Gram-negative bacteria group recognition is investigated in detail.

2. Methods

2.1. Preparation of adhesin and bacteria

2.1.1. Preparation of freeze-dried bacteria

Bacterial strains used in the experiments were obtained from the Polish Collection of Microorganisms (PCM) of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wroclaw, Poland). Bacterial strains were cultivated by shaking at 37°C in a Luria–Bertani (LB) broth medium (10 g/L Bacto Tryptone (Difco), 5 g/L yeast extract, 10 g/L sodium chloride). After 24 hours of cultivation (in the exponential phase of growth), bacterial cells were harvested by centrifugation (5000 x g, 20 min. at 4°C) and washed three times with phosphate-buffered saline (PBS). Next, the bacteria were frozen and lyophilized. The vitality of the bacteria in the dry weight after lyophilization process reaches up to 70%.

Expression and purification of gp37 adhesin

The method for expression and purification of adhesin was reported previously by Brzozowska et al. (2015). The concentration of proteins was determined using the BCA

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method and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% gels according to the method reported by Laemmli et al. [15,16].

2.2. Adhesin-bacteria binding tests

2.2.1. Whole-cell ELISA

The sandwich ELISA was used as a screening method to determine which bacterial strain is bind by the adhesin. Bacteria were used to coat the plate, next, the adhesin solution was added. The polyclonal antibodies (called the first antibodies) were used to detect the adhesin bound to bacterial cells. The first antibodies were detected by commercial antibodies conjugated with horseradish peroxidase (HRP). A 96-well plate (Maxisorp, Nunc) 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. The solution in each well was discarded, then 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. The plate was then washed using PBS-T and the adhesin solution was added (0.25 µg/well). The incubation step with adhesin was performed at 37°C for 1 hour. Next, the plate was washed and rabbit polyclonal anti-gp37 antibodies (1:4000) were added and incubated at 37°C for 1 hour. After a third washing, anti-rabbit Immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) was added (1:10000) and incubated at 37°C for 45 minutes. Finally, a 3, 3′, 5.5′-tetramethylbenzidine (TMB) substrate was added to yield a color reaction measured at λ = 490 nm using a Biotec microplate reader. All the trials were performed in triplicate and the mean value was calculated within the standard deviation range (Graphpad prism 6 software http://www.graphpad.com).

2.2.2. SPR-based BIACORE analysis

The bacterial cell binding was measured with a BIACORE T200 system which employs an SPR effect. The experiments were performed using a Series S Sensor Chip NTA (GE Healthcare)which is used for immobilization of histidine-tagged molecules. The adhesin containing a histidine-tag was immobilized to the chip surface and then the bacteria solutions were used to determine the interactions. The experiments were performed using 0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v Surfactant P20 (HBS-P) buffer (GE Healthcare), 0.5 mM nickel solution, 30 µg/ml adhesin solution, 100 µg/ml bacterial suspensions, and 350 mM ethylenediaminetetraacetic acid (EDTA) as a regeneration solution. Nickel activation and capture with histidine-tag protein were done using a constant flow of 10 µl/min for one minute. The contact time with analytes was 2 minutes with a flow rate of 30 µl/min at 25°C.

2.3. Biofunctionalized LPG-based sensor

2.3.1. Fabrication of LPGs

The LPGs were written in hydrogen-loaded Corning SMF28 fiber using a Pulse Master 840 high-power KrF excimer laser (λ = 248 nm) from Light Machinery. The pulse repetition rate was set to 100 Hz, the pulse duration to 12 ns, and peak pulse energy was about 10 mJ. The UV exposure was performed through an amplitude chromium mask (Λ = 226.8 µm) for about 7 minutes. The grating length was 4 cm. In order to stabilize their optical properties, the LPGs were annealed after exposure at 150°C for ~4 hours to release the excess of hydrogen. The LPGs were monitored in the spectral range from 1100 to 1700 nm, and had a single resonance wavelength (λ_R) near λ = 1245 nm. In order to enhance and to tune the sensitivity of the gratings to the properties of the external medium, i.e., the thickness of the forming layer and its RI, the gratings were etched in hydrofluoric acid for about three hours. The etching procedure resulted in a final sensor operation at the vicinity of the dual resonance regime

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[17]. The optical transmission of the fiber was monitored during the LPG fabrication process in order to obtain the desired spectral attenuation notch at λ_R~1650 nm. The RI sensitivity highly depends on the external RI (n_{ext}) and is about 2600 up to 6900 nm per RI unit (RIU) for measurements farthest from and closest to the dispersion turning point, respectively [13,18]

2.3.2. Biofunctionalization of LPGs

The biofunctionalization process applied here is intended for recombinant proteins containing histidine (His) tag. The His tag consists of five or six His residues added to N- or C- terminus of the protein molecule and is required for protein purification after biosynthesis Biofunctionalization of the LPG surface was performed according to the method described by Kalisz et al. with further modifications [18]. First, the LPG was incubated in 0.01% acetic acid and 2% (3-glycidyloxypropyl) trimethoxysilane solution at 90°C for three hours. After washing in $NaHCO_3$ solution (0.01 M, pH = 10), the LPG was incubated with 20 mM n-(5-amino-1-carboxypentyl) iminodiacetic acid in $NaHCO_3$ buffer at 20°C for 16 hours. Next, the nickel ions were immobilized on the surface of the sensor using 10 mM $NiCl_2$ and 5 mM glycine solution. At the end, the solution of the recombinant phage protein containing additional N-terminal six-histidine tag (2.5 μg/ml) was used to obtain the final fiber's surface.

In the bacteria detection experiment, the *E. coli* B and *L. casei* LOCK919 were used as positive and negative controls respectively. The concentration of bacteria in solution was 100 μg/ml and the adhesin – bacteria contact time was 1 hour, 30 minutes. The regeneration step was based on washing using 0.05 M NaOH and 350 mM EDTA.

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. The ambient temperature during the measurements was set to 22°C and monitored with an HP 34970A Data Acquisition Unit equipped with a thermocouple. The tension of the LPG was held constant throughout the experiment.

2. Results and discussion

In a previous paper, we showed that an LPG sensor coated with T4 phage adhesin recognizes the LPS of *E. coli* B with a high specificity. The next goal of our studies was to determine its specificity against the whole bacteria.

2.1. Adhesin-bacteria binding reference tests

First, reference binding tests were performed using the whole-cell-ELISA and BIAcore methods described above in sections 1.2.1 and 1.2.2. In the ELISA test, the bacteria were immobilized on the plate surface and then incubated with the adhesin. The results obtained after the assay are presented in Fig. 1. 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.

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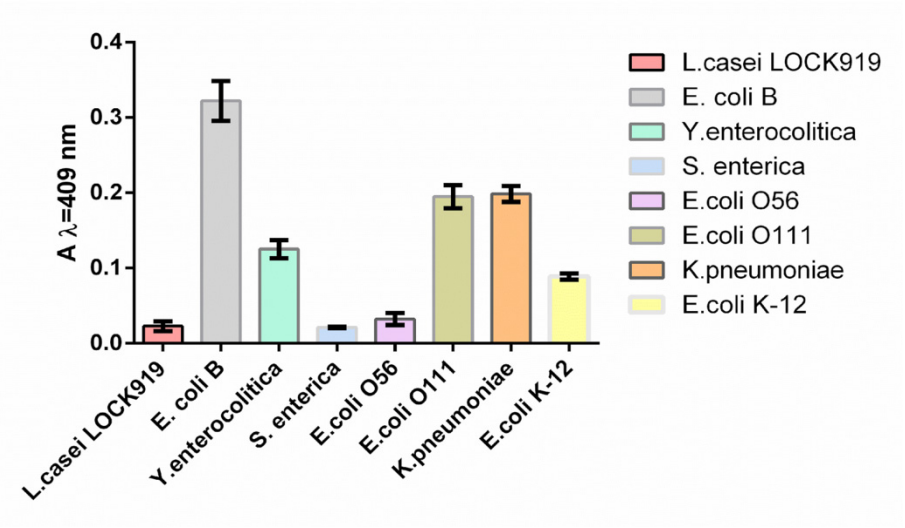

Fig. 1. The results of the whole-cell ELISA showing the adhesin gp37 binding to the bacterial cells. The freeze-dried bacteria (5 μg/well) were immobilized on the plate surface and incubated with 0.25 μg/well of the adhesin. To detect the adhesin-bacteria cell interaction, we used rabbit polyclonal anti-gp37 antibodies (1:4000) first, and then anti rabbit IgG-HRP (1:10000) antibodies.

The negative control used in this experiment was *L. casei,* a Gram-positive bacterium which is without LPS molecules in its cell wall. Its response was almost at the same level as for the blank sample containing only the reagents. The mean value of the response for the negative control was almost negligible – 0.023 (SD = 0.006). The highest reactivity was observed for the adhesin with *E. coli* B bacteria (the mean value was 0.322, SD = 0.027). This result was expected due to the fact that the adhesin specifically binds to its LPS, which was the reason we used the *E. coli* B strain as the positive control [12]. The results of the assay show that the adhesin also binds to *E. coli* O111 (mean value 0.195, SD = 0.016), *K. pneumoniae* (mean value 0.198, SD = 0.011), *Y. enterocolitica* (mean value 0.125, SD = 0.012) and *E. coli* K-12 (mean value 0.089, SD = 0.004). However, the adhesin does not bind to *S. enterica* nor does it bind to *E. coli* O56 under these test conditions.

SPR-based analysis using the BIAcore system was performed next. In this part of the experiment the adhesin was immobilized on the surface of an NTA sensor chip. The chip binds to histidine-tagged biomolecules such as the adhesin by chelation of Ni^{2+} through nitrilotriacetic acid on the surface and histidine residues in the protein tag. Bacteria were used here as the analytes. The experiment was performed according to the method described in section 1.2.2. The sensograms obtained after the SPR analysis are presented in Fig. 2. To determine which bacterial strain binds to the adhesion, we compared the Response Units (RU) before bacteria injection and 17 minutes after injection (the contact time of the adhesin with bacteria was 2 minutes). See Table 1.

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Table 1. The BIAcore analysis of binding bacteria to gp37. The RU difference was obtained after bacteria injection (washing for 15 minutes plus contact time for 2 minutes) and before their injection

Bacterial strain	RU before bacteria injection	RU after 15 minutes washing	The difference RU value
Lactobacillus casei (LOCK 919)	No response		
E.coli B*(*PCM1935)	38432,7	38496.3	**63.6**
E. coli O111(PCM418)	38532,1	38575.4	**43.3**
E. coli O56 (PCM2372)	No response		
E.coli K-12 (PCM2560	38520	38526.6	6.6
Klebsiella pneumoniae O1 (PCM1)	38502,7	38553.7	**51.0**
*Salmonella enteric*a O21 (PCM1879)	No response		
Yersinia enterocolitica O1 (PCM1593)	38538	38568.9	**30.9**

These studies prove that the adhesin recognizes and binds to *E. coli* B (the RU difference is 63.6), *E. coli* O111 (RU difference of 43.3), *K. pneumoniae* O1 (RU difference of 51.0), *Y. enterocolitica* O1 (RU difference of 30.9) and *E. coli* K-12 (PCM2560) (RU difference of 6.6). Our studies also show that T4 phage adhesin does not bind to either *E. coli* O56 or *S. enterica* ssp. *arizonae* strain PCM1593.

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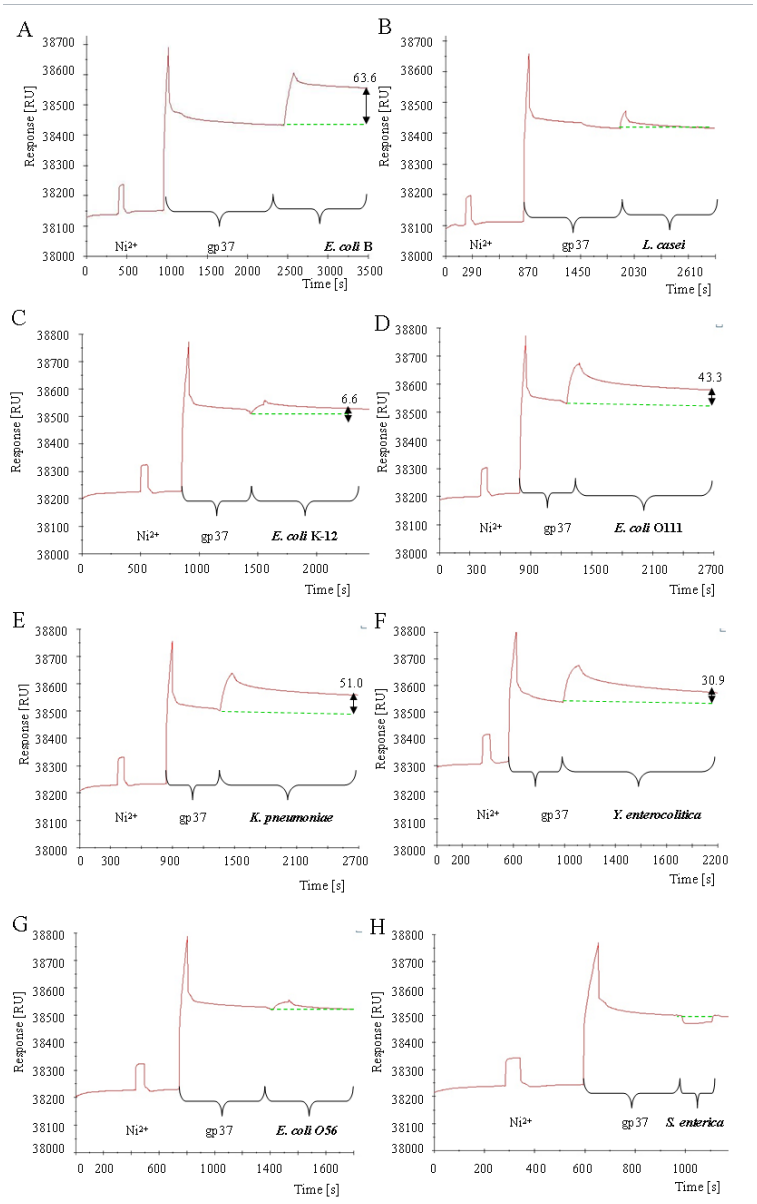

Fig. 2. BIAcore sensograms obtained from analysis of the gp37 adhesin interaction with bacteria. A) *Escherichia coli* B (PCM 1935), B) *Lactobacillus casei* (LOCK919), C*) Escherichia coli* K-12 (PCM2560), D) *Escherichia coli* O111 (PCM418), E) *Klebsiella pneumoniae* (PCM1), F) *Yersinia enterocolitica* (PCM1879), G*) Escherichia coli* O56 (PCM2372), H) *Salmonella enterica* (PCM1593). The bacterial concentration in the experiment was 100 µg/ml.

In the BIAcore analysis the obtained results for *E. coli* O56 show no binding effect, in contrast to ELISA results where the binding could occur (the negative control was a little lower than the response for *E. coli* O56). In the ELISA test, it is possible that a nonspecific interaction between reagents may occur, especially when polyclonal antibodies are used. The

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results obtained with the BIAcore system thus can be considered more reliable than the ELISA results. Moreover, the BIAcore method allows for real-time analysis and enables observation of the sensogram profiles. The profile for the adhesin – *E. coli* B binding shows that the binding mechanism is irreversible (Fig. 2(A)). The obtained signal value is constant in comparison to the signals obtained for *K. pneumoniae*, *Y. enterocolitica* and *E. coli* O11, where the responses decrease during washing. This suggests that the adhesin binds to the bacteria reversibly and that the strength of the binding is lower than in the case of the *E. coli* B strain. The low response for adhesin – *E.coli* K-12 binding correlates well with the results obtained in the ELISA test.

Although the principles of the ELISA and SPR (BIAcore) methods are different, the results obtained are consistent. The specificity of the bacteria binding by the phage adhesin depends on the composition, nature and structural peculiarities of receptors on the bacterial surface. In addition, a vital role is played by receptor localization on the cell surface, as well as the number and density of receptors at various cell wall sites [20]. *E coli* B is known as an R-strain, containing rough lipopolysaccharide (R-LPS) which is devoid of the O-specific chain [21]. The LPS of *E. coli* B appears to contain glucose, heptose and 3-deoxy-D-mannooctulosonic acid (KDO) in molar ratio 2:3:3 [22]. Prehm et al. suggest that the two glucoses are necessary for phage and bacteria interaction [22]. However, the two distal glucose residues are not present in cell-wall polysaccharides of the other bacteria which bind to the phage adhesin in the tests described here (based on comparative analysis using the bacterial carbohydrate structure database http://csdb.glycoscience.ru/bacterial/index.html). It seems most likely that a major role in the receptor formation is played by a spatial configuration around the terminal glycosidic bond rather than by terminal residue in the polysaccharide chain of the core. Moreover, the mechanism of the adhesin – LPS (purified molecules) interaction may be quite different than the mechanism of the adhesin – whole bacteria binding, especially when we consider that in the case of Gram-negative bacteria, various LPS sites as well as proteins localized in the membrane may serve as the adhesin receptors. In many cases, both types of molecules, LPS and proteins, are required for adhesin – bacteria interaction [20]. The adhesin used in our studies utilizes the cell-wall LPS as a receptor in combination with the outer-membrane protein C (OmpC). The phage adhesin is made up of a large amount of histidine residues (His-boxes), which are responsible for OmpC recognition [23]. Yu and Mizushima suggest that in the presence of the OmpC protein, the glucose region in the LPS molecule is not essential for expression of the receptor function and that the OmpC protein is not required at all when the glucose region is exposed at the distal end of the LPS molecule [24]. It is likely that the OmpC protein and the glucose residue of LPS in the adhesin in T4 phage interaction can replace each other.

3.2. Detection of bacteria with the LPG-based sensor

In the following study, the LPG-based sensor functionalized with adhesin was used to determine the interaction with the whole bacteria. For these experiments we chose the *S. enterica* strain PCM1593 as our Gram-negative bacteria, which the above reference tests show does not bind to the phage adhesin. We also chose *E. coli* K-12 (PCM2560) to determine if the bacteria interacts with the adhesin, since in the ELISA reference test it gave questionable positive results, which could be due to nonspecific cross-reactions. Our choice of *E. coli* K-12 was motivated by the fact that the phage adhesin may use its Omp as a receptor [25]. Finally, the whole bacterial cells of *E coli* B and *L. casei* were used as positive and negative controls, respectively.

The highly sensitive LPG-based sensor was fabricated and biofunctionalized according to the procedure described by Brzozowska et al. with one small modification – the concentration of N-(5-amino-1-carboxypentyl) iminodiacetic acid solution was changed from 10% to 0.5% solution, which significantly lowered the costs of the biofunctionalization procedure while keeping its linking properties [13]. The experiment was performed according to the protocol

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described above in section 1.3.2. We improved the protocol with an additional wash step using the NaOH solution. During the experiment the sensor was first immersed in PBS, then in the bacteria suspension and finally extensively washed in PBS. The most representative spectra obtained at each stage of the experiment are shown in Fig. 3, the corresponding measurement data are shown in Fig. 4. Results shown in Fig. 4 are obtained at three final steps of the experiment (covering washing in PBS, dry weight incubation, and final washing in PBS). During every step the resonance wavelength shift was monitored. Measured data were averaged in terms of mean square error.

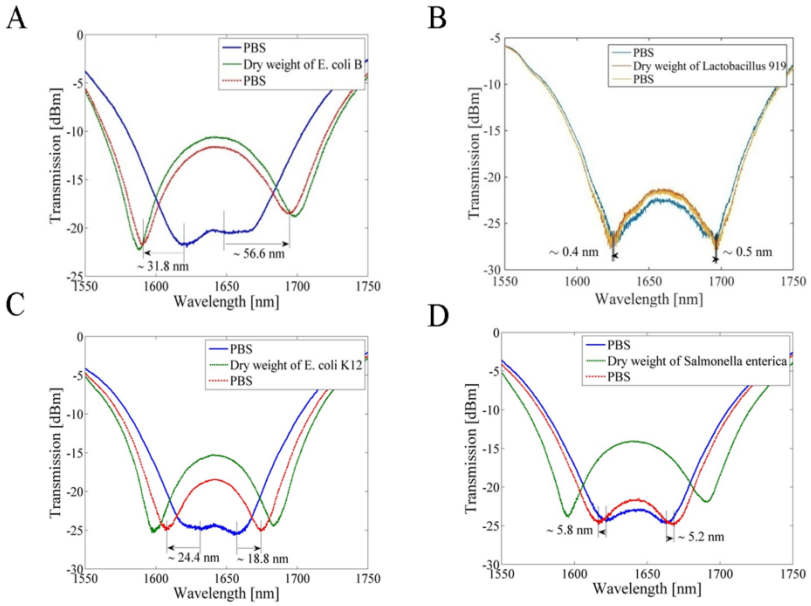

Fig. 3. Transmission spectra of the LPG at each stage of the experiment, showing (A) response with positive control *E. coli B*, (B) response with negative control *L. casei*, (C) interaction with *E. coli* K-12, and (D) interaction with *S. enterica.*

When the thickness of the high-RI LPG bio-overlay increases, it is followed by an increase in spectral distance between the resonances [7]. Since the measurements were taken at different stages of the experiment while maintaining the same external conditions, i.e., LPG immersion in PBS, the effects can be compared and considered to be solely dependent on the thickness and on the RI (corresponding to overlay density) of the bio-overlay.

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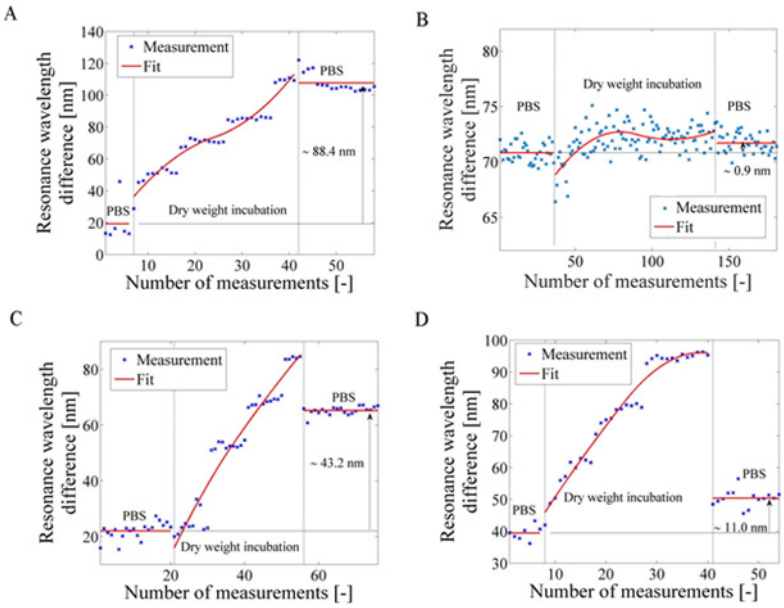

Fig. 4. Resonance wavelength shift for tested LPG induced by incubation in (A*) E. coli* B, (B) *L. casei*, (C) *E. coli* K-12, and (D) *S. enterica.*

The spectral responses for the LPGs immersed in PBS before and after incubation in bacteria solution are shown in Fig. 3. It can be seen in Fig. 3(A) that the reactions between the adhesin and *E. coli* B significantly shift the resonant wavelength, in contrast to the negative control, where the shift is negligible (Fig. 3(B)). For the *E. coli* B, an insignificant change of the spectrum as a result of sample washing in PBS is apparent. This effect is in line with well-established bacteria-adhesin interactions. The resonant wavelength shift in the case of *E. coli* B is almost three times higher than the shift for *E. coli* B LPS, most probably due to the difference in size of this bacterium and its LPS [13]. When adhesin binds to a bacterium, the bio-overlay formed at the surface of the LPG is significantly thicker than it is for the adhesin-LPS bio-overlay. The effect can be also attributed to the fact that more adhesin receptors (including LPS and OmpC) are located on the bacterial cell wall than in the LPS solution. The resonant wavelength shift of *S. enterica* is higher than for the negative control used here (Fig. 3(D)), but it is almost at the same level as for the negative control (LPS of *H. alvei*) used previously by Brzozowska et al. [13]. A significant effect of the sample washing in PBS after incubation in the bacteria solution should be noted here. Our result proves that no interaction occurs between the adhesin and *S. enterica*, but some slight non-specific interactions do take place. These background interactions may be characteristic only of Gram-negative bacteria, which contain LPS molecules on the bacterial surface. Gram-positive bacteria such as *L. casei* used in our studies as the negative control are devoid of LPS on the external side of the cell wall, explaining why the wavelength shift is negligible (no background interactions). The obtained results also suggest that the adhesin binds to *E. coli* K-12 (Fig. 3(C)). However, the binding is not as efficient as in the case of the positive control, i.e., the resonant wavelength shift is about half the size of the shift for *E. coli* B, but is still evident. This result agrees with the earlier findings (ELISA and BIAcore). In the case of *E. coli* K-12, OmpC could serve as a receptor for the phage adhesin, though at low efficiency. The *E. coli* K-12 OmpC homologous have been found (BLAST analysis using http://www.uniprot.org/blast/uniprot software) in about 228 different *E. coli* strains – including *E. coli* O111 interacting with the adhesin. However, the protein is not present in *E. coli* O56 which does not interact with the adhesin. It can be therefore suggested that the adhesin may recognize not only the bacterial strains

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containing OmpC, but also those without OmpC that have specific binding sites involving LPS.

3. Conclusion

The bacteriophage T4 adhesin was applied in this work for the first time for sensing specific whole bacteria. The label-free sensing effect was demonstrated using LPGs and the results were compared to those obtained with other methods, which include ELISA and BIAcore. The LPGs sensor is more sensitive than BIAcore method and gives unquestionable results in respect of *E. coli* K-12 by the adhesin binding The sensor coated with adhesin can be used not only for selective detection of *E. coli* B bacteria, but also for detecting a wider group of Gram-negative bacteria containing OmpC and/or in special spatial configurations around the terminal glycosidic bond in LPS structures. Moreover, the results prove the possibility of using adhesins in place of bacteriophages for specific bacteria strain recognition. This approach could well be used in commercial applications given that adhesins are more stable and can be stored longer than bacteriophages.

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