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Dear editor,

On behalf of the authors I would like to submit a manuscript: " ***Impedimetric label-free sensor for specific bacteria endotoxin detection by surface charge registration"*** based on the work carried out in Instituto de Microelectronica de Barcelona, Centro Nacional de Microelectronica (IMB-CNM), CSIC, Campus UAB, 08193 Bellaterra, Barcelona, Spain for publishing in Electrochimica Acta. Corresponding author Andrey Bratov Tel.: +34 935947700. *E-mail address*: andrei.bratov@imb-cnm.csic.es

The paper presents results of an applied research on a new type of impedimetric sensor used to control interfacial phenomena in concanavalin A - endotoxin system of biological origin, having particular relevance to the medical and food fields. The paper presents new endotoxin sensor with a short response time and detection limits much lower than reported in other publications. The article counts in 6160 total number of words, 8 figures and a supplementary material with one table and one additional figure.

The highlights of the article may be summarized as follows:

- A new impedimetric sensor with concanavalin A for bacteria endotoxins is proposed
- The sensing layer is deposited by layer-by-layer method
- The mechanism relies on registration of surface charge changes produced by biochemical reaction
- New method for blocking non-specific interactions of polyethyleneimine is proposed
- The sensor shows short detection time (20 min) and low detection limit (1.5 µg/mL)

The work described has not been published previously and is not under consideration for publication elsewhere. Its **publication is approved by all authors** and by the responsible authorities where the work was carried out (IMB-CNM, CSIC).

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Impedimetric label-free sensor for specific bacteria endotoxin detection by surface charge registration

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Keywords: impedimetric sensor; surface charge; interdigitated electrode array; bacterial endotoxin; concanavalin A.

Abstract

An impedimetric sensor based on a three dimensional electrode array modified with concanavalin A (Con A) was used for label-free detection of bacterial endotoxin: lipopolysaccharide (LPS) from *Escherichia coli.* The transducer permits the detection of the surface charge changes due to interaction of immobilized Con A biorecognition element and LPS of *E. coli* in test solution. The deposition of Con A on the surface was carried out using the layer-by-layer method with polyethyleneimine (PEI) polycation as an initial layer. The sensor surface characterization by means of electrochemical impedance spectroscopy technique allowed registering variations in superficial resistance provoked by surface charge changes and is demonstrated as an effective method to monitor sensor parameters at each modification step as well as to follow Con A – LPS reaction. In order to prevent non-specific adsorption of LPS on PEI covered surface different blocking strategies were tested to achieve the specific response between Con A and LPS. Results obtained in this work clearly show that blocking with bovine serum albumin (BSA) is not sufficient to prevent non-specific interactions of PEI and to ensure the selective biorecognition of LPS by Con A. To achieve more efficient PEI blocking a new method was proposed based on consecutive deposition of Con A-glycogen-Con A layers. Sensors modified with PEI-(Con A-Gly)$_2$-Con A multilayers are shown to be highly sensitive, selective and reproducible. Presented biosensor is able to detect bacterial LPS in a very short

detection time (20 min) with 1.5 µg/mL limit of detection, which is much lower than reported for other biosensors with Con A.

1. Introduction

Endotoxins, also known as lipopolysaccharides (LPS), are ubiquitous markers of gram-negative bacteria considered as contaminants habitually found in food, environment and clinical products [1]. Lipopolysaccharides are the major structural component of external membrane of gram-negative bacteria composed of three distinct regions: O-antigen oligosaccharide that is specific to bacterial serotype, a hydrophilic core polysaccharide chain, and the lipid A - hydrophobic lipid section responsible for the toxic properties of the molecule [2]. Endotoxins can induce immune response on the internalization of mammalian cells, producing fever, multi organism failure or sepsis [3, 4].

Among food-borne pathogens responsible of many gastrointestinal diseases and the most common cause of urinary tract infections are *Escherichia coli,* well-studied gram-negative bacteria. Moreover, LPS of *E. coli* alone can cause an important number of diseases [5]. Taking into account an increasing concern in society for microbiological safety, detection of endotoxins is essential in controlling various biological and food products.

Although there are well established techniques for determination and quantification of endotoxins, like the standard limulus amoebocyte lysate (LAL) method [6], they are relatively complex assays of multiple stages that require skilled operators [7]. Hence, the development of new detection strategies and techniques as well as rapid, compact, simple, highly sensitive, selective and high-throughput devices are required [8]. In this regard biosensors, and particularly electrochemical biosensors, have been demonstrated as a promising alternative to classical techniques and the number of publications related to biosensors for endotoxin detection increased considerably during last years [1, 8, 9].

In biosensors to achieve selectivity against endotoxins a specific biorecognition element should be immobilized on a sensor surface. The biological recognition elements that affect the selectivity of biosensors should possess a number of essential features: high specificity to the target, invariability under storage and detection conditions. More importantly, the reactions of recognition elements and targets should be accurate, rapid, reliable and reproducible. A large variety of different biorecognition elements

were tested for LPS biosensor applications [1, 8-10] including natural and synthetic proteins [11] and peptides, antibodies [12, 13] and aptamers [14]. However, the most widely used biorecognition element in LPS biosensors is concanavalin A (Con A) [15-21]. It is lectin-type carbohydrate-binding protein obtained from jack beans (*Canavalia ensiformis*) with well-characterized structure that recognizes specifically α-D-glucose and α-D-mannose groups [9]. It may be noted that lectins are more stable and smaller than antibodies and their size allows obtaining higher densities of carbohydrate sensing elements on a sensor surface [22]. Con A may be used in different biosensing applications as biosensing moiety basically for glucose and other carbohydrate detection [23]. Furthermore, Con A and some other lectins have also been employed in various biorecognition processes due to its affinity for some bacteria [19, 20, 24], viruses [25], cells [26, 27] and for endotoxin [8, 9, 28] detection .

Interactions of Con A with analytes may be registered in a label-free mode using different experimental techniques, like surface plasmon resonance (SPR) [24, 29, 30], quartz crystal microbalance [15, 19-21, 31] or electrochemical impedance on a Con A modified metal electrodes [12, 32-35]. Biosensors based on the last two techniques are the most widely reported.

In this regard, electrochemical impedance spectroscopy (EIS) is a powerful technique permitting to control changes at the solid/liquid interface of surface-modified electrodes produced by chemical, physical and biological interactions during the recognition events [36]. Typically impedance measurements are performed in a Faradaic mode [17, 33, 34, 37] in the presence of the redox probe $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ registering changes in a charge transfer resistance associated with biorecognition processes at the modified metal electrode surface. However, capacitance changes produced by surface biochemical reactions may also give additional information [38].

In the case when a redox pair is absent in the electrolyte solution, the impedance is termed nonfaradic and depends on the conductivity of the supporting electrolyte and impedimetric electrode interfacial properties (interfacial capacitance [39] or surface conductivity [40]). For interfacial capacitance measurements just simple planar macro-electrodes may be used [39], while the surface conductivity plays important role in the case of interdigitated electrode array (IDEA), in which a pair of comb-like metal electrodes are formed on a planar insulating substrate [41]. IDEA present promising advantages compared

to other impedimetric sensors such as small size, rapid detection kinetics, increase of signal-to-noise-ratio and fast establishment of a steady state response [42].

To enhance the sensitivity of IDEA sensors a three-dimensional (3D-IDEA) device, in which the electrodes digits are separated by insulating barriers, was proposed [43]. In this case under applied potential the main portion of the current goes close to the surface of barrier and this permits to enhance the sensitivity compared with standard planar structures [44]. The principles of 3D-IDEA sensor operations are based on registration of the surface conductivity changes produced by variation in the surface charge and were discussed in detail earlier [40, 45]. This design has been demonstrated to be highly sensitive to changes in the electrical charge distribution at the solid/liquid interface produced by chemical and biochemical reactions [46]. Thus, it is a promising sensitive transducer for label-free biosensor development.

The functionalization of the IDEA surface to achieve selectivity against endotoxins is a crucial step in the optimization of the sensor. Efficient immobilization and stability of biorecognition elements without decreasing their binding affinity in the performance process are essential.

Immobilization of Con A may be performed using different assembling techniques like absorption [25, 27, 32], or chemical grafting via self-assembled monolayers (SAM) on gold [15, 21, 38]. However, Con A directly adsorbed of a solid surface show low stability [47] and readily desorbs [31]. Layer-by-layer (LBL) technique is another well-known method for the assembling of oppositely charged polyions that has been demonstrated useful for immobilization of biomolecules like proteins, antibodies or lectins [48, 49]. For this purpose polyethyleneimine (PEI), a positively charged polycation, is widely used in layer-by-layer (LBL) assembly of oppositely charged polyions for the formation of thin multilayer coatings [50]. As many large biomolecules are negatively charges at neutral pH, PEI also has been demonstrated effective for the immobilization of proteins layers [51, 52] , enzymes [46], lectins [53] and bacteria [54].

Immobilization of Con A biorecognition element on a 3D-IDEA sensor surface using LBL method may be advantageous to establish robust methodologies for endotoxin detection and new biosensing strategies. However, to demonstrate the effectiveness of the proposed method thorough surface characterization of each surface modification step and interaction of PEI and Con A is necessary. Additionally, Con A lectin

stability after the immobilization is crucial to maintain its specific recognition ability for carbohydrates and consequently, bacterial LPS.

Another important aspect to take into consideration regarding performance of biosensors based on their surface reactions with analyte is possible non-specific binding of the corresponding target or other molecules present in the sample with the sensor surface layer. To prevent non-specific binding and enhance the selectivity in many cases it is required to use inert blocking reagents that cover the sensor surface without altering the specificity of a biorecognition element. As PEI polycation was shown effective in binding endotoxins [55, 56] that possess net negative charge due to phosphorylated groups of carbohydrates, it is necessary to block the effect of positive charges of PEI in order to enhance the selectivity of Con A to endotoxins.

The aim of this work is the study of sensitivity and response of impedimetric sensors based on 3D-IDEA transducers to detect bacteria endotoxins, more concretely lipopolysaccharides (LPS) extracted from bacteria *E. coli*, using the impedance technique. The biorecognition element for bacterial endotoxin detection used in this study is the lectin concanavalin A, immobilized on the sensors surface by its interactions with positively charged polyethyleneimine polycation. To increase specificity of the bacteria recognition event it is proposed to study different strategies of using blocking reagents to eliminate possible non-specific interactions between underlying PEI layer and the analyte.

The sensor modification strategies are schematically presented in Figure 1.

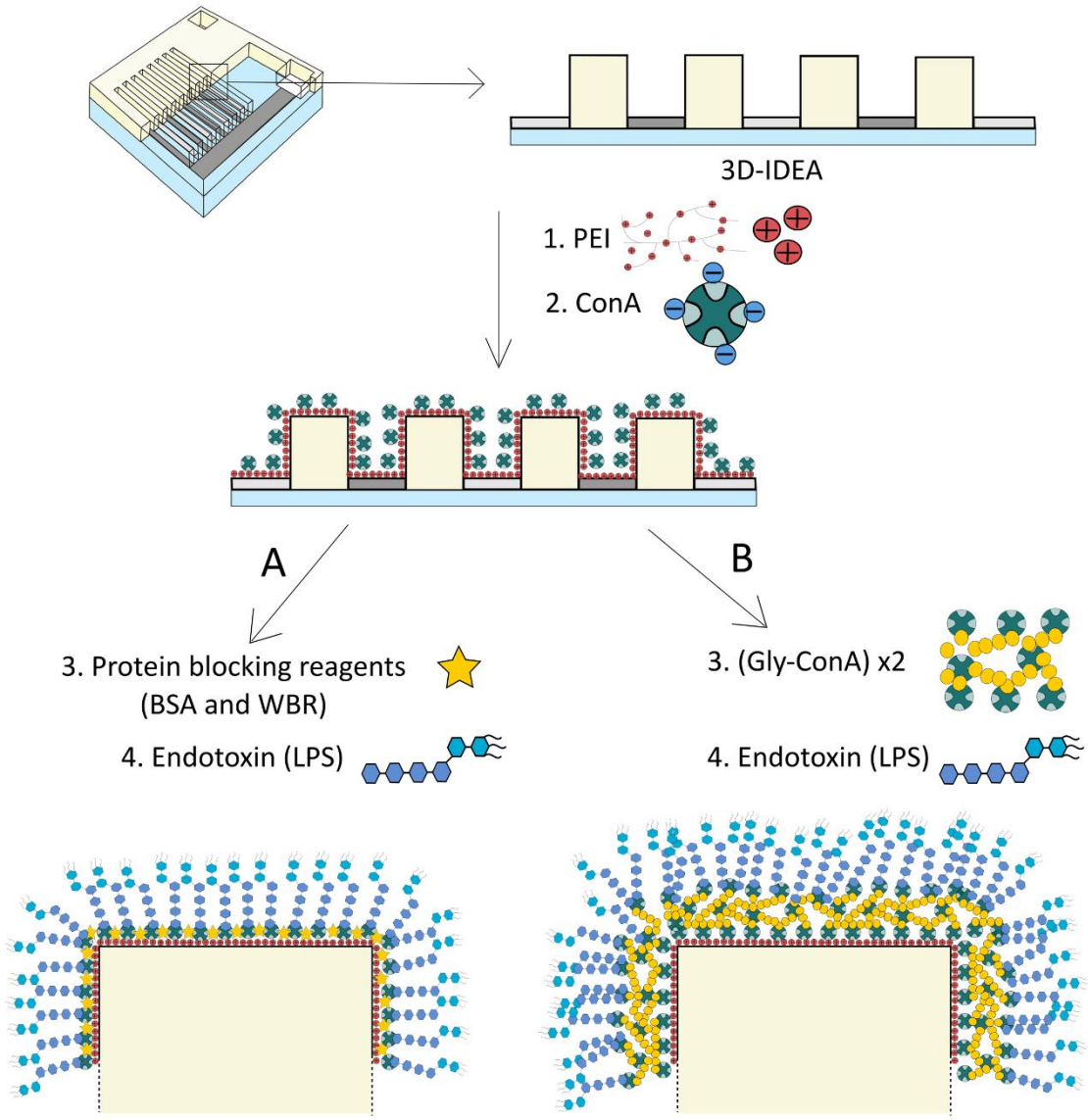

Figure 1. Sensor disign and schematic representation of the sequential steps of the sensor surface biofunctionalization.

2. Experimental

2.1. Electrode design and fabrication

The three-dimensional interdigitated electrode array (3D-IDEA) was fabricated using conventional microelectronic techniques. A silicon wafer oxidized by "wet" oxidation at 950 ^{o}C was employed as substrate to give a good quality of silicon dioxide layer of 2500 nm. As electrodes material a 230 nm layer of a highly conductive tantalum silicide ($TaSi_2$) was deposited using magnetron sputtering. The first photolithographic step defines collector bars and digits of the two electrodes. The pattering is done by reactive etching technique resulting in an interdigitated electrode array with 216 digits of 3 µm width and 3 µm gap between the adjacent electrodes. The aperture between the electrodes is 1.4 mm and the total length between them is 301 mm. To form contact pads 1 µm of aluminum is deposited and patterned by standard photolithographic and etching steps leaving metal only at extremes of the two collector bars.

The final step is the barrier formation. To do this the wafer is covered with a 4µm thick silicon oxide (SiO_2) layer deposited by a low pressure chemical vapor deposition (LPCVD). Photolithography is also used to define the openings in the oxide layer over the electrode digits and the contact pads. The aperture of these zones is carried out by deep reactive ion etching (DRIE) to obtain barriers with nearly vertical walls. The barrier separating the adjacent electrodes are 3 µm, 4 µm wide and 162 mm long and are opened at the top.

Finally, the electrodes cut from the wafer were glued to a PCB substrate and wire bonded for electrical connections. Contact pads and wires were encapsulated using epoxy resin. Complete technology is described elsewhere [43, 44, 46] and schematic design of device is also presented in *Fig. 1*.

2.2. Chemicals, solutions and reagents

Polyethyleneimine (PEI, branched, average M_w 25000, water-free) polycation and anionic poly(sodium 4-styrenbesulfonate) (PSS, average M_w 70000, water-free) were both dissolved in deionized water at 1.5 mg/mL and 2 mg/mL, respectively. These concentrations have been chosen in accord with previously published data [52]. Concanavalin A (Con A, from *Canavalia ensiformis*) lectin used as a biorecognition

element was prepared at 25 µg/mL in 0.05 M Tris-HCl buffer (pH=7.4). Compounds employed as blocking reagents were: bovine serum albumin (BSA) (from Sigma-Aldrich)), Western Blocking Reagent (WBR) (from Roche Life Science) and glycogen (Gly), a polyglucose-type, all prepared in 0.05 M Tris-HCl buffer (pH= 7.4). The optimal concentration of Con A and the blocking reagents (BSA, WBR and Gly) were studied in additional assays. LPS from *Escherichia coli* 055:B5 were prepared using Tris-HCl buffer solution at the required concentrations. All chemicals were purchased from Sigma-Aldrich, Spain.

All the solutions were prepared with deionized MilliQ water (18 MOhm·cm) which was also used for the cleaning and rinsing processes. All chemicals were of analytical grade and were used as received without further purification.

2.3. Preparation and modification of electrodes for LPS detection

Prior to use, 3D-IDEA sensors were first cleaned with isopropanol for 10 minutes, rinsed with distilled water and dried under nitrogen flow.

2.3.1. Immobilization of Con A by LBL method

To perform the deposition of corresponding layers on the sensor surface, PEI polycation was employed as the initial assembling layer. First of all, the sensors were immersed into the PEI solution for 20 minutes to form a homogeneous self-assembled monolayer on SiO_2. The immobilization method of Con A as a biorecognition moiety on the 3D-IDEA surface was carried out over the initial layer of PEI. As at pH 7.4 Con A behaves as a polyanion, it was deposited by LBL method over PEI layer by immersing sensors in Con A solution during 60 minutes. After each modification step the sensors were thoroughly rinsed with water to remove non-bound molecules.

2.3.2 Immobilization of blocking reagents

To avoid unspecific binding of LPS to the sensor surface with PEI, different compounds were used to block the surface not occupied by Con A. Protein-based blocking reagents (BSA and WBR) and Gly multiple layers were employed to ensure the specific biorecognition interaction between Con A and LPS. For BSA treatment electrodes with PEI-Con A were immersed for 20 minutes into a BSA in Tris-HCl buffer solution with the BSA concentration in the range of 10 to 100 µg/mL . In the same way, the treatment with WBR

was carried out in Tris-HCl buffer solutions with WBA concentrations in the range of 5 to 100 µg/mL and the treatment lasted up to 60 minutes.

In the experiments with glycogen as blocking-reagent, the immobilization was done taking advantage of the specific concanavalin-glucose interaction. Gly solutions were also prepared in Tris-HCl buffer at the concentration of 100 µg/mL according to procedure reported by Lvov et al. [52]. In this case Con A and Gly layers were alternatively deposited by immersing sensors into corresponding solutions during 60 minutes until the desired number of layers was obtained.

2.3.3 LPS detection assays

For LPS detection functionalized 3D-IDEA were incubated at room temperature in solutions of *E. coli* LPS with various concentrations ranging from 0 to 50 µg/mL. After this treatment electrodes were thoroughly rinsed with water to remove unreacted LPS and to reduce possible influence of adsorbed ions from Tris-HCl buffer with high salt concentration on the impedance measurements.

2.4. Impedance Measurements

For impedance measurements a QuadTech 7600 Plus highly precision LCR Meter analyzer was employed. Measurements were performed in a 10^2 Hz – 10^6 Hz frequency range with 100 mV (amplitude) voltage excitation. Impedance data treatment and equivalent circuit fitting was performed using the Z-Plot/Z-View software package (Scribner Associates, Southern Pines, NC, USA). All experiments were done in duplicates at least on three electrodes under the same conditions.

Impedance measurements were carried out at controlled room temperature in KCl 10^{-5} M solutions. To guarantee the reproducibility of the bulk solution conductivity for each single measurement a fresh portion of solution was used and its conductivity of 2.50 µS/cm was controlled with a commercial conductimeter (EC-Meter GLP 31+, Crison).

3. Results and discussion

3.1. Sensor characterization by impedance measurements

The impedance response of a 3D-IDEA in low conductivity solutions in the absence of faradaic processes can be emulated by an equivalent circuit [45] presented in Fig.2A formed by the following components: R_C is the contact resistance introduced by wires and collector bars of thin film electrodes; C_G is the geometrical capacitance between two interdigitated electrodes in a aqueous solution; R_S is the resistance between two electrodes of the array; and CPE is a constant phase element associated with the capacitance of the electrical double layer at the electrode – water solution interface. The impedance of the CPE can be expressed as:

$$Z_{CPE} = \frac{1}{(j\omega)^{-\alpha}\, C_{DL}} \quad , \tag{1}$$

where j= -1 (imaginary unit), ω is angular frequency (rad·s $^{-1}$), C_{DL} (F) is the capacitance of the double layer and α is an empirical constant representing the behavior of the CPE. When the exponent α is equal to 1 the CPE behaves similarly to a capacitor. If the value of α becomes 0 the CPE will behave as a resistor. Typical values for 3D-IDEA with $TaSi_2$ electrodes of CPE α parameter in low conductivity solutions are between 0.85 and 0.9 [44].

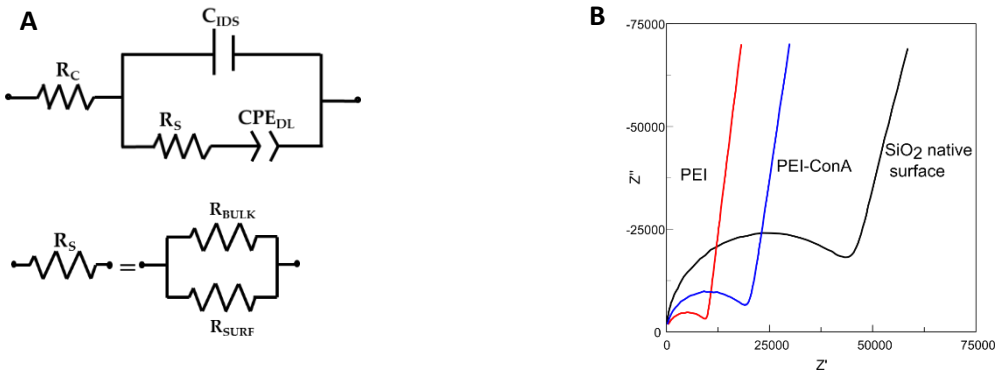

Figure 2. (A) Electrical equivalent circuit used for impedance spectra fitting. (B) The Nyquist plot of the 3D-IDEA measured in KCl 10^{-5}M solution with bare electrodes (SiO_2 native surface), after PEI deposition and with PEI-ConA layer.

Previously it has been reported that in low conducting solutions surface conductivity plays an important role in this kind of sensors [45]. Therefore, R_S is a parallel combination of bulk solution resistance (R_{BULK}) and the surface resistance (R_{SURF}) (Figure 2A). It is important to note that under experimental conditions used it is not possible to distinguish these two elements in the impedance spectra. However, if we fix the bulk solution resistance, R_{BULK}, then all changes in R_S may be attributed to surface resistance, R_{SURF}, variations associated with the surface charge changes at the barriers surface due to surface (bio)chemical reactions [45, 54].

The impedance spectra presented in the Nyquist plot (Z' vs Z'') in Figure 2 allow to observe the formation of a semicircle at high frequencies corresponding to resistance R_S in parallel with the sensor geometrical capacitance. The intercept with the Z' axes at high frequencies on the left side of the plot gives the R_C value, while the intercept on the right side gives the value of R_S (the parallel combination of R_{BULK} and R_{SURF}). As all the experiments were performed in KCl solutions with controlled conductivity, the solution resistivity was kept constant and the observed changes in R_S are attributed to surface resistance variations. The linear response at low frequencies in the Nyquist plot (Fig. 2B) is due to the CPE element of the interfacial capacitance.

As follows from the spectra in Figure 2B, modification of native SiO_2 of the barriers surface with highly positively charged PEI causes decrease in the surface resistance producing the attraction of mobile ions from the water solution bulk to the charged surface. Accordingly, when Con A and other corresponding modification components are deposited on the surface the positive charge introduced by PEI is progressively compensated, producing significant increases in R_S.

To characterize the sensitivity of sensors to *E. coli* LPS their response was defined as changes in R_S of the modified sensor before (R_S^0) and after (R_S^{LPS}) reaction with LPS:

$$\mathbf{\Delta} R_S = R_S^{\mathbf{0}} \times R_S^{LPS} \quad (2)$$

It has to be taken in consideration that in the low frequency region of the Nyquist plots (Figure 2B), the surface modification not only provokes changes in R_S but also alters the interfacial capacitance and the CPE α parameter. This is due to the formation of an additional layer over the electrodes, which results in an increase in C_{DL} and decrease in α parameter as a result of the non-ideality of the interfacial capacitor.

Subsequent experimental values (R_S, C_{DL}, α) obtained by fitting the spectra to the equivalent circuit in Figure 2 are presented in Table S1 (see supplementary information). All these parameters depend on the LPS concentration, however, ΔR_S was chosen as the main parameter due to its large scale changes and higher reproducibility.

3.2. Evaluation of the nonspecific binding on the electrode surface

The first step on the assembling process of different layers on the 3D-IDEA surface is immobilization of PEI, which is very fast and nearly irreversible. Colloid chemistry experiments show that PEI which bears positive charge adsorbs strongly on silicon dioxide surface due to the presence of hydroxyl groups [57] and increases the surface conductivity. In our experiments performed in low conductivity KCl 10^{-5} M solutions this provokes the surface resistance, R_S, decrease [45] as shows Figure 2B. This effect is used to control changes in surface charging at liquid/solid interface due to adsorption process or surface chemical reactions. Employment of 3D-IDEA device allows to improve significantly the sensitivity to surface charge changes in comparison with traditional flat devices [58].

Multiple publications confirm that branched PEI used as the first layer over silicon dioxide in the LBL process acts as a uniform anchoring network for the formation of consecutive layers, resulting in homogeneous films [50]. PEI was also employed previously [52] to assemble multilayers of Con A and branched polyglucose by LBL method. In the present work the LBL method was used for immobilization on the sensor surface of Con A as a biorecognition element for the detection of bacterial endotoxins.

In addition, an important aspect to be considered is possible non-specific interactions of positively charged PEI on the IDEA surface with negatively charged molecules in test solution that may result in non-selective sensor response.

In order to study the effect of PEI in the nonspecific binding of LPS on 3D-IDEA sensors, a preliminary comparative experiment with electrodes modified with PEI and PEI-Con A was carried out. For this, both types of sensors were immersed in 25 µg/mL solutions of *E. coli* LPS and, subsequently, the impedance response was studied at different incubation times. Obtained results are presented as ΔR_S changes in Figure 3.

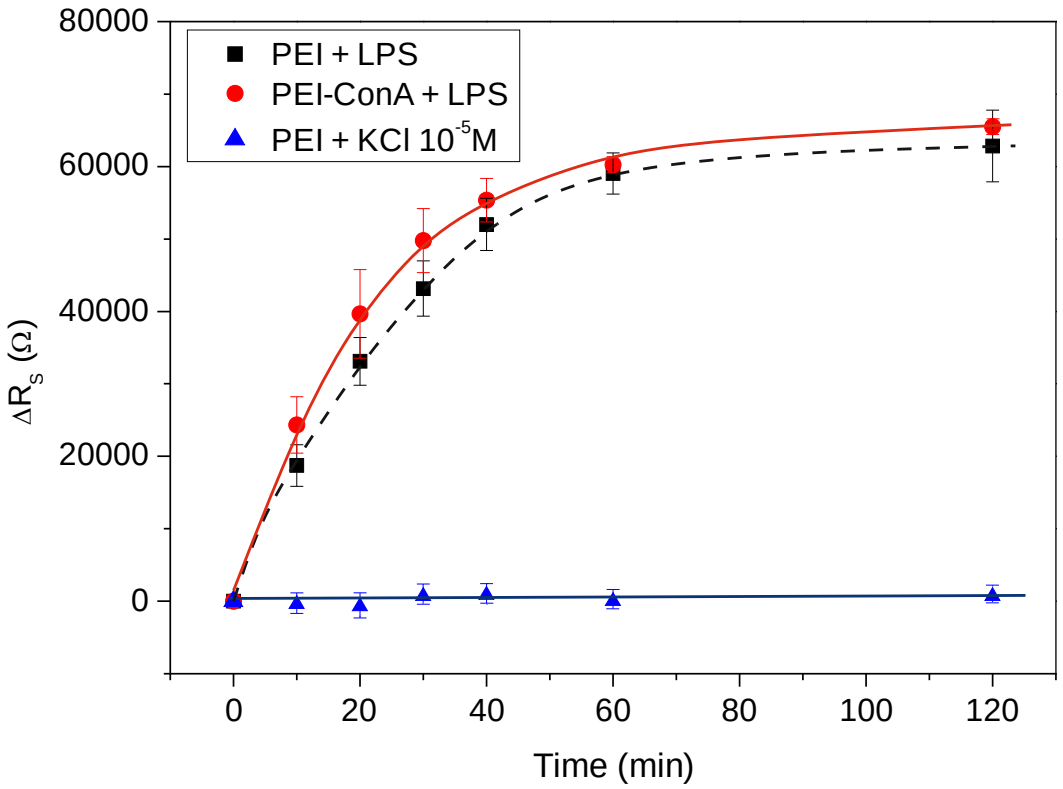

Figure 3. Resistance variations for sensors modified with PEI (squares) and PEI-Con A (circles) in the presence of *E. coli* LPS at 25 µg/mL. Response of a PEI modified sensor in the absence of LPS is also presented (triangles). Error bars show standard deviation (n=3).

It can be observed that the impedance response in the presence of the LPS changes rapidly in time in both cases, provoking significant increase in R_S parameter. Although in the case of PEI-Con A the response of modified sensor showed slightly higher sensitivity, the differences with PEI modified electrodes are practically imperceptible. The saturation of the signal for both types of sensors occurs within 2 hours. Thus, obtained results indicate that PEI and PEI-Con A modified 3D-IDEA have similar binding affinity for LPS. In contrast, in the control experiments without LPS in solution the signal of PEI modified sensor remained stable in time.

As previously described, the bacterial LPS are formed by carbohydrates with phosphorylated groups that contribute to the negative charge of this molecule. Hence, its interaction with polyethyleneimine is produced by electrostatic attraction. Therefore, obtained results are in accordance with other publications [55] confirming the ability of PEI to react strongly with endotoxins. On the other hand, the biorecognition process of Con A is based on the specific reaction with the glycosyl residues of LPS.

Obtained results clearly show the necessity of effective blocking of the sensor surface modified with PEI to prevent its non-specific interactions and to ensure the selective biorecognition of LPS by Con A. The following experiments based on the study of superficial properties were performed to test different blocking strategies aimed on reduction of the effect of the highly positive charges of PEI to ensure selective endotoxin detection by Con A.

3.3. Bacterial endotoxin detection

In order to establish a robust and reproducible methodology to avoid the direct reaction of PEI with LPS and, simultaneously, to guarantee the specific binding of LPS with Con A, two modification approaches using different surface blocking reagents were tested. Firstly, two protein-based blocking reagents, BSA and WBR (PEI-Con A-BSA and PEI-Con A-WBR) were utilized and, secondly, the application of glycogen as intermediate layer for Con A immobilization (PEI-(Con A-Gly)$_2$-Con A) was employed.

3.3.1. PEI-Con A and protein blocking reagents

Protein-based blocking reagents, especially bovine serum albumin (BSA), are widely used in different applications [59] and here BSA was employed due to its ability to interact with PEI [60]. It is well known that the isoelectric point of BSA lies between 4.7 and 5.6 [61], so in solutions close to neutral pH as in our case (pH=7.4), the net charge of BSA is negative. Thus, we suggest that BSA will strongly adsorb on PEI surface not covered by Con A lectin. To study the effectiveness of BSA as a blocking agent to isolate the electrostatic attraction of LPS produced by PEI, 3D-IDEA sensors modified by PEI and PEI-ConA were subjected to BSA blocking as presented in experimental section. The effect of the BSA blocking was studied by incubation of modified sensors in 10 µg/mL LPS solution during 1 hour. Subsequent results are presented in Figure 4.

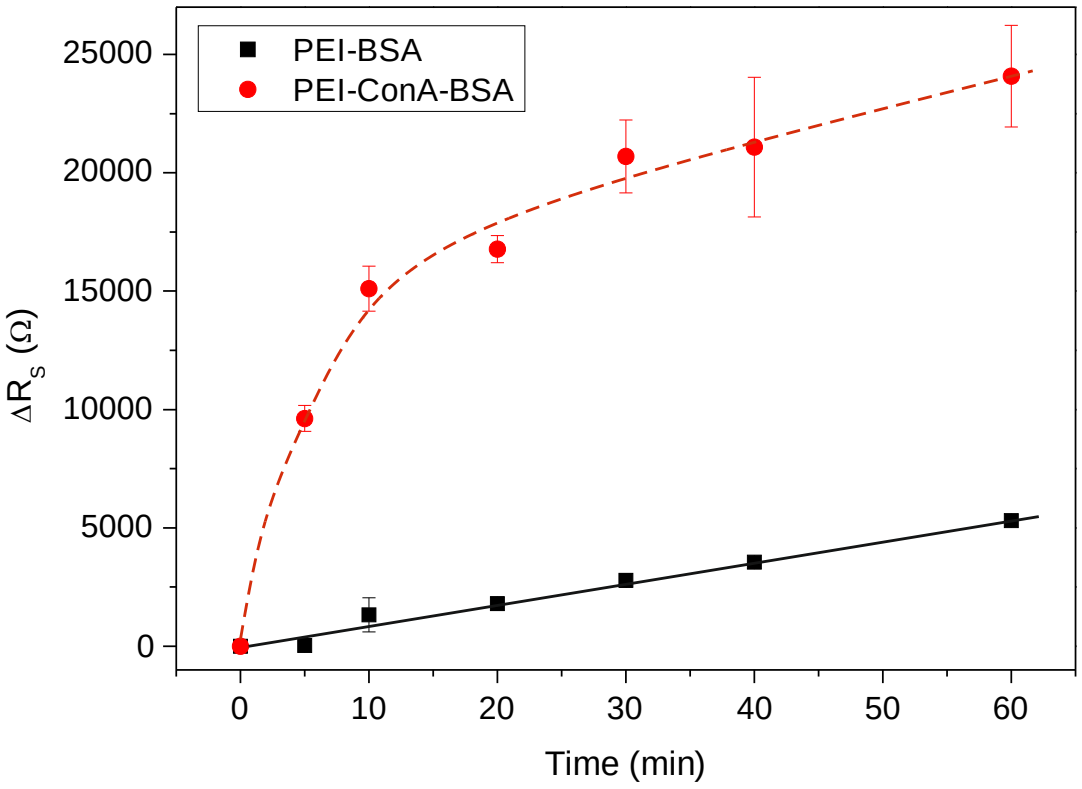

Figure 4. Variations in R_S of sensors functionalized with PEI and PEI-Con A after blocking with BSA. Response is measured in the presence of 10 µg/mL LPS in time. Error bars show standard deviation (n=3).

Figure 4 demonstrates that BSA acts as an effective blocking reagent in the case of PEI modified 3D-IDEA sensors. When the surface of PEI modified sensors is blocked with BSA the increase in R_S parameter in the presence of the LPS is rather small, especially in a short time, demonstrating low absorption of LPS. On the other hand, PEI-Con A sensors blocked with BSA show high sensitivity of the impedance response in presence of LPS which is stable after 1 hour of incubation.

Obtained results also demonstrate that Con A in PEI-Con A-BSA structures preserves its biological activity to recognize endotoxins. As previously reported [62] Con A can bind sugars, glycoproteins and glycolipids containing reduced terminal α-D-mannosyl and α-D-glucosyl groups and at pH above 6.9 exists in a tetrameric form, while below 5.9 as a dimer [63]. Thus, the solution at pH = 7.4 employed for Con A immobilization allowed to maintain the tetrameric structure with four binding sites for carbohydrates and, consequently, can stimulate the binding affinity to bacterial endotoxins.

Sensor response of PEI-Con A-BSA 3D-IDEA was measured in *E. coli* LPS solutions in 0 – 20 µg/mL concentration range during 1 hour. Subsequent results are presented in Figure 5. The R_S response increases proportionally with the LPS concentration with the sensitivity of 1.7 kOhm per mg/mL in the 5-20 mg/mL range. It may also be noted that the saturation phase is reached within 40 minutes at all concentrations. Significant differences may be noted between control tests without *E. coli* LPS in the solution and sensors response at low LPS concentration of 1-2 µg/mL. These results confirm that BSA blocking method may be used as an effective strategy to ensure specific response of PEI-Con A layer in LPS biorecognition process.

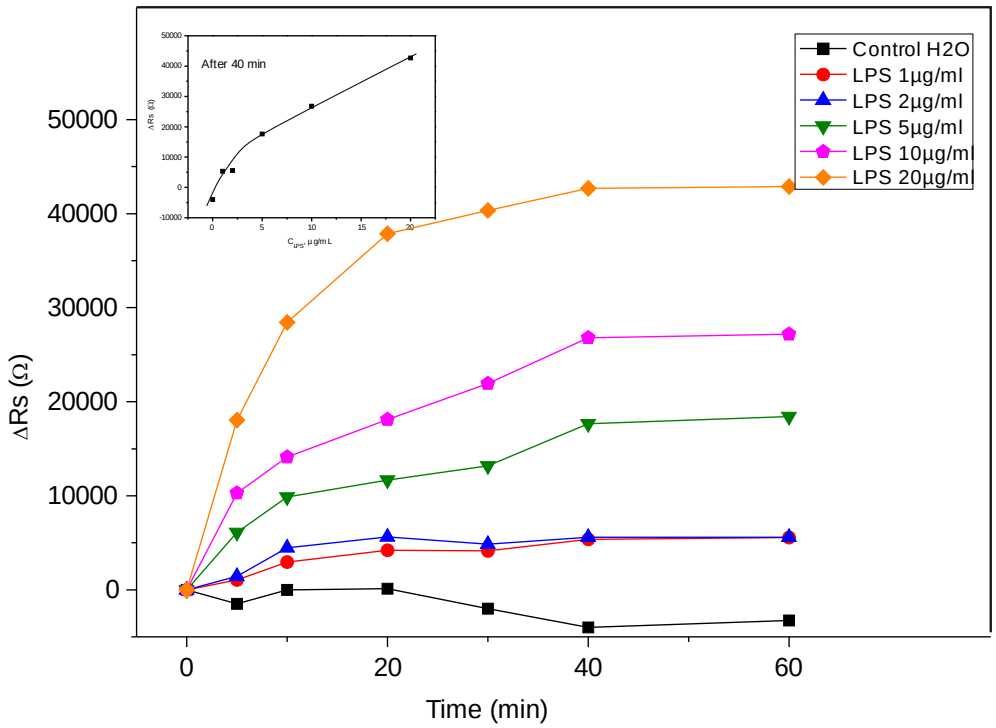

Figure 5. R_S variation in time of sensors modified with PEI-Con A-BSA at different LPS concentrations (0 –control—, 1, 2, 5, 10 and 20 µg/mL). In the inset the sensor response versus LPS concentration is shown.

Similar tests were carried out with WBR as a protein-based blocking reagent. 3D-IDEA sensors functionalized with PEI and PEI-Con A were subjected to blocking using solutions with different WBR concentrations (5, 10, 20, 40 and 100 µg/mL). Afterwards, sensors were immersed in a solution of 10 µg/mL of LPS for 1 hour to study the blocking effect of WBR. However, in the case of PEI-WBR modified

sensors the blocking of PEI was not complete, especially at low concentrations of WBR (from 5 to 40μg/mL). These test results are presented in Figure S1.

In the case of WBR similar to BSA blocking effect was achieved only at WBR concentration of 100 μg/mL. However, when this concentration of blocking reagent was employed for PEI-Con A sensors, no response to LPS was observed, demonstrating that WBA also blocks the activity of Con A making it impossible to detect bacterial endotoxins by means of Con A interaction.

3.3.2. Sensors with PEI-(Con A-Gly)$_2$-Con A multilayers

Though the BSA blocking methodology of PEI-Con A modified sensors described previously provides a good response and sensitivity to detect bacterial endotoxins, still a part of the R_S signal is produced by direct interaction of LPS with PEI. This may affect the selectivity in the presence of some other negatively charged molecules present in a test sample. Thus, an alternative approach of a robust methodology to immobilize Con A by means of PEI is required to prevent non-specific binding of bacterial LPS.

The second strategy to assemble Con A as a biorecognition element on the IDEA sensor surface is based on the use of glycogen to immobilize Con A and also to block undesirable activity of the underlying PEI layer. Glycogen (Gly) is a highly branched polysaccharide which consists of α-D-(1→4) linked D-glucose residues with α-D-(1→6) branch points and reacts easily with Con A at physiological pH [64]. The deposition of multiple layers of Con A and glycogen was performed by layer-by-layer method. As in the previous case the first two layers immobilized on the sensor surface were PEI and Con A. After this the sensor was treated alternately with glycogen and Con A to assemble the desired number of Con A-Gly layers on the device surface. Formation of Con A-Gly layers compensates the surface charges introduced by PEI and produces increase in the surface resistance as shown in Figure 6.

In order to test that thus constructed multilayer structure blocks efficiently the PEI layer positive charge, reaction with polystyrene sulfonate (PSS) polyanion was used. Being able to react with PEI by electrostatic interactions and to reverse the surface charge [50, 52], PSS is widely applied in the layer-by-layer method for deposition of polyelectrolyte multilayers. To demonstrate that the effect of PEI is completely blocked

PEI-Con A-Gly-Con A and PEI-(Con A-Gly)$_2$-Con A structures were formed on the 3D-IDEA surface and were immersed in a solution of PSS for 20 minutes.

As follows from Figure 6, where R_S values determined after each modification step are presented, different response was observed in each of the cases. PEI-Con A-Gly-Con A modified electrodes showed a significant increase in R_S on addition of PSS (Fig. 6A) indicating that PEI positive charges were not totally compensated by Con A-Gly-Con A layers and maintain ability to react with LPS. On the other hand, for sensors modified with additional Con A-Gly double layer (PEI-(Con A-Gly)$_2$-Con A, fig. 6B) no changes in impedance were observed after the PSS addition which confirms the complete isolation of PEI in this case.

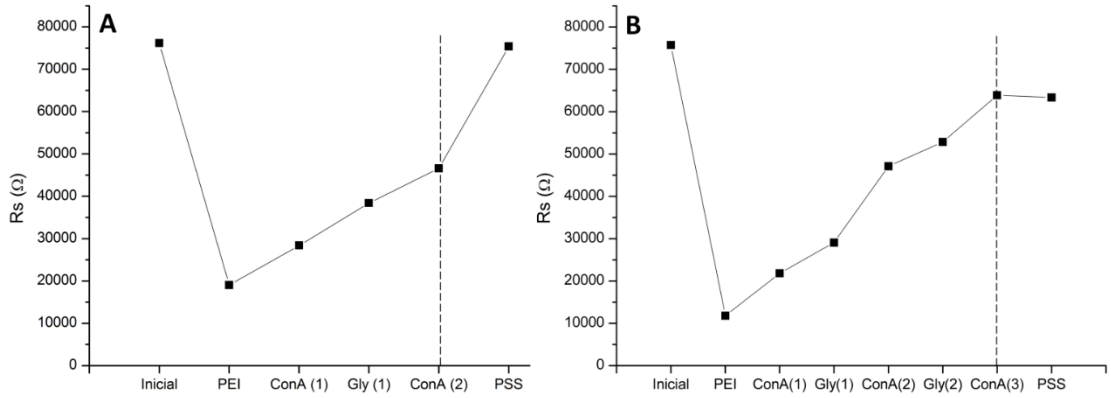

Figure 6. R_S values of the IDEA devices after the successive deposition of surface layers and final treatment with PSS. (A) PEI-ConA-Gly-Con A and(B) PEI-(Con A-Gly)$_2$-Con A.

Therefore, obtained results give the evidence that PEI-(Con A-Gly)$_2$-Con A multilayer structure completely blocks the PEI positive charges. This alternative approach of Con A immobilization may be regarded as a useful robust methodology for a selective bacterial LPS sensor development as presented below.

3D-IDEA sensors with PEI-(Con A-Gly)$_2$-Con A multilayered structure were used for detection of *E. coli* LPS. Figure 7 shows the sensors response at various concentrations of LPS (0-50 µg/mL) in time. It should be noted that kinetics of Con A – LPS interaction is quite fast and the saturation phase is reached in 20 minutes regardless of the LPS concentration. Developed sensors show high sensitivity and even at the lowest tested concentration of 1 µg/mL give a significant response. Modified sensors employed as control in the absence of LPS showed no changes in time, demonstrating the stability of the used multilayer structure.

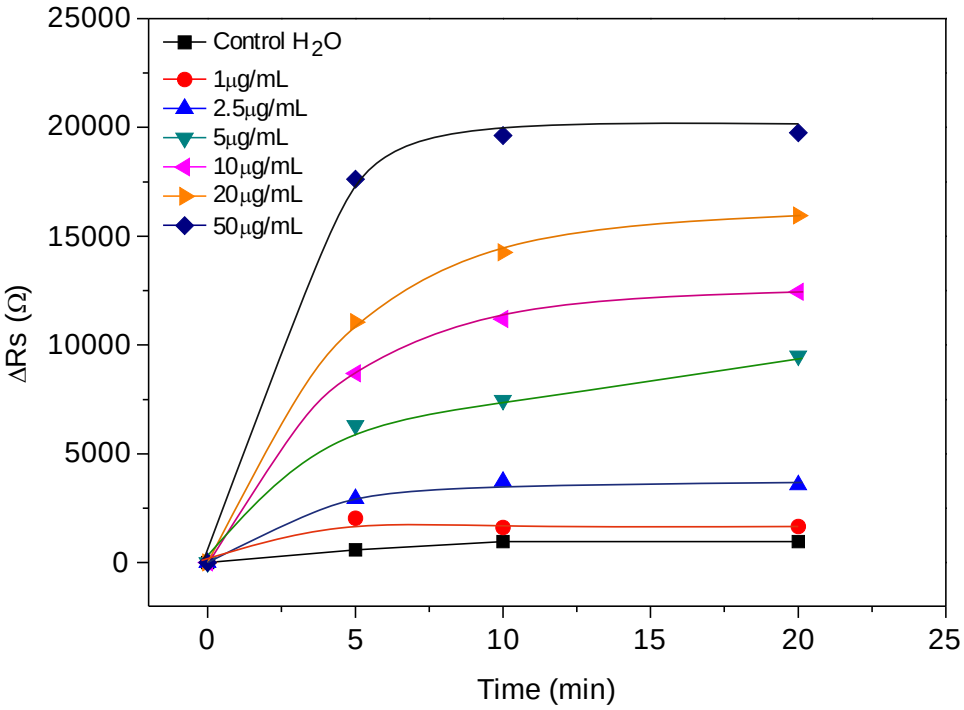

Figure 7. Variation of PEI-(Con A-Gly)$_2$-Con A 3D-IDEA sensors R_S values in time during incubation in solutions with different LPS concentrations (0 (control), 1, 2.5, 5, 10, 20 and 50 μg/mL).

Presented results confirm that the blocking is a crucial step in the development of functionalized surfaces for a biorecognition process in order to avoid non-specific interactions and enhance the selectivity and performance of biosensors based on label-free detection.

3.3. Sensor sensitivity and reproducibility

To evaluate the performance of developed devices 3D-IDEA sensors with PEI-(Con A-Gly)$_2$-Con A layers were used to test sensitivity, reproducibility and the limit of detection (LoD) of bacterial endotoxin from *E. coli*. The interaction between Con A and *E. coli* LPS were monitored during 20 minutes of incubation to reach the saturation phase at which the maximum response is obtained. The resulting response curve is presented in Fig. 8 showing significant sensitivity of the sensor to different LPS concentrations. The sensors response obeys the Langmuir adsorption isotherm typical for irreversible adsorption of analyte species on a solid surface that is usually presented as:

$$n = n_m \frac{bC}{1+bC} \quad (3)$$

where *C* is the concentration of the adsorbate in the solution, *n* is the amount adsorbed species, n_m is the amount of *n* in saturation and *b* is a coefficient. However, Langmuir equation is based on simplified assumptions that do not take into account the possible interaction of the adsorbed molecules and the possible dependence of the chemical activity of the surface active sites with the number of adsorbed molecules. Often adsorption experimental results are fitted with an empirical Hill function [65] which is expressed as:

$$y = Rs_{\max} \frac{x^n}{k^n + x^n} \quad (4)$$

,

where:

- *x* is the concentration of the adsorbate in the solution
- *n* is the Hill coefficient that describes the cooperativity of the ligand binding. If it is more than 1 it means that once one ligand molecule is bound to the surface site, its affinity for other ligand molecules increases.
- $R_{s\ max}$: The maximum R_S value at which saturation occurs and maximum surface concentration is achieved.
- *k*: Ligand concentration producing half occupation.

As shows Fig. 8 the obtained experimental points can be perfectly fitted by the Hill function. The fitting of experimental data with the Hill's equation gives the following parameters: R_S max = 21.2±2 kOhm *n* = 1.2±0.2; *k* = 7.4±1.6 µg/mL. Langmuir type adsorption isotherms may be presented in a semilogarithmic plot thus giving a linear calibration regression of the sensor response. Results presented in the inset of Fig. 8 show that in the studied concentration range of 1 – 50 µg/mL the sensors response is proportional to the logarithmic value of LPS concentration (µg/mL) with a correlation coefficient of 0.975 and with the sensitivity of 11.3 kOhm per LPS concentration decade.

The LoD may be defined as LoD=$3\ S_a/b$, where S_a is the standard deviation of the response and *b* is the slope of the calibration line [66]. The limit of detection of the studied sensors calculated in this way was as low as 1.5 µg/mL, practically the same as the lowest experimental concentration employed. It should be noted that this value is substantially lower than previously published for Con A impedimetric sensors

[16] which showed sensitivity starting from 50-100 μg/mL of LPS or QCM piezoelectric sensor (>50 μg/mL) [21] .

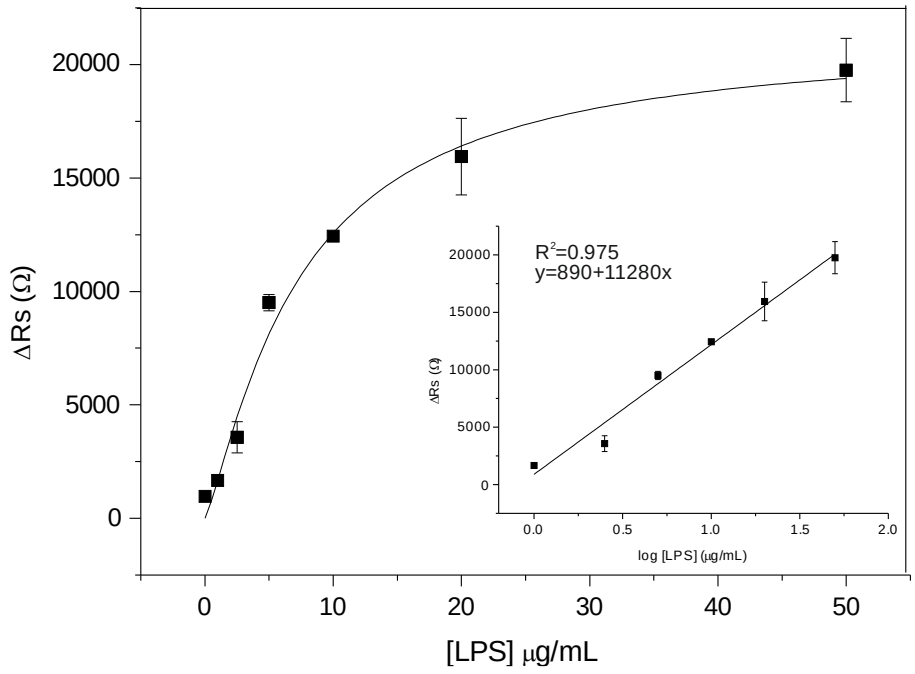

Figure 8. Response of sensors modified with PEI-(Con A-Gly)$_2$-Con A in solutions with different concentration of LPS (squares) and fitted by Hill's equation (line). In the inset - linear calibration regression of electrodes modified with PEI-(Con A-Gly)$_2$-Con A in LPS solutions. Error bars show standard deviation (n=3).

Finally, the reproducibility of the biofunctionalization strategy of the sensor with PEI-(Con A-Gly)$_2$-Con A was evaluated. In each experiment at least three sensors were used. Taking into consideration that the standard deviation of signals of different sensors was ± 3.3 kΩ we may speak about high reproducibility of the developed methodology of sensor preparation.

4. Conclusions

Presented study is focused on the development of new sensing strategies of endotoxins detection using impedimetric transducers with special consideration of the selective response between the biorecognition element and the target analyte.

In this work an impedimetric transducer based on three dimensional interdigitated electrode array was employed as a tool for the detection of the surface charge changes due to interaction of immobilized concanavalin A lectin biorecognition element and bacterial endotoxins (lipopolysaccharides, LPS) of *E. coli*

in test solution. The deposition of Con A on the surface was carried out using the layer-by-layer method with polyethyleneimine polycation as an initial layer. The sensor surface characterization by means of electrochemical impedance spectroscopy technique allowed registering variations in superficial resistance provoked by surface charge changes and is demonstrated as an effective method to monitor sensor parameters at each sensor modification step as well as to follow Con A – LPS reaction. In order to prevent non-specific adsorption of LPS on PEI covered surface different blocking strategies were tested to achieve the specific response between Con A and LPS. The typical blocking compound in many biochemical applications is BSA. However, its effectiveness is not always thoroughly studied. Results obtained in this work clearly show that blocking with BSA is not sufficient to prevent non-specific interactions of PEI and to ensure the selective biorecognition of LPS by Con A. To achieve more efficient PEI blocking a new method was proposed based on consecutive deposition of Con A-glycogen-Con A layers. Sensors modified with PEI-(Con A-Gly)$_2$-Con A multilayers are shown to be highly sensitive, selective and reproducible. The response of thus functionalized impedimetric sensors follows the Langmuir adsorption curve that is perfectly fitted by Hill's equation. The developed sensor permits to detect bacterial LPS in a very short detection time (20 min) with the limit of detection as low as 1.5 µg/mL.

Acknowledgements

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Supplementary Information

Impedimetric label-free sensor for specific bacteria endotoxin detection by surface charge registration

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Table S1. Values of the equivalent circuit parameters obtained from the fitting of impedance spectra. Data are shown as means ±SD (n=3).

	[LPS] (µg/mL)	Rs (kΩ)	CPE-T (nF)	CPE-P (α)
Native SiO2	-	53.6±1.9	3.7±0.9	0.91±0.02
PEI	-	13.6±1.3	3. 8±0.9	0.94. ±0.01
PEI-ConA	-	20.4±2.9	3.9±1.1	0.94±0.01
PEI-ConA-BSA- LPS	-	31.3±4.2	4.2±1.1	0.93±0.01
PEI-ConA-BSA- LPS	1	31.5±1.1	3.8±1.5	0.92±0.03
PEI-ConA-BSA- LPS	2	33.3±0.9	4.9±0.9	0.92±0.01
PEI-ConA-BSA- LPS	5	50.7±4.8	5.3±0.2	0.90±0.01
PEI-ConA-BSA- LPS	10	59.8±3.3	5.9±1.2	0.90. ±0.01
PEI-ConA-BSA- LPS	20	77.8±0.4	5.7±0.9	0.89±0.01
PEI-(ConA-Gly)$_2$-ConA	-	64.6±0.4	5.1±0.3	0.89±0.01
PEI-(ConA-Gly)$_2$-ConA	1	66.2±0.2	5.5±0.1	0.89±0.01
PEI-(ConA-Gly)$_2$-ConA	2,5	68.1±0.6	5.4±0.1	0.89±0.01
PEI-(ConA-Gly)$_2$-ConA	5	74.1±0.3	5.3±03	0.89±0.01
PEI-(ConA-Gly)$_2$-ConA	10	77.1±1.7	5. 6±0.1	0.89±0.01
PEI-(ConA-Gly)$_2$-ConA	20	80.5±2.6	5.7±0.1	0.88±0.01
PEI-(ConA-Gly)$_2$-ConA	50	84.3±1.4	6.8±0.4	0.85±0.01

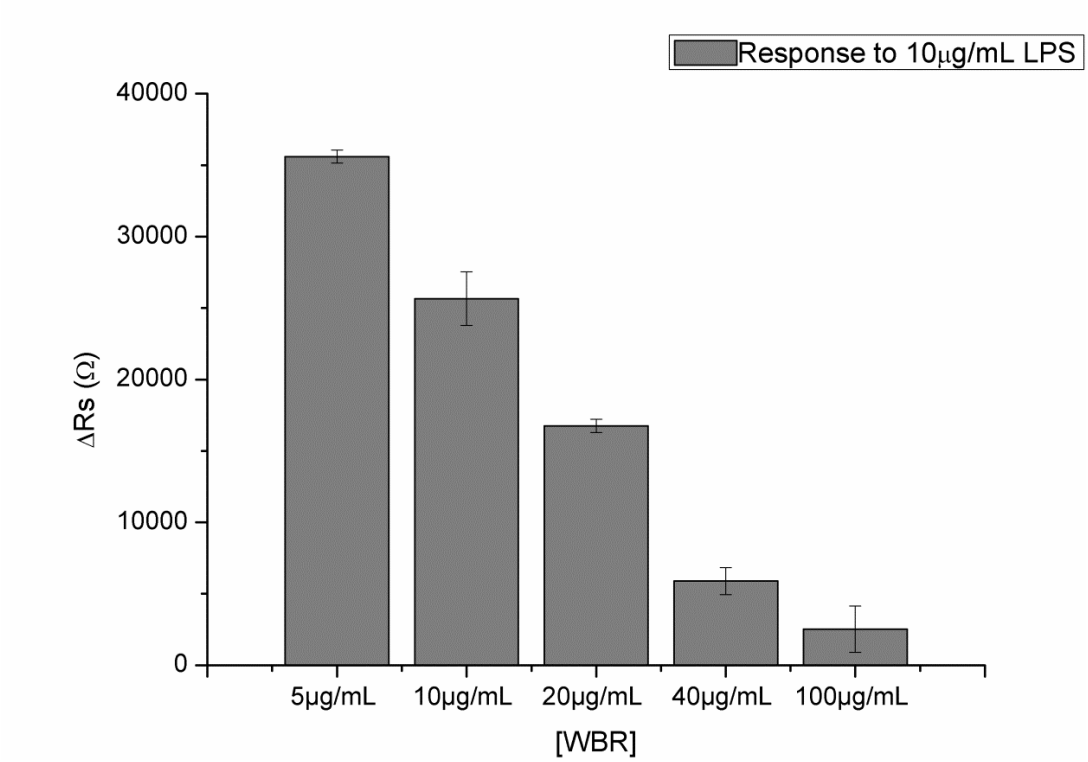

Figure S1. Response of PEI-WBR modified 3D-IDEA with different WBR concentration after 1-hour incubation in a 10 μg/mL *E. coli* LPS solution. Error bars show standard deviation (n=3).