P. L. Cabot; A. Bratov; N. Abramova

Dr. Pere L. Cabot Julià
Departament de Química Física

Dr. Andrei Bratov
IMB-CNM-CSIC

Dra. Natalia Abramova

IMB-CNM-CSIC

Grau de Química

Treball Final de Grau

Sensivity and response of modified impedimetric transducer for bacteria toxins detection

Sensibilitat i resposta de transductors impedimètrics modificats per a la detecció de toxines de bactèria

Dmitry Galyamin

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Universitat de Barcelona

Campus d'Excel·lència Internacional B:KC Barcelona Knowledge Campus

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REPORT

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1. SUMMARY

In this work, an impedimetric transducer based on a three dimensional interdigitated electrode array (3D-IDEA) was used to study interactions with gram-negative bacteria endotoxins from *Escherichia coli* in a sample solution. Bacteria endotoxins mainly belong to a lipopolysaccharide (LPS) chemical class. LPS immobilization on the sensor surface affects the surface charge and produces changes in the superficial resistance which is possible to detect using impedance spectroscopy technique. The sensor surface was chemically modified with polyethilenimine (PEI) or epoxysilane. As a biorecognition element for bacterial endotoxin detection, lectin Concanavalin A (ConA) was deposited on the IDEA surface through PEI-ConA or epoxysilane-ConA interactions. To prevent non-specific adsorption of LPS different ways of surface blocking were studied. However, different types of blocking agents tested did not permit to enhance the specificity of the sensor.

The best results on selective detection of LPS were achieved by modifying surface with Concanavalin A – Glycogen multilayers. Detection limit of the sensor with PEI-(ConA-Gly)$_2$-ConA was found to be as low as 1µgLPS/mL in *E. coli* endotoxins with response time around 20 minutes. The sensor responses follow the Langmuir adsorption curve (type I) and can be perfectly fitted by Hill's equation.

Keywords: Biosensors, Impedimetric sensors, Bacteria endotoxins, Surface modification, Electrochemical impedance, Interdigitated electrode array

2. RESUM

En aquest treball s'ha utilitzat un transductor impedimètric basat en un elèctrode interdigitat de tipus tridimensional (*3D-IDEA*) per estudiar la interacció amb endotoxines del bacteri gram-negatiu *Escherichia coli* en solució. Les endotoxines dels bacteris són d'un tipus de compost químic anomenat lipopolisacàrids (LPS). La immobilització del LPS en la superfície del sensor afecta a la càrrega de la superfície i produeix canvis en la resistència superficial, de manera que és possible detectar aquest canvis emprant la tècnica d'espectroscòpia per impedància. La superfície del sensor ha sigut modificada mitjançat polietilenimina (PEI) o epoxisilà. Com a element de bioreconeixement per a la detecció de l'endotoxina s'ha utilitzat Concanavalina A (ConA), que ha estat depositada a la superfície dels elèctrodes mitjançant una interacció PEI-ConA o epoxisilà-ConA. Per a prevenir adsorcions inespecífiques dels LPS s'ha estudiat diferents vies per a bloquejar la superfície. Alguns agents bloquejants estudiats no permeten millorar l'especificitat del sensor.

Els millors resultats per a la detecció selectiva de LPS es van aconseguir modificant la superfície amb multicapes de Concanavalina A – Glicògen. El límit de detecció del sensor amb PEI-(ConA-Gly)$_2$-ConA va ser d'1µg LPS/mL amb endotoxines de *E. coli* detectades en un temps de 20 minuts. La resposta del sensor té una tendència de tipus Langmuir i pot ser perfectament ajustada amb una equació de Hill.

Paraules clau: Biosensors, Sensors Impedimètrics, Endotoxines de bacteris, Modificació de superfície, Impedància electroquímica, Elèctrodes interdigitats

3. INTRODUCTION

The detection of molecular interactions at the solid/liquid interface play an important role in many systems, even so when we go to micro- or nano-scale and is a great interest for a wide range of bioanalytical applications in medicine, drug industry of biosensors, genetic, etc. [1,2]. Many of these applications require rapid detection of low concentrations of microorganisms in liquid samples for water contamination control, food safety or biological control [3]. Conventional methods based on biological assays which include whole-animal tests, part-animal tests and cell culture or some methods based on immunological assays which include immunodiffusion test, commercial agglutination kits, Enzime-linked immunosorbent assay (ELISA), etc. are time consuming and non-cost-effective [4], so, there is a great demand for rapid, cheap and effective biosensors. Among different bioanalytical techniques, electrochemical impedance spectroscopy (EIS) has gained much interest of biochemists due to its sensitivity to biomolecular recognition that occurs on the surface of metal or semiconductor electrodes modified with enzymes, antibodies or DNA molecules [5].

3.1. IMPEDANCE TECHNIQUE

Impedance Spectroscopy (IS) is a method generally used for characterization of the electrical properties of materials and their interfaces. It may be used to investigate the dynamics of bound or mobile charge in the bulk or interfacial regions of any kind of solid or liquid material [6].

Electrical resistance is the ability of a circuit element to resist the flow of electrical current. Ohm's law defines resistance in terms of the ratio between voltage *V* and current *I*.

$$R = \frac{V}{I} \tag{1}$$

It is virtually always assumed that the properties of the electrode-material system are time-invariant, but the real world contains circuit elements that exhibit much more complex behavior. These elements force us to abandon the simple concept of resistance. In its place we use

impedance. Electrochemical impedance is usually measured by applying an AC potential to an electrochemical cell and measuring the current through it [7].

The excitation signal, expressed as a function of time, has the form:

$$V(t) = Vo\cos(wt) \tag{2}$$

V(t) is the potential at time *t*, *Vo* is the amplitude of the signal, and *w* is the radial frequency. The relationship between radial frequency *w* (radians/second) and frequency *f* (Hertz) is:

$$w = 2\pi f \tag{3}$$

The response signal, *I(t)*, is shifted in phase and has different amplitude, *Io*:

$$I(t) = Io\cos(wt - \varphi) \tag{4}$$

An expression of impedance analogous to Ohm's law is:

$$Z = \frac{V(t)}{I(t)} = \frac{Vo\cos(wt)}{Io\cos(wt-\varphi)} = Zo\ \frac{\cos(wt)}{\cos(wt-\varphi)} \tag{5}$$

Using Euler's relationship and Fourier transform it is possible to express the impedance with a real and an imaginary part [6].

$$Z = \frac{V(t)}{I(t)} = Zo\exp(j\varphi) = Zo\ (cos\varphi + j\ sin\ \varphi) \tag{6}$$

In all cases φ gives the phase difference between voltage and current. If the real part is plotted on the Z axis and the imaginary part on the Y axis of a chart, we get a "Nyquist plot". See Fig. 1.

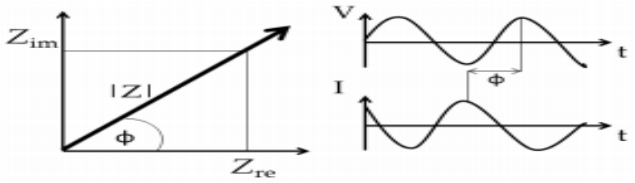

Figure 1. Complex impedance plane and relation of voltage, current and phase.

The most difficult part of IS is the correct interpretation of spectra which can be analyzed with an equivalent circuit (EC). This circuit consists of different resistors and capacitors

combined in serial or in parallel, and typically more than one circuit can be fitted equally well. However, it is possible to choose the correct EC for a studied physico-chemical system by the variation of certain system properties finding correlation of individual EC component impedance changes [8]. Each component of the EC presents some concrete parameter of the system or the occurring processes.

3.2. IMPEDIMETRIC TRANSDUCER

There are three different types of electrochemical transducers applied for biosensors: potentiometric, amperometric and impedimetric. Potentiometric sensors based on ion-selective and ion-sensitive electrodes, monitor ion concentration changes resulting from an enzymatic bioreaction. Amperometric biosensors, working in a two or three electrodes configuration, measure at a certain applied potential the resulting current proportional to concentration of electroatractive species generated by some biochemical reaction. Impedimetric transducers register impedance changes that a biochemical reaction may produce on the surface of the sensor electrode affecting resistance (*R*) and/or capacitance (*C*).

Basically, for IS performed on a metal electrode in an electrolyte solution and in the presence of electroactive compounds, the elements of the EC are well known from general electrochemistry and include: ohmic resistance of electrolyte, R_S (the bulk medium resistance), double layer capacitance, C_{DL}, charge transfer resistance; R_{CT}, and the Warburg impedance, Z_W, as it is presented in Fig. 3A. For more complex experimental systems additional components, like dielectric capacitor, polarization resistance, constant-phase element, interfacial impedance, coating capacitance, stray capacitance and virtual inductors, may be required to include.

Impedimetric detection can be achieved either in a direct manner in an analyte solution or in the presence of an additional redox probe used as a marker. In the presence of electron mediator as $Fe(CN_6)^{3-/4-}$ (ferricyanide/ferrocyanide) or $Ru(NH_3)_6^{3+/2+}$ (hexaammineruthenium III/II ions), the impedance is termed Faradaic impedance. In Faradaic impedance measurements the main parameter is the charge transfer resistance that depends on the interface blocking by surface products of biochemical reactions and thus may be used to measure concentration dependencies.

In the absence of a redox pair or if its charge transfer rate on the electrode is very slow no Faradaic process occurs and subsequent electron-transfer is not produced. In these cases, the interfacial capacitance changes are often studied. These capacitance changes occur when the dielectric constant or the thickness of the interfacial capacitance layer on the transducer surface change their values due to surface chemical reactions. Formation of biochemical reaction products may be represented by an additional capacitor that depending on the process may be included in parallel or in series with the double layer capacitor. Different kind of electrodes, especially interdigitated electrodes arrays (IDEA), differing in their geometry and immobilization strategies can be used as impedimetric biosensors.

IDEA transducers present promising advantages compared to other impedimetric biosensors as rapid detection kinetics, increase of signal-to-noise ratio, fast establishment of a steady-state response, potential low cost and ease of miniaturization. Moreover, IDEA eliminates the requirement of a reference electrode compared to three or four electrodes systems or potentiometric and amperometric devices. IDEA devices consist of a pair of comb-like metal electrodes formed on a planar insulating substrate, in which a series of parallel microband electrodes are connected together by a common bus, forming a set of interdigitating electrode fingers. (see Fig. 2) [1-2, 9-10].

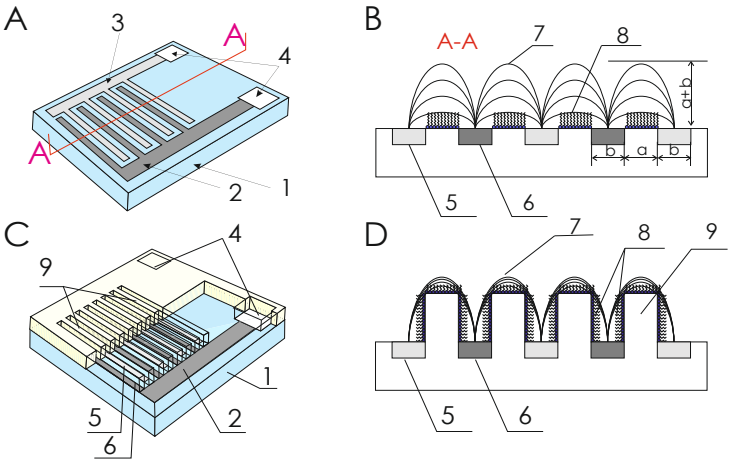

Figure 2: Planar interdigitated electrode array(A) and its cross-section(B) and 3-dimentional interdigitated electrode array (C) and its cross-section (D).1 - insulating substrate; 2,3 - electrodes collector bars; 4 - contact pads ;5,6 - electrode “digits”; 7 - electric field lines; 8 – immobilized biomelecules; 9 – insulating barrier. [13]

Parameters of impedimetric [11-13] sensors based on interdigitated electrodes depend on the electrodes geometry, i.e. dimensions and interspacing (parameters a and b on Fig. 2B). It is generally accepted that 80% of the total signal is enclosed in a certain region close to the electrode surface. Electric field penetrates within the distance equal to the distance between centres of two adjacent electrode digits (5 and 6, Fig.2), as shown in Fig. 2B, where the electric field lines are schematically marked. Typical biomolecule length is within the range of 1-100 nm, so the gap between the IDEA digits should be of the same order of magnitude or the effect of this layer properties on the sensor parameters will be negligible.

Taking into consideration the distribution of the electric field between adjacent electrodes of an IDEA sensor it seems reasonable to separate the electrodes with an insulating barrier making a three-dimensional interdigitated electrode array (3D-IDEA) so that under applied electric potential difference the main portion of the current will go not through the surrounding solution but close to the surface of the barrier as it is shown in Fig.2D. This permits [13] to enhance the sensitivity of the device for biochemical reactions of biomolecules attached to the surface of the barrier. This 3D-IDEA sensor presented considerable improvements in sensitivity compared with a standard planar IDEA design, resulting in a viable option for integrated biosensing applications. This type of sensors typically reacts on the surface charge changes, because the main part of the signal depends mainly on close the surface (Fig. 2). IDEA are widely used as impedimetric biosensors for bacterial detection because bacteria cells are normally negatively charged [8].

3.2.1 Advantages and disadvantages of IDEA transducers

IDEA transducers present promising advantages compared to other impedimetric biosensors as rapid detection kinetics, potential low cost, easy miniaturization… IDEA transducers present advantages in front of potentiometric or amperometric sensors as they do not require for measurements a stable reference electrode [9] because both IDEA electrodes have the same surface area and are made of the same material, so it is assumed that there is no DC voltage difference between the electrodes that may cause additional electrochemical reactions in the system. However, these devices present also some disadvantages, as biomolecules immobilized on electrodes surface suffer lack of stability in contact with harsh

environments reducing the lifetime of biofuncionalitzed systems. Another problem is to establish a uniform and reproducible layer of bioreceptors on the sensor surface.

3.2.2 Equivalent circuit and sensors parameters

In a 3D-IDEA the AC potential difference applied between two electrodes causes electrical current to flow between the electrodes from one capillary, formed by the barrier walls, to another. The impedance response of an IDEA device in low conductivity solutions may be emulated by an electrical equivalent circuit presented in Fig. 3C. It is formed by the following components: R_c is the contact resistance introduced by wires and collector bars of the electrodes; C_{ids} is the geometrical capacitance between two interdigitated electrodes in a water solution; R_s is the resistance of the water solution between two electrodes of the array; CPE_{dl} which is a constant phase element associated with the capacitance of the electrical double layer at the electrode water interface [9].

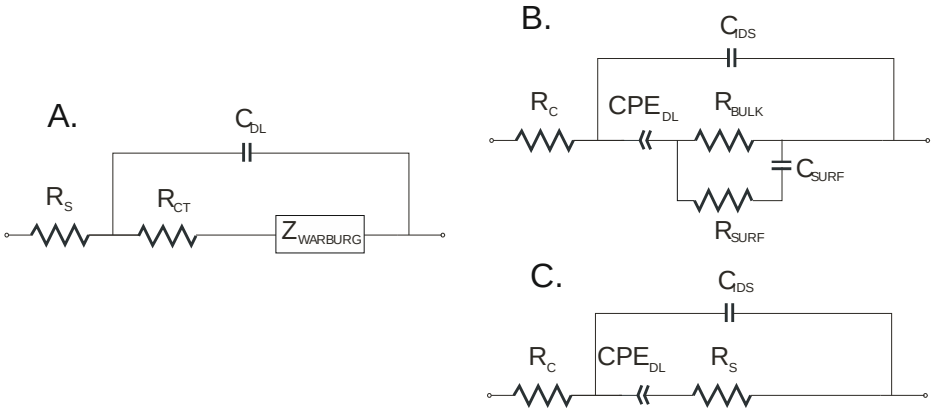

Figure 3: Electrical equivalent circuits: Randels (A) and 3D-IDEA device in and highly (B) and low conducting(C) solutions. EC elements are defined in the text.

Bratov et al. [9] have shown that the 3D-IDEA equivalent circuit contains additional surface elements, R_{surf} and C_{surf}; (Fig. 3B). In highly conducting solutions it is possible to determine R_{sol} and R_{surf} independently [14]. It is because when electric field is applied, due to a higher concentration of ions within the electrical double layer, the local electric surface current can be higher than typical of the bulk, and it alters the surface conductivity. But in low conducting

solutions it is not possible to resolve the surface components from the spectra. However, it should be always taken into account that the determined value of R_s (Fig. 3B, 3C) is the parallel combination of R_{bulk} and R_{surf}. If we perform the measurements at a fixed bulk solution conductivity (R_{sol} = constant), any changes in R_s should be attributed to R_{surf} changes.

Fig. 4 shows Nyquist plot of experimental impedance spectra. Semicircle at high frequencies appears due to resistance Rs in parallel to the stray capacitance. Its interception with the Z' axes on the left side gives the value of Rc but the interception on the right side gives the value of Rs. As an example, in Fig. 4 we can see a modification of native SiO_2 sensor surface with highly charged polyelectrolytes that gives rise to lower surface resistance. The sensor response was determined as changes in resistance Rs of a sensor before and after the surface reaction, for example:

$$\Delta Rs = Rs^{Nat.} - Rs^{PEI} \qquad (7)$$

When interdigitated sensors are used for conductivity measurements in solutions it is assumed that the dependence of the measured resistance Rs on the solution resistivity is linear in the whole range with a slope defined by the sensor cell constant [2]. As revealed data presented in the insert in Fig. 5 this dependence is linear only when the devices are measured in solutions with reasonably high conductivity. However, in our case the initial device with clean SiO_2 surface shows significant nonlinearity of its response in KCl water solutions of low conductivity. As follows from Fig. 5 adsorption of PEI on the sensor surface notably affects the resistance Rs decreasing its absolute value in solution with low conductivity. However, it may be noted that in solutions with high conductivity, the adsorption of polyion does not affect the overall resistance Rs determined from the semicircle in high frequency range. Obtained results clearly show that surface conductivity becomes dominant when the bulk solution conductivity is low. For that reason, all the experiments will be measured in 10^{-5}M of KCl (2.5μS/cm) as in this conductance range the sensitivity of the system to surface charge changes is the highest. If the surface charge of the sensor is close to zero, then the Rs – ρ dependence will be linear, as presented in Fig. 5, and only highly charged molecules attached to the surface may cause significant changes in impedance.

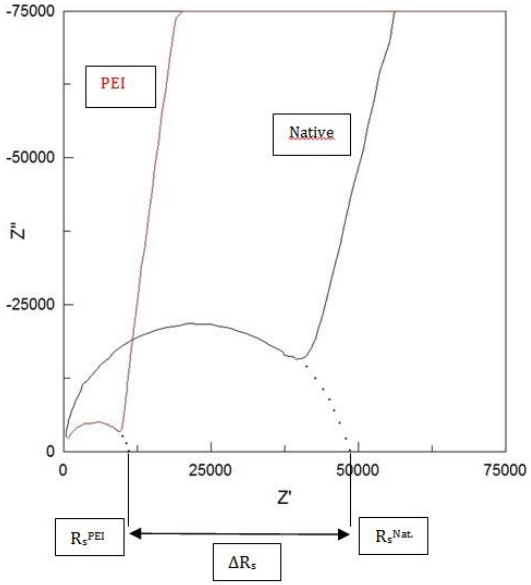

Figure 4: Impedance spectra of the 3D-IDEA surface after modification step measured in 10^{-5}M KCl solution

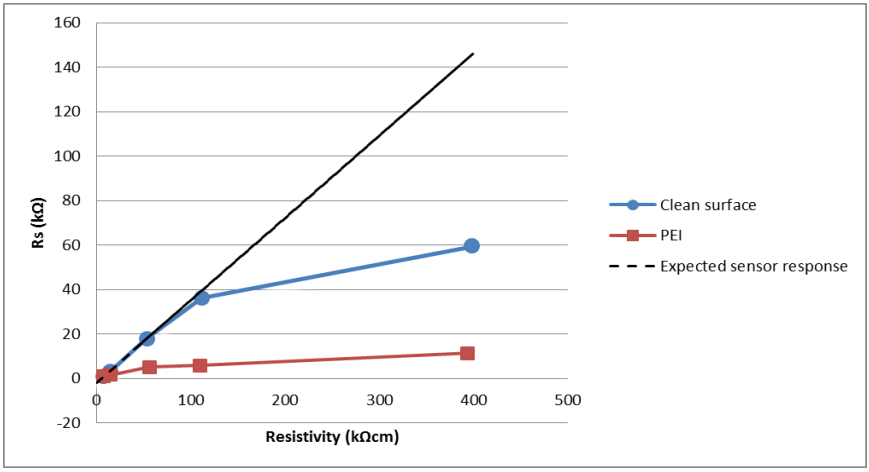

Figure 5: Resistance Rs determined from the impedance spectra in KCl solutions with different concentration by fitting the spectra to the equivalent circuit. Blue line corresponds to a sensor with clean SiO_2 surface, red line correspond to a sensor modified with PEI, dashed black line is the expected sensor response under zero surface charge conditions.

3.3. BACTERIA TOXIN DETECTION

Lipopolysaccharide (LPS) is the main component in the outer membrane of Gram-negative bacteria and it is known as a major factor of responsible for toxic manifestations [15]. To make LPS detection more selective the use of *Concanavalin A* (*ConA*), a glucose-binding lectin that reacts specifically with terminal LPS, was proposed as a biosensor biorecognition element, which is highly selective to *E. coli* bacterias [16-20].

The capture of bacteria on a solid surface by electrostatic interactions with positively charged polyethylenimine (PEI) polyelectrolyte was studied previously, and it improves bacteria immobilization by electrostatic interaction [3,21]. PEI has also been studied on IDEA transducers to detect target charged analytes in solution. The surface of $TaSi_2$ and SiO_2 barrier is negatively charged due to the presence of ionized surface OH groups. From the electroneutrality, the negative surface charge has to be compensated by cations from the bulk solution and that is the reason why PEI, which is positively charged at pH 10 and below, interacts with the surface [22].

Another method of immobilization is a surface grafting using a (3-glycidyloxypropyl)trimethoxysilane (epoxysilane), which is widely used for chemical surface modification of metal oxide surfaces forming a dense, homogeneous and complete self-assembled monolayer (SAM) due to reaction between OH groups of the oxide surface and the silane etoxy groups. This method was used to graft the sensor surface with anti-*Escherichia coli* antibody because epoxysilane has an epoxy group that readily reacts with amino groups of biological compounds [23].

One of the problems in a biosensor development is the lack of selectivity due to non-specific adsorption on the sensor surface. Normally, the grafted specific receptor molecules do not cover the entire sensor's surface and molecules from the sample solution may attach to these non-specific sites of PEI or epoxysilane in our case. There are a lot of different ways to block these non-specific sites and the reagents are typically chosen empirically, but one can choose a blocking reagent basing on the type of the surface, the biomolecule that will be immobilized and the experimental conditions [24]. Several studies use different agents that are proteins, as Bovine Serum Albumine (BSA) [24-25] or commercial Blocking solution mixture. Another way to

protect non-occupied sites is to use a negatively charged electrolyte as Poly(sodium-4-styrene sulfonate) (PSS), which has been studied directly to PEI [2]. A different possibility to protect non-occupied sites is to add Glycogen (Gly). Glycane is a branched poly(glucose) and it interacts with ConA and in this way it is possible to construct multi-layers of Gly-ConA. This kind of layers with ConA have been studied previously [23, 26-28].

4. OBJECTIVES

The main goal of this project is to study sensitivity and response of electrochemical transductors based on IDEA transducers to detect bacteria endotoxins, more concretely lipopolysaccharides (LPS) which are extracted from bacteria *E. coli*, using the impedance technique. In order to introduce a biorecognition element for bacterial detection it is proposed to use lectin Concanavalin A. This goal may be achieved by modifying the sensors' surface with polyethilenimine (PEI) or with epoxysilane and immobilize Con A either by electrostatic interaction or by direct covalent grafting. To increase specificity of the bacteria recognition event it is proposed to study different strategies of using blocking agents or the use of different layers with ConA and glycan. Finally, the designed sensor should be characterized in terms of its sensitivity, detection limit and adjust of equation.

5. EXPERIMENTAL SECTION

5.1. ELECTRODE FABRICATION

Our device consists on a chip part, package and connectors. The chips are made on an oxidized silicon wafer as a substrate with a 2.5μm thick thermally grown SiO_2 layer and it is fabricated in a clean room in IMB-CNM-CSIC. The chips were designed and fabricated by the researchers of the BIOMEMs group of the IMB. On every wafer there are IDEA chips. Each chip with IDEA sensor has 216 electrode fingers of $TaSi_2$, every finger are separated by a 4μm

height dielectric barrier made of SiO_2 (see Figs. 6 and 7A). The gap between two adjacent electrode fingers as well as the width of an individual electrode finger amounted to be 3µm [1,13].

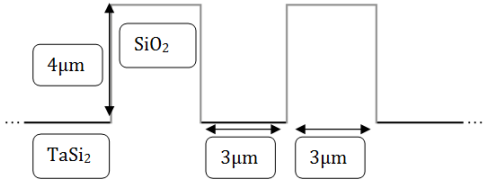

Figure 6: Scheme of barriers in 3D-IDEA.

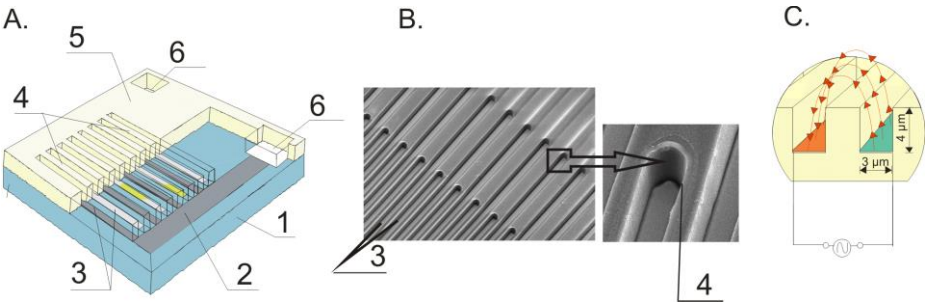

Figure 7: Scheme of a 3D IDEA. 1- SiO_2 substrate. 2- Connectors. 3- electrodes made of $TaSi_2$. 4- SiO_2 barriers. 5- SiO_2. 6- Aluminium contact pads. (image from Bratov et al. [13])

Finally, after being cut from the wafer, the sensor are glued to a PCB substrate and are wire bonded for electrical connections. Contact pads and wires were encapsulated using epoxy resin.

5.2. CHEMICAL MODIFICATION OF THE SENSORS

5.2.1. Surface modification

Cationic polyethylenimine (PEI, branched, average molecular weight 25000, water-free from Sigma-Aldrich) and (3-glycidyloxypropyl)trimethoxysilane (epoxysilane, liquid from Sigma-

Aldrich) were used in some experiments to modify the surface. Before each deposition IDEA sensor was cleaned in distilled water. To perform deposition of PEI on sensors surface they were immersed once into a PEI solution (1.5 mg/mL in distilled water, weighing 15 mg of PEI onto beaker in analytical balance and adding twice 5mL of H_2O with µ-pipette). After each modification with PEI sensors were rinsed with water and dried with a nitrogen flow.

To deposit an epoxysilane monolayer a solution of epoxysilane was transferred to a beaker and heated to 50ºC; then the electrodes were fixed close to epoxysilane solution but without immersing them. Finally, the beaker was covered with a parafilm and was left for 60 minutes at 50ºC.

5.2.2. Immobilization of Concanavalin A

In the experiments, Concanavalin A (ConA, from *Canavalia ensiformis*, from Sigma-Aldrich) was used from two different commercial lots that most probably differed in their activity. ConA from both lots was deposited in the same way. However, optimized time and concentration of ConA solution was different in each case.

Sensors with a PEI layer were put in contact with ConA (100µg/mL solution in TRIS-HCl buffer in 1mL Eppendorf tube, pH 7.4 for 90 minutes in case of ConA with lot SLBC65 and 25µg/mL solution in TRIS-HCl buffer in 1mL Eppendorf, pH 7.4 for 60 minutes in case of ConA with lot SLBL3798V).

5.2.3. Surface blocking with PSS, BSA, LPS, commercial "Blocking" reagent and Gly

After PEI or epoxysilane surface modification and reaction with ConA, different compounds were used to block the non-occupied surface by ConA, among different blocking agents: anionic poly(sodium 4-styrenesulfonate) (PSS, average molecular weight 70000, water-free from Sigma Aldrich), Bovine Serum Albumine (BSA, from Sigma Aldrich), "Blocking" (proteins from milk, Sigma Aldrich) and Glycogen (Gly, from Sigma Aldrich).

Treatment with PSS was peformed by immersing the electrodes into a PSS solution (2mg/mL in distilled water) for 20 minutes. For BSA treatment the electrodes were immersed into a BSA solution in TRIS-HCL buffer (pH 7.4), the solution concentration varied in the experiments and was in the range of 10 to 100µg/mL. The time of immersion was studied previously and took 20 min to stabilize the response. In the same way the "Blocking" solution

was prepared, the mixture was dissolved in TRIS-HCL buffer pH (7.4) at 50ºC. Studied concentrations were in the range of 10 to 100µg/mL and the treatment lasted up to 60 minutes. Experiments with glycogen were performed in Gly solutions (100µg/mL in TRIS-HCl buffer pH 7.4 in Eppendorf tube during 60 minutes) was according to procedure reported by Y. Lvov et al. [21]. There is no need to use buffer solution for Gly deposition; however, the buffer was added to guarantee that ConA molecular structure is not affected.

Sensors response to lipopolysaccharide (LPS, from E. Coli 055:B5 from Sigma Aldrich) was studied in LPS solutions prepared in TRIS-HCl, pH 7.4.

5.2.4 Preparation of KCL·10^{-5}M and TRIS-HCl pH 7.4

10^{-5}M KCl solution was prepared each time before the measurements by the dilution from the 10^{-3}M of KCl solution, putting with µ-pipette 2 mL of 10^{-3} M KCl into a 200mL volumetric flask and adding distilled water.

The solution of TRIS-HCl was prepared weighing 3g of TRIS on analytical balance and dissolving it in 200 mL distilled water. After that, 15 mL of 0.1M HCl were added and the solution was brought to the volume of 500mL with distilled water.

5.3 MEASUREMENT

As KCl 10^{-5}M increases conductivity very easily, before each experiment conductivity of KCl was always measured ranging from 2.50 to 2.52 µS/cm. The conductivity was controlled with a commercial conductimer EC-Meter GLP 31+ (Crison).

All experiments were carried out by impedance measurements in a 100Hz-1000kHz frequency range with 100mV (amplitude) voltage excitation using QuadTech 7600 Plus LCR Meter. Z-Plot/Z-View software package was applied for impedance data treatment and an equivalent circuit fitting. After that, Excel (Microsoft Office) was used to arrange and plot the data.

All experiments were performed in parallel at least on two electrodes. Corresponding results presented in this work are mean values obtained in each case.

6. RESPONSE OF THE SENSOR MODIFIED WITH PEI AND CONA

Modification of sensors with PEI and its interaction with bacteria *E. coli* were studied previously [3]. Sensors modified with PEI-ConA layers react with LPS, how it is shown in Fig. 8. However, without ConA, sensors show the same response to LPS, which means that the addition of ConA does not lend specificity to the sensor response and that non-occupied sites of PEI have to be blocked to guarantee specificity. The first method that we have applied was to modify the surface with PEI, then with a ConA monolayer and after that with a blocking agent (see Fig. 9). Finally, the sensor response to LPS was studied.

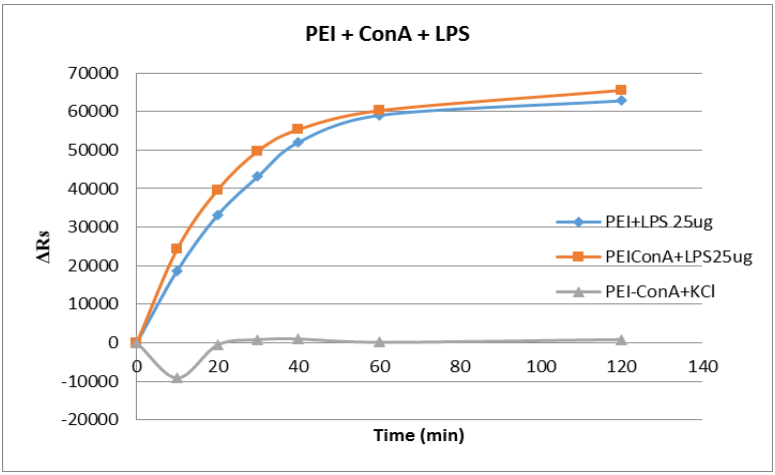

Figure 8: PEI (blue) and PEI-ConA (orange) sensors response (ΔRs) in 25μg/mL LPS solution Grey points refer to PEI+ConA sensor in the absence of LPS

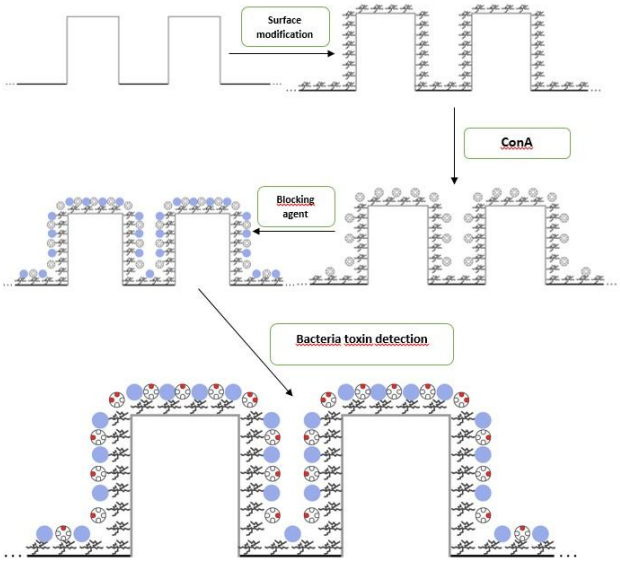

Figure 9: Scheme of steps used for sensor surface modification, blocking of non-occupied sites and LPS detection.

6.1 USE OF BSA TO BLOCK NON-OCCUPIED SITES

Bovine Serum Albumin (BSA) is commonly used as a blocking agent in biochemical experiments [24-25]. To assure the quality of the blocking we should guarantee that BSA does not interact directly with the analyte, LPS in our case. To study LPS-BSA interaction the sensor with native SiO_2 surface, without being modified with PEI, was immersed directly into BSA solution and then in to LPS. Fig. 10 presents ΔRs changes in time. After the first 45 min sensors modified with BSA were put in contact with LPS solutions of two different concentrations, 10µg/mL and 100µg/mL, during 20 minutes. The experiment confirmed that there is no LPS-BSA interaction, because after the stabilization of the sensors response in BSA solution and subsequent exposure to LPS, there are no Rs changes. It may be noted poor reproducibility of BSA adsorption on the silicon oxide surface

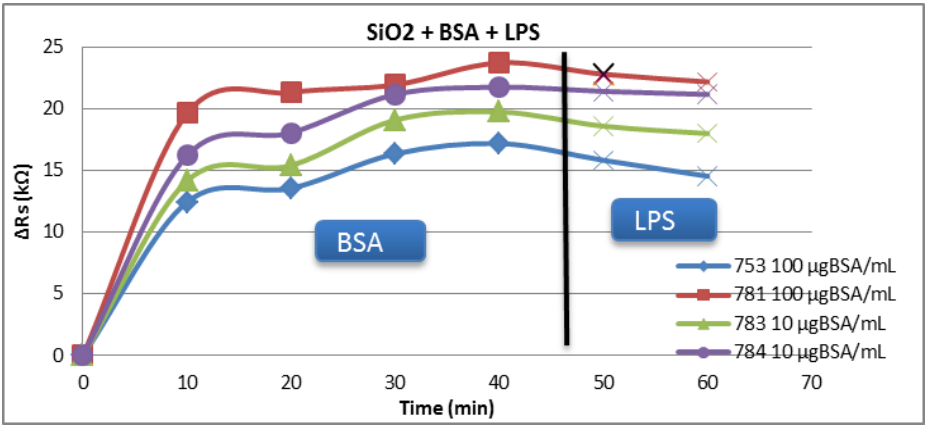

Figure 10: Sensors response, ΔRs, in time during modification with BSA (t 0-40 min) and reaction withLPS. Blue and Red lines correspond to electrodes modified with 100µg/mL of BSA and red and violet with 10µg/mL of blocking.

To check the blocking effectiveness of BSA in the presence of the PEI, the electrodes modified with PEI-ConA layers and only with PEI were subjected to blocking with BSA. Afterwards, these sensors were brought in contact with LPS (100µg/mL) solution. Fig. 11 shows the sensors response, ΔRs, to LPS in time from which it follows that both types of sensors show sensitivity to LPS. This means that blocking effectiveness of BSA in case of PEI modified surface is poor. However, it can be seen that sensors modified with ConA show higher sensitivity than those only with PEI. It could be possible to increase BSA concentration or deposition time, but this will decrease significantly the sensitivity of the IDEA device. Also, as it was noted earlier, a problem with reproducibility of BSA adsorption in different experiments was observed.

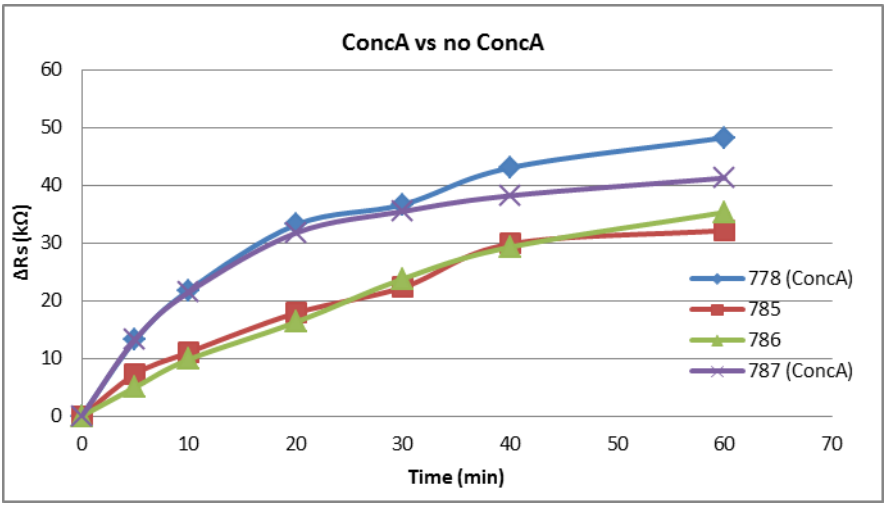

Figure 11: Response of sensors modified with PEI (red and green lines) and PEI-ConA (blue and violet) with BSA (1µg/mL and 20 minutes) used as a blocking agent.

6.2. USE OF PSS TO BLOCK NON-OCCUPIED SITES

As BSA was shown to be not suitable for blocking non-specific PEI sites and in order to find an agent that could block PEI-LPS interaction it was proposed to use poly(sodium 4-styrenesulfonate) (PSS). It was demonstrated that PSS, having an opposite charge than PEI, can be deposited on the surface modified by PEI [3]. As in the case of BSA the electrodes modified with PEI-ConA layers and only with PEI were subjected to blocking with PSS. After that, the electrode response in time was measured in 100µg/mL LPS solution.

Fig. 12 shows difference in ΔRs of the sensors blocked with PSS in the presence of LPS in time. Obtained results show that, as in the case of BSA, PSS cannot be used as a blocking agent because sensors modified with PEI-PSS show sensitivity to LPS. We also should notice that, as with the BSA, there is a difference in response between electrodes with ConA and electrodes without ConA.

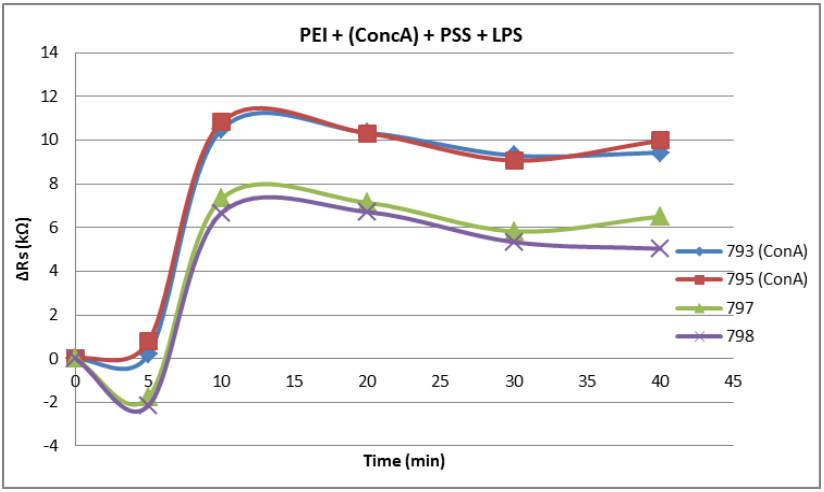

Figure 12: Response of sensors modified with PEI (violet and green lines) and PEI-ConA (blue and red) with PSS (2µg/mL 20 minutes) used as a blocking agent

6.3. USE OF "BLOCKING" TO BLOCK NON-OCCUPIED SITES

Presented experimental results show that BSA and PSS are not 100% effective in blocking the PEI modified sensor surface. In order to find a good blocking agent, it was proposed to use a new product from Sigma-Aldrich, a "BLOCKING" reagent that is a mixture of proteins from cow milk. First of all we tried to find an optimal concentration to stabilize the sensors signal when electrodes are submerged in the blocking solution. Experiments were performed with PEI modified sensors in BLOCKING solutions with 10 and 100µg/mL concentration. Fig. 13 presents absolute Rs value obtained in each measurement. In solutions of 100µg/mL the signal stabilizes in approximately 20 minutes and sensors show no response to LPS. However, the absolute R_S values become so high that the sensors can not measure possible differences in surfaces modifications. At 10µg/mL of BLOCKING saturation does not occur, and when LPS is added the positive drift still continues.

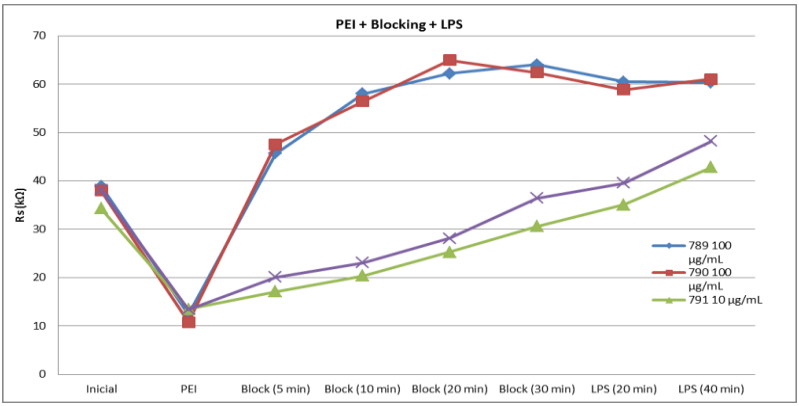

Figure 13: Absolute Rs values at different modification steps. Blue and red lines correspond to a electrodes modified with 100µg/mL of BLOCKING, and violet and green with 10µg/mL.

Then, after those results an experiment with 5 and 10µg/mL of BLOCKING solution was done. Obtained absolute Rs values are plotted in Fig. 14. With 10µg/mL of BLOCKING solution there is no stabilization as in the previous experiment. Partial stabilization of Rs for electrodes immersed in 5µg/mL BLOCKING solution occurs within 60-90 min of exposure. However, immersion in LPS containing solution results in the increase of response which means that used concentrations of blocking are no sufficient to block all surfaces in front of LPS. Also, a problem with reproducibility of BLOCKING adsorption in different experiments was observed with concentration of 10µg/mL as we can see comparing results in Figs. 13 and 14.

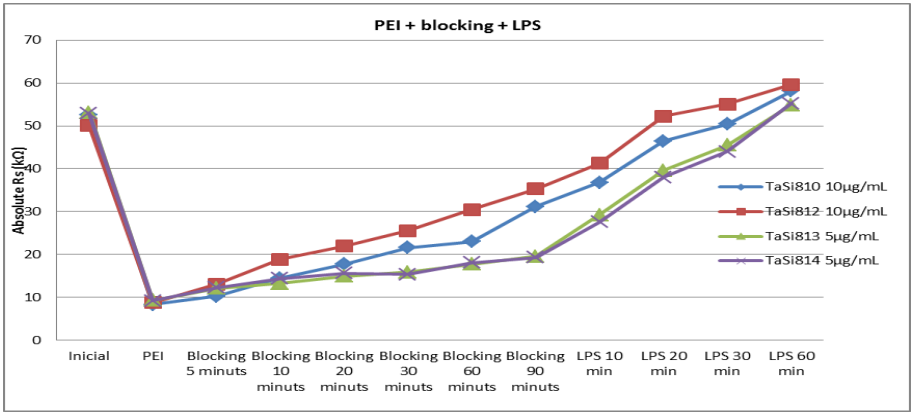

Figure 14: Absolute Rs values at different modification steps. Blue and red lines correspond to electrodes modified with 10µg/mL of BLOCKING, and violet and green with 5µg/mL.

Anyway, it was proposed to study the difference in response between sensors modified with PEI-ConA-Blocking and PEI-Blocking layers. In each case two concentrations of BLOCKING was used, 20µg/mL and 40µg/mL. Figure 15 shows results obtained in 100µg/mL LPS solution. We can see that the blocking has no effect on the sensors response as Rs increases in all of the cases regardless of surface modification type.

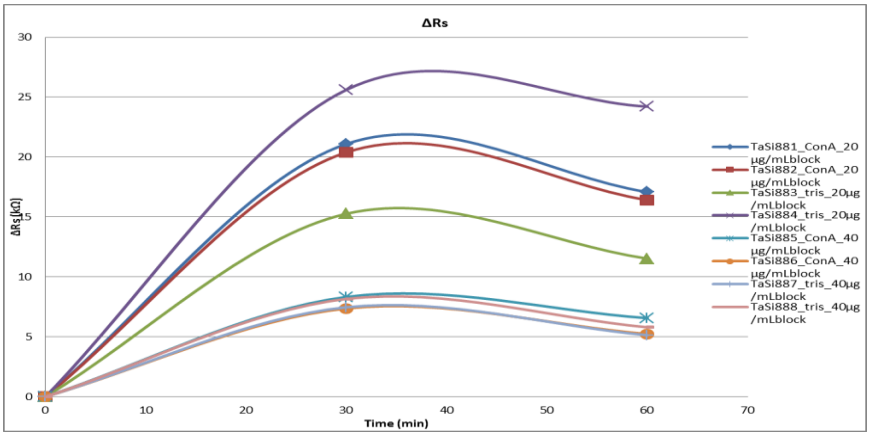

Figure 15: Response of sensors modified with PEI (violet and green lines -20µg/mL of BLOCKING; rose and grey - 40µg/mL) and PEI-ConA (dark blue and red - 20µg/mL of BLOCKING; blue and yellow - 40µg/mL) in 100 µg/mL LPS solution.

Presented results permit us to conclude that the strategy of using different blocking agents (BSA, PSS, BLOCKING) does not permit to eliminate PEI-LPS interactions which makes it impossible to make a reliable sensor with PEI-ConA layers.

7. Response of the sensor modified with epoxysilane and ConA

As sensors modified with PEI and ConA with different blocking agents were not reproducible and did not permit to obtain the required sensitivity to LPS, it was proposed to change the ConA attachment strategy. It has been demonstrated that a (3-glycidyloxypropyl)trimethoxysilane

(epoxysilane) can form a self-assembled monolayer on metal oxide surfaces and interact with amino groups of organic compounds in solution after surface modification [23].

Sensors were subjected to vapor silanization process as presented in the Experimental section. Study of LPS-epoxysilane interaction was done in solutions of 100µg/mL LPS. Absolute values of Rs after each modification step are presented in Fig. 16. After silanization, electrodes were directly measured in KCl 10^{-5}M without rinsing. Modification of the sensor surface with epoxysilane affects the impedance but overall changes are not very significant. This can be explained by the fact that epoxysilane bares no ionizable group and affects the surface charge only by reducing the surface density of Si-OH groups. Fig. 17 also shows that no interaction of LPS with epoxysilane occurs as Rs values are not affected.

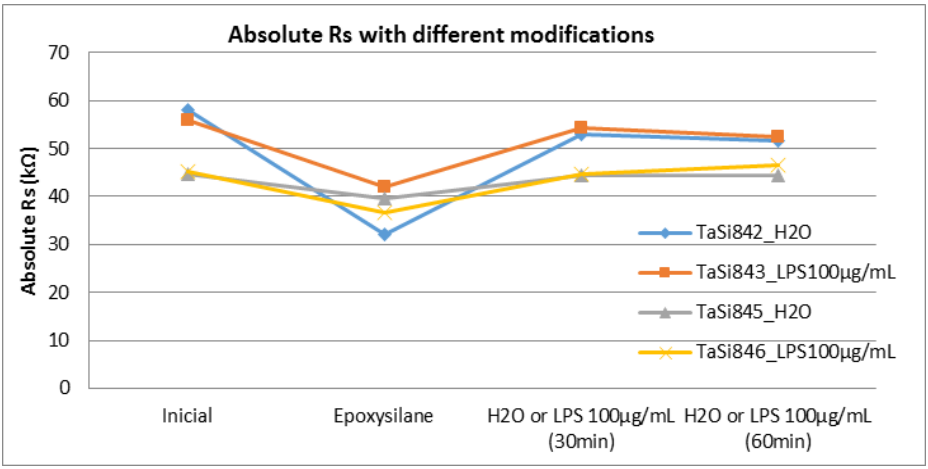

Figure 16: Chands of Rs absolute values after each modification step. Last two point – response in H_2O (blue and grey lines) or LPS solution (orange and yellow).

As LPS does not interact with epoxysilane, no blocking agent was used and the experiment with surface modified by epoxysilane-ConA was done. In this experiment after silanization electrodes were directly immersed into ConA solution. In Fig. 17 we can see absolute Rs after each modification step. We can see how after silanization and reaction with ConA Rs increases because of the deposition of the lectin. However, the response of ConA modified sensor to LPS in the concentration range 50-100 µg/mL is very low. As it was commented earlier this may be

explained by the fact that after ConA immobilization the surface charge is very low and interactions of ConA with LPS do not affect it significantly or the conformation of ConA covalently attached to the surface is not adequate for interactions with lectins [29].

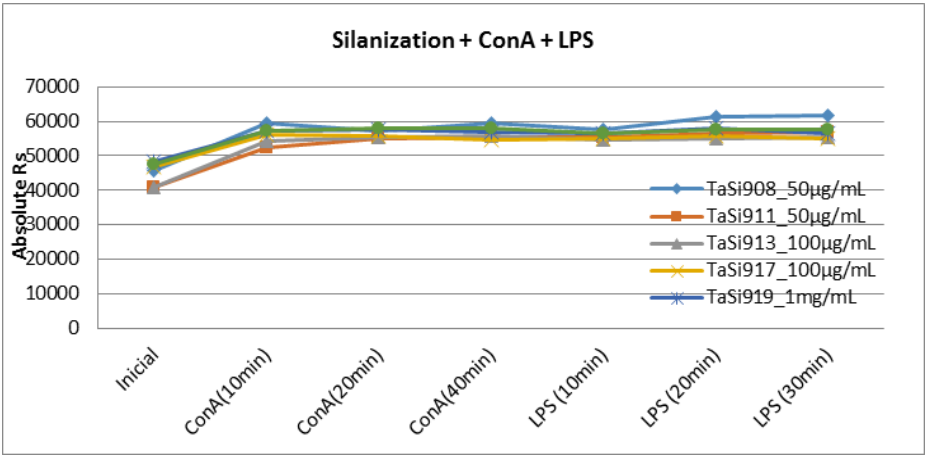

Figure 17: Sensors Rs absolute values changes at each modification step

8. LAYERS OF CONA-GLY

Y. Lvov et al. [21] studied assembly of muli-layer structures with specific interaction on a metal oxide surfaces and demonstrated that is possible to make the layers of $(ConA\text{-}gly\text{-}ConA)_n$ on a PEI modified substrate. It was decided to study this option to construst a sensor with ConA active moieties. As in previous experiments to the sensor surface was modified with PEI and ConA and Gly were deposited in a layer-by-layer (LBL) manner, thus forming a lipid/protein "sandwich". To isolate better the PEI layer and prevent its interaction with LPS two Gly-ConA "sandwiches" were deposited forming the following structure: PEI-ConA-Gly-ConA (see figure 18).

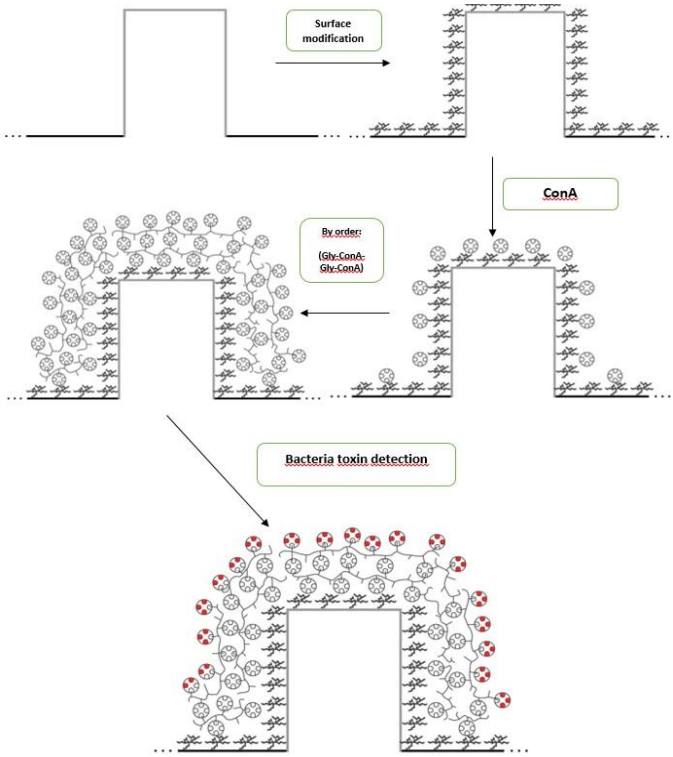

Figure 18: Scheme of ConA-Gly-ConA layers formation steps.

After each treatment step the impedance spectra were measured and determined Rs values are presented in Fig. 19.

To know if these layers block efficiently the PEI surface at the final experimental step, two electrodes were immersed in LPS solution while two other electrodes were immersed in PSS. As PSS is oppositely charged than PEI, this interaction in case of direct contact due to the blocking failure will produce a change in Rs. As reveal results presented in Fig. 19 the two electrodes modified with PEI-ConA-Gly-ConA, which was put in contact with PSS, showed no change in Rs. On the other hand, two modified sensors in contact with the LPS solution demonstrated 20 kΩ increase in Rs due to ConA-LPS interaction.

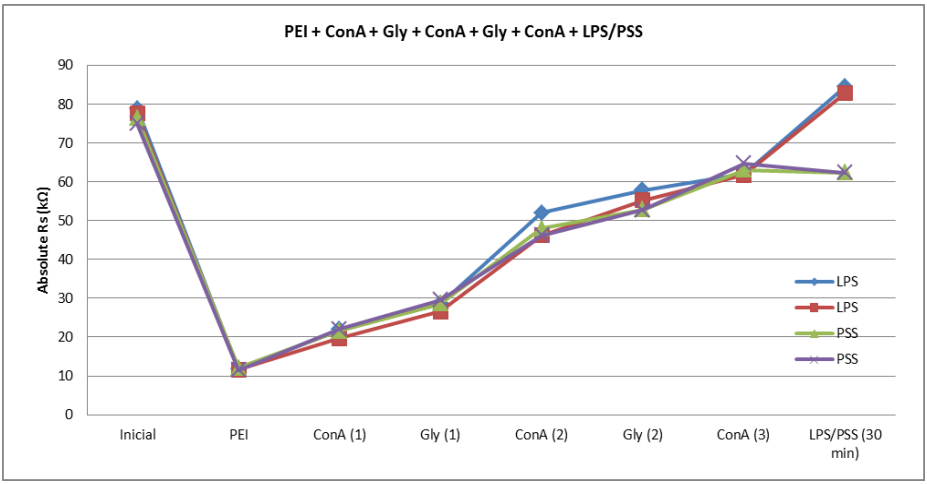

Figure 19: Absolute Rs values at each step during sensors modification with PEI-ConA-Gly-ConA. On the final step two electrodes were in contact with PSA (green and violet) and other two with LPS (red and blue).

Presented results show that the initial PEI layer is totally blocked and large Rs changes in 100µg/mL LPS solution let us expect high sensitivity of the sensor to LPS. The sensitivity of PEI-(ConA-Gly)$_2$-ConA modified 3D-IDEA sensors to LPS from *E. coli* in water solutions was performed in a wide range of LPS concentration from 1 to 50µg/mL. For each concentration a newly prepared sensor was used. In a control, experiment modified 3D-IDEA sensors were immersed in a 10^{-5}M KCl solution without LPS. Obtained results show that in all studied concentration ranges LPS adsorbs rapidly onto the sensor surface provoking significant increase in impedance. Response stabilization was around 20 minutes and Rs variation is proportional to bacteria concentration (Fig. 20).

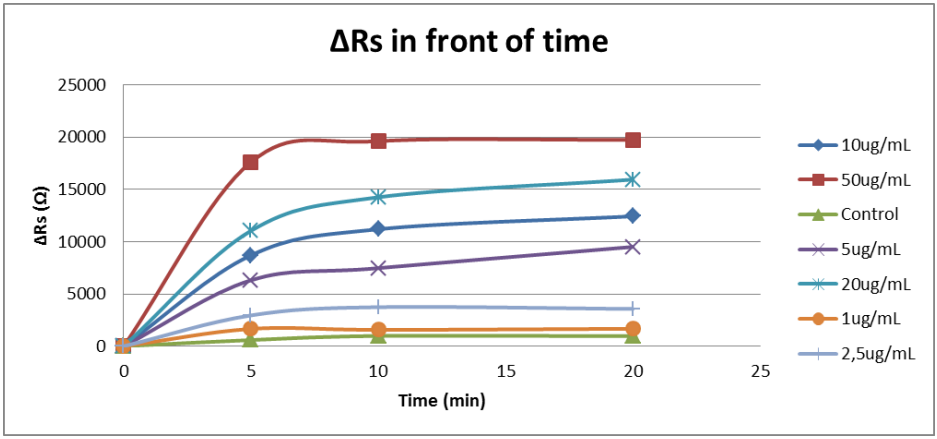

Figure 20: Response of sensors modified with PEI-(ConA-Gly)$_2$-ConA in solutions with different LPS concentration.

Obtained results demonstrate significant sensitivity of the sensor to different LPS concentrations. Irreversible adsorption of analyte species on a solid surface usually obeys the Langmuir adsorption isotherm that may be presented as;

$$n = n_m \frac{bC}{1 + bC} \qquad (8)$$

Where C is the concentration of the adsorbate in the solution, n is the amount adsorbed, 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 [30] which is expressed as:

$$y = V_{\max} \frac{x^n}{k^n + x^n} \qquad (9)$$

As Fig. 21 shows, the obtained experimental points at 20 minutes can be perfectly fit by the Hill function.

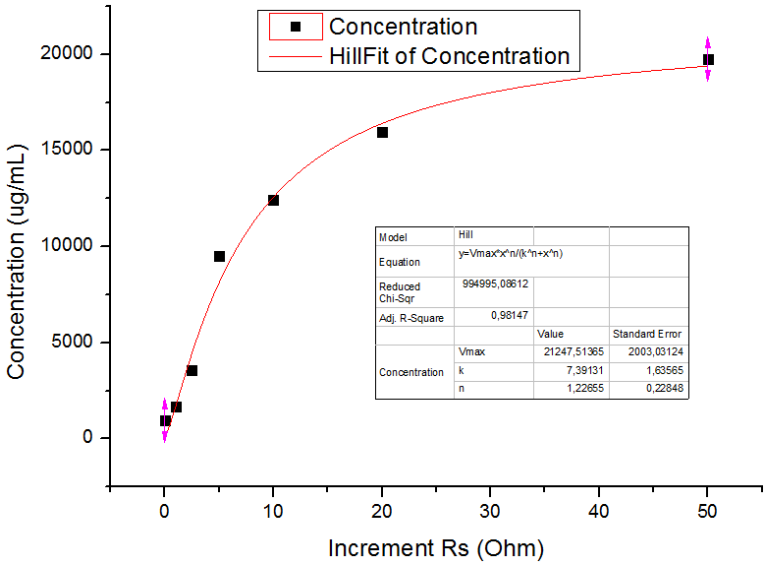

Figure 21: Response of sensors modified with PEI-(ConA-Gly)$_2$-ConA in solutions with different concentration of LPS (squares) and fitted by Hill's equation (line).

Our Hill's equation obtained was:

$$\Delta Rs = Rsmax\frac{c^n}{k^n + c^n} = 21248\frac{c^{1,2}}{7{,}4^{1,2} + c^{1,2}} = 21248\frac{1}{(\frac{7{,}4}{c})^{1,2} + 1}$$

Where:

- n: Hill coefficient describes the cooperativity of 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 Rs value at which saturation occurs and maximum surface concentration is achieved.
- k: Ligand concentration producing half occupation

As in all Langmuir type of adsorption isoterms it is possible to plot response a semilogarithmic scale (Fig. 22) thus obtaining a linear calibration regression.

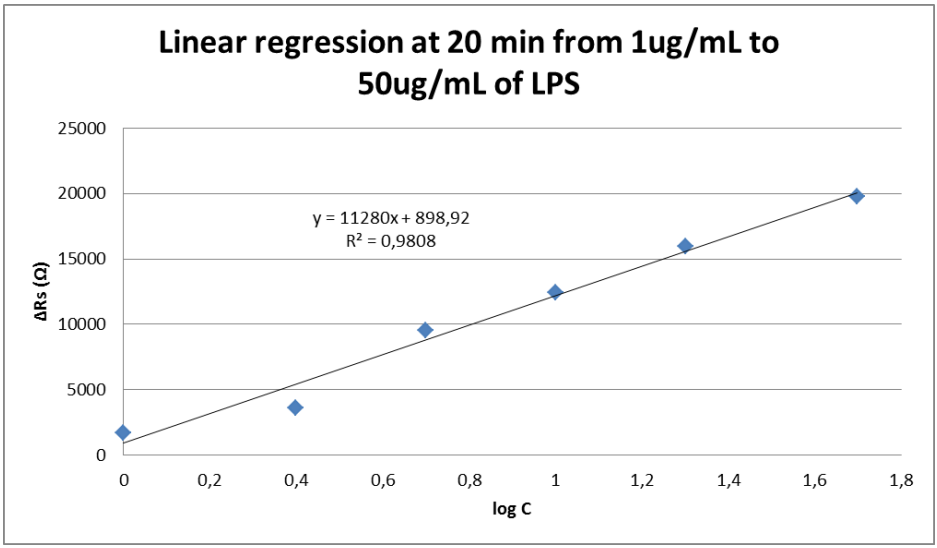

Figure 22: Linear calibration regression of electrodes modified with PEI-(ConA-Gly)$_2$-ConA in LPSA solutions.

A linear regression obtained was successfully obtained with a good adjustment. With the slope and obtained results in blank experiment it is possible to determine the limit detection of our device in LPS detection. The limit of detection (LOD) obtained was 1µg/mL coinciding with our lower experimental concentration.

9. CONCLUSIONS

The behavior of three-dimensional interdigitated electrode arrays (3D-IDEA) as a possible sensor for bacteria toxin detection was studied. The sensor surface was chemically modified with polyethilenimine (PEI) or epoxysilane. As a biorecognition element for bacterial toxin detection, lectin Concanavalin A (ConA) was deposited on the IDEA surface through PEI-ConA or epoxysilane-ConA interactions. To prevent non-specific adsorption of LPS on the PEI

different ways of surface blocking were studied. However, different types of blocking agents tested (BSA, PSS, BLOCKING) did not permit to enhance the specificity of the sensor with PEI-ConA sensitive layer.

In the case of sensors modified only with epoxysilane no response to LPS was registered. However, the sensors with epoxysilane-ConA layers did not show any response to LPS in solutions. This may be explained by the fact that after ConA immobilization the surface charge is very low and interactions of ConA with LPS do not affect it significantly, or the conformation of ConA covalently attached to the surface is not adequate for interactions with lectins.

The best results on selective detection of LPS were achieved by modifying surface with Concanavalin A – Glycan multilayers. Detection limit of the sensor with PEI-(ConA-Gly)$_2$-ConA was found to be as low as 1μgLPS/mL in *E. coli* toxins with a response time around 20 minutes. The sensor responses follow the Langmuir adsorption curve (type I) and can be perfectly fitted by Hill's equation.

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11. ACRONYMS

BSA ≡ Bovine Serum Albumin

ConA ≡ Concanavalin

ELISA ≡ Enzyme-Linked ImmunoSorbent Assay

Gly ≡ Glycogen

IS ≡ Impedance spectroscopy

LPS ≡ Lipopolysaccharide

PEI ≡ Polyethilenimine

PSS ≡ Poly(sodium 4-styrenesulfonate)

SAM ≡ Self-assembled monolayer

3D-IDEA ≡ Three dimensional interdigitated electrode array