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IMPEDANCE SENSORS MADE IN PCB AND LTCC TECHNOLOGIES FOR MONITORING GROWTH AND DEGRADATION OF PSEUDOMONAL BIOFILM

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Abstract

The suitability of low-cost impedance sensors for microbiological purposes and biofilm growth monitoring was evaluated. The sensors with interdigitated electrodes were fabricated in PCB and LTCC technologies. The electrodes were golden (LTCC) or gold-plated (PCB) to provide surface stability. The sensors were used for monitoring growth and degradation of the reference ATCC 15442 *Pseudomonas aeruginosa* strain biofilm in *in-vitro* setting. During the experiment, the impedance spectra of the sensors were measured and analysed using *electrical equivalent circuit* (EEC) modelling. Additionally, the process of adhesion and growth of bacteria on a sensor's surface was assessed by means of the optical and SEM microscopy. EEC and SEM microscopic analysis revealed that the gold layer on copper electrodes was not tight, making the PCB sensors susceptible to corrosion while the LTCC sensors had good surface stability. It turned out that the LTCC sensors are suitable for monitoring pseudomonal biofilm and the PCB sensors are good detectors of ongoing stages of biofilm formation.

Keywords: *Pseudomonas aeruginosa*, biofilm, interdigitated sensor, impedance spectroscopy.

1. Introduction

The vast majority of microorganisms exist as attached and organized communities referred to as biofilms [1]. These communities are predominantly embedded within various extracellular polymeric substances (sugars, proteins, DNA) also known as a "biofilm matrix" [2]. The matrix serves microorganisms as a shelter and a shield protecting them from not only drugs and other xenobiotics but also from activity of the immune system. The biofilms are able to form on virtually every type of surface, including tissues and abiotic biomaterials used for medical purposes [3]. It is estimated that biofilms are responsible for up to 80% of nosocomial infections [4]. Therefore, there is an urgent need of developing new tools for rapid detection of "medical biofilm" enabling to apply suitable eradication procedures.

The tools designed for rapid detection of biofilms may be helpful in virtually all flow systems endangered by the development of microbes. Among the examples of such systems in nosocomial settings are indwelling catheters, nutrition accesses or hospital water distribution pipes [5]. The usage of impedance sensors providing possibilities of non-invasive, label-free and real-time measurements is promising in the above mentioned clinical situations. Using the sensors combined with the impedance spectroscopy method for detecting attachment

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of microbial cells and biofilm formation has been already reported by other authors who tested the impedance potential using such nosocomial strains as *Escherichia coli* [6–9], *Staphylococcus aureus* [7, 10, 11], *Staphylococcus epidermis* [11, 12], *Pseudomonas aeruginosa* [5, 13–15], *Bacillus subtilis* [13] and *Salmonella typhimurium* [9, 16].

The impedance micro-sensors with interdigitated electrodes were used for this purpose by some researchers [9, 11, 12, 17] because of their advantages: a small size enabling to perform small-scale experiments using very small samples, fast establishment of a steady-state signal, a low ohmic drop of potential, an increased signal-to-noise ratio and – last but not least – higher sensitivity comparing with conventional-size macro-electrodes.

Typical sensors with interdigitated electrodes designed for microbiological sensing are usually fabricated using the lithography technique on silicon and glass substrates [18]. The main disadvantages are their high cost and complicated process of manufacturing.

The sensors made in the *printed circuit board* (PCB) technology may be a promising alternative as they are cost-effective and relatively easy to manufacture [19]. The PCB sensors were already employed in microbiological, oncologic or immunologic applications, namely for: bacteriuria screening [7], analysis of colorectal carcinoma cells [20] and Interleukin-12 detection [19, 21]. The sensors made in *low temperature co-fired ceramics* (LTCC) could be also a constructive option. LTCC devices were reported to be used in microbiological applications, *i.e.* monitoring cell cultures [22], monitoring water solutions [23], monitoring glucose concentration [24], an electronic tongue [25] and cortisol detection [26]. However, the mentioned devices used mostly voltamperometric measurement systems, avoiding the impedance spectroscopy.

In the impedance spectroscopy the electrical equivalent circuit modelling is a method of analysing impedance spectra [27]. It consists in the numerical fitting of the *electrical equivalent circuit* (EEC) impedance to the measured impedance spectrum. As specific components of the EEC are correlated with various conduction and polarisation processes, such an approach enables to identify and separate them. This method is not widely used in analysis of the impedimetric sensors' responses in microbiological applications despite of the fact that it may provide detailed information about the measured object [14, 17, 28, 29].

The aim of this work was to combine advantages of the interdigitated electrodes with the cost-effective PCB technology and the more accurate LTCC technology, and to determine whether such sensors may be used for detection of the *P. aeruginosa* biofilm presence and for monitoring particular stages of this structure formation. The presented research is related to the authors' previous work [17], in which impedance micro-sensors on glass substrates were used.

2. Materials and methods

2.1. Preparation of impedance sensors

The layout of impedance sensor interdigitated electrodes was designed using CadSoft Eagle software. Each electrode consisted of five digits with 1.9 mm length and 0.25 mm width. The distance between electrodes was 0.25 mm. Sensors in the PCB technology were fabricated in Satland Prototype (Gdańsk, Poland) on a glass-reinforced epoxy laminate (FR4 type) commonly used for electronics purposes. The copper layer on the laminate was 35 μm thick. The electrodes were electroplated with gold to improve surface stability (Fig. 1a). Sensors in the LTCC technology were formed using 4 layers of green tape (DP 951, DuPont). To obtain an appropriate shape of each layer, the laser beam cutting (LPKF Protolaser U cutting system) was performed. The metallic electrodes and their conductive paths together with contacts were screen-printed (Au conductive paste ESL 8880-H, Electroscience Laboratories) on the last ceramic layer. Additional ceramic layers were then applied to insulate electrode leads (Fig. 1b).

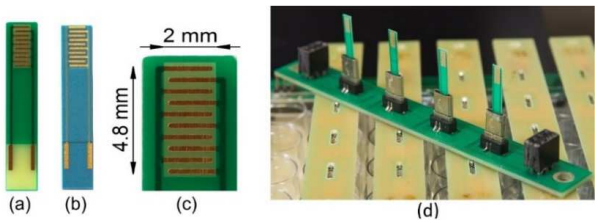


Fig. 1. An impedance sensor fabricated in: the PCB technology (a); the LTCC technology (b); a close-up view of sensing area dimensions (c); sensors inserted in sockets (d).

Due to a low cost of production all sensors were disposable. The dimensions and thickness of PCB and the distance between contacts enabled to mount a sensor directly in a micro-USB connector (Fig. 1d).

2.2. Impedance spectroscopy measurement setup

The impedance spectra were measured in a frequency range from 0.1 Hz to 100 kHz with 25 mV_{rms} excitation signal using an IMP-STM32 [30] impedance analyser and applying an accelerated method for low frequency impedance evaluation [31]. The impedance analyser was linked with a 24-channel multiplexer designed to accommodate a 24-well titrate plate and enabling sequential switching between up to 24 impedance sensors placed vertically in the titrate plate wells (Fig. 2a).

Prior to any experiments the sensors were cleaned using distilled water and acetone to remove any residue and sterilized with isopropyl alcohol. Then, they were aseptically placed into sockets of the multiplexer and irradiated with UVC light for 20 minutes.

All devices were controlled by a home-built software *ImpeDancer*. A measurement setup and an incubator in which the experiment was performed are presented in Fig. 2b.

2.3. Pseudomonal strain preparation

A reference *P. aeruginosa* ATCC14454 strain was used for experimental purposes. The ability of the aforementioned strain to form a biofilm on abiotic surfaces has been already recognized in the authors' previous work [17]. An overnight culture of the examined strain was diluted to 1 McFarland using a densitometer (Biomerieux, Poland) and subsequently diluted to 10^6, 10^4, 10^2 colony forming units per millilitre (cfu/ml) in the *Tryptic Soy Broth* (TSB, Becton Dickinson) medium.

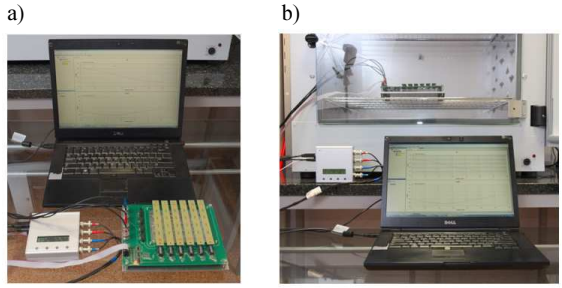


Fig. 2. A measurement setup: components of the measurement setup: a personal computer, an impedance analyser (left) and a 24-channel multiplexer with a titrate plate (right) (a); the 24-channel multiplexer placed inside an incubating chamber (b).

2.4. Impedance measurements

The measurements of impedance spectra were carried out using *P. aeruginosa* – contaminated TSB (10^6, 10^4 and 10^2 cfu/ml) and pure TSB as a reference. 4 rows of the titrate plate consisting of 6 wells each were filled with 2 ml of the medium each and placed in the multiplexer. This setup was incubated for 168 hours at 37ºC, 95% RH. A single impedance spectrum measurement took 60 s which made the measurement repetition time of approximately 24 minutes for each specific impedance sensor.

The experiment was performed separately for PCB and LTCC sensors.

2.5. Crystal violet colouring

The goal of the crystal violet colouring experiment was to visually assess the attachment of *P. aeruginosa* biofilm to the surface of PCB sensor. It was prepared in a similar way to the impedance measurements but only the 10^6 cfu/ml *P. aeruginosa* suspension was used. The sensors were incubated for 1, 4, 16 and 24 h at 37ºC. After these times of incubation the sensors were aseptically removed from the multiplexer, rinsed with distilled water and left to dry in room temperature. Next, the sensors were immersed in 1 ml of a 0.1% solution of crystal violet in water (ProLab Diagnostics) for 5 minutes, rinsed thoroughly with distilled water to remove excess stain and left in room temperature to dry.

2.6. SEM and EDS

The morphology studies were performed with a field emission gun *scanning electron microscope* (SEM) with a germanium ion source (Dual Beam, Helios NanoLab™600i, FEI). The composition of individual layers of the gold-plated copper electrode was determined using the Energy-dispersive X-ray spectroscopy (EDS, EDAX detector) on a sample that was previously milled at an angle using the focused ion beam (FIB).

3. Results and discussion

3.1. Crystal violet colouring and optical microscopy

The results of crystal violet colouring are shown in Fig. 3. The bacteria attachment and the start of biofilm formation on the surface was visible after 4 hours of incubation. After 16 hours about a quarter of the surface was covered with a biofilm, while after one day of incubation the whole surface was covered by a mature, three-dimensional thin pseudomonal biofilm structure (Fig. 3d).

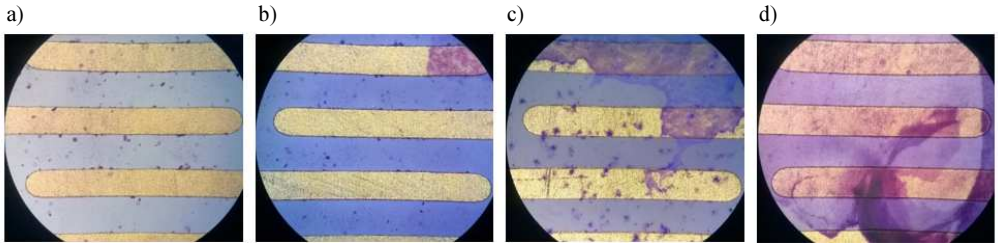


Fig. 3. Optical microscopy images (magnitude 40 x) of *P. aeruginosa* biofilm stained with a crystal violet on the surfaces of PCB sensors after: 1 hour (a); 4 hours (b); 16 hours (c) and 24 hours of incubation (d).

3.2. SEM imaging

The results of SEM imaging of LTCC sensor are shown in Fig. 4. Similarly to the PCB sensor after 16 hours of incubation the sensor's surface was partly covered with a three-dimensional biofilm structure, however the presence of a bacterial monolayer cannot be proven.

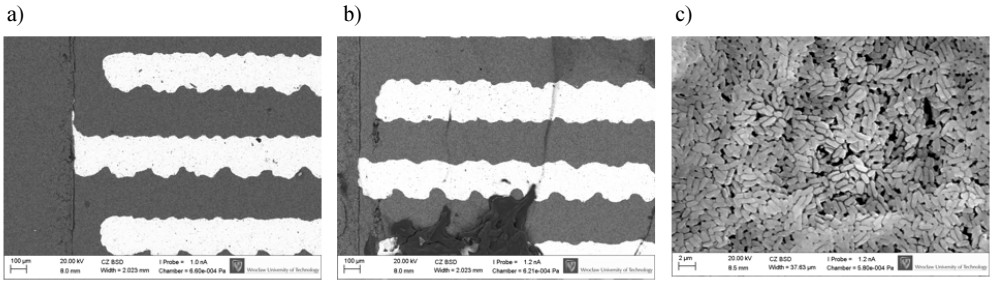


Fig. 4. SEM images of *P. aeruginosa* biofilm on the surfaces of LTCC sensors: a clean reference (a); after 16 h of incubation (b) and focused on a biofilm structure (d).

3.3. Impedance spectra

Examples of impedance spectra obtained during the experiment with *P. aeruginosa* strain with 10^2 cfu/ml initial concentration for both sensor types as well as the reference (pure TSB) during first 36 hours of incubation (biofilm formation) and last 132 hours (biofilm degradation) are shown in Table 1. Significant changes in the electric properties of sensors in both the bacterial culture and the reference were observed.

3.4. Electrical equivalent circuits

Basing on the literature [9, 14, 28, 29, 32], the measured impedance spectra and knowledge about the physiochemical properties of biofilm [5, 6, 8, 16], the *electrical equivalent circuits* (EEC) were obtained and are shown in Fig. 5.

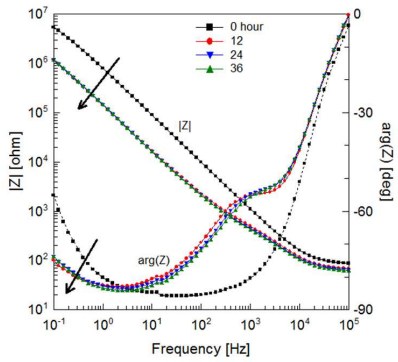
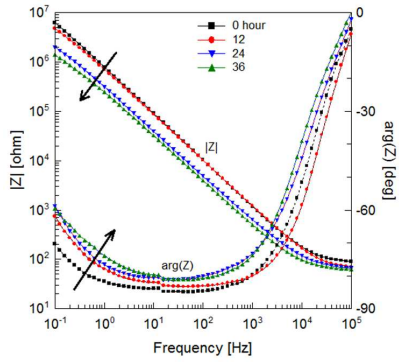
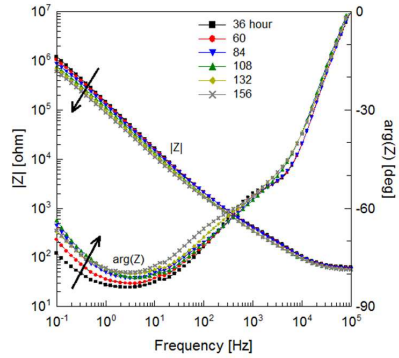
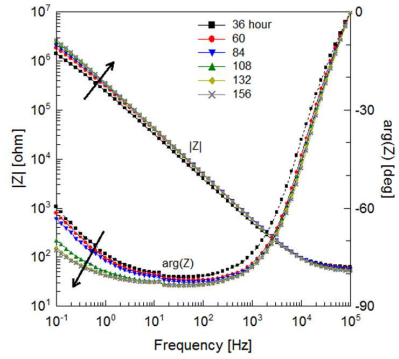
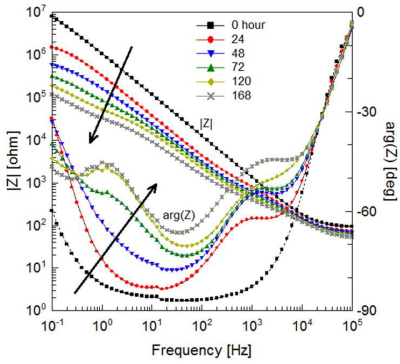
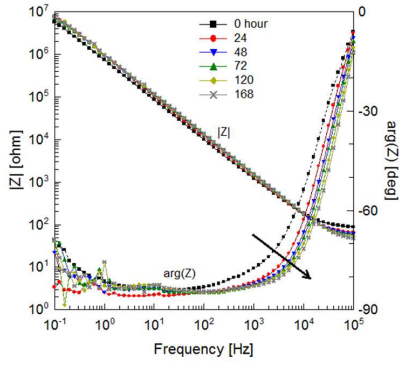
The sensors made in PCB and LTCC technologies differed in the electrode materials and morphology which caused also differences in analysis of the impedance spectra. For the LTCC sensors it was impossible to distinguish the interfacial from biofilm capacitance, therefore a simplified EEC was used (Fig 5b).

Each component of the model represents a different phenomenon of current conduction or Polarization, *i.e.* R_S – a liquid medium resistance, CPE_B – a constant phase element which models the surface of non-uniform electrodes and their coverage by the biofilm, R_B – a resistance of the surface of electrodes and the biofilm pores, C_I – an electrical double layer interfacial capacitance, R_{CT} – a charge transfer resistance. The model contains a *constant phase element* (CPE) which is widely used in the equivalent circuit modelling of impedance spectra [27]. Its admittance Y_{CPE} depends on a radial frequency ω and two parameters – Q and n:

$$Y_{CPE} = Q * (j\omega)^n . \tag{1}$$

If the parameter n goes to 1 the admittance of CPE becomes similar to the admittance of capacitor.

Table 1. Typical impedance spectra of sensors placed in a medium with *P. aeruginosa* strain (10^2 cfu/ml initial concentration). Black arrows point to changes of impedance spectra in time.

Stage\Sensor	PCB	LTCC
Biofilm formation		
Biofilm degradation		
Reference		

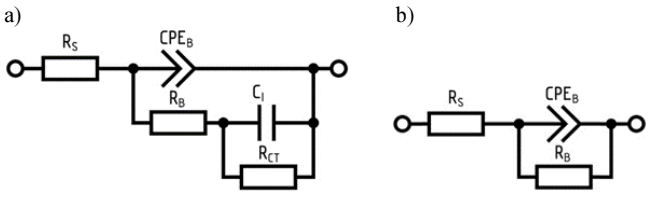


Fig. 5. Electrical equivalent circuits of PCB (a) and LTCC (b) sensors placed in a medium with bacteria.

3.5. Electrical equivalent circuit analysis of PCB sensors

The impedance spectra obtained in the experiment were analysed using ZView software (Scribner) and EEC modelling using the EEC shown in Fig. 5a. Each experiment was repeated 6 times. Changes of the calculated mean values and standard deviations of EEC parameters during 168 hours of incubation are shown in Fig. 6a-6f while a result of the impedance spectra fitting is shown in Fig 6g.

The most eye-catching EEC parameter is R_{CT}. For the PCB sensors in *P.aeruginosa*-containing wells a rapid rise of R_{CT} can be observed in the third, fifth and seventh hour of measurement for 10^6, 10^4 and 10^2 cfu/ml initial concentrations, respectively. The R_{CT} value for sensors in *P.aeruginosa* with lower initial concentrations achieves a quasi-plateau state while for the reference it slowly decreases. This parameter is a sensitive indicator of the moment, when the area of sensor's electrodes is fully covered by the biofilm because the biofilm can be regarded as an electrical insulator [29].

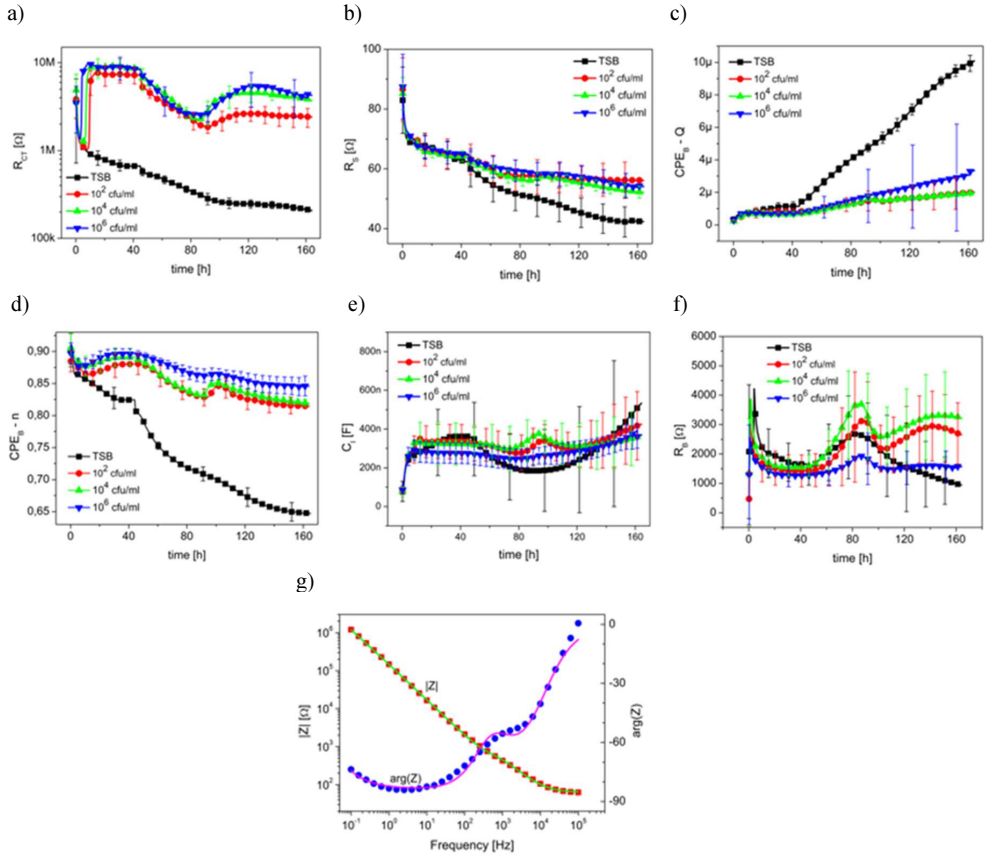


Fig. 6. Changes of mean value (points) and standard deviation (error bars) of EEC parameters in time during incubation: R_{CT} (a); R_S (b); CPE_B-Q (c); CPE_B-n (d); C_I (e); R_B (f); and examples of measured (dots) and fitted (lines) impedance spectra for 10^2 cfu/ml at 36th hour (g).

The value of liquid resistance R_S almost continuously decreases in samples with *P.aeruginosa* while for the reference it achieves a plateau state between the fourth and sixteenth hours – then it decreases too – probably because of the increased ionic concentration caused

by corrosion of electrodes. Additionally, the behaviour of CPE_B is very interesting. The values of Q and n parameters of CPE_B in the first stage of measurement fluctuate for each data series. The situation becomes stable in the eighth hour of measurement when CPE_B-Q slowly decreases for sensors in *P.aeruginosa* solution and after the thirty-second hour it increases again, while for the reference it grows continuously. The same situation – inversely – occurs for the CPE_B-n parameter values. The C_I and R_B seem to not carry any useful information as changes of their values were observed also in the reference and values of their standard deviations are quite high.

3.6. Electrical equivalent circuit analysis of LTCC sensors

As previously, the impedance spectra obtained in the experiments repeated 6 times were analysed using ZView software (Scribner) and EEC modelling using the EEC simpler than that in the case of PCB sensors, shown in Fig. 5b. Changes of the calculated values of EEC parameters during 168 hours of incubation are shown in Fig. 7a – 7d while a result of the impedance spectra fitting is shown in Fig. 7e.

In this case the most important EEC component is CPE_B, which clearly reflects a current state of pseudomonal biofilm. The adhesion stage is finished during first hours of the experiment and – depending on the initial cell concentration – goes to the growth phase which is represented by a constant growth of CPE_B-Q value and a corresponding decrease of CPE_B-n value. About the 36th hour (depending on the initial concentration) the biofilm enters the degradation phase resulting in a smooth decrease of CPE_B-Q value and a slow growth of CPE_B-n value. There should be noted that the CPE_B reference value is quasi-constant during the whole experiment.

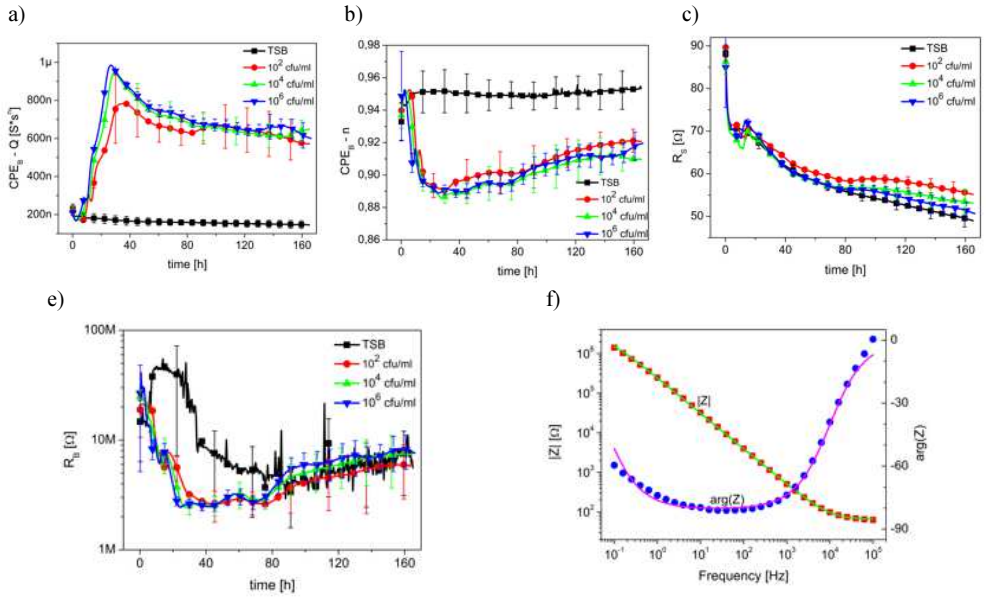


Fig. 7. Changes of mean value (points) and standard deviation (error bars) of EEC parameters in time during incubation: CPE_B-Q (a); CPE_B-n (b); R_S (c); R_B (d); and examples of measured (dots) and fitted (lines) impedance spectra for 10^2 cfu/ml at 36th hour (e).

A plot of R_S values does not show any significant difference between the biofilm and reference. Similarly, R_B with one additional phenomenon – in the first 30 hours of experiment the R_B reference value exceeds 10 MΩ, then decreases and – after about 80 hours – achieves the same level as the rest. It is caused by the lack of bacterial biofilm in the reference and probably slow adhesion of TSB nutrients to the sensor surface which creates some kind of a conductive layer between electrodes.

3.7. SEM and EDS examination of PCB electrodes

As shown in Sections 3.3 and 3.5 the impedance spectra of reference sensors and their EEC parameters varied in time. It may suggest that – despite the absence of bacteria – the sensor's surface degrades in a corrosive environment. To examine that possibility a single sensor prepared as in Section 2.2 was incubated in 0.9% NaCl aqueous solution for 48 hours. This solution in respect of a corrosive factor is very similar to TSB but does not leave any residue on the sensor.

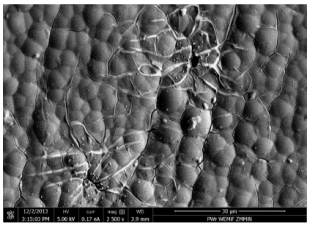


Fig. 8. A SEM image of a sensor's gold-plated electrode after incubation in 0.9% NaCl aqueous solution for 48 hours. Corrosion of metal layers under gold plates can be seen.

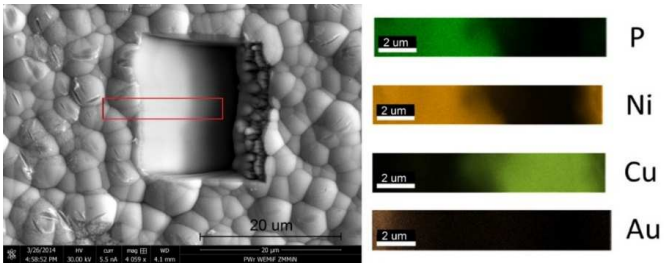


Fig. 9. A SEM image of a sensor's electrode etched by FIB and EDS place marked red (left). The elemental results of EDS analysis (right).

The sensor surface after incubation was assessed using SEM (Fig. 8). In several points the pitting corrosion of the electrodes can be seen. It means that the gold layer was not tight and in a pure corrosive environment corrosion of the electrodes can occur, influencing the EEC parameters.

A typical gold-electroplated metallic layer of PCB consists of three layers in a sandwich structure: copper, nickel-phosphorus alloy and – finally – gold [32]. The percentage of each mentioned element in the sensor's electrode was checked using the EDS analysis. Firstly, the sensor was prepared as described in Section 2.2 and then milled using the *focused ion beam* (FIB), as shown in Fig. 9. Then, the EDS analysis was performed. The obtained results confirmed a sandwich structure of metal layers – copper is the bottom one, covered by nickel-phosphorus and gold. As can be seen in Fig. 8. and in Table 2, the percentage of gold layer is very low. In effect the gold layer is not tight and the metallic layers under it are not protected from corrosion.

Table 2. The chemical composition of PCB sensor's electrodes.

Element	Orbital	Weight [%]	Atomic [%]	Error [%]
carbon	K	2.66	11.91	9.56
phosphorus	K	5.54	9.61	7.43
nickel	K	36.27	33.17	0.96
cuprum	K	52.70	44.54	1.02
gold	L	2.82	0.77	3.17

4. Conclusions

The research presented in this study concerned the application of impedance sensors fabricated using the PCB and LTCC technologies in detection and monitoring of pseudomonal biofilm development and degradation. During the experiments the impedance spectra (0.1 Hz – 100 kHz) of the sensors placed in a TSB medium with and without *P. aeruginosa* bacteria were measured in approximately half-hour intervals. The obtained data were analysed and the EEC was built (Fig. 5). It enabled to identify various processes of conduction and polarization.

For biofilm monitoring using PCB sensors the most notable element of EEC was R_{CT} which represented the charge transfer resistance. As can be seen in Fig. 6a, its value grew rapidly after a few hours of incubation of the sensor in the medium with bacteria while R_{CT} of the reference (pure medium) was slowly decreasing. This moment was strictly connected with the complete biofilm coverage of the electrodes which caused a decrease of the charge transfer. These results were coherent with the images of crystal violet stained biofilm obtained using the optical microscopy (Fig. 3). Due to limitations of the traditional PCB technology the rest of EEC parameters were highly influenced by additional phenomena occurring during the experiment. The most notable side-effect was corrosion of Ni and Cu layers placed under a non-tight gold electroplating of the electrodes. Evidently, the quality of the gold layer on the PCB was worse than it can be achieved *e.g.* by sputtering [33]. The effects of corrosion were assessed using SEM (Fig. 8). Despite that, the research results presented in this report show that the impedance sensors fabricated using the cheaper and simpler PCB technology are still very useful for marker-less in-situ detection of the biofilm formation.

There were interesting the capacitive parameters of EEC components for PCB sensors: the Q and n parameters of CPE_B and C_I capacitance (Fig. 6c, 6d and 6e). These values became quasi-stabilised after approximately eight hours for sensors in the bacterial medium, whereas for the reference they are changing all the time. CPE_B and C_I depended mostly not on the bacterial biofilm state but on corrosion of the electrodes – in this case the bacterial biofilm serves as an corrosion inhibiting layer.

For biofilm monitoring using LTCC sensors the most notable element of EEC was CPE_B which clearly reflects a current state of pseudomonal biofilm – the adhesion, growth and degradation stages (Fig. 7a and 7b). Thanks to the lack of corrosion effect this kind of sensor can be successfully applied to such purposes.

The obtained results were coherent with the authors' previous work [17], where micro-sensors fabricated using the thin film technology with pure gold interdigitated electrodes on a glass substrate were employed. The common features were a characteristic step-growth of the value of parallel resistance EEC element – like in analysis of PCB sensors, and a change of CPE element modelling electrodes' surfaces when the biofilm entered the growth phase – like in analysis of LTCC sensors.

Acknowledgements

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