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Abstract. The aim of the study was to specify by an impedimetric method the changes observed on the inner wall of central venous catheters for hemodialysis leading to the formation of a biological film. To evaluate these changes a patient-dialyzer model was built in which experimental parameters were kept closely similar to the clinical conditions of hemodialysis. The impedance spectra and SEM/EDS analysis of the biological layer deposited on the inner surface of the distal part of the catheter gave an insight into the structure of film formation and its chemical composition. Since an early detection of biofilm formation inside the distal part of the catheter is crucial for the safety of medical treatment and it usually prompts the implementation of antibiotic therapy. Developed impedimetric method can minimize the risk of infection and ensure the continuity of treatment.

Keywords: central venous catheters, electrochemical impedance method, biological layer, scanning electron microscopy

1 Introduction

Recently, the number of patients undergoing hemodialysis treatment with central venous catheters [1]-[3] has grown. For instance, in the US, it amounts to about 6-150 million each year [4]-[6]. Insertion of the catheter is an invasive procedure associated with infectious and thrombotic complications [1],[3],[7]-[13], predisposing to the occurrence of a number of complications. It is estimated that every year 12-25% of patients die due to complications and infections [14], [15]. Moreover, the number of such cases increases approximately by 1.5% per year [15] and the cost of complications amounts to more than 2.3 billion dollars [16]. The majority of pathogens that cause infections are microorganisms inhabiting the skin, mainly *Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Enterococcus spp., Acinetobacter spp., Klebsiella*

pneumonae, Enterobacter cloaca [2], [10]. Thromboses are formed by molding on the surface of the catheter a fibrin sheath comprised of numerous proteins, including albumin, fibrin, fibronectin, collagen, and laminin [12]. As a result of the formation of a bacterial film serious human catheter-related bloodstream infections (CRBSI) are observed. Thrombosis can cause problems with blood withdrawal and contribute to the partial or full occlusion [8]. A too-late diagnosis of catheter blockage is most risky during the treatment [7]. Clinical symptoms are often non-specific or characteristic for other diseases [7]. Therefore, for a long time they are not linked to the catheter infection. It leads to increased costs, interruption of therapy as well as it adversely affects the patient's condition [4], [8]. Current prevention methods which include modifications of the inner surface by antithrombotics and antibacterial agents [2], [7], [15]-[18] are so far not sufficiently effective [1]. Only recently [19] the use of the electrochemical impedance spectroscopy has led to the development of a biosensor which can monitor the formation of a biofilm layer at the port of a venous catheter, located just under the skin. The catheter with a port similar to a tunneled catheter left entirely under the skin helps to prevent the infection which is often observed in catheters described by the authors of this study. However, such type of catheter has a different construction (the type and shape of the side holes and the tip). According to numerous scientific reports [20], [21] and the present study the most threatened part is the tip of the catheter, which is placed in the right atrium of the heart and thus is in constant contact with blood. The fibrin sheath formed mainly by proteins on the surface of the catheter within 24 hours of its insertion into the patient's circulation promotes the subsequent adhesion of pathogens [1]. However, numerous scientists [19], [22]-[25] used bacterial cultures (neglecting the influence of the proteins) for providing a characterization of biofilm growth, e.g. Paredes et al. used *S. epidermidis* [19],[22] and *S. aureus* [23], Ben-Yoav et al. used *E. coli* [24], and Taeyoung et al. used *Pseudomonas aeruginosa* [25]. Examinations of the deposition and the growth of biofilm were recorded for frequencies range from 0.01 Hz to 400 kHz with an AC amplitude of 10 to 100 mV [19], [22]-[25]. In most of the studies a standard three-electrode configuration [24], [25] or a label-free interdigitated electrode (IDAM) biosensor were used [19], [22], [23]. The aim of this research was to develop an impedimetric method to monitor the initial conditioning layer formed on the inner surface of a hemodialysis catheter made of carbothane. The process of a biological layer formation was monitored for the tip of the catheter which is most vulnerable to the formation of biofilm structures. The bovine serum albumin was used as a biological factor. This protein is a part of the fibrin sheat which forms on the surface of the catheter within 24 hours from placing it into the bloodstream. In order to monitor film formation by the electrochemical impedance spectroscopy (EIS) method and to check its performance, a patient-dialyser system was elaborated. The film layer formation was analyzed during the flow of the PBS solution (0.01 M, pH 7.4) containing albumins, which account for about 60% of the total protein in human blood. The bovine serum albumin (BSA) has a negative net charge (isoelectric point - $I_{ep} = 4.7$) in human serum, similarly to the bacteria

causing catheter-related bloodstream infections. Due to the above and a positive charge of the tested surface the bovine serum albumin was used as a biological factor.

2 Materials and methods

The central venous catheters type MAHURKAR Maxid^{TM}, Covidien company, were used. This type of catheter is built with a double lumen catheter and laser cut side holes at tip. The process of biofilm formation was monitored on the distal tip of the catheter. It is made of polycarbonate-based thermoplastic polyurethanes - carbothane. The phosphate buffered saline (PBS, 0.01 M, pH 7.4), bovine serum albumin (BSA), glutaraldehyde and acetone were purchased from Sigma-Aldrich. In order to simulate clinical conditions, the system parameters were kept closely similar to clinical conditions of hemodialysis, i.e. pH=7.4, temperature of solution - 36.6 ± 0.1^oC and flow rate of solution through the dialysis apparatus (Fresenius Medical Care 4008s) - 300 ml/min.

2.1 Electrochemical measurement

The open circuit potential (OCP) values and electrochemical impedance spectroscopy (EIS) scans were recorded using a standard three-electrode configuration, with a platinum wire as a working electrode, a standard Ag/AgCl silver chloride electrode ($E_{Ag/AgCl} = 0.222$ V) as a reference electrode, and a platinum electrode as an auxiliary electrode. The electrodes were placed in the arterial lumen of the catheter. The Atlas 0531 potentiostat/galvanostat was used for all tests.

All electrochemical measurements were performed in the PBS solution (0.01 M, pH 7.4) containing 200 mg/ml BSA for different times of flow ranging from 0 h $\div$ 5 days. The measurement of the OPC values were recorded for 300 s. The EIS spectra were recorded for frequencies from 100 kHz to 0.1 Hz at zero DC potential with an AC amplitude of 10 mV.

2.2 Microscopic analysis of catheters

The microscopic analysis SEM/EDS of the brand new catheter and the catheter tested by the EIS rendered it is possible to compare changes on surfaces of catheters resulting from the flow of the PBS/albumin solution. Additional energy dispersive X-ray spectroscopy (EDS) analyses provided information on the elemental composition of every sample.

The microscopic analysis was performed by the scanning electron field emission microscope JEOL JSM 7600F equipped with an X-ray analyser INCA OXFORD. The microscopic observation of the biological layer required the use of an additional sample preparation procedure which includes: the immersing of samples in a 25% solution of glutaraldehyde in a phosphate buffer (pH=7.2) and rinsing (3 times) in a phosphate buffer solution at room temperature. Samples

were then dehydrated in 10 ml portions of acetone-water solutions with concentration rising from 10% to 100%. Drying was carried out at the critical point of CO_2, using the critical point E3000/E3100 drying apparatus (CPD). The catheter surface was covered with a chromium layer with the thickness of 2 nm.

The roughness measurements of the brand new catheter and catheter immersed in the PBS/BSA solution for 5 days were performed by an atomic force microscope (AFM) Nanosurf EasyScan 2. The catheters were subjected to the AFM scanning in air using static mode with setpoint 20 nN. Optimal scanning parameters determined experimentally to avoid protein damage or tip contamination were as follows: scan rate, 0.4 line/s with 256 points per line; integral gain. The measurements were carried out in three selected locations on the tip of the catheter.

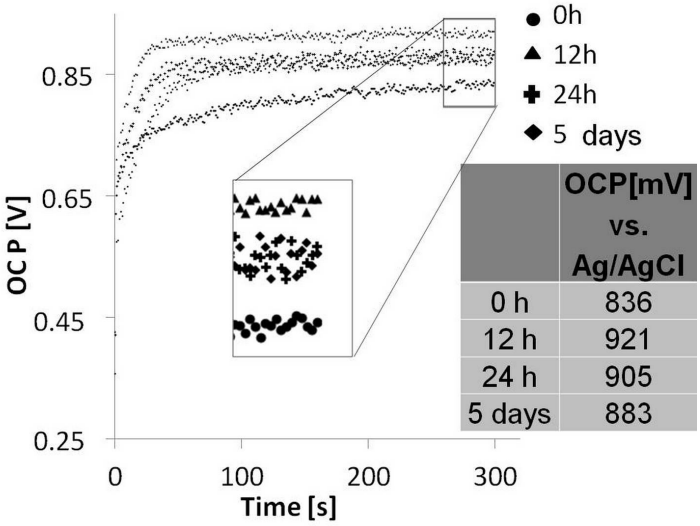
3 Results and discussion

3.1 The open circuit potential values (OCP)

The stability of the electrode potential is evaluated by recording the OCP in the PBS solution (0.01 M, pH=7.4) before and after addition of the BSA (200 mg/ml) in the standard three-electrode configuration described in the Materials and methods section for 300 s.

FIG. 1 presents the OCP curves recorded for different times of flow, i.e., 0 h ÷ 5 days. The open circuit potential of the central venous catheters made of carbothane covered with barium sulphate was 839 mV, and its values are compatible with the measurement carried out by Wei [26].

Fig. 1. Open circuit potential values (OCP) measured for 300 s in the PBS/albumin solution (0.01 M, pH 7.4) for different times of flow.



During the first 12 hours of immersion of the catheter in the PBS/BSA solution, the shift of the OCP to more positive values, from 836 mV to 921 mV, was observed. However, during the period from 1 day to 5 days a decrease in the measured values, from 905 mV to 803 mV, was noticed. The OCP values increase as a result of the additional layer formation on the catheter surface, which consists of the components of the PBS solution (mainly K^+ and Na^+).

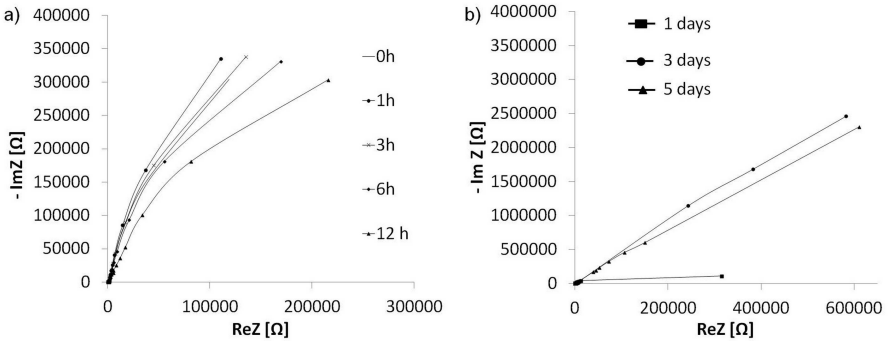
On the other hand, a slight decrease in the OPC values is related to the phosphate ($PO_4^{3^-}$) adsorption, which is favourable in the BSA adsorption mechanism [27]. The presence of the above-mentioned elements was confirmed in further EDS analyses. A decrease in the OPC values recorded on days 1-5 confirms the adsorption of the bovine serum albumin on the catheter surface. The BSA has an I_{ep} of 4.7-5.2 and is hence negatively charged in a pH neutral fluid such as the PBS [28]. I_{ep} indicates the physisorption and causes a decrease in positively charged potential of the surface. In addition, small current oscillations seen on the recorded OPC curves may suggest ongoing deposition processes and prove the metastable nature of biological layers.

In general, the OCP values reflect not only the electric properties of the electrode but also the potential change due to the oxidation or reduction of immobilized organic molecules on the electrode surface. On the basis of the above considerations, it can be stated that the first stage of biofilm formation consists of two steps: first, the ions adsorption and then biomolecules adsorption.

3.2 Impedance characteristics

The EIS spectra were recorded for frequencies from 100 kHz to 0.1 Hz at zero DC potential with an AC amplitude of 10 mV in the PBS/BSA solution (0.01 M, pH 7.4). FIG. 2 presents the Nyquist plots for film layers and their course correlates with open circuit potential measurements.

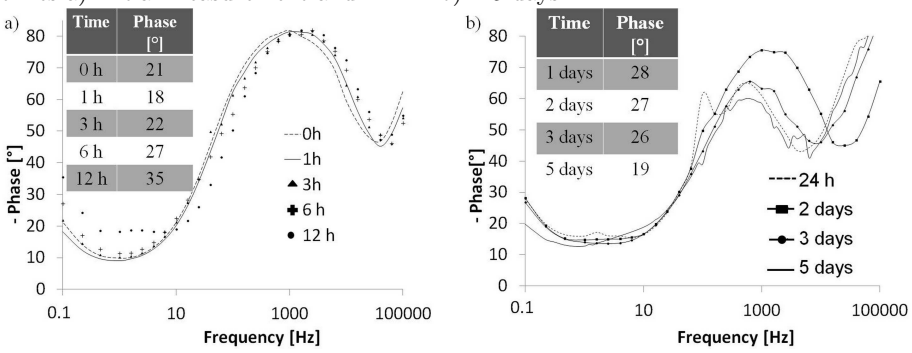
Fig. 2. Nyquist plots recorded in the PBS solution (0.01 M, pH 7.4), the frequency $0.1 - 10^5$ Hz with amplitude 10 mV vs. $E_{Ag/AgCl} = 0.222$ V, for different flow times a) spectra for initial measurement and for flows lasting from 1 to 12 h, b) spectra recorded for days 1-5 of flow through the catheters.



Because of the Pt/Pt configuration in contact with the polyurethane films, it shows only one semi-circle in the Nyquist plots throughout the whole immersion process. In the initial 12 hours, the resistivity of the platinum electrode increases due to the sedimentation of K^+ and Na^+ ions on the surface of catheters. With the immersion testing going on from day 1 to 5, the impedance and the resistance of the layers increase with the increase of flow time. Analyzing the results in FIG. 4 one can see that the impedance increased over time because of the BSA fouling on the electrode surface, which induces an increase in the charge transfer resistance. The above relation is well known in the literature [29],[30].

Bode spectra (FIG. 3), illustrating the relation between phase angles and frequencies, show that for the lowest frequency 0.1 Hz the phase angles range from 18 to 35^o. The highest heterogeneity of the tested surface (number of components of the PBS solution) was observed for the flow lasting 12 hrs (phase angle 35). A decrease in the phase angle values from 28 to 19^o for the flow lasting from 1 to 5 days is related to the formation of a protein layer. Due to the size of albumin (3.8 nm in diameter, 15 nm long) the surface heterogeneity decreases [31], [32].

Fig. 3. Bode plots recorded in the PBS solution (0.01 M, pH 7.4) over the frequency range $0.1 - 10^5$ Hz with amplitude 10 mV vs. $E_{Ag/AgCl} = 0.222V$, for different flow times a) initial measurement and 1-12 h b) 1-5 days.



3.3 Microscopic analysis of the surface of catheters

After the electrochemical experiment the analyzed samples were inspected microscopically in order to confirm the presence of BSA and PBS components. FIG. 4 shows the microstructures of surface layers deposited inside the catheters. SEM images illustrate the deposition of fine precipitates covering the inner surface of the vascular catheter. The layer spreads over the venous and arterious lumen and the central partition of the catheter. The EDS analysis (Table 1), confirms that the layer consists of deposits of the solution of phosphate buffered saline (PBS) components, i.e.: carbon (C), oxygen (O), sodium (Na), potassium (K), and phosphorus (P).

Fig. 4. SEM images of the layer on the inner surface of the catheter for its different parts characterised by the impedance spectroscopy method: a) venous lumen, b) arterial lumen, c) the central partition.

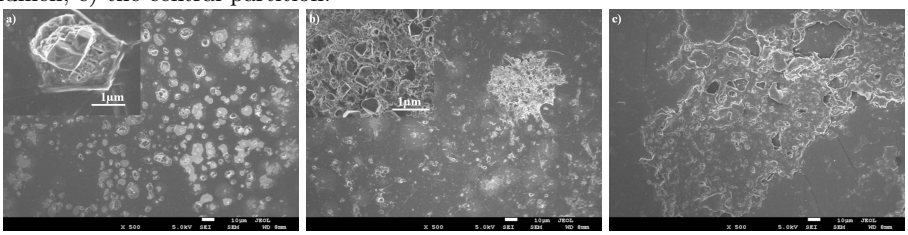
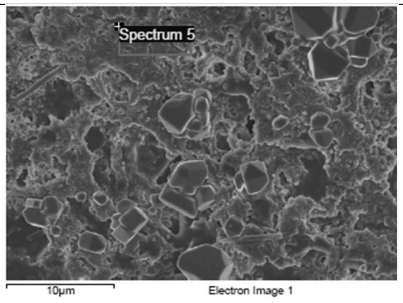
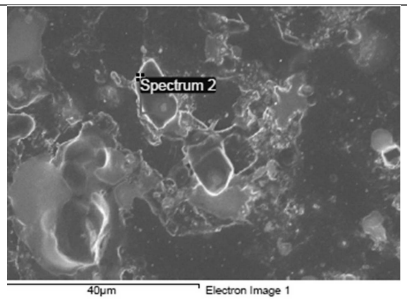
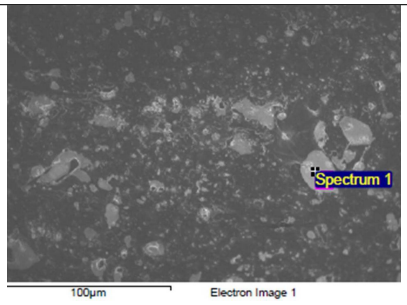


Table 1. EDS analysis of the structure of the catheter characterized by the electrochemical impedance spectroscopy.

Part of the catheter	SEM	Element	Weight [%]
Venous lumen		O Na P K	42.91 37.45 5.98 13.66
Arterial lumen		C O Na P	42.23 8.55 16.10 33.12
Central lumen		C O P	28.37 53.31 18.32

The micrographs of the brand new vascular catheter (FIG. 5) showed that the tested surface had many irregularities. The EDS analysis presented in Table 2 confirms that it is covered with barium sulfate ($BaSO_4$) - the compound used as a contrast agent which enables to localize the catheter tip after placing it in the patient's bloodstream.

Fig. 5. SEM images of the microstructure of the brand new catheter: a) x25, b) x500.

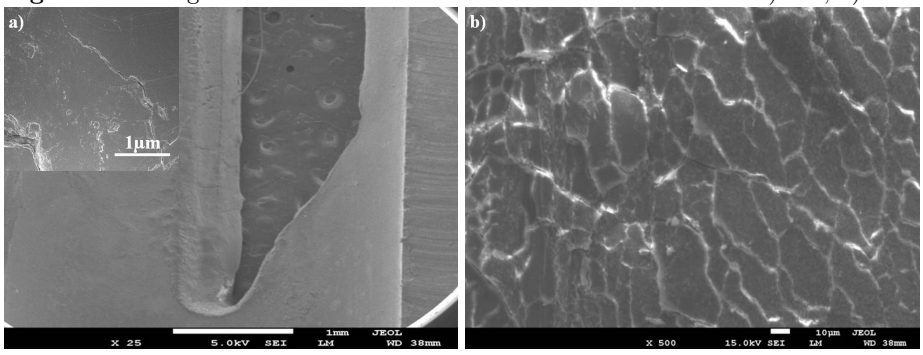
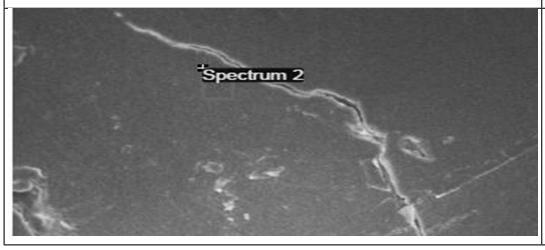


Table 2. EDS analysis of the structure of the brand new catheter.

Part of the catheter	Element	Weight [%]
	O	28.18
	C	45.76
	S	4.52
	Cr	3.07
	Ba	18.47

The AFM analysis showed that the catheter studied by EIS had the highest average roughness ($R_a = 19.61 \pm 1.96$ nm) compared to the brand new catheter ($R_a = 17.99 \pm 5.55$ nm). Higher values of R_a confirm electrochemical characteristics and analysis performed on the SEM images.

4 Conclusions

The results of the impedance analysis combined with microscopic observation confirmed the possibility of monitoring the growth of the initial biological layer on the inner surface of the catheter tip in vivo. The layer consists of carbon, oxygen, sodium, potassium and phosphorus, i.e. the components of the solution flowing through the catheter.

The surface of the brand new catheter is covered with a heterogeneous layer of barium sulfate. Such an irregular coating favours the adhesion of components of the solution used during hemodialysis as well as pathogens and biological elements. The most important is that the elaborated method offers the chance of early detection of biofilm formation in vivo, thus giving the opportunity to protect against the infection during long-term use of catheters.

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