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IMPEDANCE SPECTROSCOPY OF PROTEIN SOLUTIONS

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ABSTRACT

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Impedance spectroscopy of protein solutions

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The development and research of new types of materials is one of the most important steps ensuring technological progress. However, the development of society leads to a change in the approach to development and to the requirements to put forward to new materials. Modern society needs materials that not only have better characteristics, but are also safe and environmentally friendly. Spider silk satisfies all these requirements. This Master`s thesis is devoted to the study of protein solutions, which can be used to create artificial spider silk fibers, which properties are close to the properties of natural spider silk fibers. The study was carried out by using Electrochemical Impedance Spectroscopy. A special measuring cell was developed for the study of protein solutions. The dependencies of impedance on concentration of proteins in the solution, time and temperature were obtained. Several theories about processes of adsorption and aggregation and their impact on the impedance of the system were created on the basis of these dependencies. In addition, an equivalent circuit was selected and a test fitting was performed.
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<tr>
<td>A</td>
<td>Alanine</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CMM</td>
<td>Capillary Membrane Model</td>
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<tr>
<td>EIS</td>
<td>Electrochemical Impedance Spectroscopy</td>
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<tr>
<td>GCE</td>
<td>Glassy Carbon Electrode</td>
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<tr>
<td>G</td>
<td>Glycine</td>
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<tr>
<td>HMM</td>
<td>Homogeneous Membrane Model</td>
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<tr>
<td>ITO</td>
<td>Indium Tin Oxide</td>
</tr>
<tr>
<td>LiTFSI</td>
<td>Lithium bis(Trifluoromethanesulfone Imide), LiN(SO$_2$CF$_3$)$_2$</td>
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<tr>
<td>MaSp</td>
<td>Major ampullate Spidrons</td>
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<tr>
<td>MPTS</td>
<td>(3-MercaptoPropyl) Trimethoxysilane</td>
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<tr>
<td>NRC</td>
<td>Non-Repetitive Carboxy</td>
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<tr>
<td>NRN</td>
<td>Non-Repetitive Amino</td>
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<tr>
<td>PAH</td>
<td>Poly (Allyl amine Hydrochloride)</td>
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<tr>
<td>PBC</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PEM</td>
<td>Polyelectrolyte Multilayers</td>
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<tr>
<td>PEO</td>
<td>Polyethylene Oxide</td>
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<tr>
<td>P</td>
<td>Proline</td>
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<tr>
<td>PSS</td>
<td>Poly (Styrene Sulfonate)</td>
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<tr>
<td>Q</td>
<td>Glutamine</td>
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<td>UV</td>
<td>Ultraviolet</td>
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I. INTRODUCTION AND LITERATURE PART

1. Spider silk

The creation and research of new types of materials is an important step in the development of modern society. Spider silk is an extremely promising material, due to its excellent mechanical characteristics [1]–[3]. This work is devoted to the investigation of the behavior of protein polymer chains in an aqueous solution. As the test samples are proteins, which, subsequently, can be used to create artificial spider silk.

The advantages and problems of using spider silk were studied in the article [4]. Possible areas of application of spider silk include application in the textile industry, use as a light and durable material in aviation and body armor [5] and use in medicine [6], [7]. Materials made out of spider silk are environmentally friendly and does not cause allergic reactions. Then why is spider still not widely used when silk from silkworms has been used for thousands of years? Unfortunately, this is due to the impossibility of industrial spider breeding for the purpose to obtain silk, as spiders are lone predators by their nature and show aggression to all who invade to their territory, even to their conspecifics. One way to approach this problem is to create an artificial analog.

However, this task is far from trivial. There are thousands of spiders possessing different types of silk in the world. Even the same spider uses several types of silk for various purposes: creation of a web, nest, and cocoon. There are three major types of silk [4]: major ampullate, which is used by spiders as the framework of their future structures, it has high rigidity and medium elasticity; minor ampullate, the strength of this type is comparable with the strength of major ampullate, but the elasticity is much lower, it is used as a reinforcement of the construction; flagelliform silk is less durable, but has an extremely high elasticity. All of this allows spider silk to compete in mechanical characteristics not only with silkworm silk, but even with Kevlar and steel [8].

Spider silk consists of repeating amino acid sequences. The repetitive sections are divided into five groups [4]. GPGXX (in many cases X is Q), which probably provides the elasticity, because of the presence of b-turn spiral. Second are alanin stretches (\(A_n\) or \((GA)_n\)), which form b-sheet stacks and provide tensile strength. GGX is also responsible for elasticity. Spacers divide repeating sequences into clusters and the final one are non-repetitive terminal domains, which can be described as polypeptide chains with size more than 100 amino acids.
Eisoldt et al.\cite{9} studied the role and influence of non-repetitive terminal domains on the assembly of proteins. The repetitive sequences determine the mechanical properties of the fiber. However, also important role is played by terminal domains, which are supposed to determine the elongation and parameters at which the assembly begins. Eisoldt et al.\cite{9} considered two types of terminal domains: amino and carboxy. Despite all the variety of types of silk, they all have a common structural principle (figure 1).

**Figure 1.** Spider silk protein consist of big repetitive part with non-repetitive amino-(NRN) and carboxy-(NRC) terminal domains \cite{9}.

A prerequisite for understanding the role of terminal domains was the creation of an atomic models of these areas. As an example, Eisoldt et al.\cite{9} cited the structure of the NRN terminal domain of MaSp proteins. These domains consist of 5 α-helices and have different folds. They are monomeric above pH 6.8 (figure 2 B). When the pH is shifted towards more acidic environment, the domains from the monomers are converted into dense, antiparallel dimmers (figure 2 A). Moreover, the surface is predominantly hydrophobic. Unlike NRN domain, NRC is initially a dimer bound by a disulfide bond (figure 2 C and D). Such a complex and unique structure of terminal domains provides necessary behavior of the system under various external parameters.
Figure 2. Model of terminal domains. A) NRN dimer B) NRN monomer C) top-view and D) side-view of NRC dimer [9].

It is also necessary to understand the processes in which the proteins from the dissolved form become solid, forming fibers [10]. The whole process of forming silk fibers by a spider can be divided into four steps. The first step is the secretion of protein. The second is storage in an ampulla at a pH of about 7 in the presence of the sodium chloride. The third is an assembly in the spinning duct, at a lower pH and the last one is fiber stretching.
Interesting fact is that in nature the spiders store protein at extremely high concentrations, in a metastable form to prevent aggregation process [11]–[13]. But, when necessary, the spider can form fibers without any difficulties. Understanding the phenomena occurring in this process is a prerequisite for achieving industrial production of artificial spider silk, the parameters of which will not be worse than the parameters of natural spider silk. There is a theory that proteins would be stored in a two-phase system in the ampulla due to high protein concentration. Separation into two phases is called coacervation. Coacervates are areas with a higher concentration in comparison with the surrounding solution. Investigation of the mechanisms of formation of coacervates is an important step in the direction of creating artificial spider silk.

2. Coacervation

The coacervation process is widely known and has been studied for various types of systems. For example, the results of studying of tropoelastin coacervation was given by Yeo et al.[14].

Tropoelastin is a soluble monomer that participates in the formation of elastic fibers. When tropoelastin leaves the intracellular space, self-aggregation occurs, resulting in the formation of globules - coacervates. As a result of coacervation, tropoelastin becomes ordered and an increase in concentration occurs (inside the coacervate). Coacervation depends on the concentration of the
protein in the solution, and also on the temperature. When the transition temperature is exceeded, the monomer is assembled into the polymer due to hydrophobic interactions. And the liquid-liquid phase separation (coacervation) could be observed. This process is reversible, but with prolonged exposure of the temperature it becomes irreversible.

Figure 4. Steps of tropoelastin self-assembly. The initial phase separation include formation of 1-2 um spherules, which later combine to coacervates about 6 um size. The maturation step consist of coalesced species, which later form fibrillar structures [14].
The ability to form coacervates depends on many parameters: the structure of the molecule, the sequence of amino acids, the hydrophobicity (the transition temperature is inversely dependent on hydrophobicity) and the hydrophilicity of the domains (the effect of hydrophilicity is indirect) and also solution conditions like protein concentration, presence and concentration of salt, pH, presence of organic solvents. An increase in the protein concentration leads to an increase in the rate of coacervation and decreasing of the transition temperature. Reducing the concentration reduces the coacervation process until the process is completely prevented. The presence of salt reduces the temperature at which coacervation is possible. Increased ionic strength contributes to self-aggregation. This is due to the fact that the salt increases the effective concentration of protein. Coacervation is also sensitive to temperature and pH. Coacervation can occur when the temperature threshold is reached. An increase in pH in turn leads to decrease in temperature necessary for coacervation.

Coacervation is an advantageous process from the thermodynamic point of view, since coacervation is a transition to a more ordered state with a lower Gibbs energy. The transition depends on the parameters under which it is carried out. At subcritical parameters, the process is slow and is provided by electrostatic forces. When critical parameters are exceeded, the process is fast and it is provided by hydrophobic interactions.

It follows from all written above that the behavior of proteins in solution is a very complex process and requires a comprehensive study. For these purposes, the use of Electrochemical Impedance Spectroscopy (EIS) is very promising.

### 3. Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) is important tool for investigating the electrophysical properties of materials [15]. It allows to obtain information about charge transfer processes and allows to characterize systems whose electrochemical behavior follows from several inextricably related processes, each of which can occur at different speeds or through different channels. This method is suitable for investigating any types of solid and liquid materials, such as ionic, mixed, semiconductor and insulators. The method is especially important
for studying charge transfer in heterogeneous systems[16], [17], including phase and electrode boundaries and elements of microstructures.

As name EIS indicates, this method deals with electrical impedance or in other words with complex resistance. Complex resistance is the sum of active resistance, which could be characterized by resistor, and reactive resistance, which could be described by inductor and capacitor. It is important to note that reactive part of the complex resistance appears only when ac potential is applied.

EIS enables to research different types of processes occurring in the volume of electrolyte and on the border of electrolyte/electrode, for example, processes of corrosion and charge transfer, and various types of samples from metallic coatings to organic samples. The last one is the key theme of this thesis.

One example of using EIS as a tool for researching of organic interactions is work written by Huang et al [18]. Interaction of immobilized allergen dust mite Der f2 and murine monoclonal antibody was studied.

Huang et al.[18] chose a three-electrode scheme for measurement. Scheme consist of reference Ag/AgCl electrode, pt flag as counter electrode and modified glassy carbon electrode (GCE) as a working electrode. Measurement conditions were room temperature, range of frequency \(10^{-1} – 10^5\) Hz and voltage amplitude 5 mV. The working electrode consist of layers of (3-Mercaptopropyl) trimethoxysilane (MPTS), colloid Au, allergen and antibody. Firstly GCE was placed into 2% solution MPTS in methanol for 2 hours, which allowed to form a film of MPTS on the surface of the electrode. After that GCE with adsorbed layer of MPTS was placed in Au colloid suspension for 12 hours. This step allowed to form the layer of gold on MPTS. The next step was immobilization of allergen. And finally electrode was exposed to 0.2 ml murine monoclonal antibody solution with various concentration for 1 hour. All process is depicted in Figure 5.
The impedance measurements were based on the charge transfer kinetics of the \([\text{Fe(CN)}_6]^{3-/4-}\) redox pair, compared with different steps of the electrode assembly and were carried out at all these steps. The measurements showed, that during assembly of electrode, charge transfer resistance was increasing, especially after addition of antibody. This effect was interpreted as formation of insulating layer on the assembled surface.

Modified Randle’s equivalent circuit was chose for data fitting (Figure 6). This circuit includes: the ohmic resistance of electrolyte solution (\(R_{\text{sol}}\)); \(C_{\text{dl}}\) is element describing double layer; the electron charge transfer resistance (\(R_{\text{ct}}\)); and Warburg element (W), which describes process of diffusion.

From the results obtained it was concluded that gold nanoparticles are perfectly suitable for immobilizing allergen molecules simultaneously with the preservation of its immunoactivity. Moreover was shown, that EIS is effective method to investigate such systems. A lot of works is devoted to the use of EIS as a biosensor or tool for creation of biosensors [19]–[27].
EIS is a valuable tool to investigate interfacial interactions and phenomena. This type of interactions was studied on base of aptamer-thrombin couple by Li et al [20]. Special biosensor was constructed for exploration of the interactions. Aptamers played role of sensing element due to their high affinity and selectivity to certain types of substances. This method is label-free. Like in the work by Huang et al [18], the impedance measurements were based on the charge transfer kinetics of the [Fe(CN)₆]³⁻/⁴⁻ redox pair. Another goal of Li et al [20] was improvement of sensitivity aptamer-based biosensor. They decided to use gold nanoparticle.

![Nyquist plot](image)

**Figure 7.** Nyquist plot of the impedance spectrum was obtained on each step of GCE assembly. Equivalent circuit which was used for data fitting is represented on inset. a) bare GCE; b) GCE with gold nanoparticles; c) GCE+nanoparticles+thrombin-binding aptamers; d) + hexanethiol; e) + 0.6 nM thrombin; f) 6 nM thrombin [20].

Production of biosensor had two steps. First step was precipitation of gold nanoparticles. Glassy carbon electrode, which was previously washed and polished, was immersed in 0.5M H₂SO₄ containing 0.5mM HAuCl₄. Second step included immobilization of thrombin-binding aptamers. The electrode was immersed in thrombin-binding aptamers solution for 16 hours. After that, the electrode was rinsed by binding buffer.
As already noted, Li et al. used human a-thrombin as a sample [20]. Thrombin is the most important component of blood coagulation systems. Thrombin plays main role in a number of cardiovascular diseases and regulates many processes of inflammation and restoration of tissues of the vessel wall.

Measurements occurred with amplitude of potential 5 mV in frequency range 1 – 100 000 Hz.

Measurements were made on all stages of construction of electrode (Figure 7). Bare glassy carbon electrode demonstrate small semicircle in high frequencies. After precipitation of gold nanoparticles semicircle disappears and spectrum shows almost linear behavior in high frequencies. Addition of aptamers led to rising in $R_{ct}$ up to 920 Ohm and $R_{ct}$ was increasing up to 1450 Ohm during the process of self-assembly of aptamers on the electrode surface. It was suggested that it is connected with decreasing charge transfer by $[\text{Fe(CN)}_6]^{3-}/4-$ redox couple.

The data was processed by commercial software (CHI 660). As in earlier articles the Randle`\s equivalent circuit was chose for data fitting. The circuit showed good agreement with experimental data.

The relationship between $R_{ct}$ and concentration of thrombin was found, moreover the accuracy of data is higher than in earlier studies, which indicates high sensitivity of the manufactured sensor.

The work of Li et al.[20] showed simplicity and high accuracy of EIS based biosensor and the effectiveness of using EIS to study the interfacial processes.

One advantage of EIS is possibility to use measuring cells of different designs. It allows to use different approaches to sample measurements. Only modified glassy carbon electrodes were discussed before, cells of this construction are usually used as biosensors to detect certain types of reagents.

Affanni et al.[28] offered different approach in their study of K-casein protein aggregation. K-casein is a milk protein. This protein forms fibrils in similar way like Amyloid-β, which is connected to Alzheimer's Disease. New construction of measuring cell was created.

This cell consist of a large amount golden electrodes, located at the distance of 5 um between each other. The gold electrodes were sputtered on quartz. On the top of electrode Poly Dimethyl Siloxane reservoir was placed. Volume of reservoir was 60 uL. This system was connected to
LCR-meter, which was controlled by special software. The operation principle of such a cell is based on a change in response due to the formation of a layer of fibrils on the electrode surface.

![Diagram of protein fibrils sediment on surface of gold electrode](image)

**Figure 8.** Scheme of formation of protein fibrils sediment on surface of gold electrode [28].

The measuring procedure included two parts. First was measurement of solution of K-casein. Sodium phosphate was used as a buffer pH 7.2. Second part was measurement of protein solution after addition of fibrillation inhibitor (doxycycline). The measurements were carried out at a voltage of 100 mV in the frequency range 20 Hz - 2 MHz every 5 minutes for 18 hours.

For data interpretation authors used the equivalent circuit, which is depicted below.

![Equivalent circuit diagram](image)

**Figure 9.** Equivalent circuit, where: \(L_0\) and \(C_0\) are initial induction and capacitance of the system; CPE is constant phase element, which describes properties of double layer; \(C_{FIB}\) is capacitance due to fibril precipitation; \(R_{SOL}\) and \(C_{SOL}\) are resistance and capacitance of solution [28].

As a result, relationship between capacitance of fibril’s layer on the surface of electrode \(C_{FIB}\) and time with and without of inhibitor was obtained.
Figure 10. Dependence of $C_{\text{FIB}}$ on time with (red line) and without (blue line) inhibitor addition.

The points above and below the curves are standard deviation, that were extracted from three experiments [28].

Comparison of this method with optical fluorescence was also presented in this article. Optical fluorescence is usually used for detection of aggregation of K-casein. Main advantage of EIS was called absence of fluorescence tags, which could influence on process of protein aggregation by itself.

Oliveira and Melo [29] concluded the same. They studied the process of aggregation of surfactants and dyes in aqueous solution. Surfactants can be imagined as molecules that have a hydrophobic head and a hydrophilic tail. They tend to the interface in an aqueous solution, thereby reducing the surface tension, but at a concentration above the critical one, aggregates are formed. Moreover, the concentration, at which aggregation occurs, depends on temperature and the transition from one state to another is accompanied by a fundamental change in the physical and chemical properties of the surfactants.

Optical fluorescence is usually used to obtain the properties of the surfactants (for example critical micelle concentration), as well as in the study of milk protein by Affanni et al.[28].
However, it was difficult to estimate the accuracy of the data, due to the presence of organic dyes, which, as it was written earlier, can affect the results of the experiment by themselves. This leads to significant difficulties in the interpretation of the data. It is the reason why it was decided to use EIS to study surfactants micelle formation and organic dyes dimerization.

The measuring cell capacity was of 20 mL in which two steel plates with an area of 220 mm$^2$ were located parallel to each other. The distance from the edges of the vessel to the plates was 22 mm. The measurements were carried out at a temperature of 25 °C in the frequency range from 1 Hz to 1 MHz, the amplitude of the applied voltage was 100 mV. Data processing was performed using Z view software. To describe the process of polarization of the electrode, i.e. changes in the electrical response near and on the electrode surface, an equivalent circuit was chosen which can be described as a parallel connection of a resistor and a constant phase element, the so-called R-CPE circuit (Fig. 11).

![R-CPE circuit](image)

**Figure 11.** R-CPE circuit, where $R_{CT}$ is charge transfer resistance and CPE is constant phase element, which describes properties of double layer [29].

Impedance of the constant phase element can be described by equation:

$$Z_{CPE} = \frac{1}{(j\omega)^{\alpha}C_{pseudo}}$$

(1)

where $j$ is imaginary unit, $C_{pseudo}$ is pseudo capacitance, $\omega$ is angular frequency and $\alpha$ is constant that shows the deviation of arc from ideal one.
CPE is a non-ideal circuit element, in contrast to the ideal resistor and capacitor. Moreover, these ideal elements can be easily represented as CPE:

Ideal resistor: $\alpha=0$, $C_{\text{pseudo}} = 1/R$.

Ideal capacitor: $\alpha=1$, $C_{\text{pseudo}} = C$.

This element is excellent for describing depressed semicircles, where parameter $\alpha$ characterizes the degree of ellipticity.

The measurements were carried out for different concentrations of the surfactants in the solution. Concentration was changed by adding more amounts of surfactants to the solution. Increasing the concentration of the surfactants led to decreasing of resistance. Also one can observe the part responsible for ion transport in the obtained spectrum at low frequencies, the shape of which does not change or varies slightly with the change of the concentration. The radius of the semicircle decreases with increasing of the concentration, and the relaxation frequency shifts to higher values.

![Nyquist plot](image)

**Figure 12.** Nyquist plot of the surfactants in the solution at different concentrations [29].
Oliveira and Melo [29] also demonstrated the advantages of using a method that does not require the presence of probe molecules whose interaction with surfactant is unavoidable and have a big impact on the quality of the data obtained.

The change in the pseudo-capacitance value can also be used to determine the rate of adsorption, which was successfully done in the article by Nahir and Bowden [30]. In this paper, the interaction (adsorption) of horse-heart cytochrome c to a carboxythiol modified electrode was studied. There are various ways of obtaining data on the rate of adsorption [31]–[33]. But the goal of work [30] was to obtain data using EIS and cyclic voltammetry.

The process of creating a carboxythiol modified electrode is described in a earlier paper by Nahir and Bowden [34]. Concentrations of the protein solution under study are in the micromolar range. Such a low concentration in the opinion of the author allows not to take into account the solution related faradaic activity. In other words, it makes it possible not to include the Warburg element in the equivalent scheme. The equivalent circuit that was used to interpret the data is depicted below.

![Equivalent Circuit](image)

**Figure 13.** This equivalent circuit includes solution resistance $R_s$, charge transfer resistance $R_{ct}$, capacitance of the protein film $C_f$ and pseudo capacitance $C_a$ [30].

A Cole-Cole graph was chosen to display the obtained data. This graph differs from the Nyquist graph in that instead of the $Z$ plane we have $1 / jwZ$ plane. The authors of the article derived an equation by which it is possible to calculate $Z_{redox}$ if the impedance before the addition of the protein and the total impedance are known. The total impedance is the data that we get during the experiment.

Two situations were considered during the experiment. The first situation was when there was a big difference between solution resistance and charge transfer resistance. This lead to a difference in time constants $\tau = RC$, which in turn made it possible to distinguish between the two
seemicircles in the graph, which are related to two different processes: interaction between buffer solution and electrode, and interaction that was related to layer of cytochrome on the surface of the electrode. The second situation was when the values of solution resistance and charge transfer resistance are close to each other or even equal. This lead to overlapping of semicircles. The graphs below show these two situations and are modeled using the equation mentioned earlier.

Figure 14. Cole-Cole plot of modified gold electrode. a) Response from interaction of modified electrode with inert buffer (small semicircle) and with confined cytochrome c (big semicircle) can be resolved, and b) the parts of plot overlapped [30].

In addition, Nahir and Bowden [30] received a dependence of the number of adsorbed molecules on time which was well approximated by a straight line. From the data obtained, it was concluded that the time constant and adsorption were large and a diffusion coefficient was obtained.

In this paper, the possibility of using EIS to describe the adsorption process on the electrode surface was shown, limitations were obtained for describing this process. The limitations are that to detect the adsorption process, a sufficiently low concentration of protein in the solution is necessary, which will make it possible to distinguish two processes on the graph. However, it should be noted that all these conclusions were obtained on the assumption that the rate of adsorption is dominated by diffusion limitations.

In addition to organic samples, EIS is widely used to study metal coatings, liquid crystals and battery types. Initially, this method was developed for such systems, and only after some time its effectiveness was proved for the study of biological samples. It is worth to note that the process of describing the obtained data and the significance of a particular section of the spectrum is similar for all types of systems. Therefore, the article by Bouchet et al. [35] is quite valuable in terms of interpreting the various elements of the spectrum.
In the article [35], Bouchet explored electrolyte based on polyethylene oxide PEO-LiTFSI, which can be used for polymer lithium batteries. The measuring cell consists of two lithium foils to which the contacts are connected and a polymer electrolyte layer placed between them. The film thickness is 380 μm, and the thickness of the electrolyte is from 30 to 120 μm. For the measurement, a range of frequencies from 1 Hz to 1 MHz was chosen, the amplitude of the applied voltage was 3 mV. The results were processed using the Z view program. The temperature range was 40 - 120 °C.

In the process of aging research, the authors of the article obtained the spectrum shown below.

![Graph](image)

**Figure 15.** Dependence of spectrum of a cell Li/PEO-LiTFSI/Li on time at 90 °C, numbers show frequency [35].

They were able to identify four frequency bands (Fig. 15 and 16), each of which was responsible for the behavior of a particular section of the chain or process.
1) The high frequency range ($10^5$ Hz) was responsible for processes in the sample volume and is represented as $R_{\text{bulk}}$ or $R_{\text{solution}}$. Also it was necessary to include inductance of wires which appeared at very high frequencies.

2) The kilohertz range ($10^5$ - 100 Hz) appeared as a semicircle on the Nyquist plot. This semicircle describes the formation of the surface layer on the electrode and describes the processes occurring at or near the surface of the electrode.

3) The mid-frequency limit was not reproducible. It is hard to say if there is any particular physical meaning in it, or it is just the area of transition between the two processes.

4) The low-frequency range was responsible for ion transport and was described by Warburg element.

![Correction of spectrum for inductance](image)

**Figure 16.** Division of the spectrum into four frequency bands and correction of spectrum for the inductance [35].

For each of these ranges, a time dependence was found. High and low frequency regions of the spectrum do not depend on aging. The kilohertz region in turn depends, the resistance on the surface increasing with time, but the capacitance decreases and it took 5 days to stabilize these parameters in the studying sample. It was also shown that changes in the excitation signal from 3 mV to 250 mV do not lead to a significant change in the spectrum. Consequently, it does not matter what voltage is applied, naturally having in mind the standard limits for the impedance analysis (millivolt range).
As an equivalent scheme, the scheme shown below was chosen, which is a consecutive connection of the elements responsible for the previously mentioned processes.

**Figure 17.** Equivalent circuit, where $R_C$ and $L_C$ are resistance and inductance of connectors, $R_b$ and $C_b$ are resistance and capacitance of bulk, $R_i$ and $CPE_i$ are resistance and constant phase element of interface, $R_c$ and $CPE_c$ are resistance and constant phase element, which describe behavior of spectrum at middle frequencies, $R_d$ and $\tau_d$ are parameters of short Warburg element [35].

It can be seen from Bouchet *et al.* [35] article that the response of the system from the electrode surface changes with time. What the reason is for this behavior was not said. Such behavior could be caused by adsorption, in other words like some sort of assembly process on the surface of the electrode.

Adsorption processes have been studied in articles about which it was written earlier. However, it is worth mentioning the work of Barreira *et al.*[36], in which the process of assembling polyelectrolyte multilayers (PEM) was studied and two models were proposed to describe the behavior of the system during the assembly process. EIS was used to determine the electro activity of the redox couple $[\text{Fe (CN}_6\text{)]}^{3^-}/[\text{Fe (CN}_6\text{)]}^{4^-}$.

For the measurements [36], a three cell electrode scheme was chosen where the reference electrode was Ag / AgCl, the counter electrode was platinum, and the working electrode was represented as a modified gold disc. PEM consisted of poly (styrene sulfonate) as the polyanion and the poly- (amino acids) poly-L-histidine (pK$_a$~7), poly-L-lysine (pK$_a$~10), and poly-L-arginine (pK$_a$~12) as the polycations. Na$_2$SO$_4$ was used as the support electrolyte. The measurements were carried out in the frequency range from 100 MHz to 15 kHz, the amplitude of the applied voltage was 10 mV. For processing the data, the scheme depicted below was used.
This equivalent scheme consists of solution resistance ($R_s$), film capacitance ($C_f$), film resistance ($R_f$), capacitance of double layer ($C_{dl}$), charge transfer resistance ($R_{ct}$), diffusion impedance ($Z_d$), and resistance ($R_m$), which describes ohmic conduction in the film.

The authors of the article [36] developed two models to describe the obtained data as a result of assembling the multilayer structure. The first is the Capillary Membrane Model (CMM). In this model, a layer by layer assembly was assumed. At the beginning, some of the electrode was covered with a film, while the other remained bare or covered with only 1-2 layers. Gradually, the size of these bare zones decreased or they become covered with polyelectrolyte. But even then, these places could be determined, since the density of the layers in these areas is less than in the rest of the film. Thus, a porous coating model was obtained. This formulation of the problem required a different approach to the determination of diffusion, because of possible significant deviations from the standard linear model, which is used in the conventional definition of the Warburg element. The authors of the paper proposed equations (2) - (5) [36] to determine the diffusion impedance within the framework of this model.

\[
Z_d^{(c)} = \sum_k \frac{\sigma_k^{(c)}}{\sqrt{\omega}} \left[ 1 + \frac{\theta}{(1 - \theta)} \left( \frac{1 + (q_k/\omega)^2}{} \right)^{1/2} + \frac{q_k/\omega}{1 + (q_k/\omega)^2} \right]^{1/2} + j
\]

\[
\sigma_k^{(c)} = \frac{RT}{n^2F^2A\sqrt{2D_k^f c_k^f}}
\]
\[ q_k = \frac{2D_k^f}{r_b^2 \theta (1 - \theta) \ln \left( 1 + 0.27/\sqrt{1 - \theta} \right)} \quad \text{for} \quad 1 - \theta > 0.1 \]  

(4)

\[ q_k = \frac{D_k^f}{0.36r_a^2} \quad \text{for} \quad 1 - \theta \leq 0.1 \]  

(5)

Where: \( Z_d^{(c)} \) is diffusion impedance of circular active area of radius \( r_a \); \( \theta \) is coverage; \( R \) is ideal gas constant; \( T \) is temperature; \( n \) is number of electrons involved in the electrode reaction; \( F \) is Faraday constant; \( A \) is electrode area; \( D_k^f \) is diffusion coefficient; \( c_k^f \) is concentration; \( r_b \) is radius of inactive area.

The second model is the Homogeneous Membrane Model (HMM). Within the framework of this model, it is assumed that the multilayer polyelectrolyte forms a uniform membrane on the surface.

During the measurement, EIS spectra were obtained at different stages of film formation, in other words for different number of layers. It was revealed that the overall structure of the spectrum does not change for the first six layers and was represented as a semicircle at high frequencies and a straight line at low frequencies. The size of the semicircle increased with the number of layers. With a further increase in the number of layers, a fundamental change took place in the structure of the spectrum. The size of the semicircle at high frequencies increases significantly, and the straight line at low frequencies become similar to the second semicircle, which was explained as the dependence of diffusion on effective coverage. As the number of layers increased, the thickness and density of the film increased. The authors showed that the CMM described the experimental data well, while the HMM become applicable only for a large number of layers.
Figure 19. Nyquist plot of of 5 mM [Fe(CN)$_6$]$^{3-/4-}$ in 0.1 M Na$_2$SO$_4$ at the PEM-modified gold electrode (pSS/pLys films) for different numbers of layers [36].

Barreira et al.[36] showed that changes in the spectrum during assembly of layers on the surface of the electrode are associated with a decrease in the active zone of the electrode, with the active zone being understood not only as a bare electrode, but also zones through which ion transport is possible.

One year later the article [37] was published where the effect on the spectrum of various supporting electrolytes and temperature was studied on the basis of the obtained models. Most often, great attention is paid to the conditions before and during the assembly of the film, and changes after assembly remain unaccounted for. The aim of work [37] was to correct this injustice and show the effect of supporting electrolyte and temperature on data.

As the test sample was used poly (styrene sulfonate) / poly (allyl amine hydrochloride) (PSS / PAH) multilayers. The measurements were carried out at a temperature of 20 °C, the same type of cell construction was used as in the previous article [36]. The following electrolyte was used: 0.5 M NaClO$_4$, 1 mM K$_4$Fe(CN)$_6$, and 1mM K$_3$Fe(CN)$_6$. Frequency range was 0.1 Hz - 10 kHz.

The circuit in the figure 18 was used as an equivalent circuit. Here $Z_d$ is determined in accordance with the previously proposed models: CMM when the coating is not complete and at
the initial stages of growth there remain active islets, and HMM when the number of layers is large enough to represent the film as a homogeneous structure (N> 20).

As factors influencing the structure of the film, the authors singled out: penetration of small ions into the multilayer, charge screening effect, and increasing ionic strength that lead to extrinsic compensation.

Spectra of a multilayer with different supporting electrolytes were obtained (Fig. 20).

![Figure 20. Nyquist plot of a multilayer with different supporting electrolytes.](image)

According to these data, it was concluded that salts with greater ionic strength have lower impedance and coverage decreases with increasing valence of cations.

The study of the effect of temperature showed a decrease in impedance with increasing temperature (Fig. 21). The temperature range in which the study was conducted was 15 – 45 °C. In the course of data processing, the authors found that an increase in temperature also leads to an increase in the diffusion coefficient and a decrease in the resistance of the layers, while at the same time it does not affect the values of the degree of coverage and the radius of the pores.
In this article [37], the authors demonstrated the possibility to study the process of assembling polyelectrolyte using EIS. The viability of the models advanced by them was shown, as well as the great influence on the measurements of such parameters as the temperature and composition of the supporting electrolyte.

Obviously, there are a large number of parameters that can affect the EIS data received. And this is not only the temperature or composition of the surrounding electrolyte, but also the availability of redox probes, the frequency interval, the design of the measuring cell, etc. Even when one try to correlate the data with the work of other people, it is needed to take into account that each researcher uses those measuring parameters that are most convenient and customary for him. Moreover, these parameters can differ, starting from the temperature and applied voltage up to the design of the measuring cell. It was the problem of the data linking and the influence of the initial experimental conditions on the final result that was considered in the article by McDonald and Andreas [38].

In their article, McDonald and Andreas[38] studied the influence of such parameters as: the presence of an initial measurement in the phosphate buffered saline (PBC), the presence of a redox probe, the magnitude of the applied potential, the magnitude of the DC and AC current in
the formation of the film. As a test sample, Bovine Serum Albumin (BSA) was chosen, this protein was extensively studied using EIS.

The measurements were carried out at a temperature of 22 °C, using a measuring cell with a three-electrode configuration. The working electrode was made of a platinum wire. The counter electrode was a coiled platinum wire. Reference electrode was Ag / AgCl. The film was applied by incubation for 30 minutes in BSA solution. The measurements were carried out in the frequency range 0.1 Hz - 0.5 MHz and at a voltage of 10 mV, also redox probe were used for the measurement.

As an equivalent scheme for data processing, the scheme shown below was used.

![Equivalent scheme for processes of adsorption](image)

**Figure 22.** Equivalent scheme for processes of adsorption. Where Rs is solution resistance, CPE is constant phase element representing the protein film, Rf is film resistance, Cdl is double-layer capacitance, Rct is charge-transfer resistance and W is Warburg impedance [38], [39].

The authors of the article [38] compared the spectrum obtained by adsorption of BSA to a pure electrode and the spectrum obtained by adsorption of BSA on an electrode that was earlier used for initial measurement, that is, for measuring protein-free electrolyte. It was concluded that the adsorption on the electrode previously used for the initial measurement passes less efficiently than on a clean electrode, as evidenced by the difference in the dimensions of the semicircle at high frequencies and significant differences in the value of the charge-transfer resistance.

An increase in the size of the semicircle at high frequencies was also observed during the studying the influence of the potential on the structure of the BSA film. The growth of the film was carried out at two different potentials of 0.171 V and 0.35 V, measurements were made at the same potentials. The change in the size of the semicircle in comparison with the blank
experiment indicates the process of BSA adsorption on the electrode surface. It is also seen that the size of the semicircle is larger for a larger potential, which can be explained by the more effective adsorption of BSA (Fig. 23).

Figure 23. Nyquist plots of films obtained from incubation at 0.171 V (dot red) and 0.35 V (solid black), as well as a blank (dot-dash blue) experiment [38].

On the basis of the data obtained by the authors of the article [38], it was concluded that it is necessary to be extremely careful when choosing the measurement potential and the potential at which the film grows, the most correct is to make them the same. In addition, it was shown that the presence of a variable potential does not affect the formation of the BSA film.

Thus, the authors of the article [38] concluded that the measurement conditions influenced the obtained data. The factors that exerted the greatest influence, was the presence of an initial measurement in the PBS solution, the second most important was the potential at which the film grows, the least influence on the results of the experiment was exerted by the presence of a variable potential.
Studying of the influence of different parameters on the output data is an important step to understanding how to correctly use EIS and to obtain reliable information about the processes occurring during the experiment, but no less important is the process of processing the data. The problem of interpreting data is one of the most important when working with EIS.

The process of interpreting the obtained data with EIS involves the development of an electrochemical model that is capable of adequately describe the obtained data. As such models, so-called equivalent schemes[40], [41] are widely used, which have already been mentioned many times. They are electrical circuit analogues. However, before developing such a scheme, it is necessary to understand whether the system is stationary and how many processes are represented in the system under study.

If the model does not accurately describe the experimental data, there are several reasons for this. The first is noise, error, deviation in the experimental data, and the second possible reason is the incorrectly selected model. There are ways how to get almost complete agreement of the model with experiment, however, the use of such methods leads to the impossibility to obtain physical justification for the elements of the model.

The authors of the article [42] divide the models into two classes: process model, which purpose is to predict the response of the system for a particular physical phenomenon, and the measurement model, which is constructed by successively approximating the model line to the experimental data. The measurement model is used to obtain initial parameters, which can further help in creating the process model.

As the studied measuring model, the authors chose the Voight model [42]:

\[
Z(\omega) = Z_0 + \sum_k \frac{\Delta_k}{(1 + j\tau_k \omega)}
\]

(6)

where \(Z_0\) is ohmic resistance, \(\tau_k\) is time constant of element \(k\), and \(\Delta_k\) is equivalent to \(R_k\).

The advantages of using the measuring model in the opinion of the authors are: the number of elements is smaller than for the usual polynomial fit, the data can be immediately checked for compliance with the Kramers-Kronig equations, and it does not require extrapolation.
The purpose of the paper [42] was to demonstrate the applicability of the measurement model for describing complex electrochemical systems that include mass transfer phenomena (through Warburg elements), pseudo-capacitive behavior, frequency dispersion (through constant phase elements), and kinetic control.

For this demonstration, five equivalent circuits were used, which were used to interpret various systems (Fig. 24).

![Equivalent circuits for various systems](image)

**Figure 24.** Equivalent circuits for: 1) hydrogen evolution on LaNi₅; 2) corrosion of carbon steel in NaCl solution; 3) corrosion of iron in H₂SO₄; 4) corrosion of a model pit electrode in NaCl and 5) corrosion of a painted metal [42].

The data obtained with these equivalent circuits were approximated using the proposed measurement model.
The following is an example of a data fitting for circuit 1 from figure 24.

Figure 25. Data fitting of circuit 1 with three Voight elements [42].

The model was constructed by the sequential addition of elements, and sufficient accuracy was achieved with three Voight elements. A further increase in the number of elements also increased the accuracy, but it was not significant compared to the noise level in the system.

For the remaining schemes, good approximations have also been obtained. Thus, the authors of the article [42] showed that the Voight model can be used for a statistically adequate fit, and can also be used as a means for verifying conformity to the Kramers-Kronig equations.
II. MATERIALS AND METHODS

4. Cell manufacturing

The right configuration for the measuring cell was selected at the beginning. Various options were considered, including the cell design presented in the article by Affanni et al.[28]. As a result, a two electrode configuration of the cell was chosen, where the working electrode was a gold film, the counter electrode was Indium Tin Oxide (ITO) glass and the gap between two electrodes was 100 μm. This type of cell was chosen because of the ease of manufacturing and the ability to create a large amount of the cells at a time.

![Figure 26. Scheme of the measuring cell.](image)

The manufacturing process includes several stages. In the first stage, the substrate is prepared for the deposition of a gold electrode. The substrate is a conventional 1 mm thick glass microscope slides. These slides are cut into 25x25 mm pieces. After the pieces of the desired size have been obtained, this glass pieces were cleaned. The cleaning process includes (in order) in itself: washing with ordinary water and detergent, rinsing with ethanol and milli-q water. After that, the glass is dried with nitrogen under pressure (2 bar). As additional cleaning, the glass is treated with UV source for 15 minutes.
The purification process is necessary to ensure the maximum degree of adhesion of gold to the glass.

When cleaning is completely done, it is necessary to apply a mask to the surface of the glass to obtain the desired shape of the electrode and to create contacts. The mask is a plain sticky tape, placed between two strips of double-sided adhesive tape with a sticky side up. After the tape has been fixed, the shape of the electrode is cut out, in our case it is a circle with a diameter of 10 mm and a contact track 2 mm wide. This shape of the electrode was chosen because of the availability of suitable tools that allow to cut out the desired shape without much effort. When all the necessary operations are completed, the glass is placed on the sticky tape, after which the glass and sticky tape are disconnected from the double-sided tape. Surpluses of the tape are cut off.

The next step in the manufacturing process is sputtering. For this, the prepared glasses (four at a time) are placed in the sputtering machine (Emitech K100X). The sputtering occurs at 50 mA current for 4 minutes. As a result, several tens of nanometers thick film is formed. After sputtering, the sticky tape is removed from the glass surface.

After sputtering, measuring cell was assembled. To create a gap between the electrodes, soda lime glass microspheres 100 μm in diameter mixed with UV cured glue (Norland UV Sealant 91)
are used. The glue / sphere ratio was chosen experimentally. This mixture is mixed in a syringe and then applied through the needle to the surface of the glass in four places. The glue application process should be carried out in a dark room, since the presence of sunlight can lead to premature hardening of the glue. A needle is used in order to apply a small amount of the gluing mixture. The concentration of spheres in the glue is selected from the considerations that at each point where the mixture was applied, it is necessary to have at least one microsphere. Failure to do so will result in a change in the signal received during the measurement or in the complete inoperability of the cell, since in the absence of microspheres in all four points, the electrodes are in direct contact with each other, which leads to the inability to use this measuring cell.

After applying the glue with microspheres onto the glass with the deposited electrode, ITO glass with conductive side down is placed on top of it. ITO glasses were also pre-cut into 25x25 mm pieces and cleaned. Next, this construction goes to the drying. Drying was done with UV source for 10 minutes. It is important to be careful during moving cells from the assembly place to the UV source, since the glasses slide easily relative to each other, which can lead to cell damaging.

![Figure 28. Picture of assembled measuring cell.](image)

After the drying process is completed, all cells are checked for presence of microspheres by using a optical microscope. The example of such a study is given below.
When all cells are checked, wires are attached to them. The length of the wire is about 7 - 10 cm. The wires are attached using a silver paste. Silver paste dries at room temperature in 30-40 minutes. After that the cell is ready for measurements.

5. Measuring setup

The measurements were carried out using HF2IS Impedance Spectroscope from Zurich Instruments. This spectroscope has two differential measurement units with a wide frequency range and 4 dual-phase demodulators. The possible frequency range is 0.7 µHz - 50 MHz, and the applied voltage is up to 10 volts.
Figure 30. HF21S Impedance Spectroscope.

In the complete set with the equipment there is a convenient software (Zi control) which allows to trace changes in an output signal in a mode of real time.

Figure 31. Zi control software.

All measurements were made with the following parameters: frequency range 5 Hz - 10 MHz, applied voltage 100 mV and number of points 200. Averaging of the signal varied depending on the type of measurement.
Also, HF2IS Impedance Spectroscope was used in conjunction with the heating / cooling stage LTS 350, which was controlled by temperature and a cryogenic controllers. The temperature controller TMS 94 was used in the measurements together with the Cryogenic controller LNP 94/2, the manufacturer of all these devices is the Linkam Company. These devices allow to change the temperature of the sample in the range from -196 to 350 °C. The temperature is controlled by means of a platinum thermocouple.

![Image](figure32.jpg)

**Figure 32.** Heating (upper) and cooling (lower) controller from Linkam Company.

The interface of these instruments allows to set the rate of temperature change, the time during the temperature must be kept unchanged, and even create cycles through which the temperature will change. The samples are cooled by liquid nitrogen.
6. Sample preparation

Protein samples were produced in *Escherichia Coli* (*E. Coli*) bacteria. Magic Media was used as the growth media. Purification of proteins was carried out with heat treatment in 70°C for 30 min and then size exclusion chromatography (Econo-Pac 10DG desalting columns were used for this). After that majority of the impurities are already removed and sample can be concentrated with centrifugal concentrators (30 kDa cut off in the filter).

The studied recombinant silk protein has a dumbbell structure; it has globular terminal groups and silk sequence in the middle. Similar structure is depicted in figure 1. Size of the protein is 85288 Da.

All samples were in MilliQ-water.

Samples are stored frozen in liquid nitrogen. This method of storage prevents the aggregation of proteins and allows to maintain a two-phase state, if they were so initially. For the experiment, the samples are thawed, after which it is necessary to place them in a centrifuge for 90 seconds at a rotation speed of 7000 rpm. For this purpose Eppendorf centrifuge 5424 was used. This procedure is carried out to separate and remove aggregated protein from the solution. Unfortunately freezing also affects the sample, but this effect is much less than if stored at room temperature. After centrifugation, these proteins form a film precipitate on the wall of the vessel. The remaining solution is moved to a new vessel, and it is ready for measurements.

7. Insulation of measuring cell

Aqueous protein solutions were used for the measurements. This type of specimens is subject to rapid evaporation, which in turn can lead to strong deviations in the experimental data. Therefore, it was decided to isolate the sample inside the cell from the external environment. The most simple and effective way was to apply glue around the perimeter of the cell. The glue was UV cures Norland Optical Adhesive 81. This type of glue was chosen because of the absence of components that could damage the sample, and also because of the fast drying speed, which in turn minimizes the effect of UV radiation on the sample. The process of applying the adhesive consists of three steps: the first is the application of glue from the contacts side and the subsequent drying, the second is the measurement of the empty cell. This stage makes it possible
to make sure that the cell is assembled correctly and it can be used for measurements. The third stage is the sample loading followed by a full insulation with glue. This cell design prevents evaporation of the sample. The effect of glue on the cell has also been studied (Fig. 33) and it manifests itself only in a small change of capacitance. Capacitance of the cell without glue was 11 pF, with glue 16 pF.

**Figure 33.** Bode plot of the empty cell with (red-blue line) and without (black line) glue.
8. Influence of storage on measuring cells

During the work it was discovered that empty cells cannot be stored for a long time (several months).

![Bode plot](image)

**Figure 34.** Bode plot of the empty cell after assembly (black line) and after storage during two months (red-blue line).

From the figure 34 it can be seen that the spectrum of the cell differs radically from what it was two months earlier. There are several possible explanations for this change. One is that dust or something else got into the gap between the electrodes and as a result we see the spectrum of this dust, because the cell was washed with milli-q water.

This procedure helped, but spectrum of this cell still showed deviation from the usual one. This could be the result of the formation of an oxide film on the surface or the result of damage of the electrode. In any case this cell could not be used for measurements.
9. Loading of sample

The process of loading a sample into the cell has several nuances. First, the sample is placed in a syringe, after which the sample droplets are placed on the interface between the glass with the deposited electrode and ITO glass, after which the sample penetrates into the gap between the glasses due to the action of the capillary forces. However, periodically there was a situation when an air bubble remains on the working electrode.

Figure 35. Measuring cell with big air bubble (bright area on electrode).

The bubble could be removed from the electrode by centrifugation. However, this method is suitable only for samples with a low concentration of protein, because at a high concentration centrifugation makes redistribution of proteins in space and it is unknown how long it will take to restore the initial state of the system. Therefore, it was decided to ignore the presence of small bubbles on the surface of the electrode. Their impact on the output is not great.
III. RESULTS

10. Initial experiments and first results

Experiments were carried out on three samples with protein concentrations of 9, 15 and 100 g/l. Sample 100 g/l contains coacervates, as well as fluorescent molecules. The presence of coacervates was previously checked by means of a microscope.

![Figure 36. Coacervates in the solution with 100 g/l protein concentration.](image)

All three samples were measured at room temperature with averaging of 32, which means that each point of the graph is obtained by averaging the 32 measured values at the same frequency. On the basis of the obtained data, a graph of the imaginary part of the impedance dependence on the real part (also known as the Nyquist plot) was plotted.
Figure 37. Nyquist plot of the samples with concentrations 9, 15 and 100 g/l. Left plot is the full spectrum, right plot is the part of spectrum responsible for the surface layer behavior.

All samples were re-measured after nine days. The obtained data showed clear change in the spectrum of the samples (figure 38).

Figure 38. Nyquist plot of the 9 g/l sample after storage.
Moreover, it was found that after some time water appeared in the air bubbles condensation of water occurred, which led to the formation of small drops inside the bubble. Whether the change in the spectrum was related to the formation of droplets or the something else, we just had to find out.

This batch of samples was a trial. It was used to determine the possibility to study the properties of protein solutions by EIS, and also allowed to work out an experimental technique. The first measurements showed to which direction to guide the research and what tasks should be solved.

For example, one of these tasks was to determine the influence of the experiment by itself on the behavior of the system. For this purpose, the same sample was measured several times in a row without interruptions between measurements. The data showed only a small deviation in the region responsible for the inductance of the wires. This lead to the conclusion that there is no influence of several repeated experiments on the output data.

11. Equivalent scheme and fitting results

On the basis of the data obtained from the first experiments, an equivalent scheme presented in figure 39 was chosen. This circuit seemed to be the most suitable for our system.

![Equivalent circuit](image)

**Figure 39.** Equivalent circuit, which was used for data fitting. Where: \( R_S \) is solution resistance, \( R_{sl} \) and \( CPE_{sl} \) are resistance and constant phase element of surface layer, \( CPE_{dl} \) is phase element of double layer, \( R_{ct} \) is charge transfer resistance and \( W \) is Warburg element.
Equivalent circuits are used for interpretation of obtained data and to extract parameters of circuit elements, which farther could help in determination of physical properties of studied system. It is the reason why it is necessary to chose right equivalent circuit.

The scheme (Fig. 39) almost coincides with the scheme proposed in the article by McDonald and Andreas [38]. One difference is that $C_{dl}$ replaced by CPE$_{dl}$. Moreover, the behavior of the data obtained by the modeling processes using this scheme satisfies the Kramers-Kronig equations, which was proved in the article by Agarwal et al.[42]. Using the standard Randles scheme does not allow to describe the experimental data with sufficient accuracy. In the approximation, significant deviations occur, especially in the low-frequency region. Due to this fact scheme was complicated by sequentially adding elements until good agreement was received.

The plotting and fitting of the data was carried out in the Z view program. This software was mentioned earlier in the first chapter as it was used by Oliveira and Melo [29]. The scheme shown in the figure 39 was created in this program. The selection of the parameters of the elements was carried out manually.

![Equivalent Circuit](image)

<table>
<thead>
<tr>
<th>Element</th>
<th>Freedom</th>
<th>Value</th>
<th>Error</th>
<th>Error%</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>Free(s)</td>
<td>187</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CPE1-T</td>
<td>Free(s)</td>
<td>4.9E-7</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CPE1-P</td>
<td>Free(s)</td>
<td>0.68</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>R2</td>
<td>Free(s)</td>
<td>49</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CPE2-T</td>
<td>Free(s)</td>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CPE2-P</td>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>R3</td>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
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<td>N/A</td>
</tr>
<tr>
<td>W1-P</td>
<td>Free(s)</td>
<td>0.3</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Figure 40.** Equivalent circuit and parameters, which was used for data fitting in Zview software.
The parameters were chosen so that the theoretical curve obtained as a result of calculation by an equivalent circuit passed as close as possible to the experimental plot. In addition, the selection was carried out taking into account all three plots (Nyquist plot, modulus of Z and theta versus frequency), in order to ensure the most accurate and adequate result. The result of the fitting is shown in figure 41.
Figure 41. Result of fitting. Red-blue curve is spectrum of 9 g/l sample and green line is fitting curve.

In addition, the experimental data were processed in the Matlab software. The same equivalent circuit was used for the data fitting. The equation of impedance for fitting was derived based on the elements included in the equivalent scheme.
Figure 42. Result of fitting in Matlab. Red dots are experimental data (9 g/l sample), blue line is fitting curve.

Where the diffusion element was presented in the standard form:

\[ Z_W(\omega) = \frac{\sigma_D(1 - j)}{\sqrt{\omega}} \]  

(7)

Where \( \sigma_D \) is determined the same way like in equation (3).

From figure 42, it can be seen, that this approach also showed acceptable results.

The approximation using the proposed scheme (figure 39) was carried out only for one set of data obtained by measuring the 9 g/l sample. In the future, additional calculations will be needed to ensure that the selected circuit correctly describes the experimental data.
12. Main measurements: dependence on concentration, time and temperature

To study more clearly the dependence of the spectrum on time and on the concentration, it was decided to make the batch of solutions of proteins with different concentrations. The proteins in these solutions are the same since they were prepared at the same time under the same conditions. Such an approach to the choice of samples allow to avoid the effect of differences in the composition of proteins on the resulting EIS spectrum. Also, cells made at the same time were used to measure this batch of samples.

The second batch consists of samples with the concentrations presented in the table 1.

Table 1. Concentrations of the samples in the second batch

<table>
<thead>
<tr>
<th>Concentration g/l</th>
<th>5.2</th>
<th>16.4</th>
<th>30.6</th>
<th>41.88</th>
<th>60.15</th>
<th>76.3</th>
<th>98.1</th>
</tr>
</thead>
</table>

Before carrying out a series of experiments with these samples, the milli-q water spectrum was measured. Milli-q is the supporting electrolyte in this system.
After measuring the spectra of milli-q water, the spectra were measured for each sample. Based on the obtained data, a spectrum was plotted in which the dependence on concentration is clearly visible.

**Figure 43.** Nyquist and Bode plots of milli-q water.
The most surprising was the behavior of the protein solution with concentration 98.1 g / l. Possible interpretation of the obtained data will be described in discussion part.

As mentioned earlier, the spectrum of the sample changes over time, so an experiment was carried out to determine the dependence of the EIS output parameters on time. For this purpose a sample with the lowest concentration of 5.2 g / l was used. Three measurements were made at intervals of 20 minutes and two further measurements after 4 and 11 days.
Figure 45. Nyquist plot of the 5.2 g/l sample. Dependence of the spectrum on time.

From the plotted graph it can be seen that the system is extremely dependent on time. This is very important because due to the technological features of the assembly of the cell and loading of the sample, the time from when the sample was loaded into the cell prior to the start of the experiment is more than 40 minutes.

Second type of dependence, which was interesting to study was the temperature dependence. Two samples with the lowest (5.2 g/l) and the highest (98.1 g/l) concentrations were used for this experiment. Control and temperature changes were carried out with the help of controllers and heating / cooling stage. Zero degrees Celsius was used as a starting point, samples were heated to 80 °C and then cooled down to the starting temperature. Before cooling, both samples were measured at room temperature. Cooling to zero degrees was carried out at a rate of 10 degrees per minute. Averaging was 16.
Sample with the concentration of 5.2 was measured in the temperature range from 0 to 50 °C with the step of 5 °C, the heating rate was 5 °C per minute and time between measurements was 5 minutes. In the temperature range from 50 to 80 °C the step was 10 °C, but speed and time did not change. After that, cooling followed from 80 to -10 °C followed, with step 10 °C, speed and time did not change. The total time of the experiment was 2 hours. The temperature step was changed since it became clear that there was no need for so much data.

**Figure 46.** Temperature dependence of the 5.2 g/l sample during heating. Step was 5 degrees below 50 °C and 10 degrees above 50 °C.
Figure 47. Temperature dependence of the 5.2 g/l sample during cooling. Step was 10 °C.

Sample with the concentration of 98.1 g / l was measured in the temperature range from 0 to 80 °C: with the step of 10 °C, the rate of change of the temperature was 5 °C per minute, the time between measurements was 5 minutes. After that, the sample was cooled to a temperature of -10 °C, at a rate of 5 °C per minute. The measurements were carried out at temperatures of 65, 50, 35, 20, 10 and -10 °C. The total time of the experiment was 80 minutes.
Figure 48. Temperature dependence of the 98.1 g/l sample during heating.

Figure 49. Temperature dependence of the 98.1 g/l sample during cooling.
Such temperature differences can lead to different effects on proteins, so it was decided to check the condition of the solution under the microscope. The first sample (5.2 g/l) does not have any visible differences or defects, however, the study of the second sample (98.1 g/l) revealed the presence of aggregates.

![Figure 50](image)

**Figure 50.** The 98.1 g/l sample under microscope (bright field).

Unfortunately, the sample was not studied by a microscope prior to measurement, so it is unknown at what stage the aggregation occurred.

**13. AFM and SEM measurements**

To ensure the most accurate interpretation of the EIS data, it was decided to make an additional study of the surface of the working electrode by using AFM and SEM. Interest is in the surface of both the clean, unused electrode, and the surface of an already used electrode. Several cells were disassembled for the study. One of them was never used, so it was decided to use it for the studying of the surface of a pure electrode. The second had been used for measurements and contained material with the concentration of 60.15 g/l, this cell was stored for 14 days until the time of disassembly. After the used cell was disassembled, it was dried at room temperature.
From the results of a pure electrode, it can be said that the electrode is sufficiently pure to carry out a qualitative analysis of the samples. However, in the future, it may be necessary to take into account the porous structure of the electrode, which was clearly visible at a higher resolution.

Figure 51. SEM images of the gold electrode (unused cell). Surface of the electrode (upper) and border between glass (dark area) and electrode (bright area) with higher magnification (lower).
The results of the study of the used cell showed the presence of a kind of protein islands on the surface of the electrode. This, in turn, indicates the presence of adsorption processes, which have a great influence on behavior of impedance spectrum.

**Figure 52.** Protein islands on the surface of the electrode. SEM (upper) and AFM (lower) images.
IV. DISCUSSION PART

14. Discussion

The most difficult problem to be solved in the framework of this study was the task of interpreting of the obtained data. Before proceeding to direct data processing using software and using equivalent circuits, it was necessary to evaluate the spectra obtained qualitatively. Namely, determine which part of the spectrum is responsible for which process and verify that this is indeed the case. For a more accurate interpretation, not only the dependence of the imaginary part of the impedance on the real part (Nyquist plot, which shows values of impedance at different frequencies), but also the graphs of the dependence of the impedance module and phase angle on frequency (Bode plot) was analyzed. Spectrum analysis was carried out in accordance with the article by Bouchet et al.[35], in other words, the spectrum was divided into four parts. The separation was demonstrated on the spectrum of sample with the concentration of 9 g/l. The sample is from the first batch.

![Figure 53. Division of the spectrum into regions depending on the physical interpretation.](image)

The first part of the spectrum describes the behavior of the system at high frequencies and is not informative, since at high frequencies the imaginary part of the impedance is in the positive region and can be interpreted as the inductance of the wires. However, the value at the point at
which the spectrum intersects the real axis can be taken as the bulk resistance of the solution. In figure 53 this is 165 Ohm at frequency 1.6 MHz.

The second part of the spectrum is one of the most informative, since this part is responsible for the response of the surface layer. However, this part of the spectrum is present in only some measurements.

The third part of the spectrum is the part of transition process and it is non-reproducible and does not carry any information [35]. However, in our case, a loop on the Nyquist plot was noticed at a frequency range 60-200 kHz. At first it was thought that this artifact appeared as a result of insufficient averaging, but an increase in the number of measurements at each point followed by averaging did not lead to the disappearance of this loop. The next assumption was that perhaps this is the part of the data that describes some process in the sample, and this process has an inductive nature from the point of view of equivalent elements of the system. This assumption also turned out to be incorrect, since the behavior of this element did not depend on the sample being measured. Moreover, the same loop appeared also during the measurements of liquid crystal samples, which indicates that this is only an artifact of the system, a measurement error that does not belong to the investigated sample.

Figure 54. Middle-high and middle frequency regions. The loop on the spectrum in the middle frequency region in Nyquist plot (left) and in Bode plot (right).
The fourth part of the spectrum describes mass transport processes or in other words diffusion of charged particles. In the standard model, a straight line at an angle of 45 degrees represents this element of the spectrum. Bouchet \textit{et al.}\cite{35} supplemented the line with a semicircle starting at extremely low frequencies, but the angle was still 45 degrees. This approach is not suitable for describing our system, since the angle of the diffusion element with respect to the real axis is much greater than 45 degrees. It was decided to complicate the standard equivalent circuit by sequentially adding elements until good agreement between experimental and fitting curve will be achieved.

Starting with the standard Randles equivalent circuit, a gradual complication was made up to the scheme shown in the figure 39. The obtained scheme showed a good agreement with the experimental data and is a scheme with the smallest number of elements in its composition that ensures such a result.

However, it is important to note that this fitting procedure has been tested only on one data set and it is unknown how it will behave when used with other experimental data. It should also be taken into account that the data processing procedure does not end with obtaining a good approximation of the fitting curve to the experimental data. A physical explanation of the obtained values should be found, as well as an explanation of the processes taking place in the system, which in turn is an extremely time-consuming task.

The equivalent circuit that was used for the data fitting was already described in the first chapter in framework of studies of adsorption processes \cite{38}. This suggests that there are adsorption processes in the studying system. It can be seen from the figures 38, 45 and 55 that the spectrum of the system depends strongly on time. What can also be explained by adsorption processes, i.e. over time, the film grows on the surface of the electrode and consequently, the response of the system changes. Moreover, it should be noted that the low-frequency part of the spectrum does not change with time. Bouchet \textit{et al.}\cite{35} wrote that there is no dependence of the Warburg element (element responsible for diffusion) on time and it completely agrees with our results. Thus, the straight line at low frequencies in the spectra obtained is indeed a diffusion element.
Figure 55. Nyquist and Bode plots of the 5.2 g/l sample. Dependence of the spectrum on time.

Over time, the semicircle on the Nyquist plot decreases. Oliveira and Melo obtained similar result [29]. They investigated the processes of aggregation of surfactants. Perhaps, in addition to the adsorption processes, aggregation processes also occur in the studying solutions. This theory should be verified in the future.

Extremely interesting results were obtained after the study of the disassembled cell by using AFM and SEM. The figure 56 clearly shows like a protein droplets, the size of which is only about one micrometer. Probably, the change in the response of the system during the time is associated with the formation of these droplets. Moreover, the obtained images look similar as the images obtained in the study of coacervates. Therefore, it is possible, that the studied proteins form coacervate-like structures on the surface of the electrode.
However, it is necessary to be careful when interpreting the obtained results, since other possible cause of the formation of coacervates can be the evaporation process. In other words, after the cell was disassembled, the electrode was dried. During the drying process, the amount of water in the solution decreased and as a result the protein concentration increased. Theoretically, increasing the concentration could lead to the formation of coacervates. Therefore, in order to be sure of the correctness of the proposed theory, it is necessary to investigate the electrode in the absence of unadsorbed particles. For this, if initially it is assumed that there is an adsorbate layer on the electrode, it is needed to come up with a method for removing the solution without removing layers on the electrode surface. This study will be carried out in the future.

In addition, indirect evidence of the presence of adsorption are the graphs of the dependence on concentration (figures 37, 44 and 57). It can be seen from these graphs, that semicircle can be observed at middle high frequency only at low enough concentrations of protein in the solution. This can be explained as follows. From the moment the sample is placed in the cell and up to the moment of measurement it takes about 30-40 minutes. This is due to the features of the cell
assembly. As can be seen from the time dependence graphs, the spectrum changes quickly even at low concentrations, and at high protein concentrations the formation of the adsorbed layer on the electrode surface occurs even faster. In other words, the spectrum drastically changes during the time that passes from the moment the sample is placed into the cell until the moment of measurement.

![Graphs depicting the dependence on concentration](image)

**Figure 57.** Dependence on concentration. The direction of spectrum change with concentration is showed by arrows (from smallest to highest).

Moreover, it is necessary to reconsider the approach to the calculation of the Warburg element, since the presence of adsorption processes should complicate the description of the spectrum at low frequencies. The use of the models proposed by Barreira *et al.*[36] seems very promising.

One unexpected result was obtained after the measurement of milli-q water, when water inside the cell was isolated from the environment as well as during the measurement of the protein solutions. After measurements, the next morning it was discovered that the gold electrode was destroyed.
Similar situation has never occurred with cells in which protein solutions are enclosed, regardless of the amount of time. In an attempt to understand what happened, milli-q water was placed in another cell, but it was not isolated. As a result, the water evaporated after a few days, and the electrode was left undamaged. Several theories have been put forward, which will still need to be checked. For example, the destruction of the electrode can be caused just by poor adhesion of gold to the glass, that is, the poor quality of the cell. This seems most likely. But the adsorption process might be an explanation to why the cell is destroyed in pure water and is not destroyed in the presence of proteins. This can be explained by the fact that the adsorbate on the electrode surface prevents the interaction of water with the electrode, i.e. it creates a kind of film that protects the electrode from destruction.

Discussing the concentration dependence of the spectra behavior, it is easy to see one very strange result. At a protein concentration 98.1 g / l in the solution, the line, that describes diffusion processes turned into a small semicircle. Of course, initially one can consider the line as a part of a large semicircle [35]. But then this means that at a concentration of 98.1 g / l there was a strong decrease in the size of the semicircle. Moreover, before this, was measured the sample
with concentration of 100 g / l and the presence of coacervates, but line did not turn into semicircle.

**Figure 59.** Comparison of 98.1 g/l (red-blue line) and 100 g/l (black line) samples spectra.

Comparing the spectra of these two samples, nothing common between them was found. A possible explanation for this behavior of the spectrum will be given a bit later.

The study of the dependence of the spectrum behavior on temperature yielded some interesting results. Figure 60 shows the temperature dependence upon heating and cooling of the sample with the concentration of 5.2 g / l. With increasing temperature, the semicircle at medium-high frequencies decreases. This agrees well with the article by Silva et al.[37] and is explained by the decrease in the resistance of the surface layer. However, sufficient unusual behavior was detected in the low-frequency region. According to the equations (3), (7) and Silva et al.[37], the slope of the site responsible for the diffusion should increase with increasing temperature, but the results show that the slope decreases with temperature. What is the reason of such behavior is unknown. Moreover, the behavior of the second sample (with a concentration of 98.1) demonstrates an
increase of the impedance of the diffusion element with increasing temperature, which corresponds to the previously written principles. But it is worthwhile to be careful when using the data obtained during the measurement of this sample, since strong fluctuations in the low-frequency region were observed.

Comparing the spectra obtained by heating and cooling the sample, as expected, a difference in the values of the impedance at the same temperature points was found. The difference is due to the fact that the response of the system depends strongly on time. However, it was mentioned before that the spectrum changes with time only at medium high frequencies, in the region responsible for the surface layer. The region responsible for diffusion remains unchanged, which can easily be seen in the figure 60 and 61.

Figure 60. Comparison of the 5.2 g/l sample spectra during heating and cooling. a) Full Nyquist plot; b) Nyquist plot at low frequencies.
Figure 61. Comparison of the 5.2 g/l sample spectra during heating and cooling at middle-high frequencies (h heating means and c means cooling)

After the measurements, both samples were examined with a microscope and it was found that aggregates were formed in the 98.1 g/l sample. The first explanation was that the aggregation occurred as a result of exposure to high temperature. This seems quite logical, since even heat shocking purification is carried out at the temperature of 70 degrees. On the other hand, the impedance spectrum did not show any strong changes that should appear with aggregation, it is unlikely that such sensitive technique as EIS could not detect the change in the studying sample. Then there was a completely logical assumption that it is possible that aggregation occurred before the measurement. This could explain the strange kind of spectrum and the fact that the spectrum showed no significant change. Moreover, this situation could well have occurred considering that proteins at high concentrations are more prone to aggregation and that more than five hours have passed since the sample was taken out of the freezer until the sample was placed in the measuring cell.
15. Conclusions

In this Master's thesis, the process of manufacturing and assembling of measuring cells for EIS was developed and tested. Data on the effect of storage on the operability of the measuring cells were obtained. In addition, the process of destruction of the sputtered gold electrode was detected. Destruction occurred when there was water without any proteins inside the isolated cell.

Samples with different concentrations of proteins in solution were measured. Also a sample containing coacervates was measured. The presence of coacervates was confirmed by an optical microscope. Also, the dependence of the sample spectrum on time was obtained. It was shown that the spectrum of the sample changes very rapidly with time. In addition, samples with concentrations of 5.2 g/l and 98.1 g/l were measured at different temperatures (during heating and cooling). The temperature range was from -10 to 80 degrees Celsius. After measurement, both samples were examined by an optical microscope. The presence of aggregates in a more concentrated sample was detected.

On the basis of the all obtained data, it was concluded that there were adsorption processes, and a possible explanation of the spectra behavior in connection with these processes was proposed. To confirm the presence of adsorption, several cells were disassembled and surfaces of electrodes were studied by AFM and SEM. As a result, the presence of coacervate-like structures on the electrode surface was demonstrated.

The equivalent circuit describing the behavior of the system was proposed. The fitting of the data was performed.

In the future, work in this direction will continue. It is necessary to check the correctness of the used equivalent circuit, to associate the obtained parameters with the real parameters of the sample by processing the spectrum, and to perform series of experiments that will contribute to a clearer understanding of the system.
V. REFERENCES


[31] C. M. Roth and a M. Lenhoff, “Electrostatic and Van-Der-Waals Contributions to Protein Adsorption - Comparison of Theory and Experiment,” *Langmuir*, vol. 11, no. 6, pp. 3500–


