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FORMATION OF METAL-BIOMATERIALS NANOCOMPOSITES AND THEIR APPLICATION FOR THE DEVELOPMENT OF IMMUNOSENSORS

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METALO IR BIOMEDŽIAGŲ NANOKOMPOZITŲ FORMAVIMAS IR TAIKYMAS IMUNINIUOSE JUTIKLIUOSE

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Abstract

Immunosensors are renowned for their high sensitivity and specificity, making them commonly used in diagnostics. Immunosensors are utilized to identify cancerous conditions, conduct histological studies, and as a tool to help control the spread of viral diseases. However, creating quantitative immunological sensors often involves challenges such as the denaturation of the biological recognition element or label, insufficient signal strength, or the need for expensive equipment to convert the signal into a measurable one. The dissertation focuses on the development of a quantitative electrochemical immunosensor by creating a new method to detect labeled antibodies and replacing traditional labels with denaturation-resistant nanoparticles.

The dissertation aims to create a prototype of a quantitative electrochemical immunosensor using a metal-biomaterial composite as the recognition element and scanning impedance microscopy for signal conversion.

The introduction presents the problem formulation, the object, and the relevance of the dissertation. It describes the research methodology, scientific novelty, defending statements, and the structure of the dissertation.

The first chapter provides an overview of immunosensors. It discusses biosensors and focuses on electrochemical immunosensors, their history, and types. The use of nanomaterials, i.e., gold, platinum, reduced graphene, and conductive polymers, in immunosensors is described. The working principles of scanning electrochemical microscopy and electrochemical impedance spectroscopy, as well as their application in immunological sensors, are analyzed. Surface characterization methods are also briefly discussed.

The second chapter reviews the methods and materials used, solution preparation, sample immobilization, and electrode modification protocols. The construction of electrochemical cells and the parameters used are briefly described. This chapter also outlines the mathematical models used to determine reaction kinetics and surface parameters.

The third chapter presents the results of experimental studies on the immunological sensor. It details the advantages and disadvantages of scanning electrochemical impedance microscopy, modifications to ultramicroelectrodes to address high resistance encountered, and the evaluation of a prototype quantitative immunosensor based on gold nanoparticle-labeled antibodies and scanning electrochemical impedance microscopy as the signal converter.

The dissertation’s results have been published in seven scientific articles in journals indexed in the Clarivate Analytics Web of Science database. The dissertation topic has been presented at eleven international conferences.
Reziumė


Šios disertacijos tikslas yra sukurti kiekybinio elektrocheminio imuninio jutiklio prototipą taikant metalo ir biomedžiagos kompozitą kaip atpažinimo elementą ir skenuojamą susidarymo impedanso signalui keisti.

Įvade pristatoma darbo problematika, tyrimo objektas, disertacijos aktualumas ir svarba, aprašoma tyrimo metodika, moksliniai teiginiai ir disertacijos struktūra.


Antrajame skyriuje apžvelgiama taikytų metodų ir naudotos medžiagos, tirpų paruošimo bei mėginio imobilizavimo ir elektrodo modifikuavimo protokolai. Pateikiama elektrocheminių celių konstrukcija ir trumpai aprašomi naudoti parametrai. Šiame skyriuje taip pat aprašomi matematiniai modeliai, taikyti reakcijų kinetikai ir paviršiaus parametrams nustatyti.

Trečiajame skyriuje pateikiami imuninio jutiklio eksperimentinių tyrimų, kurių metu buvo nustatyta skenuojamosios elektrocheminės impedanso mikroskopijos privalumai ir trūkumai, rezultatai, modifikuoti ultramikroelektrodai, kurių siekiama išspręsti registruojamą didelės varžos problemą. Taip pat įvertintas prototipinis kiekybinis imuninis jutiklis, paremtas auksno nanodalele žymėtai antikūnas ir skenuojama elektrocheminio impedanso mikroskopija kaip signalo keitikliu.

Disertacijos rezultatai pristatyti septyniuose moksliniuose straipsniuose, publikuotose Clivinate Analytics Web of Science duomenų bazėje esančiuose žurnaluose. Disertacijos tema perskaityta 11 pranešimų tarptautinėse konferencijose.
Notations

Symbols

\( A \) – electrode’s surface area (liet. elektrodo paviršiaus plotas);
\( C \) – capacitance (liet. elektrinė talpa);
\( C_{PCE} \) – double capacitance (liet. dvigubo sluoksnio elektrinė talpa);
\( D \) – diffusion coefficient of the redox mediator (liet. elektронų tarpininko difuzijos koeficientas);
\( E \) – potential (liet. potencialas)
\( E(t) \) – potential at time \( t \) (liet. potencialas, priklausantis nuo laiko);
\( E_0 \) – amplitude of the oscillating potential (liet. svyruojančiopotencialo amplitudė);
\( F \) – Faraday’s constant (liet. Faradėjaus konstanta);
\( I \) – current (liet. srovė);
\( I(t) \) – current strength at time \( t \) (liet. srovės stipris, priklausantis nuo laiko);
\( I_T \) – normalized ultramicroelectrode tip current (liet. normalizuota ultramikroelektrodo srovė);
\( I_T^{\text{ins}} \) – model for the insulating surface (liet. matematinė srovės išraiška artėjant prie nelaidaus paviršiaus);
\( I_T^{\text{cond}} \) – model for the conductive surface (liet. matematinė srovės išraiška artėjant prie laidaus paviršiaus);
\( I_0 \) – amplitude of the oscillating current (liet. kintamosios srovės amplitudė);
\( L \) – normalized distance to the sample surface (liet. normalizuotas atstumas iki paviršiaus);
NZ – normalized impedance magnitude (liet. normalizuotas kompleksinės varžos modulis);

\( P(x) \) – function describing the surface profile (liet. funkcija, apibūdinanti paviršiaus profilij);

\( R \) – resistance (liet. varža);

\( R_g \) – ratio between the total radius and the radius of the conducting disk of ultramicroelectrode (liet. ultramikroelektrodo laidžios ir nelaidžios dalies santykis);

\( R_s \) – solution resistance (liet. tirpalo varža);

\( R_p \) – charge transfer resistance (liet. krūvio pernašos varža);

\( S_a \) – arithmetical average of surface profile roughness (liet. aritmetinis profilio šiurkštumo vidurkis);

\( S_T \) – maximum height of the profile (liet. moksimalus profilio aukštis);

\( S_v \) – minimum profile valley depth (liet. giliausia profilio iduba);

\( S_p \) – maximum profile peak height (liet. auksčiausias profilio iškilimas);

\( Z_I \) – imaginary part of the complex impedance (liet. impedanso menamoji dalis);

\( Z_r \) – real part of the complex impedance (liet. impedanso realioji dalis);

\( Z_w \) – Warburg impedance (liet. Varburgo impedansas);

\( Z_{cdl} \) – double layer impedance (liet. dvigubo sluoksnio impedansas);

\( Z(\omega) \) – complex impedance (liet. kompleksinis impedansas);

\(|Z_d|\) – impedance at distance \( d \) from the sample surface (liet. impedanso modulis esant atstumui \( d \) nuo paviršiaus);

\(|Z_{\infty}|\) – impedance far from the sample surface (liet. impedanso modulis esant begaliniam atstumui nuo paviršiaus);

\( a \) – radius of ultramicroelectrode exposed metal disk (liet. ultramikroelektrodo laidžios dalies spindulys);

\( c \) – concentration of electroactive species (liet. elektrochemiškai aktyvios medžiagos koncentracija);

\( h \) – profile length (liet. profilio aukštis);

\( i_T \) – experimentally measured tip current (liet. eksperimentiškai gauta srovė elektrodo paviršiuje);

\( i_{\infty} \) – steady-state current (liet. nuostovioji srovė);

\( i_0 \) – transfer current density (liet. perduodamos srovės tankis);

\( j \) – an imaginary number \( \sqrt{-1} \) (liet. menamas skaičius \( \sqrt{-1} \));

\( l \) – distance between the electrodes (liet. ststumas tarp elektrodų);

\( n_e \) – number of electrons involved in the electrochemical reaction (liet. reakcijoje dalyvaujančių elektrų skaičius);

\( r_g \) – total radius of ultramicroelectrode (liet. ultramikroelektrodo laidžios dalies spindulys);

\( \alpha \) and \( \beta \) – variable dependant only on \( R_g \) for feedback mathematical model (liet. kintamieji, priklausantys nuo \( R_g \) vertės taikant grįžtamojo ryšio matematinį modelį);

\( \delta \) – thickness of the Nerst diffusion layer (liet. Nersto difuzijos sluoksnio storis);

\( \varepsilon_r \) – dielectric constant (liet. dielektrinė konstanta);

\( \varepsilon_0 \) – electric constant (liet. elektrinė konstanta);

\( \kappa \) – solution’s conductivity (liet. tirpalo elektrinis laidumas);

\( \lambda \) – kinetic constant (liet. kinetinė konstanta);
\( \rho \) – solution’s resistivity (liet. tirpalo savitoji varža);
\( \phi \) – phase shift (liet. fazės kitimas);
\( \omega \) – cyclic frequency (liet. kampinis dažnis).

**Abbreviations**

Ab-AuNP – goat anti-human IgG labeled by 6 nm gold nanoparticles (liet. ožkos anti-žmogaus IgG, žymėtas 6 nm aukso nanodalelėmis);
Ab-HRP – horseradish peroxidase labeled antibodies (liet. krięńų peroksidaže žymėti antikūnai);
AFM – atomic force microscopy (liet. atominių jėgų mikroskopija);
AuNPs – gold nanoparticles (liet. aukso nanodalelės);
BioPPy – biosynthetic polypyrrole particles (liet. polipirolo dalelės, susintetintos pasitelkiant mikroorganizmus);
DNA – deoxyribonucleic acid (liet. deoksiribonukleorūgštis);
EIS – electrochemical impedance spectroscopy (liet. elektrocheminio impedanso spektroskopija);
ELISA – enzyme-linked immunosorbent assay (liet. imunofermentinės analizės tyrimai);
FB – feedback mode in scanning electrochemical microscopy/scanning electrochemical impedance microscopy (liet. grįžamojo ryšio režimas, taikomas skenuojamosiose elektrocheminėje ir impedanso mikroskopijose);
FcCOOH – ferrocene monocarboxylic acid (liet. feroceno-mono-karboksių rūgštis);
GOx – glucose oxidase enzyme (liet. gliukozės oksidazė);
LOD – limit of detection (liet. atpažinimo riba);
PABS – phosphate-acetate buffer solution (liet. fosfatūs acetatinis buferis);
PPy – polypyrrole (liet. polipirolas);
PtNPs – platinum nanoparticles (liet. platinos nanodalelės);
RC – redox competition mode in scanning electrochemical microscopy/scanning electrochemical impedance microscopy (liet. konkurencinis režimas, naudojamas skenuojamosiose elektrocheminėje ir impedanso mikroskopijose);
rGO – reduced graphene oxide (liet. redukuotas grafeno oksidas);
rpm – rotations per minute (liet. apsisukimai per minutę);
SECM – scanning electrochemical microscopy (liet. skenuojamoji elektrocheminė mikroskopija);
SEIM – scanning electrochemical impedance microscopy (liet. skenuojamoji elektrocheminio impedanso mikroskopija);
SEM – scanning electron microscopy (liet. skenuojamoji elektronų mikroskopija)
UME – ultramicroelectrode (liet. ultramikroselektrodas);
YPD – yeast extract-peptone-dextrose (liet. mielių ekstraktas-peptonas-dekstrožė).
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Introduction

Problem Formulation

Immunosensors are analytical devices known for their high sensitivity and specificity. They play a crucial role in modern medicine by facilitating the diagnosis, monitoring, and managing oncological diseases, and aiding drug development through the precise evaluation of therapeutic efficacy. These devices operate based on the affinity interactions between antibodies and antigens, translating complex biological responses into measurable signals. In recent years, electrochemical immunosensors have gathered substantial interest in their inherent ease of use and their capacity for miniaturization into compact, portable devices capable of conducting real-time measurements.

Several challenges arise in the engineering of quantitative electrochemical immunosensors. Typically, antibodies/antigens are labeled by redox enzymes to amplify electrochemical signals and then immobilized on the electrode. Posing several problems during conjugation, an enzyme, antibody, or both could be inactivated. After immobilization, the electrode has restricted diffusion of redox mediators due to the formed biomaterial layer. Furthermore, some enzyme labels use highly oxidative substrates, which, when working in turbid samples, may cause sample destruction. Finally, used immunosensors are hard to reuse as electrode regeneration may be partial.
Relevance of the Dissertation

The increasing focus on fast and ultra-sensitive electrochemical immunosensors underscores the persistent challenges of signal amplification and reusability. Addressing these scientific challenges is crucial for the adoption of quantitative immunosensors in everyday life. By replacing traditional redox enzyme labels with catalytically active nanoparticles, immunosensors can achieve enhanced stability while maintaining sensitivity. Additionally, the use of scanning electrochemical microscopy (SECM) for transduction enables the immobilization of antibodies/antigens on cost-effective, non-conductive substrates like plastic and glass, facilitating the miniaturization of immunosensors. The use of ultramicroelectrodes for sample probing also supports reusability, advancing immunosensors toward more economically viable and accessible diagnostics.

Research Object

The object of the present research is an electrochemical immunosensor with scanning electrochemical microscopy as a transducer.

Aim of the Dissertation

The dissertation aims to develop a quantitative electrochemical immunosensor based on metal and biomaterial composite as a sensing element (an antibody labeled by enzyme-mimicking nanoparticle) and SECM as a transducer.

Tasks of the Dissertation

To achieve the aims of the dissertation, the following tasks had to be solved:

1. To develop a combined analytical method that leverages the strengths of SECM in redox competition mode and electrochemical impedance spectroscopy (EIS) for quantitative assessment of immunosensors where horse radish peroxidase is used as a label.

2. To evaluate the detection limit of glucose oxidase enzyme labels using different modes of SECM and SEIM. Glucose is less oxidative compared to hydrogen peroxide.

3. To create an immunosensor based on a metal biomaterial composite, antibodies labeled with the metal nanoparticles, and evaluated using scanning electrochemical impedance microscopy (SEIM).
4. To modify the ultramicroelectrode’s surface by engineering a metal/biomaterial composite layer and evaluate it as a potential application as a probe for immunosensor design.

5. To create a prototype immunosensor using the most suitable composite for ultramicroelectrode modification and compare immunosensor performance to non-modified immunosensors.

Research Methodology

The research methodology employed in the dissertation implements a mixed-method approach. It begins with a literature analysis to identify current trends and patterns to address problems with current transducer techniques and signal amplification methods. Then, the research delves into experimental studies using a novel combination of SECM and EIS to assess immunosensors with horseradish peroxidase and glucose oxidase labels. The research introduces a more stable immunosensor design using gold nanoparticle-labeled antibodies instead of redox enzymes. Experimental data indicated higher impedance, necessitating transducer modification using synthesized composite materials via electrochemical and biosynthesis methods. Visualization was achieved through atomic force and scanning electron microscopy. Ultimately, the acquired data were analyzed, facilitating a comprehensive discussion of the results and the formulation of recommendations.

Scientific Novelty of the Dissertation

The research on the dissertation topic yielded novel insights that contribute significantly to the field of material engineering. The enhanced sensitivity SEIM in redox competition mode over traditional SECM for analyzing the catalytic activity of immobilized redox enzymes on different surfaces showcases the practical applicability of the dissertation’s developed method. Enhanced charge transfer kinetics from biomaterials towards the electrode were observed by using biosynthetic polypyrrole particles, synthesized by leveraging yeast’s metabolism, compared to electrochemically produced polypyrrole. The formation of platinum microstructures on the active part of the ultramicroelectrode surface reduces the charge transfer resistance of the system while maintaining the localized nature of the measurements. This might be caused by platinum-filling ultramicroelectrode defects. A prototype of a quantitative electrochemical immunosensor based on SEIM was developed by using an ultramicroelectrode modified with platinum
microstructures as a probe and antibodies labeled with gold nanoparticles as a recognition element.

**Practical Value of the Research Findings**

The findings presented in this research significantly advance the development of sustainable electrochemical immunosensors by using enzyme-mimicking metal nanoparticles and a SEIM technique. This approach enhances immunosensor sensitivity beyond traditional methods and simplifies sample preparation, reducing costs via the use of inexpensive materials for disposable sensors. The combination of antibody-nanoparticle conjugates, modified ultramicroelectrodes, and SEIM holds promise for creating more efficient, rapid, and cost-effective diagnostic tools in various settings.

**Defended Statements**

The following statements on the results of the presented research may serve as the official statements to be defended:

1. SEIM is applicable and highly effective in immunosensor studies as it exhibits greater sensitivity than conventional SECM. This effectiveness stems from SEIM’s advanced capability to detect subtle changes in electrochemical impedance. This increased sensitivity is pivotal in detecting lower concentrations of analytes, making SEIM a more effective tool for detailed and accurate immunosensor studies.

2. More sensitive and accurate SEIM measurements can be performed by using enhanced ultramicroelectrode by platinum microstructures. Platinum microstructures significantly reduce the charge transfer resistance in prototype immunosensor systems up to ten times.

3. The developed immunosensor, which incorporates metal-biomaterial nanocomposite consisting of antibodies labeled with gold nanoparticles and utilizes SEIM as a transducer, exhibits a notably higher sensitivity compared to conventional impedimetric immunosensors because of the localized nature of the SEIM.
Approval of the Research Findings

Research results were published in seven scientific articles in Clarivate Analytics Web of Science journals, four with impact factors (Bironaite et al., 2023; Zinovicius et al., 2021, 2024; Zinovičius et al., 2023; Zinovicius et al., 2022; Zinovicius et al., 2022; Zinovicius et al., 2022). The author gave presentations at eleven scientific conferences:

- 11th International Workshop on SECM and Related Techniques 2023. Montreal, Quebec, Canada.
- The 4th World Congress on Electroporation & Pulsed Electric fields in Biology, Medicine, Food and Environmental Technologies, 2022. Copenhagen, Denmark.
- The 64th International Open Readings Conference for Students of Physics and Natural Sciences, 2021. Vilnius, Lithuania.

Structure of the Dissertation

The dissertation structure consists of an introduction and three primary chapters. The first chapter provides an overview of immunosensor design and current trends. The second chapter reviews the methods and parameters and addresses calculations for reaction kinetics and surface characterization. The third chapter
details the research results on metal-biomaterial composite applications in immunosensors. Finally, the research is summarized by a general conclusion and recommendations for further studies. The dissertation closes with an extensive list of references a list of publications by the author on the dissertation topic and a summary in Lithuanian. The total scope of the dissertation is 105 pages, with 30 equations, 30 figures and one table.
Overview and Analysis of Immunosensors

The first chapter introduces biosensors, their history, design, and standard classifications, focusing on immunosensors. It reviews the working principle of immunosensors and how nanomaterials are used to improve sensor characteristics, focusing on the use of gold and platinum nanoparticles, reduced graphene oxide, and conductive polymer when developing electrochemical immunosensors. It further discusses scanning electrochemical microscopy, electrochemical impedance spectroscopy, and combination methods for biosensor transducer use. Additionally, it reviews surface characterization techniques: atomic force microscopy and scanning electron microscopy. Seven scientific articles on the topic of this chapter were published by the author (Bironaite et al., 2023; Zinovicius et al., 2021, 2024; Zinovičius et al., 2023; Zinovicius et al., 2022; Zinovicius et al., 2022; Zinovicius et al., 2022).

1.1. From Biosensors Towards Immunosensors

In the ever-evolving landscape of modern medicine, the pursuit of better life quality, extended life expectancy, faster treatment, and other health benefits hinges upon the development and integration of novel sensing methodologies and
devices. These methods must have excellent sensitivity and specificity while providing reliable and reproducible results. Additionally, a device must be user-friendly and cost-effective. To meet these demands, a new trend is emerging: an increasing number of sensors rely on biological compounds as their primary sensing elements. These innovative devices, known as biosensors, harness the power of biological components for precise and efficient detection, diagnosis, and monitoring.

1.1.1. History of Biosensors

The inception and development of biosensors can be traced back to ancient civilizations, where humans initially relied on innate sensory abilities to perceive and respond to changes in their surroundings. Early societies adeptly used their senses of taste, smell, and touch as rudimentary biosensing tools to evaluate the safety and quality of food and water (Pan & Tang, 2021). This primal form of biosensing set the starting point for the evolution of sophisticated sensor technologies. However, only in the mid-20th century, the concept of modern biosensors began to take shape.

Leland C. Clark Jr. is considered the “father of biosensors”, as in 1956, he developed a biosensor for oxygen detection. His invention – the oxygen electrode – is called the “Clark electrode” (Severinghaus & Astrup, 1986). Furthermore, in 1962, Clark demonstrated the first glucose oxidase enzyme-based amperometric biosensor for glucose detection (Clark & Lyons, 1962). In 1969, Guilbault and Montalvo developed the first potentiometric biosensor for urea detection (Guilbault & Montalvo, 1969). In 1971, Engvall and Perlman independently discovered the enzyme-linked immunosorbent assay method (ELISA) for detecting hormones and viruses, a technique widely utilized to this day (Alfie et al., 2023; Engvall & Perlmann, 1971; Tapela et al., 2023; Van Weemen & Schuurs, 1971). Subsequently, the first commercial biosensor entered the market in 1975, introduced by Yellow Spring Instruments (Yoo & Lee, 2010). Another groundbreaking development was the i-STAT handheld blood sensor, which sparked significant progress in biosensor technology (Mock et al., 1995).

Now, the biosensor field is a multidisciplinary research area that merges chemistry, physics, biology, material engineering, micro/nanotechnology, electronics, and medicine.

1.1.2. Biosensor Definition and Classification

International Union of Pure and Applied Chemistry defines a biosensor as a device that uses specific biochemical reactions that are mediated by isolated enzymes, immunosystems, tissues, organelles, or whole cells to detect chemical compounds
by electrical, thermal, or optical signals (Nagel et al., 1992). In other words, biosensors are analytical devices designed to detect specific substances, known as analytes. A biorecognition element and a transducer are essential components for the functioning of these devices (Fig. 1.1). A biorecognition element is a biological component (enzyme, antibody, nucleic acid, organelle, whole cell, or molecular imprint) that can specifically interact with the analyte and produce a biological response (Chambers et al., 2008). A transducer can convert biological responses into useful quantitative or semi-quantitative information (rather than providing exact concentration tests that show low, medium, or high).

**Fig. 1.1.** Schematical representation of biosensor components (made by the author)

Biosensors can be classified by their two functional features: transducer and biorecognition element (Fig. 1.2). According to the transducer, biosensors are divided into optical, piezoelectric (mass transport), thermoelectric, and electrochemical. Optical biosensors transform biological responses into signals that can be detected based on absorption, refractive index, light scattering, or luminescence (Chen & Wang, 2020). Piezoelectric biosensors transform biological response – change in mass bound to the surface – into a shift in oscillations (Pohanka, 2018). Thermoelectric biosensors register heat from biorecognition reactions, as these reactions tend to be either endothermic or exothermic (Nestorova et al., 2016). Electrochemical biosensors transform the biological response to electric signal change in generated current, potential, or impedance (Ramanavičius et al., 2006).
Another way to classify biosensors is according to their biorecognition elements. These elements fall into two main categories: catalytic and affinity-based. In catalytic biosensors, biorecognition element reacts with the analyte. One of the most commonly used catalytic bioreognition elements is enzymes, which show great catalytic activity and specificity toward analytes (Gupta et al., 2022). In some cases, organelles or whole cells are used when designing a catalytic biosensor (Wahid et al., 2023). Additionally, catalytic biosensors can be fashioned using nanozymes, i.e., nanoparticles exhibiting catalytic activity similar to enzymes (Wang et al., 2023).

Affinity-based biosensors exploit a high degree of attraction between analyte and biorecognition elements. These sensors typically boast high selectivity, and their interaction with the analyte is non-destructive (Feng et al., 2024). Affinity biosensors are divided into receptor sensors, genosensors, and immunoensors. Receptor sensors utilize cellular or membrane proteins, or even entire membrane parts, as their recognition elements (Cancino-Bernardi et al., 2023). Genosensors, also known as DNA sensors, incorporate nucleic acids as biorecognition elements (Kowalczyk, 2020). Immunoensors leverage antibodies or antigens as their biological recognition elements due to their high affinity and specificity toward the analyte (Liustrovaite et al., 2022).
An effective biosensor must possess several key characteristics to be considered useful (Tetyana et al., 2021):

1. Selectivity is one of the most essential features of a biosensor, as it is highly dependent on the chosen biorecognition element. Selectivity is the ability of a biorecognition element to distinguish a specific analyte amidst a complex matrix of various substances and contaminants in the sample.

2. Sensitivity shows the minimal amount of analyte detectable by a biosensor and is often defined by the limit of detection (LOD). In essence, it serves as the resolution of biosensors.

3. Stability in biosensors refers to their resilience against external interferences that might induce signal drifts, potentially leading to inaccuracies in concentration measurements. Stability is vital for prolonged measurements or monitoring continues. Additionally, degradation of analyte and biorecognition elements can influence stability over time.

4. Linearity denotes a biosensor’s ability to exhibit a proportional relationship between its measured response and varying analyte concentrations, ideally represented by a linear trend in the biosensor’s output.

5. Reproducibility can be described through two distinct yet interconnected components: precision and accuracy. Precision refers to the biosensor’s capability to consistently reproduce the results of the measured sample, while accuracy signifies how closely these results align with the “true” value.

6. Response time defines the duration required for a biosensor to generate an output response.

In addition to the characteristics mentioned above, a commercial biosensor needs to be cost-effective and user-friendly.

1.1.3. Electrochemical Immunosensors

Biosensors have become integral in various fields, such as medicine, environmental monitoring, food safety, drug discovery, and many more (Aydin et al., 2021). Monitoring and detecting pathogens, viruses, biomarkers, and other specific molecules that are indicators of a disease or drug targets helps diagnose or prevent them. In this regard, biosensors must be simple to operate, have good sensitivity and selectivity, and generate fast output. One of the best examples can be depicted by immunosensors. In addition to the mentioned characteristics, immunosensors can work in complex mediums such as blood serum, whole blood,
and milk (Ding et al., 2008; Jiang et al., 2016; Kilic et al., 2020; Vasanatham et al., 2020).

In immunosensor design, antibodies or antigens are immobilized on the surface of a transducer. Antibodies and immunoglobulins can be separated into two types based on the epitope. An epitope is a region of antigen and antibody interaction. The first type – monoclonal antibody has high specificity – typically recognizes a single epitope, while polyclonal antibody can bind with multiple epitopes (Byrne et al., 2009). Each of them has its advantages and disadvantages for analytical applications. A monoclonal antibody is chosen when extremely high specificity is needed. However, the price is much higher than that of polyclonal. On the other hand, when polyclonal antibody binds more than one epitope, even with low concentrations of analyte, the generated signal is amplified.

Various sensing techniques have been reported since the introduction of immunosensors, and electrochemical immune sensors have recently gained considerable interest (Chen et al., 2023) as it has relatively simple instrumentation that can be scaled down at the circuit board level (Drobysh et al., 2022; Morkvenaite-Vilkonciene et al., 2023). This simplifies the creation of inexpensive, disposable devices for point-of-care diagnostics, offering high sensitivity with small sample consumption (Carneiro et al., 2022; Kongkaew et al., 2023).

Affinity interaction between antibody and antigen typically does not generate a significant detection signal. Therefore, to amplify the signal from affinity interaction, it is possible to conjugate a label molecule or a material to an antibody/antigen. For this conjugation, mainly amine (-NH₂), thiol (-SH), or hydroxyl (-OH) group is used as it does not hinder the epitope (Shrestha et al., 2012). As a label, redox-enzymes are commonly conjugated to an antibody/antigen; one of the most popular enzymes is horseradish peroxidase as it is used with ELISA (Abuknesha et al., 2009; Gu et al., 2022; Kumari & Dhir, 2003; Zinovicius et al., 2021). Even though redox enzymes significantly increase analytical signal, further efforts have been made to improve it. One such is integrating nanomaterials to modify the surface of the transducer-electrode. However, with the progress of material engineering and synthesis of nanomaterials, another solution has allowed the use of other types of labels such as metals, alloys, semiconductors, and composite materials and combination of both techniques (Ahirwal & Mitra, 2010; Popov et al., 2021; Ramanaviciene et al., 2022; Ramanaviciene & Plikusiene, 2021; Spain et al., 2016).

Quantitively immunosensors that do not use labels are typically based on the measured impedance signal, these sensors can be categorized into two distinct groups. First, capacitance-based immunosensors employ an electrode system that functions as an electrical insulator, enveloped by the electric double layer, devoid of any redox pairs. The measurement focus is on changes in the double layer's
1. OVERVIEW AND ANALYSIS OF IMMUNOSENSORS

diameter and its properties, influenced by biological interactions, notably antigen-antibody interactions that typically decrease the double layer's capacitance (Mehrotra et al., 2015). These sensors operate at lower frequency ranges to capture capacitance metrics. Such systems are often modeled using a constant phase element rather than a conventional capacitor, a concept first proposed by Pethig and Kell in 1987 (Guan et al., 2004). However, in ion-rich environments such as electrochemical cells, this model proves inadequate. To address this, the biolayer is frequently coated with insulating polymers, effectively functioning as parallel resistors to each capacitor.

Second, Faradaic impedimetric sensors, which are not coated with insulating layers, operate via redox reactions at the electrode-solution interface, measuring charge transfer resistance (Prodromidis et al., 2009). In these, antigen-antibody interactions usually heighten the charge transfer resistance, restricting analyte access to the electrode, with sensor performance dependent on the redox-active compounds. Various electrodes, including those coated with thiol-based monolayers or silane layers on metal and semiconductor oxides, and those covered with polymer layers or electrochemically polymerized semi-insulating layers, are employed for biomolecule immobilization (Guan et al., 2004). Impedance-based immunosensors have also been developed for detecting diverse entities such as cancer biomarkers, lectin bacteria, and various toxins, with one primary challenge being the low dissociation constant between antigens and antibodies, complicating protein separation post-measurement (Bahadir et al., 2014; da Silva et al., 2020). Regeneration of these sensors often involves treatments with varying pH buffers or detergents affecting the biolayer's integrity and sensor reproducibility. Recent innovations have included the use of materials mimicking antibody functionality, and in capacitance-based sensors, enhancing sensitivity through materials that catalyze the production of insulating compounds at the electrode interface.

1.2. Nanocomposites in Immunosensors

The progression of technology has led to the creation of innovative nanomaterials and composites, presenting a promising approach to enhancing the sensitivity and performance of biosensors. Nanomaterials can come in different shapes, forms, and sizes tailored to specific applications: nanoparticles, nanowires/nanorods (diameter from 1 to 100 nm), nanocrystals (single crystal with least one side ≤ 100 nm), nanotubes, dendrimers, clusters (quantum dots) (Joudeh & Linke, 2022; Lobnik et al., 2011). Nanomaterials possess unique catalytic, magnetic, optical, mechanical, and electric properties related to their surface, small size, or macroscopic tunneling effects (Pan et al., 2017).
The distinctive high surface-to-volume ratio of nanomaterials enables efficient conjugation with biomolecules or polymers while retaining their catalytic activity. This characteristic facilitates the electron transfer between their redox center and the electrode surface (Zhang et al., 2020). These properties have rendered nanomaterials indispensable in the engineering of electrochemical biosensors.

Electrochemical immunosensors that integrate nanomaterials can be categorized into the nanostructure platform as the transducer to improve sensitivity and the nanomaterial as a label for signal enhancement (Ma et al., 2019). A diverse array of nanostructures is currently under active investigation for their applicability in immunosensors. Notably, noble metal nanoparticles, reduced graphene oxide, and conducting polymers stand out prominently and will be expounded upon in further detail.

### 1.2.1. Gold and Platinum Nanoparticles

Classical natural enzyme labels in immunoassay provide high selectivity toward analyte and high catalytic activity. For these reasons, ELISA is considered the gold standard in immunoassays (Aruna & Ramalingappa, 2022). However, enzyme preparation, purification, conjugation with another biomolecule, and storage are typically expensive and time-consuming. In addition, enzyme activity is susceptible to external conditions: temperature, pH, and mechanical stress. To overcome these restrictions, the search for new catalytic labels began. It was found that nanoparticles, notably noble-metal nanoparticles (gold, platinum, and palladium), exhibit enzyme-mimicking oxidation, reduction, hydrogenation, or dehydrogenation (Chen et al., 2021). These nanoparticles can be used as an excellent label-designing non-enzymatic immunosensor as it can offer higher stability, broader temperature, and pH range for catalytic activity and less complex preparation procedures when compared to biomolecules.

Gold nanoparticles (AuNPs) are one of the most frequently used in nanoparticle-based immunosensors. AuNPs can be found in a variety of immunoassays. In optical immunosensors, AuNPs are used in lateral flow assays as colloidal AuNPs possess a distinct deep red color, and in lateral flow assays, sensitivity is defined by the accumulation of AuNP-bio-conjugate on the target molecule on the surface (Borse & Konwar, 2020). The deep red color of colloidal AuNPs is caused by localized surface plasmon resonance, which can be used for plasmonic sensing (Eddin & Fen, 2020).

In the early 2000s, it was discovered that AuNPs exhibited dual enzyme-mimetic properties. It can catalyze glucose oxidation when dissolved oxygen is present in a very similar fashion to glucose oxidase enzyme (GOx), and later, it was found that it also had peroxidase-like activity (Chen et al., 2021; Wang et al.,
The catalytic properties of AuNPs were found to be dependent on several parameters: size, shape, temperature, and pH (Suchomel et al., 2018; Zinovicius, Rozene, Sataite et al., 2022).

In electrochemical immunosensors, AuNPs play an essential role. AuNPs can be used for labeling or decorating transducers as not only AuNPs have catalytic activity but also allow fast electron transfer between electroactive species and electrodes, high surface-to-volume ratio, high conductivity, and biocompatibility (Guo & Wang, 2007). Two positive effects are eliminating the necessity to use an electron transfer mediator and decreasing double-layer capacitance (Medhi et al., 2022), giving the ability to design faster, less expensive, and easier-to-use immunosensors. Additionally, using AuNPs increases the immobilized number of antibodies/antigens without losing stability. Stability is retained due to the conjugation process being based on three phenomena (Fig. 1.3) (Jazayeri et al., 2016).

![Fig. 1.3. Protein adsorption types onto AuNP surface.
A – ionic interaction; B – hydrophobic interaction;
C – dative binding (made by the author)](image)

Fig 1.3 shows the ionic interaction (Fig. 1.3. A) with AuNP, which is negatively charged with positively charged protein sites; the hydrophobic interaction (Fig. 1.3 B) between the AuNP surface and protein, and dative binding (Fig. 1.3. C) via covalent bond (Jazayeri et al., 2016).

The first synthesis of AuNPs was reported by Faraday in 1857. Today, AuNPs can easily be synthesized by chemical reduction from HAuCl₄ using citrate or electrochemical synthesis via fast cyclic voltammetry reducing from Au(III) to Au (Kochane et al., 2017; Rahman & Rebrov, 2014; Zhao & Liu, 2019; Zinovicius, Rozene, Sataite et al., 2022).
Platinum is often one of the most used materials for catalytic techniques, such as catalytic converters to transform harmful substances produced by internal combustion engines (Saidani et al., 2019). Platinum nanoparticles (PtNPs) are no exception. Gold and platinum nanoparticles exhibit dual catalytic activity – catalase and peroxidase mimetic – depending on size, shape, temperature, and pH (Fan et al., 2011; Mostafa et al., 2010). Also, such nanoparticles can be conjugated with biomolecules via amine (-NH$_2$) and thiol (-SH) groups (Kuniyasu et al., 2007). Often, PtNPs are used in colorimetric immunoassays as PtNPs can produce colorful products where the sensing mechanism is based on the 3,3',5,5'-tetramethylbenzidine-radical cation (blue) is reduced by PtNPs and produces yellow color depending on analyte concentration (Ali et al., 2019). These biosensors could be engineered even on paper, creating simple, cost-effective point-of-care devices (Nilghaz et al., 2021; Sun et al., 2019). PtNPs are also used in electrochemical immunosensors. Developed sensors show great promise as results are obtained fast, e.g., in 5 minutes, and the possibility to detect low concentrations of analyte 2.2 pg/mL within a long linear range (Cui et al., 2014; Dutta et al., 2017).

It is important to note that even when nanoparticles are used as labels or as a decoration of transducers in immunosensors, engineered immunosensors tend to have higher stability than conventional ones can function across a wider range of temperatures and pH levels, and have similar preparation processes to traditional biomolecules. However, such sensors are still limited by the stability of the antibody or antigen.

### 1.2.2. Reduced Graphene Oxide

In electrochemical immunosensor design, having an efficient transducer and a biorecognition element is equally important. Graphene is a new promising candidate offering excellent conductivity, fast electron transfer kinetics, biocompatibility, and ease of functionalization for fabricating immunosensors (Mao et al., 2016; Mehrali et al., 2014; Reiner-Rozman et al., 2016). One commonly used graphene derivative is reduced graphene oxide (rGO). rGO gained much attention due to its structure, even though fewer oxygen-containing groups are available for modification. Other characteristics are comparable to pure graphene (Malik et al., 2021). When graphene oxide is reduced to rGO, obtaining a near-flawless structure is possible, allowing a more straightforward production process than flawless graphene. There are a few advantages when rGO is used in electrochemical immunosensors. rGO offers a high surface area with numerous sites for the immobilization of antibodies/antigens, increasing the loading capacity of the surface (Zhang et al., 2019). Additionally, it can be used for signal amplification by attaching to other nanomaterials, leading to higher sensitivity and...
lower detection limits. For example, reduced graphene oxide in tandem with gold nanoparticles showed a linear range (0.0001→300 U/mL) than traditionally labeled with AuNPs (0.01→400 U/mL) (Sangili et al., 2020). Finally, easy rGO integration allows for the development of miniaturized microsensors, paving the way for practical applications in healthcare, environmental monitoring, and other fields requiring rapid and accurate detection.

1.2.3. Conductive Polymers in Biosensing

Electrically conductive polymers were a breakthrough in the field of polymers and gained significant interest in immunosensor applications. Conductive polymers can be classified according to their conductivity type (Balint et al., 2014):

1. Electron-conducting polymers,
2. Proton-conducting polymers,
3. Ion-conducting polymers.

One of the most researched conductive polymers in electrochemical biosensors is polypyrrole (PPy) (Fig. 1.4). PPy has saturated single $\sigma$-bonds and unsaturated double $\sigma$-bond and $\pi$-bond in its structure, enabling electric conductivity. Other unique characteristics of PPy include high biocompatibility and stability at room temperature, which can be easily synthesized (Ghaffari-Bohlouli et al., 2023; Qu et al., 2022). PPy is commonly used as a matrix for biorecognition element immobilization on a transducer; one such application is immunosensors (Kwon et al., 2019; Ramanaviciene & Ramanavicius, 2002). Using PPy as a matrix is very attractive as it provides incredible control over many aspects, such as layer thickness, stabilization of biomolecules, and easy preparation procedures.

![Fig. 1.4. Structure of polypyrrole (made by the author)](image)

Polymerization of pyrrole can be induced in a variety of methods. Chemical initiation by oxidative agent provides the highest control or particle size and the possibility to oxidize formed polymer. However, using the chemical polymerization method, it is extremely hard to cover surfaces as formed PPy has poor adhesive capabilities and is almost insoluble in non-organic solvents.
Biosynthesis of PPy is assisted by cells and forms PPy particles in cells’ periplasm; depending on pyrrole concentration, cells can form different size particles or homogenous layers (Zinovicius et al., 2022). The main disadvantage of this method is the difficulty extracting synthesized polymer. Finally, electrochemical PPy synthesis – PPy deposition on the electrode. This method allows high surface coverage, control over layer thickness, almost 100% yield, and high conductivity (Borges et al., 2023).

PPy matrix can incorporate biomolecules and additional signal amplifiers such as AuNPs, PtNPs, and rGO to increase electron transfer while providing a biocompatible and stable matrix for immobilizing biomolecules, such as antibodies or antigens.

1.3. Scanning Electrochemical Microscopy for Immunosensing

Scanning electrochemical microscopy (SECM) is a relatively new technique developed in 1989 by Professor Allen J. Bard (Kwak & Bard, 1989). SECM is a versatile surface characterization technique that merges principles of electrochemistry with microscopy and enables the research of materials and interfaces at high spatial resolution. It probes electrochemical processes at the micro- or nanometer scale, providing spatially resolved information about surface electrochemical reactivity and topography (Nasri et al., 2022; Traxler et al., 2022). SECM maps and characterizes surfaces, offering a unique possibility to analyze corrosion, material interfaces, and biological systems locally (Bironaite et al., 2023; Nasri et al., 2022; Traxler et al., 2022). Scanning electrochemical microscopy has found applications in fields ranging from fundamental electrochemistry to developing novel materials, biomedical diagnostics, and biosensors, promising insights into complex systems with unparalleled spatial resolution.

1.3.1. Working Principles

SECM is one of the scanning probe microscopy techniques. In this technique, an ultramicroelectrode (UME) is used as a scanning probe (in some literature instances, called a tip). UME is typically fabricated as a disk of a noble metal or carbon and shrouded in an insulating material like glass or polymer. Its main feature is that the diameter of the conducting disk is less than 25 µm (Bard et al., 1992).

Scanning electrochemical microscope consists of two main parts: a positioning system and an electrochemical cell controlled by a computer (Fig.
1.5). Probe – UME is attached to the positioning system, which is used to move UME. An electrochemical cell usually consists of a 3-electrode system where UME is used as a working electrode and connected to a potentiostat along with reference and counter electrodes. In some cases, when it is desired for the surface of interest to be polarized, it may be connected as the second working electrode. To avoid environmental noise caused by vibrations and electromagnetic fields, SECM can be set inside the Faraday cage and on the antivibration table.

During the experiment, the biased probe is in close proximity to a surface of interest yielding data about electrochemical processes and topographical features of the sample. This data is recorded as a Faradaic current, dependent on several parameters – properties of the sample surface, distance sample to UME, and UME characteristics (Bard & Mirkin, 2012). The sample surface can induce current changes as electroactive species diffuse toward the electrode surface during scanning. As this process depends on diffusion, UME to the sample distance plays a vital role; by approaching the surface with UME, diffusion becomes burdened, thus restricting the movement of electroactive species toward UME. Diffusion is not only dependent on the distance between UME and the sample surface but also
on UME characteristics. One such is $R_g$, a ratio between the total radius of UME ($r_g$) and the radius of the exposed metal disc ($a$), represented as equation 1.1 (Lefrou & Cornut, 2010; Skaanvik et al., 2022).

$$R_g = \frac{r_g}{a}.$$  \hspace{1cm} (1.1)

When the $R_g$ value is low, diffusion transitions from linear to radial. As the diffusion layer extends beyond the outer edges of the insulating shroud, an influx of electroactive species follows, thus increasing the current (Barroso-Martínez et al., 2022; Lefrou & Cornut, 2010). $R_g$ value highly impacts steady-state current ($i_{T\infty}$), which can be expressed as follows (Bard & Mirkin, 2012):

$$i_{T\infty} = 4n_e FDca.$$  \hspace{1cm} (1.2)

Where $n_e$ is the number of electrons involved in the reaction, $F$ is the Faraday’s constant, $D$ is the diffusion coefficient, $c$ is the concentration of electroactive species, and $r$ is the radius of the UME.

SECM has several advantages compared to other microscopy techniques, such as scanning electron microscopy (SEM) and fluorescence microscopy. Samples can be studied in situ, allowing for the analysis of the samples in their native medium or under specific conditions. Additionally, unlike SEM, which often requires sample preparation, especially bio-samples, scanning electrochemical microscopy is non-destructive, and samples can be measured repeatedly (Morkvenaite-Vilkonciene et al., 2019; Stratmann et al., 2015). SECM can provide information about chemical reactions that are not feasible with SEM or fluorescence microscopy. Finally, SECM can be combined with other techniques and methods, such as atomic force microscopy (AFM) or electrochemical impedance spectroscopy (EIS), to achieve higher resolution or superior sensitivity (Cheng et al., 2021; Ramanavicius et al., 2021; Zinovicius et al., 2021).

### 1.3.2. Modes of Scanning Electrochemical Microscopy

Scanning electrochemical microscopy can be operated in several modes: feedback (FB), generation/collection, and redox competition (RC). These modes are chosen based on the system and depend on several factors such as sample conductivity and catalytic activity, product or reactant electrochemically active.

Feedback mode can be divided into negative-FB and positive-FB (Fig. 1.6). Typically, FB mode requires one form of quasi-reversible redox mediator couple (Kai et al., 2018). When the UME is far from the sample surface, the current depends on diffusion and concentration of redox species and can be calculated according to Equation 1.2.
Fig. 1.6. Principle scheme of feedback mode of scanning electrochemical microscopy. A – positive feedback; B – negative feedback (made by the author)

When UME is nearing the conductive surface, positive-FB occurs, and current increases as the redox mediator generated on the UME is oxidized/reduced on the conductive surface, causing a reversible redox process (Fig. 1.6 A). However, this process is only observable when UME is close (1-2 radii of UME) to the conductive surface (Morkvenaite-Vilkonciene et al., 2019; Zinovicius et al., 2022).

When UME approaches the insulating surface, the distance between the sample surface hinders diffusion, and UME current decreases, showing negative-FB behavior as portrayed in Fig. 1.6 B. The current is solely dependent on the distance from the surface (Bard & Mirkin, 2012).

Biological samples are not typical conductors nor insulators but rather something in between; thus, intermediate behavior is obtained. In these cases, data obtained on current dependency on the distance to the surface are fitted to analytical expression (Cornut et al., 2009; Cornut & Lefrou, 2008; Lefrou & Cornut, 2010). Reaction kinetics can be obtained and evaluated. However, FB mode has limitations, as the current is directly proportional to the concentration of redox species.

Generation/collection is a highly sensitive mode and can be divided into substrate generation-tip collection and tip generation-substrate collection. The substrate generation-tip collection mode solution contains a redox mediator form that
UME cannot detect (Bard & Mirkin, 2012). When this form undergoes a reaction at the substrate, it can be detected by UME. Even though this mode has high sensitivity, quantifying generated species can be challenging as generation occurs continuously, independent of whether UME is present. While in tip generation-substrate collection mode, UME generates redox active species similarly to feedback mode, but a feedback loop cannot be formed as when this species reacts with substrates, it generates a product that is not regenerated at the UME (Sun et al., 2007).

Another mode that provides high-resolution imaging capabilities is redox competition. In RC-SECM mode, both the UME and the substrate participate in the consumption of the same redox species, as shown in Fig. 1.7 (Eckhard et al., 2006). This unique characteristic of RC-SECM leads to a decrease in the measured current signal when the UME approaches the substrate. This reduction in current is a result of the electrochemically active species consumed by the electrode and the sample in the narrow gap between them.

**1.3.3. Use for Immunosensors**

Since the very beginning of the SECM technique, researchers have been tempted to research biological samples. One of the first attempts was the investigation of horseradish peroxidase in 1993 to determine hydrogen peroxide concentration (Horrocks et al., 1993). SECM offers numerous advantages as methods developed to evaluate enzymatic reactions can be adapted to detect antigen-antibody interactions. In traditional electrochemical immunosensors, the biomolecule is
immobilized on the surface of the electrode – the same surface affinity interaction occurs. Additional layers of biomolecules may burden or even block the diffusion of redox species toward the electrode surface (Ciani et al., 2012; Cordeiro et al., 2019; Ramanavicius et al., 2010; Sargent & Sadik et al., 1999; Yun et al., 2007). It is difficult to reuse electrochemical immunosensors as regeneration after use may be partial, or the creation of single-use systems is too expensive. On the contrary, SECM allows local detection of the biomolecules immobilized on both conductive and non-conductive supports. The UME is used as a transducer and does not touch the surface of interest; thus, the biomolecules are unaffected, the diffusion of redox species is not blocked, and UME can be used many times. Therefore, scanning electrochemical microscopy seems to be a more powerful tool for developing biosensing systems, using inexpensive glass or plastic substrates to immobilize biomolecules.

In various applications, SECM effectively detects immune complexes formed on different surfaces. Simultaneous detection of four lung cancer tumor markers was developed by Pingang He research group using a biochip with four immobilization spots for antibodies, scanning electrochemical microscopy in generation-collection mode, sensors linear range was 5 ng/mL – 1 μg/mL for target markers, detection limit 0.40 ng/mL of AFP, 0.42 ng/mL of CEA, 0.67 ng/mL of Cyfra21-1, 0.69 ng/mL of NSE (Ning et al., 2018). Tomokazu Matsue group developed dual pepsinogens 1 and 2 (PG1 and PG2) immunosensor on glass substrate, SECM in generation-collection mode, sensors linear range was 12.8–50.5 ng/mL for PG1 and 12.8–40.4 ng/mL for PG2 (Yasukawa et al., 2007). Overall, SECM emerges as a powerful and versatile tool for the precise visualization, detection, and quantification of biomolecular interactions on various surfaces, offering solutions to key challenges encountered in traditional immunoassay techniques.

1.4. Scanning Electrochemical Impedance Microscopy

Scanning electrochemical impedance microscopy (SEIM) is a combined SECM and electrochemical impedance spectroscopy (EIS) technique, which can be used for the electrochemical detection of low molecular weight analytes without applying potential (Morkvenaite-Vilkonciene et al., 2017; Valiuniene et al., 2019). SEIM enables the measurement of the complete electrochemical impedance spectra, within the desired frequency range, at any point of interest within 3D spaces. Therefore, SEIM is particularly sensitive to minor variations in impedance since measurements can be made close to the surface of interest. And obtained data from the electrochemical system can be evaluated by applying equivalent electrical
circuits. Moreover, SEIM offers high spatial resolution, making it an excellent option for analyzing microelectrodes.

### 1.4.1. Electrochemical Impedance Spectroscopy

EIS is an electrochemical analysis method measuring the combined effects of resistance and reactance of an electrochemical system to a current (Bard et al., 2001; Rubinstein, 1995). Emili Gabriel Warburg first developed it at the end of the 19th century; it describes the systems’ response to current or potential perturbation as a function of frequency. Today, it has become a staple method for characterizing coatings, batteries, corrosion, electrode kinetics, semiconductors, and sensors (Laschuk et al., 2021; Magar et al., 2021; Single et al., 2019; Vivier & Orazem, 2022).

Electrochemical systems often exhibit a non-linear response, specifically where doubling the potential does not result in a doubled current (Liu et al., 2023). Hence, for impedance measurements, a low-amplitude alternating current is chosen. This aids in obtaining more reliable results as the polarization curve remains within the linear part. In this pseudo-linear system, the response oscillates at the same frequency as the input; however, at certain moments, the system’s current may lag or lead the potential by a phase shift angle, thus not exhibiting harmonic oscillation. The entire spectrum is obtained by conducting measurements within a selected range of frequencies, with points arranged logarithmically at equal intervals.

Before analyzing impedance, it is essential to define resistance, which can be described by Ohm’s law, where resistance is the ratio of potential to current strength:

$$ R = \frac{E}{I} $$  \hspace{1cm} (1.3)

Where $R$ represents resistance, $E$ stands for potential, and $I$ denotes current. However, this dependency only describes a single element of the circuit — ideal resistance, characterized by several simplified assumptions (Christensen, 2009):

1. Ohm’s law applies regardless of the magnitude of potential and current strength;
2. The resistance value is independent of frequency;
3. The phase of the variable voltage and current strength align.

Impedance is not limited by these simplified properties, allowing for the analysis of more complex systems. When analyzing input and output signals, a generalized system impedance is obtained. The electrochemical impedance can be described as a complex or comprehensive system resistance. A complex resistance can be divided into real and imaginary (Lisdat & Schäfer, 2008).

The input potential can be expressed in terms of Cartesian or polar variables:
Where $E(t)$ represents an oscillating potential at time $t$, where $E_0$ is the signal amplitude, and $\omega$ is the cyclic frequency.

The strength of the response current can be expressed as:

$$I(t) = I_0 \cos(\omega t - \phi) = I_0 \exp(j \omega t - \phi). \quad (1.5)$$

Where $I(t)$ is the oscillating current strength at time $t$, $I_0$ is the signal amplitude, and $\phi$ is the phase shift.

By introducing the newly described variables into Ohm’s law, the resistance needs to be replaced with complex impedance and then, the new functional dependency has to be rewritten:

$$Z(\omega) = \frac{E(t)}{I(t)} = \frac{E_0 \cos(\omega t)}{I_0 \cos(\omega t - \phi)} = Z_0 \frac{\cos(\omega t)}{\cos(\omega t - \phi)} = Z_0 \frac{\exp(j \omega t)}{\exp(j \omega t - \phi)} = Z_0 (\cos(\phi) + j \sin(\phi)) = Z_r + jZ_i. \quad (1.6)$$

Where $Z_r$ and $Z_i$ are the real and imaginary parts of the complex impedance.

### 1.4.2. Displaying Electrochemical Impedance Spectroscopy Data

When representing the impedance in a Nyquist plot, it is expressed in complex numbers, with the real components of the impedance ($Z_{real}$) depicted along the ordinate axis and the reactive components – imaginary ($-Z_{imag}$), envisioned along the abscissa axis. The obtained graph provides insights into the ongoing process and kinetics. For instance, the presence of a perfect semicircle suggests a charge transfer process. The semicircle extends into a line in a typical Nyquist plot (Fig. 1.8). A-line observed at low frequencies indicates a process limited by mass transfer, whereas a semicircle at high frequencies suggests a process limited by charge transfer (Bond et al., 1988). However, in a more complex plot, where there are loops or multiple semicircles, analyzing the process involves more than a one-time constant (Ferrigno & Girault, 2000).

The chosen approach to depict impedance often involves this style due to its ability to quickly overview the collected data and facilitate an initial assessment of its quality. However, to ensure an accurate evaluation of the acquired data, the axes must be maintained at equal lengths to prevent any distortion in the shape of the curve. The Nyquist plot, which is used in this context, does not inherently display frequency information; therefore, it becomes necessary to manually annotate frequencies on the graph for clarity. Consequently, it becomes necessary to manually annotate frequencies onto the graph to enhance clarity and understanding. Additionally, the graphical representation of data on the Nyquist plot tends to significantly compress at higher frequencies. This compression can pose
substantial challenges in conducting detailed analysis, as it leads to reduced visibility of subtle changes and trends, which are critical for a thorough interpretation of the data.

In investigating frequency dependency, Bode plots are the most commonly chosen method. Graphically represents the logarithmic interplay between phase shift and the logarithm of impedance magnitude as a function of the logarithm of frequency, as depicted in Fig. 1.9. Bode plots are particularly useful for observing the behavior of a system at high frequencies, where the impedance magnitude typically diminishes. As the frequency shifts from lower to higher values, the slope of these Bode curves provides critical insights into the characteristics of the process under study. Furthermore, the graph depicting phase shift is noted for its
1. OVERVIEW AND ANALYSIS OF IMMUNOSENSORS

high sensitivity to variations in the parameters of the equivalent circuit. This sensitivity enables a more accurate alignment and comparison of empirical data with theoretical models, enhancing the understanding of the system's dynamics.

1.4.3. Electrical Equivalent Circuit for Electrochemical System

Interpreting the obtained impedance data, an electrical equivalent circuit model is commonly designed based on the studied system (Rubinstein, 1995). This model disassembles the investigated system into individual electrical circuit components consisting of elements with similar characteristics that operate within a chosen frequency range (Eckhard et al., 2008).

Creating an equivalent circuit necessitates a comprehensive understanding of the electrochemical system and avoiding the addition of unnecessary components (Suh et al., 2005). This caution is essential because converging complex equivalent circuitry with experimental data does not guarantee that the designed system will yield useful and meaningful information. These equivalent circuits are composed of passive elements, such as resistors, capacitors, inductors, constant phase elements, and Warburg elements.

When modeling the equivalent circuit for an electrochemical system, it is crucial to consider the solution resistance between the reference and working electrodes. This is because ions act as resistors, impeding the movement of electrons and directly influencing the impedance of the electrochemical cell. The resistance between the reference and working electrodes is typically compensated for by the potentiostat itself (only in more modern potentiostats) (Bard, Inzelt, 2012).

Solution resistance depends on several factors (Harrington, 2004):

1. Ion concentrations;
2. Ion types;
3. Electrode shapes and surface areas;
4. Temperature.

The solution resistance can be described by the equation (Kadan-Jamal et al., 2020):

\[ R = \rho \frac{l}{A} = \frac{1}{\kappa} \times \frac{l}{A}. \]  \hspace{1cm} (1.7)

Where \( \rho \) represents the solution’s resistivity, \( \kappa \) stands for the solution’s conductivity, \( A \) denotes the electrode’s surface area, and \( l \) signifies the distance between the electrodes.

In reference books, it is difficult to find the solution’s resistance, but it is possible to find the conductivity of solutions in Siemens per meter, which is the reciprocal of specific resistance (Weiner, 2012). However, during experiments, it is extremely challenging to ensure ideal conditions. Thus, the equation represents
only the theoretical electrolyte resistance, which may deviate from experimentally obtained data.

Another important aspect of performing EIS measurements on the electrode surface is the absorbed ions from the electrolyte, which form a double electric layer (Fig. 1.10) (Bard, Inzelt, 2012). The accumulated charge is directly proportional to the size of the electrode and the size of the ions, implying that the larger the electrode, the greater the capacitance of the double layer. The double electric layer forms when there is a potential difference in the system, causing ions to move toward an opposite charge. This resulting layer behaves like a capacitor, storing charge, and discharges when the current moves in the opposite direction, repelling ions away from the electrode.

![Fig. 1.10. Formation and disappearance of a double electric layer. A – the movement of cations and ions toward polarized electrodes; B – ion absorption on the surface; C – ion desorption (made by the author)](image)

The charge transfer process occurs when a metal \((\text{Me})\) in an electrochemical system comes into contact with an electrolyte. During this interaction, electrons are transferred from the electrolyte to the metal surface. This transfer results in the formation of metal ions, denoted as \(\text{Me}^{n+}\), which subsequently disperse throughout the surrounding solution. This phenomenon is indicated by the following reaction:

\[
\text{Me} \Leftrightarrow \text{Me}^{n+} + n_e. \tag{1.8}
\]

This reaction (Eq. 1.8) illustrates not only the movement of electrons but also the dynamic changes occurring on the metal’s surface as it interacts with the electrolyte, highlighting the fundamental steps involved in the charge transfer process.

The rate at which charge is transferred depends on several factors: the type of reaction, temperature, concentration of reaction products, and the applied
potential. As the electrochemical system achieves equilibrium, the resistance to this charge transfer can be quantitatively described by equation (Wang et al., 2005):

$$ R_p = \frac{RT}{n_eF_i_0}. $$ (1.9)

Where $R_p$ is the charge transfer resistance, $R$ is the gas constant, $T$ is temperature, $n_e$ is the number of electrons involved in the reaction, $F$ is Faraday’s constant, and $i_0$ is the transfer current density.

Equation 1.9 helps to understand the dynamics of charge flow under varying conditions of the system in equilibrium.

The coating capacitance can effectively be likened to the characteristics of a typical capacitor. In essence, a capacitor is formed by inserting a dielectric between two conductor plates. The overall capacitance of such a capacitor depends on several factors including the physical dimensions of the conductive plates, the spacing between these plates, and the inherent properties of the dielectric material inserted between them. This dependency can be described by an equation (Kadan-Jamal et al., 2020):

$$ C = \frac{\varepsilon_0 \varepsilon_r A}{l}. $$ (1.10)

Where $\varepsilon_0$ represents the electric constant, $\varepsilon_r$ denotes the dielectric constant, $A$ signifies the plate size, and $l$ represents the distance between them.

Diffusion also influences impedance, which is known as Warburg impedance. As previously mentioned, impedance relies on the low-amplitude potential change at selected frequencies. At higher frequencies, Warburg impedance remains low, primarily because the particles involved in the reaction have less distance to travel, thus requiring minimal movement. Conversely, at lower frequencies, the impedance sees a noticeable increase. This is due to the necessity for reacting particles to diffuse over greater distances, substantially amplifying their influence on the overall impedance measure. This diffusion-induced variation in impedance highlights the intricate interplay between particle mobility and frequency within electrochemical systems.

In the case of “infinite” Warburg impedance, it can be expressed by equation (Bard et al., 2001):

$$ Z_W = \sigma (\omega)^{-\frac{1}{2}} \times (1 - j). $$ (1.11)

Where $\sigma$ represents the Warburg coefficient and $\omega$ stands for the cyclic frequency.

This equation holds true only under the condition that the diffusion layer extends infinitely. However, it is important to note that in practical scenarios
involving real samples, the diffusion layer is typically finite, as described by (Bisquert & Compte, 2001):

\[ Z_0 = \sigma (\omega)^{\frac{1}{2}} \times (1 - j) \times \tanh \left( \frac{\delta}{D} \right)^{\frac{1}{2}}. \] (1.12)

Where \( \delta \) represents the thickness of the Nerst diffusion layer, where \( D \) stands for the average diffusion coefficient value for diffusing particles.

### 1.5. Electrode Surface Evaluation Techniques

SECM is a formidable technique for probing interfacial processes and for mapping out electrochemical activities with high-resolution detail. Despite its numerous benefits, SECM faces certain obstacles, particularly the distortions related to diffusion that can affect the accuracy of topographical imaging. In order to gain a more precise examination of surface topology, researchers frequently complement their studies with atomic force microscopy and scanning electron microscopy. These complementary techniques offer a robust means to explore and characterize surface structures with enhanced precision, providing a comprehensive understanding of nanoscale surface features and properties.

#### 1.5.1. Atomic Force Microscopy for Composite Layer Morphology Evaluation

Atomic Force Microscopy enables the examination of nearly any surface, including polymers, ceramics, glass, and biological samples (Bellotti et al., 2022). Over the past five years, numerous new approaches for utilizing AFM have emerged, allowing for the investigation of morphological and mechanical properties of specimens (Bucinskas et al., 2020; Zinovicius et al., 2022).

Similar to scanning electrochemical microscopy, AFM is part of scanning probe microscopy methods and utilizes a scanning probe. This probe consists of a flexible beam called a cantilever. At the end of this cantilever, an extremely sharp micro-needle is mounted, which is pivotal to its function. The quality of the images produced by AFM depends heavily on the sharpness of this needle, a critical component in achieving high-resolution imaging (Bellotti et al., 2022). In terms of operation, AFM utilizes the various interaction forces that occur between the sample being examined and the tip of the needle. These interactions are primarily governed by forces such as Van der Waals, electrostatic, and capillary forces, which play a significant role in the imaging process (Zhang & Geng, 2024).

Contact mode scanning is one of the simplest and most widely adopted modes for Atomic Force Microscopy. Compared to other AFM scanning methods,
the advantage of this method is the controlled force, resulting in faster image acquisition, higher resolution, and the lowest ratio of extraneous noise to signal (Munz et al., 2024). However, contact mode scanning is not without its limitations. Particularly when examining biological specimens, there exists a risk that the probe tip may snag and unintentionally dislodge the sample from the substrate. This incident can lead to contamination of the tip, which in turn may produce false signals and lead to unreliable surface analysis results (Rousso & Deshpande, 2022).

1.5.2. Scanning Electron Microscopy for Composite Layer Evaluation

Scanning Electron Microscopy is a powerful imaging technique widely utilized in various scientific fields, particularly materials science, biology, and nanotechnology. Its fundamental principle involves directing a focused beam of electrons onto a sample surface. This interaction produces high-resolution images by capturing the various reactions between the electrons and the material surface (Zhou et al., 2007). The sample’s surface is scanned point by point. The detection of emitted signals, such as secondary electrons or backscattered electrons, allows for the assembly of detailed, three-dimensional visual representations of the sample. SEM is renowned for its superior magnification and resolution capabilities, which permit the detailed observation of nanoscale structures and reveal complex aspects of surface morphology, elemental composition, and topographical features of specimens (Sukackienė et al., 2022). Due to its versatility and ability to examine diverse materials, SEM finds applications in metallurgy, semiconductor research, biomedical studies, forensics, and various other scientific investigations, contributing significantly to our understanding of microscopic structures.

1.6. Conclusions of the First Chapter and Formulation of the Dissertation Tasks

Literature analysis led to the following conclusions:

1. Biosensors have found extensive applications across various fields, including medicine, environmental monitoring, food safety, and drug discovery. Their ability to monitor and detect pathogens, viruses, biomarkers, and other molecules makes them invaluable in diagnosing and disease prevention. However, the specificity and sensitivity of these devices are often challenged by complex biological samples, where interference from various substances can lead to false positives or negatives, underscoring the need for continued innovation.
Developed biosensors that meet all required characteristics, such as selectivity, sensitivity, stability, linearity, reproducibility, and a reasonable response time, are often complex and expensive.

The field of immunosensors, a subtype of biosensors, has seen significant advancements, particularly in electrochemical immunosensors. The development of reliable and robust electrochemical immunosensors that can be easily manufactured and deployed on a large scale is still an area that needs further research.

The incorporation of nanomaterials in immunosensors has enhanced their performance. This includes the use of noble metal nanoparticles. Metal nanoparticles have emerged as effective labels in immunosensors due to their enzyme-mimicking properties and stability. Similarly, using graphene derivatives like reduced graphene oxide and conductive polymers in electrochemical immunosensors has shown promising results in increasing sensitivity and lowering detection limits. Yet, these benefits come with the challenge of integrating these materials into devices in a way that maintains their enhanced properties while ensuring design remains cost-effective, accessible, and biocompatible.

Emerging techniques in immunosensor analysis, such as scanning electrochemical microscopy and electrochemical impedance spectroscopy, offer more precise detection and analysis capabilities in biosensing applications. Further integration of these novel techniques requires extensive research to ensure reliability and consistency across diverse samples and conditions.

After reviewing the literature and considering the aim of the dissertation, it is necessary to accomplish the following tasks:

1. To develop a combined analytical method that leverages the strengths of scanning electrochemical microscopy in redox competition mode and electrochemical impedance for sensitive and specific detection of immunolabels.

2. To compare the sensitivity of scanning electrochemical impedance microscopy in redox competition mode versus conventional scanning electrochemical microscopy in the quantification of typical immunolabels.

3. To identify methodological, technical, and application-based shortcomings of the scanning electrochemical impedance microscopy method and propose innovative solutions to address these challenges.

4. To design a prototype immunosensor using nanoparticles as a label for antibodies instead of conventional redox enzyme label and leveraging scanning electrochemical impedance microscopy for the detection of
target analytes, focusing on parameters such as detection limit, linear range, and reproducibility.

5. To assess the effectiveness of a modified ultramicroelectrode design using biocompatible metal and biomaterial composites in addressing previously identified shortcomings within electrochemical measurement techniques.
This chapter describes all the materials used, the methodologies for preparation, the experimental setup, and the calculations performed. The materials chapter covers the preparation of ultramicroelectrodes, detailing both bio and electrochemical synthesis of polypyrrole and the formation of matrices. It then describes the electrochemical deposition of platinum structures and the immobilization techniques utilized for biorecognition elements. The experimental setup is explained with an emphasis on the electrochemical procedures. Furthermore, this chapter provides a comprehensive overview of the parameters for surface characterization methods. Finally, it discusses the calculations used to analyze reaction kinetics, electrochemical impedance, and surface morphology. Six scientific articles on the topic of this chapter were published by the author (Bironaite et al., 2023; Zinovicius et al., 2021, 2024; Zinovičius et al., 2023; Zinovicius et al., 2022; Zinovicius et al., 2022).
2. PREPARATION OF MATERIALS AND METHODOLOGY

2.1. Materials for Immunosensor

For electrochemical experiments, a 0.1 M phosphate-acetate buffer solution (PABS) was prepared by dissolving NaH₂PO₄ from Fluka Chemie GmbH (Bucharest, Romania), Na₂HPO₄ from Carl Roth GmbH&Co (Karlsruhe, Germany) and CH₃COONa from Merk (Tokyo, Japan) in deionized water. To improve conductivity, 0.01 M KCl from Scharlau (Barcelona, Spain) was added to the PABS solution. PABS pH was adjusted to 6.5 with CH₃COOH or NaOH from Merk (Steinheim, Germany).

Biorecognition elements: Ab-HRP was obtained from an ELISA kit produced by Institut Pourquier (Montpellier, France). Glucose oxidase was obtained from Merk (Saint Louis, USA). Goat Anti-Human IgG labeled by 6 nm gold nanoparticles (Ab-AuNP) were obtained from Abcam (Cambridge, United Kingdom).

Substrates for the biorecognition elements 30% Hydrogen peroxide was obtained from Carl Roth GmbH&Co (Karlsruhe, Germany), glucose (D- (+)-glucose (99%)) from Carl Roth GmbH&Co (Karlsruhe, Germany) were prepared in PABS to sustain stable pH. The prepared glucose solution was left to mutarotate overnight before use.

For electrode surface cleaning and modification sulfuric acid (98%) was purchased from Merk (Darmstadt, Germany), and 98.5% ethanol solution from “Vilniaus degtinė” (Vilnius, Lithuania). 25% glutaraldehyde solution was purchased from Fluka Chemie GmbH (Buchs, Switzerland). YPD-broth, pyrrole (98%), reduced graphene oxide powder, 2 nm and 10 nm gold nanoparticle suspension, and 2 nm platinum nanoparticle suspension were purchased from Merck (Darmstadt, Germany). K₂PtCl₆ from Merk (Darmstadt, Germany) solution was prepared in 0.5 M H₂SO₄, and to remove all dissolved oxygen, nitrogen was bubbled for 1 hour. Dry yeast (*lot. Saccharomyces cerevisiae*) was purchased from a food supplier “Dr Oetker Lietuva” (Vilnius, Lithuania).

Mediators for the electrochemical systems potassium ferrocyanide/potassium ferricyanide were purchased from Carl Roth GmbH&Co (Karlsruhe, Germany), ferrocene monocarboxylic acid (FcCOOH) from Merk (Darmstadt, Germany) from Carl Roth GmbH&Co (Karlsruhe, Germany) was prepared in PABS.

2.2. Glucose Oxidase and Antibody Conjugate Immobilization

A poly (methyl methacrylate) Petri dish with a diameter of 3 cm for Ab-HRP and GOx for Ab-AuNP glass was chosen as an immobilization substrate. The substrate was first cleaned thoroughly by washing it with 98.5% ethanol, rinsed with deionized water, and dried. Next, to activate the substrate surface, it was placed in a
closed vessel above a 25% glutaraldehyde solution for 15 minutes, forming the initial glutaraldehyde layer. Afterwards, 0.5 µL drop of the desired solution, GOx solution ranging from 10 pg/mL to 40 mg/mL in concentration or 40 mg/ml Ab-HRP solution or Ab-AuNP solution ranging from 10 µg/mL to 100 fg/mL, was deposited onto the surface and allowed to dry at room temperature. Subsequently, the sample was exposed to the 25% glutaraldehyde solution for an additional 15 minutes to cross-link the attached GOx/Ab-HRP/Ab-AuNP to the substrate’s surface. Finally, the modified surface was rinsed with a PABS solution to complete the preparation process.

2.3. Polypyrrole Synthesis

Polypyrrole synthesis was performed in two different ways using baker’s yeast, biosynthesis, and utilizing controlled potential pulses, electrochemical synthesis.

In polypyrrole biosynthesis, the first step is to grow yeast cells. YPD-broth was mixed with PABS to get a 50 g/l concentration of YPD-medium. Next, dry yeast (lot. Saccharomyces cerevisiae) was added to the prepared medium for a 10 mg/mL concentration. The medium with yeast was incubated at 32 ºC on an orbital shaker at 250 rpm for 17–24 hours (until yeast reached the logarithmic phase). Yeast was then harvested by centrifugation at 5600 rpm for 3 min and washed with PABS 3 times. The wet mass was weighed and suspended in PABS to the concentration of 1 g/mL.

For yeast (lot. Saccharomyces cerevisiae) modification with PPy, the solution containing 0.3 M of pyrrole, 0.4 M of potassium ferrocyanide, 1 M of glucose, and PABS was used and incubated at 32 ºC temperature on an orbital shaker at 250 rpm for 24 hours. Cell harvesting and suspension preparation parameters and conditions are the same as those applied for yeast (Zinovicius, Rozene, Merkelis et al., 2022).

In the process of isolating PPy particles from the suspension of modified yeast cells, a concentration of 390 mM hydrogen peroxide was added to the solution. Following the addition of hydrogen peroxide, the suspension was subjected to gentle agitation using an orbital shaker at 250 rpm for 24 hours. To distinguish residual cell components from the PPy particles, the suspension was subjected to centrifugation at 5600 rpm for 3 minutes. The resulting supernatant containing PPy particles was then collected by subjecting it to further centrifugation at 16000 rpm for 15 minutes.

Electrochemical pyrrole polymerization was carried out using a series of controlled potential pulses, as shown in Fig. 2.1. 10 seconds at 0 V for resting and then 10 seconds at 1.1 V for pyrrole polymerisation, resulting in the formation of the polypyrrole layer after each potential pattern application.
2.4. Electrode Modification by Composite Materials

Ultramicroelectrode modifications can be separated into two types. First by forming polypyrrole matrix layer according to methodology in 2.3. electrochemical polypyrrole synthesis. After the matrix was formed, an additional layer containing different nanomaterials: polypyrrole nanoparticles, AuNPs, PtNPs, and rGO. These layers are formed by adding nanoparticles to the electrochemical cells and undergoing the electrochemical formation of an extra PPy/Nanoparticle layer.

The second type of UME modification was done by electrochemically forming platinum structures on the conductive part of the electrode. UME is emersed into 2mM K$_2$PtCl$_6$ solution, and cyclic voltammetry was applied under a nitrogen atmosphere with a range from -200 mV to 1.3 V, step 50 mV, ten cycles, start was chosen 1 V to induce nucleation (González-González et al., 2011).

2.5. Electrochemical Cell Design for Scanning Electrochemical Microscopy

A local electrochemical investigation was performed using a scanning electrochemical microscope from Sensolytics (Bochum, Germany). A 10 µm diameter platinum UME from Sensolytics (Bochum, Germany) with an $R_g$ value of 10 was used as the probe. A platinum wire with a substantial surface area, which was at
least 100 times larger than UME, served as the counter electrode, while an Ag/AgCl in 3M KCl Ag/AgCl\textsubscript{(3M KCl)} electrode from Sensolytics (Bochum, Germany) was used as the reference electrode. The distance between every electrode was 10 mm. Before the experiments, the UME was thoroughly cleaned through an electrochemical process following the manufacturer’s guidelines, which involved the application of potential cycling in 0.5 M H\textsubscript{2}SO\textsubscript{4}, in a potential range between 0 V and 1.2 V \textit{vs} Ag/AgCl\textsubscript{(3M KCl)}, followed by rinsing with ethanol and deionized water.

The approach curves were obtained at feedback mode using a 120 µM solution of FcCOOH as a redox mediator. The sample surface was probed by approaching it at a 1 µm step with a 1 µm/s speed, 10 ms wait time, and an applied potential of +400 mV \textit{vs} Ag/AgCl\textsubscript{(3M KCl)}. The experiments were conducted repetitively, with a total of five iterations.

### 2.6. Scanning Electrochemical Impedance Microscopy Measurements

EIS operates on the principle of measuring the current response elicited by a sinusoidal excitation signal of low voltage amplitude applied to an electrochemical system. This process results in an alternating current with a phase shift, which the working electrode detects. The observed signal is a complex impedance, reflecting various electrochemical properties, such as the solution resistance, the double-layer capacitances at both the working and counter electrodes, and the charge-transfer resistance across these electrodes. The UME is capable of scanning in three dimensions, enabling the investigation of a specific 3D space within the solution.

To perform scanning electrochemical impedance microscopy measurements, a frequency range of 100 mHz to 50 kHz was applied with a root mean square amplitude of 10 mV and a chosen direct current potential bias. A mixture based on 1 mM of K\textsubscript{3}[Fe(CN)\textsubscript{6}] and 1 mM of K\textsubscript{4}[Fe(CN)\textsubscript{6}] was utilized as a redox mediator. Measurements were performed at different distances between the probe and the modified surface. The distance between the probe and the sample surface was determined by recording and fitting current vs distance dependencies as the UME was brought closer to the plastic surface of the Petri dish. The obtained data were then fitted to an equivalent circuit, which describes the system of interest.

To perform RC-SEIM, the modified surface and probe would have to consume the same electrochemically active species. Therefore, the competition between the modified surface and UME electrode for this form of redox mediator occurs.
The FB-SEIM technique utilizes a mediator diffusing within the space between an altered surface and an ultramicroelectrode. During this process, the mediator undergoes oxidation or reduction at the UME. Consequently, the measurements obtained through this approach mirror the electrochemical behavior of the sample. Specifically, heightened electrochemical activity of the sample corresponds to diminished impedance levels, creating a direct relationship between activity and impedance.

For both approaches, appropriate potential can be determined from cyclic voltammetry.

### 2.6.1. Horseradish Peroxidase – Labeled Antibodies

The scheme of the experiment with Ab-HRP is shown in Fig. 2.2. The oxidation of $[\text{Fe(CN)}_6]^{4-}$ is possible in two instances:

1. On the surface of UME
2. During enzymatic HRP reaction.

**Fig. 2.2.** Schematic representation of Ab-HRP detection by electrochemical impedance microscopy in the presence of potassium ferrocyanide ($K_4[\text{Fe(CN)}_6]$), which serves as a redox mediator. HRP$_{\text{ox/red}}$ – oxidized and reduced forms of horseradish peroxidase (Zinovicius et al., 2021) (made by the author)

Reaction on the UME:

$$2[\text{Fe(CN)}_6]^{4-} - 2e^{-} \xrightleftharpoons{+0.4 \text{ V}} 2[\text{Fe(CN)}_6]^{3-}. \quad (2.1)$$
Reaction, catalyzed by HRP:

\[ H_2O_2 + 2[Fe(CN)_6]^{4-} + 2H^+ \rightarrow 2H_2O + 2[Fe(CN)_6]^{3-}. \] (2.2)

This way, the UME registers the consumption of \([Fe(CN)_6]^{4-}\), the concentration of which is related to the activity of horseradish peroxidase found in the Ab-HRP conjugate. To optimize the detection accuracy, a direct current potential bias was specifically set at +400 mV. This value was selected based on the peak current potential identified through the technique of cyclic voltammetry, which is instrumental in determining the optimal operating conditions for electrochemical measurements.

### 2.6.2. Glucose Oxidase

The scheme of the experiment with GOx is shown in Fig. 2.3. The reduction reaction of \([Fe(CN)_6]^{3-}\) simultaneously occurs at the interface with the UME as well as the surface of the substrate during the enzymatic reaction of glucose oxidase.

![Fig. 2.3. Schematic representation of the redox competition mode for the electrochemical impedance microscopy measurements of glucose oxidase modified surface in the presence of glucose and potassium ferricyanide (K₃[Fe(CN)_6]), which serves as a redox mediator (made by the author)].

Reaction on the UME:

\[ [Fe(CN)_6]^{3-} + e^- \overset{-0.2V}{\longrightarrow} [Fe(CN)_6]^{4-}. \] (2.3)
Reaction, catalyzed by GOx:

\[
\text{Glucose} + [\text{Fe(CN)}_6]^{3-} + O_2 \Rightarrow \text{Gluconolactone} + H_2O_2 + [\text{Fe(CN)}_6]^{4-}. \quad (2.4)
\]

A direct current potential bias of -200 mV was chosen based on the peak current potential identified through the technique of cyclic voltammetry.

### 2.6.3. Gold Nanoparticle – Labeled Antibodies

The experimental setup involving antibody-functionalized gold nanoparticles is shown in Fig. 2.4. The process begins with the reduction of \([\text{Fe(CN)}_6]^{3-}\) to \([\text{Fe(CN)}_6]^{4-}\) at the surface where Ab-AuNPs are immobilized. Subsequently, the newly formed \([\text{Fe(CN)}_6]^{4-}\) undergoes oxidation when it reaches the UME. To facilitate this electrochemical reaction, a direct current potential bias of +400 mV was applied. This specific voltage was selected based on the optimal peak current potential that was previously determined through the method of cyclic voltammetry.

This setup ensures efficient electron transfer processes at the electrode surfaces, crucial for the accurate analysis and measurement in the experiment.
2.6.4. Approach Curves and 3-Dimensional Scanning

The approach curves were obtained at RC-SEIM using a 1 mM solution of \( K_3[Fe(CN)_6] \), and 1 mM of \( K_4[Fe(CN)_6] \) was utilized as a redox mediator. The procedure involved carefully probing the sample surface by systematically approaching it in incremental steps of 1 µm at a consistent speed of 1 µm/s, while implementing a brief pause of 10 ms at each step. During this process, an extensive frequency range from 100 mHz to 50 kHz was applied. The applied signals had a root mean square amplitude of 10 mV, and a potential bias of +400 mV versus Ag/AgCl\(_{\text{3M KCl}}\) as potential bias at each point. These experiments were conducted repetitively, completing a total of five iterations for each test to ensure consistency and reliability of the results. The collected data were subsequently analyzed and differentiated according to frequency.

The dependencies of EIS’s imaginary and real components on distance from the surface are similar to the feedback mode in SECM. In order to acquire accurate and reliable data pertinent to specific chemical reactions, it is essential to select an appropriate frequency that correlates well with the dynamics of the reaction being studied. Consequently, graphical representations of the SEIM approach curve are typically illustrated in the form of normalized impedance values plotted against normalized distances. These plots are aligned with the methodologies outlined in Equations 2.5 and 2.6, facilitating a clear visualization and analysis of the impedance behavior relative to the proximity to the surface.

\[
Z = \frac{|Z_d|}{|Z_\infty|}, \quad (2.5)
\]

\[
L = \frac{d}{r_T}. \quad (2.6)
\]

Where \( |Z_d| \) – impedance at a distance \( d \) from the surface, \( |Z_\infty| \) – impedance far from the surface, \( r_T \) – tip radius, \( d \) – the distance between the tip and the sample.

A 3-dimensional scanning procedure was carried out at a 3×3 mm area, with a distance of 4 µm between the UME and the surface of interest. The scanning was executed with a step size set at 200 µm, proceeding at a consistent speed of 200 µm/s, coupled with a settling time designated at 100 ms. Operational parameters included a frequency set at 10 Hz and an amplitude of 10 mV, with an applied potential bias fixed at -200 mV. The data acquired from this process was subsequently normalized according to established methodologies detailed in the AC versus distance dependency equations (Equations 2.5, 2.6). Employing this standardized procedure facilitated a direct comparison between sample surfaces that were either with or without immobilized enzymes, ensuring a consistent and accurate assessment of the enzymatic presence on the surfaces under investigation.
2.7. Sample and Electrode Surface Characterization

Atomic force microscopy measurements were performed by using the BioScope II AFM combined with an inverted optical microscope developed by Veeco Instruments Ltd. (Santa Barbara, USA). The setup included the utilization of an NP-D cantilever (Bruker, USA). For the imaging of surface topography, the AFM was operated in contact mode at a scanning speed of 0.30 Hz. During these measurements, a precise surface area of 500×500 nm surface area was scanned to accurately assess and calculate the surface roughness parameters.

Samples were prepared on 3 mm diameter graphite electrodes obtained from Sigma-Aldrich (Steinheim, Germany). These electrodes were prepared following the same protocols used for ultramicroelectrodes modification as physical restrictions do not allow for high-length samples to be measured with this technique.

Scanning electron microscopy was used in addition to AFM to investigate modified electrode surfaces further. Experiments were conducted using a Helios Nanolab 650 scanning electron microscope (FEI, Eindhoven, the Netherlands). The SEM operated at an electron beam voltage of 2 kV and a beam current of 0.4nA, settings that are optimized to image the polymer layers deposited on the electrodes without causing thermal damage. Similar to the constraints faced in AFM, the SEM sample preparations had to adhere to the physical limitations, with modifications being applied directly to the surface of the graphite electrodes.

2.8. Mathematical Models

Mathematical models serve as theoretical frameworks that enable researchers to dissect and understand the intricate relationships present within complex systems by transforming real-world challenges into mathematically solvable equations. Utilizing these models, scientists can apply the scanning electrochemical microscopy approach curve experimental data to calculate the reaction kinetics of a system. Moreover, by fitting the data obtained from electrochemical impedance experiments, researchers can construct an electrical equivalent circuit that mirrors the characteristics of the system under study. This process facilitates the indirect calculation of several crucial parameters, such as solution resistance, charge transfer resistance, and capacitance. These calculated parameters provide a deeper insight into the electrochemical properties of the system. Additionally, by analyzing data acquired through atomic force microscopy, researchers can quantify surface roughness parameters, further enhancing the understanding of the material properties at the microscopic level. Through these methodologies, mathematical models prove invaluable in advancing our comprehension of complex systems in a scientifically rigorous manner.
2.8.1. Reaction Kinetics from Approach Curves

Ultramicroelectrodes parameters were calculated using the mathematical model fitted to the experimentally obtained approach curves (Cornut & Lefrou, 2008). To ensure a comprehensive comparison of the data sets collected, each set of approach curves was normalized. Subsequently, these normalized curves were methodically presented in a format that showcases the normalized tip current as a function of the normalized distance. This presentation adhered strictly to the specifications outlined in Equations 2.2 and 2.7, thereby facilitating a consistent and clear interpretation of the data (Lefrou & Cornut, 2010):

$$I_T = \frac{i_T}{i_\infty}$$ (2.7)

Where $i_T$ is the experimentally measured tip current, and $i_\infty$ is the steady-state current described according to Equation 1.2.

Glucose oxidase exhibits properties that are not typical of a conventional conductor, nor do they align strictly with those of an insulator; instead, this enzyme demonstrates a unique amalgamation of properties from both categories. Consequently, it is feasible to utilize a mathematical model designed to integrate the distinctive aspects of both conductors and insulators. This model would effectively govern the tip current by accounting for the hybrid nature of the surface types influenced by the enzyme's activity. (Cornut & Lefrou, 2008; Bironaite et al., 2023):

$$I_T(L, \lambda, R_g) = I_T^{\text{cond}}(L, R_g) + \frac{i_T^{\text{ins}}(L, R_g) - 1}{(1 + 2.47R_g^{0.31}L\lambda)(1 + R_g^{0.006R_g + 0.113\lambda - 0.236R_g + 0.91})},$$ (2.8)

$$I_T^{\text{cond}}(L, R_g) = \alpha(R_g) + \frac{\pi}{4\beta(R_g)\text{ArcTan}(L)} + \left(1 - \alpha(R_g) - \frac{1}{2\beta(R_g)}\right)\frac{2}{\pi} \text{ArcTan}(L),$$ (2.9)

$$\alpha(R_g) = \ln 2 + \ln 2 \left(1 - \frac{2}{\pi} \text{ArcTan} \left(\frac{1}{R_g}\right)\right) - \ln 2 \left(1 - \left(\frac{2}{\pi} \text{ArcCos} \left(\frac{1}{R_g}\right)\right)^2\right),$$ (2.10)

$$\beta(R_g) = 1 + 0.639 \left(1 - \frac{2}{\pi} \text{ArcCos} \left(\frac{1}{R_g}\right)\right) - 0.186 \left(1 - \left(\frac{2}{\pi} \text{ArcCos} \left(\frac{1}{R_g}\right)\right)^2\right),$$ (2.11)

$$i_T^{\text{ins}}(L, R_g) = \frac{2.08}{R_g^{0.358}} \left(L^{0.145} - 1.585\right) + \frac{2.08}{R_g^{0.358}} \left(L + 0.0023R_g\right) + 1.57 + \ln \frac{R_g}{L} + \frac{2}{\pi R_g} \ln \left(1 + \frac{\pi R_g}{2L}\right).$$ (2.12)

Where, $I_T^{\text{cond}}(L, R_g)$ is a model for the conductive surface described by equation 2.9, $I_T^{\text{ins}}(L, R_g)$ – a model for the insulating surface described by equation 2.10, $\lambda$ – the kinetic constant, $R_g$ – the ratio between the radius of the insulating shroud and the radius of the conductive surface, and L – the normalized distance.
2.8.2. Electrochemical Impedance Data

The obtained data from experimental electrochemical impedance measurements were then fitted to an equivalent circuit, as depicted in Figs. 2.2., 2.3., 2.4. which describes the systems of interest. The impedance observed in these systems is quantitatively expressed as a function of their respective electrical characteristics:

\[
Z = \frac{Z_{cd}(R_p)}{Z_{cd} + (R_p)} + R_s.  \tag{2.13}
\]

Where \(Z_{cd}\) is the double layer impedance (Eq. 2.14), \(R_p\) – charge-transfer resistance, and \(R_s\) – the ohmic resistance of the solution.

Double layer impedance is expressed as a constant phase element:

\[
Z_{cd} = \frac{1}{Q(j\omega)\alpha}.  \tag{2.14}
\]

Where \(Q\) represents the capacitance of a CPE with an \(\alpha\) of 1, \(j\) – the imaginary unit, \(\omega\) – the angular frequency, \(\alpha\) represents the angle by which the CPE impedance is rotated.

Solution resistance between the UME and other electrodes was uncompensated.

In this way both the resistive and capacitive components inherent to the system’s behavior are displayed. His mathematical formulation allows for a more thorough understanding of the system’s dynamics and interactions.

2.8.3. Surface Roughness Parameters

Roughness can be described by several parameters: height, wavelength, spacing, and hybrid parameters (Gadelmawla et al., 2002). Among these, the most significant parameter is height. This parameter encompasses a variety of elements (Oliveira et al., n.d.; Qi et al., 2015; Astakhov, 2010):

\(S_\alpha\) is the arithmetical average:

\[
S_\alpha = \frac{1}{h} \int_0^h |P(x)| \, dx.  \tag{2.15}
\]

\(S_p\) is the maximum profile peak height:

\[
S_p = |maxP(x)|.  \tag{2.16}
\]

\(S_v\) is the minimum profile valley depth:

\[
S_v = |minP(x)|.  \tag{2.17}
\]

\(S_T\) is the maximum height of the profile:

\[
S_T = S_p + S_v.  \tag{2.18}
\]
Where $P(x)$ is the function describing the surface profile in terms of height ($P$) and position ($x$) over the evaluated length ($h$).

2.9. Conclusions of the Second Chapter

This chapter presents all the information about the materials and equipment used and a description of the chosen research methods. First, solution and biorecognition element preparation detailed the procedures for immobilizing the biorecognition element onto the desired surface, described the methods used for polypyrrole synthesis, and provided a detailed overview of electrochemical techniques, complete with schematic representations of the experiments. It also provides a chapter on surface characterization using SEM and AFM. The chapter closes with a separate chapter dedicated to mathematical models, researching reaction kinetics from approach curves, impedance data, and surface roughness parameters from obtained AFM data.

The chapter led to the following conclusions:

1. Immobilization is achieved by cross-linking the biorecognition element with the substrate surface using glutaraldehyde. This approach is simpler and cost-effective as it uses only glutaraldehyde. Additionally, it ensures complete utilization of the biorecognition element. However, it does not offer oriented immobilization of the biorecognition element.

2. Yeast cells can assist in polypyrrole particle synthesis and provide an eco-friendly approach to the polymerization process. On the other hand, electrochemical synthesis offers enhanced control over the formation of the polypyrrole matrix, thereby allowing for a more precise manipulation of its properties and characteristics.

3. The ultramicroelectrode was modified by polypyrrole and gold/platinum/reduced graphene oxide composites or platinum microstructures using cyclic voltammetry, a method that ensures control over layer formation on its active part of UME with minimal material use. Thus, this technique is simpler and more efficient compared to chemical reduction.

4. Antibody labels can be effectively assessed using scanning electrochemical microscopy and scanning electrochemical impedance microscopy. Although these methods introduce complexity in probe manipulation and data interpretation, however, it enables the design of immunosensors on more cost-effective substrates and facilitates the reuse of electrodes. This contrasts with the conventional approach in
electrochemical immunosensors, which often relies on single-use electrodes.

5. Electrode surface can be characterized by SEM and AFM, such studies are widespread and provide insights beyond surface topography but also allow to have additional surface parameters to be calculated, such as roughness.
The third chapter presents an experimental part of the research. The research was carried out according to the tasks set in the dissertation. According to the tasks, the plan of experiments was set (Fig. 3.1).

The plan consists of three main parts: development and application of scanning electrochemical impedance microscopy, engineering, and evaluation of composite materials for signal amplification, and transducer modification. Finally, new methods and engineered materials are applied to develop and characterize a prototype electrochemical immunosensor system that uses antibodies labeled with gold nanoparticles as a bioreognition element.
Fig. 3.1. Plan of experiments is divided into three segments (made by the author)

Four scientific articles on the topic of this chapter were published by the author (Bironaite et al., 2023; Zinovicius et al., 2021, 2024; Zinovičius et al., 2023).

3.1. Detection of Antibody Labeled by Horse Radish Peroxidase

To detect antibodies labeled by horseradish peroxidase, the experimental setup illustrated in Fig. 2.2 was used. In this setup, the working electrode is a UME, which plays a critical role in the measurement process. The reaction products generated from reaction 2.1 and Ab-HRP (Reaction 2.2) accumulate near the UME, consequently affecting the conductivity of the surrounding solution. These
products, originating from the Ab-HRP modified surface, gradually diffuse into the solution, making SEIM with a mobile UME an optimal method for examining the diffusion of reaction products, which allows for precise local examination of the diffusion behavior of reaction products in the vicinity of the modified surface.

Electrochemical impedance spectra were recorded at varying distances from both non-modified and Ab-HRP-modified Petri dishes (Fig. 3.2). The collected experimental data were then correlated with the mathematical model of the Randles circuit (Equations 2.13 and 2.14). Subsequently, all three parameters were graphed as a function of distance from the sample surface. This analysis was essential to determine the most advantageous distance for subsequent measurements of Ab-HRP.

The EIS spectra, recorded at varying distances from the non-modified Petri dishes, exhibited consistent results with no observable changes, as anticipated (Fig. 3.2 A).

**Fig. 3.2.** Impedance measurements performed at different distances from A – unmodified and B – Ab-HRP-modified Petri dish in PABS with 1 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] using 4.2 mM H₂O₂ concentration; C – dependences of solution resistance; D – charge transfer resistance; E – double-layer capacitance on the distance between UME and the surface of Petri dish (Zinovicius et al., 2021)
In contrast, the EIS spectra obtained from the Ab-HRP-modified Petri dishes demonstrated a notable increase in charge transfer resistance when examined at closer proximities to the surface. This increase is attributed to the depletion of $[\text{Fe(CN)}_6]^{4-}$ and the impeded diffusion toward the UME (Fig. 3.2 B Notably, despite these modifications, the solution resistance displayed uniformity, showing no significant difference between the non-modified and Ab-HRP-modified Petri dishes, regardless of the proximity in which measurements were taken (Fig. 3.2.C). Moreover, a clear correlation was observed between the charge transfer resistance and the spatial separation of the unmodified from the Ab-HRP-modified Petri dishes (Fig. 3.2 D). Specifically, it was discovered that the charge transfer resistance at the Ab-HRP modified interface was significantly reduced, ranging from 1.5 to 2.6 times lower than that on the non-modified surfaces, with resistance decreasing from a 1.5-fold at a minimal 2 µm gap to a 2.6-fold reduction at an extended 20 µm distance. This finding led researchers to select a 2 µm distance for subsequent analyses due to the significant rise in resistance measured at this range, approximately 0.34 GΩ. Additionally, the observed pattern of resistance changes in relation to the distance closely mirrored the trends seen in SECM approach curves, which were recorded using either negative FB or RC-SECM modes. This similarity suggests that as the distance to the surface decreases, the concentration of $[\text{Fe(CN)}_6]^{4-}$ diminishes, unequivocally indicating the consumption of the redox mediator at the surface pre-modified with Ab-HRP.

To explore the feasibility of employing redox competition mode in analyzing surfaces modified with Ab-HRP, EIS spectra were recorded at a 2 µm distance from the surface under varying $\text{H}_2\text{O}_2$ concentrations (Fig. 3.3). It was observed that the solution resistance remained consistent across all $\text{H}_2\text{O}_2$ concentrations for both non-modified and Ab-HRP-modified Petri dishes (Fig. 3.3 C). For the non-modified dishes, no significant relationship was found between the charge transfer resistance and $\text{H}_2\text{O}_2$ concentration. However, a linear correlation was observed for dishes with immobilized Ab-HRP (Fig. 3.3 D). Furthermore, the double-layer capacitance exhibited a decrease with increasing $\text{H}_2\text{O}_2$ concentrations up to 4.2 mM, stabilizing at 10 mM (Fig. 3.3 E). These findings suggest that the charge transfer resistance linearly correlates with $\text{H}_2\text{O}_2$ concentration. This indicates that surfaces modified with Ab-HRP can be effectively studied using RC-SEIM and charge transfer resistance as a key parameter.

Measurements demonstrated the detection of an Ab-HRP, utilizing the combined method of EIS and SECM in redox competition mode. This approach facilitated an in-depth exploration of HRP’s enzymatic activity through the strategic variation of substrate concentrations. EIS data were analyzed employing the Randles circuit model. This analysis yielded insights into three crucial parameters, plotted concerning $\text{H}_2\text{O}_2$ concentration or spatial distance from the reaction site.
A significant finding was the emergence of charge transfer resistance as a pivotal parameter.

![Graph showing impedance measurements](image)

**Fig. 3.3.** Impedance measurements performed at 2 µm distance from A – unmodified Petri dish and B – Ab-HRP modified Petri dish in PABS with 1 mM K$_3$[Fe(CN)$_6$]/K$_4$[Fe(CN)$_6$] using different concentrations of H$_2$O$_2$; dependence of C – solution resistance, D – charge transfer resistance E – double-layer capacitance on H$_2$O$_2$ concentration using unmodified and Ab-HRP modified Petri dish (Zinovicius et al., 2021)

It effectively described the dynamics of the HRP-catalyzed reaction occurring at the modified surface, capturing essential aspects, such as substrate consumption and diffusion patterns. Notably, the research unveiled a linear correlation between charge transfer resistance and H$_2$O$_2$ concentration. This relationship highlights the potential of employing RC-SEIM for the detailed investigation of surfaces modified not only with HRP but also with other immunolabels.
3.2. Detection of Glucose Oxidase

HRP is a commonly employed antibody label in immunosensor development. However, its reliance on H$_2$O$_2$ as a substrate raises concerns due to H$_2$O$_2$’s highly oxidative nature, which poses potential risks to the integrity of the sample. It is, therefore, crucial to choose an enzyme for antibody labeling that relies on a less oxidative substrate to avoid potential damage. In this regard, GOx emerges as a viable alternative in this context, primarily due to its specificity for glucose, e.g., a substrate that, unlike H$_2$O$_2$, does not pose oxidative threats to the sample. This attribute positions GOx as a promising candidate for antibody labeling. Additionally, the well-documented kinetic properties of GOx further bolster its suitability for application in immunosensors, enhancing its utility.

Another innovative option to consider in the realm of antibody labeling is the use of metal nanoparticles often termed nanozymes, which represent a cutting-edge approach. These nanozymes are engineered to emulate the catalytic activities of enzymes and bring several benefits over traditional enzymatic labels. They are notably more robust against abrupt changes in environmental conditions such as pH, temperature, and pressure, which contributes to their increased stability and minimizes the risk of denaturation, thereby extending their operational lifespan in varied analytical conditions (Zinovicius et al., 2022; Zinovicius et al., 2022). However, it is critical to acknowledge the limitation in specificity associated with nanozymes, a factor that must be carefully considered in their application. Despite these advantages, the specificity of nanozymes does not always match that of traditional enzymes, which poses a challenge for their application in precision-dependent settings like immunosensor development. This limitation underscores the importance of a thorough evaluation of nanozymes' performance relative to conventional enzymatic labels but might bring new possibilities when developing immunosensors.

3.2.1. Detection Limits of Glucose Oxidase by Scanning Electrochemical Microscopy in Feedback Mode

Further, the relationship between the concentration of immobilized GOx on an insulating surface and its activity using SECM in FB mode was investigated. This technique was particularly effective for assessing the activity of GOx at low concentrations (Zinovicius et al., 2022). As depicted in Fig. 3.4 A, approach curves for surfaces modified with different concentrations of immobilized GOx (ranging from 10 mg/mL to 40 mg/mL) exhibited typical positive feedback responses. Further decreasing GOx concentrations on the surface resulted in reduced catalytic activity when performing an approach with UME.
3. RESEARCH OF METAL-BIOMATERIAL NANOCOMPOSITES AND APPLICATION ...

Fig. 3.4. A – fitted FB-SECM approach curves toward surfaces modified by different GOx concentrations from 0 to 40 mg/mL containing solutions. B – dependency of $\lambda$ on GOx concentrations from 0 to 1 mg/mL. The working solution contained 120 µM ferrocene mono-carboxylic acid and 50 mM $\beta$-D-glucose. Potential bias was +400mV vs Ag/AgCl

The gathered experimental data was analyzed using a mathematical model formulated by Renaud and Christine Lefrou. This analysis facilitated the calculation of the kinetic constant, $\lambda$, allowing for a comparative evaluation as shown in Equation 2.8. Fig. 3.4 B illustrates that the minimum detectable concentration of GOx was 100 ng/mL, corresponding to a $\lambda$ value of 0.16. A further reduction in GOX concentration to 10 pg/mL, which is indicated by the orange dashed line in Fig. 3.4 A, resulted in a $\lambda$ value of 0.14. Intriguingly, this value aligns with the $\lambda$ of a non-modified surface, as indicated by the purple line, highlighting the sensitivity limits of the technique in detecting low GOx concentrations.

In the initial experiments, GOx at a 100 ng/mL concentration was employed to determine the detection limit and linear range in response to varying glucose concentrations. However, the results were inconclusive, as no significant changes were observed. This lack of response could be attributed to GOx’s insufficient catalytic activity at this concentration. Consequently, subsequent experiments employed a more sensitive method, referred to as RC-SEIM, previously used to detect Ab-HRP, as illustrated in Fig. 2.3. In these experiments, the redox couple $[\text{Fe(CN)}_6]^{3-}/[\text{Fe(CN)}_6]^{4-}$ served as a mediator. The reduction of $[\text{Fe(CN)}_6]^{3-}$ occurred concurrently at both the UME interface and the surface of the substrate during the enzymatic reaction of GOx, as depicted in chemical Reactions 2.3 and 2.4.
3.2.2. Scanning Electrochemical Impedance Microscopy for Glucose Oxidase Measurements

In applying the RC-SECM to assess the activity of GOx, EIS spectra were recorded at varying distances from both non-modified and modified Petri dish surfaces. These experiments were carried out in an electrolyte solution consisting of 1 mM of the redox couple 1 mM of $\text{[Fe(CN)}_6\text{]}^{3-}/\text{[Fe(CN)}_6\text{]}^{4+}$ along with 20 mM of glucose. The research findings showed no significant trends in impedance values in the vicinity of the unaltered Petri dish surface, as illustrated in Fig. 3.5 A. Additionally, fitting these data to a mathematical model did not reveal any noteworthy changes in either the charge transfer resistance or the capacitance, as depicted in Figs. 3.5 C and 3.5 D, respectively.

![Fig. 3.5](image)

**Fig. 3.5.** Impedance measurements with glucose oxidase and a Petri dish in different distances of 2 µm, 4 µm, 6 µm, 10 µm, 20 µm, and 50 µm, respectively. Glucose concentration was 20 mM during all measurements. A shows impedance measurements with the Petri dish and used analogue element scheme, B shows the impedance measurements with GOx-modified (100ng/mL) surface, C shows the calculated charge transfer resistance concerning the distance, and D shows the estimated double layer capacity, calculated from CPE parameters concerning the distance.
In contrast, the modified Petri, by GOx, surface demonstrated a distinctly different behavior. Notably, as the distance between the probe and the surface was decreased from 20 µm to a mere 2 µm, there was a significant rise in the charge transfer resistance, which increased by approximately 690 MΩ, as shown in Fig. 3.5 C. In contrast, the Constant Phase Element (CPE) parameters remained relatively unchanged, as evidenced in Fig. 3.5 D.

The impact of varying glucose concentrations, ranging from 0 to 20 mM, while keeping a constant distance from the sample (2 µm), is detailed in Fig. 3.6.

![Fig. 3.6](image_url)  
**Fig. 3.6.** Impedance measurements with glucose oxidase and a Petri dish at a constant distance of 2 µm from the sample with different glucose concentrations in solution. Glucose concentrations were 0 mM, 2 mM, 6 mM, 10 mM, 15 mM, and 20 mM. A shows impedance measurements registered at the non-modified surface; B shows impedance measurements of the GOx-modified (100ng/mL) surface; C shows the calculated charge transfer resistance concerning glucose concentration; and D shows the estimated capacity from CPE parameters concerning the concentration of glucose.
This variation led to a significant increase in charge transfer resistance by 343 MΩ and a corresponding decrease in estimated capacitance by 11 F. These changes were particularly notable at the interface with GOx, as illustrated in Figs. 3.6 C and D. Concurrently, the charge transfer resistance and capacitance values on the unmodified Petri surface exhibited minimal fluctuations, maintaining relative consistency (Figs. 3.6 C and D).

In the control experiment, a marginal decrease in estimated capacitance was observed, which could be attributed to an increase in the solution’s viscosity following the addition of glucose.

### 3.2.3. Approach Curves at Different Frequencies

In the comparative analysis, measurements were conducted at various frequencies from 50 kHz to 10 Hz, as illustrated in Fig. 3.7, for two distinct cases: the Petri (Fig. 3.7 A) and the GOx-modified surface (Fig. 3.7 B).

**Fig. 3.7.** Normalized impedance values concerning the normalized distance in different frequencies. The glucose concentration in both solutions was 20 mM. A – measurement approaching Petri dish; B – measurement approaching GOx (100 ng/mL)
A noteworthy observation emerged while recording approach curves, particularly at a frequency of 10 Hz, where the most substantial variance in normalized impedance values was detected. This observation suggests a heightened sensitivity of measurement outcomes at lower frequencies to the glucose oxidation reaction. Subsequent experimental observations, as depicted in Fig. 3.8, involved the application of varying glucose concentrations at a constant frequency of 10 Hz.

![Graph showing normalized impedance at different glucose concentrations](image)

**Fig. 3.8.** Normalized impedance measured at GOx-modified surface (100ng/mL) dependencies on the normalized distance at different glucose concentrations. Measurements were performed at a frequency of 10 Hz, increasing glucose concentration from 0 to 20 mM glucose.

These experiments revealed a notable enhancement in the normalized impedance value. Specifically, an increase of 1.77 was recorded in the normalized impedance when the glucose concentration was elevated from 0 mM to 20 mM. Furthermore, a detailed analysis of the range from 2 mM to 20 mM indicated a continuous upward trend in the normalized impedance value.

### 3.2.4. 3-Dimensional Imaging at Single Frequency

The observed data indicate a significant elevation in normalized impedance proximate to the active surface, as previously shown. To further investigate this phenomenon, a three-dimensional scan was conducted employing the RC-SEIM technique. This scan aimed to generate a detailed map elucidating the electrochemical activity in the area. The scan was performed in the vicinity of the Petri dish, as shown in Fig. 3.9 A, did not reveal any notable changes. Contrasting normalized
impedance near the surface of immobilized GOx indicated a significant increase, as depicted in Fig. 3.9 B.

It was noted that a significant concentration of the enzyme predominantly accumulated near the periphery of the droplet during the drying process. Interestingly, the distribution of catalytic activity was not uniform around the edge, indicating a non-homogeneous pattern.

**Fig. 3.9.** Electrochemical activity images of A – the non-modified surface and B – 100 ng/mL GOx-modified surface. Measurements were performed at a frequency of 10 Hz, a distance from the surface of 4 µm, a step of 200 µm, a UME movement speed of 200 µm/s, a waiting time of 1 ms, and a glucose concentration of 20 mM.

This observation underscores the necessity of conducting a comprehensive three-dimensional scan of catalytic activity prior to selecting the most suitable location for conducting approach experiments. Notably, the maximal NZ value observed at a distance of 4 µm near the edge of the droplet was 5.6. This contrasts with the peak NZ value of 4.2 obtained from approach curve analyses, further highlighting the importance of 3D scans as the variability of enzyme concentration during immobilization remains an important issue.

This research investigated the activity dependence of a GOx-modified insulating surface using FB-SECM. Mathematical modeling determined the minimal detectable GOx concentration at 100 ng/mL, yet this level did not significantly respond to glucose concentration changes, indicating a lack of catalytic activity. To enhance sensitivity, the research utilized RC-SEIM with \([\text{Fe(CN)}_6]^{3-}/[\text{Fe(CN)}_6]^{4+}\) as a mediator, observing increased charge transfer resistance and decreased capacitance correlating with glucose concentration increases. This contrasted with the stable impedance of the unmodified surface, underscoring the
importance of GOx concentration in surface modification and the effectiveness of RC-SEIM in glucose detection when low concentrations of the enzyme are used. Moreover, measurements focus on glucose oxidation reaction response to frequency variations, focusing on impedance measurements at different frequencies. Our findings revealed that lower frequencies, especially at 10 Hz, exhibited increased sensitivity to this reaction. Subsequent experiments consistently demonstrated a rise in normalized impedance with increasing glucose concentration, a trend that was particularly pronounced at the 10 Hz frequency. 3D scans utilizing a 10 Hz frequency revealed an irregular distribution of catalytic activity, primarily at the periphery of the enzyme drop. This uneven activity distribution underscores the importance of thorough 3D catalytic activity mapping in identifying the most effective sites for further experimental procedures. Additionally, it highlights the importance of frequency selection in impedance measurements and the necessity of precise 3D mapping of catalytic activity to ensure accurate experimental outcomes.

3.3. Decoration of Ultramicroelectrode

Glucose oxidase has shown significant promise as a label for antibodies. When used in conjunction with RC-SEIM, it enables the detection of concentrations as low as 100 ng/mL. However, employing the RC-SEIM technique introduces a notable challenge: the emergence of charge transfer resistance at the level of giga-ohms. This issue is reminiscent of the one encountered during the Ab-HRP measurements. Such high resistance levels can adversely affect the accuracy of measurements. This is because the system’s impedance may reach or surpass the inherently high impedance of the potentiostat and cables, leading to considerable distortions in the recorded measurements. One potential solution to address the issue of elevated resistance during measurements is the modification of the probe’s conductive surface. This could be achieved through the creation of composite structures, which may reduce resistance while maintaining the benefits of localized measurements.

In addressing these challenges, this research employed seven distinct electrode modifications. Each modification involved using polypyrrole as the foundational matrix, further enhanced by forming a composite layer. This composite layer consisted of polypyrrole embellished with various nanomaterials. The initial application of these modifications was on a graphite electrode with a diameter of 3 mm. To evaluate their effectiveness, cyclic voltammetry experiments were carried out. This experiment solution is composed of 1 mM $\text{[Fe(CN)}_6\text{]}^{3-}/\text{[Fe(CN)}_6\text{]}^{4-}$ as a mediator and 50 mM of glucose, as depicted in Fig. 3.10. The objective of this experimental arrangement was to mimic the conditions typically encountered
in localized impedance measurements. This approach provided a platform to investigate the implications of the electrode modifications under research.

![Graph](image)

**Fig. 3.10.** Cyclic voltammograms of modified and non-modified graphite electrodes. Measurements were performed in a phosphate buffer solution with 1 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] and 50 mM glucose.

The research findings revealed a notable augmentation in the electrical current near the surfaces that underwent modification. Specifically, the incorporation of a polypyrrole matrix led to an increase in the current generated near the oxidation peak, surpassing that of the bare graphite electrode by over 35 µA. Furthermore, the integration of biosynthetically derived polypyrrole particles contributed to an additional rise in the current, reaching 157.53 µA. In line with expectations, smaller AuNPs demonstrated superior performance to those measuring 10 nm in diameter, enhancing the generated current by a factor of four compared to the bare electrode. However, 2 nm PtNPs displayed a reduction in current generation relative to 2 nm AuNPs. Notably, the most pronounced increase in current was observed with electrodes-modified rGO, which generated over five times higher current compared to the control and 69 µA greater than that achieved with 2 nm AuNPs.

Furthermore, SEM was utilized to evaluate the morphology of the newly formed structures. This analysis was conducted at two distinct magnifications: 10,000 times and 5,000 times, as depicted in Fig. 3.11. This approach provided
detailed insights into the microstructural characteristics of the samples under investigation.

**Fig. 3.11.** Scanning electron microscopy images under 10,000 and 5,000 times magnification. A – polished graphite electrode; B – polypyrrole matrix; C – polypyrrole matrix with polypyrrole particles; D – polypyrrole matrix with 10 nm-diameter gold nanoparticles; E – polypyrrole matrix with 2 nm-diameter gold nanoparticles; F – polypyrrole matrix with 2 nm-diameter platinum nanoparticles; G – polypyrrole matrix with reduced graphene oxide
The surface morphology analysis revealed that the polypyrrole matrix exhibits a granular structure, with granule diameters ranging from 0.125 to 0.526 µm (Fig. 3.11 B). Upon introducing biosynthesized PPy particles produced using yeast cells, a notable decrease in the average top-layer granule size was observed, from 0.249 to 0.09 µm (Fig. 3.11 C). The decoration of PPy with 10 nm gold nanoparticles led to a more uniform formation of the PPy layer, with an average granule size of 0.105 µm and a standard deviation of 0.015 (Fig. 3.11 D). Reducing the size of the gold nanoparticles to 2 nm resulted in increased cavitations in the top layer and a granule size range of 0.065 to 0.442 µm, exhibiting a significantly higher standard deviation of 0.136 (Fig. 3.11 E).

Switching the decoration to 2 nm PtNPs induced a noticeable fuzziness in the image, possibly due to the platinum nanoparticles adhering to the PPy matrix. This alteration caused the PPy structure to resemble towers composed of PPy granules, with top-layer granule sizes ranging from 0.111 to 0.05 µm (Fig. 3.11 F). Lastly, the decoration of the PPy matrix with rGO necessitated a 5000-fold magnification for clearer imaging. This revealed reduced graphene blocks randomly distributed on the electrode’s surface, with an average width of 3.63 µm and length of 4.94 µm (Fig. 3.11 G).

Fig. 3.12. Cyclic voltammograms of modified and non-modified platinum ultramicroelectrode (diameter – 10 µm). Measurements were performed in phosphate buffer solution with 1 mM K₃[Fe(CN)₆]/ K₄[Fe(CN)₆] and 50 mM glucose.

The formation of the PPy matrix with rGO on the UME was conducted similarly to the modification of graphite electrodes, and cyclic voltammetry was performed as a standard procedure to verify the UME’s modification. Subsequently,
a significant reduction in current near the modified UME was observed, as illustrated in Fig. 3.12. This reduction suggests that the decorated PPy matrix may be impeding the conductive surface of the electrodes, potentially leading to an increase in impedance during localized impedance measurements. The UME was further electrochemically modified to mitigate this issue by introducing platinum microstructures on its conductive surface. The success of this modification was assessed using cyclic voltammetry as before, with the results detailed in Fig. 3.13.

![Cyclic voltammograms of modified and non-modified platinum ultramicroelectrode by platinum structures (diameter – 10 µm). Measurements were performed in phosphate buffer solution with 1 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] and 50 mM glucose](image)

Fig. 3.13. Cyclic voltammograms of modified and non-modified platinum ultramicroelectrode by platinum structures (diameter – 10 µm). Measurements were performed in phosphate buffer solution with 1 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] and 50 mM glucose

![Scanning electron microscopy images under 10,000 times magnification of A – polished graphite electrode; B – graphite electrode modified by platinum microstructure (Zinovicius et al., 2024)](image)

Fig. 3.14. Scanning electron microscopy images under 10,000 times magnification of A – polished graphite electrode; B – graphite electrode modified by platinum microstructure (Zinovicius et al., 2024)
The research findings revealed a marked augmentation in the electrical current produced near the modified surface. Specifically, there was a 4.3-fold enhancement in current at the anodic peak potential compared to the current generated by the unmodified UME. This substantial increase strongly suggests the successful modification of the electrode. Furthermore, platinum microstructure formation was confirmed by using SEM and AFM to investigate surface morphology and surface properties.

Fig. 3.14 presents Scanning Electron Microscope images that compare the surfaces of non-modified and modified materials magnified 10,000 times. A detailed analysis of these images reveals the emergence of platinum structures on the graphite electrode’s surface (Fig. 3.14 B). These structures are characterized by a non-uniform, globular morphology with varying diameters, ranging between 2.5 and 35 µm. This finding implies that analogous morphological transformations are likely to have occurred on the conductive surface of the UME. Such changes could contribute to a decrease in impedance resistance during subsequent measurement processes.

AFM was utilized to examine the analysis of the surface characteristics further. Data were gathered over a 500×500 nm area to provide a detailed characterization of the surface roughness parameters.

![Graph showing surface roughness parameters](image)

**Fig. 3.15.** Comparison of surface roughness parameters between a polished graphite electrode and graphite electrode modified by platinum microstructure.

A – $S_a$ arithmetical average; B – $S_T$ maximum height of the profile

Fig. 3.15 A illustrates a minimal change in the $S_a$ surface roughness parameter, approximately 1.15 nm. However, it is notable that the standard deviation is over three times higher for the non-modified graphite electrode compared to its modified
counterpart. Similarly, Fig. 3.15 B shows that the difference in the $S_T$ parameter is only 13.37 nm. Yet, the standard deviation is more than sevenfold higher for the non-modified electrode than the modified one. The analysis of this data suggests that the deposited platinum structures on the electrode surface act to fill in the gaps inherent in the graphite structure. Consequently, this implies that forming platinum structures on UME could remedy surface defects from mechanical damage.

### 3.4. Gold Labeled Antibody Measurements

The subsequent chapter delves into the use of Ab-AuNP as a biorecognition element and UME as a transducer. Focusing on a comparative evaluation of ultramicroelectrodes, both in their modified and non-modified forms. A uniform experimental setup was utilized for these assessments applying FB-SEIM, as illustrated in Fig. 2.4. The inclusion of Ab-AuNP facilitated enzyme-like reactions, resulting in the reduction of $[\text{Fe(CN)}_6]^{3-}$ to $[\text{Fe(CN)}_6]^{4-}$, similar to GOx catalyzed reaction. This reduced form can then be re-oxidized at the surface of the UME. Employing this methodology enables the UME to detect and measure the production of $[\text{Fe(CN)}_6]^{4-}$, providing a direct correlation to the catalytic activities of the gold nanoparticles in the Ab-AuNP conjugate. For a comprehensive understanding, EIS spectra were recorded across a wide range of Ab-AuNP conjugate concentrations, from 1 mg/mL down to 100 fg/mL. A glass substrate was used as a control in these experiments, as detailed in Fig. 3.16.

**Fig. 3.16.** Impedance measurements were performed at a 2 µm distance from the sample A – non-modified platinum ultramicroelectrode and B – modified platinum ultramicroelectrode, near different concentrations of immobilized Ab-AuNP, starting from 1 mg/mL to 0 mg/mL. Measurements were performed in phosphate buffer solution with 1 mM $K_3[\text{Fe(CN)}_6]/K_4[\text{Fe(CN)}_6]$ and 50 mM glucose (Zinovicius et al., 2024)
The research findings showed that, as hypothesized, the non-modified UME displayed a markedly higher resistance in comparison to its modified equivalent. This observation was consistent across both systems, with impedance variations observed up to a concentration of 100 fg/mL. To quantitatively analyze this phenomenon, the Randles circuit model, as delineated in Equations 2.9 and 2.10, was applied to the experimental data. This approach facilitated the computation of the charge transfer resistance, providing a deeper insight into the electrochemical behavior of the systems under investigation.

In the unmodified electrode setup, measurements indicated a charge transfer resistance surpassing 1 GΩ, as illustrated in Fig. 3.17 A. Conversely, when employing a modified electrode, the charge transfer resistance was notably reduced to slightly above 100 MΩ. This alteration signifies a reduction in resistance by an order of magnitude greater than 10-fold.

![Image](image.png)

**Fig. 3.17.** Charge transfer resistance logarithmic dependency on immobilized Ab-AuNP concentration. A – comparison between modified and non-modified electrodes B – results from modified electrodes fitted to a linear model. Measurements were performed in PABS with 1 mM K₃[Fe(CN)₆]/ K₄[Fe(CN)₆] and 50 mM glucose (Zinovicius et al., 2024)

In the research, the application of the modified electrode (Fig. 3.17 B) yielded results with reduced deviation compared to its non-modified counterpart. Notably, the linear detection range of the modified electrode extended to a lower limit of 100 fg/mL, a significant improvement over the 1 pg/mL maximum attained by the non-modified electrode. Furthermore, the modified system demonstrated an enhanced lower limit of detection at 100 fg/mL. These outcomes highlight the substantial benefits of electrode modification, including decreased resistance and improved measurement accuracy. This enhancement is particularly valuable for evaluating catalytic activities in biological samples, where precision is critical.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Used electrode</th>
<th>Linear range</th>
<th>Limit of detection</th>
<th>Analysis time, min</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IgG</td>
<td>platinum UME modified with platinum microstructures</td>
<td>$0.001-10^3$ ng/mL</td>
<td>0.001 ng/mL</td>
<td>20</td>
<td>This research</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>eight gold microelectrodes functionalized with 4-aminophenylacetic acid, then the structure of magnetic nanoparticles coated with poly (pyrrole-co-pyrrole-2-carboxylic acid and crosslinked with specific polyclonal antibody for tetracycline</td>
<td>$0.0001-1$ ng/mL</td>
<td>0.0012 ng/mL</td>
<td>60</td>
<td>(El Alami El Hassani et al., 2019)</td>
</tr>
<tr>
<td>Microalbuminuria</td>
<td>carbon interdigitated microelectrode modified with gold nanocrystals with trapped nanoparticles (polystyrene nanoparticle core with silver nanoshells covalently conjugated to HSA antibodies)</td>
<td>$30-300$ μg/mL</td>
<td>30μg/mL</td>
<td>-</td>
<td>(Shaikh et al., 2019)</td>
</tr>
<tr>
<td>Dengue virus</td>
<td>screen printed carbon electrode coated with oxidized bovine serum albumen and functionalized by anti-non-structural monoclonal antibody</td>
<td>1–200 ng/mL</td>
<td>0.3 ng/mL</td>
<td>30</td>
<td>(Nawaz et al., 2018)</td>
</tr>
<tr>
<td>Mucin 4</td>
<td>carbon-based screen-printed electrode functionalized with phenylacetic acid with immobilized human partial recombinant MUC4 protein and mouse monoclonal antibody</td>
<td>1–15 μg/mL</td>
<td>0.33μg/mL</td>
<td>30</td>
<td>(Hosu et al., 2017)</td>
</tr>
<tr>
<td>Cry1Ab</td>
<td>indium tin oxide electrodes modified by 3-aminopropyltrimethoxysilane monolayer with covalently immobilized Anti-Cry1Ab polyclonal antibodies</td>
<td>1–10 ng/mL</td>
<td>0.37 ng/mL</td>
<td>60</td>
<td>(Freitas et al., 2016)</td>
</tr>
</tbody>
</table>
Upon contrasting with conventional impedimetric immunosensors, as outlined in Table 3.1, the SEIM employing modified UME demonstrates a superior linear range, spanning from 0.001 to $10^3$ ng/mL, expedited analysis time of 20 minutes, and, notably, one of the most sensitive detection thresholds of 0.001 ng/mL. This highlights the method’s versatility and the broad array of potential applications it can accommodate. This combination of features underscores the SEIM immunosensor's advanced efficiency and adaptability in comparison to traditional models. However, it is pertinent to acknowledge that the evaluations of our system were conducted exclusively in controlled, idealized conditions and did not encompass the analysis of real-world samples.

This research marks a notable advancement in scanning electrochemical impedance microscopy, enhancing the examination of biological samples with greater precision and accuracy. The modification of UME significantly reduces charge transfer resistance, outperforming standard impedimetric immunosensors in simplicity without compromising efficiency. These advancements could have wide-ranging implications in scientific and biomedical research. Further exploration of this method promises deeper insights into biological processes, potentially advancing our understanding of complex biological mechanisms.

### 3.5. Conclusions of the Third Chapter

The third chapter delves into experimental studies where the following objectives were pursued: (1) to investigate the possibility of using combined SECM and EIS methods to detect typical antibody conjugate immobilized on isolator substrate; (2) to explore potential benefits of SEIM compared to conventional SECM researching standard antibody labels; (3) to mitigate high resistance by decorating
UME; (4) to apply enzyme-mimicking nanoparticles and decorated UME to engineer an immunosensor; (5) to evaluate immunosensor characteristics.

From the results obtained in these studies, the following conclusions could be drawn:

1. By leveraging the localization of scanning electrochemical microscopy and the high sensitivity of electrochemical impedance spectroscopy, a new method of scanning electrochemical impedance microscopy was applied to detect common immunosensor labels and determined that the most suitable parameter for detection is charge transfer resistance.

2. Comparative analysis highlighted the limitations of Scanning Electrochemical Microscopy (SECM) in feedback mode for detecting low concentrations of GOx. Even though 100 pg/mL of GOx could be detected, it does not respond to changes in glucose concentration. Leading to the adoption of more sensitive techniques like RC-SEIM. Distinct changes in impedance were observed near surfaces modified with GOx (100 pg/mL), particularly concerning glucose concentration, with a linear range from 2 to 15 mM. Underlining the effectiveness of this method for measuring GOx label catalytic activity.

3. Frequency sensitivity in measurements showed that lower frequencies, specifically 10 Hz, are more sensitive to glucose oxidation reactions, indicating the importance of frequency choice in such measurements. Choosing low frequency to observe glucose oxidation could help further simplify the design of electronic components, as there is no need to use complex potentiostat, as well as decrease costs when miniaturizing immunosensors.

4. 3D imaging and catalytic activity distribution were measured utilizing 3D scans to map out electrochemical activity, revealing the uneven distribution of catalytic activity, especially around the edges of immobilized enzymes. Suggesting that immobilization method, even though simple and cost-effective, may cause irregularities when measuring. To solve this problem, a future-orientated immobilization method should be used.

5. Ultramicroelectrode was modified with polypyrrole to address high resistance issues in SEIM measurements (Ab-HRP over 2 GΩ and GOx over 1.2 GΩ), and UME was modified with polypyrrole and other nanomaterials. Modification blocked the UME, thus significantly increasing resistance. This modification is not suitable for UME. A different approach to high resistance issues was electrochemically depositing platinum on the conductive surface of the ultramicroelectrode.
This solution decreased charge transfer resistance more than ten times compared to non-modified systems. It is suggested that this modification can be used further when designing immunosensors.

6. The prototype immunosensor demonstrated the efficiency of modified ultramicroelectrodes in detecting gold nanoparticle-labeled antibodies, showing an increase in a linear range from 0.01–10³ ng/mL to 0.001–10³ ng/mL and a decreased limit of detection from 0.1 to 0.001 ng/mL compared to unmodified ultramicroelectrode. It is suggested that application in medical diagnostics is possible, though it was only tested in an ideal scenario and needs to be further validated with more turbid and real-life samples.
The present work aims to contribute to the further development of electrochemical immunosensors based on metal and biomaterial nanocomposites. Based on the literature review results of the experiments and analysis of the data, the following conclusions can be drawn:

1. Scanning electrochemical impedance microscopy shows great promise in studies of biological samples as it inherits a non-destructive nature and the ability to probe the localized property of the sample. The first application of redox competition mode for scanning electrochemical impedance microscopy was to detect and evaluate antibodies labeled by horseradish peroxidase. This research showed that charge transfer resistance is the key parameter evaluating redox activity. However, horseradish peroxidase consumes hydrogen peroxide as a substrate, which might cause oxidation of the analyte and loss of sensitivity and accuracy. Thus, alternative labels such as glucose oxidase and enzyme-mimicking nanoparticles had to be investigated.

2. According to data obtained by scanning electrochemical microscopy in feedback mode, the detection limit of glucose oxidase is 100 pg/mL. However, no changes were observed when glucose concentration was changed; on the other hand, utilizing scanning electrochemical impedance allowed for the observation of changes dependent on glucose
concentration. Afterwards, it was determined that the glucose oxidation reaction near the enzyme could be probed at a low alternation frequency of 10 Hz within 2 radii of the surface. Superior sensitivity allows scanning electrochemical impedance microscopy to be used to detect and image the electrochemical activity of the sample.

3. Probing Ab-HRP and GOx, systems display extremely high charge transfer resistance (Ab-HRP over 2 GΩ and GOx over 1.2 GΩ); thus, feedback instead of redox competition mode was employed. Changing the modes did not have a significant impact on charge transfer resistance when measuring the system based on antibody-conjugated to the gold nanoparticle (Ab-AuNP over 1.2 GΩ) and suggesting the need for additional system modifications such as ultramicroelectrode decorations.

4. Application of electrode modifications, polypyrrole matrix incorporating gold/platinum nanoparticles or reduced graphene oxide, showed an increase in generated current compared to the non-modified graphite electrodes; however, when ultramicroelectrode was modified with any composite material, which included the polypyrrole matrix, the UME displayed no activity. Thus, only modification with platinum microstructures showed a significant increase in registered current at the peaks when cyclic voltammetry was performed. Using a modified ultramicroelectrode, maximal charge transfer resistance decreased to 120 MΩ. With this decrease, more accurate and reliable results can be obtained from the prototype immunosensor.

5. Developed prototype electrochemical immunosensor, based on scanning electrochemical impedance microscopy with modified ultramicroelectrode as a transducer and antibody labeled by gold nanoparticle as a biosensing element showed a linear range of 0.001–10³ ng/mL, with a limit of detection being 0.001 ng/mL, and response time of 20 minutes. It is suggested that application in medical diagnostics is possible, though it was only tested in an ideal scenario and needs to be further validated with more turbid and real-life samples.

Future studies in this area should be related to examining engineered sensors’ performance in real-life samples and their stability over time. Additionally, ultramicroelectrode decoration with reduced graphene oxide should be considered without the use of a polypyrrole matrix. Further, miniaturization possibilities should be considered by using single frequency to detect an analyte and simplified potentiostat to make fast and accurate quantitative immunosensors for consumers.
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List of Scientific Publications by the Author on the Topic of the Dissertation

Papers in the Reviewed Scientific Journals


**Papers in Other Editions**


Įvadas

Problemos formulavimas

Nuolat tobulėjančioje šiuolaikinėje medicinoje analizė ir diagnostika vaidina labai svarbų vaidmenį. Diagnostikos dažniausiai yra naudojami imuniniai jutikliai, kurie garsėja dideliu jautrumu ir specifiku. Dideliu jautrumu pasižymintys imuniniai jutikliai gali aptikti labai žemas patogenų koncentracijas, tai padeda diagnozuoti ligą dar prieš atsirandant simptomams, o toks ankstvyvas ligos nustatymas leidžia užkirsti kelią patognumui plisti. Imuniniai jutikliai plačiai naudojami onkologiniams susirgimams nustatyti, kuriant naujus vaistus, nustatant terapinių preparatų specifiku ir efektyvumą, taip pat kuriant personalizuotos gydymo metodus. Histologiniose tyrimuose imuninių jutiklių panaudojimas leidžia nustatyti audinių sudėtį, praplečiant supratimą apie procesus lastelių lygmeniu.

Kuriant elektrocheminius imuninius jutiklius dažnai yra naudojami antikūnai ir antigenai, kurie žymimi fermentais, siekiant sustiprinti elektrocheminį signalą, o tada pritvirtinti ant elektrodo paviršiaus. Toks elektrocheminių imuninių jutiklių naudojimo būdas gali sumažinti jų jautrumą ir specifikuos. Konjugacijos metu gali būti inaktyvinti fermentas, antikūnas ar abu, po pritvirtinimo ant elektrodo paviršiaus elektronų pernašos tarpininko difuzija link elektrodo aktyvaus ploto yra suformuota biologinio sluoksnio. Be to, kai kuriems fermentų žymenims naudojami labai oksiduojantys substratai, kurie gali sugadinti mėginį dar prieš analizę. Galiausiai, tokiu būdu panaudoti imuniniai jutikliai dažniausiai yra vienkartiniai, nes ne visada elektrodo regeneracija yra sėkminga. Šios problemos yra aktyviai sprendžiamos naudojant naujas nanomedžiagas elektrodo paviršiui modifikuoti ir kuriant naujus signal keitimo metodus. Pritaikius šias naujos, ateityje tikimasi sukurti kiekybiniai imuniniai jutiklius, kurie būtų daugkartinio naudojimo, efektyvūs ir prieinami.

**Darbo aktualumas**

Didelis dėmesys šiuo metu yra skiriamas itin jautriems kiekybiniam elektrocheminiams imuniniams jutikliams, kuriuos kuriant susiduria su jų daugkartinio panaudojimo problema ir silpną signalą.

Tyrinėjant katalitiškai aktyvias platinos ir aukso nanodaleles atrandama galimybė jomis paveikti tradicinius fermentinius antikūnų ir antigenų žymenys sudarant metalo ir biomedžiagos kompozitą. Šis kompozitas leistų užtikrinti didesnį sistemos stabulumą nepaprastai signalo stiprumo, o taikant skenuojamąją elektrocheminę mikroskopiją kaip alternatyvų signalo keitiklį, atsiranda galimybė bio-atpažinimo dalį pritvirtinti ant pigaus ir prieinamo paviršiaus, pavyzdžiui, plastiko ir stiklo, taip pat sumažinti jrenginių ar sudaryti imuninių jutiklių matricią. Bet, ultramikrolektrodos galėtų būti naudojamos pakartotinai, taip papildomai sumažinant imuninio jutiklio kainą. Integravus šią strategiją, imininiai jutikliai taptų ekonomiškesni ir prieinamesni.

**Tyrimo objektas**

Disertacijos tyrimų objektas yra elektrocheminis imuninis jutiklis ir jo tyrimai taikant skenuojamąją elektrocheminę mikroskopiją.

**Darbo tikslas**

Disertacijos darbo tikslas yra sukurti kiekybinio elektrocheminio imuninio jutiklio prototipą, pagrįstą metalo ir biomedžiagos kompozitą (antikūną, žymėtą nanodalele, kuri turėtų panašų katalitinės veikimo kaip fermentas), skirtu bioatpažinimui, ir skenuojamiosios elektrocheminės mikroskopijos kaip keitiklio pritaikymu.

**Darbo uždaviniai**

Darbo tikslui pasiekti buvo sprendžiami šie uždaviniai:

1. Sukurti naują analitinį metodą, kuris jungtų skenuojamiosios elektrocheminės mikroskopijos konkurenčinio režimo stipriąją savybę su elektrocheminio
impedanso spektroskopija ir kurį būtų galima taikyti tiriant krienų peroksidaze žymėtus antikūnus.

2. Įvertinti gliukozės oksidazės aptikimo ribas taikant skenuojamąsias elektrocheminę ir impedanso mikroskopijas, nes gliukozė turi mažiau oksidacinių savybių nei vandenilio peroksidas.

3. Sukurti imuninio jutiklio prototipą, kuris būtų pagrįstas metalo ir biomedžiagos kompozitu (antikūnas, pažymėtas katalitiškai aktyvia nanodalele), skirtu bioatpažinimui, ir įvertintas taikant skenuojamąja elektrocheminio impedanso mikroskopiją.

4. Modifikuoti ultramikroelektrodo paviršių, sudarant metalo ir laidas polimero kompozitinį sluoksnį, ir įvertinti galimybes šį paviršių pritaikyti kuriant imuninio jutiklio prototipą.

5. Sukurti imuninį jutiklį, taikant tinkamiausią ultramikroelektrodo modifikaciją, įvertinti sukurto imuninio jutiklio parametrus ir palyginti juos su ne-modifikuotu imuninio jutiklio prototipu.

Tyrimų metodika


Darbo mokslinis naujumas

Gauti tyrimų rezultatai, leidžiantys pateikti naujas įžvalgas apie imuninius jutiklius ir priśidėti prie medžiagų inžinerijos srities vystymo, yra šie:

1. Sukurtas skenuojamosis elektrocheminės impedanso mikroskopijos metodas, taikytas konkurencinių režimų, buvo jautresnis tiriant fermentų aktyvumą, palyginti su tradicinių skenuojamosių elektrocheminės mikroskopijos metodu.

2. Pagerėjusi krūvio pernašos kinetika iš biomedžiagos link elektrodo naudojant biosintetines polipirolio daleles, sintetintas iš naudojant mielių metabolizmą, palyginti su elektrochemiškai pagamintu polipiroliu.
3. Platinos mikrostruktūrų formavimas ant ultramikroelektrodo laidžios dalies leidžia sumažinti krūvio pernašos varžą, išlaikant lokalizuotų matavimų pobūdį.

4. Sukurtas kiekybinio elektrocheminio imuninio jutiklio prototipas, pagrįstas skenuojamajame elektrochemine impedanso mikroskopijos, naudojant ultramikroelektrodu, modifikuotą platino mikrostruktūromis, kaip zondą ir antikūnus, pažymėtus aukso nanodalelėmis, kaip atpažinimo elementą.

**Darbo rezultatų praktinė reikšmė**


**Ginamieji teiginiai**


2. Jautresni ir tiksliai skenuojamiosios elektrocheminės impedansos metodus gali būti atliekami naudojant platinos mikrostruktūras, suformuotas ant laidžios ultramikroelektrodo dalies, kadangi modifikacija leidžia sumažinti krūvio pernašos varžą daugiau nei 10 kartų, padidinant elektrodo našumą.

3. Sukurtas kiekybinio elektrocheminio imuninio jutiklio prototipas, kuriame naudojami aukso nanodalelės žymėti antikūnai bei skenuojamiosios elektrocheminės impedanso mikrostruktūromis, naudojant ultramikroelektrodu, dėl lokalizuoto matavimų pobūdžio pasižymi didesniu jautrumu, palyginti su tipiniais impedimetriniais imuninius jutiklius.

**Darbo rezultatų aprobavimas**

Septyni moksliiniai straipsniai disertacijos tema atspausdinti žurnalose, esančiuose Clarivate Analytics Web of Science duomenų bazėje (Bironaitė et al., 2023; Zinovicius et al., 2021, 2024; Zinovičius et al., 2023; Zinovicius et al., 2022; Zinovicius et al., 2022; Zinovicius et al., 2022).
Disertacijoje atliktų tyrimų rezultatai buvo paskelbti dvylikoje konferencijų Lietuvoje ir užsienyje:
- Tarptautinėje konferencijoje „11 International Workshop on SECM and Related Techniques 2023“ 2023 m. Monrealyje, Kvebeke, Kanadoje;
- Tarptautinėje konferencijoje „The 66th International Open Readings Conference for Students of Physics and Natural Sciences 2023“ 2023 m. Vilniuje, Lietuvoje;
- Tarptautinėje konferencijoje „The COINS 2023: International Conference of Life Sciences“ 2023 m. Vilniuje, Lietuvoje;
- Tarptautinėje konferencijoje „The 4th World Congress on Electroporation & Pulsed Electric fields in Biology, Medicine, Food and Environmental Technologies“ 2022 m. Kopenhagoje, Danijoje;
- Tarptautinėje konferencijoje „The 18th International Conference on Electroanalysis“. 2022 m. Vilniuje, Lietuvoje;
- Tarptautinėje konferencijoje „The Advanced Nanomaterials Conference, 19th International Conference on Advanced Nanomaterials“ 2022 m. Aveire, Portugalijoje;
- Tarptautinėje konferencijoje „The 16th International Conference: Mechatronic Systems and Materials“ 2021 m. Vilniuje, Lietuvoje;
- Tarptautinėje konferencijoje „The 64th International Open Readings Conference for Students of Physics and Natural Sciences 2021“ 2021 m. Vilniuje, Lietuvoje;
- Tarptautinėje konferencijoje „The 14th International Conference The Vital Nature Sign“ 2020 m. Kaune, Lietuvoje;

**Disertacijos struktūra**

Disertaciją sudaro įvadas ir trys pagrindiniai skyriai. Pirmajame skyriuje skiriau švietimo ir mokslo reikšmę, pagrindinius imuninių jutiklių funkciniai savybės, skatindami kitų bangų ir švietimo teorijas. Antrajame skyriuje tyrinėti imuninių jutiklių funkcijas, taip pat jų fizinio ir cheminio veikimo principus. Trečiajame skyriuje tyrinėti imuninių jutiklių funkcijas, taip pat jų fizinio ir cheminio veikimo principus. Šiame steigti konkrečius gydymo ir profilaktikos methodus.
1. Imuninių jutiklių apžvalga ir analizė


Biologiniai jutikliai yra naudojami ne tik medicinoje, bet ir kitose srityse, pavyzdžiui, aplinkos bei vandens ir maisto kokybei stebėti (Feng et al., 2024).

![S1.1 pav. Biologinio jutiklio veikimo schema](image)

Disertacijoje pateikiamos norimos biologinių jutiklių charakteristikos: lengvai naudojami, jautrūs, selektyvūs ir teikiantys greitus rezultatus. Puikus to pavyzdys yra imuninių jutikliai. Imuninių jutiklių bioatpažinimo elementa dažniausiai yra antikūnas arba antigenas. Jie pasižymi nepaprastai dideliu jautrumu ir selektyvumu, gali veikti sudėtingose terpėse, pavyzdžiui, kraujyje ir piene (Tetyana et al., 2021). Pats imuninis jutiklis veikia afiniškumo pagrindu, antikūnai sudaro stabilių kompleksą su antigenu, o gautas biologinis signalas paverčiamas į mums naudingą (Liustrovaite et al., 2022).

Vieni iš perspektyviausių keitiklių yra elektrocheminei. Jie pasižymi ganėtinai nesudėtinga konstrukcija, kuri esant reikalui gali būti supaprastinta ir miniatiūrizuota.
Sumažinant ne tik pažįstamų, tačiau ir analizei skirtą aparaturą. O tai leistų kurį nesudėtingus, sąlyginai pigius, mažai reagentų naudojančius imuninius jutiklius.

Atsižvelgiant į tai, kad imuninio komplekso sudarymas neišskiria jokio elektrocheminio signalo, norint sustiprinti atpažinimo signalą ir antigenai yra žymimi fermentais (Ding et al., 2008; Jiang et al., 2016; Kilic et al., 2020; Vasantham et al., 2020). Dažniausiai antikūnų konjugacija su fermentu vyksta per aminų (-NH2), tiolo (-SH), ar hidroksi (-OH) grupes, kad nebūtų užblokuojamos antikūnų epitopos.

Disertacijos darbe nagrinėjami fermentų žymėliai imuno tyrimuose, pvz., ELISA, kuris pasižymi aukštu selektyvumu ir katalitiniu aktyvumu, bet yra brangus ir jautrus aplinkos sąlygoms: temperatūrai ar pH. ELISA tyrimuose dažnai naudojami redoks fermentai toki kaip krienų peroksidazės.


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Platinos nanodaleles imituoją reakcijas panašias į krienų peroksidazės fermentą, šioms reakcijoms reikalingas vandenilio peroksidas kaip substratas. Kadangi vandenilio peroksidas pasižymi stipria oksidacinius savybėmis buvo toliau nagrinėjama aukso nanodaleles. Jos pasižymi panašiomis savybėmis į gliukozos oksidazę ir naudoja gliukozę kaip substratą. Analizuojant medžiagas bei kompozitus elektrodų modifikacijoms buvo apžvelgiami didelio laidumo medžiagos tokios kaip redukuotas grafeno oksidas, platinos, aukso nanodaleles bei jų pritvirtinimo metodai bei mikrostruktūrų formavimas ant elektrodo paviršiaus.

Vienas iš perspektyvių, bet retai naudojamų keitiklių yra skenuojamoji elektrocheminė mikroskopija. Disertacijoje yra detaliai analizuojami skenuojamosios elektrocheminės mikroskopijos režimai:


Taip pat nagrinėjama kaip sugyvenus skenuojamąjį elektrocheminę mikroskopiją su elektrocheminio impedanso spektroskopiją galima gauti naują analizės metodą, kurį taikant pasirinktoje mėginio vietoje galima išmatuoti elektrocheminių impedansų. Nagrinėjami gautų duomenų vaizdavimo būdai, bei elektrinės ekvivalentinės schemas sudarymo principai (Bironaite et al., 2023; Nasri et al., 2022; Traxler et al., 2022, Sukackienė et al., 2022).

Galiausiai aptariamos atominių jėgų ir skenuojamojo elektronų mikroskopijos, kurios yra plačiai taikomos medžiagų moksle tirti paviršiaus morfologijai ir sudėčiai.

2. Imuninių jutiklių tyrimams naudojamos medžiagos, jų paruošimas ir metodai

Antrajame disertacijos skyriuje pateikiamos naudotos medžiagos, apžvelgiamas tirpalų paruošimas bei skirtingi bioatpažinimo elemento tvirtinimo ant nelaidžių paviršių protokolai. Apaščioma vienas iš lengviausių pritvirtinimo būdų nelaidus paviršius aktyninamas glutaro aldehido („Carl Roth“, Vokietija) garais 15 min. Tada užlašinama biologiškai aktivyta medžiaga ir palaukiama iki skysčio išdžiūvimo ir pakartotinai paveikiama glutaro aldehido garais 15 min.

Taip pat aprašoma polipirolo dalelių biosintezę, kuriai atliekamą mėsinės naudodamos gliukozą Vidulasteliniam procesams, kai terpėje yra piolo bei K₄[Fe(CN)₆] šalia membranos inifijuojamo polimerizacija. Taip pat polipirolo electrocheminė sintezė formuojant matricę elektriniais pulsaus bei aukso, platininos nanodalelių ir redukuoto grafeno oksido integravimas į polipirolo matricą ir platininos mikrosrūtūrų formavimas bei jų panaudojimas modifikuojant ultramikroelektrodo aktivyvų paviršių (González-González et al., 2011). Galiausiai yra pateikiamas electrocheminių tyrimų ir paviršiaus charakterizavimo aprašymas.

Didžiąją dalį tyrimų sudarė electrocheminiai matavimai, kurie buvo atliekami ske- nuojamojo elektrcheminiu mikroskopu „Sensolytic“ (Bochumas, Vokietija). Naudojant šį įrenginį buvo atliekami priartėjimai prie paviršiaus grižtamajai rešimo režimu, paviršiaus skenavimai, matuojamas lokalizuotas electrocheminio impedanso atsakas. Siekiant gauti tikslesnius matavimus, buvo parenkami atstumai iki paviršiaus bei tinkamiausias potencialas (Zinovicius et al., 2021, Zinovicius et al., 2024).

Metodikos dalyje taip pat aprašomi matematiniai modeliai, kuriuos taikant iš gautų eksperimentinių priartėjimo kreivių duomenų, padaugantys interpretuoti stebimos reakcijos kinetiką. Kadangi mėginys pasižymi ne tik laidininko, bet ir dielektriko savybėmis sudarytas modelis apjungia šias dvi dalis. Pagal gautos electrocheminio impedanso duomenis yraapskaičiuojami papildomai sistemoms parametrai, kurių negalima pamatuoti tiesiogiai. Atskirus sistemos parametrai yra tirpalo varžą, krūvį pernašos varžą ir susidariusią dvi-gubos sluoksnio talpą (Zinovicius et al., 2021, Zinovicius et al., 2024). Šie parametrai padaeda stabthi sistemoje vykstančias reakcijas. Galiausiai pateikiamos paviršiaus parametrų
skaičiavimo metodai, kuriuos naudojant galima apdoroti atominių jégų mikroskopijoje gautus duomenis ir aprašyti paviršiaus šiurkštumą.

3. Eksperimentiniai metalo ir biomedžiagos nanokompozito taikymo tyrimai imuniniams jutikliams

Trečiajame skyriuje pateikiama eksperimentinių tyrimų dalis. Tyrimai buvo atliekami pagal numatytus disertacijoje uždavinius. Atsižvelgiant į disertacijos uždavinius ir tikslą buvo sudarytas eksperimentų planas (S.3.1 pav.).

S.3.1 pav. Atliktų tyrimų planas

Planas susideda iš trijų pagrindinių dalių. Pirmoji dalis apima skenuojamosios elektrocheminio impedanso mikroskopijos metodo kūrimą ir taikymą bei ultramikroelektrodo
modifikavimą, pasitelkiant kompozitinų medžiagų formavimą, naudojant laisžius polimerus, aukso, platinos nanodaleles, redukuotą grafeno oksidą ir platinos mikrostruktūras. Antroji dalis apima antikūnų žymenų tyrimus. Trečioje dalyje numatyta naujo metodo ir modifikuoto ultramikroelektrodo taikymas elektrocheminio iminio jutiklio prototipui kurti ir charakterizuoti, naudojant aukso nanodalelėmis žymetus antikūnus kaip bioatpažinimo elementą.

Pirmieji eksperimentai taikant sujungtą skenuojamąjį elektrocheminę mikroskopiją ir elektrocheminio impedanso spektroskopiją buvo atlikti konkurenciniu režimu matuoja
t antikūnus, konjuguotus su krienų peroksidazės fermentu (klasikinis antikūnų žymėjimas, dažnai atliekamas signaui sustiprinti).

Paveiksle S.3.2 pateikiami elektrocheminio impedanso matavimai 2 µm atstumu nuo mėgėjo paviršiaus, esant fosfatiniams acetatiniam buferiniams tirpalams su 1 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] elektronų perniašos tarpininko koncentracija ir keičiant vandenilio peroksido koncentraciją. A – nemo
difikasiota Petri lėkštėlė, B – krienų peroksidazės žymëtasis antikūnais modifikuota Petri lėkštelo, C – tirpalo varžos, D – krūvio pernašos varžos ir E – dvigubo sluoksnio talpos priklausomybė nuo vandenilio peroksido koncentracijos (Zinovicius et al., 2021)

Paveiksle S.3.2 pateikiami elektrocheminio impedanso matavimai 2 µ atstumu nuo mėgėjo paviršiaus, taip užtikrinant, kad atsakui didžiausią reikšmę turės pritvirtintas antikūno ir krienų peroksidazės konjugatas. Šioje sistemoje krienų peroksidzė naudoja vandenėlio peroksidą kaip substratą. Vandenėlio peroksidas pasižymi stipriomis oksidacinėmis savybėmis, ir jeigu matavimai būtų atliekami ne tobulomis sąlygomis, kai tirpalo
yra tik substratas ir analitė, o pavyzdžiui su kraują vandenėlio peroksidas galima jį koaguliuotų taip sunaikindamas mėginį. Todėl buvo išbandoma kiti imininių jutikliuose plačiai naudojamų žymenys.
Imuniniuose jutikliuose plačiai naudojamas gliukožės oksidazės žymuo, kadangi šio fermento reakcija yra gerai ištyriama užtikrinti substratas nepasियżmi oksidaciniu poveikiu, todėl jį būtų galima naudoti realiuose mėginiuose.

Disertacijos metu buvo palyginta skenuojamosios elektrocheminės mikroskopijos grižtamojo ryšio režime jautrumas su skenuojamą elektrocheminio impedanso mikroskopijos kontrastu. Rezultatai parodė, kad skenuojamajį elektrocheminio impedanso mikroskopiją aptiko 100 pg/mL gliukožės oksidazės koncentraciją bei naudojant šią fermento koncentraciją buvo galima nustatyti gliukožės koncentraciją nuo 2 iki 15 mM diapazone. Tačiau reikia pastebėti, kad abejuose tirtose sistemose skenuojamą elektrocheminio impedanso mikroskopiją krūvio varža yra labai didelė, viršijanti 2 GΩ. Kas gali sukelti paklaidas bei yra artėjama prie aparato galimybių ribos. Dėl šios priežasties tolimiausiuose tyrimuose, naudojant metalų ir biologinės medžiagos kompozitą kap bioatpažinimo elementą buvo naudojamas grižtamojo ryšio režimas. Norint papildomai sumažinti išmatuotą varžą, taip padidinat sistemos stabilitumą, buvo modifikuotas ultramikroelektrodas.

Visu pirmą pagal literatūros apžvalgoje pastebėtas tendencijas ultramikroelektrodas buvo modifikuojamas suformuojant polipirolų matricą ir jos paviršiui pritvirtinant skirtingas laidumų gerinančias medžiagas: 2 nm aukso nanodaleles, 10 nm aukso nanodaleles, 2 nm platinos nanodaleles, biologiškai susintetintas polipirolo daleles ir redukuota grafeno oksidą. Atliekant pilotiniai tyrimai naudojant didelio skersmens grafitinį elektrodą parodė, kad visos modifikacijos pagerina laidumų, didžiausią pokytį turėjo polipirolų matrica su redukuota grafeno oksidą. Atlirkul ultramikroelektrodo modifikaciją naudojant polipirolo ir redukuotą grafeno oksidą ultramikroelektrodas buvo užblokuotas. Visos modifikacijos, kuriose buvo polipirolas blokavo ultramikroelektrodą. Todėl buvo atsiskyrę šio tipo modifikacijos ir pereita prie modifikavimo formuojant mikrostruktūras ant laidžios elektrodo dalies. Suformuotos platinos mikrostruktūros buvo ištirtos atominių jėgų mikroskopija bei skenuojamajį elektronu mikroskopija. S.3.3 paveiksle matome sėkmingą paviršiaus modifikavimą platinos mikrostruktūromis. Suformuotos struktūros paviršiui sudaro netolygios globules formos debesis, šiurkštumo tyrimai parodė, kad elektrodo paviršiui suformuotos struktūros sumažina šiurkštumą, bei elektrodo paviršius tampa tolygesnis.
Toliau buvo kuriama prototipinė imuninio jutiklio sistema, kurioje fermentinį žymenį pakeičiame 6 nm auksno nanodalele, pasižyminčia panašiomis savybėmis kaip glukozės oksidazės fermentas.

S.3.4 paveikslė. Krūvio pernašos priklausomybė nuo antikūno, žymėto auksno nanodalele.
A – palyginimas, kai sistemoje yra naudojamas nemodifikuotas ir modifikuotas ultramikroelektrodas, B – platinos mikrostruktūromis modifikuoto elektrodo krūvio pernašos charakteristika (Zinovicius et al., 2024)

S.3.4 paveiksle pateikiamas krūvio pernašos charakteristikos palyginimas modifikuotos (naudojančios ultramikroelektrodą su suformuotomis platinos mikrostruktūromis) ir nemodifikuotos (naują iš gamintojo gautą ultramikroelektrodą) prototipinės imuninio jutiklio sistemoje. Mikrostruktūros suformavimas sumažino krūvio pernašos aržu sunaikinimas su standartiniu ultramikroelektrodu. Modifikuota sistema pasižymi ilgesne linijine priklausomybe ir žemesne antikūnų koncentracijos aptikimo riba bei didesniu stabilumu.

Bendrosios išvados
Remiantis literatūros apžvalga ir eksperimentinių duomenų rezultatais, galima padaryti šias išvadas:

1. Skenuojamoji elektrocheminio impedanso mikroskopija yra perspektyvus biologinių mėginių tyrimų metodas, nes jų metu mėginių nėra sunaikinamas ir tyrimus galima atlikti lokalai. Pirmasis konkurencinio režimo taikymas skenuojamajai elektrocheminio impedanso mikroskopijai buvo antikūnų, pažymėtų krienų peroksidaze, aptikimas ir įvertinimas. Šiuo tyrimu buvo nustatytas, kad krūvio pernašos varža yra tinkamas parametras aptikti fermento aktyvumui. Tačiau krienų peroksidazei kaip subtiratą naudojamas vandenilio peroksidas, kuris gali sukelti analitės oksidaciją ir lemėti jautrumo bei tikslumo praradimą, todėl buvo tiriami alternatyvūs žymenys, tokie kaip glukozės oksidazė ir fermentinį aktyvumą imituojančios nanodalelės.

2. Remiantis skenuojamosios elektrocheminės mikroskopijos duomenimis, esant grįžtamojo ryšio režimui glukozės oksidazės aptikimo riba yra 100 pg/mL.
Keičiant gliukožės koncentraciją, srovės pokyčių nebuvo pastebėta. Tačiau tai-
kant skenuojamąjį elektrocheminio impedanso mikroskopiją buvo galima paste-
bėti priklaušomybė nuo gliukožės koncentracijos. Vėliau nustatyta, kad gliuko-
žės oksidacijos reakcija šalia fermento gali būti tiriama esant žemam kintančios
srovės dažniui 10 Hz, kai ultramikroelektrodo atstumas yra per 2 spindulius nuo
paviršiaus. Tai leidžia taikyti skenuojamąją elektrocheminio impedanso mikros-
kopiją elektrocheminiams aktyvumui mėginyje aptikti ir vaizduoti.

3. Tiriant Ab-HRP ir GOx sistemų krūvio pernašos varža virš Ab-HRP buvo dau-
giau nei 2 GΩ, o virš GOx – daugiau nei 1.2 GΩ, todėl vietojė konurencinio
buvo taikomos grįžtamojo ryšio režimas. Režimo pakeitimas nesukėlė reikš-
mingo krūvio pernašos pokyčio sistemoje, kai atpažinimo elementas buvo
antikūnai, konjuguoti su auksno nanodalele (Ab-AuNP daugiau nei 1.2 GΩ), tai
patvirtino papildomą sistemos modifikacijos poreikį. Vienas iš galimų šios prob-
lemos sprendimo variantų buvo modifikuoti naudojamą zondą.

4. Modifikuojant grafito elektrodo, polipirolo matrica su paviršiupe esančiais
aukso, platinos nanodalemis ar redukuotu grafeno oksidu padidino elektrodo pa-
viršišių generuojamą srovę, tačiau, atlikus modifikacijas su ultramikroelektrodu,
jis buvo užblokuojamas. Perėjus prie zondo modifikavimo platinos mikrostruktūromis, užfiksotas reikšmingas registruotos srovės padidėjimas atliekant ciklinę voltametrą. Naudojant modifikuotą ultramikroelektrodu, maksimalus krūvio
perdavimo pasipriešinimas sumažėjo iki 120 MΩ. Sumažinus krūvio
pernašos varžą atliekant tyrimų rezultatai yra patikimesni ir jautresni.

5. Sukurtas elektrocheminis imuninis jutiklis, grįstas skenuojamąja elektrochemi-
nio impedanso mikroskopiją su modifikuotu ultramikroelektrodu kaip keitikliu ir
antikūnais, žymėtai akuo nanodalemis kaip atpažinimo elementu, parodė li-
nijinį diapazoną nuo 0,001 iki 10^3 ng/mL, esant aptikimui ribai 0,001 ng/mL ir
atsako laikui 20 minučių. Šie rezultatai patvirtina, kad sukurtas prototipas gali
būti taikomas diagnostikoje.

Ateities tyrimuose šioje srityje rekomenduojama imuninio jutiklio bandymai rea-
luose mėginiuose, tiriant jų stabilumą ir toliau bandant sumažinti krūvio pernašos varžą,
dekoruojant ultramikroelektrodu redukuotu grafeno oksidu be polipirolo matricos. Taip
pat turėtų būti apsvystyta jutiklio sumažinimo galimybė taikant vieno dažnio matavimus,
taip supaprastinant potensiosostato konstrukciją ir sukūriant greites, tikslius ir vartotojams
prieinamus kiekvienius elektrocheminius imuninius jutiklius.
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