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MODIFICATION OF GLASSY CARBON ELECTRODES WITH ELECTRODEPOSITED GOLD OR 2D-NANOMATERIALS: CHARACTERIZATION AND APPLICATIONS

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I. Introduction

One of the main aims of the world's scientific efforts is to improve the quality of life. Achieving this goal is directly related to the availability of methods for rapid diagnosis of common diseases, food quality control, and environmental monitoring. The scientific studies are aimed at developing analytical methods that meet these requirements. Based on their working principle, they can be optical (absorption, luminescence, chemiluminescence, and surface plasmon resonance), bulk (piezoelectric and magnetoelectric), and electrochemical.

In the past few decades, electroanalytical methods have attracted the attention of many researchers due to their experimental simplicity, relatively low cost, and low detection limits. Other important prerequisites for their high popularity are their easy digitization and the compact size of the equipment, the latter allowing in situ monitoring. For the reasons listed, they are widely used in clinical, industrial, environmental, and agricultural analysis fields.

Electrode materials play a major role in the development of susceptible electroanalytical methods for the determination of target analytes. The modification of electrode surfaces is carried out with one main goal - increasing the sensitivity of the determination, which in turn is achieved either by increasing the electrochemically active surface or by applying catalysts.

This dissertation examines the preparation of modified electrode surfaces, their characterization using a variety of electrochemical methods, and demonstrates their successful application in:

- quantitative analysis of fast-degrading active components of drugs;
- components of vegetable oils harmful to the human body.

II. PROPOUSE AND TASKS

The aim of this dissertation is to create two types of sensitive and selective electrochemical sensors for the quantitative analysis of two different groups of biologically significant compounds - on one hand, the catecholamines dopamine and L-epinephrine, by means of appropriate modification of electrodes made of catalytically inactive conductive material, which are fast-degrading components of medicinal forms, and on the other hand - of hydrogen peroxide and its organic analogues, the latter being practically insoluble in aqueous solutions.

In fulfillment of the set goal, the following research tasks have been specified:

1. Obtaining a modified glassy carbon electrode by electrochemical deposition of gold nanostructures. This will serve as a basic signal converter in the development of an enzyme electrode for the quantitative analysis of the catecholamines dopamine and adrenaline, in which:

   • The gold deposits should be obtained by two alternative procedures with a view to selecting a modified electrode with a larger electrochemically accessible surface and characterizing its surface morphology.

   • The laccase enzyme should be covalently immobilized on the gold nanoparticle-modified glassy carbon electrode, then the enzyme electrode is obtained and characterized in terms of the operating parameters of the biosensor in the quantification of the catecholamines dopamine and L-epinephrine.

2. Obtaining an electrode-catalyst based on a glassy carbon electrode, modified with 2D-nanomaterial graphite carbon nitride - pure or doped with metal oxides, by studying the electrocatalytic activity of the modified electrode in the process of electrochemical reduction of water-soluble peroxide compounds hydrogen peroxide and tert-butyl hydroperoxide, wherein:

   • The selection and optimization of the most suitable composition of the modifying phase should be conducted in order for the electrocatalyst to be created.

• The influence of the binding polymer and the acidity of the medium on the catalytic activity of the developed electrode in the electrochemical reduction of hydroperoxides should be investigated.

• The possibility of the peroxide electrode to determine the enzymatic activity of catalase in a neutral aqueous medium should be investigated.

3. The catalytic activity of the developed glassy carbon electrode, modified with 2D-nanomaterial graphite carbon nitride, doped with Co_3O_4 electrode-catalyst in a medium of polar organic solvent with respect to water-insoluble organic peroxides should be investigated, as well as the possibility of determining the peroxide content in a mixture of vegetable oils.

III. EXPERIMENTAL PART

III.1. Methods

III.1.1. Preparation of modified electrode materials

III.1.1.1. Electrochemical deposition of gold on glassy carbon

Modification of glassy carbon electrodes with gold was performed by direct electroreduction of tetrachloroaurate ions from electrolytes containing: 50 mM $HAuCl_4$ dissolved in 0.1 M hydrochloric acid or 3.5 mM $HAuCl_4$ dissolved in 0.1 M HCl. Au deposition was performed in the potentiodynamic method in two alternative ways: procedure 1 (EDP1) cycling between 0 and −0.6 V (vs. Ag|AgCl, sat. KCl) for 1 cycle at a scan rate of 0.1 V s^{-1}; and procedure 2 (EDP2) from a cycling between of -0.6 V to 0 V for 1 cycle at a scan rate of 0.1 V s^{-1}.

III.1.1.2. Preparation of modified glassy carbon electrodes with graphite carbon nitride g-C_3N_4 – pure and doped with transition metal oxides

The preparation of the modifier was carried out as follows: the required amount of the catalyst (Co-Mg-g-C_3N_4, Co-Bi-g-C_3N_4, Co-g-C_3N_4, Co_3O_4 or g-C_3N_4) was weighed into a suitable container and added to added the required volume of the polymer suspension (Nafion 117) with the selected concentration. After obtaining the dispersion, the modifying phase was applied to the cleaned glassy carbon surfaces by dropwise aliquots of 5 µl and dried at room temperature for 16 h.

In comparison, an alternative methodology of modifying the glassy carbon electrodes was carried out as follows: The Co-g-C_3N_4 catalyst evaluated as the most active was dispersed in an aqueous medium containing 5% ethylene glycol by sonication for 30 min (twice). The deposition was carried out as described above, and after drying at room temperature for 24 h, a Nafion polymer coating with a concentration of 0.2% or 5 mM aqueous glutaraldehyde was drop-coated on the modified electrode surface and then air-dried for 24 h.

III.1.1.3. Immobilization of laccase enzyme on gold (Au) modified glassy carbon electrodes

Prior to the enzyme immobilization gold-modified GC electrodes were cleaned electrochemically in 0.5 M H_2SO_4 by cyclic voltammetry (CV, scan rate 0.1 V s^{-1}) over the potential range from −0.2 to 1.5 V (vs. Ag|AgCl, sat. KCl) for at least 20 cycles, then thoroughly rinsed with ultrapure water.

Laccase was immobilized as described previously[1], through crosslinking of enzyme to cystamine moieties self-assembled on gold-coated glassy carbon using a bi-functional reagent, glutaraldehyde. The self-assembly of cystamine was carried out under static conditions by immersing the electrodes in its 10 mg mL^{-1} aqueous solutions. The duration of the sorption process was 2 h. After completing the chemisorption, the loosely bound alkanethiol was removed

[1]N. Dimcheva and E. Horozova, "Improved operational stability of a laccase-based electrode applicable in biofuel cells," 2018.

from the surface by rinsing with ultrapure water. Then, a 3 µL drop of laccase solution (16 mg mL^{-1}) was cast on the electrode surface and 1 µL of glutaraldehyde (45 mM aqueous solution) was mixed with it and allowed to react for at least 30 min at ambient temperature, then another 3 µL drop of laccase solution was cast to which a portion of 1 µL glutaraldehyde was added and allowed to react for another 30 min at ambient temperature. The prepared enzyme electrodes were stored in 0.05 M sodium citrate buffer, pH 4.0, in a refrigerator at 4 °C until use.

III.1.1.4. Immobilization of catalase enzyme on glass pads

For this purpose, glass slides for microscopic preparations are used, which before enzyme immobilization are cleaned by washing with ethanol, washed with ultrapure water and subjected to ultrasonic treatment for 3 minutes, after which they are air-dried. A freshly prepared solution of catalase with a concentration of 5 mg.ml^{-1} in a phosphate buffer with pH 7.0 was applied dropwise to the prepared substrates and left in a refrigerator at 4◦C for 2 hours until the volume of the liquid decreased threefold. Then, 0.5 mM glutaraldehyde was added to the catalase-treated support, keeping a ratio of 2:1 with the enzyme solution (for every 10 µl of enzyme solution, a portion of 5 µl of the crosslinking agent was added) [2]. The resulting reaction mixture was allowed to react in a refrigerator at 4◦C for 24 hours.

III.1.2. Characterization of the modified glassy carbon electrodes

All electrochemical experiments were performed in a conventional three-electrode cell with working volume of 20 mL or 10 mL, connected to a computer-controlled electrochemical workstation, Autolab PGSTAT 302 N (Metrohm-Autolab, Utrecht, The Netherlands) equipped with NOVA 2.1.5 software. Unmodified or modified glassy carbon electrode was used as working, Ag|AgCl, sat. KCl or Ag|Ag+ (Metrohm, Utrecht, The Netherlands)—as a reference—and a platinum foil as an auxiliary electrode. When necessary, the buffers were purged with either chemically pure argon (99.99%) or air during measurements. The modified electrodes were characterized using different electrochemical methods such as: cyclic voltammetry, differential pulse voltammetry, chronoamperometry, electrochemical impedance spectroscopy, etc. The surface morphology of the deposits on the glassy carbons was investigated using scanning electron microscopy.

III.3. Determination of the electrochemically active surface of the modified electrodes

The electrochemically accessible surface area of the electrodes (unmodified or modified) was determined using cyclic voltammetry, at low scan rates (2–10 mV.s^{-1}). For this purpose, the electrochemical cell in a three-electrode configuration is filled with an electrolyte of 0.1 M KCl, in which $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ are dissolved, taken in an equimolecular ratio (usually 5 mM solutions). On the cyclic voltammetry curves recorded under the given conditions, two peaks appear - one of oxidation and one of reduction. To calculate the electrochemically active surface, the height of the reduction peak (current strength, A) is determined, taking into account the baseline from which the height is determined.

The obtained peak current value is substituted into the Randles- Sevcik equation, from which the electrochemically accessible electrode surface is calculated:

$$i_p = 0.446.\frac{(nF)^{3/2}}{(R.T)^{1/2}}.A.C.D^{1/2}.v^{1/2}, \qquad \textit{(Equation 1)}$$

where i_p – peak current, A; A – surface of the working electrode, cm^2; C – concentration of $K_3[Fe(CN)_6]$ or $K_4[Fe(CN)_6]$, or $[Ru(NH_3)_6]Cl_3$, (redox probe), mol.dm^{-3} ; D – diffusion coefficient, cm^2 .s^{-1}, which for

[2]NB Milosavić, RM Prodanović, D. Velićković, and A. Dimitrijević, Enzyme Stabilization and Immobilization, vol. 1504. New York, NY: Springer New York, 2017.

$K_3[Fe(CN)_6]$ has a value of 7.6.10 $^{-6}$, for $[Ru(NH_3)_6]Cl_3$ is 8.43.10 $^{-6}$ cm^2 .s^{-1} ; n – number of electrons; R – universal gas constant (8.314 J.mol^{-1}. K^{-1}) ; T – the absolute temperature, K; F – Faraday's constant, (96485 C.mol^{-1}) ; v– scan rate, V.s^{-1}.

Electrochemically, the active surface of the gold-deposited glassy carbon electrodes was also determined by an alternative methodology based on the properties of gold in highly acidic media to form a surface oxide layer at potentials higher than 1.2 V, as well as to desorb oxygen from its surface with subsequent electrode polarization in the range 0.95 – 0.85 V [3]. A series of at least 20 consecutive cyclic voltammetry curves in 0.5 M H_2SO_4 were recorded, and the area of the surface-bound oxygen desorption peak was determined for the last recorded curve. The electrochemically active surface *A* of the electrodes is determined by the formula:

$$A = \frac{S_p}{vQ_{O_2}} \qquad \textit{(Equation 2),}$$

where: S_p is the peak area [μA.V] determined by software; v is the scan rate [Vs^{-1}]; Q_{O_2} = 400 μC.cm^{-2} is the amount of electricity required to desorb 1 mol of oxygen from the gold surface.

IV. RESULTS AND DISCUSSION

IV.1. Electrodeposited Gold Modified Glassy Carbon Electrode - Preparation, Characterization and Applications

This section illustrates the advantages and opportunities afforded by modifying a glassy carbon material with electrochemically deposited gold to increase the electrochemically active surface area. Our goal in developing the gold-modified glassy carbon electrode is to obtain a signal transducer with a well-developed surface, which can subsequently be functionalized with a molecular recognition enzyme, and the combination of the two elements results in an electrochemical biosensor for quantification of biologically important compounds.

IV.1.1. Preparation and characterization of glassy carbon electrodes modified with electrochemically deposited gold

IV.1.1.1. Preparation of glassy carbon electrodes with electrodeposited gold

Our preliminary studies have shown that the electrochemical reduction of gold on glassy carbon proceeds at an appreciable rate at potentials of -0.3 V and more negative. In this connection, two alternative gold deposition procedures were tested, the aim of which is to obtain a modified electrode with a highly developed electrochemically accessible surface. In the first deposition procedure (Fig. 1A, curve 1), the glassy carbon electrode is polarized at a starting potential of 0 V, where no oxidation or reduction processes occur. Gradually, the polarization potential shifted at a rate of 0.1 V s^{-1} in the negative direction to -0.6 V, then again returned to the initial potential of 0 V. The second gold electrochemical deposition procedure was carried out in the same range of potentials and with the same rate of change of the polarization potential of the working glassy carbon electrode, with the difference that the initial potential is -0.6 V, at which the reduction of tetrachloroaurate ions proceeds at a significant rate (Fig. 1A, curve 2). The type of cyclic voltammetry curve recorded under these conditions shows that with a shift of the polarization potential in the positive direction, the strength of the reduction current gradually

[3]Álvarez-Martos and EE Ferapontova, "Electrochemical Label-Free Aptasensor for Specific Analysis of Dopamine in Serum in the Presence of Structurally Related Neurotransmitters," Anal. Chem., vol. 88, no. 7, pp. 3608–3616, 2016, doi: 10.1021/acs.analchem.5b04207.

decreases and reaches a practically constant value in the range of potentials of the polarizer from –0.4 V to 0 V. At in the reverse cycle, the presence of Faraday currents is again not observed in the range of potentials from 0 V to – 0.35 V, but the reduction currents noticeably increase when the polarization potential is shifted in the negative direction and reach their highest value at – 0.6 V. The voltammetric curve has a pronounced hysteresis character and on it, you can observe areas in which the reduction of tetrachloroaurate ions proceeds at a high rate.

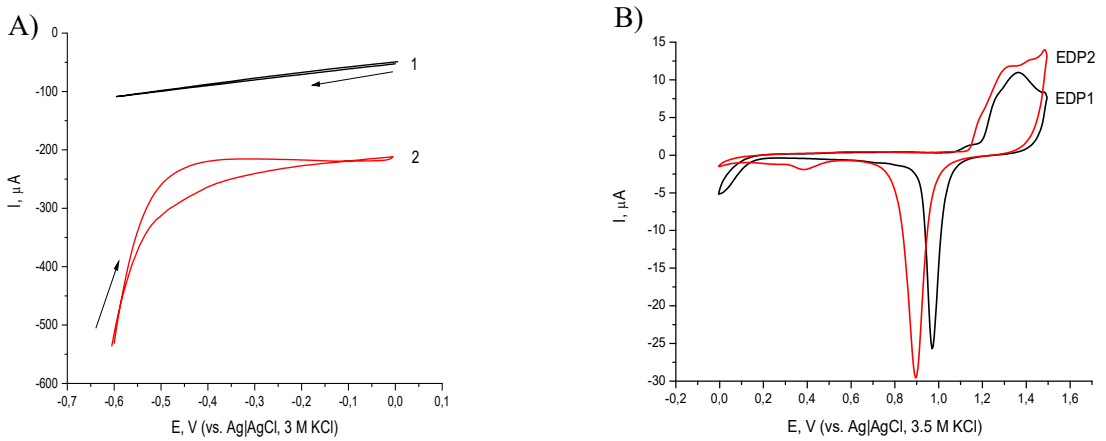


Figure 1. *Cyclic voltammetric curves recorded on: A) glassy carbon electrode in a solution of 0.1 M HCl containing 50 mM $HAuCl_4$ (pH = 1) in the range of potentials from -0.6 to 0 V: starting potential 0 V (1); starting potential -0.6 V (2) and B) glassy carbon electrodes modified according to procedure 1 (EDP1, red) and according to procedure 2 (EDP2, black) in 0.5M H_2SO_4, reference electrode Ag|AgCl (sat. KCl); scan rate 100 $mV.s^{-1}$*

Immediately after their preparation, the gold-modified glassy carbon electrodes were subjected to electrochemical cleaning in 0.5 M H_2SO_4 solution**.** In addition to the desorption of impurities from the surface of the modified electrodes, during the anodic cycle of the cyclic voltammogram curve, a surface oxide layer is formed on the gold deposits, which is desorbed during the cathodic cycle with the formation of a deep peak with a maximum potential of around 0.9 – 1.0 V (vs. Ag|AgCl, sat. KCl) (Fig. 1B). The voltammetric curves of glass carbon modified according to the second procedure are distinguished by a deeper and wider desorption peak compared to the one obtained according to EDP1, which testifies to the presence of a more developed electrochemically active surface of the electrode materials. These dependencies are observed for both investigated concentrations of the tetrachloroaurate ions used in modifying the electrodes. The area of the desorption peaks obtained with the electrode materials modified with electrodeposited gold by the two alternative procedures using 3.5 mM $HAuCl_4$ solution was found to be between 3.8 and 4.1 times smaller than the area of the desorption peaks obtained with the modification of the glassy carbon electrodes with a 50 mM $HAuCl_4$ solution. These data unequivocally show that the electrochemically accessible surface of the gold deposits is several times larger when the more concentrated solution of tetrachloroaurate ions is used for modification, regardless of which of the two electrodeposition procedures is used.

The determined electrochemically accessible surface area for the gold-modified electrode materials obtained by the second procedure is 0.312 ± 0.009 cm^2, which is nearly 51% larger

than that obtained by the first procedure (0.207 ± 0.018 $cm^{2)}$ and ~5 times larger than the geometric surface of a gold electrode of the same diameter (0.071 cm^2).

IV.1.1.2. Characterization of the gold-modified electrodes by electrochemical impedance spectroscopy (EIS) and scanning electron microscopy

In Fig. 2A shows the dependences of the imaginary component of the impedance on the real one for an unmodified glassy carbon electrode (curve 1) and the same electrode coated with gold electrodeposited according to the second procedure (curve 2). The Nyquist plot for the pure glassy carbon electrode consists of a semicircle, characteristic of the occurrence of charge transfer resistance, followed by a linear region with a slope of 45 ^{0}, characteristic of diffusion-limited processes occurring in the medium- and low-frequency range. The impedance spectrum of the gold-modified glassy carbon electrode has an arc-shaped course in the high-frequency region, and in the mid-and low-frequency region, it passes into an almost linear dependence, concluding an angle between the X-axis and the linear part of the spectrum greater than 45 degrees. Simulations performed based on the experimental data resulted in the equivalent circuit (Randles circuit, Figure 2B) containing a constant phase element (CPE) connected in parallel to the charge transfer resistance (Rp). The calculated value for Rp (11.9 k Ω) is much smaller than that for the clean electrode (315 k Ω), which means that electron transfer to and from the gold-modified surface is greatly facilitated compared to the unmodified glassy carbon. In the specialized literature, CPE is often presented as a typical feature of imperfect capacitors (e.g.,

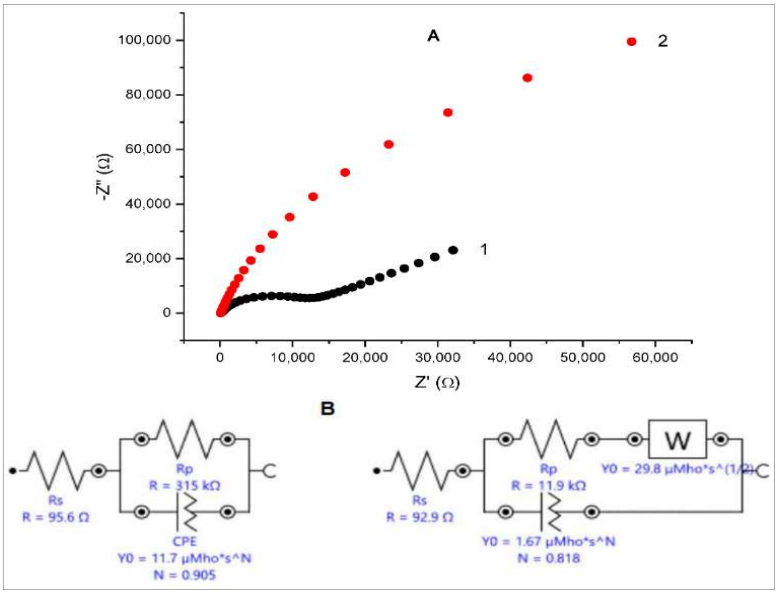


Figure 2. *Nyquist plot—dependence of the imaginary vs. the real part of the impedance for bare glassy carbon electrode (black squares) and the same electrode after electrodeposition of nanoporous gold (red circles); reference electrode: Ag|AgCl, sat. KCl; electrolyte: 0.1 M KCl containing 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ as redox probe; frequency range from 100 kHz to 1 Hz. (**B**) Equivalent Randles circuits for the bare (left) and gold-modified (right) glassy carbon electrode.*

the electric double layer) and implies significant surface roughness due to increased porosity or the presence of irregularities on the electrode surfaces [4].

The electron microscopic image of the gold deposits obtained by the first procedure shows that they are unevenly distributed and in some places form clusters with sizes exceeding 100 nm (Fig. 3A). The morphology of the gold structures deposited by the second procedure (Fig. 3B) is characterized by a dense population of the surface with volumetric formations resembling rosettes with diameters ranging from several tens to hundreds of nanometers. It is evident from the microscopic image that the surface coating resembles some porous film rich in micro- and mesoporous formations. Studies with scanning electron microscopy confirm the hypothesis stated above about the presence of unevenly distributed formations due to the deposition of gold. These observations explain the significant differences obtained in the electrochemically determined contact surface of the modified electrode materials.

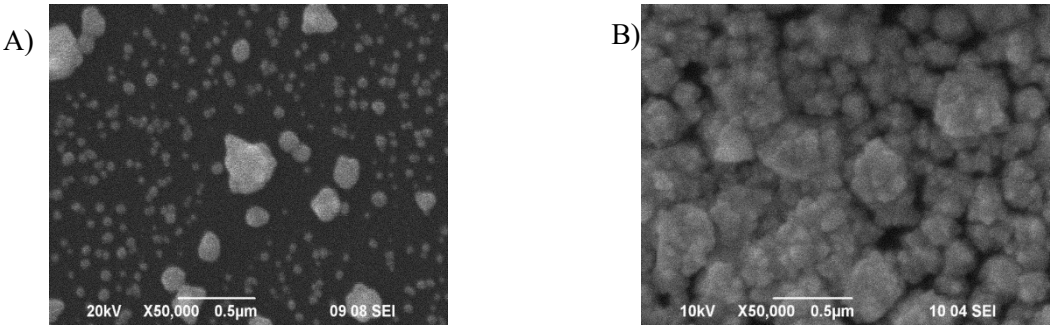


Figure 3. *Electron microscopic image of the surface morphology of glassy carbons modified with 50 mM $HAuCl_4$; EDP1 – A and EDP2 -B*

Based on the obtained results for the electrochemically accessible surface of the modified electrodes and the type of their surface morphology, all subsequent studies were performed with electrodes modified with gold deposited by the second procedure.

IV.1.2. Preparation and electrochemical characterization of a laccase enzyme electrode

Laccase immobilization on electrode surfaces is of key importance when aiming at a lasting operational stability of resulting bioelectrodes. To immobilize laccase on the Au electrodeposits, a positively charged at the working pH, self-assembled monolayer, SAM, of 2-aminoethane thiol was created (resulting from the chemisorption of cystamine on gold. This electrode preparation is further denoted in the text as cystamine-modified electrode). Laccase was further confined on the electrode surface by binding it to the thiol layer using the bi-functional agent, glutaric aldehyde.

The electrochemical behavior of the immobilized enzyme was then studied by means of cyclic voltammetry (CV). Comparison of the CVs of the gold-modified electrode with immobilized laccase recorded in the absence and the presence of dioxygen (Fig. 4) reveals laccase-catalyzed electrochemical reduction of dissolved oxygen. On the CV recorded with a low scan rate in deaerated buffer (Fig. 4, solid line) two broad oxidative peaks appear: at ca. 0.2 V and ca. 0.4 V, whereas on the reverse scan the corresponding reductive peaks were less visible and appeared at potentials of ca. 0.1 V and 0.350 V, respectively. The two pairs of redox peaks are not present on the CV recorded in aerated buffer solution (Fig. 4, dash line), however, a clearly expressed

[4] Song *et al.* , "Enhanced Electrochemical Impedance Spectroscopy Analysis of Microbial Biofilms on an Electrochemically in Situ Generated Graphene Interface," *ACS Sensors* , vol. 5, no. 6, pp. 1795–1803, 2020, doi: 10.1021/acssensors.0c00570 .

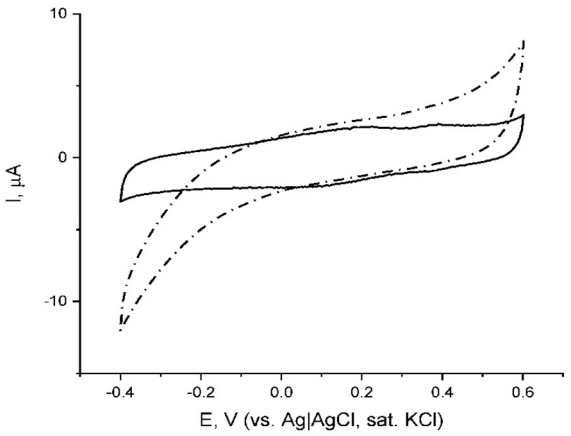


Figure 4. *Cyclic voltammograms (second scan) of laccase immobilized on chemisorbed monolayer of cystamine in background electrolyte in aerated (dash) and de-aerated with Ar medium (solid), 0.05 M citrate buffer containing 0.1 M $NaClO_4$, pH = 4.0; scan rate, v = 5 mV/s, reference electrode Ag|AgCl, sat. KCl.*

reductive wave starts at ca. 0.1 V, which is due to the electrochemical reduction of dissolved oxygen catalyzed by the immobilized enzyme. As already mentioned, laccase is one of the few oxidoreductases capable of exchanging electrons with underlying electrode surfaces without the need for additional electron shuttles (mediators), with the efficiency of the electrical communication controlled by both enzyme orientation and the distance between its active site and the electrode surface. It is hypothesized that the positively charged electrode surface electrostatically attracted the negatively charged laccase active site this way orienting the enzyme in a conformation favorable for electron exchange with the underlying electrode surface, and the voltammetric studies revealed its ability for working in DET mode (direct electron exchange between the enzyme active site and the electrode).

Differential pulse voltammogram (DPV) of the same laccase electrode is depicted in Figure 5 (Fig. 5A, curve 1). For comparison, an identically prepared laccase electrode with a SAM of cysteine to which laccase is similarly attached (Fig. 5A, curve 2) was also subjected to differential pulse voltammetry. The DPV of laccase immobilized over a cystamine-modified surface (Fig. 5A, curve 1) shows a peak at a potential of 0.18 ± 0.02 V that can be assigned to the redox transformation of the T1 copper site, responsible for binding benzenediols, as these observations are in conformity with the literature data [5] [6]. In support of this hypothesis is the finding that the DPV of the electrode does not change in the absence of oxygen (deaerated buffer). For the laccase immobilized on the cysteine monolayer (Fig. 5A, curve 2), the peak flattens and turns into a shoulder, suggesting a much less efficient electron exchange between the immobilized enzyme active site and the underlying electrode surface. The comparison of the DPVs implies the surface charges affect the orientation at which the laccase enzyme lies on the electrode upon its immobilization, and most probably in the case of laccase attached to 2-aminoethane thiol SAM its orientation is favorable for electron exchange with the underlying electrode surface, whereas when attached to a cysteine monolayer the enzyme might experience some steric hindrances when exchanging electrons with the electrode surface, as represented schematically in Figure 5B. The values of the open circuit potentials for the two electrode preparations were found to be similar and around 0.3 V (vs. Ag|AgCl, sat. KCl) at the operating temperature and pH.

[5] DM Ivnitski, C. Khripin, HR Luckarift, GR Johnson, and P. Atanassov, "Surface characterization and direct bioelectrocatalysis of multicopper oxidases," *Electrochim. Acta* , vol. 55, no. 24, pp. 7385–7393, 2010, doi: 10.1016/j.electacta.2010.07.026 .
[6] G. Gupta, V. Rajendran, and P. Atanassov, "Bioelectrocatalysis of oxygen reduction reaction by laccase on gold electrodes," *Electroanalysis*, vol. 16, no. 13–14, pp. 1182–1185, 2004, doi: 10.1002/elan.200403010.

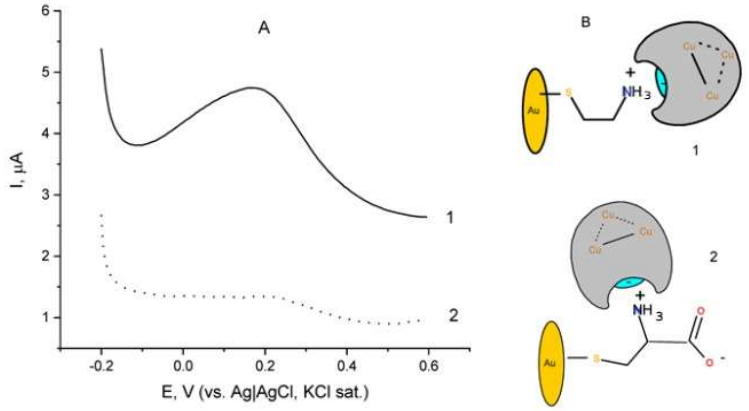


Figure 5. *Differential pulse voltammograms of laccase immobilized on chemisorbed monolayer of (1) cystamine and (2) cysteine; background electrolyte: 0.05 M citrate buffer, pH 4 containing 0.1 M $NaClO_4$; (**B**) Schematic of laccase orientation upon immobilization over a self-assembled monolayer of (1) cystamine and (2) cysteine residues.*

The appearance of a clearly expressed peak at the DPVs of laccase immobilized on the cystamine-functionalized gold layer, motivated us to further explore the voltammetric behavior of laccase in the presence of two structurally similar catecholamines—dopamine and L-epinephrine. The addition of dopamine aliquots to the buffer followed by the record of the resulting DPV (Fig. 6A) caused a notable increase in the peak height even for dopamine concentrations as low as 2 μM. Upon the increase of dopamine concentration, the peak sharpened and shifted to the negative direction. A linear dependence between the peak height and dopamine concentration was observed over the range from 2 up to 40 μM (Fig. 6A, Inset), which deviates from linearity at higher concentrations. Under the same conditions, the addition of L-epinephrine (Fig. 6B) did not substantially affect the shape or position of voltammetric maxima and the relationship between the peak height and analyte concentration was not consistent.

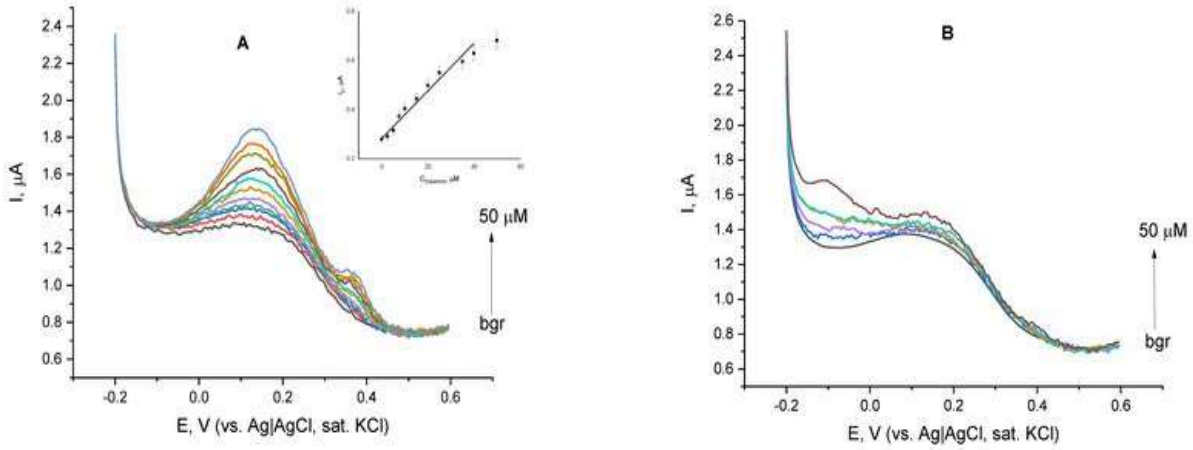


Figure 6. *Differential pulse voltammograms of laccase immobilized on cystamine-modified gold in the presence of dopamine (A**)** and L-epinephrine (B**)** with concentrations ranging from 0 to 50 μM; background electrolyte - constantly aerated 0.05 M citrate buffer containing 0.1 M $NaClO_4$ pH = 4.0. In (A**)** dependence of peak height on dopamine concentration.*

Further studies of the laccase electrode by cyclic voltammetry (CV), recorded in the presence of each catecholamine (Figure 7) showed additional dissimilarities between the two catecholamines. The presence of both laccase substrates, dioxygen and either catecholamine, caused a reductive current to flow at potentials less positive than 0.4 V (i.e., at much lower overpotentials than the oxygen reduction catalysed by only laccase), thus indicating the ability of these substances to facilitate the electron transfer between the immobilized enzyme and the electrode surface—a phenomenon known as mediated bioelectrocatalysis [7]. Schematically, the mechanism of catecholamine-mediated bioelectrochemical oxygen reduction can be presented as follows:

$$E_{red} + O_2 + 4e^- + 4H^+ \rightarrow E_{ox} + 2H_2O$$
$$E_{ox} + M_{red} \rightarrow E_{red} + M_{ox}$$
$$M_{ox} + e^- \xrightarrow{electrode} M_{red},$$

where E_{red} and E_{ox} stands for the reduced/oxidized form of enzyme and M_{red} and M_{ox} the reduced and oxidized forms of catecholamine. The proposed mechanism was confirmed by comparative studies—in the absence of oxygen, no electroreduction of either catecholamine was observed.

The efficiency of the mediated electron transfer (MET process) was found to depend on substrate structure. Immobilized laccase is capable of reducing oxygen in the presence of dopamine at potentials more negative than 0.4 V rather efficiently (Fig. 7—red, solid) and reaches a limiting current over the region from 0.3 to 0 V (backward scan) followed by a second reduction wave overlaping the one of enzymatic oxygen reduction in DET mode, however, with higher current intensity. In the presence of L-epinephrine, the efficiency of the MET process over the range 0.4–0 V is far lower (Fig. 7—red, dashed line) with the starting potential for the mediated oxygen reduction below 0.3 V (i.e., at ca. 0.1 V less positive potential than with dopamine), and reaching a plateau region below 0 V.

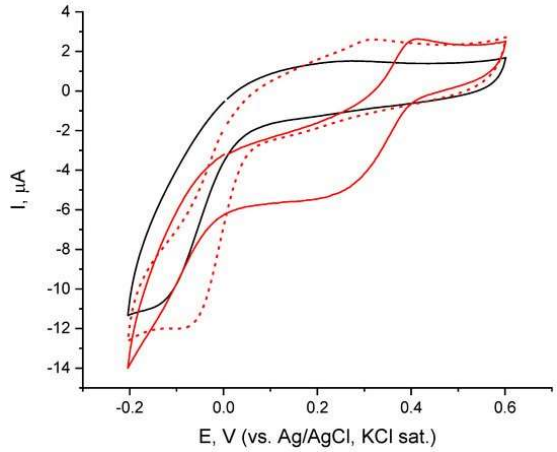


Figure 7. Cyclic voltammograms (second scan) of laccase electrode immobilized on chemisorbed monolayer of cystamine in background electrolyte (black, solid) and in dopamine (red, solid) and L-epinephrine (red, dash) present; concentration of catecholamines, C = 49.8 µM.

[7] K. Kano and T. Ikeda, "Fundamentals and Practices of Mediated Bioelectrocatalysis," *Anal. Sci.* , vol. 16, no. 10, pp. 1013–1021, Oct. 2000, doi: 10.2116/analsci.16.1013.

IV.1.4. Quantification of dopamine and L-epinephrine with a laccase biosensor

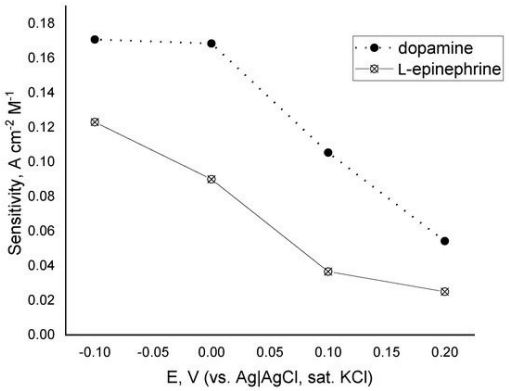


Figure 8. *Sensitivity of the determination of dopamine (closed circles) and L-epinephrine (open circles) vs. operating potential over the potential region from −0.1 to 0.2 V (vs. Ag|AgCl, sat. KCl); electrolyte 0.05 M citrate buffer with 0.1 M $NaClO_4$, pH = 4.0; 20 ± 1 °C.*

Based on these findings a detailed study on the oxygen reduction mediated by either dopamine or L-epinephrine by means of constant-potential amperometry was performed over the potential region starting from 0.2 down to −0.1 V (vs. Ag|AgCl, sat. KCl). The potential region was selected so that its start point was the potential at which the DPV peak appears, and the end point is the potential at which the reductive current in the presence of L-epinephrine reaches a plateau. As the applied potential shifted to the negative direction, the electrode response to catecholamines increased, reaching the highest electrode sensitivities at potential of 0 V for dopamine and −0.1 V for L-epinephrine. At an applied potential of 0.2 V the biosensor response to L-epinephrine was found to be about twice as low as the one for dopamine, whereas at −0.1 V its sensitivity was 76% of that for dopamine. Such a steep increase in the sensitivity might be a cumulative result from two parallel electrode reactions—mediated oxygen reduction and electroreduction of enzymatically generated L-epinephrine semi-quinone. Electrode sensitivities as a function of the applied potential are presented in Figure 8. It is obvious that the biosensor sensitivity towards dopamine is practically the same at applied potentials of 0 V and −0.1 V and then goes down with increasing operating potential. The same parameter for L-epinephrine gradually decreases with increasing the operating potential over the studied range. As it can be concluded from the discussed findings, the laccase can react with both catecholamines, but possesses different affinity towards them, and therefore their simultaneous determination is not plausible with a single biosensor. However, by using a differential approach, e.g., two identical biosensors poised at different potentials, seems to provide a realistic prospective for discriminating between them.

IV.1.5. Determination of kinetic constants of the immobilized enzyme and operating parameters of the laccase biosensor

The electrochemical Michaelis–Menten plots (Fig. 9) were drawn on the basis of chronoamperometric records obtained upon additions of catecholamine solutions at a constant potential of –0.1 V (vs. Ag|AgCl, sat. KCl), as the highest electrode sensitivity was achieved at this operating potential. The hyperbolic shape of both dependencies of the current density on substrate concentration suggests that the process is controlled by Michaelis-type enzyme kinetics. For both enzyme substrates, initially the current density raises linearly with catecholamine concentrations up to ca. 120 μM for dopamine and up to ca. 200 μM for L-epinephrine with constant sensitivities of 0.18 μA L mol^{-1}cm^{-2} (dopamine) and 0.12 μA L mol^{-1}cm^{-2} (L-epinephrine). This initial linear trend corresponds to the diffusion-controlled region typical for the low substrate concentrations. At higher catecholamine concentrations, the

dependence deviates from linearity (i.e., the reaction is controlled by the very enzyme kinetics) and the enzyme reaches saturation at dopamine concentrations exceeding 300 μM, whereas for L-epinephrine the saturation concentration was found to be a bit higher (above 350 μM).

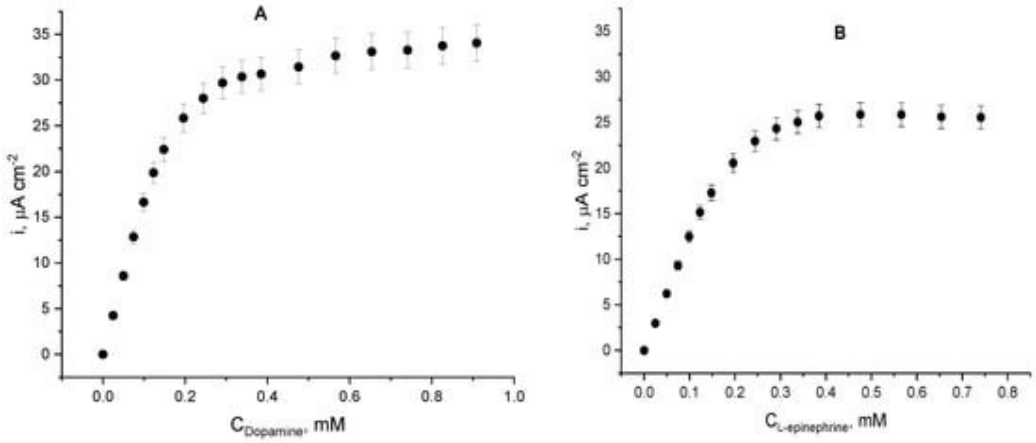


Figure 9. *Dependence of the current density on catecholamine concentrations (electrochemical Michaelis–Menten plot); working electrodes: laccase immobilized on cystamine monolayer in the presence of dopamine (**A**) and L-epinephrine (**B**); citrate buffer, pH = 4.0; working potential −0.1 V (vs. Ag|AgCl, sat. KCl); 20 ± 1 °C.*

The calculated apparent kinetic constants, maximum velocity (V_{max}^{app}) and Michaelis-Menten constant (K_M^{app}) differed for the two catecholamines. The value of K_M^{app} for dopamine (0.116 ± 0.015 mM) is close to that determined spectrophotometrically for the free enzyme in solution with the same substrate, K_M^{app} = 0.0918 mM [8], and the plausible reason for the observed finding is that the immobilized enzyme possesses enough conformational mobility, but the diffusion of the substrate through the enzyme layer most probably enlarges it.

The apparent Michaelis constant for L-epinephrine (0.245 ± 0.031 mM) determined under the same conditions was found to be more than twice as large, indicating a weaker interaction between enzyme and substrate. This is also confirmed by the maximum velocity, which for dopamine is greater (4.08 ± 0.16 × 10^{-5} A cm^{-2}) compared to that obtained for adrenaline (3.36 ± 0.17 × 10^{-5} A cm^{-2}). At concentrations exceeding 400 mM, a slight decrease in current density was observed, which could be explained by enzymatic inhibition by L-epinephrine.

Operational parameters of the laccase biosensor when either dopamine or L-epinephrine were used as analytes are presented in in Table 1. Data analysis suggests greater laccase affinity towards dopamine, which is manifested not only by the kinetic constants, but also by the 1.45-times higher biosensor sensitivity and 1.30-fold larger maximal current density than the for L-epinephrine. The linear dynamic ranges for both analytes span over 0.1 mM, which makes the biosensor a convenient analytical tool for monitoring catecholamines' concentrations in pharmaceutical products. The limits of detection were calculated as three times the dispersion (σ) of the blank response divided by the slope of the calibration graph (linear part): LOD = 3 σ/slope. The limits of quantification for both analytes were calculated as LOQ = 10 σ/slope, where σ has

[8] M. Deng *et al.* , "High catalytic activity of immobilized laccase on core–shell magnetic nanoparticles by dopamine self-polymerization," *J. Mol. Catal. B Enzym.* , vol. 112, pp. 15–24, 2015, doi: 10.1016/j.molcatb.2014.11.012.

the same meaning. Both values lay in the sub-micromolar range, which makes the biosensor potentially applicable in biological liquids, as the dopamine physiological levels (10 ng mL^{-1}) fall within this range.

Table 1. Operating parameters of the laccase electrode for dopamine and L-epinephrine present as substrates; working potential −0.1 V (vs. Ag|AgCl, sat. KCl); temperature: 20 ± 1°C.

Operating parameters	***Dopamine***	***L-epinephrine***
Maximum current density i_{max} (Acm^{-2})	$(33.76 \pm 1.2) \times 10^{-6}$	$(25.80 \pm 1.3) \times 10^{-6}$
sensitivity, AL mol^{-1} cm^{-2}	0.178 ± 0.005	0.123 ± 0.002
Linear dynamic range, mM	0.12	0.19
Limit of detection, LOD, M	3.74×10^{-8}	5.41×10^{-8}
Limit of quantification, LOQ, M	1.25×10^{-7}	1.80×10^{7}

Validation of the laccase biosensor was implemented by analyzing pharmaceutical products with known concentrations (Table 2). The determined mean values of 41.2 ± 1.5 mg mL^{-1} for dopamine (40 mg mL^{-1}) and 0.948 mg mL^{-1} for L-epinephrine (1 mg mL^{-1}) support the biosensor applicability for the determination of these two analytes in pharmaceutical products.

Table 2. Biosensor validation with real samples—injection solutions with known concentration of dopamine (40 mg mL^{-1}) and L-epinephrine (1 mg mL^{-1}).

Analyte	***Spiked Volume, µL***	***Concentration Determined, mg mL^{-1}***	***Recovery, %***
Dopamine	10 20 30	39.6 42.3 41.5	99 106104
L-epinephrine	20 40 60	0.89 1.046 0.909	89 105 91

IV.1.6. Interference Studies and Stability

Having in mind that gold nanoparticles are an excellent catalyst for ascorbate electrochemical oxidation at near zero potential, the presence of L-ascorbic acid in the assayed sample might potentially interfere with the electrode response to either catecholamine. Hence, the laccase electrode response to L-ascorbate was examined under the same experimental

conditions at the operating potentials of 0 V and −0.1 V. It was found that at 0 V, the addition of L-ascorbate causes a reductive current to flow at an increased noise level. If present at equal concentrations with L-ascorbic acid the biosensor response to L-epinephrine will add 1.1%, whereas its response to dopamine will be increased by 0.8%, provided that the concentrations were chosen over the linear region of the calibration plots (i.e., up to 0.12 mM). If operating at −0.1 V, the presence of L-ascorbate will affect the biosensor response to dopamine by 1.4%, whereas the response to L-epinephrine will be overestimated by 2.2%. It has to be pointed out that these operating potentials ensure practically interference-free catecholamine determination, since only the presence of L-ascorbate in the sample can potentially interfere with the electrode response to either catecholamine. Calibration of the laccase electrodes towards either catecholamine was performed under isothermal conditions, at 20 ± 1 °C. The current density of the laccase biosensor in the presence of either catecholamine was found to be highly sensitive to temperature changes: an increase in the temperature by 6–7 deg. caused a drastic decrease in the electrode response. A plausible explanation is the fast auto-oxidation of catecholamines under ambient conditions and thus the substrate solutions were prepared immediately before measurement and kept in an ice bath during chronoamperometric measurements. Some contribution to the decreased reaction rate from the decreased oxygen solubility in buffers as temperature increases is also probable.

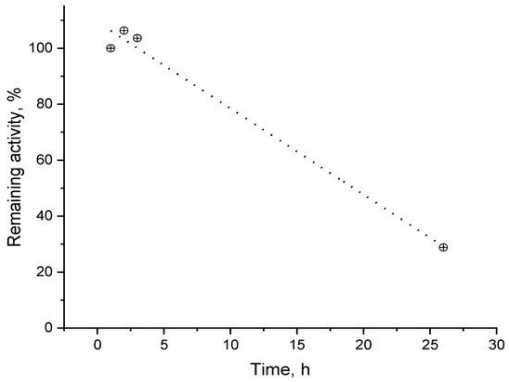


Figure 10. *Remaining catalytic activity as a function of the time passed since biosensor fabrication. The data were normalized to the first measurement at E = −0.1 V in citrate buffer, pH = 4.0: substrate—dopamine*

Stability of fabricated biosensor was tested over a 26-hour period (Fig. 10) using dopamine as the analyte. It was found that the activity of the freshly prepared laccase biosensor remains intact within the next 3 h (consecutive measurements). The maximum current density was accepted as an activity indicator (biosensor response at saturating analyte concentration divided by electrochemically accessible electrode surface). After a 21 h pause, when the biosensor was kept refrigerated in humid atmosphere, the maximum current density decayed to 1/3 of the initial one. It has to be pointed out that a slight increase of the biosensor response to dopamine can be observed after the first use that is most probably due to the facilitated analyte access to the electrode surface as a result of the swelling of the protein-containing layer.

It should be noted that in our preliminary experiments with laccase immobilized in a layer of the Nafion polymer on an identically prepared modified glassy carbon, the sensitivity of the biosensor decreased by more than 10% at each subsequent measurement within 4 h. Therefore, the chosen immobilization method, although labor-intensive, provides much higher operational stability of the laccase biosensor compared to other methods, such as immobilizing the enzyme by incorporating it into a polymer film.

IV.2. Glassy carbon electrode modified with two-dimensional nanomaterials based on graphite carbon nitride (g-C_3N_4)

IV.2.1. Electrocatalytic Activity of Pristine and Metal Oxide—Doped g-C_3N_4

Having in mind their heterogeneous-catalytic activity in peroxide bond cleavage, the metal oxide doped g-C_3N_4 composites, as well as the pristine g-C_3N_4 and spinel Co_3O_4, have been examined as electrocatalysts in the process of electrochemical reduction of two water-soluble hydroperoxides: hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide (t-BHP). For this purpose, a series of glassy carbon electrodes modified with each of the catalysts dispersed (by sonication) in Nafion aqueous suspension have been examined in the peroxide electroreduction process (Fig. 11). The electrocatalytic activity has been measured under mild conditions (neutral pH, room temperature) as the electrode sensitivity, determined at a constant potential, where peroxide electroreduction takes place. Surprisingly, the electrochemical activity of these catalysts did not follow the same rationalities as in the heterogeneous catalysis. In the electroreduction process of either hydroperoxide, the highest electrocatalytic activity has been observed for Co_3O_4-doped g-C_3N_4 (Co-g-C_3N_4). It has to be mentioned that its activity is much higher than the one of pristine g-C_3N_4 or Co_3O_4, which suggests that there is a synergistic effect upon combining the two components of the composite catalyst. Not surprisingly, the most active composite (Co-Mg-g-C_3N_4) in the heterogeneous peroxide bond disruption which possesses the highest specific surface area shows very low activity in hydrogen peroxide electrochemical reduction (Fig. 11, dark blue series) and a lack of such in the electroreduction of t-BHP (Fig.11, blue series). Upon its addition to concentrated (e.g., 6%) hydrogen peroxide, aqueous solution causes vigorous release of oxygen gas. This finding can explain its apparently low activity in hydroperoxides electrochemical reduction due to the purely heterogeneous-catalytic decomposition the actual concentration near the electrode surface sharply decreases, which, in turn, results in much lower electrode sensitivity. The second composite catalyst, Co-Bi-g-C_3N_4, the surface area of which is more than twice as high as the one of Co-doped g-C_3N_4, demonstrates ca 30% lower electrocatalytic activity at the electroreduction of hydrogen peroxide, and 60% lower activity at t-BHP electroreduction than the latter, which can be explained similarly; the heterogeneous-catalytic decomposition of these hydroperoxides decreases its concentration near the electrode surface and apparently causes lower sensitivity.

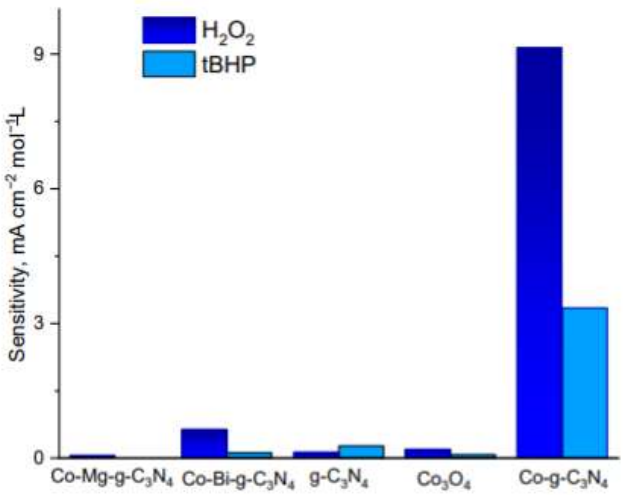


Figure 11. *Sensitivity of the catalytic electrode upon electrochemical reduction of H_2O_2 (dark blue series) and t-BHP (blue series) in a neutral aqueous medium; working potential -0.2 V (vs. Ag\|AgCl, sat. KCl).*

Therefore, based on the above findings and its superior catalytic performance in electroreduction of the two hydroperoxides, the Co-g-C_3N_4 has been selected for further studies.

IV.2.1.1. Optimization of the Polymer-Catalyst Ratio

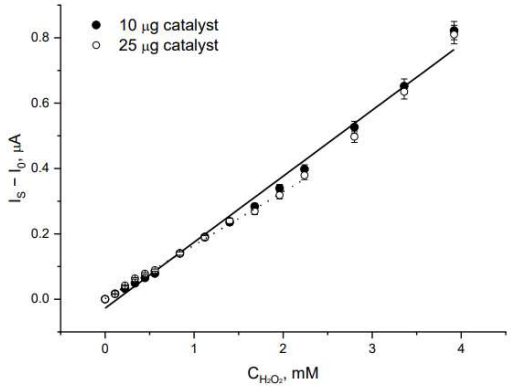


Figure 12. *Dependence of the electrode response on peroxide concentration at a constant operating potential for GCE modified with Co-doped g-C_3N_4 suspensions with catalyst load 0.01 mg (closed circles) and 0.025 mg (open circles)*

Knowing that the catalyst is a large band-gap semiconductor that, if loaded in large amounts, can impede the electrocatalytic process, we aimed close to the monolayer surface coverage. For the purpose of electrode modification, Co-g-C_3N_4 was pre-dispersed in aqueous suspension of Nafion in amounts gradually decreasing from 10 mg.mL^{-1} to 2 mg.mL^{-1}, followed by the assay of electrode sensitivity. It should be pointed out that all dispersions were highly unstable, tending to sediment within several minutes after stopping sonication, which is most probably a consequence of the strong attraction forces acting between the g-C_3N_4 layers that trigger particle agglomeration in the absence of ultrasound treatment. It has been found that current variation depends linearly on peroxide concentration (measured at a constant potential) for the electrodes prepared with 2 mg.mL^{-1} (0.01 mg catalyst load) and 5 mg.mL^{-1} suspensions (0.025 mg catalyst load); however, the electrode sensitivity (slope of the dependence) decreases with increasing the catalyst's amoun as the amount of catalyst increases (Fig. 12). The highest sensitivity was achieved when modified with a suspension containing 2 mg.mL^{-1}, therefore this composition was selected for our further studies.

The content of the second component in the modifying phase, the polymer Nafion 117, has also been optimized in order to warrant not only good analyte penetrability and high sensitivity, but also durability of the modifier. The concentration of polymer suspension was varied from 0.1 to 1%. By means of polarization microscopy (Fig. 13A), it has been observed that by decreasing polymer concentration from 1 to 0.1%, the catalyst's nanoparticles tend to agglomerate due to decreased viscosity of suspension. The catalyst particles were unevenly distributed within the modifying layer, since the hardness and Young's modulus (both measured by nanoindentation) showed very different values at different areas of the same sample. As a common feature, the larger the polymeric content, the more structures are disposed in the periphery of the studied sample (Fig. 13B) that leads to the formation of cracks in the modifying phase and its destruction during the measurements.

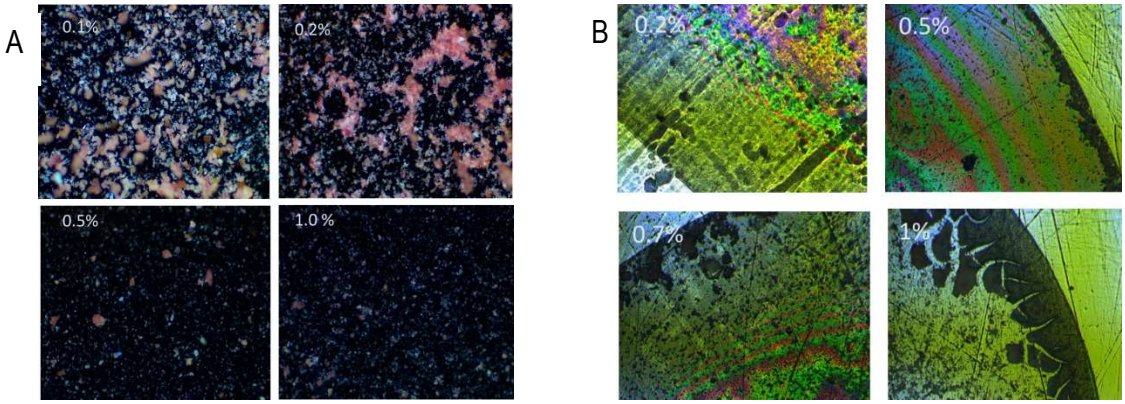


Figure 13. *Microscopic images of glassy carbon surface modified with Co-g-C_3N_4 (0.01 mg) dispersed in polymer suspension with different Nafion™ content. Magnification × 20; A) polarized light microscopy; and B) reflected light photographs of the boundary regions.*

Moreover, with increasing the amount of the binding polymer in the modifier, the deviations from linearity of the dependence of the electrode response on hydroperoxide concentration become substantial. It becomes even more noticeable upon continuous utilization of the modified electrode since the electrode response was found to gradually change to some extent during several measurements which most probably comes from the swelling of the polymer film. The sensitivity of electrocatalytic hydroperoxide reduction at a constant potential of -0.2 V (vs. Ag|AgCl sat. KCl) is virtually identical for the modifier with polymeric content of either 0.1% or 0.2%, whilst the preparation with 0.5% polymeric content demonstrates a decrease of the electrode sensitivity with ca 1/4 as compared with the former, the obvious reason for which is the troubled analyte penetration through a thicker modifying layer. These findings, together with the non-linear dependence of response on concentration, makes this polymer content non-optimal for analytical applications despite the mechanical stability of the polymer-enriched modifier is greater than the one with 0.1%-polymer content and a bit more stable than the electrode prepared with 0.2% Nafion as a binder. Therefore, for fabrication of catalytic electrodes applicable in hydroperoxide assay, a 2 mg. mL^{-1} Co-g-C_3N_4 dispersed in 0.2% Nafion aqueous suspension was selected as an optimal composition.

IV.2.2. Electrochemical characterization of the modified electrodes

The electrochemical impedance spectroscopy (EIS) is a valuable alternating current technique that supplies information about the charge transfer at the electrode-solution interface, while changing the frequencies from high to low during the measuring process. The Nyquist plots (Fig. 14) of a glassy carbon electrode modified with a layer of Nafion™ (Fig. 14, series 1) and with Co-g-C_3N_4—Nafion™ composite (Fig. 14, series 2) prepared with the optimized polymer-to-catalyst ratio is depicted. The first region in Figure 14, series 1 represents a semicircle, which appears within the high-frequencies range, followed by a linear “tail” sloped at ca. 45°, typical for the medium and low-frequencies region of the spectrum. The semicircular part of the EIS spectrum indicates the occurrence of charge-transfer resistance (R ~ 3.9 kΩ), whilst the linear part can be assigned to the diffusion process. Since the EIS spectra have been obtained in the presence of $Fe(CN)_6^{3-/4-}$ as redox probe, the appearance of the charge transfer resistance can

be associated with the electrostatic repulsion between the hexacyanoferrate anions and the polymer-coated glassy carbon surface, as the Nafion polymer contains numerous negatively charged fluoride/sulfonate side groups, providing it with a negative surface charge. The EIS spectrum of glassy carbon electrode modified with Co-g-C_3N_4—Nafion™ composite in 0.1 M KCl with 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ has been recorded over the same frequency range: from 10^5 Hz to 1 Hz (Fig. 14, series 2). On the EIS spectrum of Co-g-C_3N_4—Nafion™ modified electrode the semicircle region on the Nyquist plot is not detectable, as it represents a straight line tilted at an angle larger than 45°, which corresponds to the occupation of the electrode surface with numerous imperfect capacitors, similar to the behavior of a graphene modified surface, as reported in [9]. Apparently, the charge transfer is not restricted over the high-frequencies region; however, the data analysis and simulations returned a value of the charge-transfer resistance, which is approximately 3–4 orders of magnitude higher, as can be expected due to the semiconductive nature of the catalyst Co-g-C_3N_4.

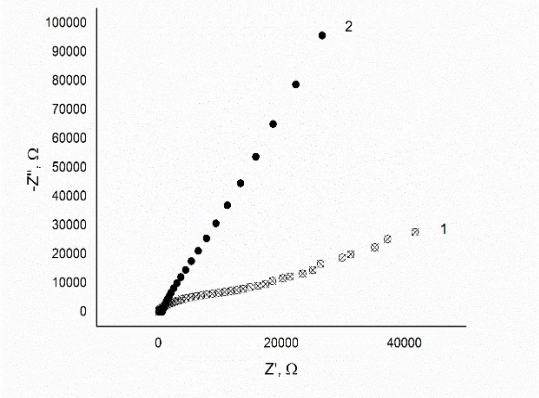


Figure 14. *EIS spectra (Nyquist plot) of GC electrode, modified with Nafion™ (1) and with Co-g-C_3N_4—Nafion composite (2); reference electrode Ag|AgCl, sat KCl; electrolyte 0.1 M KCl with 5 mM $Fe(CN)_6^{3-/4-}$ redox probe.*

The voltametric response of Co-g-C_3N_4/Nafion™-modified glassy carbon electrode in the absence and the presence of both water-soluble hydroperoxides: hydrogen peroxide (Fig. 15A) and t-BHP (Fig. 15B) shows much more pronounced electroreduction waves starting at ca. +0.2 V (vs. Ag|AgCl, sat KCl) and going down to −0.4 V vs. (Ag|AgCl, sat KCl) and further. The most clearly expressed difference between the background current and the current in the presence of either hydroperoxide was noticed at ca. −0.2 V (vs. Ag|AgCl, sat KCl). Comparative studies performed in a completely deaerated medium have shown that over the modified electrode, the oxygen reduction starts at potentials more negative than −0.25 V vs. (Ag|AgCl, sat KCl), and hence there is an overlap of the hydroperoxide reduction wave and the oxygen reduction one. This is an important finding because oxygen is produced during the hydroperoxides electroreduction process and, as such, it affects both the noise level and the sensitivity of the

[9] JD Huffstutler *et al.* , "High performance graphene-based electrochemical double layer capacitors using 1-butyl-1-methylpyrrolidinium tris (Pentafluoroethyl) trifluorophosphate ionic liquid as an electrolyte," *Electron.* , vol. 7, no. 10, pp. 1–11, 2018, doi: 10.3390/electronics7100229.

determination (the latter being taken as a measure of the electrocatalytic activity of modifying phase).

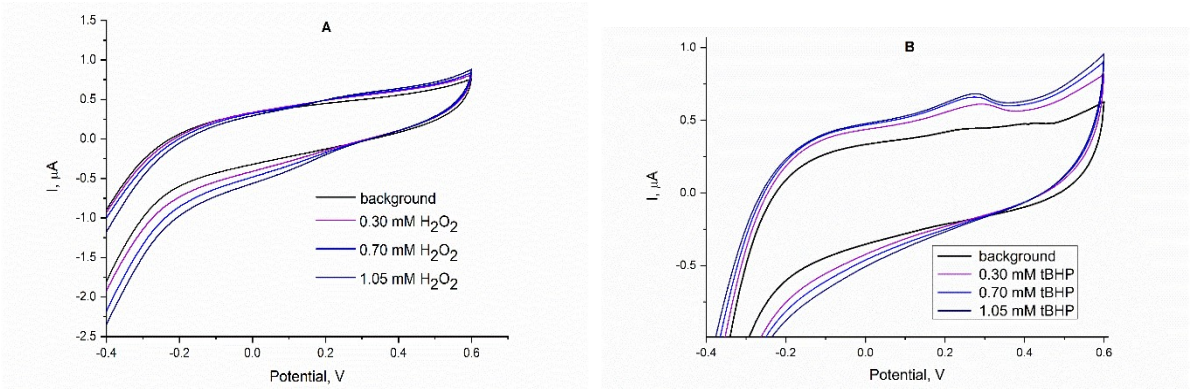


Figure 15. *Cyclic voltammograms of GC electrode modified with Co-g-C_3N_4/Nafion™ in the absence and presence of H_2O_2 (**A**); in the absence and presence of t-BHP (**B**); background electrolyte: phosphate buffer, pH = 7, reference electrode Ag|AgCl (sat. KCl); scan rate 20 mV/s; catalyst load 0.01 mg.*

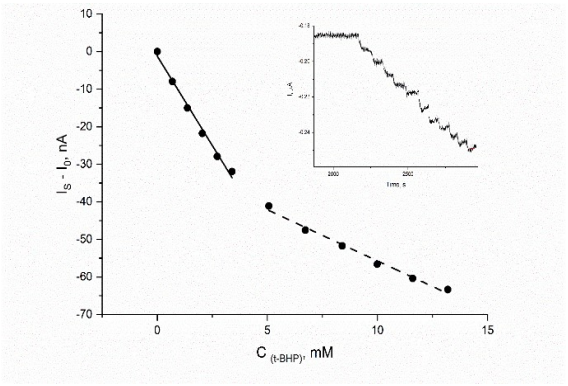


Figure 16. *Dependence of current variation on t-BHP concentration at a constant potential of –0.3 V vs. Ag|AgCl. Inset: chronoamperometric record of GC electrode modified with Nafion™ upon addition of aliquots of t-BHP; background electrolyte: phosphate buffer, pH = 7.0, reference electrode Ag|AgCl (KCl sat); operating potential: –0.3 V; catalyst load – 0.01 mg; room temperature*

By means of constant potential amperometry (at an applied potential of –0.3 V vs. Ag|AgCl) it has been observed that a reductive current flows upon the addition of hydroperoxide aliquots that reaches a steady – state value in 35-40 seconds (Fig.16, Inset). When adding the next hydroperoxide aliquot the current changes stepwise until a new steady-state is reached. It shall be noted that the record is rather noisy and the noise level vastly increases with shifting to the less-negative operating potentials (e.g. –0.2 V) so that it was not possible to discriminate between the noise and the signal at potentials less-negative than –0.3 V. The corresponding calibration plot drawn on the basis of chronoamperometric data (Fig. 16, Inset) is composed of two linear sections – a steeper segment (Fig. 16, solid line) going up to 3.5 mM, followed by a linear dependence with ca. 3 times smaller slope spanning over the region from 5 to 13 mM. The existence of two linear parts of the plot most probably results from the hampered penetration of t-BHP through the polymer layer, most visible at concentrations exceeding 3.5 mM.

By means of constant potential chronoamperometry, the electrode response has been examined as a function of hydroperoxides concentration at −0.2 V, −0.1 and 0 V (vs. Ag|AgCl) in a neutral medium (Fig. 17). It can be seen that as the working potential goes in the negative direction, the electrode sensitivity increases slightly between 0 and −0.1 V (vs. Ag|AgCl), however

the slopes of the linear dependencies at −0.1 and -0.2 V (vs. Ag|AgCl) differ drastically. At all these potentials, the current changes stepwise when introducing hydroperoxide aliquot and the noise levels were kept low. At potentials more negative than −0.2 V the sensitivity was found to increase, however, with a higher noise level. Therefore, further chronoamperometric experiments were performed at −0.2 V where the linear dynamic range determined spans over the interval from 0.4 to 14 mM peroxide concentration. At all three working potentials upon the addition of an analyte, the signal was several times higher compared to that of the Nafion-only coated electrode (at −0.3 V). These results testify that the electrocatalytic activity is the result of the synergistic action of the two components making up the modifying phase – the Co-g-C3N4 catalyst and the Nafion ionomer, which defines this system as a composite.

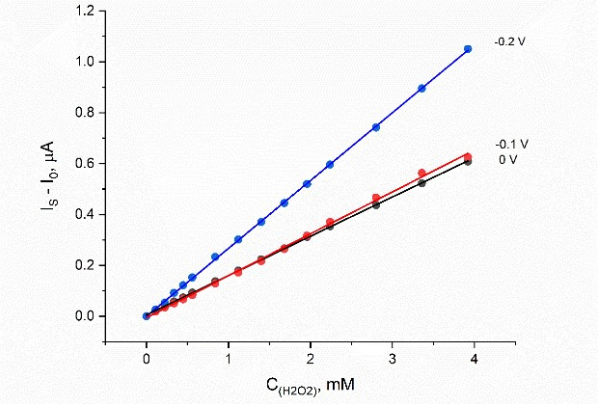


Figure 17. *Calibration plots of GC electrode modified with Co-g-C_3N_4/Nafion™ dependence of electrode response on H_2O_2 concentration; background electrolyte: phosphate buffer, pH = 7.0, reference electrode Ag|AgCl (sat. KCl); operating potentials: −0.2 V (curve 1); −0.1 V (curve 2) and 0.0 V (curve 3).*

To justify the role of each component of the modifying phase, a series of chronoamperometric experiments with electrodes modified with only polymer, with only Co-g-C_3N_4 and with Co-g-C_3N_4/Nafion have been performed (Fig. 18) at the potential where the highest sensitivity has been detected (−0.2 V vs. Ag|AgCl, sat. KCl) in the presence of both studied hydroperoxides. The lowest electrocatalytic activity has been detected for the polymer-coated GCE, that can be assigned to the delayed penetration of the hydroperoxides through the polymer layer, as the studies with a bare GCE showed higher electrode sensitivity for the polymer-coated GCE. The solely catalyst-modified electrode manifests higher activity than the Nafion/GCE (however comparable with the one achieved on bare GCE), whilst the Co-g-C_3N_4/Nafion modified GCE shows a dramatic change in the H_2O_2 electroreduction rate which exceeds more than 10 times the sensitivity registered over only Co-g-C_3N_4-modified GCE, and is about two orders of magnitude higher than the one found on polymer-coated GCE. All of these point toward a pronounced electrocatalytic effect of the modifying phase in the process of hydrogen peroxide electroreduction. Similarly, the response to t-BHP at a constant potential of −0.2 V (vs. Ag|AgCl, Sat. KCl) of the Co-g-C_3N_4/Nafion™/GCE confirms the conclusion for electrocatalytic effect of the composite modifier, as its sensitivity is more than 50 times bigger than the one achieved with only

the polymer coated electrode, and almost 5 times higher than the one of Co-g-C_3N_4-coated GCE. The catalytic electrode signal is stable and noise-free with increasing analyte concentration, and a wide linear dynamic range is strictly observed from concentrations up to 14 mM of the hydroperoxide with correlation coefficients of $R^2 = 0.997$.

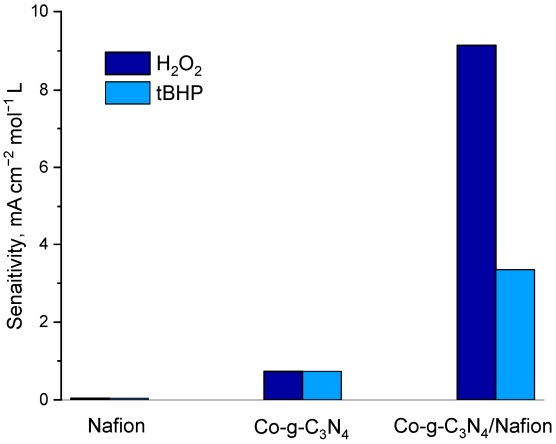


Figure 18. *Electrode sensitivity determined at a constant potential of −0.2 V (vs. Ag|AgCl, sat. KCl) for different electrode modifications: 0.2% Nafion—coated GCE; electrode modified with Co-g-C_3N_4 layer (2 mg/mL dispersed in water); and electrode modified with Co-g-C_3N_4/Nafion suspension (catalyst load 0.01 mg); pH = 7.0, room temperature.*

IV.2.3. Influence of the binding polymer

Analogous to the above studies were also carried out with an electrode modified with Co-g-C_3N_4, which was retained on the surface by cross-linking with glutaraldehyde. The purpose of this study is to determine how much the binding polymer affects the catalytic activity of the modifying phase. Similar to the phenomena observed in the previous experiments, the electrochemical reduction of water-soluble hydroperoxides on the electrode-catalyst in a medium of 0.1 M phosphate buffer is established in the range of potentials from 0.2 to about - 0.3 V. The difference between the background signal and the signal in the presence of peroxide is the most – distinct at a potential of -0.2 V, which determined the working potential in the following studies.

Figure 19 compares the results of the study of the electrocatalytic activity at a constant potential -0.2 V (vs Ag|AgCl, sat. KCl) of pure glassy carbon electrode (GCE) and modified glassy carbon surfaces with: glutaraldehyde (GA/GCE); Co-g-C_3N_4 /GA/GCE and Co-g-C_3N_4 /Naf/GCE. The analysis of the obtained data shows that the signal obtained with the electrode-catalyst in the electrochemical reduction of H_2O_2 and t-BHP is 4 times higher than that of the unmodified glassy carbon and more than 2 times higher than the signal of the modified only with glutaraldehyde. Compared to the electrodes modified with the Co-g-C_3N_4/Nafion composite, the signal of glutaraldehyde cross-linked Co-g-C_3N_4 obtained under equivalent experimental conditions is about 3% lower, which is most -probably due to differences in the permeability of the modifying phase with respect to the analyzed peroxide.

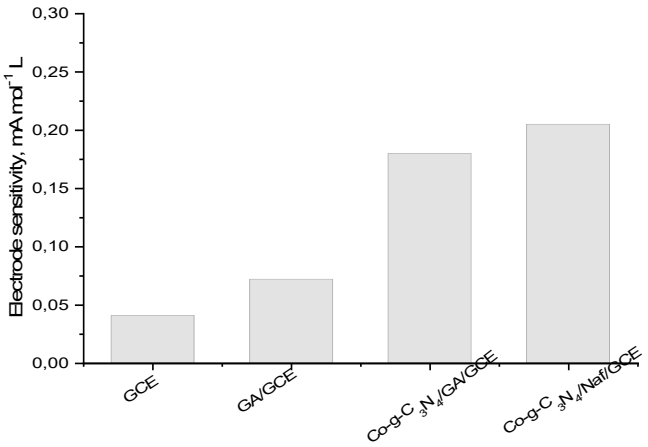


Figure 19. *Plot of electrode sensitivity determined at a constant potential of −0.2 V (vs. Ag|AgCl, sat. KCl) for different electrode coatings: pure glassy carbon GCE electrode; GA/GCE glutaraldehyde-only coating; coated with a layer of Co-g-C_3N_4 (2 mg. mL^{-1} dispersed in water) and glutaraldehyde or coated with the composite Co-g-C_3N_4/Nafion; pH = 7.0, room temperature*

IV.2.3.1. Influence of the acidity of the medium

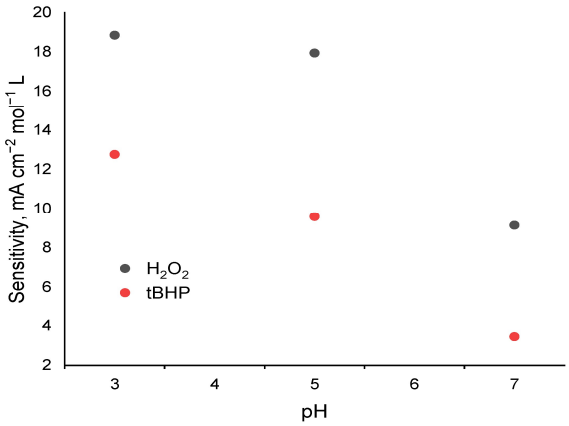


Figure 20. *Dependence of the sensitivity of modified with Co-g-C_3N_4/Nafion glassy carbon electrode on pH of the operating medium; background electrolyte: phosphate buffer (pH = 3, pH = 5 and pH = 7), reference electrode Ag|AgCl (sat. KCl); operating potential: −0.2 V vs. Ag|AgCl (sat. KCl).*

Keeping in mind that Nafion is an ionic conductor widely used in proton-exchange membranes for PEM, fuel cells, the effect of proton concentration on the electrode response has been explored. By increasing the concentration of H^+ 100 times (to pH = 5), the electrode sensitivity almost doubled the one at neutral pH upon hydrogen peroxide electroreduction (Fig. 20), whilst for the other analyte, t-BHP the difference in the sensitivity was even bigger: ca. 3 times. A further decrease of the pH of the working medium to pH = 3 has led to an insignificant augmentation in the electroreduction rate of H_2O_2, and approximately a 30% rise of the one for t-BHP electroreduction. The studies carried out unequivocally show that the binding agent in the modifying phase has an effect on both the sensitivity of the modified electrodes and its behavior in non-neutral pH medium. The differences in the electrocatalytic activity of the two types of modifying phases testify that the Nafion proton exchange polymer is not only a binding agent in the modifying phase, but also an essential element of the composite, providing a higher catalytic activity of the modified electrodes not only in neutral but also in acidic

media. Therefore, all further studies were conducted with *Co-g-C_3N_4/Nafion*- modified glassy carbon.

IV.2.3.2 Stability of the Co-Doped C_3N_4/Nafion Modified Electrode

Stability of the modified electrode with optimized polymer-catalyst ratio, has been found to depend strongly on the duration of the drying process (i.e., on the residual moisture in the polymer-composite deposit). Upon continuous drying (48–72 h.at ambient temperature) the resulting catalytic electrode is stable for more than two weeks; however, an additional "activation" takes place, the electrode sensitivity raises with ca. 15–20% after 3–4 uses and decays again after 2–3 days of "dry" storage at ambient atmosphere. Most probably, the "activation" effect is due to the swelling of the polymer layer, which makes it more permeable for the analyte. The reproducibility of the response of already "activated" electrode is 96.5–97.0%.

IV.2.4. Application of the developed electrochemical electrode-catalyst for tracking enzyme activity in a neutral aqueous medium

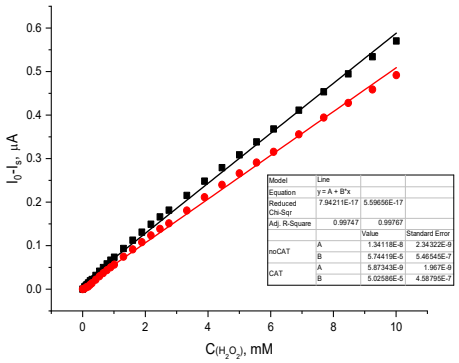


Figure 21. *The calibration plots when adding aliquots of H_2O_2; In the absent (black) and the present (red) of immobilized catalase; phosphate buffer, pH = 7.0, working potential – 0.2 V (vs. Ag\|AgCl, sat. KCl).*

In enzymology, two main methods are used to monitor the catalytic activity of catalase enzyme, one of which is spectrophotometric and the other is titrimetric. The catalytic peroxide electrode we developed provides a third alternative to the above two methods for following the kinetics of catalase enzymatic action.

Figure 21 shows the dependence of the signal of the catalytic peroxide electrode as a function of the concentration of hydrogen peroxide in a pre-deaerated solution of neutral pH, under continuous stirring. The peroxide concentration was gradually increased by adding aliquots of freshly prepared H_2O_2 solution to the working medium, which was kept in an ice bath during the measurements to avoid its spontaneous decomposition at room temperature. In the same way, the experiment was carried out in the presence of immobilized catalase from the mold culture *Penicillium chrysogenum*. Studies have shown that the signal of the peroxide catalytic electrode is higher when immobilized catalase is absent in the electrochemical cell. In the presence of an immobilized enzyme (bioreactor), the signal weakens, which is due to a decrease in the concentration of hydrogen peroxide in the solution as a result of the enzyme-catalyzed decomposition of the peroxide.

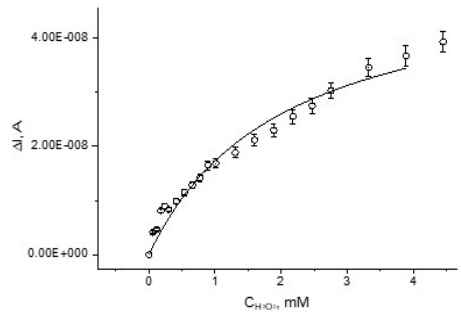


Figure 22. *Dependence of the signal chance on H_2O_2 concentration (Michaelis-Menten plot); working electrode: Co_3O_4-g-C_3N_4 - modified GCE; phosphate buffer, pH = 7.0; working potential – 0.2 V (vs. Ag|AgCl, sat. KCl)*

The analysis of the obtained data shows that the signal obtained in the presence of immobilized catalase when adding portions of the peroxide follows a hyperbolic course typical of the Michaelis-Menten kinetic model. Through the nonlinear regression of the obtained hyperbolic dependence of the rate of the enzymatic-catalytic decomposition of H_2O_2 , the kinetic constants of the enzymatic-catalytic process were determined from its concentration: the apparent Michaelis constant K_M^{app} = 2.03 mM and the maximum velocity of the enzyme-catalyzed reaction V_{max}^{app} = 5.30.10^{-8} A (Fig. 22). Conducting a parallel spectrophotometric study testifies that, compared to the Michaelis constant determined in the heterogeneous-catalytic decomposition of H_2O_2, a certain increase in the value of the apparent Michaelis constant is observed, which can be explained by the increased diffusion limitations. The low maximal reaction rate is due to the extremely small amount of enzyme immobilized on the solid support ~ 1.6 μg. After recalculating the enzyme activity based on the obtained dependences, the activity of the immobilized catalase was found to be: 102.15 U/mg, i.e. a two-fold decrease in enzyme activity was observed as a result of immobilization. The obtained results testify that electrochemical methods (such as amperometric detection at constant potential) can be used as an alternative to the classical - spectrophotometric and titrimetric methods for determining the enzyme activity, and the obtained results have no analog so far in those described in the scientific literature.

IV.3. Electrocatalytic activity of modified Co-g-C_3N_4 /Nafion electrode in non-aqueous medium

The electrochemical behavior of the Co–g-C_3N_4/Nafion modified electrode was studied in a medium of aprotic organic solvent acetonitrile (ACN), and the inert electrolyte 0.1 M tetrabutylammonium perchlorate ($TBAClO_4$) was added to increase the electrical conductivity. Figure 23 shows the cyclic voltammetry curves taken on the catalyst electrode in the absence and presence of benzoyl peroxide (BPO). The resulting dependences testify that when portions of BPO solution are added to the electrolyte solution in the cell, an electrochemical reduction of peroxide takes place. In the reverse course of the cyclic voltammetry curves obtained in the presence of benzoyl peroxide, a reduction wave is observed, starting at a potential of 0.3 V (vs. Ag|Ag+ pseudo-reference electrode) and reaching a peak value at -0.2 V, which upon further variation of the potential in the negative direction passes into a second wave of reduction, which is not observed in the cyclic voltammograms obtained in a de-aerated medium. As the concentration of benzoyl peroxide increases, the reduction maximum gradually shifts to more negative potentials. The obtained polarization dependences give reason to assume that it is possible to quantify the organic peroxide by means of chronoamperometry at a constant operating potential of -0.2 V, based on a calibration graph.

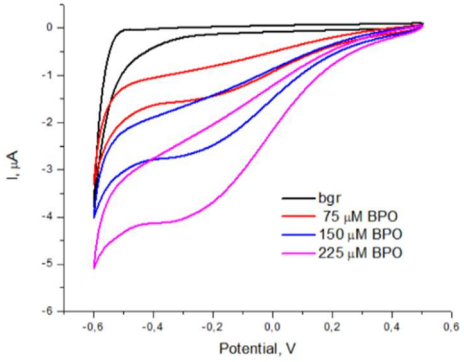


Figure 23. *Cyclic voltammograms of GC electrode modified with Co-g-C_3N_4/Nafion™ in the absence and presence of BPO/ACN; background electrolyte 0.1 M $TBAClO_4$ /ACN, reference electrode Ag|Ag+; scan rate 20 mV.s^{-1}.*

Experiments carried out at a constant potential of -0.2 V (vs. Ag|Ag+) with the chronoamperometry method showed that the reduction current changes stepwise when portions of the peroxide are added, reaching steady-state values within 10 seconds. The dependence of the signal on the peroxide concentration was found to be linear up to a concentration of 50 μM and higher. It should be noted that at low concentrations (up to about 2 μM) a deviation from linearity is observed, which is probably due to the low values of the electrode signal at these concentrations, which in turn leads to a large error in its reading.

IV.3.1. Application of the electrode - catalyst in a non-aqueous medium

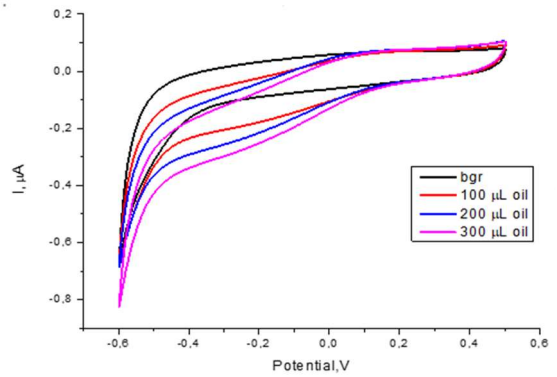


Figure 24. *Cyclic voltammograms of GC electrode modified with Co-g-C_3N_4/Nafion™ in the absence and presence of oil/$CHCl_3$; background electrolyte 0.1 M $TBAClO_4$ /ACN, reference electrode Ag|Ag+; scan rate 20 mV.s^{-1}*

Based on the received in results, the catalytic peroxide electrode was used to determine the concentration of peroxides in a real sample – highly oxidized vegetable oil (anti-cellulite massage oil). The electrochemical behavior of the electrode-catalyst upon the addition of anti-cellulite oil dissolved in chloroform ($CHCl_3$) was investigated to investigate the possibility of determining peroxides in the sample under the already established experimental conditions. Cyclic voltammetry curves resemble in appearance the behavior of the catalytic electrode obtained during research in the presence of the model organic peroxide BPO - the addition of aliquots of the solution of the real sample to the working medium leads to a pronounced reduction wave starting at a potential of 0.18 V and reaching a plateau in the interval -0.2 V to -0.4 V. The presence of this reduction wave gave us the basis for constructing a calibration graph that would be applicable in determining the amount of organic peroxides in the real sample.

Chronoamperometric measurements were carried out (Fig. 25, inset) at a constant potential of -0.2V (vs. Ag|Ag+) while adding aliquots of the standard peroxide electrode calibration

solution, based on which a calibration graph was constructed showing a linear dependence of the BPO concentration signal with a correlation coefficient of 0.993. To account for the influence of the matrix of the real sample, the standard 0.003 M BPO solution was dissolved in chloroform to which 0.4 g of unoxidized oil was added. At each measurement, the peroxide concentration in the real sample was compared to the BDS-measured peroxide number of those samples.

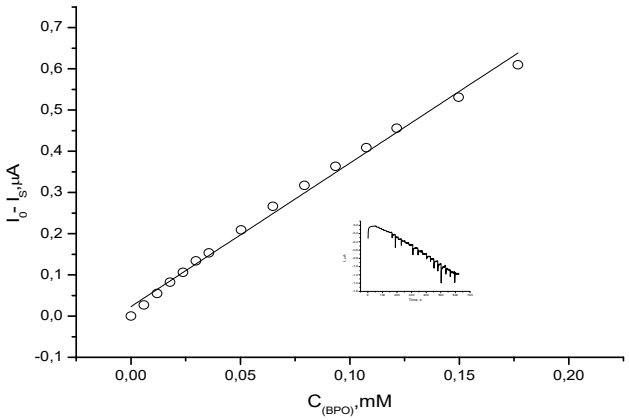
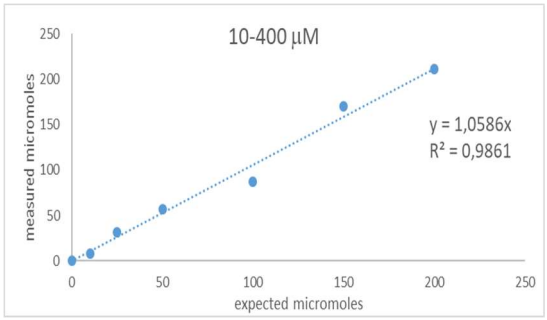


Figure 25. *Chronoamperometric record (inset) and calibration plot of an electrode modified with Co –g-C_3N_4/Nafion upon addition of aliquots of 0.003M BPO (in $CHCl_3$/oil); background electrolyte: 0.1 M $TBACIO_4$/ACN, reference electrode Ag| Ag+; potential: - 0.2 V.*

The experimental results obtained in the study of samples with a known concentration of peroxides in the range of high (10 to 400 μM) and low (0.5 to 10 μM) concentrations are presented in Figure 26. Based on the experiments conducted at a constant operating potential of -0.2 V (vs. Ag|Ag+), it was calculated (in micromoles O_2) the concentrations of peroxides in the real sample. The obtained results testify to a good coincidence of the experimentally determined values with the theoretically calculated ones, with a high correlation coefficient (respectively $R^2 = 0.9861$ and $R^2 = 0.9885$).



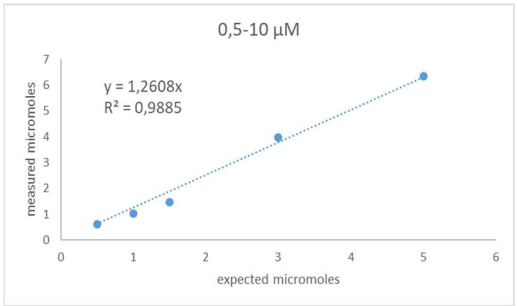


Figure 26. Measured versus expected micromeles of O_2 in a real oil sample enriched with peroxides in a wide (10-400 μM) and narrow (0.5-10 μM) concentration range.

Based on the obtained dependences, the peroxide value of the real sample containing a mixture of easily oxidizable vegetable oils (anti-cellulite massage oil) was also determined. Figure 27 presents the results of determining the degree of peroxidation of the real sample by the standard addition method. According to this method, the intercept of the calibration line corresponds to the signal of the real sample. Dividing this signal by the slope of the line, we obtain

that the concentration of peroxides in the electrochemical cell is 1.25.10 $^{-6}$ M. After recalculation, taking into account the dilution and mass of the real sample taken, we obtain that the real sample contains 276 µmol of peroxides, which corresponds to POV = 739.75 meq O_2 /kg. The conclusion we can draw from the obtained POV value is that the vegetable oils contained in the real sample are highly peroxidized.

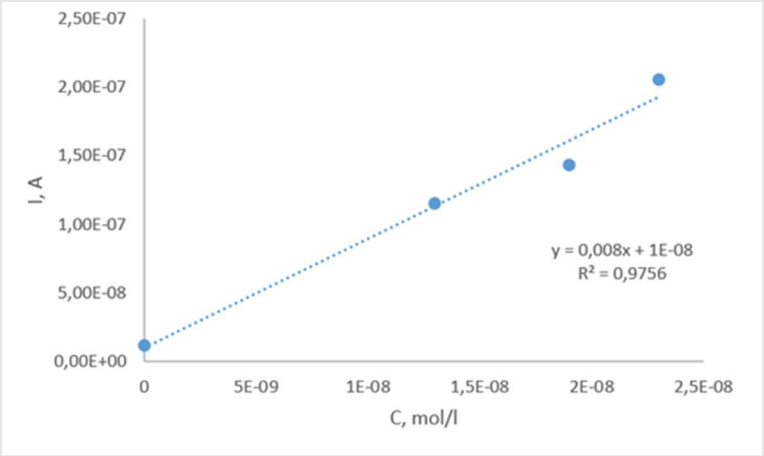


Figure 27. *Dependence of the measured catalytic electrode signal upon the addition of a standard peroxide solution (BPO) on the peroxide concentration in the electrolysis cell. The right was obtained by dissolving a precise amount of BPO in chloroform to which the real sample was added.*

The obtained results show that the developed modified electrode has a significant potential to be used as an alternative method for the analysis of the peroxide number of vegetable oils.

V. CONCLUSIONS

The experimental results discussed so far can be summarized as follows:

1. An electrochemical biosensor based on glassy carbon modified by electrochemical deposition of gold nanostructures and covalently bound laccase was obtained, where it was found that:
    - The electrochemically accessible surface area of gold-deposited glassy carbon electrodes modified is 51% larger when potentiodynamic procedure 2 is used for modification for deposition and solution of tetrachloroaurate ions with concentration 50 mM; the surface morphology of the deposits is characterized by the presence of micro-porous formations;
    - Laccase exhibits electrochemical activity in an immobilized state: in the absence of oxygen, oxidation-reduction peaks of the enzyme are observed, and in the presence of oxygen, a reduction wave is observed; differential pulse voltammetry studies showed the presence of a peak in the absence of substrate, with the peak current increasing upon addition of the substrate dopamine over the concentration range of 2 to 40 µM. It was found that with the substrate epinephrine no such dependence was observed;
    - The quantification of the catecholamines dopamine and epinephrine is carried out by the amperometric method at a constant potential of -0.1V

and is characterized by a detection limit of 0.037 μM for dopamine and 0.054 μM L-epinephrine and a very good analytical performance in terms of sensitivity; the apparent Michaelis constants differ for the two substrates, for dopamine it is twice as small as for adrenaline, which proves that the structure of the substrate influences its interaction with the enzyme;

- Interference studies have shown that at an operating potential of 0 V the presence of L-ascorbate will affect the laccase electrode response to L-epinephrine by 1.1% and to dopamine by 0.8%, whereas at an operating potential of −0.1 V the interference from L-ascorbate will contribute a 2.2% increase in the response to L-epinephrine, and 1.4% to dopamine.
- The calculated limits of detection and limits of quantification of both substrates are in the sub-micromolar concentration range, making the enzyme electrode potentially applicable in biological fluids. Its applicability was demonstrated by quantifying dopamine and L-epinephrine in ampoules of the injectable solution, with the assay for dopamine showing values of 41.2 ± 1.5 mg. mL^{-1} and 0.948 mg. mL-1 for adrenaline; the analytical yield for dopamine ranges from 99% to 106% and from 95 to 105% for adrenaline.

2. Modified electrodes based on glassy carbon modified with different catalysts were obtained: g-C_3N_4, Co-Mg-g-C_3N_4, Co-Bi_2-g-C_3N_4, Co-g-C_3N_4, and Co_3O_4 dispersed in Nafion polymer, and were determined the electrochemically accessible surfaces, where it was found that:
    - In the process of electrochemical reduction of hydrogen peroxide and tert-butyl hydroperoxide, the electrode modified with Co-g-C_3N_4 and Nafion exhibits the highest electrocatalytic activity. The optimal composition of the modifying phase was determined - 2 $mg.ml^{-1}$ catalyst and 0.2% polymer; electrode sensitivity is highest at an applied constant potential of -0.2 V (vs. Ag|AgCl, sat. KCl);
    - The high electrocatalytic activity of the modified electrode is the result of the synergistic effect of the two components making up the modifying phase – the Co-g-C_3N_4 catalyst and the Nafion ionomer, which defines the system as a composite. The Nafion polymer is an essential element in the composite phase, ensuring high catalytic activity of the modified electrodes in the electrochemical reduction of hydroperoxides not only in a neutral but also in an acidic medium; the stability of the modified electrode strongly depends on the residual moisture in the composite film;
    - The developed peroxide electrode is applicable for the determination of catalase enzyme activity in a neutral medium, and the obtained results are unprecedented in the scientific literature.
3. The electrocatalytic activity of the developed electrode-catalyst was investigated in a medium of aprotic organic solvent acetonitrile concerning benzoyl peroxide, where it was found that:
    - At a constant potential of -0.2 V (vs. Ag|Ag+), the dependence of the signal of the catalytic peroxide electrode on the concentration of the analyte benzoyl peroxide is linear up to a concentration of 50 μM;

- The applicability of the electrode-catalyst for determining the concentration of peroxides in a real sample - a highly oxidized mixture of vegetable oils - is shown, and the peroxide value of the real sample is determined.

The analytical method developed based on the catalytic peroxide electrode has a significant potential to be used as an alternative to titrimetric methods for determining the peroxide value of vegetable oils.

LIST OF THE RESURCH PAPERS ON THE DISSERTATION

Publications in refereed and indexed publications:

1. 2D Nanomaterial—Based Electrocatalyst for Water Soluble Hydroperoxide Reduction. Pimpilova M, Ivanova-Kolcheva V, Stoyanova M, Dimcheva N. „Catalysts" vol. 12, no. 8, p. 807, 2022. JCR - Q2 (Chemistry, Physical) / CiteScore - Q1 (General Environmental Science).
2. Biosensing dopamine and L-epinephrine with laccase (Trametes pubescens) immobilized on gold modified electrode Mariya Pimpilova, Kalina Kamarska, Nina Dimcheva. „Biosensors" vol. 12, no. 9, p. 719, JCR - Q1 (Chemistry, Analytical) / CiteScore - Q1 (Engineering (miscellaneous).

Patent application for invention:

1. "Electrochemical method for quantification of peroxide compounds" with authors V. Kolcheva, N. Dimcheva, M. Stoyanova, M. Pimpilova. Patent application for invention No BG|P|2023|113803, registered with the Patent Office of the Republic of Bulgaria on 27.10.2023

LIST OF PARTICIPATIONS IN SCIENTIFIC FORUMS ON THE DISSERTATION WORK

Posters:

1. M. Pimpilova, N. Dimcheva, "Modification of electrode materials with Pd, Pt and Au ", Fifth scientific conference for students, PhD students and young scientists "Challenges in Chemistry", November 22-23, 2019, Paisii Hilendarski University of Plovdiv. Plovdiv Bulgaria.
2. Mariya Pimpilova, Vanina Kolcheva, Maria Stoyanova, Nina Dimcheva, "Investigation of the catalytic activity of immobilized catalase (Penicillium chrysogenum 245) using an electrochemical peroxide sensor", Sofia Electrochemistry Days (SED2022) national conference with international participation, May 12 - 14, 2022., Sofia, Bulgaria.
3. Mariya Pimpilova, Nina Dimcheva, Kalina Kamarska, "Modification of electrode materials with Au", VI Scientific conference for students and PhD students "Challenges in chemistry", October 7-8, 2022, Paisii Hilendarski University of Plovdiv, Plovdiv, Bulgaria.
4. Angel Peshkov, Mariya Pimpilova, Iliya Iliev, Nina Dimcheva, *"Electrochemical approach for the monitoring of immobilized catalase activity"*, Novel enzymes 2023, March 28-31, 2023, Greifswald, Germany.

Reports:

1. Mariya Pimpilova, Vanina Ivanova – Kolcheva, Maria Stoyanova, Nina Dimcheva, " *Development of a new* Co_3O_4*–g-*C_3N_4*-NAFION™ composite catalyst for the electrochemical reduction of peroxide compounds in aqueous* medium ", XIX National

Chemistry Conference for students and PhD students, June 2 - 4, 2021, SU "St. Kliment Ohridski, Sofia, Bulgaria.

2. Nina Dimcheva, Mariya Pimpilova, Vanina Kolcheva, Maria Stoyanova "Development of novel Co_3O_4-g-C_3N_4 – Nafion™ composite catalyst for electrochemical reduction of peroxide compounds", 3RD Virtual Congress on Materials Science & Engineering: Outlining the Importance of Materials Science for a Better Future; SEP 27 - OCT 01, 2021.
3. Maryia Pimpilova, Vanina Ivanova - Kolcheva, Maria Stoyanova, Nina Dimcheva, *"Comparative study of electrocatalytic activity of Co-g-C_3N_4 modified electrode - catalyst for electrochemical reduction of peroxide compounds in aqueous medium",* XX National Conference on Chemistry for students and PhD students, May 18 - 20, 2022, "St. Kliment Ohridski, Sofia, Bulgaria.

Scientific and applied scientific contributions

1. An electrochemical biosensor has been successfully developed that can quantify dopamine using two different electrochemical techniques - differential pulse voltammetry and constant potential amperometry, the latter being also suitable for the analysis of L-epinephrine.

2. The practical application of the laccase electrode for quantitative analysis of dopamine and L-epinephrine in ampoules of injection solution is demonstrated.

3. For the first time, an electrocatalytic effect of Co-g-C_3N_4 in combination with a conductive Nafion polymer was experimentally proven in the electrochemical reduction of hydrogen peroxide and tert-butyl hydroperoxide in a wide range of concentrations from 0.4 to 14 mM.

4. The developed peroxide electrode is applicable for the determination of catalase enzyme activity in a neutral medium, and the obtained results are without analog so far in those described in the scientific literature.

5. An electroanalytical method is presented as an alternative to titrimetric methods for determining the peroxide number of vegetable oils based on the catalytic peroxide electrode.

Acknowledgments: Bulgarian National Science Foundation – grant KP-06 N39/8 "Chemo-enzyme catalysis in a non-aqueous medium" (2019/2024) and to the project "Personalized Innovative Medicine PERIMED", OP "Science and education for smart growth "BG05M2OP001-1.002-Q8 0005-C01 (2019/2023).