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Study program – Pharmaceutical chemistry
Master thesis

Application of Prussian Blue in Developing Catalase Inhibition-based Cyanide Ion Biosensor

Fermento katalazės pagrindu veikiančio biojutiklio formavimas cianido jonų nustatymui

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Evaluation: _____
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Content

Introduction

Cyanide compounds are very dangerous and at the same time useful compounds. It is widely used in human life and unfortunately can be accumulated in biological objects as plants, which subsequently can cause great changes in bioecosystems. Monitoring of cyanide concentration in the environment program is acute and urgently needed for solution action which is needed to be provided by governments of industrial countries. Modern methods of cyanide detection still are expensive and difficult in application. One of the potent methods of cyanide detection is electrochemical biosensing. This method has proved its sustainability, not very high cost and simplicity of using. Electrochemical biosensing is widely applied in monitoring of various pollutants. [49] Various electrochemistry-driven biosensing approaches for inexpensive and small analytical devices for on-site examination have recently been introduced. This trend can be used to replace commercial lab instruments made by well-known in vitro diagnosis (IVD) businesses that advertise great sensitivity and automation when measuring analytes. However, designing an appropriate on-site biosensor that meets the needed sensitivity while maintaining excellent reproducibility is still a challenge. One method of electrochemical biosensor modification is using of conductive reagent as Prussian Blue. Prussian blue is used for signal transducing Prussian blue and its analogs are a popular class of sensors and biosensors. Their electrochemical activity is directly linked to the intercalation of alkaline ions due to their open framework structure. Furthermore, these compounds have a strong peroxidase activity, making them ideal transducers in biosensors that measure H_2O_2. Combining electrochemical responses with Prussian blue and immobilized enzyme as Catalase can follow to creation of the efficient biosensor for cyanide detection. This sensor can be used in a various ways to detect a lot of agents, inhibiting work of catalase or other oxidoreductases.

Aim of the research is to develop knowledge about possibility of biosensor of this type on the model of cyanide detection using the method of potentiometry. Potentiometry is an analytical chemistry technique for determining the concentration of a solute in solution. A high-impedance voltmeter is used to detect the potential between two electrodes in this technique. There can be applied to methods for the purpose of work. The first one is cyclic voltammetry for the sustainable deposition of Prussian blue and its stabilization. The second one is chronoamperommetry for the investigation of changing current decrement and increment according to the reactions on hydrogen peroxide and cyanide. The final aim of the study is to present effective biosensor for cyanide detection in the environment, what and can be applied in medicine, ecology or chemical analysis.

The aim of the study:

To investigate a possibility of application of electrochemical biosensor with immobilized catalase on FTO glass electrode covered with Prussian blue for cyanide detection using the method of potentiometry.

The objectives of the study:

- To examine the electrochemical activity of the electrochemical biosensor with immobilized catalase on FTO glass electrode covered with Prussian blue on Hydrogen peroxide and cyanide.

- To determine suitable conditions for FTO/PB electrode preparation.
- To examine the possibility of multiple use of the investigated biosensor and find peculiarities of the process.

1. Literature review

1.1 Prussian blue

Prussian blue is electrochemically active compound with a chemical formula ($Fe^{III}_4[Fe^{II}(CN)_6]_3$). [1] Prussian blue has found to have electrochemical properties at [2] investigation and it has opened new horizons to the biosensoring possibilities. Prussian blue film, deposited on a specific conductive material can conduct electric flux by the way of transport both electrons and ions[3]. Those properties are mostly determined by a specific structure of a deposited film. The iron(III) ions are surrounded octahedrally by nitrogen atoms, while the iron(II) ions are surrounded by carbon atoms as Itaya et al has shown on a Figure 1 [4].

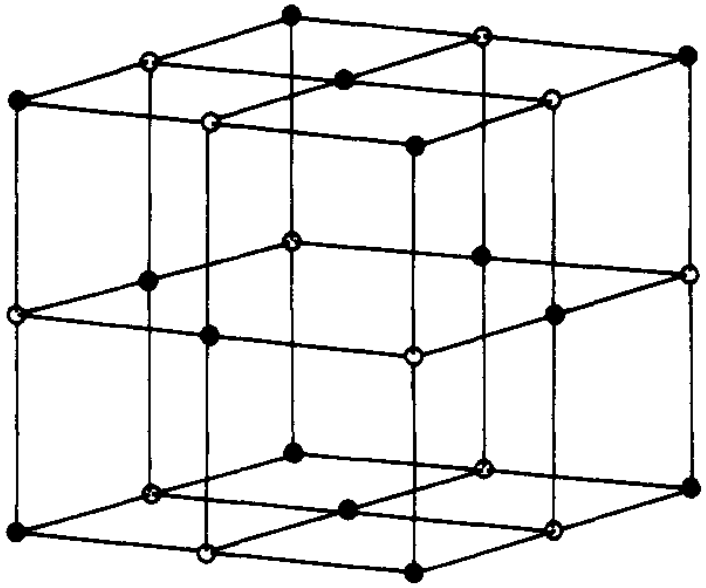

Figure 1. Illustrative depiction of the unit cells of Prussian Blue structure(• Fe^{+3}; o Fe^{+2})[4].

Depending on the oxidating state of the pigment, usually are highlighted two states of PB. The reduced state, also called Prussian white, due to its color can be formed by electrochemical reduction, and oxidized form – Prussian green is formed by the oxidation of the native Prussian blue. All these reactions are followed by changing of color because of properties of iron ions depending on the charge of formula unit [4]. It is important to keep in attention the oxidative states of Prussian blue, because Lundgren and Murray [5] mentioned that reduced Prussian blue films are more electrochemically stable, during deposition. But much more interesting is Prussian blue state. There are few ways to transform Prussian white into Prussian blue. For

example it can take electrons from active oxygen forms like a Hydrogen peroxide, or take electrons from electrolyte in solution under the stable flux. On the 0.5-0.8 on glassy carbon electrode conditioning potential film exist in oxidized Prussian blue state [4]. This state can easily take electrons from the surrounding and show stable reduction current potential and can be used for electrochemical experiments for investigation of enzymatic activity for enzymes immobilized on the Prussian blue film.

1.2 Cyanide toxicity

Cyanides are compounds containing structure the –C≡N in their molecule. These substances can be found in both natural and processed foods. Cyanogenic glycosides, which can be found in apricot kernels, cassava roots, and bamboo shoots, are natural sources of cyanide ions. Hydrogen cyanide and other cyanides are employed in a variety of sectors, including silver and gold mining. They're also utilized in the manufacturing of all kinds of colors in plastic, as well as in chemical laboratories [5]. Mines, metallurgical industries, and automobile exhaust gas are some of the sources of pollution in the environment. The largest source of cyanide ions in the environment is wastewater. These chemicals can also be released into the environment as a result of fires in factories and homes, as well as cigarette smoke.

The high toxicity of cyanide, as well as environmental worries about its continuous industrial usage, have sparked interest in simple and sensitive cyanide detection technologies. In recent years, there has also been increased awareness of HCN toxicity from smoke inhalation, as well as the possible use of cyanide as a poison by terrorists [7].

Cyanide is a biological poison that affects all cells in the body, but the central nervous system and heart are especially vulnerable. In most cases, the mechanism of death is central apnea caused after respiratory center in the brain is depressed by cyanide. The rapid onset of symptoms is due to the ease with which cyanide diffuses through cell barriers. Endogenous rhodanese converts small quantities of cyanide to thiocyanate, which is then eliminated in the urine [6]. Cyanide has a strong affinity for ferric iron (Fe^{3+}), which is found in enzymes like cytochrome C oxidase, which is a key electron transport chain enzyme. Cyanide binds to cytochrome c oxidase in mitochondria and inhibits it irreversibly. This causes cellular respiration to stop, which is followed by cellular death. Blast furnaces, gas plants, and coke ovens all produce hydrogen cyanide gas. X-ray film, wool, silk, nylon, paper, nitriles, rubber, urethanes, polyurethane, and other synthetics all contain cyanide in their combustion products. It is a common cause of mortality in industrial and home fires, along with carbon monoxide.

1.3 Cyanide detection

The most popular ways for the determination of cyanides are titration, spectrophotometry, potentiometry with cyanide-selective electrodes [8].

1.3.1 Naked-eye methods of cyanide detection

It is possible to detect cyanide ions even by a naked eye. There are available reagent kits for visualizing of presence of cyanide in biological samples. Simple visual confirmation of the presence or absence of a material is sufficient, and in many cases, comparison with a color chart can yield at least semi-quantitative information, such as with strips used to test urine glucose [9].

Some sensors require organic solvents, such as methanol or acetonitrile, dimethylsulfoxide (DMSO) [10]. The color of metal indicator complexes can be affected by cyanide, which is a potent ligand for many metal ions; this principle has been applied copper-indicator complexes [11]. Solution in totally aqueous environment was proposed by Hamza et al. [12], in which a new imine reagent combines with cyanide to create a brown-red result. Männel-Croisé et al. [13] proposed another direct technique in which cyanide replaces the water coordinated to cobalt in a complex. Zelder [14] proposed the color response of Vitamin B12 with cyanide, which allows for quick visual detection of cyanide at mM levels in water.

1.3.2 Fluorimetric methods

Niu et al. [18] demonstrated that cyanide quickly attaches to the carbon in the iminium cation, causing the dye Cy5 to lose color and fluorescence as negative response. The color reaction's naked eye detection limit can be as low as 1.5 uM, but it necessitates a two-phase system (dichloromethane holding the dye and an aqueous buffered cyanide carrying material in the presence of a tetrabutylammonium salt as a phase transfer agent). Shang and Dong [18] present a fascinating method of detecting cyanide that relies on the fact that in an oxygen-bearing solution (for example from enough air exposure), causes cyanide quickly attacks gold and forms the aurocyanide complex. Considering a solution containing a fluorescent dye and gold nanoparticles, in the case if gold nanoparticles are of a size allowing them efficiently absorb the fluorescence emission. When cyanide is introduced to this solution, it destroys the gold nanoparticles quickly, reducing their size and detuning the size-controlled absorption, resulting in increased fluorescence emission. The minimum approximate value of detection is 0.6 μM. It also was recently tested by Chakraborty et. al [19] with different modifications of sensors and have shown very high level of detection.

1.3.3 Spectral and colorimetric methods

One of the most well-established standard methods for quantifying cyanide is the Chloramine-T/Pyridinebarbituric acid colorimetric method [7]. Afkhami et. al [15] have discovered that if the initial reaction with Chloramine-T is carried out at pH 4, thiocyanate decomposes to CNCl at a different pace than Chloramine-T. This can be used to kinetically distinguish between SCN and CN, as well as to determine both at the same time. Zvinowanda et. al [16] found that if p-nitrobenzaldehyde and o-dinitrobenzene react with cyanide in a very alkaline media, it leads to creation of a bright purple molecule. They used this procedure on mining wastes, determining cyanate using an independent method, and concluding that cyanide is oxidized to cyanate in the environment when exposed to air. Papezová and Glatz [17] described the detection of cyanide by capillary electrophoresis employing a thiosulfate-based background electrolyte and an in-capillary reaction with rhodanese, a mitochondrial enzyme that converts cyanide to the considerably more strongly absorbing thiocyanate, which is measured at 200 nm.

1.3.2.1 Potentiometry and amperometry

Ion selective electrodes (ISEs) are extensively used because they are convenient, do not need chemistry, and have a quick response time; commercial ISEs for cyanide are available. The cyanide ISE, on the other hand, includes a plenty of possible agents, including halides, pseudohalides, sulfides, and other metals complexed by cyanide, such as cadmium, silver, zinc, copper, nickel, and mercury [20]. Abbaspour et al. [21] created a chemically modified carbon paste electrode using 3,4-tetra pyridinoporphirazinatocobalt(II) that had no reactivity to halides, pseudohalides, or oxalate. They were able to accurately detect spiking cyanide in tap water. The electrode has a Nernstian slope (60±1.5 mV decade^{-1}) from 0.015–10mM CN− and a level of detection of 9 uM.

Amperometry is another popular technique that can be extremely sensitive. A new cyanide sensor was created by Taheri et al. [22] Self-assembly of a sol–gel network and silver nanoparticles were used to make a silver doped silica nanocomposite. To create a three-dimensional structure around the gold electrode (GE/sol–gel/AgNPs), a precleaned gold electrode (GE) was immersed in a hydrolyzed mercaptopropyltrimethoxysilane (MPS) sol–gel solution containing Ag nanoparticles (AgNPs). Between the Ag nanoparticle and the CN, an electrochemical reaction occurs. The LOD was said to be 14 nM, yet the linear range's lower limit was >100x at 1.5 uM. The results of several industrial electroplating waste fluids were statistically indistinguishable from those obtained using established standard procedures. Christison and Rohrer [23] used sodium hydroxide to stabilize cyanide before using a cation-exchange cartridge to remove transition metals. The LOD of this sample was less than 40 nM

cyanide after IC–PAD analysis which. Those level of detection values make amperometery probably the most attractive method of cyanide detection.

1.3.4 Mass spectrometry

One of the most frequent types of mass spectrometry is electrospray ionization tandem mass spectrometry (ESI–MS–MS). Minakata et al. [24] produced the dicyanogold anion, $Au(CN)_2$, by reacting cyanide in biological fluids with NaAuCl4. The extract was fed directly into a negative ion mode ESI–MS–MS equipment after being extracted into methyl isobutyl ketone in the presence of tetramethylammonium ion as a paring agent. The transition 248.9 –>26.2 $Au(CN)2^-$ -> CN^- was investigated. Level of detection was 1.04 ug L^{-1}. The use of isotope dilution to improve quantification was not pursued. By the way Dumas et al. [25] has shown that the level of sensitivity can be increased by using radioactive isotopes in analyte with specific signatures to the Gas Chromatography(GC). He developed the isotope dilution mass spectrometry (IDMS) method for cyanide testing in blood. The later group employed a cryogenically cooled oven, which allowed them to better focus the HCN, increasing sensitivity and shortening the analytical cycle time. Loger et al. [26] used a headspace GC–IDMS technique, and automated needle flushing between each analysis avoided cyanide carryover by absorption on the needle surface. For the detection of cyanide in plasma and urine, Liu et al. [27] used solid-supported liquid–liquid extraction (SLE) and a two-step derivatization process prior to GC–MS. To make the cyanohydrin, the sample was buffered and benzaldehyde was added. As an internal standard, 1,3,5-tribromobenzene was also included at this time. The combination was then absorbed on a diatomaceous earth column and then eluted with n-hexane containing 0.4 percent heptafluorobutyryl chloride, yielding the final derivative and analyte, -cyanobenzyl heptafluorobutyrate. According to the authors, converting cyanide to a higher mass molecule enhances sensitivity and eliminates interference from thiocyanate and N_2 (m/z 28, identical to HCN at m/z 27), (such derivatization will also considerably boost sensitivity for detection by an electron microscope).

1.4 Biosensors

1.4.1 Biosensors classification

Biosensors are mechanisms of detection of some substance, using in its construction biological object. Biosensors are classified by two main parameters signal transduction and the biorecognition element type. Biosensors currently are well developed school of engineering and biotechnology and they are widely used in almost all spheres of human life [29].

1.4.2 Biosensors by the signal transduction classification

By the signal transduction parameter biosensors are divided on thermal sensors, electrochemical, sensors, optical sensors and mass sensitive sensors [30]. The most popular biosensor type is electrochemical biosensors, due to their reliability, swiftness of signal gaining, low cost and compatibility with modern technologies as nano particles [31]. Similar to it are optical biosesnors, also allowing to detect a chemical without any additional efforts [32]. Mass sensitive sensors as for example microcantilever, can be applied in specific conditions for example vacuum, or air environment [33]. Thermobiosensors are usually applied in clinical or industrial environments for detection of chemical influence on temperature of bioobject [34].

1.4.3 Biosensors by the biological recognition element classification

Based on the biological recognition element biosensors are divided into four: enzymatic, protein receptor-based, immunosensors, DNA biosensors, and whole-cell biosensors [29].

Enzymatic biosensors work on the idea of the enzyme catalysing reaction with interesting agent into electrically measureable signal or reverse. A lot of enzyme reactions work out of immobilized enzyme on a suitable transducer to gain a signal [35]. The most widespread enzymes that are used in enzymatic biosensors are oxidoreductases and hydrolases [36].

For protein-based biosensor, what is mean using of protein which not catalyze any reaction, are usually used cell membrane proteins, which are able to act as receptors. For this can be used oestrogen receptors, ion channel proteins, ion binding proteins and others [37].

Similar to non-catalitic protein biosensors can be used immunosensors. Solid-phase immunoassays, in which antigens and antibodies are immobilized on a solid support, are used to build most immunosensors. Antigen-antibody interaction occurs as a result of this contact at the solid-liquid interface. Immunosensors are appealing because of their high sensitivity and selectivity, as well as the possibility of improving their affinity and selectivity by inventing new recombinant antibodies [38].

Because of their great stability, specificity, and low cost, DNA-aptamers-based biosensors have been created as an alternative to antibodies [30]. Aptamers are defined as short single-stranded DNA (ssDNA) or RNA sequences of less than 100 nucleotides. The aptamers fold into a novel 3D assembly due to the unique intramolecular interactions between these nucleotides. Biosensors based on DNA-aptamers may preferentially bind to a specific bacteria, virus, protein, hormone, analyte, and even tiny molecules and ions with high specificity and affinity. Hydrogen bonds and Van der Waals forces are the most common types of bonds used in this binding [30].

When compared to immunosensors, the development of aptamers allows for the creation of new biosensor devices with high stability and specificity, as well as reduced costs and simpler detection procedures [39].

Microbial biosensors as a whole cell biosensors based on luminescence reporter genes have been widely applied in a variety of medicinal applications. In a real-time and cost-effective manner, a genetically engineered whole cell microbial biosensor uses either prokaryotic or eukaryotic cells to detect chemical composition, toxicity, carcinogenicity, and mutagenicity [40].

It was designed to not only indicate the presence or absence of the chemical under research, but also to precisely measure the sublethal concentration that causes toxicity or mutation [41].

Furthermore, whole cell biosensors provide highly detailed information about the bioavailable and bioaccessible fraction of substances and/or pharmaceuticals under study. It's crucial to figure out how much analyte concentration can be absorbed by the human gastrointestinal system and get into the bloodstream, showing immeasurable importance of whole cell biosensors [43].

1.4.4 Biosensors for cyanide detection

The information is known about several types of biosensors, for cyanide ions detection.

Virender et.al [44] have developed the whole cell cyanide dihydratase biosensor based on cells of *Flavobacterium indicum* combined with an ammonium ion selective electrode to create a potentiometric system. Response time and cyanide concentration were shown to have a linear relationship. With a response time of 2 minutes, this potentiometric biosensor could detect cyanide concentrations of 0.06 ppm. The developed potentiometric biosensor could be utilized to detect cyanide in food samples and industrial wastes.

Zhen-Zhen Dong et.al [45] created an Ag/Au core–shell nanoparticle (NP)/iridium(III) complex-based sensing platform for sensitive cyanide ion "turn-on" sensing. The assay is based on the dampening impact of AgAu NPs on complex 1 emission, however luminescence is restored when cyanide anions are added because they can dissolve the Au shell. With a detection limit of 0.036 M, sensing platform demonstrated high sensitivity toward cyanide anions, as well as high selectivity for cyanide over 10-fold excess quantities of other anions. The sensing technology has also been used to monitor cyanide anions in drinking water and living cells with great success.

Martinkova et.al [46] nn a flow injection study made a CynD and a commercial FDH and paired it with an amperometric sensor. Amperometry was used to identify NADH generated in

the reaction catalyzed by FDH. A flow-through system was used to include the enzymes immobilized in cartridges. This sensor had excellent long-term stability and could be used to analyze complicated substances like plant extracts. The sensor is likely to interfere heavily with formate (the reaction product). Only the interference with thiocyanate (0.1–0.5 mM) was confirmed, whereas sulfide or nitriles had no effect on the results at the same doses.

Aisha Attar et. al [47] made nine enzyme biosensor designs using three different electrodes: Sonogel-Carbon, glassy carbon, and gold electrodes were discussed, as well as new biosensors based on inhibition for the detection of cyanide. Three different horseradish peroxidase immobilization techniques were investigated, including those with and without gold sononanoparticles. The amperometric measurements were carried out in a 50 mM sodium acetate buffer solution pH = 5.0 with an applied potential of 0.15 V vs. Ag/AgCl. In the absence of an inhibitor (cyanide), the apparent kinetic parameters (Kmapp, Vmaxapp) of immobilized HRP were computed using caffeic acid, hydroquinone, and catechol as substrates. The addition of gold sononanoparticles boosted the biosensors' analytical performance by enhancing the electron transfer process. HRP kinetic interactions reveal non-competitive cyanide binding with a Ki of 2.7 M and an I50 of 1.3 M. The detection limit of 0.03 M, which is lower than those reported by prior investigations, may be reached in a dynamic range of 0.1–58.6 M. As a result, this biosensing system can be utilized to detect cyanide in a new and promising way.

Coelho et. al [50] designed a biosensor for cyanide detection, based on the amperometric inhibition.. Using chitosan and acrylamide, horseradish peroxidase was immobilized on the working electrode's surface. The experiment was carried out at a 100 mV applied voltage vs Ag/AgCl in a 0.1 M phosphate buffer at pH 7.5 using hydroquinone as an electron mediator. Also tuned were the effective parameters on the performance of the inhibitory biosensor, such as applied potential, electrolyte pH, mediator concentration, and substrate amount. The cyanide concentration was measured over a linear range of 1.64–13.13 M, with a detection limit of 0.43 M. The kinetic parameters Michaelis–Menten constant Kapp and maximum current I_{max} were determined without and with inhibitor, and the mechanism was investigated.

1.5 Enzymes as biological agents for biosensors for cyanide detection

The strong affinity of cyanide for the iron atoms of haemoproteins such as peroxidase, ferric haemoglobin, and myoglobin, as well as catalase, is responsible for the majority of its harmful effects [47]. It also binds to the ferric form of cytochrome-c, blocking the mitochondrial electron-transport chain, preventing oxygen transmission to the brain, heart, and lungs, and disrupting the entire organism's process. It is proven that cyanide decreases ability of oxydases to

bind oxygen. For example Leavsley et. al [52] has published a dynamics of oxygen consumption after cyanide adding to the cell culture as it shown on the Figure 2.

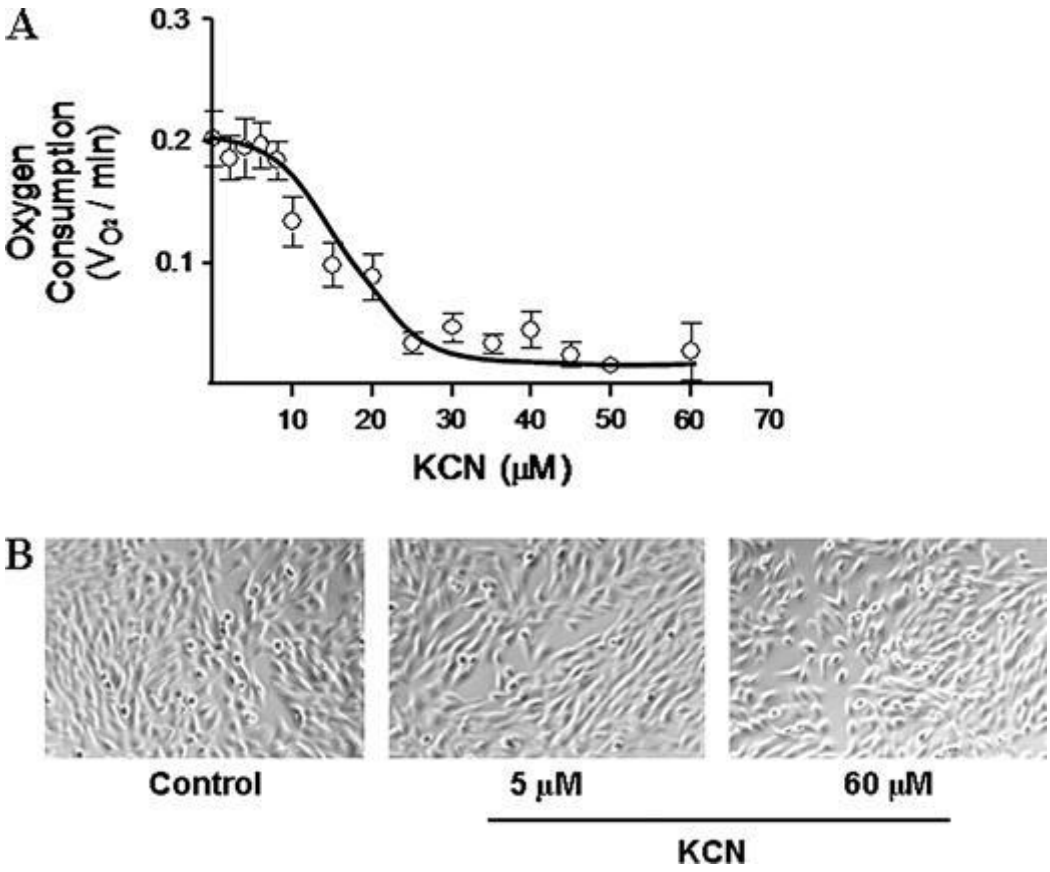

Figure 2. A)Dynamics of oxygen consumption after adding of 2–60M KCN to N27 strain of cells [52].

Reaction of cyanide with catalase was opened by Chance [53] in 1948 and has a formula, presented on Figure 3.

$$Cat(OH)_4 + 4HCN \underset{k_6}{\overset{k_5}{\rightleftharpoons}} Cat(CN)_4 + 4H_2O$$

Figure 3. Scheme of reaction of catalase enzyme with cyan acid [53].

As we can see CN^- ion can substitute hydroxyl group on the enzyme and limit its function of reduction of oxygen containing molecules as hydrogen peroxide. Accordingly to this the scheme

of reaction needed for potentiometrical detection of cyanide is performed in the Figure 4.

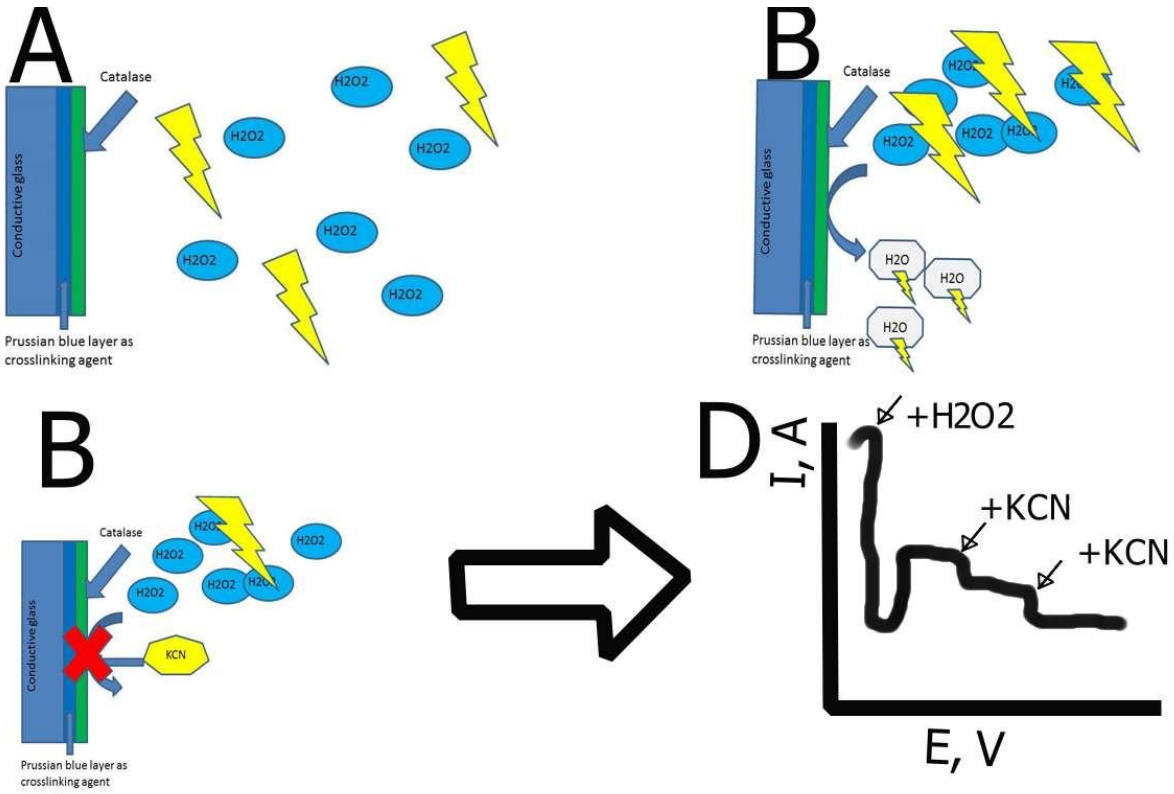

Figure 4. Hypothetical scheme of work of biosensor. A) Hydrogen peroxide causes strong negative increment of current in the cell; B) Catalase reduces hydrogen peroxide to water, causing a negative decrement of current around the electrode; C) Potassium cyanide inhibits catalase, increasing concentration of hydrogen peroxide around the electrode and causes subsequent negative increment of current; D) Hypothetical scheme of how chronoamperometry should look.

2. Materials and methods

2.1 Materials and equipment

2.1.1 Materials

Distilled water	
Potassium hexocyanoferrate	ROTH
Ferrum threechloride*6H_2O	ROTH
Pottasium chloride	Scharlau
Sodium dihydrogen phosphate dihydrate	Fluka
Sodium hydroxide	ROTH
Hydrochloric acid 35%	Sigma Aldrich
Catalase crystal dust	Sigma Aldrich
Pyrrol	Alfa aesar
FTO glass	Sigma Aldrich
Glutaric aldehyde	ROTH

Potassium cyanide	ROTH

2.1.2 Equipment

Analytical scales	RADWAG
Potentiostat	μAutolab PGSTST30
Optical microscope	Levenhuk
Mechanical pipettes	Eppendorf
Magnetic stirrer	Labochema
Ultrasonic bath	Codyson

2.1.2 Electrochemical cell

Working electrode	FTO glass/Prussian blue layer/ Catalase
Reference electrode	Ag/AgCl/KClsat
Counter electrode	Platinum/titanium electrode*

*****During experiment wasn't noticed significant difference between using platinum or titanium electrode as a counter.

2.2 Electrode preparation

1. Cutting electrode from conductive glass on plates for 1 on 2.5 cm. Marking the conductive side, checking it with the voltmeter by the resistance parameter.

2. Wash the electrode at micro 90 two times, acetone two times, using ultrasonic bath on 480 seconds. Then 2 times with acetone. Then 2 times in distillated water, the level of water must be lower than glass cover for stability. If it is not -> remove the water, refill the cleaner with distillate.

2.2.1 Prepare solution of Prussian blue

Prussian Blue preparation for 50 ml solution

Concentration:

- $K3[Fe(CN)_6]$ – 1 mmol/l – 0.01647 g.
- $FeCl_3$ – 1 mmol/l 0.0135 g.
- HCL 0.1 mol/l 0.45 ml.

Building the conductive cell For Prussian Blue deposition, fill the cell with solution and turn on the regime of cyclic voltammetry (staircase) which is should be normal.

Parameters of pretreatment for deposition of cyclic voltammeter are presented in Table 1.

First conditioning potential (V)	0.4
Duration (s)	0
Equilibration time (s)	1
Number of scans	40
Standby potential (V)	0.4

Start potential (V)	0.4
First vertex potential (V)	0.8
Second vertex potential	0.4
Step potential (V)	0.00405
Scan rate V/s	0.040002

Table 1. Parameters for cyclic voltammetry for deposition of Prussian blue on conductive glass

Scheme of correct placing of electrodes is presented on Figure 5.

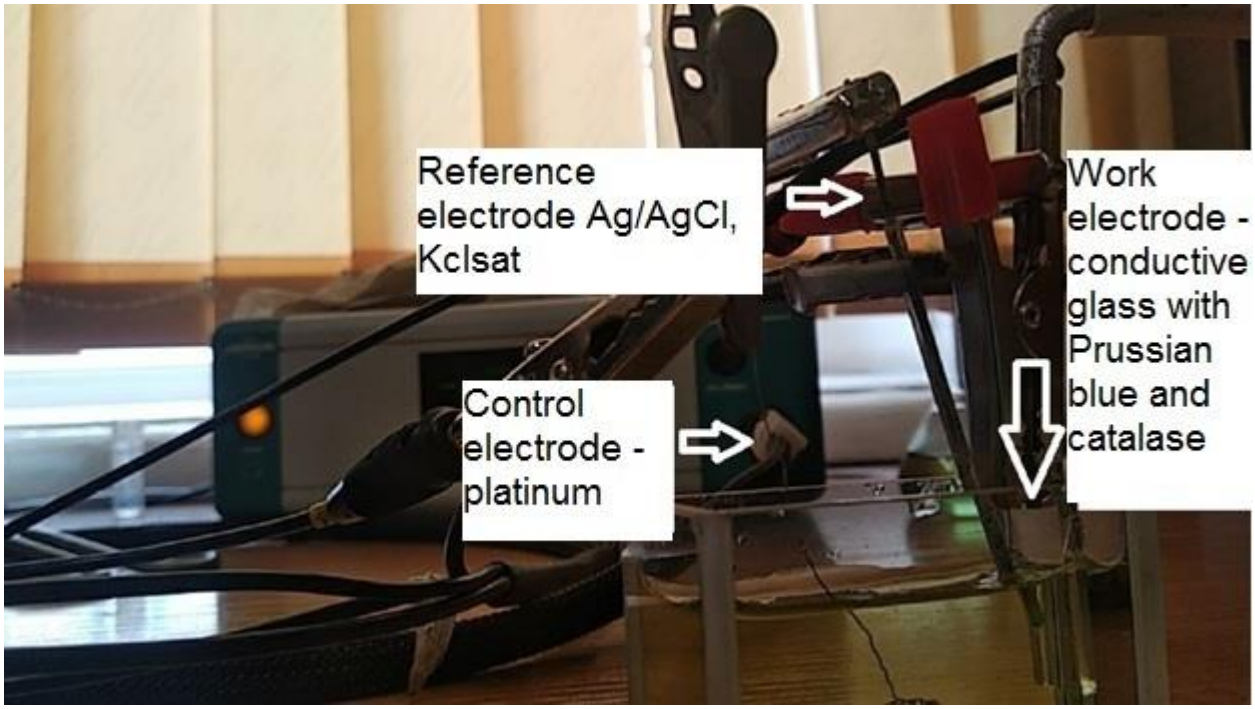

Figure 5. Correct position of electrodes for cell working

2.2.2 Prepare the Stabilization solution

After the deposition is needed to stabilize the Prussian Blue on the electrode. For that we need to get rid of the used pigment solution and fill the rinsed cell with Stabilization solution with next concentration for 200 ml solution

- 0.1 M KCl (1.48 g)
- 0.1 M HCl (1.8 ml)

After that turn on the Cyclic Voltammetry mode with next regime, presented on the Table 2

First conditioning potential (V)	0.45
Duration (s)	0
Equilibration time (s)	1

Number of scans	20
Standby potential (V)	0.45
Start potential (V)	0.45
First vertex potential (V)	0
Second vertex potential	0.45
Step potential (V)	0.00405
Scan rate V/s	0.040002

Table 2. Parameters for cyclic voltammetry for stabilization of Prussian blue on conductive glass

2.2.3 Prepare the solution of Catalase.

The base of our biosensor is the enzyme, which is called Catalase. The enzyme should be dissolved in buffer with next concentration.

- 500 ml H20
- 0.5 M KCl – 3.7231 g.
- 0.15 M – NaH2PO4 0.78 g.

After that should be taken **5 mg** of dry Catalase (weighting in Eppendorf) and diluted with **1 ml of Buffer.**

Then we should cover with completely dissolved enzyme our glass electrode, covered with Prussian blue and wait until it dries. Take 15 ul of solution and cover 1cm^2 of electrode

After that Enzyme should be stabilized on the glutaraldehyde vapor for 15 min to cross-link Catalase on the surface of optical slides (do not drown glass in the solution!) as shown on Figure 6.

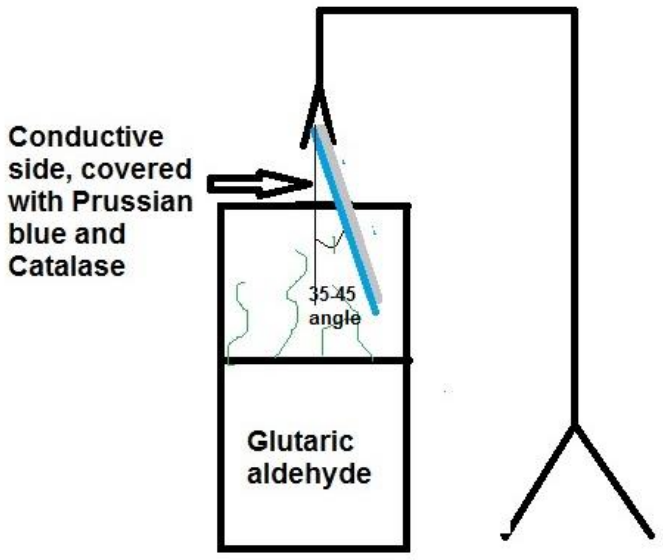

Figure 6. Stabilization of Catalase with Glutaric aldehyde vapor

2.2.4 Immobilization with pyrrole

Alternatively to the crosslinking with glutaraldehyde was examined a possibility of immobilization of catalase on pyrrol. The procedure was taken from Valiuniene et. al.[51] and parameters for cyclic voltammetry can be seen on Table 3. This procedure including the same deposition and stabilization stages as in 2.1 – 2.3 chapters, but after that instead of simple drying of catalase solution and crosslinking with glutaraldehyde vapor, there is added the third cyclic voltammetry in the next solution for 20 ml.

- $K3[Fe(CN)_6]$ – 1 mmol/l – 0.01647 g.
- $FeCl_3$ – 1 mmol/l 0.0135 g.
- Pyrrol 30 mmol/l 15 ml.
- Catalase 15 mg
- H_2O – 5 ml (appr.)

First conditioning potential (V)	0.4
Duration (s)	0
Equilibration time (s)	1
Number of scans	20
Standby potential (V)	0.4
Start potential (V)	0.4
First vertex potential (V)	0.8
Second vertex potential	0.4
Step potential (V)	0.00405
Scan rate V/s	0.04

Table 3. Parameters for deposition of immobilized catalase on pyrrole

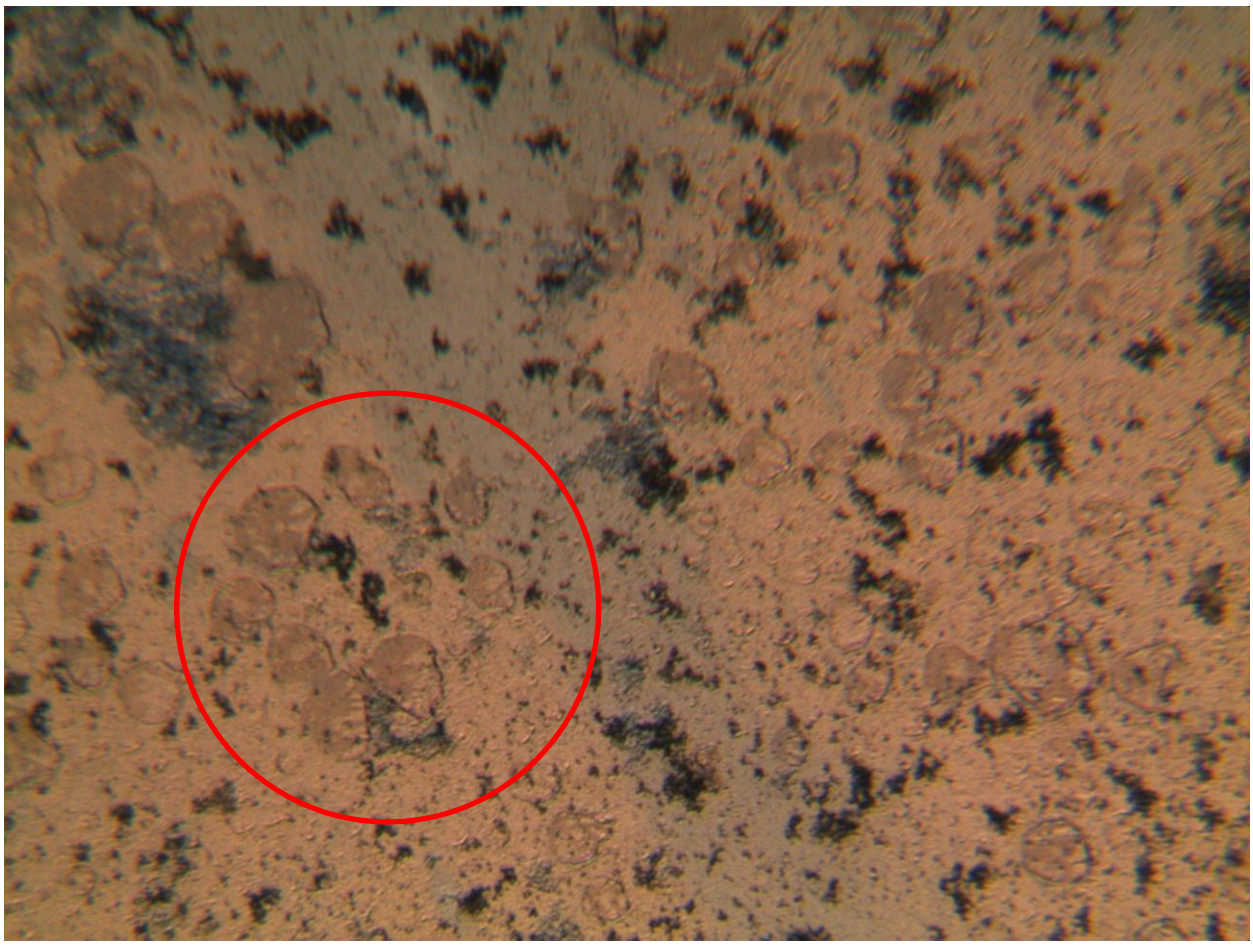

Figure 7. Micropicture of pyrrol immobilized biosensor (x20) with distinguishable pyrrol/catalase "bubbles"

After immobilization pyrrole produces a bubble structures of immobilized catalase as shown on the micropicture Figure 7.

2.2.5 Prepare the experiment

Choose the Chronoamperometry mode at GPES Manager and put all the needed parameters, which are presented on the Table 4.

First conditioning potential (V)	0
Duration (s)	0
Equilibration time (s)	0
Interval time	1
Standby potential (V)	0
Number of potential steps (1-10)	1

Table 4. Parameters for chronoamperometry for investigation

Build conductive cell again and fill it with 20 ml of Buffer, used for Catalase dissolving , put the cuvette on the magnetic stirrer, put magnet inside the cuvette. Check if the equivalent

current is stable (shouldn't be changed). If it is changed check all the electrodes and crocodiles. If you don't see the E (equilibrium potential value), turn off the sleep mode of autolab (Zzz button)

2.2.6 Starting experiment

After stabilizing the current on the plateau on after 200 seconds should be added reduction current substance, H_2O_2 in our case as shown on Figure 8.

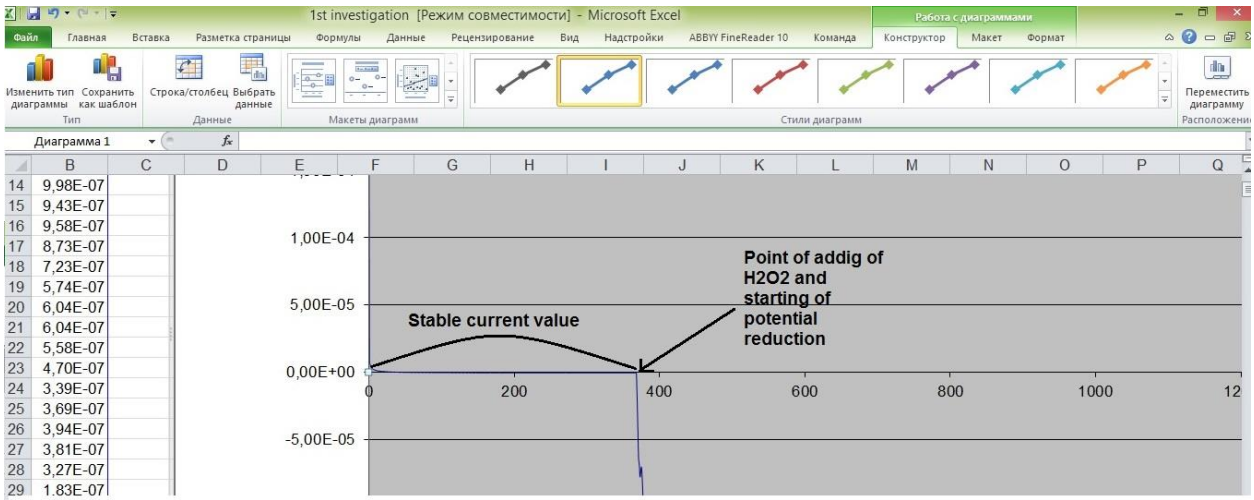

Figure 8. Current stabilization and point of Adding of H_2O_2

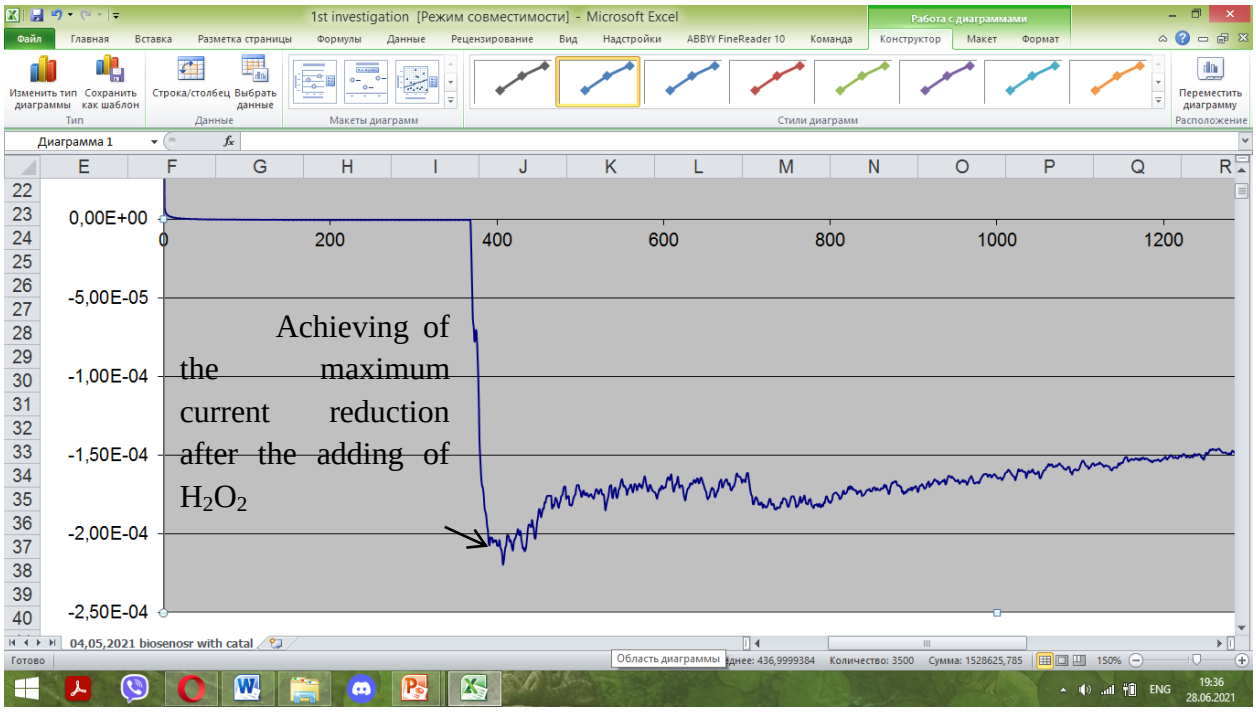

Figure 9. Achieving of the current reduction maximum

After that with a stable period of time (every 100 seconds for example) and mixing on the magnetic stirrer, should be added the solution of Cyanide ions with stable concentration as shown on the Figure 9.

3. Results and discussion

3.1 Experimental data

The electrochemical experiment has consisted of three parts, deposition of Prussian Blue (Fig. 10), its Stabilization (Fig.13), control experiment with no Catalase (Fig.14) and Chronoamperometrical investigation (Fig.17). On the Figure 10 is presented plot of cyclic voltammetry where a solution of Prussian blue is depositing on FTO glass. This process requires 40 cycles of current and two main peaks of the plot what points the normality of the process. It has the parameters pointed in the Table 1 of Materials and Methods chapter. After deposition look of the electrode changes to the cyan blue, as shown on the Figure 11. Also with the optical microscope can be seen islands of crystalized catalase, as can be seen on micropicture on the Figure 12.

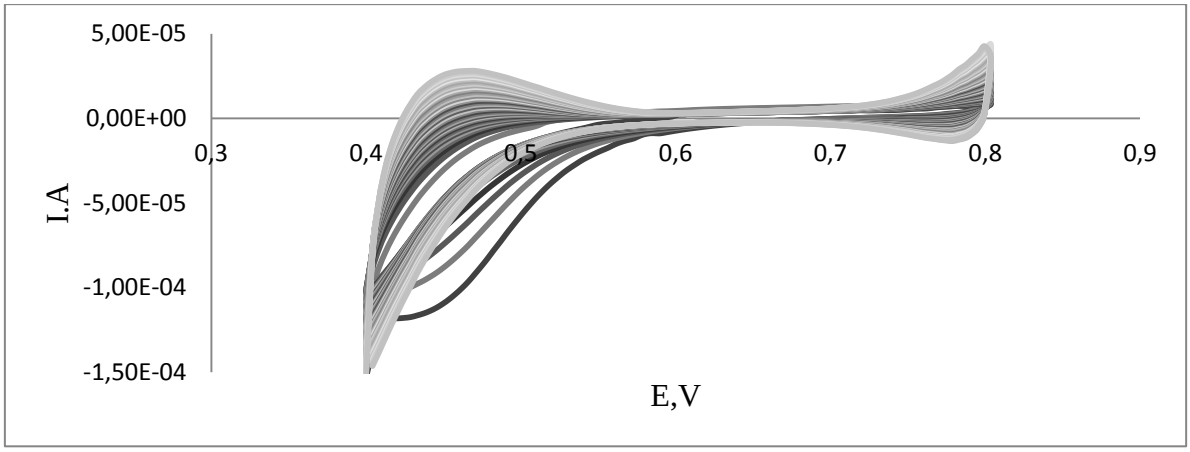

Figure 10. Typical cyclic voltametric plot of the deposition of Prussian blue on FTO glass.

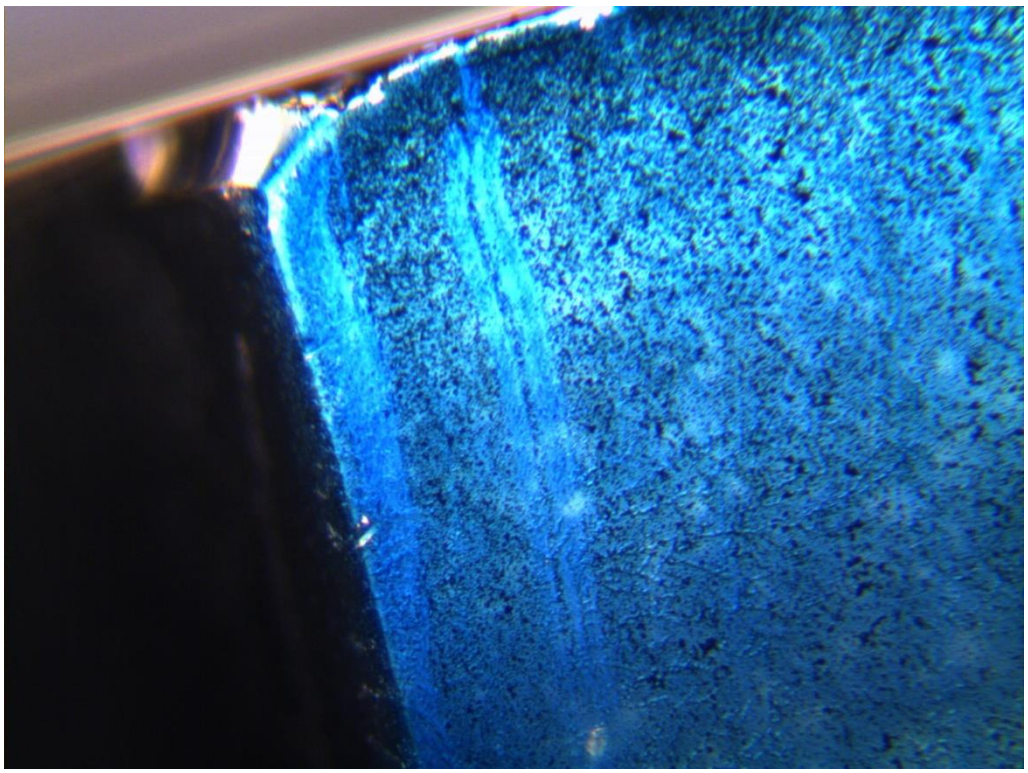

Figure 11. Micropicture of electrode with deposited Prussian blue on x10 zooming

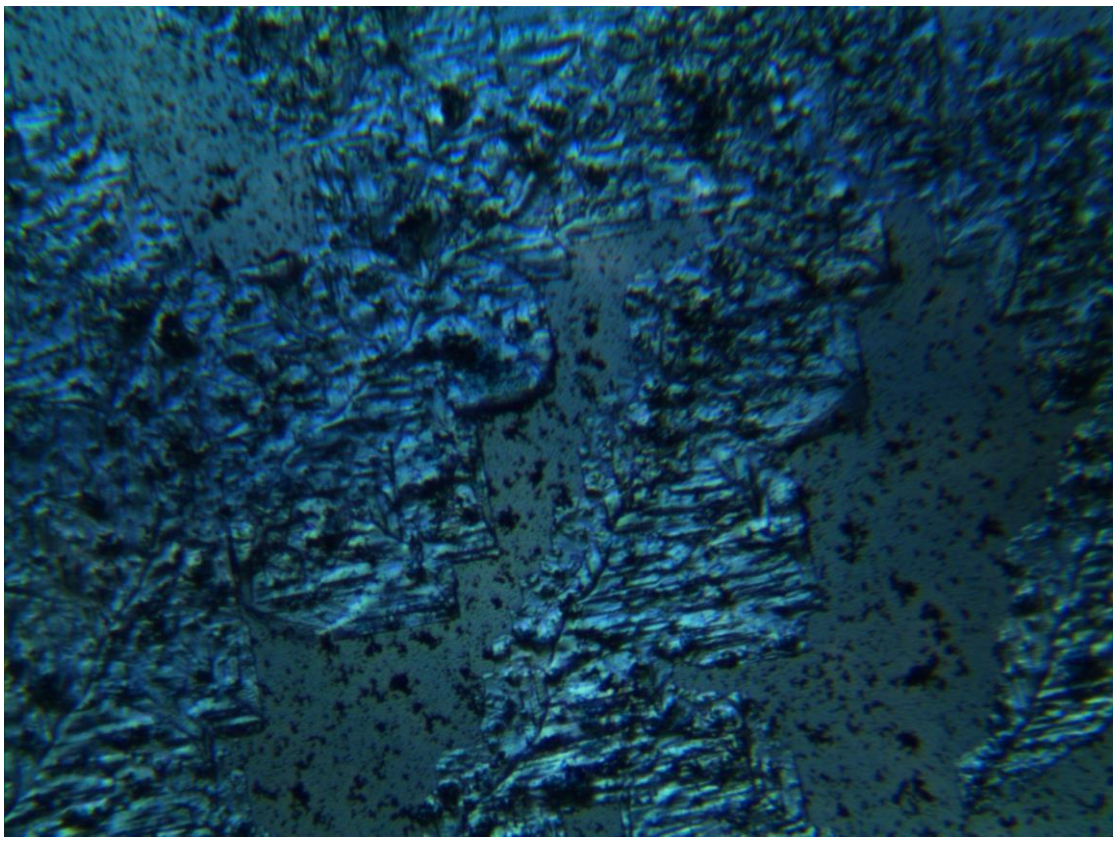

Figure 12. Micropicture of crystalized catalase on the electrode on x20 zooming

The Figure 13 plot is similar to the deposition but there is different potential and it is occurred in a specific solution of KCl and HCL, its parameters are presented in Table 2 of Materials and Methods chapter.

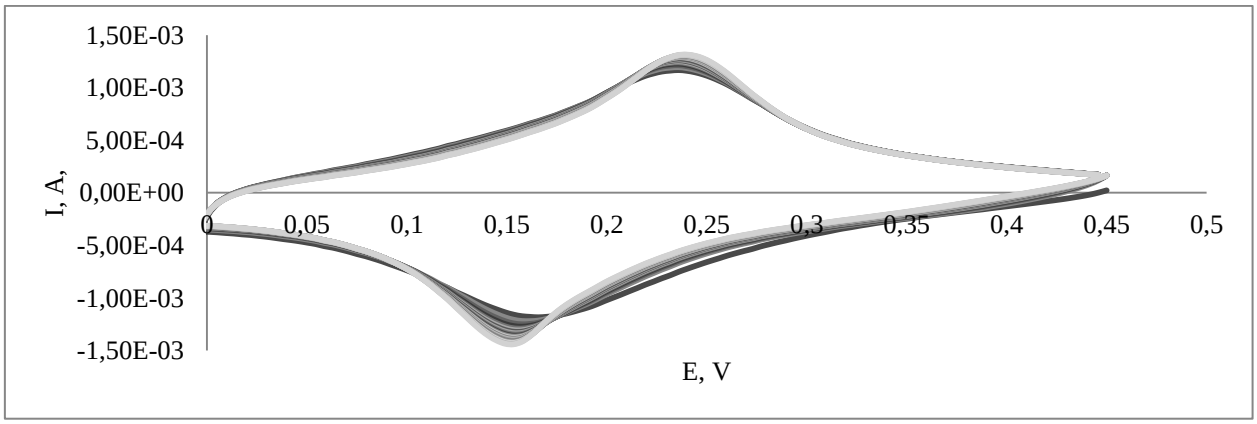

Figure 13. Typical cyclic voltametric plot for the stabilization of Prussian blue on FTO

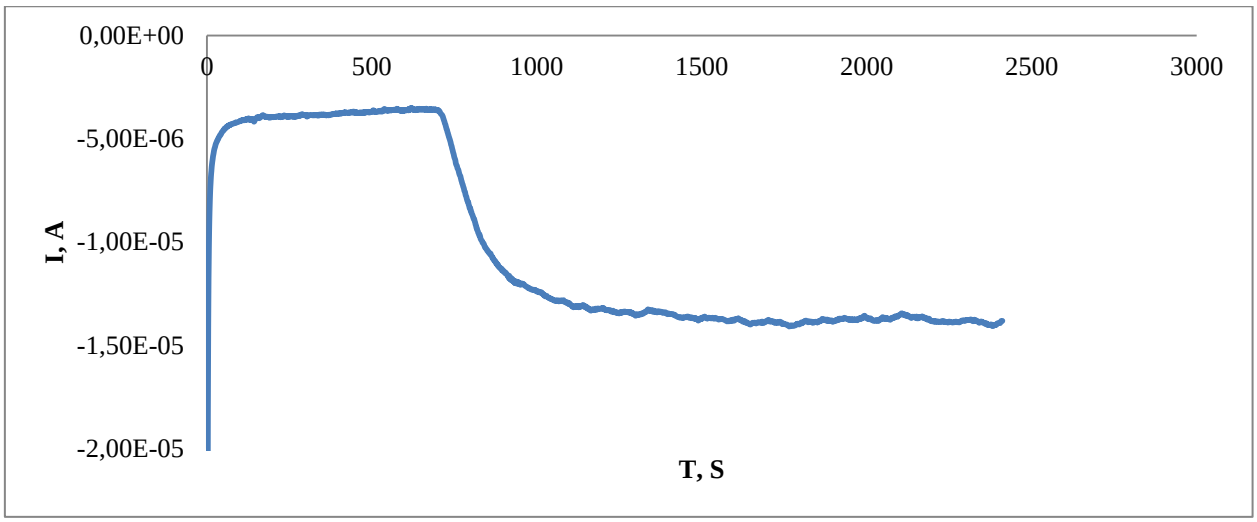

Figure 14. Chronoamperometric plot for control experiment without catalase on the electrode

t,s	I, uA	C(H_2O_2) Mol/L	C(KCN) Mol/L
100	-10	0	0
200	-20	0	0
300	-20	0	0
400	-20	0	0
500	-20	0.0000075	
600	-25	0.0000075	4.9975E-05
700	-25	0.0000075	7.49438E-05
800	-25	0.0000075	0.000175
900	-25	0.0000075	0.000175
1000	-25	0.0000075	0.000175
1100	-25	0.0000075	0.000175

1200	-25	0.0000075	0.000175
1300	-25	0.0000075	0.000574819
1400	-25	0.0000075	0.00049975
1500	-25	0.0000075	0.000749438
1600	-25	0.0000075	0.000999001
1700	-25	0.0000075	0.001248439

Table 5. Datasheet for chronoamperometry for investigation of control electrode without catalase

Plot presented on the Figure 14 is a control experiment with the working electrode with cover of Prussian blue on it, but without a solution of catalase what shows us a clean reaction on Hydrogen peroxide on the 800 seconds and no responses on cyanide ions, what can be seen on the datasheet in the Table 5.

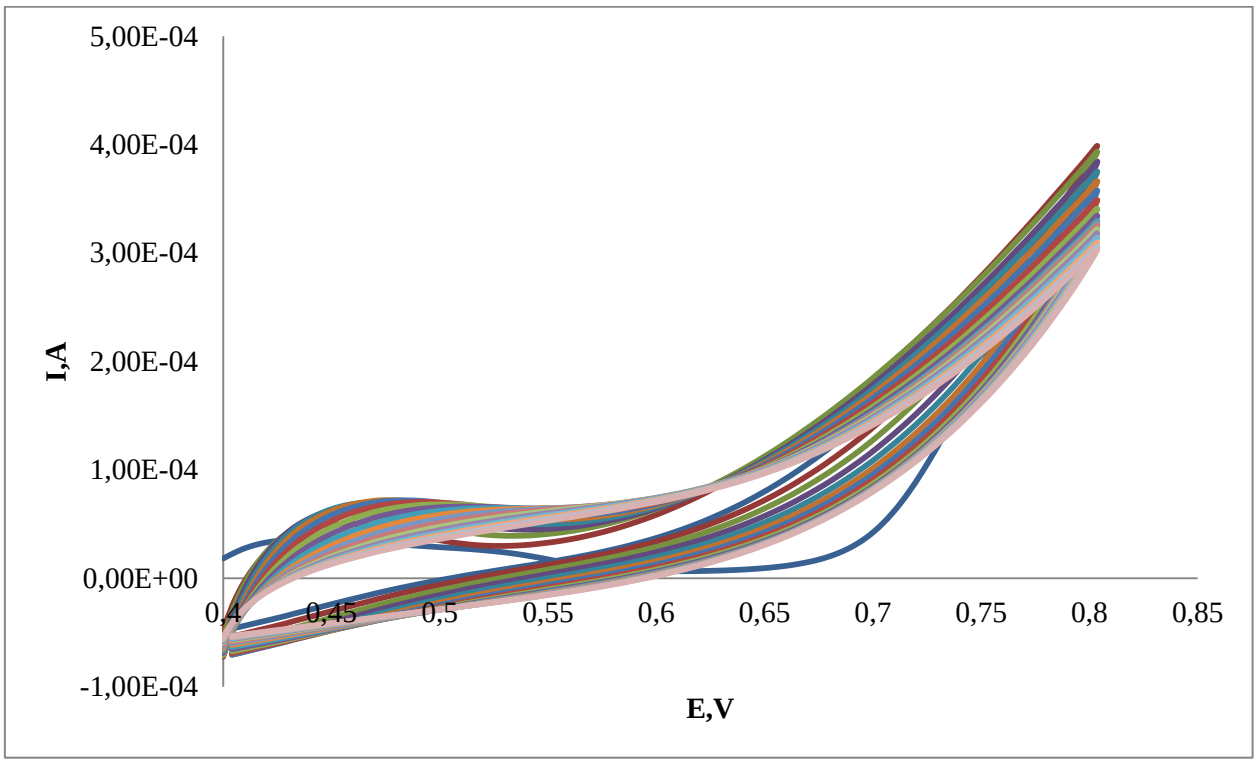

Figure 15. Cyclic voltametric plot for the deposition of pyrrole/prussian blue immobilized catalase on Prussian blue/FTO electrode

The Figure 15 shows the normal process of deposition of Pyrrole/PB/catalase solution on FTO electrode covered with Prussian blue. It requires 20 cycles of current and two main peaks of the plot what points the normality of the process. It has the parameters pointed in the Table 1 of

Materials and Methods chapter. This part of the experiment was provided as an alternative to the glutaraldehyde immobilization.

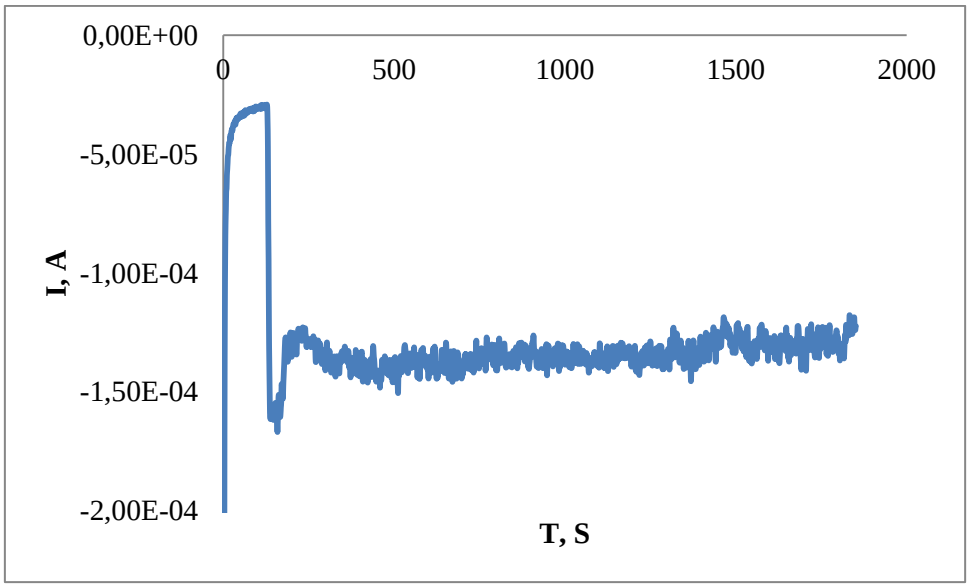

Figure 16. Chronoamperometry plot of biosensor with catalase immobilized on pyrrole

As can be seen on a Fig. 16 biosensor with pyrrol immobilizing shows very distinguishable reaction on hydrogen peroxide on 120 seconds but after that no reaction on any cyanide adding neither 0.1 mol, nor 1 mol stock solution as can be seen on the datasheet in Table 6.

t,s	I μA	C(H_2O_2 (3%) Mol/L	C(KCN) Mol/L
100	-0.028	0	0
127	-0.029	0.0000075	0
420	-0.146	0.0000075	2.49938E-05
580	-0.137	0.0000075	4.9975E-05
680	-0.138	0.0000075	7.49438E-05
800	-0.135	0.0000075	9.99001E-05
940	-0.136	0.0000075	0.000124844
1040	-0.134	0.0000075	0.000374782

1080	-0.136	0.0000075	0.000624594
1300	-0.134	0.0000075	0.000874282
1400	-0.135	0.0000075	0.001123845
1600	-0.131	0.0000075	0.001373283
1720	-0.133	0.0000075	0.001622597
1820	-0.135	0.0000075	0.001871787

Table 6. Datasheet for chronoamperometry for investigation with biosensor with pyrrole

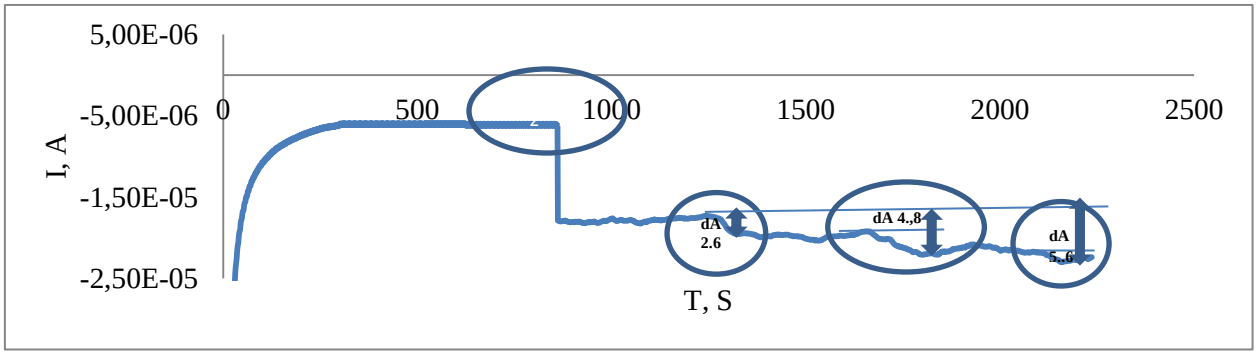

Figure 17. Typical chronoamperometric plot for the stabilization of Prussian blue on FTO with shown responses on cyanide adding in concentration of stock solution of 1 Mol/L

Figure 17 demonstrates a chronoamperometric plot, showing reaction on hydrogen peroxide and subsequent current decrement after adding of Potassium cyanide. Was achieved to detect three points of decrement after adding of Potassium cyanide, and counted dA(difference in flux from the stable reaction on hydrogen peroxide to the decremented area). All of it can be observed in the datasheet in the Table 7. It can be seen that current falls from the -6 uA, to -17.5 uA after the adding of hydrogen peroxide. In first time after first addition of cyanide was observed a distinguishable response from -17.6 uA to -18.9 uA. The second response was observed when concentration of cyanide has achieved 1.9E-04 and negative current increment was shown from -19.8 uA to -22 uA. And when concentration of cyanide has achieved 0.00029991 current increment achieved 0.7 uA from -22 uA till -22.7 as it can be seen in the Table 7.

t,s	I μA	C(H_2O_2 (3%) Mol/L	C (KCN) Mol/L
100	-9	0	0
200	-7.5	0	0

300	-6	0.0000075	0
900	-17.5	0.0000075	0
1200	-17.6	0.0000075	1.00E-04
1300	-18.9	0.0000075	1.00E-04
1400	-19.8	0.0000075	1.00E-04
1500	-20.2	0.0000075	1.00E-04
1600	-19.8	0.0000075	0.00019996
1700	-20	0.0000075	0.00019996
1800	-22	0.0000075	0.00019996
1900	-20.8	0.0000075	0.00019996
2000	-21	0.0000075	0.00019996
2100	-22	0.0000075	0.00019996
2200	-22.7	0.0000075	0.00029991
2300	-22.7	0.0000075	0.00029991

Table 7. Datasheet for chronoamperometry for investigation with biosensor with responses on cyanide adding.

Figure 18 is a calibration curve, with the points obtained by the calculation by the formula $dA_1 = (I_1-I_0)/I_1*100$, $dA_2 = (I_2-I_0)/I_2*100$ and etc. This line is showing that on this plot subsequent adding of cyanide decreases strength of responses with each addition because of high instability of sensor it wasn't possible to obtain more points because in most cases current has a tendency to destabilize with a time of using biosensor.

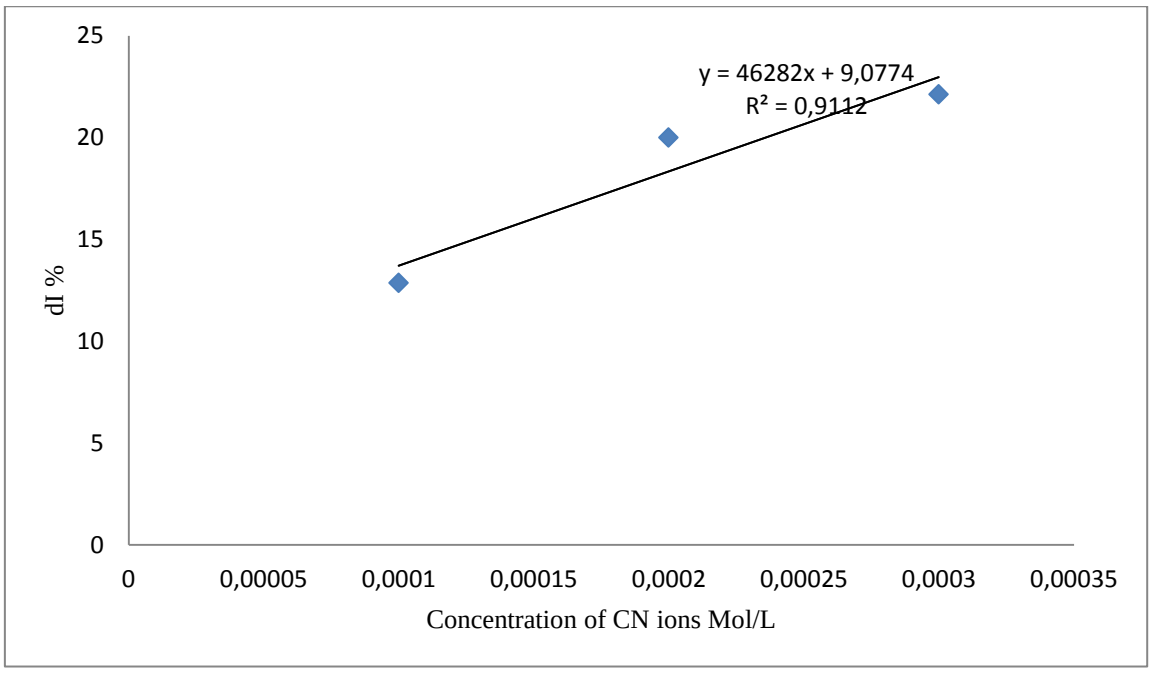

Figure 18. Dynamics of response values in % according to concentration of CN^- in 1 Mol/L stock solution of cyanide.

4. Conclusion

Result of the experiment series has shown some possibility of the electrochemical biosensor on the base of catalase for detection of cyanide ions. But method needed to be modified for the better reliability of responses and using the biosensor. This method shows a pretty unsustainable result due to the current instability on electrode and high possible influence of the human factor. In comparison with other techniques of cyanide detection this method has the following advantages:

- Relatable simplicity in preparation of procedure
- Lack of need in high equipment cost equipment beside of potentiometer
- Were detected a responses from close to nanomolar concentration of cyanide to unimolar ranges.
- Short time of response (approximately 1-3 seconds)

And following disadvantages:

- Low repeatability
- High level of current noise and low accuracy
- Possible degradation of sensor with subsequent uses
- High influence of human factor

The probable reasons of unsustainability of work of biosensor are probable fast degradation of Catalase/Prussian blue complex. Mix of cyanide and hydrogen peroxide can lead to the unwanted reaction $KCN + H_2O_2 \rightarrow KOCN + H_2$ and subsequently recruit iron, what can lead to the degradation of Prussian blue layer [54].

The cyanide inhibition in the catalase reaction has been reported to be noncompetitive in spite of the fact that cyanide and H_2O_2 compete for the same site on the catalase molecule. At high concentrations of H_2O_2, however, the inhibition became clearly competitive [55].

This unpredicted reaction can make the great influence on the biosensor work.

The way of solving this problem:

1. Probably to change the H_2O_2 on some other reduction current agent and to change the enzyme as well selective to cyanide as catalase.
2. Somehow find an additional protective agent, which can prevent the reaction of H_2O_2 with CN^- ions but not with the catalase. Maybe to mix CN- ions with some agent which can protect it from the reaction with H_2O_2, add some enzyme to the biosensor as a reactor which can unbind the CN^- ions and immediately include them in reaction with catalase.
3. To continue experiment with buffer content, stirring and potentiometery parameters
4. To decrease human factor procedure is needed to be better standardized. It can be achieved with modification of reaction cell as shown for example on the Figure 19.

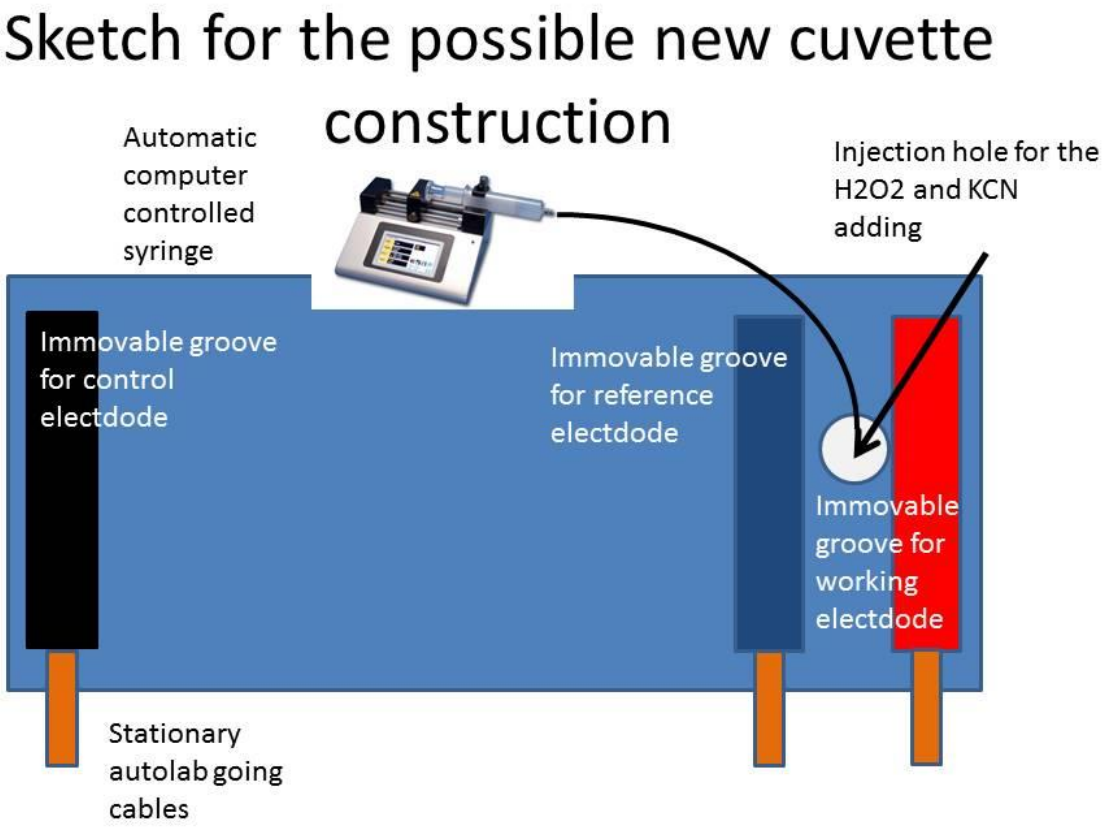

Figure 19. Possible cuvette construction

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