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Electroconductive Polymers in (Bio)chemical Sensors

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In this paper, research concerning application of electroconducting polymers in biosensors is discussed. Selection of the electropolymers has been limited to those which monomers are water-soluble, such as: polypyrrole and polyaniline. In general, experiments concerning EP utilization in dehydrogenase based biosensors were divided in four stages: (1) incorporation of mediators as dopants of the EP's layer, (2) immobilization of cofactors e.g. NAD+/NADH, (3) immobilization of enzymes and (4) immobilization of all components: mediator, cofactor and enzyme. As a dopant of the PPy water-soluble electroactive compound ferrocyanide was used. Another EP, namely polyaniline was also tested as an electrode material for NADH detection based on its electrochemical oxidation. In the case of simultaneous immobilization of mediator, NAD, and ADH in the PPy layer, as a result of the ethanol oxidation, the oxidation peaks of the mediator become smaller whereas the oxidation peaks of PPy increase. In the analyzed EtOH concentration range (0 – 2 mM), slope of calibration curve for the PPy oxidation peak was around 20 μA/mM. In the case of PAn deposition on the platinum electrode, decrease of the oxidation potential for NADH by 0.4 V was observed.

K e y w o r d s: NAD/NADH, enzymatic biosensors, electroconductive polymers, polypyrrole, polyaniline, electroactive mediator

1. Introduction

Electrconductive polymers (EP) discovered near 30 years ago have recently aroused growing interest in the design of different transducers because they offer controlling of different parameters such as conductivity and chemical, optical and mechanical properties. A very important group of these devices are biosensors – a main subject

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of this paper – including these for *in vivo* monitoring of different analytes, e.g. drugs or metabolites, because of biocompatibility of these materials and possibility of their photolithography that enables miniaturization of such devices. The importance of development of the conductive polymers was highlighted through the Nobel Prize in Chemistry for authors of this achievement – Alan J. Heeger, Alan G. MacDiarmid and Hideki Shirakawa in 2000 [1].

Electroconductive properties of EPs result from the specific single and double bonds configurations (π-conjugated system) in the main backbone chain. Electrons may be transferred along or between the backbone chains. Particularly important feature of EPs is their ability to change their conductivity in a very wide range. Depending on kind and concentration of doping compounds – *p* or *n* type as well as different physical factors (e.g. photo-doping, built-in electrical charge conductive polymer in MIS structure) the EP conductivity may be changed even by 18 orders of magnitude (Fig. 1) [2–6]. Therefore, they might exhibit conductive properties similar to metal conductors, semiconductors or insulators. Their energetic gap (forbidden band) for organic semiconductors may be matched in the range of 0.5–5 eV [3–7]. The important oxidizing dopants (*p* type) are the following: iodine (I_2), AsF_5, BF_3, $FeCl_3$, $SnCl_4$, $NOPF_6$ and acids of the HX type, where X = F, Cl, Br, I; and reducers (*n* type) – alkali metals and their compounds.

Several mechanisms explaining the charge carrier flow along or between the backbone chains of EP exist [8]. In one of them, the charge carrier flow is considered as a series of redox reactions that undergo between fragments of the EP chains. This corresponds to transportation of electrons and/or holes. The reactions determine the charge carriers' mobility [9]. An explanation of the optical properties of EPs, similarly as in inorganic semiconductors, includes: generation of charge carrier pairs by the absorbed photons, their recombination resulted in photon emission and partial transferring of energy to the EP net's vibration, and photon emission due to changes of quantum state of the electron carriers [10, 11].

The following chemical properties of EPs are utilized in construction of (bio)chemical sensors: (1) ability to easy immobilization of different, (bio)chemically active agents, e.g. ionophores, enzymes, antibodies, DNA, nucleic acids, cells fragments; (2) possibility of ion implantation into polymer; (3) ion exchange reactions between polymer and electrolyte; (4) redox mediator properties; (5) dependence of redox reaction on applied voltage; (6) possibility to resistivity adjustment in a very wide range through chemical reactions, e.g. redox; (7) photochromizm and (8) selective permeability of different substances (molecules and ions sewing) [12–19].

A large variety of physical states (liquid, gel, liquid crystal, solids) and features (water solubility, plasticity, easy in processing, springy, porosity, piezoelectricity) of EPs enables utilization them in manufacturing of components of different shapes (blocks, plates, membranes, thick and thin layers, nanotubes, nanowires) that are very useful in (bio)sensors' technology [20–23]. Although there are many of electroconductive polymers (EP) of excellent physical and chemical properties, however, in

biosensors their number is practically limited to those that are water-soluble. This limitation is mainly due to necessity of incorporation of bioreceptors into the EP-based membranes [12–18].

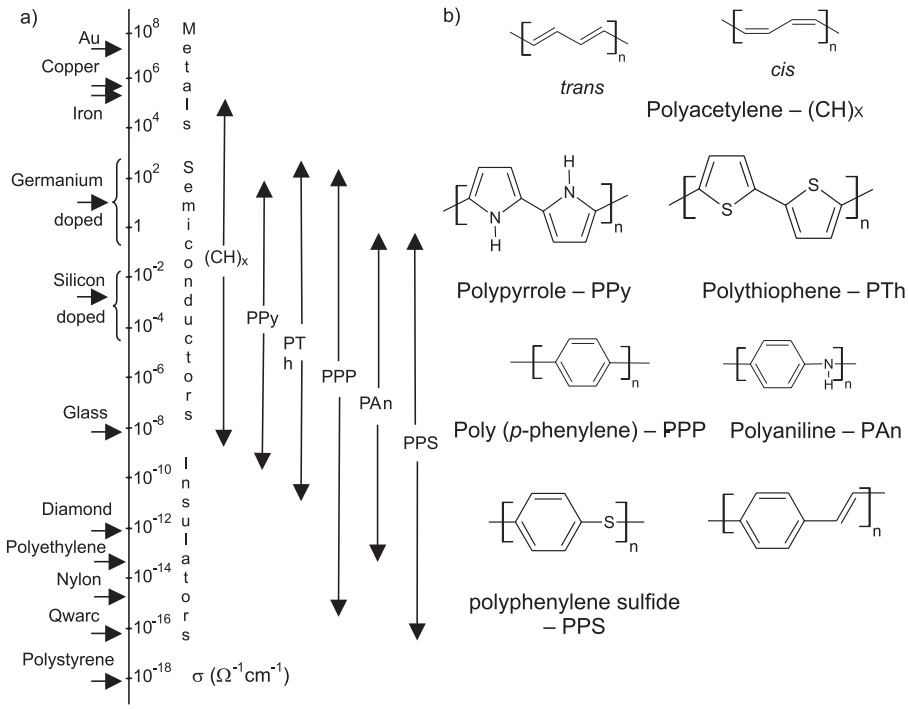

Fig. 1. Basic forms of the electroconductive polymers: a) conductivity scale and ranges, b) chemical structures

Basing on the great diversity of the electroconductive polymers, many miniaturized electronic components and devices such as: chemical sensors and biosensors, resistors, conductors, capacitors, diodes, transistors, lasers, light emitting diodes and transistors, displays, energy sources, and even electromagnetic shields have been developed [19–22]. It is expected that fully organic biosensing systems may be manufactured in the near future [22–27].

Referring to the biosensors, the EP-based biochemically sensitive membranes may be deposited on majority of known analytical transducers to design electrochemical (amperometric, voltamperometric, potentiometric, conductometric), mass, or optical biosensors, where the electroconductive polymers play the well defined roles, either directly in the sensing mechanism or indirectly through immobilization the species (bioreceptors) responsible for selective sensing of analyte in a sample. The EP-based biosensors are reviewed in many papers, e.g. in [12–15, 18, 22,

28–33]. Analysis of these papers indicates that the majority of the research works in the considered area is devoted to amperometry/voltamperometry. Enzymes used in amperometric biosensors, belong to oxidoreductases group – oxidases and dehydrogenases – that catalyse oxidation or reduction reactions.

The proper and long-term operation of the enzymatic amperometric biosensors requires regeneration of electron acceptors after the reaction. Oxygen as a mediator is used in the case of biosensors with oxidoreductases [34–38]. Dehydrogenases are the largest group in oxidoreductases [39]. Both, pyridine type cofactors, e.g. nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) participate in redox reactions in cells. In mechanisms of dehydrogenase type enzymes (DH), such coenzymes as: NADH or NADPH are involved – about 250 or 150, respectively. Stability of redox reaction with these coenzymes (cofactors) requires adequate conditions concerning the buffer composition and pH, electrode material or applied electrode polarization potential [40].

In this paper, application of EPs in the biosensors' design in which NADH cofactor is used, and exemplary utilizations of polyaniline and polypyrrole in the electrochemical biosensors for lactate detection will be reviewed. In particular, methods of the EP layers' deposition – mainly using electropolymerization – incorporation of enzymes and mediators, and application of these methods in the development of the biosensors with alcohol dehydrogenazes (ADH) is presented.

2. Amperometric Biosensors Based on Conductive Polymers and Dehydrogenases

NADH is utilized in amperometric biosensors with dehydrogenases for modification of the working electrode both, when it was made of inorganic material, e.g. gold or carbon and when EP was applied. In general, operation of these type biosensors, e.g. with DH enzymes immobilized on the working electrode, is based on the redox reactions related to the cofactor and analyte [34–39]:

$$NADH \xleftrightarrow{DH} NAD^+ + H^+ + 2e^- \tag{1}$$

$$NADH + H^+ + analyte_{ox} \xleftrightarrow{DH} NAD^+ + analyte_{red} \tag{2}$$

Depending on the working electrode construction, three generations of DH/NADH based biosensors are considered in literature [19, 34–38, 41]. In the first one (Fig. 2a), direct oxidation of NADH undergoes at conventional solid-state electrodes made of carbon, Pt or Au. The NAD is immobilized on the electrode or is a component of an analysed solution. In this case, the enzyme used in the biosensor for L-lactate determination was lactate dehydrogenase (LDH).

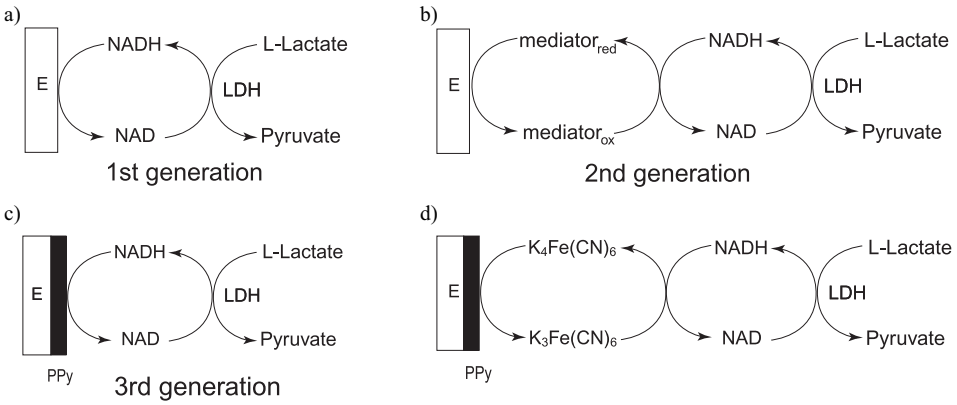

Fig. 2. Generations of amperometric biosensors with NAD/NADH cofactor

Because of high costs of NAD/NADH, the system providing regeneration of the cofactor, i.e. oxidation of NADH with the use of an electron mediator was developed (Fig. 2b). Ferrocenes (hexacyanoferrates) and metallic complexes, as well as *o*- and *p*-quinones, quinoneimines, phenylenediimines and their derivatives, are the commonly used mediators [34–38, 41]. The mediator can be immobilized on the electrode surface by different methods: adsorption, covalent attachment, inclusion in polymers and other electrode materials (for example in carbon paste [37]) and polymerization of the mediator itself, e.g. poly(methylene green) [42, 43]. In spite of rather well description of the oxidation process of NADH in literature, the redox reaction of the mediator at the working electrode is not presented in the adequate way. It was indicated that oxidation of the mediator on the electrode may be a source of extra side reactions that may influence on the biosensor stability. A competitive reaction of the mediator oxidation by atmospheric oxygen resulting in generation of hydrogen peroxide, mentioned in literature, may disturb regeneration of the mediator and the biosensor response [44].

As it was mentioned earlier, some doped EPs, e.g. polypyrrole (PPy), polyaniline (PAn) and polythiophene (PTh) exhibit mediating properties, so that they may be used as a mediator itself, resulting in the third generation of the biosensors (Fig. 2c) [12, 28, 34, 42, 45–49]. Utilization of PPy in this type of biosensors was most frequently described [45–47, 49].

In Figure 2d, a system consisting of pyrrole layer with an extra electron transfer mediator (ferricyanide) is presented. Co-deposition of ferricyanide and PPy increases conductivity of the doped polymer that results in improving of oxidation properties of the system [49]. This configuration combines advantages of both, mediators and electroconducting polymers.

It was expected that modification of the surface of sensing membrane by nanotubes in amperometric biosensors with NADH would improve their redox properties [36, 50, 51]. Despite of a large number of reports related to the

NAD/NADH-based enzymatic systems design, the practical realization of these biosensors is still under development.

3. NAD^+/NADH Type Biosensors

In this paper a research concerning application of electroconducting polymers in biosensors is discussed. Selection of the electropolymers has been limited to those that monomers are water-soluble, namely polypyrrole and polyaniline. This allows incorporation of biomolecules such as: enzymes, coenzymes, etc.

Oxidation process in polypyrrole is going from the neutral state through partially oxidized to the fully oxidized state. Charge neutrality of the deposited EP layer is maintained by counterions – anions introduced into the material. Each positive charge in the polymer chain is neutralized by the negatively charge an ion. If as a counterion an electroactive substance in ionic form is introduced into the polymer then conductivity of the polymer can be increased. This process is called doping of polymers.

In general, experiments concerning the EP utilization in dehydrogenase based biosensors can be divided in four stages: (1) incorporation of mediators as dopants of EP's layer, (2) immobilization of cofactors e.g. NAD^+/NADH, (3) immobilization of enzymes and (4) immobilization of all components: mediator, cofactor and enzyme.

In our experiments, as a dopant of PPy water-soluble electroactive compound ferrocyanide was used. Another EP, namely polyaniline was also tested as an electrode material for NADH detection based on its electrochemical oxidation.

3.1. Chemicals

Monomers of electropolymers: pyrrole, aniline, and cofactors: NAD/NADH, enzyme – alcohol dehydrogenase (ADH, EC 1.1.1.1, 451 U/mg) were purchased from Sigma-Aldrich. Mediators: hexacyanoferrates (II, III) – ferrocyanide/ferricyanide ($K_4[Fe(CN)_6]$ / $K_3[Fe(CN)_6]$) – were purchased from POCh, Poland. As a buffer 0.1 M tris(hydroxymethyl)methylamine (TRIS) solution containing 0.1 M KCl of pH 8.0 was applied. All other chemicals were of analytical grade.

3.2. Measurements

In the experiments, 3-electrode 0.2 μm-thick platinum layer amperometric sensors were utilized. The electrodes were patterned on a silicon/silicon oxide substrate and assembled on printed circuit boards. The sensors have been developed in our institute and fabricated in the Institute of Electron Technology (Warsaw). The three electrodes differed in sizes, in particular surface area of the working electrode (WE)

and the counter electrode (CE) was 1.3 mm^2 and 2.6 mm^2, respectively. As a reference electrode an electrochemically chlorided silver wire (i.e. Ag/AgCl wire) or a saturated calomel electrode (SCE) was used.

The electrochemical measurements were performed with a PalmSens potentiostat (Palm Instr. BV, The Netherlands) or a VMP2/Z potentiostat (PAR, USA). Both potentiostats were also used for electrochemical deposition of polyaniline and polypyrrole layers.

Electrochemical depositions of the EP layers were carried out in deareated solutions of monomers Py and An in 0.1 M KCl and 1 M HCl, respectively. For deposition of EP, electrochemical methods such as cyclic voltammetry (CV) and chronoamperometric method (CA) were applied.

All measurements by means of cyclic voltammetry (CV) and chronoamperometric method (CA) and deposition processes were performed at room temperature.

4. Results and Discussion

4.1. Immobilization of Mediator in PPy Layer

Conditions of electrochemical polymerization of pyrrole (Py) and Py with mediators were analyzed. Several water-soluble mediators, such as: methylene green, Meldola's blue and ferrocyanide/ferricyanide were investigated as reported in [49, 52]. In this paper, results concerning optimal conditions for immobilization of mediator, coenzyme and ADH are presented.

In the case of PPy layer deposition by means of the CV method, working electrode potential was changed from +0.95 V to –0.85 V with a scan rate 100 mV/s in 5 or 10 cycles whereas for the CA method potential was +0.85 V to +0.6 V for 1 to 5 min, respectively. Concentration of the Py monomer solution were of two types: without mediator - 0.1 M in 0.1 KCl or with a mediator – 50 mM $K_4[Fe(CN)_6]$ in 0.1 KCl.

Exemplary voltamperogramms for electropolymerization of pyrrole itself and with the mediator, in particular 50 mM $K_4[Fe(CN)_6]$ in 0.1 M KCl are shown in Fig. 3. The consecutive CV curves are wider with a number of cycles. The greater number of cycles the higher conductivity of the deposited layer and consequently, the higher oxidation/reduction peaks of pyrrole and ferricyanide. Since each CV curve corresponds to deposition of a PPy monolayer, the CV method is convenient for controlled deposition of PPy, namely layer-by-layer deposition.

Electropolymerization of pyrrole takes place at +0.25 V and +0.3 V for Py with mediator – visible as oxidation peaks (Fig. 3 (a) and (b)), which are higher when the mediator is immobilized. If the reduction peaks are considered, a single one occurs for the PPy layer, whereas two peaks are observed for PPy with the mediator layer, where the reduction peaks for PPy and PPy with the mediator occur at +0.15 V and –0.1 V, respectively.

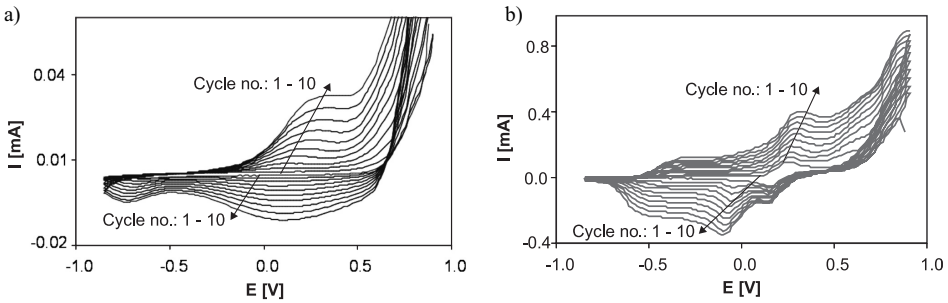

Fig. 3. Electrochemical polymerization of pyrrole (Py) without (a) and with mediator – $K_4[Fe(CN)_6]$ (b). In both (a) and (b) figures, the CV curves are wider with higher number of the cyclic voltammograms loops. Number of the CV curves reflects number of the deposited layers of the electropolymerized polypyrrole

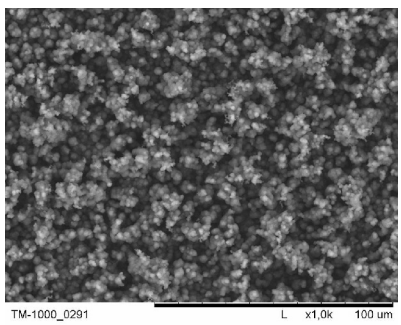

Fig. 4. SEM view of the electrochemically polymerized polypyrrole layer with the immobilized mediator ($K_4[Fe(CN)_6]$ / $K_3[Fe(CN)_6]$), magnification x 1k

Another feature of the EP modified electrode is expansion of its effective surface. SEM view of the PPy with immobilized ferricyanide is shown in Fig. 4. As can be seen, the surface is non-planar, rather 3D structure of a high roughness.

4.2. Immobilization of NAD and NADH in the PPy Layer

Next step in this study was related to immobilization of both forms of coenzyme oxidized/reduced – NAD/NADH within an electropolymeric layer by the CV method. Voltamperogramms of electrochemical polymerization of pyrrole with content of NAD and NADH are shown in Fig. 5. Immobilization of both NAD and NADH was performed in a 0.1 M KCl solution of fixed concentration pyrrole (0.1 M). Concentration of the cofactors in the Py solution varied from 1 to 10 mM. Content of NAD in the Py solution in the entire investigated concentration range affected the polymerization process in a very small extend in contrary to NADH, where decrease of conductivity of the PPy layer deposited from solutions containing above 5 mM NADH was observed (Fig. 5b).

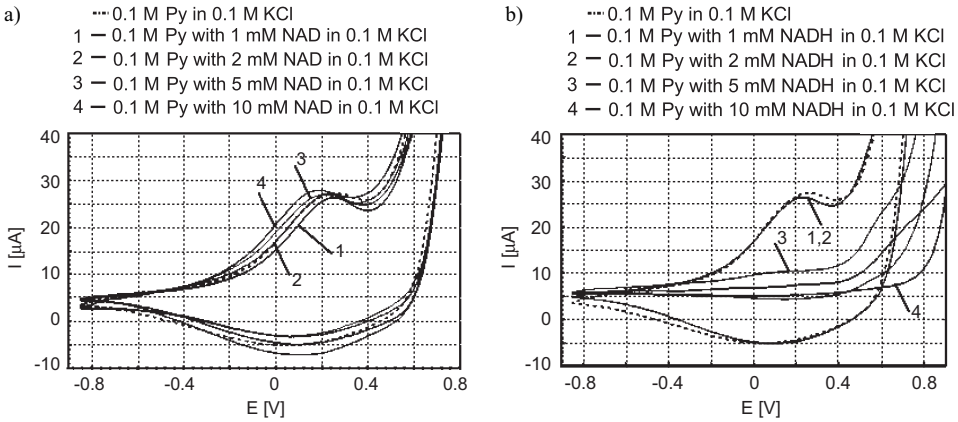

Fig. 5. Voltamperogramms (CV) for the 5th cycle of polymerization 0.1 M Py in 0.1 M KCl, containing different concentrations: 1, 2, 5 and 10 mM of NAD (a) and NADH (b). Order of appearance in legend is according to the order of the plotted CV curves in (b) and reversed in (a), excluding the doted line

Chronoamperogramms for polymerization of 0.1 M Py in 0.1 M KCl, containing different concentration of NAD and NADH by the CA method are shown in Fig. 6. The PPy/cofactor layers deposited by the CA method exhibit higher conductivity than those deposited by the CV method using the same solutions.

However, in the case of CA method used for deposition of the PPy layer containing cofactor, similar effect as for the CV method, namely drop of conductivity for the PPy/NADH layer deposited from the Py solutions containing above 5 mM NADH was observed (Fig. 6.b).

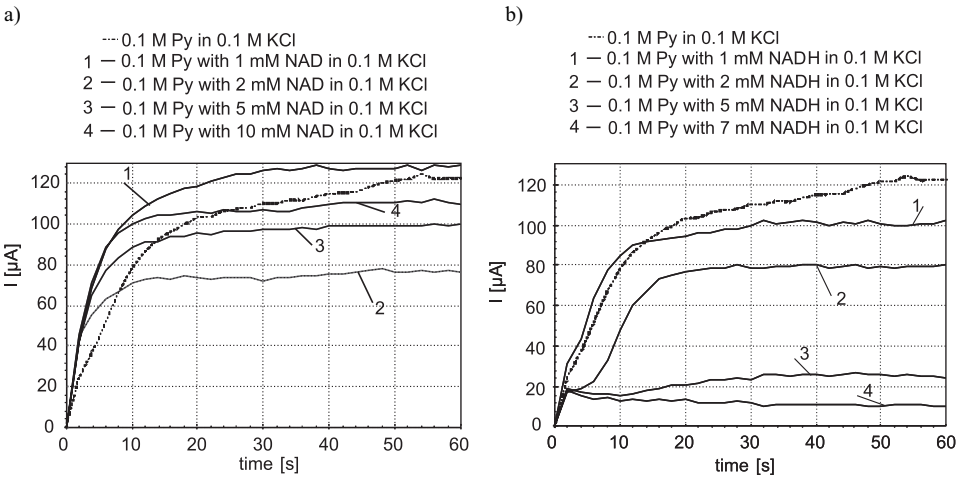

Fig. 6. Chronoamperogramms for polymerization of 0.1 M Py in 0.1 M KCl, containing different concentrations 1, 2, 5 and 10 mM of NAD (a) and NADH (b). Order of appearance in legend is according to the order of the plotted CA curves, excluding the doted line

For both CV and CA method, a high concentration of NADH (above 10 mM) in the Py solution obstructs the Py polymerization process.

4.3. Immobilization of Enzyme in the PPy Layer

The third stage of this study was devoted to immobilization of the enzyme – alcohol dehydrogenase (ADH) in the PPy layer. The enzyme was immobilized in PPy deposited in a solution of 0.1 M Py and 5 U of ADH/ml in 0.1 M KCl by the CV method in 10 cycles. As a substrate for the ADH, ethyl alcohol was used. Exemplary voltamperogramms before and after the enzymatic reaction oxidation of ethanol of concentration 4.19 and 7.99 mM in a presence of 10 mM NAD dissolved in a solution and ADH immobilized in the PPy layer are shown in Fig. 7.a. As can be seen, the oxidation current peaks at 0.1 V are smaller for higher concentration of ethanol (~8 mM).

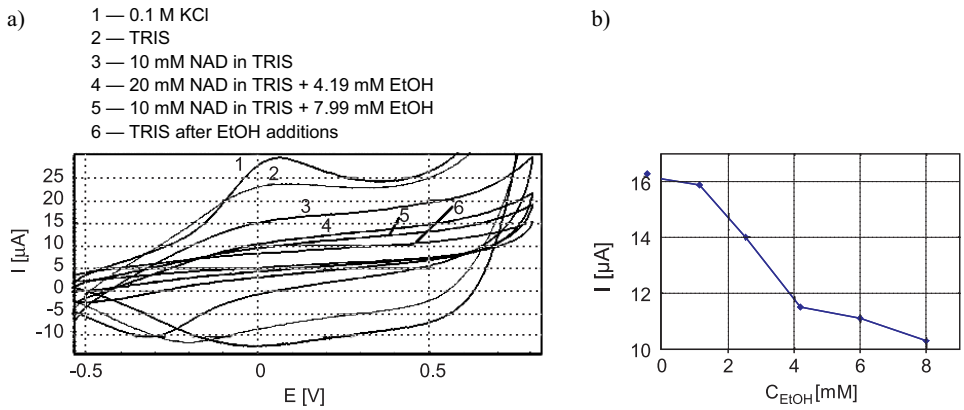

Fig. 7. Exemplary voltamperogramms before and after the enzymatic reaction oxidation of ethanol in presence of NAD dissolved in a solution and ADH immobilized in the PPy layer (a). Order of appearance in legend is according to the order of the plotted CV curves. Corresponding dependence between the oxidation current value at 0.1 V vs. SCE and concentration of ethanol (b)

Dependence between the oxidation current value at 0.1 V vs. SCE and concentration of ethanol is shown in Fig. 7b. The dependence is linear for the ethanol concentration range from 1 to 8 mM.

4.4. Immobilization of Mediator, Coenzyme and Enzyme in the PPy Layer

The third stage of this study was devoted to immobilization of all components: mediator, coenzyme and enzyme within the PPy layer. Each component was immobilized in the PPy layer by deposition from three separate solutions of 0.1 M Py in 0.1 M KCl containing 50 mM $K_4[Fe(CN)_6]$, 10 mM NAD and 20 U of ADH per 3 ml, respectively. Incorporation of the components in the electropolymerized PPy layers was performed by the CV method in 3 cycles, starting with the mediator, through

the cofactor and finally the enzyme solution. Exemplary voltamperogramms before and after the enzymatic oxidation reaction of ethanol of concentration 1 and 2 mM in the presence of ADH and cofactor – NAD immobilized in the PPy layer as well as the mediator are presented in Fig. 8.

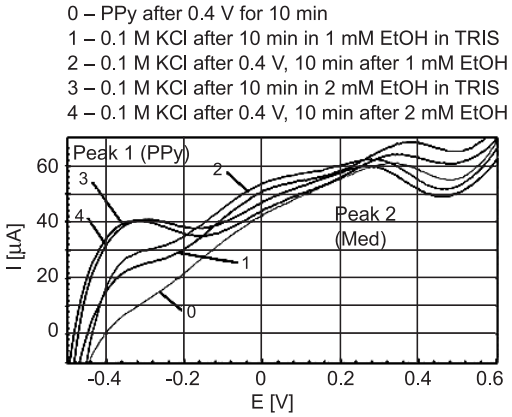

Fig. 8. Fragments of voltamperogramms before and after the enzymatic oxidation reaction of ethanol in presence of both NAD and ADH immobilized in the PPy layer

After deposition of the PPy layer, the electrode was polarized at 0.4 V vs. SCE for 10 min, which is an oxidation potential of ferrocyanide. Next, 10 µl droplets of 1 or 2 mM EtOH in the TRIS buffer were put on the sensor and left for 10 min. Afterwards, the sensors were tested in 0.1 M KCl solution. Between the measurements the sensors were rinsed with MQ water and oxidation potential of ferrocyanide was applied for 10 min.

As result of the ethanol oxidation, the oxidation peaks of mediator become smaller whereas the oxidation peaks of PPy increase. Both peaks shifts along the potential-axis but in opposite directions, in particular the oxidation peak of mediator (Peak 2) in negative from *c.a.* from 340 mV to 280 mV. In the analyzed EtOH concentration range (0–2 mM), slope of calibration curve for the PPy oxidation peak was around 20 µA/mM. For higher concentrations of ethanol (> 4 mM) – data not shown, a plateau of the calibration curve is observed. This might be due to too small amount of coenzyme immobilized in the PPy layer, which reacts with ethanol in equimolar quantity.

4.5. Polyaniline as an Electrode Material

Finally, polyaniline was used as the electrode material. The polyaniline was deposited from monomer solutions of different concentrations 0.2, 0.3 and 0.3 M in

1 M HCl by means of the chronoamperometric method at constant electrode potential +0.75 V for 3 min.

Chronoamperogramms for electropolymerization of An in the solutions of the above-mentioned concentrations are shown in Fig. 9. The inset shows dependence between concentration of the monomer and height of the oxidation current peak. During polymerization, the oxidation current increased with increase the An content in the solution. In result, the higher concentration of the monomer the higher conductivity of the obtained PAn layer was observed.

The obtained amperometric sensors with the PAn layer on the working electrode were tested to NADH. Comparison of time response and corresponding calibration curves for the modified Pt/PAn and bare Pt electrode obtained at fixed oxidation potentials of NADH are shown in Fig. 10. In the case of PAn deposition on the

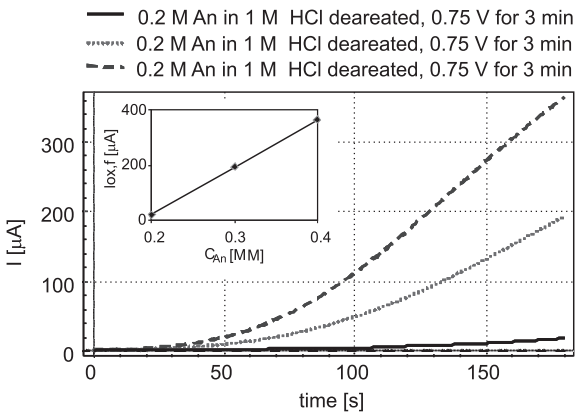

Fig. 9. Exemplary chronoamperogramms of polymerization of aniline from solutions of different concentrations of An, and dependence between the final oxidation current of polymerization and the concentration of aniline (inset)

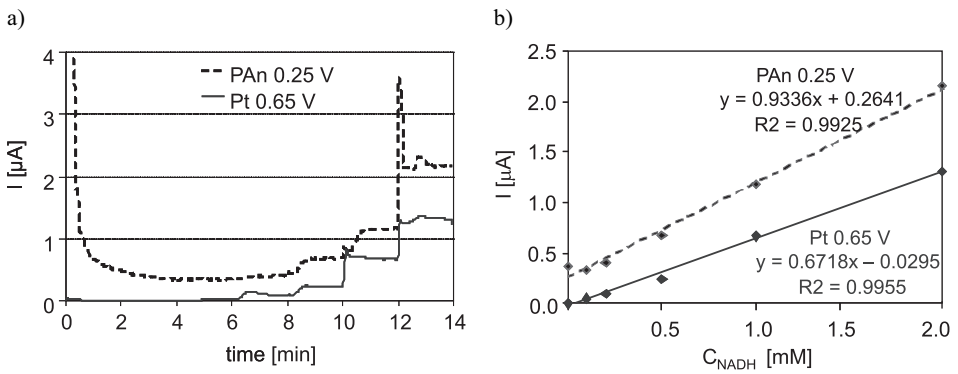

Fig. 10. Time response (a) and corresponding calibration curves (b) for the modified Pt/PAn and the unmodified Pt sensors obtained at the fixed oxidation potentials of NADH, namely +0.25 V and +0.65 V, respectively

platinum electrode, decrease of the oxidation potential for NADH by 0.4 V was observed, namely after the deposition of PAn the oxidation potential was +0.25 V whereas for the bare Pt was +0.65 V.

In the case of Pt electrode with the deposited PAn layer, the slope of calibration curve was greater than for the bare Pt one by *c.a.* 30%.

5. Conclusions

Summarizing, the electroconductive polymeric layers and their application in enzymatic biosensors based on NAD^+-dependent dehydrogenases were reviewed. Different approaches to the EP's layer preparation and technologies of the biosensors fabrication and the different methods of detection were described.

A method improving amperometric biosensors by applying electroconducting polypyrrole doped with the electroactive mediator (ferrocyanide) was presented. In the case of simultaneous immobilization of the mediator, NAD and ADH in the PPy layer, the oxidation peaks of mediator become smaller whereas the oxidation peaks of PPy increase. In the analyzed EtOH concentration range (0–2 mM), slope of the calibration curve for the PPy oxidation peak was around 20 μA/mM. In the case of PAn deposition on the platinum electrode, decrease of the oxidation potential for NADH by 0.4 V was observed.

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