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Title: Development of label-free impedimetric platform based on new conductive polyaniline polymer and three-dimensional interdigitated electrode array for biosensor applications

Article Type: Research Paper

Keywords: Interdigitated electrode array, DET, PQQ dependent enzyme, polyaniline, biosensor

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Abstract: Novel label-free impedimetric platform based on a three-dimensional interdigitated electrode array (3D-IDEA) sensor and new conductive polymer as a transducer for oxidoreductases is introduced. This platform is cost-effective, simple to construct and miniaturize. Monomer of conductive polymer N-(N',N'-diethyldithiocarbamoyl ethylamidoethyl)aniline (AnD) was deposited onto 3D-IDEA by chemical polymerisation. It was found that the polymer film resistance depends on the redox-potential of the solution. For the first time polyAnD was used as enzyme immobilisation matrix. Pyrroloquinolinequinone (PQQ) dependent alcohol and glucose dehydrogenases were immobilized on 3D-IDEA covered with polyAnD by two different methods. 3D-IDEA sensors with enzymes, which were immobilised by superficial adsorption on polyAnD layer, showed specific response in the presence of corresponding substrates with 1 μM sensitivity. Obtained results revealed that PQQ dependent dehydrogenases can re-oxidize on polyAnD via direct electron transfer (DET) from enzyme active site to the polymer surface. This process can be monitored by methods of electrochemical impedance spectroscopy (EIS) and chronoamperometry. Presented study shows that EIS method gives a useful tool for research of re-oxidation process and interaction of electroactive enzymes with conducting materials giving information required to construct and develop analytical devices.

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Editorial Office
Electrochimica Acta
Dr. Elena Ferapontova

Dear Dr. Elena Ferapontova,

We would like to thank You for giving us an opportunity to revise our submission "Development of label-free impedimetric platform based on new conductive polyaniline polymer and three dimensional interdigitated electrode array for biosensor applications".

The comments of both Reviewers were very helpful and we are grateful to them for careful reading. We have implemented all their suggestions in the revised version of the manuscript. We are sending the corrected paper for your further consideration. Please contact me if you require further information.

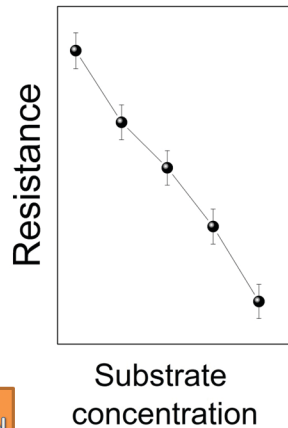
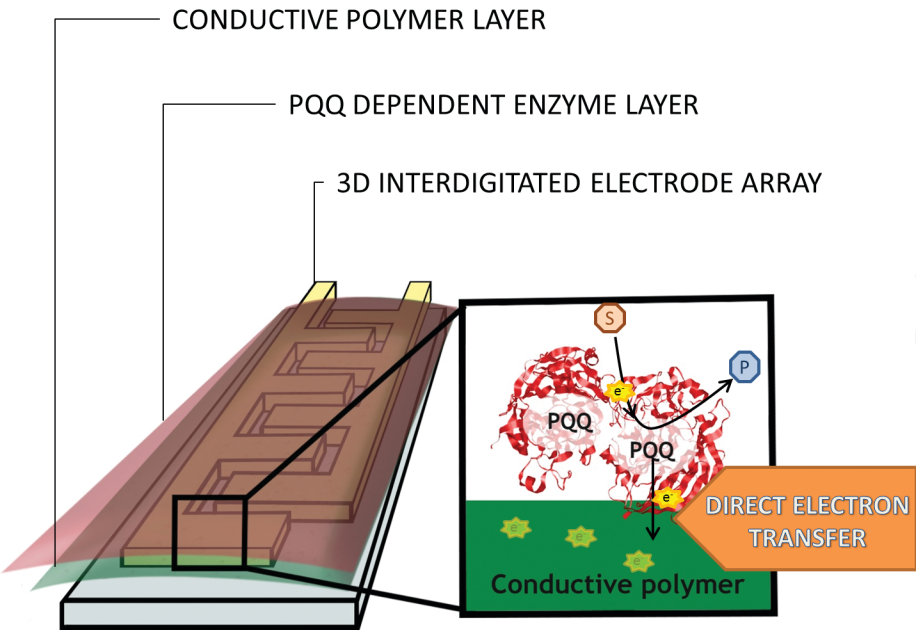
Sincerely yours,

A handwritten signature in blue ink, appearing to read 'E. Voitechovic', with a long horizontal flourish extending to the right.

Edita Voitechovic

April 15, 2015

Graphical Abstract (for review)



We are thankful to reviewers for their critical comments and interesting questions. The detailed answers for the points raised by referees are provided below.

Reviewer #2

1. The chemical structure of polyAnD should be shown.

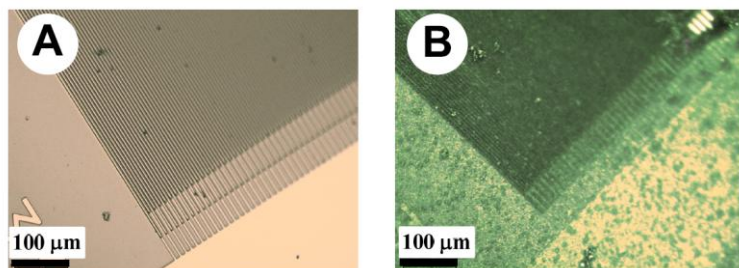
A figure with the chemical structure of polyAnD was added to the manuscript (p.8).

2. In the introduction it is mentioned that polyAnD has a dithiocarbamate functional unit that can be used for further surface modification. However, it is not clear from the manuscript to what extent this functional unit is utilized in the present work where the enzymes were immobilized by "superficial adsorption". What is the advantage of polyAnD compared to polyaniline (or some other common conducting polymer) in the present work?

Superficial adsorption or physisorption is the most simple and less destructive enzyme immobilization method. However, the biggest problems with this method are either too weak or too strong enzyme globule adhesion or improper enzyme orientation. We expected that such additional modification of aniline may result in a polymer with surface properties facilitating enzyme attachment and coordination. The main advantage of dithiocarbamate functional group is a possibility to modify the polyaniline layer with different monomers by simple UV grafting (P.K. Ivanova-Mitseva, V. Fragkou, D. Lakshmi, M.J. Whitcombe, F. Davis, A. Guerreiro, J.A. Crayston, D.K. Ivanova, P.A. Mitsev, E.V. Piletska, S.A. Piletsky, Conjugated polymers with pendant iniferter units: versatile materials for grafting, *Macromolecules*, 44 (2011) 1856-1865). This gives opportunity to modify the polymer surface in a desired way. Additionally, it was shown, that polyAnD can be used as molecular imprinted polymer for amperometric determination of some organic compounds. These features seem useful and are more advantageous in comparison with other conducting polymers for construction and directional modification of electrode matrix. However, before trying to optimize polyAnD three-dimensional structure, one should study reactions of enzymes in contact with this polymer. As it is noted in the introduction, this was the principal scope of the present work. Another advantage of polyAnD that was found recently by our colleagues (data are not shown in present article) is that conductivity of polyAnD is less dependent on pH in comparison with polyaniline and other common conducting polymers. It opens the possibility to design sensors based on conducting polymers which can work in a broad pH range. Corresponding comment was added to the revised version (p. 6).

3. Since polyAnD was chemically polymerized, how can you control where it is deposited on the 3D-IDEA? SEM pictures before and after deposition could help to illustrate this.

PolyAnD was deposited onto the whole electrode surface, which was immersed into the polymerization solution. As the polymer has green color, this can be easily controlled by optical microscopy. The electrode surface before (A) and after (B) polyAnD deposition.



These figures are not provided in the submission, since the number of plots is already quite large. Corresponding comments are implemented in the text (p. 8).

4. Please, check spelling of Nyquist (on several places in the manuscript).

We are grateful to Reviewer for the comment, this mistyping was corrected.

5. How is the resistance (R) in Figures 4-6 related to the equivalent circuit and impedance spectra? Is the value of R obtained by fitting or is it the impedance value at a certain frequency? This should be explained.

The detailed answers to these questions are provided in the section 2.4 of the manuscript. The value of R was obtained by impedance spectra fitting. The resistance (R1) of polyAnD layer, obtained by spectra fitting to one of the equivalent circuits, is presented in Fig. 4-6.

6. On top of page 14 it is stated that "Re-oxidation processes provoke partial reduction of polyAnD chain and thereby affect the electrical conductivity of the polymer layer registered by impedance measurements." This statement is in agreement with the Graphical Abstract showing electron transfer from PQQ to the conducting polymer. However, this means that the conducting polymer is reduced and therefore it is surprising that the resistance decreases (Fig. 6). This is a very crucial point that seems to contradict the suggested reaction mechanism. Unless the polymer (polyAnD) would be n-doped, one would expect an INCREASE in resistance when polyAnD is reduced, i.e. transferred to a less oxidized (less p-doped, less conducting) state. This needs to be clarified, because it is important for the whole manuscript, including the graphical abstract.

We agree with the Reviewer, that the resistance of polyAnD should increase when electrons from enzyme active site are released into the polymer layer, because conductive polyaniline form became nonconductive leucoemeraldine. However, our studies showed opposite results. This interesting phenomenon is not fully understood yet.

As it is shown in Fig. 4 of the manuscript, the resistance of polymer goes down when the concentration of $[\text{Fe}(\text{CN})_6]^{4-}$ ions, as well as the solution redox-potential, increases. The same occurs during enzyme catalyzed redox-reactions with and without potassium

ferricyanide. There are several possible explanations of this effect. 1) Pending group with dithiocarbamate moiety of polyAnD participates in redox-reactions. Pending group of polyAnD contains two nitrogen atoms, which can bound two protons and became positively charged. The protons as well as electrons can be released from enzyme active site during re-oxidation processes. Thus, the environment close to the electrode surface became more acidic. Polyaniline conductivity is known to be induced by proton doping. This effect was applied in the case of “self-doped” polyanilines derivatives, when the charge compensation occurs at the expense of proton exchange, that occurs much more faster and do not limit the rate of the redox-process (A. Malinauskas, Self-doped polyanilines, *J. Power Sources*, 126 (2004) 214-220). The first example of the sensor based on conductivity of polyaniline derivative exploiting this phenomenon was recently presented (E.A. Andreyev, M.A. Komkova, V.N. Nikitina, N.V. Zaryanov, O.G. Voronin, E.E. Karyakina, A.K. Yatsimirsky, A.A. Karyakin, Reagentless Polyol Detection by Conductivity Increase in the Course of Self-Doping of Boronate-Substituted Polyaniline, *Anal. Chem.*, 86 (2014) 11690-11695). 2) Resistance decrease can be explained by the following considerations. Properties of conducting polymers depend on where the redox-reactions with the environment occur: on the outer polymer/solution interface or within the polymer film bulk. If the reactions occur only on the polymer/solution interface without affecting the chemical composition of the bulk polymer, the environment oxygen can participate in the polymer surface oxidation processes and produce different reduced oxygen species, which can affect polymer conductivity (A. Malinauskas, R. Holze, An in situ spectroelectrochemical study of redox reactions at polyaniline-modified ITO electrodes, *Electrochim. Acta*, 43 (1998) 2563-2575).

Corresponding explanations were added to the text (pp. 14-15).

While preparing the revision of the manuscript we found that Figure 4 in the initial submission (Fig.5 in the revised version) had incorrect label (instead of $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ it has $[\text{Fe}(\text{CN})_6]^{4-}/[\text{Fe}(\text{CN})_6]^{3-}$ label). This was corrected in the revised manuscript.

Reviewer #3

1) Correct the sentence "showed specific response in the presence of corresponding substrates with 1 μM sensitivity" to "showed specific response in the presence of 1 μM of the corresponding substrates".

The sentence was corrected

2) Analytical utility of EIS is not well documented. Additional references (especially review articles) should be added. In addition, excellent examples on the use of EIS to study changes of the biocatalyst's structure are notably missing.

We are grateful for this comment and corresponding references [2-8] proving analytical utility of EIS were added to the revised version of the manuscript. Please refer to p. 3 in the document.

3) The meaning of the sentence "Oxidoreductases are the class of enzymes [14] and they catalyse selective oxidation or reduction of many organic compounds, which are of interest for various industry branches. Unfortunately, many oxidoreductases do not have DET [15] and only several electrode surface materials are suitable for DET [16]." is not clear. It needs editing.

The following reformulation was done to provide for more clarity: Oxidoreductases are the class of enzymes [20] that catalyze selective oxidation or reduction of many organic compounds and this class is of interest for various industrial applications. The redox-active moiety of most of the oxidoreductases is deeply buried within the enzyme globule [21]. Such spatial isolation protects enzyme active site from surrounding and provides effective kinetic barrier for DET. Thus, due to unique three-dimensional structure only certain types of oxidoreductases may participate in DET and only on specific electrode materials [22].

4) The sentence "2) the appropriate immobilization matrix compatible with enzyme on an electrode surface must be provided;" also needs editing.

The following correction was made: 2) the enzyme immobilization matrix must provide for enzyme adhesion, stabilization and orientation,

5) Correct "1.2-propandiol" to "1,2-propandiol" throughtout the manuscript.

This was corrected.

6) Correct the chemical formula of $(\text{NH}_4)_3\text{S}_2\text{O}_8$ to $(\text{NH}_4)_2\text{S}_2\text{O}_8$.

Corresponding correction was made.

7) The term "superficial adsorption" maybe replaced by the term "adsorption" or "physisorbed biomolecules".

The term "superficial adsorption" was replaced with physisorption.

8) Please check the upper limit of frequencies in the EIS experiments. Is that really so high 1 MHz? If yes, please explain why. Typically frequency values up to 100 kHz are sufficient to describe the phenomena take place on the biomolecule-modified electrode/electrolyte interface.

This remark holds for ordinary EIS measurements between two bulk electrodes immersed in the solution. However in the case of EIS measurements with 3D-IDEA we have measured up to 1 MHz in order to attain higher fitting precision from a broader range in Nyquist plot for the second semicircle. This is associated with the specific geometry of the sensor. Corresponding explanation was added to the text p. 9.

9) Correct the term "Nyquist" throughout the text.

Corrections were made

10) Correct to "chi-square".

Corresponding correction was made.

11) Correct to "potentiostatic"

Correction was done.

12) Correct the title of 3.2.

Reformulation: Influence of solution redox-potential on polyAnD resistance

13) The first sentence of paragraph 3.2 also needs editing.

Reformulation: Conducting polymers based on polyaniline and its derivatives are able to enhance the speed of various redox-processes as well as to participate in electrocatalytic oxidation of certain biologically important compounds, such as coenzymes and cytochromes.

14) The meaning of the sentence "his observation implies the relevance of using the same polymer material to register enzyme catalysed reactions in the conductive polymer." is not clear. Please explain or rephrase.

This sentence was rephrased as follows: "This phenomenon led us to assume that the same polymer material may be used to register the enzyme catalysed redox reactions."

15) Replace "inhibition" by "deactivation" (Page 12, line 59)

This was corrected.

16) Paragraph 3.5. The increase of the background signal after the modification of gold electrodes with polyAnD indicates an increase of the electrode surface rather than the presence of electroactive groups.

This sentence was reformulated: Modification of gold microelectrode by polyAnD with or without enzymes enhances the background current which indicates an increase of the electrode surface (curves (2) and (3)).

17) The quality of the data presented in Fig.7 is not sufficient to support DET between the enzyme and the electrode surface through the polyAnD layer. In addition, the respective text

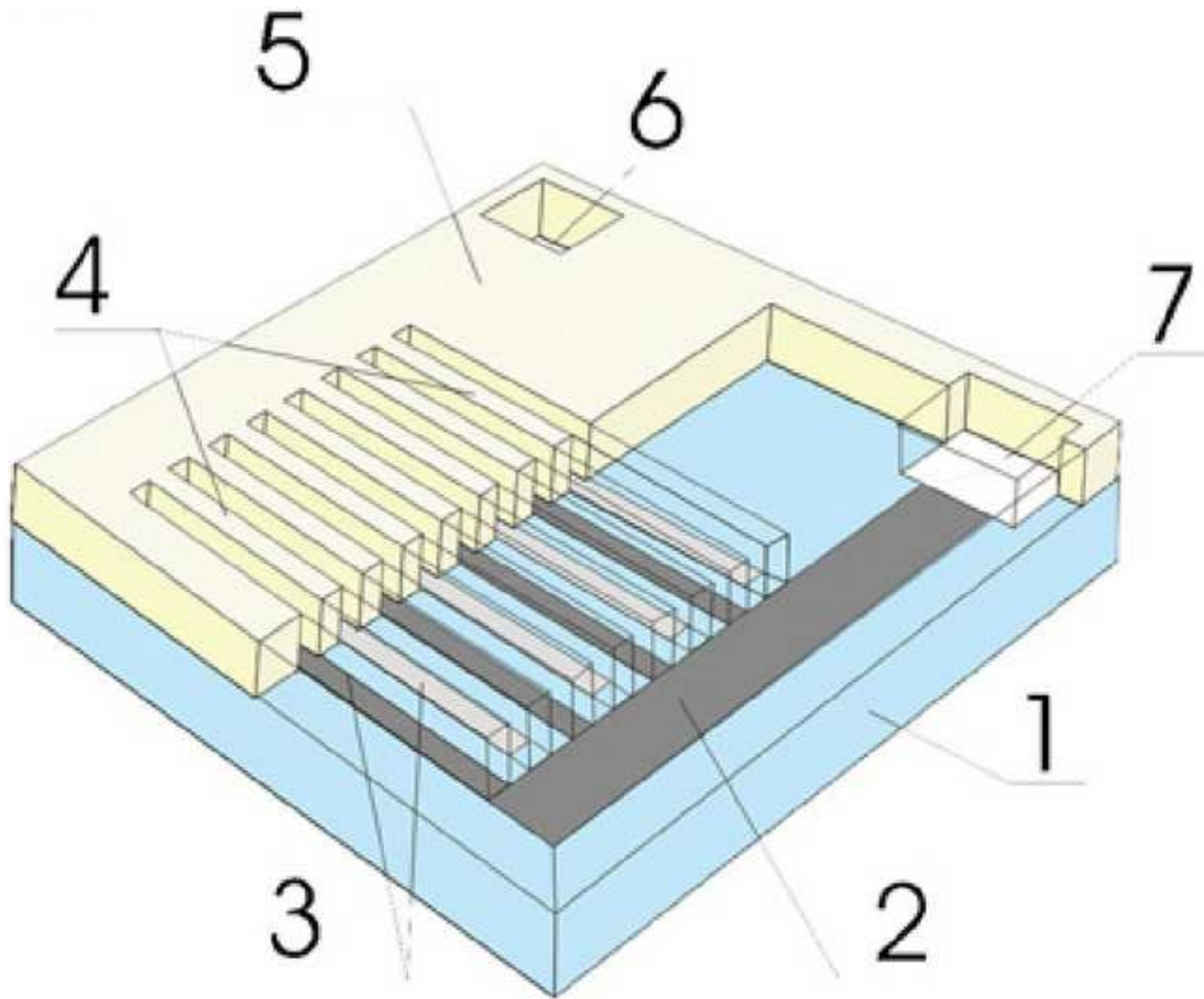
is also not clear for the reader. I would suggest authors to provide simple cyclic voltammetric experiment to document DET.

We agree with the referee that cyclic voltammetry (CV) experiments may confirm DET. We have performed CV measurements with polyAnD and enzymes to prove DET. However, our CV results contained significant noise since the electrode surface was too small to provide for clear signal (gold microelectrode area was only 1.65 mm²). Because of these technical difficulties we decided not to present the experimental graphs in the manuscript and chronoamperometry data were presented in the manuscript as alternative.

18) List of references should be checked. At least one reference is appearing twice; Biosensors Bioelectron., 24 (2008) 729-735 at Refs. 2 and 11.

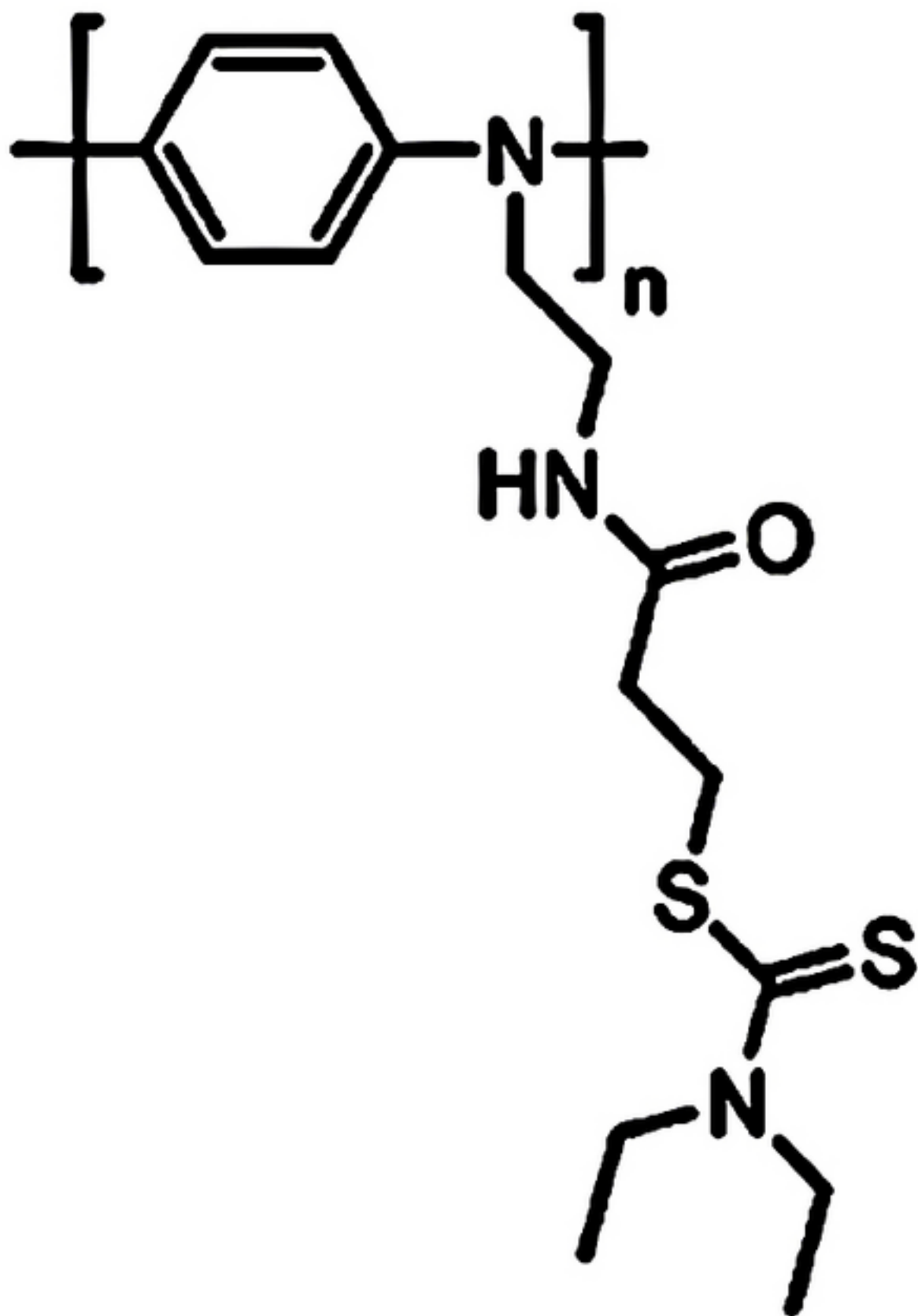
We would like to thank the Reviewer for careful reading. This issue was corrected and other references were thoroughly checked.

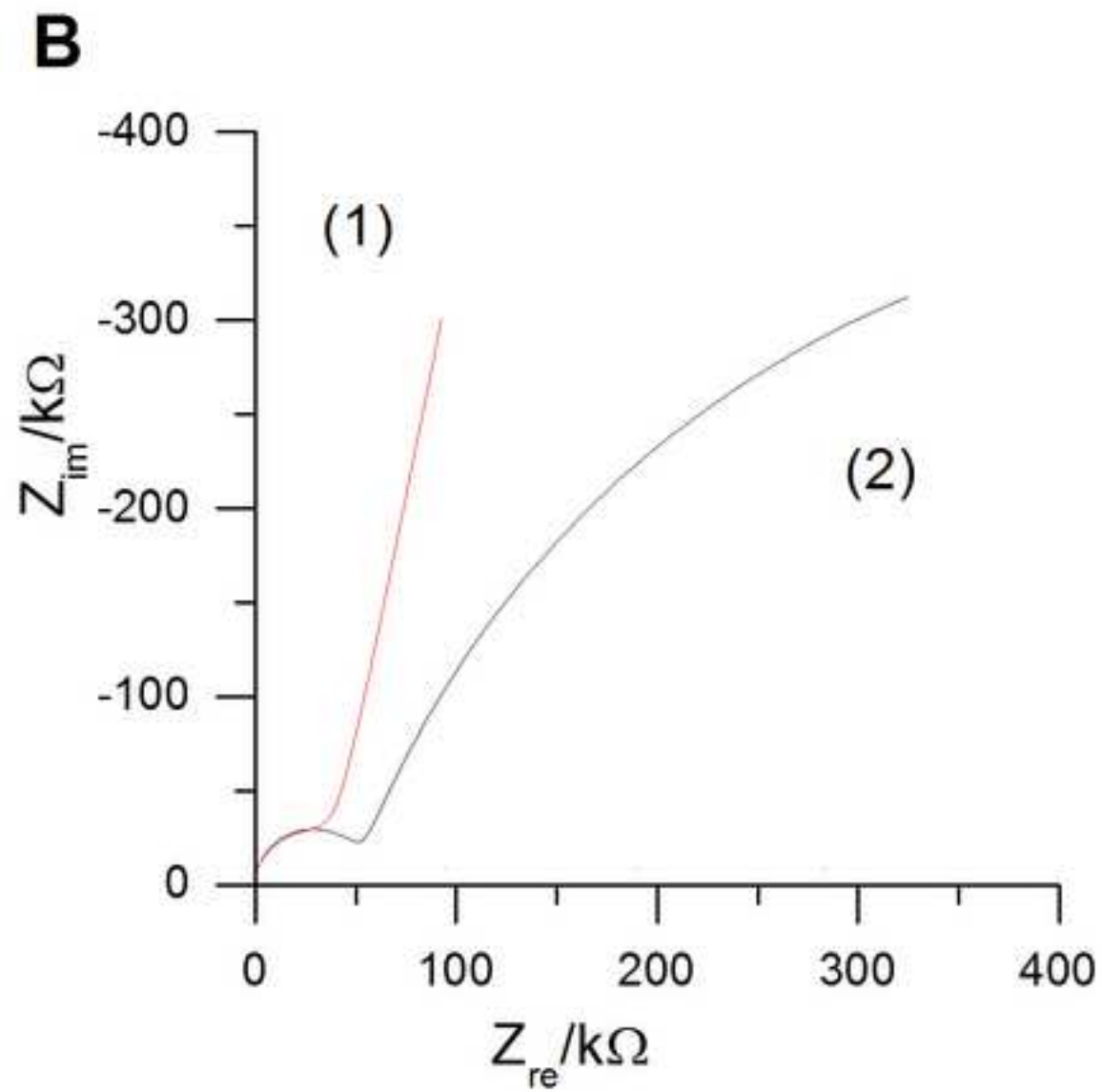
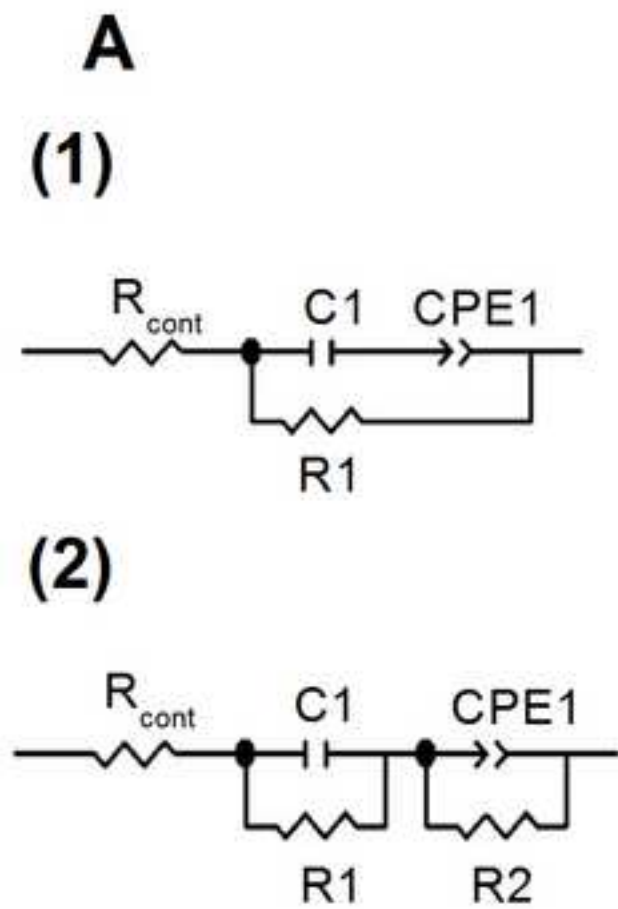
Figure(s)
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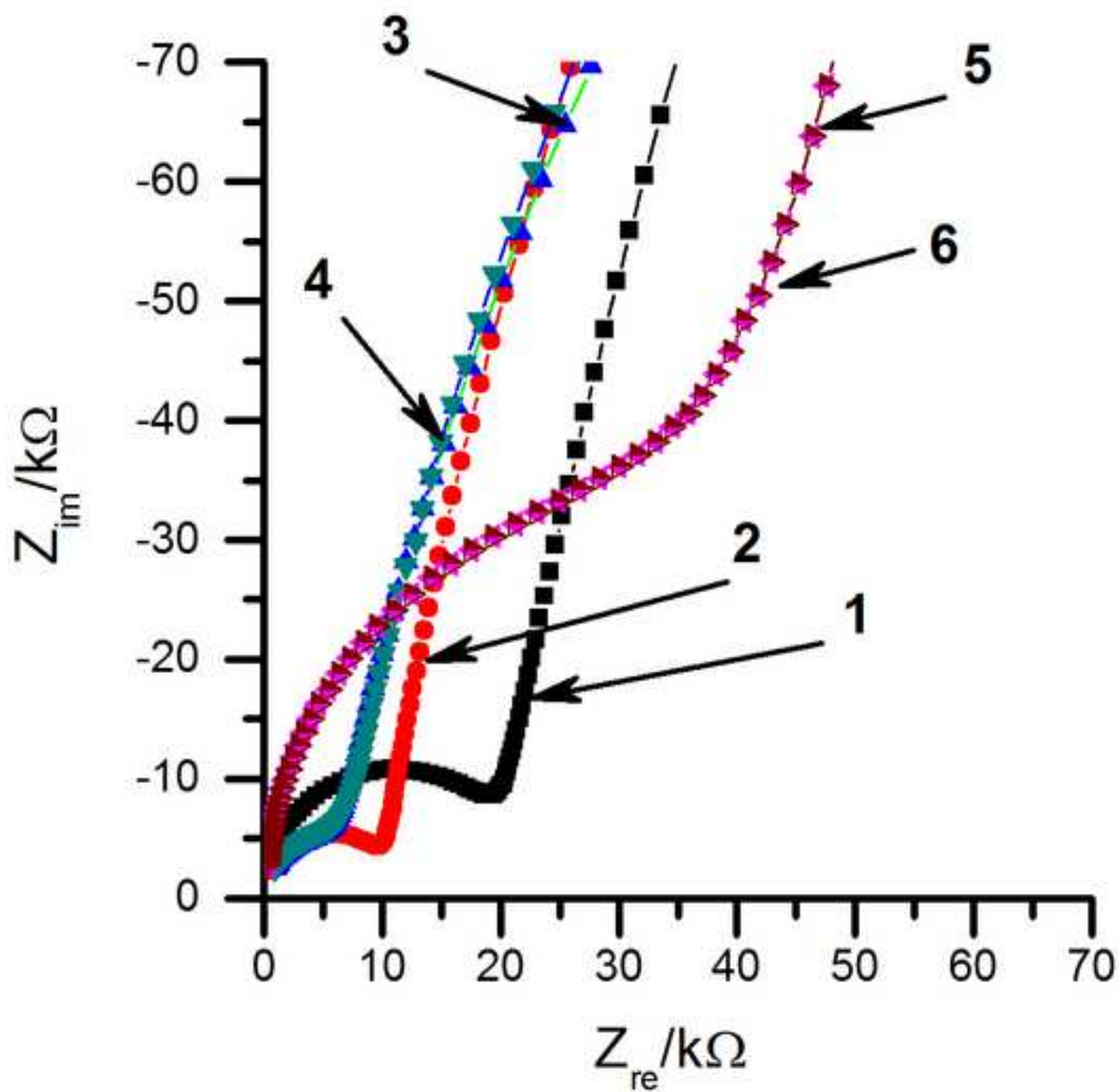
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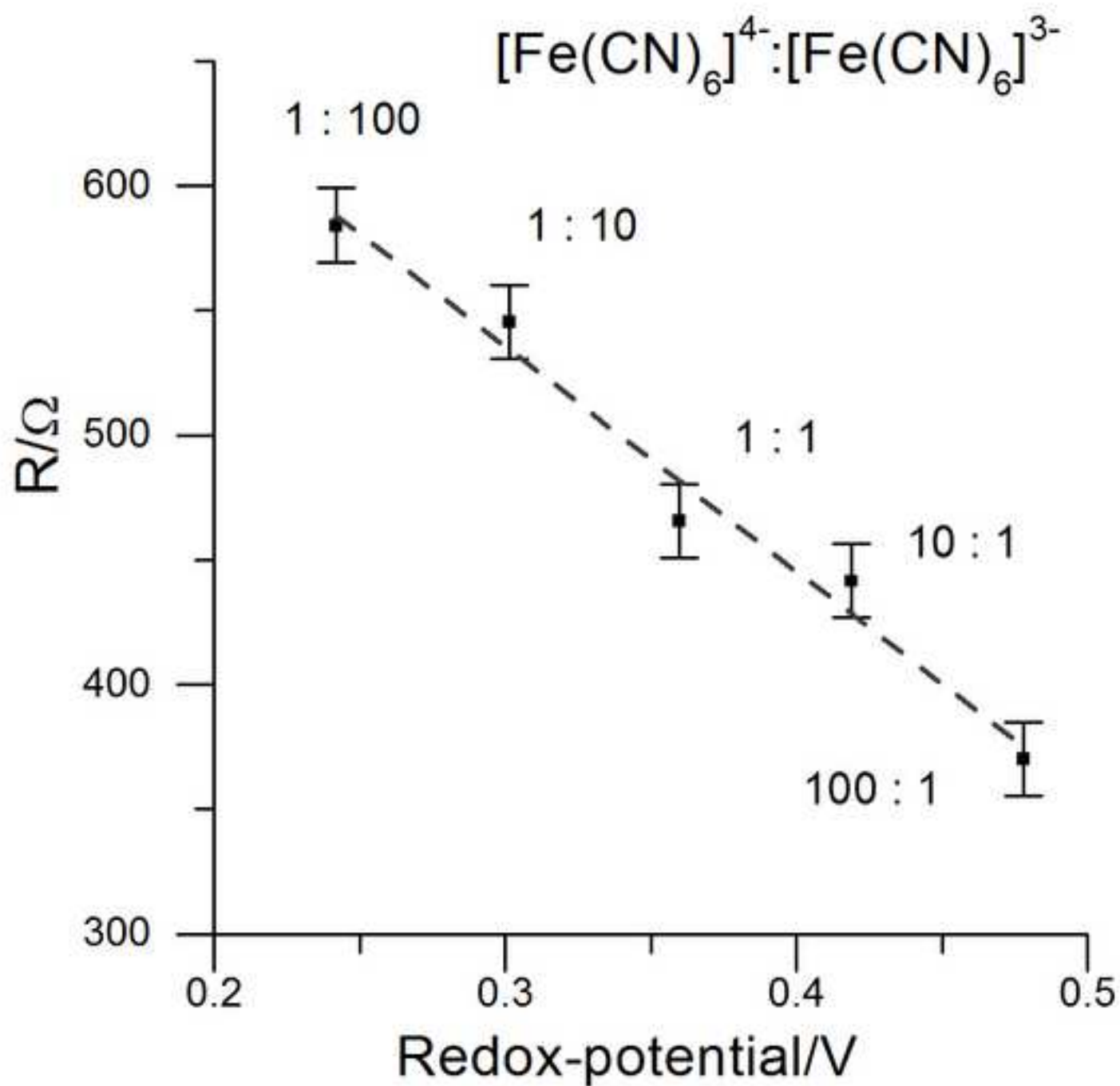
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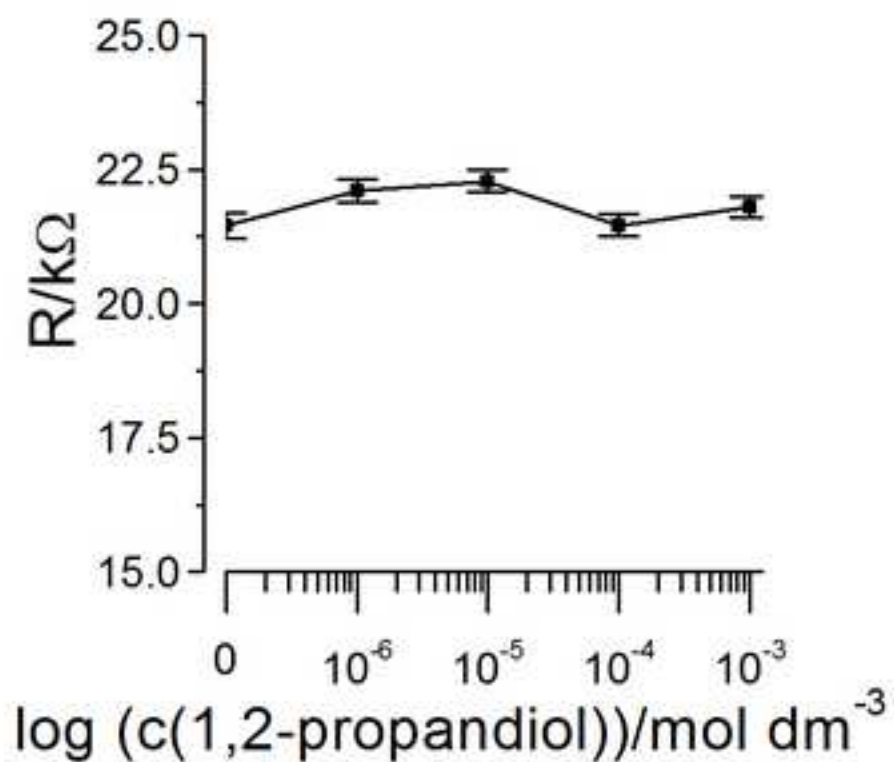
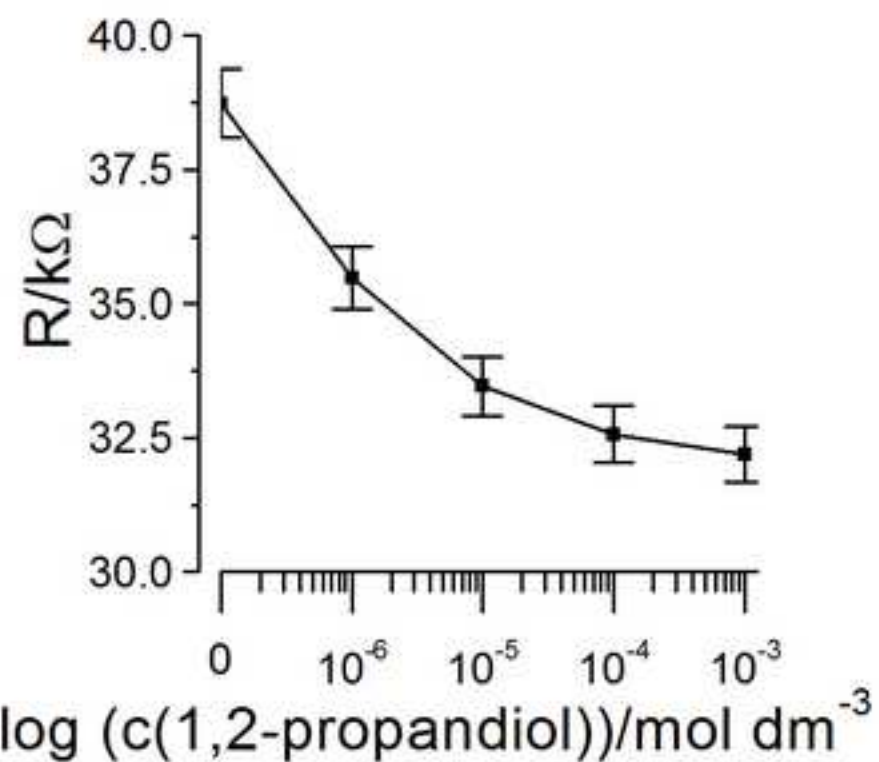


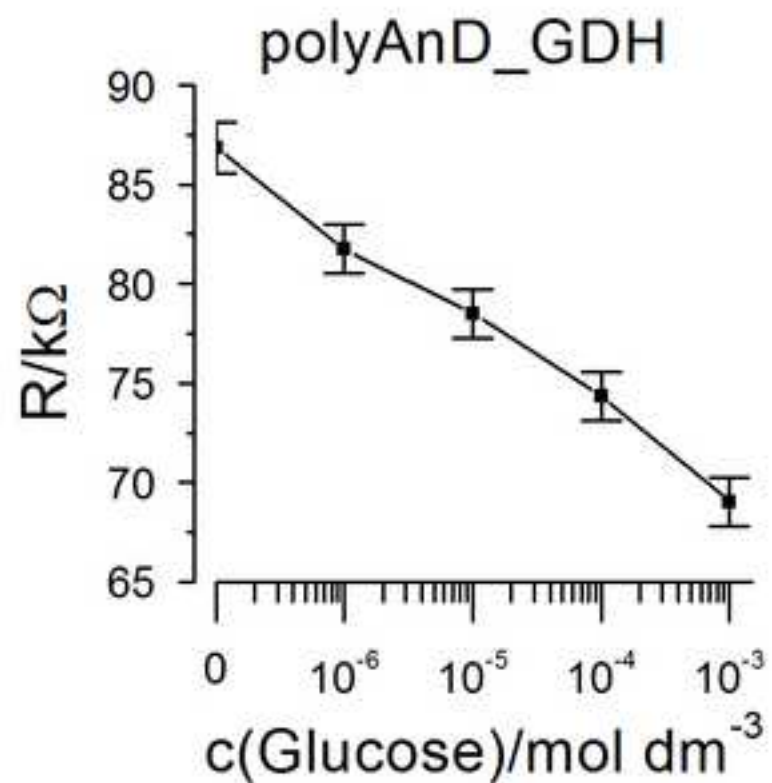
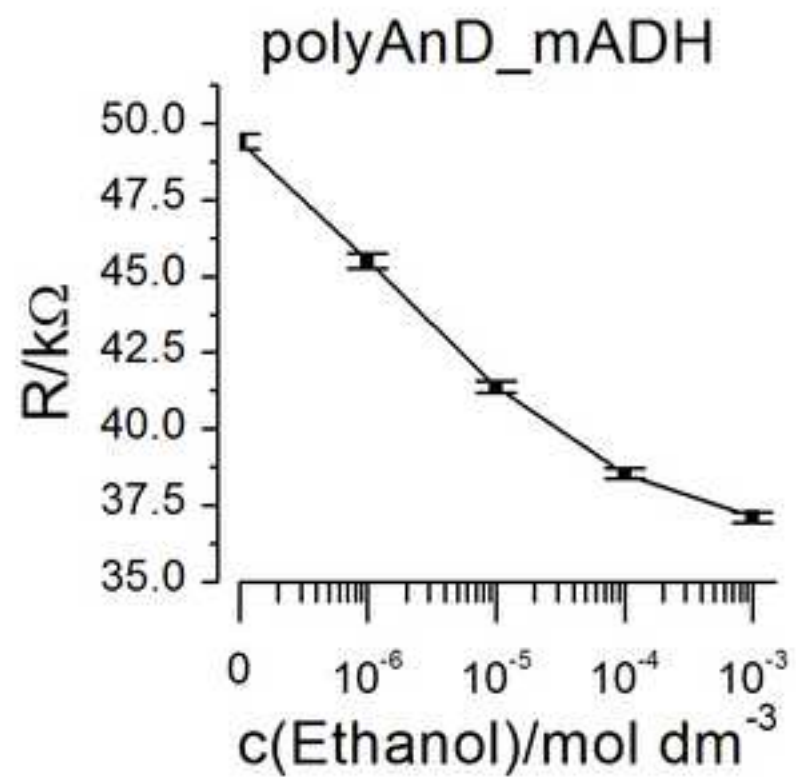


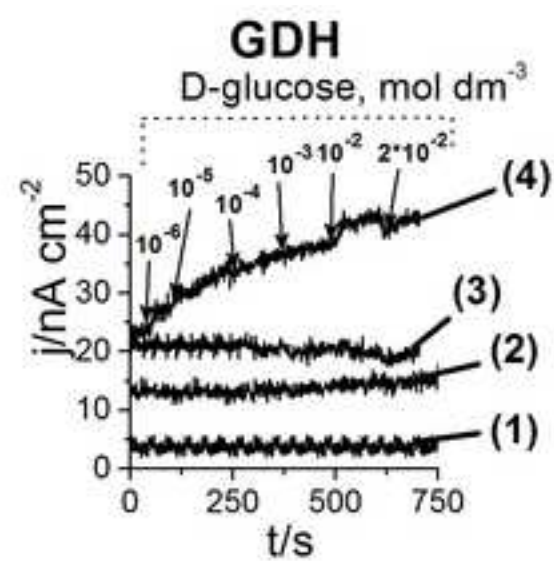
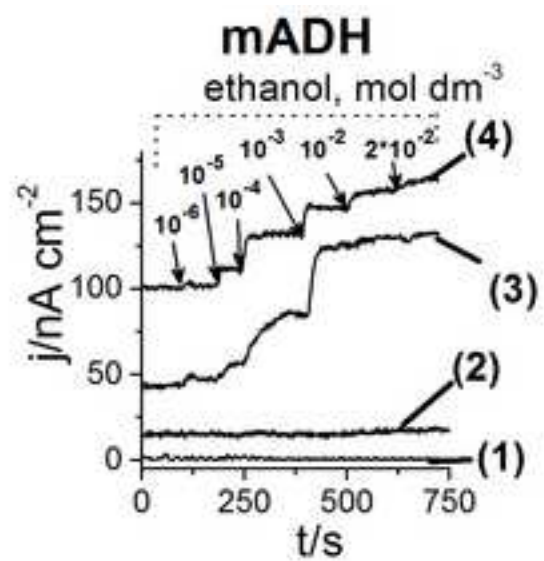
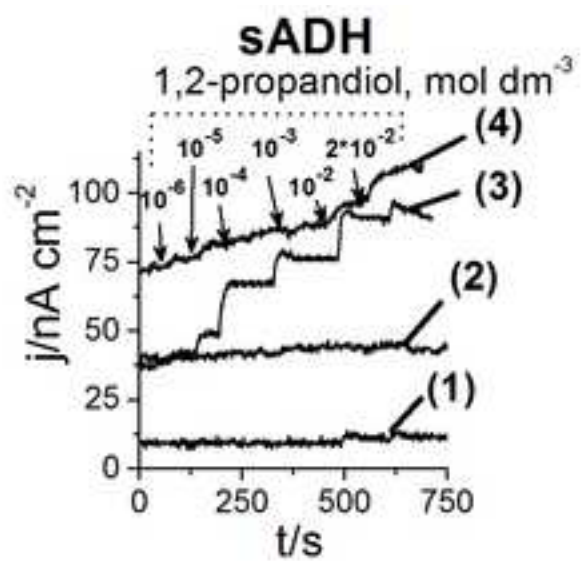
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A polyAnD+sADH
direct reoxidation on polymer**B** polyAnD_sADH
direct reoxidation on polymer





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13 **Abstract**

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16 Novel label-free impedimetric platform based on a three-dimensional interdigitated electrode
17 array (3D-IDEA) sensor and new conductive polymer as a transducer for oxidoreductases is
18 introduced. This platform is cost-effective, simple to construct and miniaturize. Monomer of
19 conductive polymer N-(N',N'-diethyldithiocarbamoylethylamidoethyl)aniline (AnD) was
20 deposited onto 3D-IDEA by chemical polymerisation. It was found that the polymer film
21 resistance depends on the redox-potential of the solution. For the first time polyAnD was used as
22 enzyme immobilisation matrix. Pyrroloquinolinequinone (PQQ) dependent alcohol and glucose
23 dehydrogenases were immobilized on 3D-IDEA covered with polyAnD by two different
24 methods. 3D-IDEA sensors with enzymes, which were immobilised by **physisorption** on
25 polyAnD layer, **showed specific response in the presence of 1 μ M of the corresponding**
26 **substrates.** Obtained results revealed that PQQ dependent dehydrogenases can re-oxidize on
27 polyAnD via direct electron transfer (DET) from enzyme active site to the polymer surface. This
28 process can be monitored by methods of electrochemical impedance spectroscopy (EIS) and
29 chronoamperometry. Presented study shows that EIS method gives a useful tool for research of
30 re-oxidation process and interaction of electroactive enzymes with conducting materials giving
31 information required to construct and develop analytical devices.
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60 **Keywords:** Interdigitated electrode array, DET, PQQ dependent enzyme, polyaniline, biosensor
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1. Introduction

Nowadays there are numerous fields such as medicine, microbiology, food and environmental protection where ultrasensitive and fast sensors for determination of various analytes are required. The sensors based on biocatalysts and electrochemical detection gained wide attention from researchers due to their unique sensitivity, cost-effectiveness, short-time analysis, capability of measurements in dispersed and nontransparent solutions. Moreover these devices are easy to miniaturize.

There are different electrochemical methods, which are employed to construct biosensors: amperometry, voltammetry, potentiometry, coulometry, conductometry and electrochemical impedance spectroscopy (EIS) [1]. EIS is a powerful analytical tool that is employed successfully in chemical and biochemical analysis [2-5], as well as to study biocatalysis on the surface of electrodes [6-8]. EIS is widely applied in physical and biological sciences especially in the field of biosensor research [9]. EIS can provide information on the changes of biocatalyst structure or electrode matrix structure [10, 11]. Moreover, in comparison with other electrochemical methods, EIS setup does not require a reference electrode and analysis can be performed without high potential treatment of the electrode surface, that can destroy thin and sensitive bioactive layer of the sensor [12]. Impedimetric biosensors are employed for quantitative and qualitative immunoanalysis [10], determination of nucleoacids, their mutations [13] and different redox-active compounds [14], e.g. H_2O_2 . However, the possibility of impedance measurements often requires the presence of redox-active species in test solution, e.g. usually ferricyanide/ferrocyanide couple is used. In this case Faradaic charge transfer resistance is measured, which may be affected by interactions of a target biomolecule with a probe-functionalized sensor surface [15]. To avoid addition of redox-agents and to be able to perform the measurements immediately on the area close to the electrode surface the capacitive

1 biosensors based on interdigitated electrode arrays (IDEA) were introduced [16]. In this case
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3 biochemical reactions at the sensor surface are registered as capacitance changes in non-Faradaic
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5 measurements. Planar microband electrodes of IDEA, between which the impedance is
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7 measured, are very closely situated, so that in this case changes in electrical properties of the
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9 interdigital space may affect the sensor impedance [15]. Several years ago the concept of a three-
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11 dimensional interdigitated electrode arrays (3D-IDEA) with electrode fingers separated by an
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13 insulating barrier was proposed [9, 17]. The specific design of this sensor structure allows for
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15 enhancement of its sensitivity for biochemical reactions taking place at the sensor surface [18].
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18 The principles and applications of 3D-IDEA sensors were recently discussed in [15].
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23 Another opportunity to avoid the addition of redox-compounds or special labels for analytes into
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25 the test solution is an application of electrochemical systems based on redox-active enzymes –
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27 oxidoreductases, since some of them can possess direct electron transfer (DET) from an enzyme
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29 active site to the electrode surface. The effective direct re-oxidation of enzyme onto electrode
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31 surface ensures the highest selectivity of analysis, independence of oxygen concentration in the
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33 media, analysis at lower potential and simplifies the sensor construction [19]. Due to this, DET
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35 became a phenomenon, which is extensively investigated during last several decades.
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40 Oxidoreductases are the class of enzymes [20] that catalyze selective oxidation or reduction of
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42 many organic compounds and this class is of interest for various industrial applications. The
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44 redox-active moiety of most of the oxidoreductases is deeply buried within the enzyme globule
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46 [21]. Such spatial isolation protects enzyme active site from surrounding and provides effective
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48 kinetic barrier for DET. Thus, due to unique three-dimensional structure only certain types of
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50 oxidoreductases may participate in DET and only on specific electrode materials [22].
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55 Broad applicability of 3D-IDEA sensors to study interactions of biomolecules [18] can be useful
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57 to apply this sensor type for investigation of oxidoreductases. A synergism of 3D-IDEA and
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59 unique selectivity of oxidoreductases can be promising to design label-free ultrasensitive new
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1 impedimetric biosensor, based on determination of the changes only in the electrical properties
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3 of immobilised enzyme layer and enzyme immobilisation matrix. In order to construct such
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5 biosensor platform several requirements must be fulfilled: 1) the enzyme must possess DET; 2)
6 the enzyme immobilization matrix must provide for enzyme adhesion, stabilization and
7
8 orientation; 3) the immobilization matrix must have reasonable conductivity [23-26].
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12 To address the first issue one can consider pyroloquinolinquinone (PQQ) dependent
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14 oxidoreductases which represent a unique group of enzymes that can act as electrocatalysts,
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16 facilitating the electron transfer between different conductive electrode materials and their
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18 substrate molecule with no mediator involved in the process [27-29]. It is important to note that
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20 PQQ dependent enzymes do not react with molecular oxygen and, consequently, they are
21
22 attractive for construction of the third generation biosensors [19] based on the DET between
23
24 enzyme and electrodes. Previous investigations showed that PQQ dependent enzymes exchange
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26 electrons with gold, silver, pyrolytic graphite, several organic polymers, glassy carbon and
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28 carbon paste electrodes [30-35]. In addition, the efficiency of the DET is highly dependent on the
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30 intrinsic properties of the electrode material.
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37 In order to address the second and the third issues one can apply various conducting polymers
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39 which are very attractive materials as their properties may be tuned chemically in a desired way
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41 to assist with electron transfer [36, 37].
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45 Conducting polymers, especially polyanilines, have a great potential in electronics and optics
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47 [38, 39] and especially in the field of chemical sensors and biosensors [3, 37, 40, 41]. Due to
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49 their peculiar electrochemical and optical properties these materials can be used as components
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51 of different transducers in potentiometric, amperometric and impedimetric/conductimetric
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53 sensors [3]. They are frequently employed as sensitive elements of “chemiresistors” for gas
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55 sensing [42]. These polymers were found to change their electronic conductivity in response to
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57 changes in pH or redox-potential of the solution in contact with the polymer [43, 44] and were
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1 suggested to be applied as chemical or redox-sensors [45]. Biosensors with conducting polymers
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3 are most commonly used in amperometric detection [3], though conductimetric sensors with
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5 different enzymes have also been reported [46-48]. Recently new *N*-substituted aniline
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7 derivatives with interesting features were reported in the literature [36, 49]. These monomers
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9 contain two essential parts: aniline and methacrylamide or dithiocarbamate. Recently one of such
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11 monomers *N*-(*N*',*N*'-diethyl dithiocarbamoyl ethyl amido ethyl)aniline (AnD) [36] is introduced.
12
13 The aniline part of this substance can be polymerized in both chemical and electrochemical way
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15 and conductivity of the resulting polymer is similar to that of aniline. An attractive feature of
16
17 polyAnD molecule is that each monomer unit has a dithiocarbamate ester moiety, and it can be
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19 used for further surface modification. UV grafting can be applied for grafting of other polymers
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21 over polyAnD via iniferter activation. Moreover, it was shown, that polyAnD can be used as
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23 molecular imprinted polymer for amperometric determination of certain organic compounds
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25 [50]. These features seem useful and are more advantageous in comparison with other
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27 conducting polymers for construction and directional modification of electrode matrix for design
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29 of bio-compatible enzyme immobilisation matrix with high surface area.
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37 In this study we introduce a novel label-free impedimetric platform based on 3D-IDEA sensor
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39 and new conductive polyaniline polymer polyAnD as a transducer for oxidoreductases. The three
40
41 different PQQ dependent dehydrogenases were applied to test suitability of the proposed
42
43 impedimetric platform for studying of electron transfer processes between enzymes and
44
45 immobilization matrix and for biosensing purposes as well.
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50 **2. Experimental**

51 **2.1. Enzymes and chemicals**

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53 Membrane bound PQQ dependent alcohol dehydrogenase (mADH) from *Gluconobacter sp.* 33,
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55 E.C. 1.1.5.5, was isolated and purified by the method reported in [51]. The activity of the
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1 enzyme solution in 5 mM Tris buffer (pH 7.5) with 1 mM CaCl₂, 0.02 % Triton-X-100 and 0.5%
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3 sucrose was 200 U/ml. Ethanol was used as default substrate for the mADH.
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5 Soluble PQQ dependent alcohol dehydrogenase (sADH) from *Pseudomonas putida* HK5, E.C.
6
7 1.1.9.1, was isolated and purified by the method reported in [52]. The activity of the enzyme
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9 solution in 5 mM Tris buffer (pH 8.5) with 5 mM CaCl₂ was 130 U/ml. 1,2-propandiol was used
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11 as the default substrate for the sADH.
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14 Soluble PQQ dependent glucose dehydrogenase (GDH) from *Acinetobacter calcoaceticus*, E.C.
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16 1.1.5.2, was isolated and purified by the method reported in [53]. The activity of the enzyme
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18 solution in 5 mM Tris buffer (pH 8.0) with 1 mM CaCl₂ was 1800 U/ml. D-(+)-glucose was used
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20 as a default substrate for the GDH.
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24 All enzymes were purified at the Department of Molecular microbiology and biotechnology
25
26 (Vilnius University, Institute of Biochemistry) (Lithuania) and were kindly provided by Dr. R.
27
28 Meškys.
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31 Monomer of conductive polyaniline polymer N-(N', N'-diethyl dithiocarbamoyl ethyl amido
32
33 ethyl)aniline (AnD) was synthesized according to the method reported earlier [36].
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37 Sodium acetate, acetic acid, CaCl₂ and 1,2-propandiol were obtained from J.T. Baker (Holland,
38
39 the Nederland's). Ethanol, KCl and D-(+)-glucose were purchased from Riedel-de Haen
40
41 (Denmark). K₃[Fe(CN)₆], K₄[Fe(CN)₆], HCl, acetonitrile, N₂, (NH₄)₂S₂O₈ (APS) were purchased
42
43 from Sigma Aldrich (Germany).
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48 Three dimensional IDEA electrodes were fabricated at the IMB-CNM, CSIC (Spain) as
49
50 described in [9]. The scheme of 3D-IDEA sensor is presented in Fig. 1.
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53 Gold microelectrodes (working area 1.64 mm²) were fabricated at the same facility by deposition
54
55 of titanium (10 nm) and gold (100 nm) onto a silicon wafer covered with 1 μm thick silicon
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57 oxide layer. Electrodes were patterned using conventional lift-off technique.
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9 **2.2. AnD polymerisation**

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11 Oxidative polymerization was performed following the method reported in [36] using APS under
12 the following conditions: 0.018 M APS, 0.025 M AnD, 0.225 M HCl, 25% acetonitrile in water.

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14 The reaction mixture was prepared by mixing 250 μ l of AnD (0.1 M in acetonitrile) with 207 μ l
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16 of an aqueous solution of APS (0.0915 M), 230 μ l of HCl (1 M), and 680 μ l of water. The
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18 solution was transferred to the Eppendorf tube where a sensor was immersed and fixed
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20 vertically. Polymerisation was carried out during 1.5 h at room temperature in the dark resulting
21
22 in the deposition of a thin, green layer of functionalized polyAnD coating over the electrode
23
24 surface. This process was controlled with optical microscopy to ensure the uniform deposition of
25
26 the polymer. PolyAnD structure is presented in Fig. 2. PolyAnD-coated transducers were washed
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34 in three cycles with de-ionised (DI) water and dried in a nitrogen flow.
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39 FIG2 here
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45 **2.3. Immobilisation of enzymes**

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47 Two methods were employed for enzyme immobilisation: entrapment in polyAnD and
48
49 **physisorption**. Immobilisation of sADH enzyme by entrapment was performed as follows. The
50
51 reaction mixture was prepared by mixing 250 μ l of AnD (0.1 M in acetonitrile) with 207 μ l of
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53 aqueous solution of APS (0.0915 M), 230 μ l of HCl (1 M), and 690 μ l of sADH solution (0.9
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55 mM). The solution was transferred to the Eppendorf tube where the sensor was immersed and
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1 fixed vertically. Polymerisation was carried out during 1.5 h at room temperature in the dark.
2
3 Enzyme and polymer modified electrodes were rinsed several times with DI water and dried in a
4
5 nitrogen flow.
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7
8 **Physisorption** of enzymes was done as follows: 2 μl of enzyme solution were dropped onto pre-
9
10 modified with polyAnD electrode surface and kept at $+4\text{ }^{\circ}\text{C}$ for 1 h. Enzyme modified electrodes
11
12 were carefully washed with DI water and dried in a N_2 flow.
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20 **2.4. Impedance measurements**

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22 Characterisation of 3D-IDEA sensors was performed by impedance measurements in a 100 Hz –
23
24 1000 kHz frequency range with a 25 mV (amplitude) voltage excitation using PARSTAT 2263
25
26 Advanced Electrochemical System (Princeton Applied Research, USA) in 0.01 mM CaCl_2 water
27
28 solution. **Such frequency range for measurements was chosen in order to attain higher fitting**
29
30 **precision from a broader range in Nyquist plot. This is associated with the specific geometry of**
31
32 **the sensor.** The enzyme substrates solutions, containing 100 mM of ethanol, 100 mM **1,2-**
33
34 **propandiol** or 100 mM of D-glucose were prepared using 0.01 mM CaCl_2 as a background. Z-
35
36 Plot/Z-View software package (Scribner Associates, Southern Pines, NC, USA) was employed
37
38 for data treatment and an equivalent circuit fitting. **Fig. 3** shows two equivalent circuit models of
39
40 3D-IDEA sensor. These models were applied for fitting of impedance spectra.
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49 **FIG3 here**

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54 The first equivalent circuit presented in **Fig. 3 A (1)** was used for spectra fitting when only one
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56 semicircle appears in a spectrum at high frequencies and the low frequency part on the **Nyquist**
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1 plot is linear. In water solutions where the studied systems showed spectra with two semicircles
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3 as presented in Fig. 3 B (2), the second equivalent circuit was used to fit them.
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5
6 The parameter R_{cont} is the resistance of the electrodes contacts. Parallel combination of the C1-
7
8 R1 represents the resistance of the polyaniline layer and the geometrical capacitance of the IDEA
9
10 sensor and CPE1 and R2 present the capacitance and resistance at the interface between the
11
12 polymer and the solution.
13

14
15 The quality of fit can be assessed with the chi-square parameter which is the sum of squares of
16
17 the ratio of the standard deviation between the original data and the calculated spectrum. For all
18
19 spectra measured in this work the chi-square parameter was typically smaller than 0.0025.
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27 **2.5. Amperometric measurements**

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29 The electrochemical system PARSTAT 2273 (Princeton Applied Research, USA) was employed
30
31 for amperometric experiments. The system has a conventional three-electrode cell with saturated
32
33 Ag/AgCl reference electrode, platinum plate auxiliary electrode and working gold
34
35 microelectrode.
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39 Before the experiments gold microelectrodes were rinsed with DI water and sonicated in DI
40
41 water for 10 min. Then they were electrochemically cleaned by potential scanning from -0.2 V to
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43 1.7 V (vs. Ag/AgCl) 10-20 times at 200 mV/s scan rate in 0.5 M H_2SO_4 water solution until
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45 reproducible gold oxide stripping peaks were obtained.
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49 Chronoamperometric measurements were employed to study the response of the biosensor to
50
51 substrate addition. The conditions were as follows: potentiostatic mode at +400 mV (vs.
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53 Ag/AgCl) in a stirred 100 mM acetate buffer solution, pH 6.0, 10 mM CaCl_2 and 10 mM KCl.
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55 The enzyme substrates were used as acetate buffer solutions, pH 6.0, containing 100 mM of
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57 ethanol, 100 mM 1,2-propanediol or 100 mM of D-glucose.
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3. Results and discussion

3.1. 3D-IDEA sensors modifications

3D-IDEA sensors were modified with polyAnD polymer by chemical polymerisation. The impedance spectra before and after modification were measured in solutions of CaCl₂ with different conductance and the results are presented in Fig. 4. As expected, spectra of the initial sensor before modification depend on the solution conductivity (curve 1 and 2). After deposition of the polymer film the sensor shows rather small interelectrode resistivity that is only slightly affected by changes in the solution conductivity (curve 3 and 4). This is due to the presence of a highly conductive film that shunts the electrodes and prevents penetration of the electric field into the solution. Adsorption of enzymes on the polyAnD provokes significant impedance changes (curve 5), which didn't decrease during months after immobilisation. The resistance of the film composed of polyAnD and enzyme becomes several times higher. This means that there is an interaction between PQQ dependent enzymes and the conductive polymer that affects its electrical properties. However, as in the previous case, the sensor impedance was not affected by the solution conductivity (curves 5 and 6) and there were no differences between impedance spectra obtained in solutions with 4.62 μS/cm and 16.8 μS/cm conductivity.

FIG4 here

3.2. Influence of solution redox-potential on polyAnD resistance

Conducting polymers based on polyaniline and its derivatives are able to enhance the speed of various redox-processes as well as to participate in electrocatalytic oxidation of certain biologically important compounds, such as coenzymes and cytochromes [54, 55].

1 The enzymes studied in this work have quinone cofactor and one or more heme c in their active
2 sites. In this case, it is possible, that polyAnD could also provoke re-oxidation of enzymes active
3 sites during substrate oxidation. It is also known that polyaniline based polymers are sensitive to
4 solution redox-potential changes. In this case electric properties of polyAnD should change in
5 the presence of soluble electron acceptor in solution, when concentration of oxidised and
6 reduced forms changes during the enzyme catalysed substrate oxidation.
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15 Firstly, the sensitivity of polyAnD to redox-potential was tested. The impedance spectra of
16 polymer modified sensor were recorded in solutions with different ratio of $[\text{Fe}(\text{CN})_6]^{3-}$
17 $/[\text{Fe}(\text{CN})_6]^{4-}$. These ions are chosen due to ferricyanide is known as good electron acceptor of
18 heme containing enzymes, as sADH and mADH are. The resistance (R1) of the sensor was
19 determined by fitting of impedance spectra by equivalent circuit model Fig. 2 A (1). The redox-
20 potentials of solutions were calculated using Nernst equation:
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$$E = E_0 + \frac{RT}{Fn} \ln \frac{[[\text{Fe}(\text{CN})_6]^{3-}]}{[[\text{Fe}(\text{CN})_6]^{4-}]},$$

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where $E_0 = 0.36$ V is the standard redox-potential of the $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ pair in water
solution. Data are presented in the Fig. 5. The polymer film resistance goes down linearly as the
redox-potential of the solution becomes more positive. This phenomenon led us to assume that
the same polymer material may be used to register the enzyme catalysed redox reactions.

FIG5 here

3.3. Response of the sensor with soluble alcohol dehydrogenase (sADH)

The bioelectrochemical systems with sADH on the polyAnD modified 3D-IDEA sensors were
constructed using enzyme immobilisation by entrapment (polyAnD+sADH) and by

1 **physisorption** (polyAnD_sADH). The response of both systems to addition of enzyme substrate
2
3 was recorded in the presence and absence of water soluble electron acceptor $K_3[Fe(CN)_6]$ in a
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5 small volume (500 μ l) of reaction solution.
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8 The polyAnD_sADH system showed specific response to addition of **1,2-propandiol** in the
9
10 presence of $K_3[Fe(CN)_6]$ resulting in the resistance decrease, similar to previous experiments
11
12 with different ratio of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$. These results show that sADH is able to change
13
14 significantly $K_3[Fe(CN)_6]$ concentration during biocatalysis. However, the (polyAnD+sADH)
15
16 system with entrapped sADH showed no specific response to the presence of **1,2-propandiol**, that
17
18 can indicate **deactivation** of sADH during polymerisation process.
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23 The response of sensors with adsorbed and entrapped sADH and 3D-IDEA sensor covered with
24
25 polyAnD to addition of **1,2-propandiol** was measured without $K_3[Fe(CN)_6]$ expecting that
26
27 polyAnD could provoke re-oxidation of sADH active site. These results are shown in **Fig. 6**.
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32 **FIG6 here**
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37 The sensor with adsorbed sADH showed specific response to addition of the enzyme substrate
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39 (**Fig. 6. B**), while the sensor with entrapped enzyme showed no response (**Fig. 6. A**) in
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41 experiments with $K_3[Fe(CN)_6]$. From this we can conclude that the enzyme activity is affected
42
43 by the polymerisation process. It was shown in ref. [56] that sADH exhibits 35 – 5% of its initial
44
45 activity after 1 h storage in 25% acetonitrile water solution. In our case, the presence of organic
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47 solvent is accompanied by low solution pH and release of oxygen during chemical reactions
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49 which provoke an irreversible denaturation of sADH globule. This method of enzyme
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51 immobilisation was not used further in experiments.
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1 **3.4. Response of the sensors with membrane bound alcohol dehydrogenase (mADH) and**
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3 **soluble glucose dehydrogenase (GDH)**
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6 Since enzyme immobilisation by entrapment in polyAnD resulted in a total loss of enzyme
7 activity, PQQ dependent membrane bound alcohol dehydrogenase and soluble glucose
8 dehydrogenase were immobilised onto polyAnD modified 3D-IDEA sensors by **physisorption**.
9
10 As in the case of sADH, we recorded impedance spectra of obtained sensors in the presence of
11 different concentration of enzymes substrates. The results of these experiments are presented in
12 **Fig. 7**. Sensors with mADH and GDH show decrease in determined resistance (R1) upon
13 addition of enzymes substrates. This suggests that all studied enzymes in this work can undergo
14 direct re-oxidation on polyAnD.
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28 **FIG7 here**
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33 **Enzymes re-oxidation means electron flow to the polymer from reduced enzyme active site.**
34 **Reduction processes of the conducting polymer chains usually result in conductivity decrease.**
35 **However, obtained results with $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ (section 3.2) and all studied enzymes**
36 **showed opposite behaviour. This interesting phenomenon is not fully understood yet. There are**
37 **several possible explanations for this effect. First one can be attributed to pending group with**
38 **dithiocarbamate moiety of polyAnD, which can participate in redox-reactions. Pending group of**
39 **polyAnD contains two nitrogen atoms (Fig. 2), which can bound two protons and became**
40 **positively charged. The protons as well as electrons can be released from enzyme active site**
41 **during PQQ re-oxidation processes [57, 58]. Thus, the environment close to the electrode surface**
42 **should become more acidic. Polyaniline conductivity is known to be induced by proton doping.**
43 **This effect was applied in the case of “self-doped” polyanilines derivatives, when the charge**
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1 compensation occurs at the expense of proton exchange inside the polyaniline molecule, that
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3 occurs much more faster and does not limit the rate of the redox-process [59]. The first example
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5 of the sensor based on conductivity increase of polyaniline derivative exploiting this
6
7 phenomenon was recently presented in [60]. Another reason for decrease in resistance can be as
8
9 follows. Properties of conducting polymers depend on where the redox-reactions with the
10
11 environment occur: on the outer polymer/solution interface or within the polymer film bulk. If
12
13 the reactions occur only on the polymer/solution interface without affecting the chemical
14
15 composition of the bulk polymer, the oxygen from the ambience can participate in the polymer
16
17 surface oxidation processes thus producing different reduced oxygen species, which can affect
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19 polymer conductivity [54].
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26 *3.5 Chronoamperometric measurements*

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29 Chronoamperometric measurements were performed using gold micro-electrodes to ensure that
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31 PQQ dependent enzymes can directly re-oxidise on polyAnD surface. Modification of electrode
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33 with polyAnD was performed by the same method as presented before (section 2.5).
34
35 Immobilisation of enzymes by **physisorption** was performed on initial gold microelectrodes and
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37 on gold microelectrodes covered with polyAnD. The response of these two types of sensors to
38
39 stepwise addition of enzymes substrates (**1,2-propanediol** for sADH, ethanol for mADH and D-
40
41 glucose for GDH) were recorded. The results are presented in **Fig. 8**.
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46 **Modification of gold microelectrode by polyAnD with or without enzymes enhances the**
47
48 **background current which indicates an increase of the electrode surface (curves (2) and (3)).** In
49
50 the absence of enzymes in the solution the background current did not change significantly
51
52 **(curves (1) and (2)).** In the cases when the enzymes sADH and mADH were immobilised
53
54 directly on gold the sensors showed response in the presence of corresponding substrate **(curve**
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56 **(3)).** The possibilities of DET from sADH and mADH to gold metal surface have been reported
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[30, 61]. Sensors with these enzymes immobilised on polyAnD also show response to their respective substrates, which may be regarded as a confirmation that the DET also occurs in this case (curve (4)). However, sensors with the GDH adsorbed on gold electrodes showed no response in the presence of glucose (curve (3)). The response appears only if the gold microelectrode is covered by the conductive polymer layer of polyAnD (curve (4)). This fact indicates that GDH can be directly re-oxidised on polyAnD, while this process is blocked on gold surface. Thus, obtained results of chronoamperometric measurements confirm the results obtained by EIS. Moreover, taking into consideration that GDH has no heme c active site in the enzyme molecule and the behaviour of all studied enzymes is similar, it may be concluded that in these enzymes – conductive polymer systems DET occurs from the PQQ moiety. However, in the case of alcohol dehydrogenases we cannot exclude that heme c also participates in the electron transfer to polyAnD as this is the energetically favorable way.

FIG8 here

4. Conclusions

Label-free impedimetric platform based on 3D-IDEA sensor covered by new conductive polyaniline-type polyAnD polymer is proposed. Introduced impedimetric platform was applied to study biocatalysis of three different PQQ dependent enzymes. For the first time polyAnD was applied as enzyme immobilisation matrix. It was shown, that enzyme immobilisation onto polyAnD by entrapment resulted in total inactivation of enzymes, while enzymes immobilised by physisorption demonstrate specific response in the presence of the enzyme substrates with and without additional electron acceptor. Obtained results show that sADH, mADH and GDH can re-oxidise on polyAnD via direct electron transfer from the enzymes active sites to the polymer

1 surface. Simple amperometric method was applied to confirm these results obtained by
2
3 impedance spectroscopy. Application of the EIS method for investigation of redox-enzymes in
4
5 contrast to amperometric method allows for obtaining not only indirect information about
6
7 processes of biocatalysis at low potential (only 25 mV excitation potential was used during EIS
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9 experiments), but also gives information about modification of electrode surface, its structural
10
11 and electrical changes. Introduced label-free impedimetric platform with PQQ dependent
12
13 enzymes shows sensitivity to typical enzymes substrates at 1 μ M level. Based on the presented
14
15 results it would be possible to design label-free impedimetric biosensors for alcohols and sugars
16
17 determination. However, the improvement and nano-scale modification of polyAnD layer onto
18
19 3D-IDEA should be done to achieve higher surface area for enzyme immobilisation and to
20
21 enhance sensor response.
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27 Presented study shows that EIS method applied to interdigitated electrode structures give a
28
29 useful tool for research of re-oxidation process and interaction of electroactive enzymes with
30
31 various conducting materials giving information required to construct and develop analytical
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33 devices.
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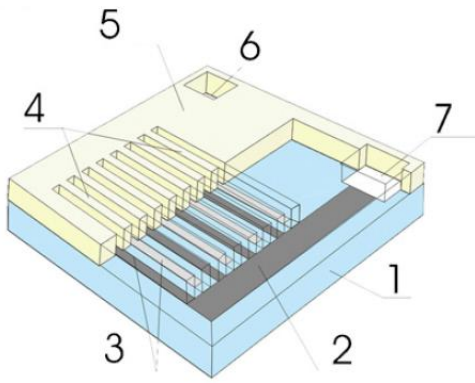


Fig. 1. Design of 3D-IDEA sensor. (1) Insulating substrate (Si/SiO₂), (2) electrode collector bar, (3) electrode digits, (4) silicon dioxide barriers between the electrode digits, (5) 4 μm thick SiO₂ layer, and (6 and 7) aluminium contact pads.

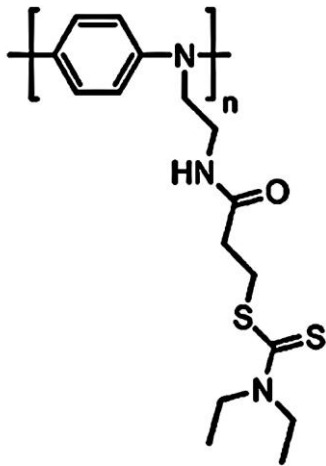


Fig. 2. PolyAnD structure.

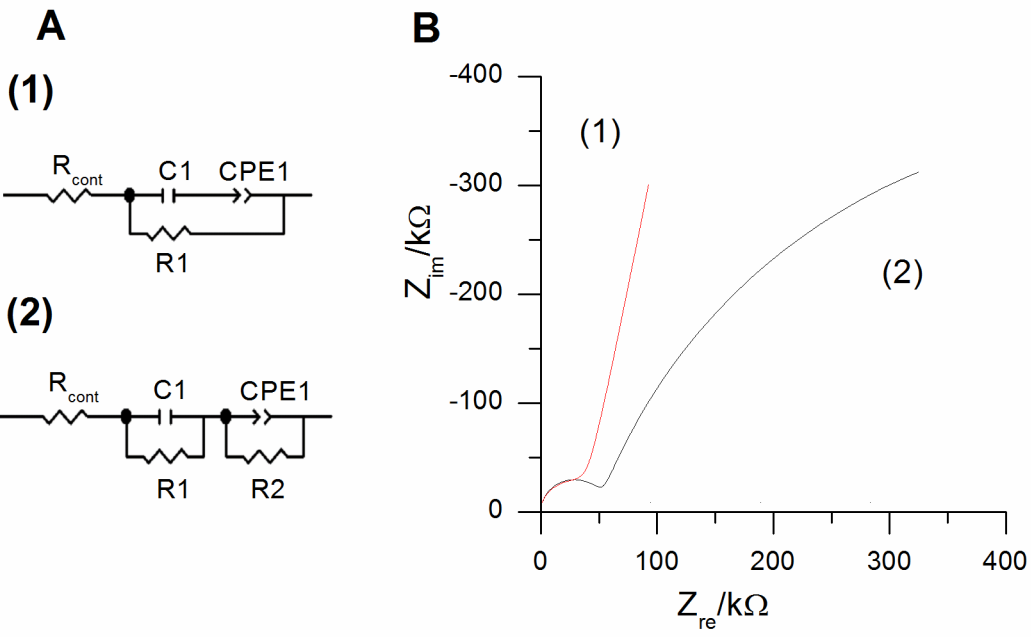


Fig. 3. Equivalent circuits of a 3D-IDEA sensor. **A** – Equivalent circuit models (1) and (2). **B** – Nyquist plot shows characteristic impedance spectra for these two equivalent circuits.

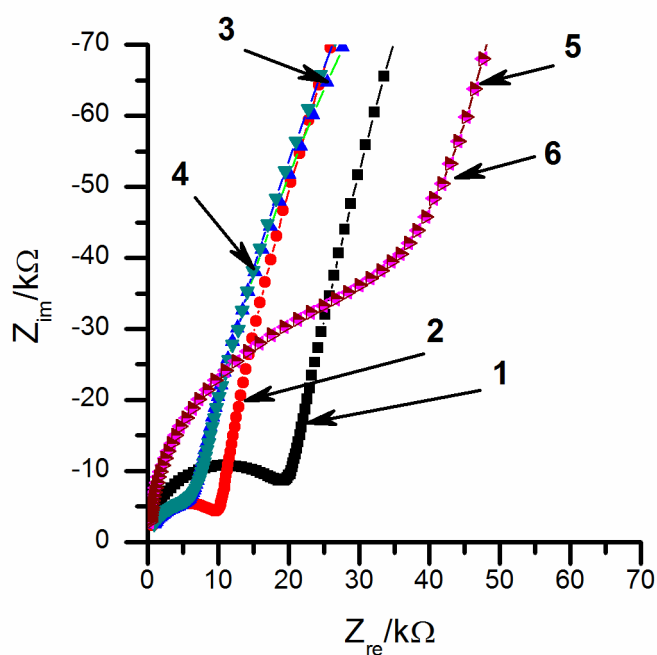


Fig. 4. The Nyquist plot of the impedance spectra of 3D-IDEA sensor modified by polyAnD and PQQ dependent enzyme in CaCl_2 solutions with different conductivity. The curves 1 and 2 represent spectra of an initial clean 3D-IDEA sensor; 3 and 4 – modified by polyAnD; 5 and 6 – modified by polyAnD and the adsorbed enzyme. Curves 1, 3 and 5 were obtained in 10^{-5} M CaCl_2 ($4.62 \mu\text{S/cm}$). Spectra 2, 4 and 6 were measured in $5 \cdot 10^{-5}$ M CaCl_2 with $16.8 \mu\text{S/cm}$ conductivity

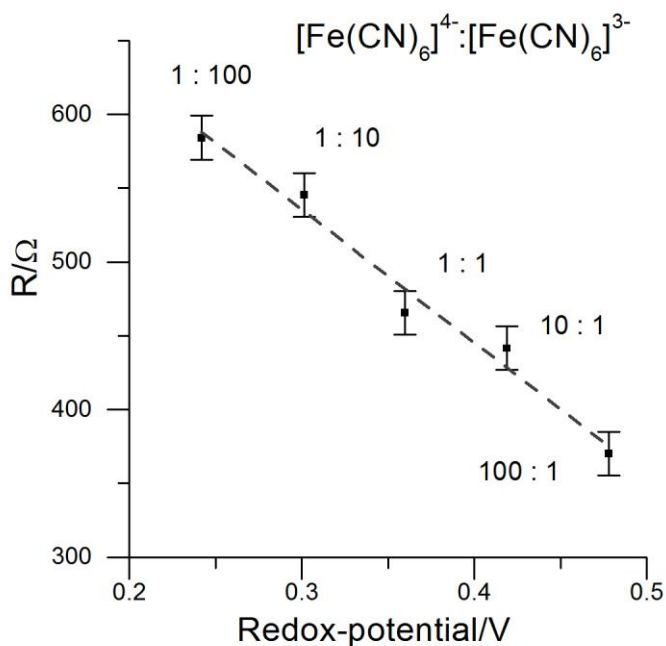


Fig. 5. The resistance of modified by polyAnD 3D-IDEA sensor dependence of solution redox-potential. Dash line represents linear fitting of data points ($r^2=0.9734$).

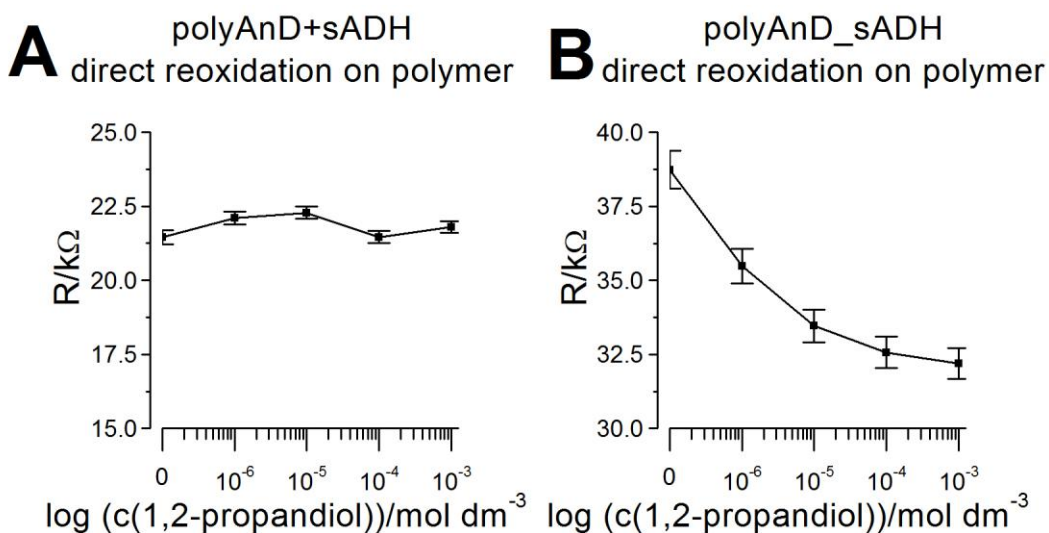


Fig. 6. Response of modified by polyAnD sensor with immobilized sADH to addition of 1,2-propandiol. Response of **A** – entrapped and **B** – adsorbed sADH to addition of substrate without soluble electron acceptor.

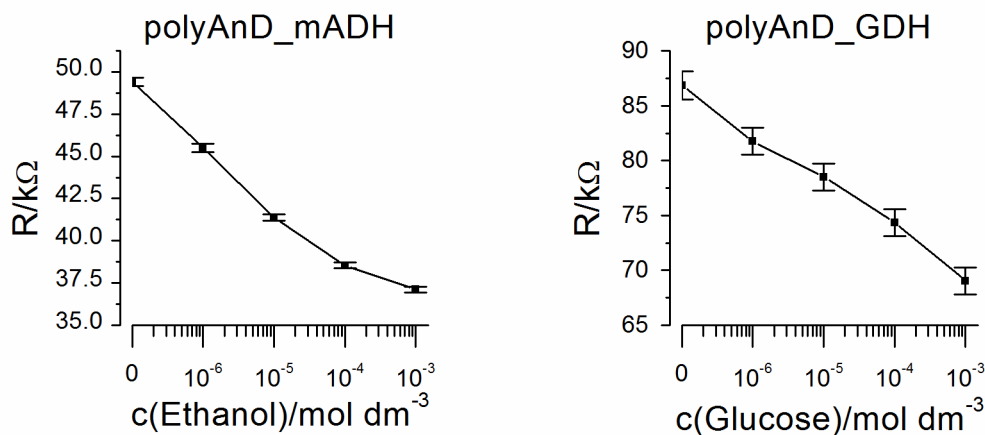


Fig. 7. Response of polyAnD modified sensor with adsorbed mADH (left) and GDH (right) to addition of enzymes substrates, ethanol and glucose, respectively.

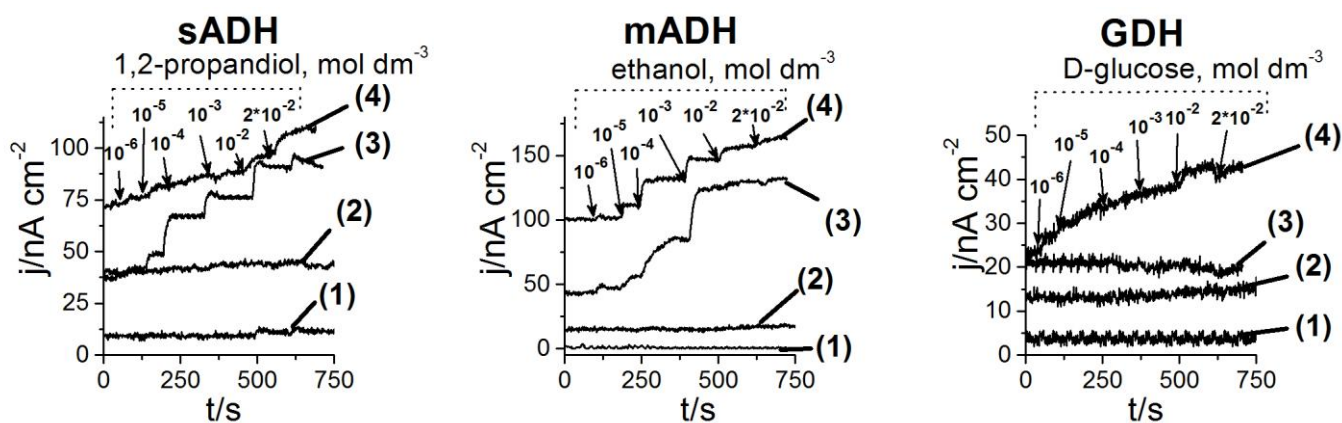


Fig. 8. Chronoamperometric measurements of gold electrodes with PQQ dependent enzymes and polyAnD. (1) – chronoamperogram of clean gold microelectrode, (2) – chronoamperogram after modification with polyAnD, (3) – chronoamperogram after modification with enzyme and (4) – chronoamperogram of gold microelectrode, which was modified by polyAnD and enzyme. The numbers in each graph show concentration of the corresponding enzyme substrate (M) after stepwise addition.