VILNIUS UNIVERSITY

EDITA VOITECHOVIČ

OXIDOREDUCTASES IN BIOELECTROCHEMICAL SYSTEMS: INVESTIGATION AND APPLICATION

Summary of doctoral dissertation
Physical sciences, biochemistry (04 P)

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VILNIAUS UNIVERSITETAS

EDITA VOITECHOVIČ

OKSIDOREDUKTAZIŲ VEIKIMO BIOELEKTROCHEMINĖSE SISTEMOSE TYRIMAS IR TAIKYMAS

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INTRODUCTION

As nowadays the industrial branches of medicine, microbiology, food and environmental protection are rapidly evolving, they require wide variety of analytes and devices determining their extremely low concentrations and reactors performing effective conversions. The efficiency and high selectivity of such devices can be ensured by using a biocatalyst. This role is usually played by enzymes. Yet, despite their uniqueness, the enzymes are expensive and short-lived. Thus in order to develop long-term and effective biosensors and bioreactors cheap and optimal enzymes immobilisation techniques are necessary.

On the other hand, the biosensors and bioreactors should work in the medium which mainly consists of dispersed and fractionated solutions with various organic compounds. Here ultrasensitive spectrophotometric methods cannot be used for complicated qualitative and quantitative detection of the material due to such diverse nature of the medium. Bioelectrochemical methods can be used instead, as they are very sensitive, fast and analysis is cheaper in comparison with imunoanalytical methods. Moreover, transduction element of bioelectrochemical systems could be the same as enzyme immobilisation matrix and as electrode material. Bioelectrochemical systems are easy to miniaturize and they can be used to design micro- sized electrode arrays or, on the contrary, to create bioreactors with high surface area depending on the need of the modern technology [1-2].

Bioelectrochemical systems are usually designed using oxidoreductases. Pyrroloquinoline quinone (PQQ) dependent enzymes are promising in development of bioelectrochemical systems as their natural electron acceptor isn't oxygen and some of them can directly re-oxidize on special electrode surfaces. Several new bioelectrochemical systems based on PQQ dependent dehydrogenases: membrane bound alcohol dehydrogenase from *Gluconobacter sp. 33* (mADH), soluble alcohol dehydrogenase from *Pseudomonas putida* HK5 (sADH), soluble glucose dehydrogenase from *Acinetobacter calcoaceticus* (GDH) and membrane bound fructose dehydrogenase from *Gluconobacter industruis* are studied and proposed in this work. These enzymes can directly re-oxidize on special electrode surfaces and possess wild range substrate specificity. These dehydrogenases are also independent on molecular oxygen. Such enzyme properties are very useful for the design of bioelectrochemical systems, which can work not only under aerobic but also anaerobic conditions.

However for successful application it is necessary to optimize enzyme behaviour in bioelectrochemical systems and to know characteristics of these systems. In this case, one of the most important parts for nano-science and technology is the modelling of the mechanisms of substrate recognition, charge transfer and estimation of limitative factors for general bioelectrocatalytic process. Only clear definition of the action of the molecule, surrounded by other molecules, creates the conditions for the targeted manipulation of those molecules and predict their performance [3].

In this case new bioelectrochemical systems were designed by aiming several goals: selecting optimal electrode surface and immobilisation method, optimizing working conditions of enzymes, estimating effects of heterogeneous, homogeneous and non-aqueous ambience to enzyme action and studying electron transfer (ET) pathways. The results were used to design amperometric biosensors for alcohols, sugars and CO detection. It also was used to construct the bioreactor based on thermophilic arabinose isomerase from *Gluconobacter lituanicus 5* (TAI) for the synthesis of rare sugar – D-(-)-

tagatose. Two bioelectrochemical methods for D-(-)-tagatose determination were proposed.

The aim of this work is to study the behaviour of PQQ dependent oxidoreductases – sADH, mADH, GDH and FDH in homogeneous and heterogeneous systems and to create new bioelectrocatalytic systems based on these enzymes.

The main objectives of the work were as follows:

1. Analysis of PQQ dependent alcohol dehydrogenases properties: effects of buffer solution pH, possibilities of ET in heterogeneous systems and influence of non aqueous solutions on the action of these enzymes in heterogeneous ambience.
2. Study of impedimetric bioelectrochemical systems based on sADH, mADH, GDH and new poly(N-(N',N'-diethyldithiocarbamoylethylamidoethyl)aniline) (poly(NDDEAEA)) conductive polymer.
3. Design and characterization of new bioamperometric systems possessed effective bioelectrocatalysis: by using proper electrode material, enzyme immobilisation technique and electron transfer mediator.
4. Application of these bioamperometric systems for detection of alcohols, carbohydrates and CO.

The novelty and significance of the work

The properties of PQQ dependent enzymes (sADH, mADH, GDH and FDH), such as wild range substrate specificity, the possibility of working under anaerobic conditions and direct re-oxidation on special electrode surfaces are very promising for the development of devices for biosynthesis or biosensors. However, the properties of these enzymes in heterogeneous systems have not been investigated thoroughly. Thus, the influence of the system heterogeneity to substrate specificity of sADH, mADH, GDH and FDH, possibilities to enzyme action in ionic liquids and ET pathways in bioelectrochemical systems with alcohol dehydrogenases were analyzed in this work. In order to create efficient and stable bioelectrochemical systems, new enzyme immobilisation techniques, new electrode materials and new mediators were tested. It was shown, that bioamperometric systems based on sADH and mADH can be applied for determination of alcohols and CO. Investigation of bioamperometric systems based on FDH and 2-(3-nitro(phenyl)amino)- cyclohexa-2,5-dien-1,4-dione permitted us to design a biosensor prototype for D-(-)-tagatose determination.

The defence statements of the work

1) Substrate specificity of immobilised PQQ dependent enzymes differs from that of native enzymes and depends on properties of heterogeneous system used.
2) It is possible to realize the direct electron transfer (DET) from reduced PQQ molecule in enzyme active site to the electrode surface in heterogeneous systems, if energetically favourable way isn't possible.
3) PQQ dependent enzyme immobilisation on polyvinyl alcohol (PVA) modified semi-permeable terylene film ensures efficient performance of enzyme for more than a half year.
4) Bioamperometric system based on FDH and 2-(3-nitro(phenyl)amino)-cyclohexa-2,5-dien-1,4-dione can be applied for D-(-)-tagatose determination.

Dissertation contents and approbation

Dissertation (in Lithuanian) contains 146 pages, 65 figures, 14 tables and 197 entries in the reference list. Dissertation results were published in four research articles and have been presented at 6 national and 8 international scientific conferences.

List of papers

Razumienė J, Gurevičienė V, Voitechovič E, Barkauskas J, Bukauskas V, Šetkus A. Fine structure and related properties of the assembleable carbon nanotubes based electrode for a new family of biosensors with chooseable selectivity. Journal of Nanoscience and Nanotechnology, 2011;11:1-9.

Voitechovič E, Razumienė J, Šakinytė I, Barkauskas J. Investigation of bioelectrocatalytic systems with PQQ-dependent GDH and carbonaceous materials. Biologija, 2010;56:83-7.

Barkauskas J, Razumienė J, Šakinytė I, Voitechovič E. Use of carbon nanomaterials for amperometric biosensors. Lectures Notes of the ICB Seminar. Micro- and Nanosystems in Biochemical Diagnosis. Principles and application, 2010;28-39.

Razumiene J, Voitechovic E, Bachmatova I, Marcinkeviciene L, Meskys R, Laurinavicius V. Electrochemical and spectrophotometric investigations of electron-transfer pathways in biocatalysis by PQQ-ADH. Biologija. 2008;54:171-173.

Voitechovič E, Razumienė J, Gurevičienė V, Bachmatova I, Marcinkevičienė L. Oksidoreduktazių panaudojimas alkoholių ir angliavandenių nustatymui. The book of articles of the conference „Organinė chemija" materials – Kaunas, 2009 m. - Kaunas: Technologija, 2009, p. 76-81. ISBN 978-9955-25-663-2.

Voitechovič E, Šakinytė I, Barkauskas J, Razumienė J. Grafito oksidacijos produktai: tyrimas ir taikymas bioelektrokatalizinėse sistemose. The 15st international conference of students and young researchers „Chemija ir cheminė technologija, 2010" (articles of the conference materials in digital media format on CD); 2010 05 07; Vilnius, LT.

Conference materials/abstracts

1. Voitechovic E, Razumiene J, Palaima A, Janciene R. New quinone-type electron transfer mediators and their application in bioamperometric systems based on PQQ dependent Fructose Dehydrogenase. 3rd International Conference on Bio-Sensing Technology. 2013 may (Spain). Poster presentation.
2. Voitechovic E, Razumiene J, Palaima A, Janciene R. Study of bioamperometric systems based on PQQ dependent FDH EC 1.1.99.11 and new quinone-type mediators. Lietuvos Biochemikų Draugijos XII konferencija "Biochemijos studijoms Lietuvoje – 50 metų". 2012 June (Lithuania). Oral and poster presentation.
3. Voitechovic E. Bioelektrocheminių sistemų su PQQ priklausomomis dehidrogenazėmis ir naujo anilino tipo laidžiu polimeru tyrimas elektrocheminio impedanso spektroskopija. Lietuvos konferencija „Bioateitis–gyvybės ir geomokslų perspektyvos". 2011 December (Lithuania). Oral presentation.
4. Voitechovic E, Bratov A, Abramova N, Razumiene J, Lakshmi D, Ivanova-Mitseva P K, Piletsky S. Electrochemical Impedance Spectroscopy as a Sensitive Method to

Study of Bioelectrochemical Systems Based on Dehydrogenases and Conductive Polymer. International conference: "Science, innovation and gender". 2011 November (Lithuania). Poster presentation.

5. Voitechovic E, Bratov A, Abramova N, Razumiene J, Lakshmi D, Ivanova-Mitseva P K, Piletsky S. The study of bioelectrochemical systems based on PQQ dependent enzymes and conductive polyaniline polymer using method of electrochemical impedance spectroscopy. 2nd International Conference on Bio-Sensing Technology. 2011 October (the Netherlands). Poster presentation.
6. Voitechovic E, Bratov A, Abramova N, Razumiene J. Investigation of bioelectrochemical systems based on the interdigitated electrode array electrodes and PQQ dependent enzymes. Lietuvos konferencija „Doktorantų stažuotės užsienio mokslo centruose". 2011 March (Lithuania). Poster presentation.
7. Razumiene J, Kuisiene N, Voitechovic E, Stankeviciute J, Marcinkeviciene L, Bachmatova I, Citavicius D, Meskys R. Amperometric tagatose determination. „61st Annual meeting of the International society of Electrochemistry". 2010 September, October (France). Poster presentation.
8. Voitechovič E, Šakinytė I, Čirbaitė E. Lacasses – promissing materials in oxidoreductases cofactor regeneration. Meeting of international COST action CM0701 „Cascade chemoenzymatic processes – new synergies between chemistry and biochemistry". 2010 September (Lithuania). Poster presentation.
9. Voitechovič E, Šakinytė I, Razumienė J, Barkauskas J. Investigation of bioelectrocatalytic systems with PQQ-dependent enzymes and nanostructurized carbonaceous materials. Lietuvos biochemikų draugijos XI-ojo suvažiavimas-konferencija „LBD 50". 2010 June (Lithuania). Poster presentation.
10. Voitechovič E, Šakinytė I, Barkauskas J, Razumienė J. Grafito oksidacijos produktai: tyrimas ir taikymas bioelektrokatalizinėse sistemose. The 15st international conference of students and young researchers „Chemija ir cheminė technologija, 2010". 2010 May (Lithuania). Oral presentation.
11. Voitechovič E, Šakinytė I. Baltymų ir anglies struktūrų suderinamumas ateities technologijoms. Lietuvos konferencija „Bioateitis–gyvybės ir geomokslų perspektyvos". 2009 December (Lithuania). Oral presentation.
12. Razumiene J, Gureviciene V, Voitechovic E, Barkauskas J, Bukauskas V, Setkus A. Carbon nanotube electrode and enzyme matrix based composite biosensor. International conference "NanoSmert2009". 2009 October (Italy). Poster presentation.
13. Voitechovič E, Razumienė J, Gurevičienė V, Bachmatova I, Marcinkevičienė L. Oksidoreduktazių panaudojimas alkoholių ir angliavandenių nustatymui. Konferencija "Organinė chemija". 2009 April (Lithuania). Poster presentation.
14. Voitechovič E, Razumienė J, Laurinavičius V, Bachmatova I, Marcinkevičienė L, Meškys R. Elektronų pernašos keliai biokatalizėje su PQQ-ADH. Fundamentiniai ir taikomieji aspektai. Biochemikų draugijos X-asis suvažiavimas-konferencija "Biochemija ir sistemų biologija". 2008 June (Lithuania). Oral presentation.

ABBREVIATIONS

1F – 2-(2-fluoro-phenyl-amino)-cyclohexa-2,5-dien-1,4-dione
1M – 2-(2-methoxy-phenyl-amino)-cyclohexa-2,5-dien-1,4-dione
2F – 2-(4-fluoro-phenyl-amino)-cyclohexa-2,5-dien-1,4-dione
2M – 2-(4-methoxy-phenyl-amino)-cyclohexa-2,5-dien-1,4-dione
3D-IDEA – three-dimensional interdigitated electrode array
AFM – atomic force microscopy
AOx – alcohol oxidase
AuME – gold microelectrode
BBen – 2,5-bis-[methyl-(phenyl)-amino]-cyclohexa-2,5-dien-1,4-dione
Ben – 2-[methyl-(phenyl)-amino]-cyclohexa-2,5-dien-1,4-dione
BQ – 1,4-benzoquinone
BSA – bovine serum albumin
ChMA – chemical modified amorphous carbon
Cst – amorphous carbon "Raven M"
CV – cyclic voltammogram
DCPIP – 2,6-dichlorophenolindophenol
DET – direct electron transfer
ET – electron transfer
FDH – fructose dehydrogenase
GDH – glucose dehydrogenase
GO – graphite oxide
IL – ionic liquid
IL_1 – tetrakis-(2-hydroxyethyl) ammonium trifluoromethane sulfonate
IL_2 – 1-iso-butyl-3-methyl imidazole dicyanamide
IL_3 – 1-iso-pentyl-3-methyl imidazole dicyanamide
mADH – membrane bound alcohol dehydrogenase
MET – mediated electron transfer
MN – 2-(3-nitro-phenyl-amino)-cyclohexa-2,5-dien-1,4-dione
NDDEAEA – N-(N, N'-diethyldithiocarbamoyl ethyl amido ethyl)-aniline
OG-1P, OG-1S, OG-2P and OG-2S – graphite oxidation products
PF – potassium ferricyanide
PMS – phenazine methosulphate
poly(NDDEAEA) – poly-(N-(N',N'-diethyldithiocarbamoylethylamidoethyl)-aniline
PQQ – pyrroloquinoline quinone
PVA – polyvinyl alcohol
sADH – soluble alcohol dehydrogenase
TAI – thermophilic arabinose dehydrogenase
Tris – tris(hydroxymethyl)aminomethane

MATERIALS AND METHODS

Enzymes: **mADH** from *Gluconobacter sp. 33*, E. C. 1.1.5.5, activity of enzyme solution 200 U/ml; **sADH** from *Pseudomonas putida* HK5, E. C. 1.1.9.1, activity of enzyme solution 130 U/ml; **GDH** from *Acinetobacter calcoaceticus*, E. C. 1.1.5.2, activity of enzyme solution 1800 U/ml; GDH mutants with different substrate specificity: **Mut33**, **Mut35** and **Mut42**; **TAI** from *Geobacilus lituanicus 5*, activity of enzyme solution 28 U/ml. MADH, sADH, GDH, Mut33, Mut35, Mut42 and TAI were isolated and purified at the Department of Molecular microbiology and biotechnology (Vilnius University, Institute of Biochemistry) (Lithuania). **FDH** from *Gluconobacter industrius*, E. C. 1.1.99.11, was purchased from "Sigma-Aldrich" (Germany), activity of enzyme solution 800 U/ml. **AOx** from *Pichia pastoris*, E.C. 1.1.3.13, was purchased from "The Merck Group" (Germany).

Electrode materials: amorphous carbon "Raven M" (**Cst**) were purchased from "Columbian Chemicals Co" (USA). Graphite rods were purchased from "Rings-dorff Werke GmbH" (Germany). Chemical modified amorphous carbon (**ChMA**) was synthesized in Department of Inorganic Chemistry (Vilnius University, Faculty of Chemistry) (Lithuania) by the method described in [4]. Graphite oxide (**GO**) was synthesized by the method reported in [5] and graphite oxidation products: **OG-1P**, **OG-1S**, **OG-2P** and **OG-2S** were synthesized by using the methods reported in [6] in Department of Inorganic Chemistry (Vilnius University, Faculty of Chemistry) (Lithuania). Monomer of conductive polyaniline polymer used N-(N, N'-diethyldithiocarbamoyl ethyl amido ethyl)-aniline (**NDDEAEA**) was synthesized in Cranfield Biotechnology Centre (Cranfield University, United Kingdom) by the method reported in [7].

Electron acceptors and mediators: water soluble mediators: potassium ferricyanide (**PF**), phenazine methosulphate (**PMS**) from "Sigma Aldrich" (Germany) and 2,6-dichlorophenolindophenol (**DCPIP**) from "Fluka Chemie GmbH" (Germany). New quinone-type mediators: 2-(2-fluoro-phenyl-amino)-cyclohexa-2,5-dien-1,4-dione (**1F**), 2-(4-fluoro-phenyl-amino)-cyclohexa-2,5-dien-1,4-dione (**2F**), 2-(2-methoxy-phenyl-amino)-cyclohexa-2,5-dien-1,4-dione (**1M**), 2-(4-methoxy-phenyl-amino)-cyclohexa-2,5-dien-1,4-dione (**2M**), 2-(3-nitro-phenyl-amino)-cyclohexa-2,5-dien-1,4-dione (**MN**), 2-[methyl-(phenyl)-amino]-cyclohexa-2,5-dien-1,4-dione (**Ben**), 2,5-bis-[methyl-(phenyl)-amino]-cyclohexa-2,5-dien-1,4-dione (**BBen**) were synthesized in Department of Bioorganic Compounds Chemistry (Vilnius University, Institute of Biochemistry).

Ionic liquids: room temperature ionic liquids (ILs): tetrakis-(2-hydroxyethyl) ammonium trifluoromethane sulfonate (**IL_1**), 1-iso-butyl-3-methyl imidazole dicyanamide (**IL_2**) and 1-iso-pentyl-3-methyl imidazole dicyanamide (**IL_3**) were synthesized in Department of Bioelectrochemistry and Biospectroscopy (Institute of Biochemistry, Vilnius University) (Lithuania).

Another chemicals: 1,2–propanediol, acetic acid, NaOH ("J. T. Baker", the Netherlands), ethanol, KCl ("Riedel – de Haen", Germany), 1,3-propanediol ("Merc", Germany), methanol, 2-propanol, allyl alcohol and 3-methyl-1-butanol ("Reachim-Pharm Ltd", Russia), 2-butanol ("Reanal", Hungary), 2-decanol, polyvinyl dichloride ("Fluka Chemie GmbH", Germany), 1,3-diamino-2-propanol, polyvinyl alcohol (PVA) ("Fluka Chemie GmbH", USA), D-(+)-glucose, D-(+)-galactose, D-(-)-tagatose, D-(-)-fructose from ("Merc", Germany), sodium acetate, $CaCl_2$ ("Lachema", Czech Republic),

Tris ("Carl Roth GmbH + Co", Germany), imidazole ("Fluka AG", Switzerland), 1,4-benzoquinone, bovine serum albumin (BSA), KCl, $K_4[Fe(CN)_6]$, HCl, acetonitrile, N_2, $(NH_4)_3S_2O_8$, dialysis tubing cellulose membrane MWCO 12000 (25 mm thickness) ("Sigma Aldrich", Germany), Na_2HPO_4, 0,1 M citric acid, chloroform, glutaraldehyde, sodium azide, methane acid (85%), concentrated sulfuric acid (95%) ("AppliChem", Germany), sandpaper, terylene film (thickness 12 μm, pore size 0.4 μm) ("Joint Institute of Nuclear Research", Russia); mica ("SPI Supplies Inc", USA); 1.5 ml and 1 cm thickness "semi-micro PMMA" cuvettes ("Brand GmbH+Co KG", Germany).

Construction of impedimetric systems

Impedimetric systems based on a three-dimensional interdigitated electrode array (3D-IDEA) as a transducer were designed by modification of the electrode with poly(N-(N, N'-diethyldithiocarbamoyl ethyl amido ethyl))-aniline (poly(NDDEAEA)). 3D-IDEA were fabricated at the IMB-CNM, CSIC (Spain), as described in [8]. 3D-IDEA electrodes were modified with poly(NDDEAEA) by NDDEAEA oxidative polymerization. Oxidative polymerization was performed following the method published in [7] using $(NH_4)_3S_2O_8$ under the following conditions: 0.018 M $(NH_4)_3S_2O_8$, 0.025 M NDDEAEA, 0.225 M HCl, 25% acetonitrile in water, 1.5 h polymerization in the dark at room temperature. Poly(NDDEAEA)-coated transducers were washed in three cycles with de-ionised water and dried in a N_2 flow.

Immobilisation of sADH by entrapment was performed as follows: the reaction mixture was prepared by mixing 250 μL of NDDEAEA (0.1 M in acetonitrile) with 207 μL of aqueous solution of $(NH_4)_3S_2O_8$ (0.0915 M), 230 μL of HCl (1 M), and 690 μL of sADH solution (0.9 mM). Polymerisation was carried out for 1.5 h at room temperature in the dark. Enzyme and polymer modified electrodes were rinsed several times with de-ionised water and dried in a N_2 flow. Such impedimetric system was named as **sADH+poly(NDDEAEA)**.

Immobilisation of sADH, mADH and GDH by adsorption was done as follows: 2 μL of enzyme solution were dropped onto pre-modified with poly(NDDEAEA) electrode surface and kept at +4 ^oC for 1 h. Enzyme modified electrodes were carefully washed with de-ionised water and dried in a N_2 flow. Such designed impedimetric systems named as **sADH_poly(NDDEAEA)**, **mADH_poly(NDDEAEA)** and **GDH_poly(NDDEAEA)**.

Construction of bioamperometric systems

Bioamperometric systems based on a gold microelectrode (**AuME**) as a transducer were designed by modification of electrode with poly(NDDEAEA). AuMEs (working area 1.64 mm^2) were fabricated at the IMB-CNM, CSIC (Spain) by deposition of titanium (10 nm) and gold (100 nm) onto a silicon wafer covered with 1 μm thick silicon oxide layer. Electrodes were patterned using conventional lift-off technique. Before experiments AuMEs were rinsed with de-ionised water and sonicated in de-ionised water for 10 min. Then they were electrochemically cleaned with potential scanning from -0.2 V to 1.7 V (*vs*. Ag/AgCl) 10-20 times, 200 mV/s scan rate in 0.5 M H_2SO_4 water solution until reproducible gold oxide stripping peaks were obtained. AuMEs were modified with poly(NDDEAEA) by NDDEAEA oxidative polymerization. Oxidative polymerization was performed by the method published in [7] using $(NH_4)_3S_2O_8$, as

described above. SADH, mADH and GDH were immobilised by adsorption: 2 µL of enzyme solution were dropped onto electrode surface and kept at +4 ^{o}C for 1 h. Enzyme modified electrodes were carefully washed with de-ionised water and dried in a N_2 flow.

Different bioamperometric systems based on carbon paste electrode (**CPE**) or graphite rod electrodes (**GE**) as transducers were designed by modification of electrode with carbonaceous materials or quinone-type mediators and immobilisation of enzymes. CPEs were fabricated in Department of Bioanalysis (Vilnius University Institute of Biochemistry). Electrode surface of rod-type CPEs (working area Ø 2.4 mm) and plate-type CPEs (working area Ø 2 mm) was formed by Cst paste, which was prepared by mixing of Cst powders with polyvinyl dichloride. Bioamperometric systems based on sADH, mADH, GDH, Mut33, Mut35, Mut42, AOx and FDH were designed using following methods:

a) Adsorption onto not modified CPEs: 2 µl of sADH, mADH, FDH or GDH were dropped onto CBE surface and kept at +4 ^{o}C for 1 h. These systems are named: **sADH_Cst**, **mADH_Cst, FDH_Cst** and **GDH_Cst**. Between the measurements the systems were stored in 50 mM acetate buffer solution, pH 6.0, contained 10 mM KCl and $CaCl_2$ at +4 ^{o}C.

b) Adsorption onto CPEs modified with carbonaceous materials. Procedure of CPE modification with carbonaceous materials: about 1 mg of ChMA, GO, OG-1P, OG-1S, OG-2P or OG-2S powders was pressed into CPE surface, then not sufficiently attached powders were removed by strong air flow. Then sADH and mADH were immobilised on modified CPEs as described above in section a). These systems are named: **mADH_ChMA-Cst**, **sADH_GO-Cst**, **sADH_OG-1P-Cst**, **sADH_OG-2P-Cst**, **sADH_OG-1S-Cst**, **sADH_OG-2S-Cst**. Between the measurements the systems were stored in 50 mM acetate buffer solution, pH 6.0, contained 10 mM KCl and $CaCl_2$ at +4 ^{o}C.

c) Adsorption onto modified with quinone-type mediators CPEs and GEs. Procedure of CPE and GE modification with mediators: 2 µl of 10 mg/ml MN, Ben, BBen, 1M, 2M, 1F, 2F or BQ solution in chloroform were dropped onto electrode surface and kept at room temperature for 30 min. FDH and GDH were immobilised on modified CPEs as described above in section a). These systems are named: **FDH_MN-Cst**, **FDH_Ben-Cst**, **FDH_BBen-Cst**, **FDH_1M-Cst**, **FDH_2M-Cst**, **FDH_1F-Cst**, **FDH_2F-Cst**, **FDH_MN-GE** and **GDH_MN-Cst**. Between the measurements the systems were stored in McIlvaine's buffer system with pH 4.6 at +4 ^{o}C.

d) Immobilisation onto 0.5% PVA coated semi-permeable terylene film. Procedure of immobilisation onto PVA coated terylene film: 2 µl of sADH or GDH were dropped onto film surface and kept at +4 ^{o}C for 1 h to 1 day. Rod-type CPEs were covered by enzymes modified PVA coated terylene film, so that the enzyme was being placed between the film and the CPE surface. These systems are named: **(PVA)-GDH/1/-Cst** (enzyme immobilisation 1 h), **(PVA)-GDH/2/-Cst** (enzyme immobilisation 2 h), **(PVA)-GDH/4/-Cst** (enzyme immobilisation 4 h), **(PVA)-sADH/1.5/-Cst** (enzyme immobilisation 1.5 h), **(PVA)-sADH/1P/-Cst** (enzyme immobilisation 1 day). Between the measurements the systems were stored in 50 mM acetate buffer solution, pH 6.0, contained 10 mM KCl and $CaCl_2$ at +4 ^{o}C.

e) Cross linking using glutaraldehyde on terylene film. Procedure of AOx cross linking: 5 µl of stock solution, which consist of mixture of 20 µl BSA solution (50 mg/ml BSA in 0.01 M phosphate buffer solution, pH 8.0) with 1.3 mg of AOx, 0.5 mg of

sodium azide and 5 µl of 5% glutaraldehyde water solution, were dropped onto terylene membrane and kept at +4 ^{o}C for 12 h.

Before experiments prepared bioamperometric systems were carefully washed with de-ionised water.

Impedance measurements

Characterisation of 3D-IDEA sensors was performed using impedance measurements in a 100 Hz – 1000 kHz frequency range with a 25 mV (amplitude) voltage excitation at 25 ^{o}C using PARSTAT 2263 Advanced Electrochemical System (Princeton Applied Research, USA). Impedance measurements and the response of enzyme modified electrodes to the addition of enzyme substrate were performed in a 0.01 mM $CaCl_2$ water solution. The enzyme substrates solutions, containing 100 mM of ethanol, 100 mM 1.2-propandiol or 100 mM of D-glucose were prepared using 0.01 mM $CaCl_2$ as a background. Z-Plot/Z-View software package (Scribner Associates, Southern Pines, NC, USA) was used for impedance data treatment and an equivalent circuit fitting. The Origin Pro 8.0 program (free trial version from http://www.originlab.com, Origin-Lab Corporation, USA) was used for data analysis and presentation.

Impedance spectra were fitted using one of the two equivalent circuit models of 3D-IDEA sensor presented in Fig.1.

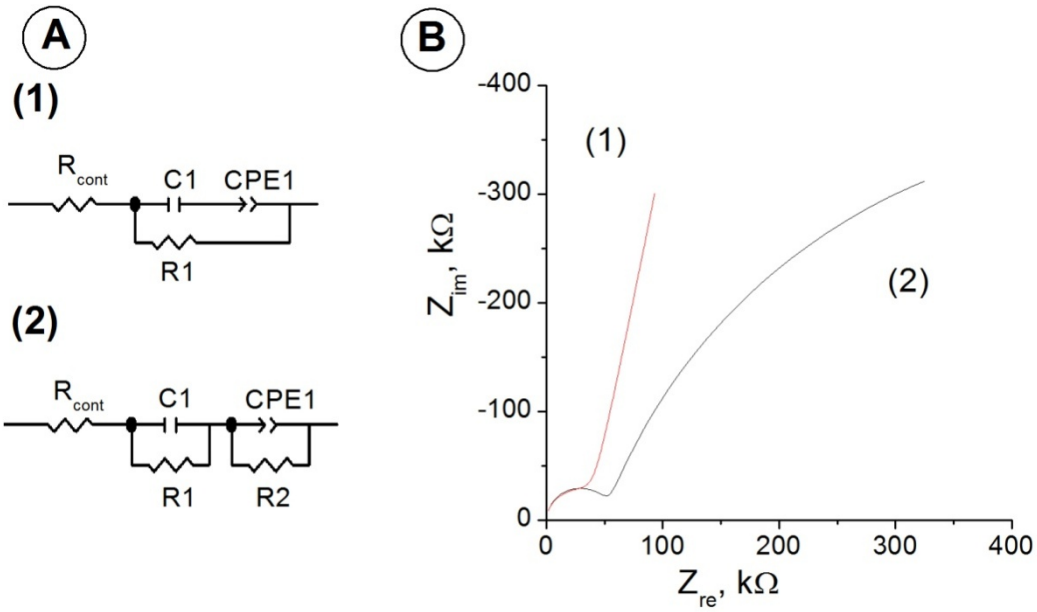

Fig.1. Equivalent circuits of a 3D-IDEA sensor. **A** – equivalent circuit models (1) and (2). **B** – Nyquist plot shows impedance spectra characteristic for each of the equivalent circuits.

The first equivalent circuit presented in Fig.1 A (1) was used for spectra fitting when only one semicircle appears in a spectrum at high frequencies and the low frequency part on the Nyquist plot is linear. In water solutions where the studied systems showed spectra with two semicircles as presented in Fig.1 (2), the second equivalent circuit was used to fit them. The parameter R_{cont} is the resistance of the electrodes contacts. Parallel

combination of the C1-R1 presents the resistance of the polymer layer and the geometrical capacitance of the IDEA sensor and CPE1 and R2 present the capacitance and resistance at the interface between the polymer and the solution.

The accurateness of the fit may be assessed using the chi-squared parameter which is the square of the standard deviation between the original data and the calculated spectrum. For all spectra measured in this work the chi-square parameter was typically smaller than 0.0025.

Chronoamperometry measurements

The potentiostat (Department of Bioanalysis, Vilnius University, Institute of Biochemistry) with a conventional four-electrode system consisting of a platinum plate electrode as an auxiliary, a saturated Ag/AgCl electrode as a reference and two AuME or CBE as a working electrodes were used for chronoamperometric experiments. Same potential is applied on both working electrodes during measurements. Chronoamperometry measurements were carried out in 1.65 ml volume electrochemical cell at 20 ^{o}C. The response of the prepared AuMEs to substrate addition were recorded under potentiostatic conditions at +400 mV in a stirred 100 mM acetate buffer solution, pH 6.0, containing 10 mM $CaCl_2$ and 10 mM KCl. The response of the systems: sADH_Cst, mADH_Cst, GDH_Cst, mADH_ChMA-Cst, sADH_GO-Cst, sADH_OG-1P-Cst, sADH_OG-2P-Cst, sADH_OG-1S-Cst, sADH_OG-2S-Cst, (PVA)-GDH/1/-Cst, (PVA)-GDH/2/-Cst, (PVA)-GDH/4/-Cst, (PVA)-sADH/1.5/-Cst and (PVA)-sADH/1P/-Cst to substrate addition were recorded under potentiostatic conditions at +400 mV in a stirred 50 mM acetate buffer solution, pH 6.0, containing 10 mM $CaCl_2$ and 10 mM KCl. The response of the systems: FDH_MN-Cst, FDH_Ben-Cst, FDH_BBen-Cst, FDH_1M-Cst, FDH_2M-Cst, FDH_1F-Cst, FDH_2F-Cst, FDH_MN-GE and GDH_MN-Cst to fructose, tagatose or galactose addition were recorded under potentiostatic conditions at +400 mV in McIlvaine's buffer system. The chronoamperometry measurements with water soluble mediators were performed by adding of 2 mM PF, 0.1 mM DCPIP or 0.2 mM PMS into electrochemical cell.

The storage stability of bioamperometric systems:

GDH_Cst, (PVA)-GDH/1/-Cst, (PVA)-GDH/2/-Cst and (PVA)-GDH/4/-Cst were checked by response to 3 mM glucose; sADH_Cst, (PVA)-sADH/1.5/-Cst and (PVA)-sADH/1P/-Cst to 1.25 mM 1,2-propanediol; mADH_Cst and mADH_ChMA-Cst to 1.25 mM ethanol; FDH_MN-Cst, FDH_Ben-Cst, FDH_BBen-Cst, FDH_1M-Cst, FDH_2M-Cst, FDH_1F-Cst ir FDH_2F-Cst and FDH_Cst to 10 mM fructose.

Chronoamperometry measurements of sADH_Cst and mADH_ChMA-Cst based on plate-type CPEs were carried out in unstirred ILs water solutions in "one-drop" (60 µl volume) electrochemical cell. The response of these systems to addition of 8 mM 1,2-propanediol or 8 mM ethanol, respectively, were recorded under potentiostatic conditions at +400 mV and 20 ^{o}C. The 5 mM PF were added into electrochemical cell to obtain mediated electron transfer (MET).

The Origin Pro 8.0 program was used for data treatment and analysis.

Voltamperometric measurements

The electrochemical system PARSTAT 2273 (Princeton Applied Research, USA) with a conventional three-electrode system consisting of a platinum plate electrode as an

auxiliary, a saturated Ag/AgCl electrode as a reference and CBE, GE or AuME as a working were used for voltamperometric measurements. Cyclic voltamperometric measurements of modified with quinone-type mediators and FDH GEs and CBEs were carried out in 0.5 M H_2SO_4 water solution or in McIlvaine's buffer system, pH 4.6 at 20 ^oC. Analysis of cyclic voltammograms (CVs) and data processing were performed by the Origin Pro 8.0 program. Determination of heterogeneous electron transfer rate constant was performed by the method described in [9].

AFM measurements

Surfaces of carbonaceous materials were analyzed by using scanning probe microscope Agilent 5500 AFM/STM (Agilent Technologies Inc, US). Standard AFM method such as acoustic AC mode surface scanning was used for visualization of the surface morphology. Imaging was done in intermittent contact mode using a rectangular 'FESP' probe (Veeco Instruments Inc., USA, 0.01 – 0.025 Ωcm Antimony (n) doped S_i with frequency f_c=60 – 100 kHz, spring constants k=1 – 5 N/m, and nominal tip radius 10 nm). Samples for AFM imaging were prepared by the standard method of mechanical exfoliation [10] of carbonaceous materials on a freshly cleaved mica surface. Surfaces of carbonaceous materials were characterized by typical roughness parameters: roughness average (S_a), ten point height (S_{10}), surface skewness (S_s) and surface kurtosis (S_k).

Surfaces of PVA coated semi-permeable terylene film without and with immobilised enzymes were analyzed by scanning probe microscope D3100/Nanoscope IVa (Veeco Instruments Inc., USA) in the State research institute Centre for physical sciences and technology. Standard AFM methods such as contact and tapping mode surface scanning were used for visualization of the surface morphology.

The data and images of scanning probe microscopes were processed by the Scanning Probe Image Processor 5.1.2 (free trial version from http://www.imagemet.com, Image metrology, DK).

Spectrophotometric measurements

Spectrophotometric measurements were performed on UV 300, UV-Visible spectrometer (Thermo Spectronic, USA). Activity of the sADH, mADH and GDH was measured in 50 mM acetate buffer solution, pH 6.0, in the presence of 2 mM 1,2-propanediol, ethanol or glucose, respectively, at 420 nm if PF (ε = 1000 $cm^{-1}M^{-1}$) (2 mM) was used or at 600 nm in the presence of DCPIP (ε = 9300 $cm^{-1}M^{-1}$) (0.1 mM).

The effect of pH to activity of sADH and mADH was measured in 50 mM acetate buffer solution (pH 4.0 – 6.2), 50 mM imidazole buffer solution (pH 6.2 – 7.0) and 50 mM Tris buffer solution (pH 7.0 – 11.0) at 420 nm using PF.

Synthesis of carbon monoxide and its influence to activity of native enzymes and bioamperometric systems

The synthesis of CO was carried out according to the published protocol [11]. The inactivation procedure was carried out by bubbling 50 mM acetate buffer solution, pH 6.0, containing native sADH, mADH, GDH or dipped mADH_ChMA-Cst and GDH_Cst with CO for different time.

Dry mADH_ChMA-Cst and GDH_Cst systems were inhibited by cigarette smoke for different time. The decrease of systems activity was measured by chronoamperometric method.

Galactose bioconversion to tagatose

Isomerisation of D-(+)-galactose to D-(-)-tagatose was catalysed by TAI in stirred phosphate buffer solution at 60 ^{o}C. The reaction was started by addition of 10 mM galactose. 100 µl of reaction mixture, which were collected for different reaction time, were analysed with FDH_MN-Cst and (PVA)-GDH_MN-Cst by the method of chronoamperometry in McIlvaine's buffer system, pH 5.0.

Determination of ethanol in alcoholic beverages

Determination of ethanol in alcoholic beverages was performed by mADH_ChMA-Cst, sADH_Cst in 50 mM acetate buffer solution, pH 6.0, and immobilised AOx on terylene film in 0.01 M phosphate buffer solution, pH 7.5, chronoamperometricaly. Determination of ethanol by mADH_ChMA-Cst and sADH_Cst was performed at +400 mV with two working electrodes potentiostat. Determination of ethanol by AOx was performed at +600 mV with analyser "Eksan A" ("Analita", Lithuania) with conventional three-electrode system consisting of a titanium electrode as an auxiliary, a saturated Ag/AgCl electrode as a reference and platinum plate electrode as a working. Before the determination of ethanol concentration in wines ("Beauvillon", "J. P. Chenet" (medium dry)), bitter ("Palanga") and liqueur ("Mariel"), the beverages samples were diluted 1000 times.

RESULTS AND DISCUSSIONS

1 Properties of PQQ dependent alcohol dehydrogenases

Soluble and membrane bound PQQ dependent alcohol dehydrogenases, which were studied in this work, are promising enzymes in application to bioelectrochemical systems. However, their properties such as pK_a, electron transfer pathways in heterogeneous systems, effect of non aqueous ambience are still unknown.

1.1 Effects of buffer solution pH

In the present work the effect of pH to the activity of native sADH and mADH was determined spectrophotometrically using PF as electron acceptor. It was found, that the variation of sADH initial velocity with pH gives a classical "bell-shaped" curve with maximum enzyme activity in pH range between 7 and 9. The dependence of mADH initial velocity on pH has two optimums: at pH 5.5 and pH 8.4. These results correspond to data, which were published in [12-13]. mADH and sADH pK_a values were determined using Dixon method as described in [14] from the plots of logarithms of initial velocity of enzymes against pH (Fig. 2). pK_{a1} and pK_{a2} of sADH were obtained 6.1 and 10.3, respectively (Fig. 2, A). pK_{a1} and pK_{a2} of mADH were obtained 4.7 and 5.7, respectively (Fig. 2, B). pH dependence of initial velocity of mADH also points that more than one form of enzyme-substrate complex can yield products. The critical point pH value of mADH at 8.4 (Fig. 2, B) corresponds maximum velocity of second enzyme-substrate complex breakdown to products. Moreover, deprotonation of enzyme-substrate complex with pH more than 8.4 irreversibly diminishes the rate of the catalysed reaction. In this case, H^+ ions are activators of mADH catalysis.

Obtained results show, that mADH and sADH activity is optimal in weakly acidic or neutral ambiences. This explained the effect of pH to activity of bioelectrochemical systems based on mADH and sADH, which were published in [4, 15-16].

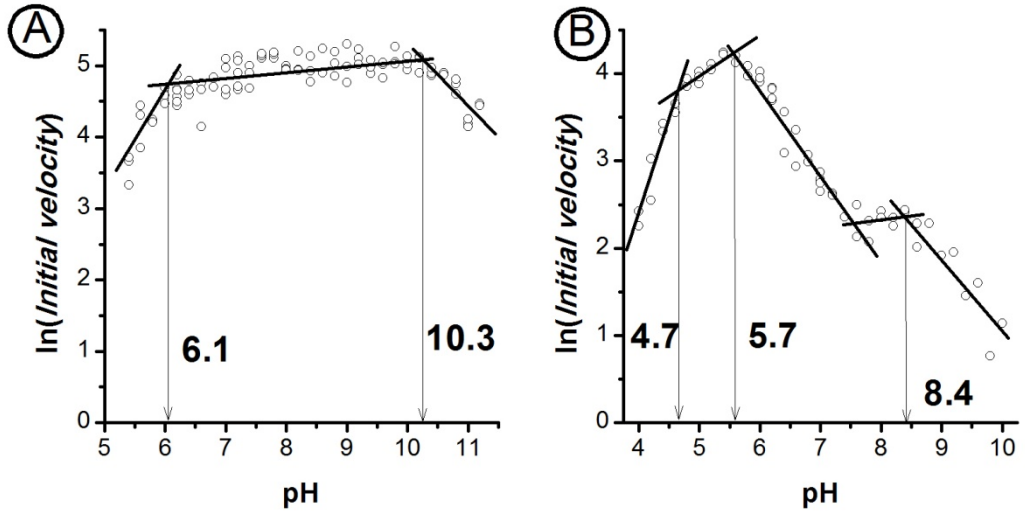

Fig. 2. Plots of logarithms of initial velocity of (A) sADH, (B) mADH against pH.

1.2 Electron transfer pathways in bioelectrochemical systems based on sADH and mADH

mADH and sADH are quinohemoproteins having prosthetic groups: PQQ and hemes c. The enzymes structures and assumed one- or two-electron transfer pathways during bioelectrocatalysis are schematically shown in Fig. 3. As was previously reported, the oxidized cofactor transforms during bioelectrocatalysis to quinol ($PQQH_2$) by accepting two electrons and two protons, and re-oxidation proceeds easily by releasing two electrons [4, 17-20]. Afterwards the electrons might be transferred to the electrode either via the hemes c groups (one-electron transfer), as it occurs in natural ambience, or via PQQ (two-electron transfer).

Aiming to investigate electron-transfer pathways in biocatalysis by sADH and mADH the inhibition of hemes c of enzymes by carbon monoxide was carried out. The inactivation kinetics was studied using one- and two-electron mediators: PF and DCPIP in homogeneous and heterogeneous systems.

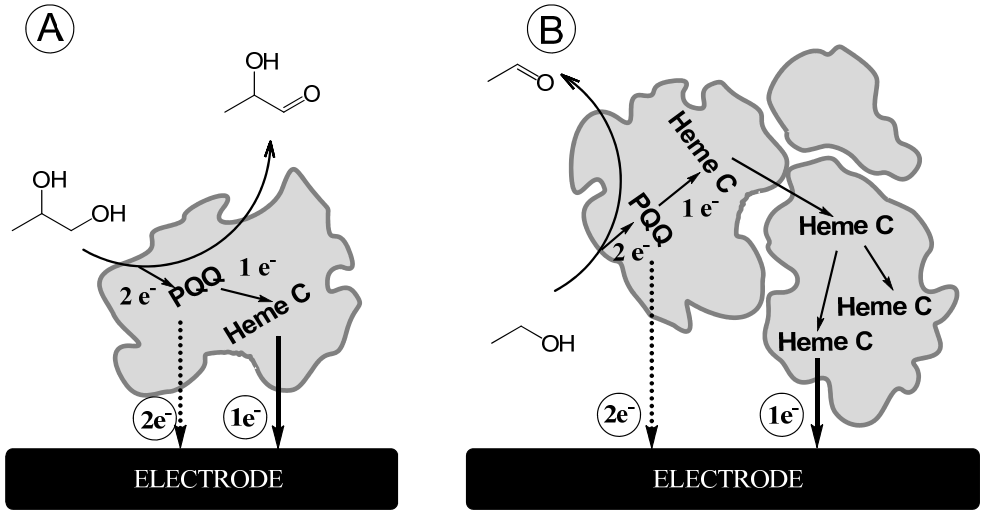

Fig. 3. Proposed electron-transfer pathways for bioelectrocatalysis by sADH (A) and mADH (B). One-electron transfer pathway: from PQQ via heme c to the electrode (pointed by black arrows), and two-electron transfer pathway: from PQQ to the electrode (pointed by dashed arrows).

sADH and mADH activity during the inhibition procedure was evaluated by analysis of responses to 1,2-propanediol and ethanol, respectively. For the first, the effect of inhibition of both enzymes by CO was studied spectrophotometrically in homogeneous system. It was found that sADH activity decreased during process of inhibition with CO and also without treatment with CO. The effect of inhibition of mADH by CO was more effective and mADH was more stable than sADH. In this case, mADH was used for the next studies of electron transfer. The inactivation kinetics of mADH during biocatalysis was found to be significantly different for one- and two-electron mediating processes, using PF and DCPIP, respectively. Besides that, the same inactivation procedure was performed on GDH, which has no hemes c, and no inactivation was observed. The calculated inactivation constant (k_{in}) observed for native mADH by using the one-electron mediator PF (k_{in} 0.344 min^{-1}) differed sufficiently from the k_{in} of the control sample (0.002 min^{-1} in the absence of CO). This indicates that hemes c were successfully inhibited and the electron-transfer mechanism via the internal mediators is hardly

possible, whereas the mediating way by using the two-electron acceptor DCPIP exhibits a significantly lower inactivation (k_{in} 0.027 min^{-1}) in comparison with the one-electron mediating way. This fact implies that an alternative electron transfer way – shunting the hemes pathway – may be switched on in the presence of two-electron mediators even if the hemes are blocked.

Aiming to shed light on the proposed alternative electron transfer pathway during biocatalysis by mADH, the electrochemical measurements were carried out as well. For this purpose, the bioelectrochemical system mADH_ChMA-Cst was constructed and inactivation kinetics using CO was studied. Inactivation constants for direct (mediatorless) bioelectrocatalysis (DET) of mADH or one- or two-electron-mediated processes were calculated in heterogeneous system. Calculated from electrochemical measurements k_{in} of the control sample in the absence of CO (0.004 min^{-1}) correlated with the k_{in} of the control sample of native mADH (0.002 min^{-1}). Furthermore, in all cases of inhibition the inactivation process of mADH dependent bioelectrocatalysis can be divided into the faster and the slower steps characterized by the different inactivation rates. The calculated k_{in} for both stages corresponded to k_{in} obtained spectrophotometrically of the native enzyme in one-electron (the faster stage) and two-electron (the slower stage) mediating pathways, respectively. Evidently, the k_{in} values of the faster and the slower inactivation processes in both cases of bioelectrocatalysis: mediatorless or mediated using one-electron mediators differed at least 10 times. Values of k_{in} are summarized in Table 1.

Table 1. Inactivation constants calculated for inactivation of biocatalysis by mADH.

Investigation:	***Spectrophotometric***	***Electrochemical***
k_{in} of one-electron transfer pathway, min^{-1}	**0.344** (using PF)	**0.442** (faster stage of inactivation of DET) **0.155** (faster stage of inactivation using PF)
k_{in} of two-electron transfer pathway, min^{-1}	**0.027** (using DCPIP)	**0.053** (slower stage of inactivation of DET) **0.026** (slower stage of inactivation using PF) **0.024** (inactivation of bioelectrocatalysis using DCPIP)
k_{in} of bioelectrocatalysis without inhibition, min^{-1}	0.002	0.004

It can be concluded that the electrochemical analysis of inactivation kinetics justifies the fact that the bioelectrocatalysis by mADH in the case of no additional two-electron mediators proceeds via an energetically favourable way of the internal mediation (Fig. 3, B one-electron transfer pathway). However, an alternative two-electron transfer pathway from PQQ to the electrode (Fig. 3, B one-electron transfer pathway) is switched on when hemes are inactivated. This electron transfer pathway can be realized due to function groups of the carbon electrode material. Furthermore, two-electron mediators promote this alternative way of bioelectrocatalysis by mADH independently of the inhibition of hemes c.

1.3 Effect of non aqueous ambience on activity of mADH and sADH in heterogeneous systems

In the present work non aqueous ambience was created with new synthesized room temperature ionic liquids: IL_1, IL_2 and IL_3. Activity of sADH and mADH was evaluated by analysis of responses to 1,2-propanediol and ethanol, respectively, in the pure ILs and ILs water solutions and compared with the responses of enzymes, which worked in typical buffer solution. Both enzymes were immobilised on CPEs. The chromoamperometry measurements were performed with sADH_Cst and mADH_ChMA-Cst bioamperometric systems. The direct electron transfer and mediated electron transfer (MET) with PF were observed.

It was found, that sADH and mADH show no activity in the pure ILs. The response of both enzymes to substrate addition was decreased by increasing ILs concentration. The main results of these studies are summarised in Table 2. For the first time it was shown, that sADH_Cst system is able to work in a 50% IL_3 water solution by maintaining up to 50% of enzyme activity.

Table 2. Effect of ILs on activity of sADH and mADH working in heterogeneous system expressed in current.

Ambience:	***50 mM acetate buffer solution***		***50% IL_1***		***50% IL_2***		***50% IL_3***	
Electron transfer:	*DET*	*MET*	*DET*	*MET*	*DET*	*MET*	*DET*	*MET*
sADH	290 ± 45 nA	630 ± 30 nA	-	-	-	130 ± 15 nA	-	210 ± 25 nA
mADH	76 ± 8 nA	1.95 ± 0.13 µA	-	-	-	7 ± 4 nA	-	2 ± 0.8 nA

2 Impedimetric systems

Electrochemical impedance spectroscopy is a sensitive tool providing information on various physical and chemical properties of materials, as well as on interaction processes occurring in the bulk or at the surface of these materials. The behaviour of PQQ dependent enzymes immobilised on 3D-IDEA impedimetric sensor covered by new conductive polyaniline-type poly(NDDEAEA) polymer is studied.

3D-IDEA sensors were modified with poly(NDDEAEA) by chemical polymerisation. After deposition of the polymer film the sensors show rather small interelectrode resistivity that is only slightly affected by changes in the solution conductivity. 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. The resistance of the film composed by poly(NDDEAEA) and adsorbed sADH, mADH or GDH becomes several times higher. This means that there is an interaction between PQQ dependent enzymes and the conductive polymer that affects the electrical properties of the latter. However, the sensor impedance also was not affected by the solution conductivity.

No specific resistance changes of 3D-IDEA covered with poly(NDDEAEA) without immobilised enzymes were observed after addition of enzymes substrates: 1,2-propandiol, ethanol and D-glucose till 1 mM their concentration in 10^{-5} M $CaCl_2$

(4.62 μS/cm) solution. The study of impedimetric systems with sADH enzyme immobilised by entrapment during polymerization of poly(NDDEAEA) showed that this method is not suitable as it results in inactive enzymes.

The sensor with adsorbed sADH, mADH and GDH showed specific response to addition of the enzyme substrates (Fig. 4). As can be seen, sensors with sADH, mADH and GDH show decrease in determined resistance (R1) upon addition of enzymes substrates. This fact permits to say that all studied enzymes in this work can undergo direct re-oxidation on poly(NDDEAEA). Re-oxidation processes provoke partial reduction of poly(NDDEAEA) chain and thereby affect the electrical conductivity of the polymer layer registered by impedance measurements.

Chronoamperometric measurements were performed using AuMEs to ensure that PQQ dependent enzymes can be directly re-oxidised on poly(NDDEAEA) surface. Modification of AuMEs with poly(NDDEAEA) was performed by using the same method as is used in impedimetric systems. Immobilisation of enzymes was performed by superficial adsorption. Results obtained on amperometric sensors confirmed results obtained by impedance measurements and show that studied enzymes can be re-oxidised on poly(NDDEAEA) via DET from the enzyme active site to the polymer surface.

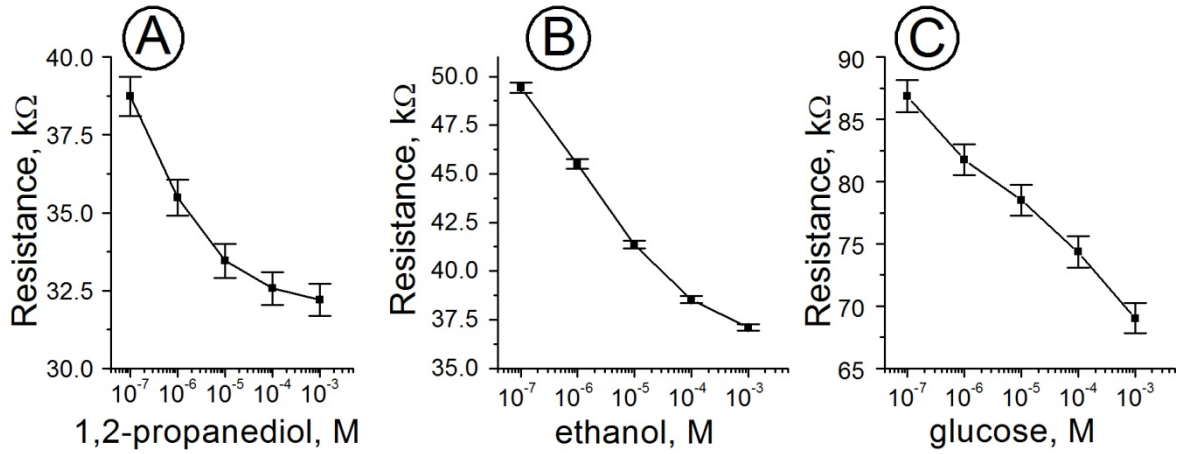

Fig. 4. Response of poly(NDDEAEA) modified sensor with adsorbed sADH (A), mADH (B) and GDH (C) to addition of enzymes substrates, 1,2-propandiol, ethanol and glucose, respectively.

3 Bioamperometric systems

Aiming to enhance the bioelectrocatalysis of PQQ dependent dehydrogenases the bioamperometric systems based on different carbonaceous materials were designed. A key to obtain effective bioamperometric system is the development of a suitable immobilisation of enzymes technique and optimisation of communication of dehydrogenases with electrode surface. In this case several new methods of enzyme immobilisation, new carbonaceous materials and new quinone-type mediators were tested.

3.1 Systems based on GDH

In the present work the immobilisation of GDH on PVA modified semi-permeable terylene film was developed. The efficiency of this immobilisation method was

compared with simple superficial adsorption on not modified CPE (bioamperometric system GDH_Cst).

Bio-recognition properties, calibration curve and long term stability of bioamperometric systems can mainly be related to porous structure of PVA modified terylene film on which enzyme is immobilised. The characterisation of surface of terylene film was performed by AFM scanning. The pictures of the front side of PVA modified film with immobilised GDH and the back side without enzyme are presented at Fig. 5 A and B, respectively. The front side of the PVA modified film (Fig. 5, A) is covered by comparatively thick GDH layer through which the surface of the membrane practically cannot be detected by the AFM scanning. Differences in height on the enzyme layer can be up to about 1 μm, as it is represented by the dark to light scale. The back side of the PVA modified film remained clear of enzyme molecules after immobilisation of GDH on the front side of the film, as it is shown in Fig. 5 B. So, after GDH immobilisation, PVA modified terylene film remained its semi-permeability and different low molecular weight molecules can easily pass through the film.

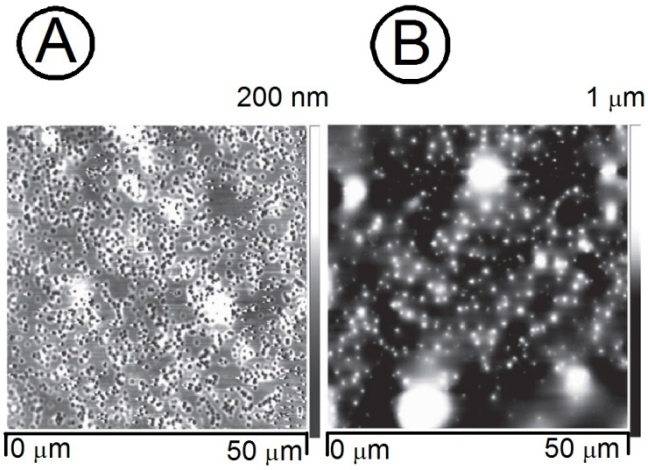

Fig. 5. AFM image of the PVA modified terylene film surfaces obtained on the front side **(A)** with enzyme GDH layer and the back side **(B)** without enzyme layer.

GDH immobilisation was performed for different periods of time to optimise the amount of enzyme on the PVA modified terylene film. The obtained bioamperometric systems were: (PVA)-GDH/1/-Cst, (PVA)-GDH/2/-Cst and (PVA)-GDH/4/-Cst. These systems were investigated using water soluble mediator: PMS. The main characteristics of (PVA)-GDH/1/-Cst, (PVA)-GDH/2/-Cst and (PVA)-GDH/4/-Cst and GDH_Cst were summarised in Table 3. Kinetic parameters: apparent Michaelis constant (K_M^{app}) and apparent maximum current (I_{max}^{app}) were determined using Michaelis and Menten equation for bioamperometric systems [21].

The systems, which were designed by immobilisation of GDH on PVA modified terylene film were at least 10 times more stable and generated 4 times higher response to glucose in comparison to the GDH_Cst system. The sensitivity of immobilised GDH was also higher than GDH_Cst. However, the immobilisation of GDH extended the response time of systems (up to more than 4 min). It also should be noted, that inactivation of all studied systems occurs in two stages, except for (PVA)-GDH/1/-Cst, its inactivation occurs in one stage. The first inactivation stage could be explained by desorption of not effective attached enzyme from film or electrode surface, and then the second inactivation stage defines slow denaturation process of GDH. Among the others (PVA)-

GDH/2/-Cst system possesses the highest stability, as it responded to addition of glucose for more than 9 months. These are the best stability results, which were obtained during the study of bioelectrochemical systems with GDH. Because of its high stability and efficiency, (PVA)-GDH/2/-Cst can be applied in commercial biosensoric systems or biological fuel cell elements. For this reason the influence of room temperature changes to the activity of systems with immobilised GDH on PVA modified terylene film were evaluated. The magnitude of response to glucose was increased approximately 2.4% by increasing temperature for 1 degree in the range of 15 – 35 ^{o}C. It was also found, that the activation energy of bioelectrocatalysis by GDH is 26 ± 2 kJ/mol (obtained as described in [22-23]) and is similar to activation energies of bioelectrocatalysis by other dehydrogenases [24]. It also should be noted, that the thermal inactivation of bioamperometric systems of immobilised GDH on PVA modified terylene film is reversible, if the measurements were done at the temperature not higher than 50 ^{o}C.

Table 3. The characteristics of bioamperometric systems based on GDH.

Bioamperometric system:		***(PVA)-GDH/1/-Cst***	***(PVA)-GDH/2/-Cst***	***(PVA)-GDH/4/-Cst***	***GDH_Cst***
Apparent maximum current density, μAcm^{-2}		45 ± 5	37 ± 1	46 ± 2	11.5 ± 0.7
K_M^{app}, mM		7.2 ± 0.6	3.6 ± 0.4	5.7 ± 0.8	2.5 ± 0.3
Sensitivity, $\mu Acm^{-2}mM^{-1}$		4.1	4.4	4.5	3.3
Time of response, min		4.2 ± 0.6	5.0 ± 2.1	5.3 ± 1.9	1.5±0.5
Linear ranges, mM		0.001 – 3	0.001 – 4.5	0.001 – 4.5	0.001 – 1
Decreasing of 50% response, days		4.6	17	20	1.7
Time of system working, days		50	265	149	15
$k_{in} \cdot 10^{-5}$, min^{-1}	***1***	-	5.2 ± 0.6	3.9 ± 0.3	21 ± 5
	2	-	0.9 ± 0.1	1.3 ± 0.7	59 ± 0.3
	3	3.7 ± 0.3	-	-	

3.1.1 Substrate specificity of GDH

In order to evaluate possible application potential, substrate specificity of GDH was studied in this work. It is known, that native GDH possesses wild range of specificity to aldoses [25]. Aiming to enhance specificity of GDH, the GDH mutants: Mut33, Mut35 and Mut42 were constructed. In the present work the substrate specificity of GDH and its mutants was studied in heterogeneous systems and compared with specificity of native GDH (in homogeneous system). Enzymes were immobilised on PVA modified terylene film and their activity was estimated by chronoamperometric measurements using PMS. The obtained results are presented in Fig. 6. Substrate specificity of wild-type GDH in heterogeneous system hasn't changed significantly comparing with the

same specificity in homogeneous system. Substrate specificity of GDH mutants was different than of wild-type GDH. Mut33, Mut35 and Mut42 possessed more than 20% lower response to same carbohydrates comparing with GDH activity in heterogeneous system. Immobilised enzymes were characterised by main kinetic parameters: K_M^{app} and I_{max}^{app}. I_{max}^{app} of GDH mutants for each substrate were at least more than 3 times lower and K_M^{app} were more than 10 times higher than I_{max}^{app} and K_M^{app} of wild-type GDH, respectively. K_M^{app} of GDH mutants for glucose was more than 20 mM, for lactose – more than 60 mM, for galactose – more than 100 mM and for maltose – more than 90 mM. This leads to conclusion, that the bioelectrocatalysis of GDH mutants was not effective. However, neither wild-type GDH nor GDH mutants did not oxidise ketoses: fructose and tagatose.

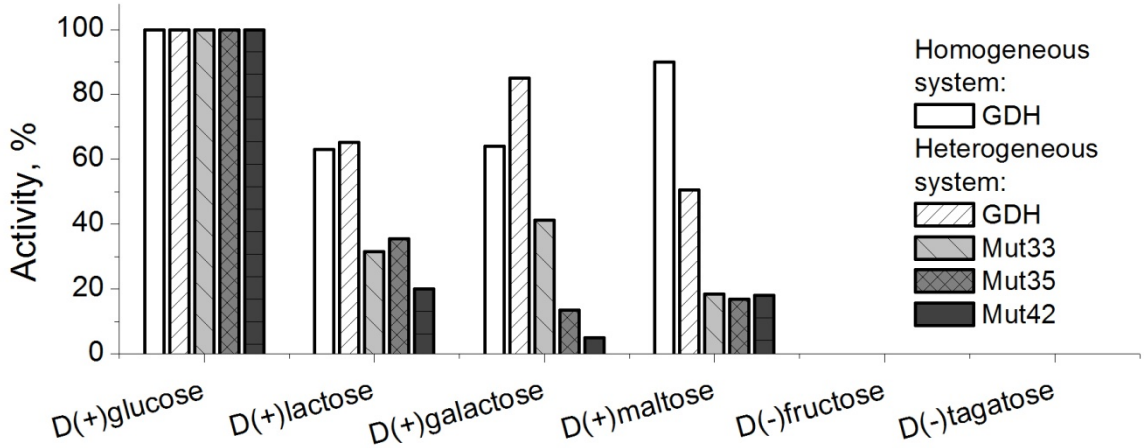

Fig. 6. Substrate specificity of glucose dehydrogenases. Substrate specificity of native GDH in homogeneous system from [25-26].

3.2 Systems based on sADH

SADH is water soluble protein and similarly to GDH possesses hydrophilic surface. Aiming to obtain stable and effective bioelectrocatalytic system the immobilisation of sADH on PVA modified semi-permeable terylene film has been tested. The efficiency of this immobilisation method was compared to simple adsorption on not modified CPE (bioamperometric system sADH_Cst). Focusing to optimise the amount of enzyme on the PVA modified terylene film, sADH was immobilised for different periods of time. It resulted in two designed bioamperometric systems: (PVA)-sADH/1.5/-Cst and (PVA)-sADH/1P/-Cst. Investigations of these systems were performed using water soluble mediator – PF. The summary of obtained main characteristics of sADH_Cst, sADH/1.5/-Cst and (PVA)-sADH/1P/-Cst were presented in Table 4Table 3. Systems based on immobilised enzyme (sADH/1.5/-Cst and (PVA)-sADH/1P/-Cst) possessed higher apparent current density and sensitivity. However system based on adsorbed sADH (sADH_Cs) was more stable: the response to addition of 1,2-propanediol was obtained for more than 160 days. As in the case of bioamperometric systems with GDH, inactivation of sADH_Cst, (PVA)-sADH/1.5/-Cst and (PVA)-sADH/1P/-Cst occurs in two stages: faster and then slower. The first inactivation stage could be explained by desorption of not effective attached enzyme, while second inactivation stage defines slow denaturation process of sADH. Thus, in this case adsorption of sADH determined more stable systems.

Aiming to enhance the efficiency of bioamperometric systems based on sADH the new carbonaceous materials – graphite oxidation products: GO, OG-1P, OG-1S, OG-2P and OG-2S were used as a transducers and immobilisation matrix of sADH as well. Enzyme was immobilised on modified by graphite oxidation products CPE surfaces by adsorption. The following bioamperometric systems: sADH_GO-Cst, sADH_OG-1P-Cst, sADH_OG-2P-Cst, sADH_OG-1S-Cst and sADH_OG-2S-Cst were obtained. The investigations of these systems were carried out without adding of any external mediator.

Table 4. The characteristics of bioamperometric systems based on sADH.

Bioamperometric system:	***sADH_Cst***	***(PVA)-sADH/1.5/-Cst***	***(PVA)-sADH/1P/-Cst***
Apparent maximum current density, μAcm^{-2}	4.6 ± 0.5	12 ± 0.2	13 ± 0.7
K_M^{app}, mM	0.80 ± 0.03	0.4 ± 0.1	2.2 ± 0.1
Sensitivity, $\mu Acm^{-2}mM^{-1}$	2.2	18.1	5.1
Time of response, min	7.4 ±1.2	1.0 ± 0.2	1.1 ± 0.3
Linear ranges, mM	0.001 – 1.0	0.001 – 0.3	0.001 – 0.7
Decreasing of 50% response, days	4.7	0.1	1.3
Time of system working, days	160	60	60
$k_{in} \cdot 10^{-5}$, min^{-1} 1	28 ± 2	40 ± 6	29 ± 2
2	1.5 ± 0.5	7 ± 1	7 ± 1

All obtained reagentless bioamperometric systems based on sADH were characterized by kinetic parameters: K_M^{app} and I_{max}^{app}, which are presented at the Fig. 7. The kinetic parameters were compared with parameters of reagentless systems based on GE and not modified CPE with adsorbed sADH. As can be seen, no response to addition of 1,2-propanediol was obtained by bioamperometric systems based on GE and GO, whereas OG-1P, OG-1S, OG-2P and OG-2S enhanced sADH bioelectrocatalysis more than 2 times in comparison with Cst. The highest activity of sADH was obtained with system based on OG-S2, which apparent maximum current density was at least higher than 2 μA/cm^2.

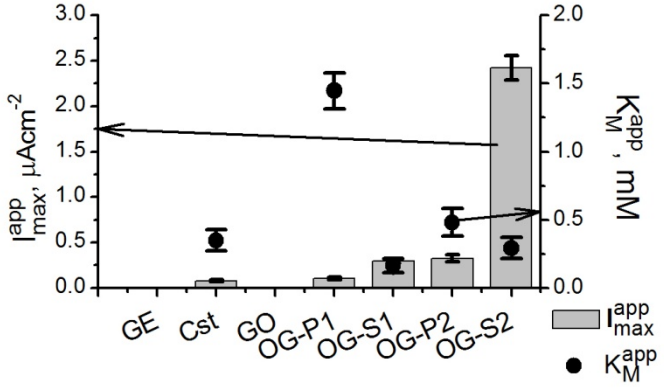

Fig. 7. Kinetic characteristics of reagentless bioamperometric systems with sADH and different carbonaceous materials.

It is known, that graphite oxidation products include different amount of basic groups. However, the efficiency of bioamperometric systems also may depend on three dimensional structures formed on electrode surfaces by carbonaceous materials.

In order to discover how three dimensional structures of electrode surfaces influence the efficiency of the bioelectrocatalysis, the analysis of morphology of carbonaceous materials was done by AFM scanning. The carbonaceous materials morphology was characterised by typical roughness parameters: roughness average, ten point height, surface skewness and surface kurtosis (see Table 5). The AFM imaging analysis revealed differences of these carbonaceous materials. It was found, that graphite oxidation products formed fine particles, which possess different roughness. The most effective bioelectrocatalysis by sADH were obtained in the system based on OG-2S, which is characterised by low S_a, S_k and S_s values. However, the clear dependence of efficiency of bioelectrocatalysis by sADH on some roughness parameters wasn't observed. This allows the conclusion, that efficiency of bioelectrocatalysis not only depends on surface morphology of the carbonaceous materials, but also the content of functional groups is substantial for creating reagentless enzyme-based bioamperometric systems.

Table 5. Roughness parameters of different carbonaceous materials.

	Graphite	*Cst*	*GO*	*OG-1P*	*OG-1S*	*OG-2P*	*OG-2S*
S_a, nm	3	20	3	4	25	4	12
S_k, nm	10	7	153	47	4	55	3
S_s, nm	0.6	-2	3.1	10.9	0.3	29.1	1.1
S_{10}, nm	70	361	225	228	175	423	74

3.2.1 Substrate specificity of sADH

The specificity of sADH to alcohols was evaluated in homogeneous (biocatalysis was performed in buffer solution) and heterogeneous (biocatalysis of immobilised enzyme on CPE) systems. It was found, that sADH catalyses oxidation of all studied alcohols in homogeneous system, while the response to addition of 1,3-diamino-2-propanol, methanol, 2-propanol and 2-decanol wasn't achieved by immobilised enzyme (see Fig. 8). The highest response of sADH was obtained to addition of 1,2-propanediol, allyl alcohol and 1,3-propanediol.

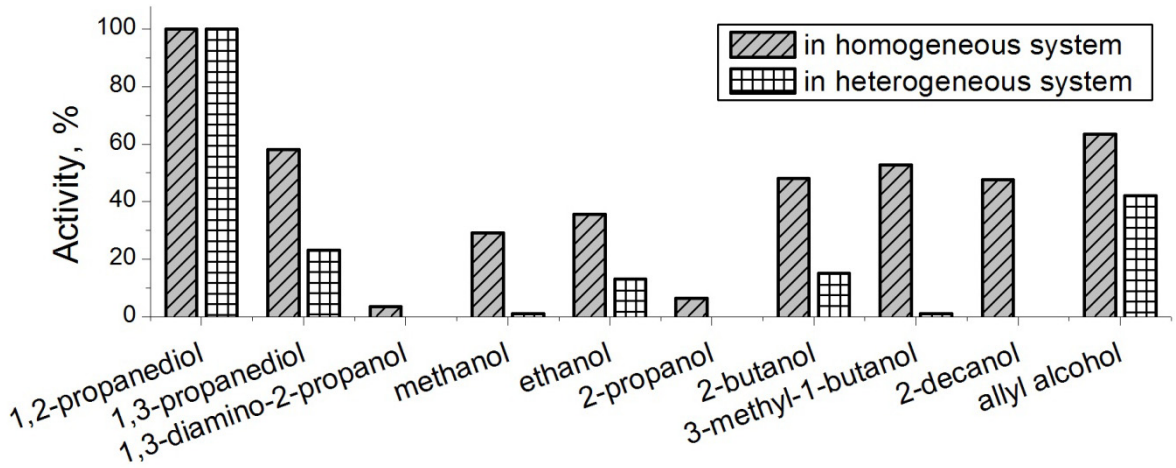

Fig. 8. Substrate specificity of sADH in homogeneous and heterogeneous systems.

It was shown, that sADH possessed specificity to wider range of alcohols in heterogeneous systems then in homogeneous system. This fact can be explained by conformational changes of sADH during immobilisation process that changed structure of enzyme active site, and by different mobility of alcohols in electric field of bioamperometric system.

3.3 Systems based on mADH

The immobilisation of mADH was performed on hydrophobic CPE surface, to obtain effective interaction of protein molecule with immobilisation matrix. The immobilisation of mADH was performed by adsorption on surfaces of modified with ChMA and not modified CPE. In consequence two reagentless bioamperometric systems (mADH_ChMA-Cst and mADH_Cst) were obtained. The determined main characteristics of mADH_ChMA-Cst and mADH_Cst are presented in Table 6. The bioamperometric system based on ChMA exhibited 6 times higher stability, 15 times higher I_{max}^{app} and was more sensitive in comparison with bioamperometric system based on not modified CPE. It can be concluded, that immobilisation of mADH on ChMA modified electrode surface is suitable for effective DET from mADH active site to the electrode surface.

Table 6. The characteristics of bioamperometric systems based on mADH.

Bioamperometric system:	***mADH_ChMA-Cst***	***mADH_Cst***
Apparent maximum current density, μAcm^{-2}	3.3 ± 0.1	0.2 ± 0.01
K_M^{app}, mM	0.48 ± 0.03	0.07 ± 0.001
Sensitivity, $\mu Acm^{-2}mM^{-1}$	3.9	0.8
Time of response, min	1.6 ± 0.2	1.8 ± 0.7
Linear ranges, mM	0.001 – 0.3	0.001 – 0.1
$k_{in} \cdot 10^{-5}$, min^{-1}	11 ± 2	60 ± 9

3.3.1 Substrate specificity of mADH

The specificity of mADH to alcohols was evaluated in homogeneous (biocatalysis was performed in buffer solution) and heterogeneous (biocatalysis of immobilised enzyme on CPE) systems. The results are shown in Fig. 9. It was found, that mADH effectively oxidised ethanol, allyl alcohol, 3-methyl-1-butanol and 1,3-propanediol in homogeneous system. However, the enzyme specificity in heterogeneous system was different. Here the highest response to addition of ethanol, allyl alcohol, 3-methyl-1-butanol and methanol were achieved by biocatalysis of mADH. This fact can be explained by conformational changes of enzyme during immobilisation process and by different mobility of alcohols in electric field of bioamperometric system.

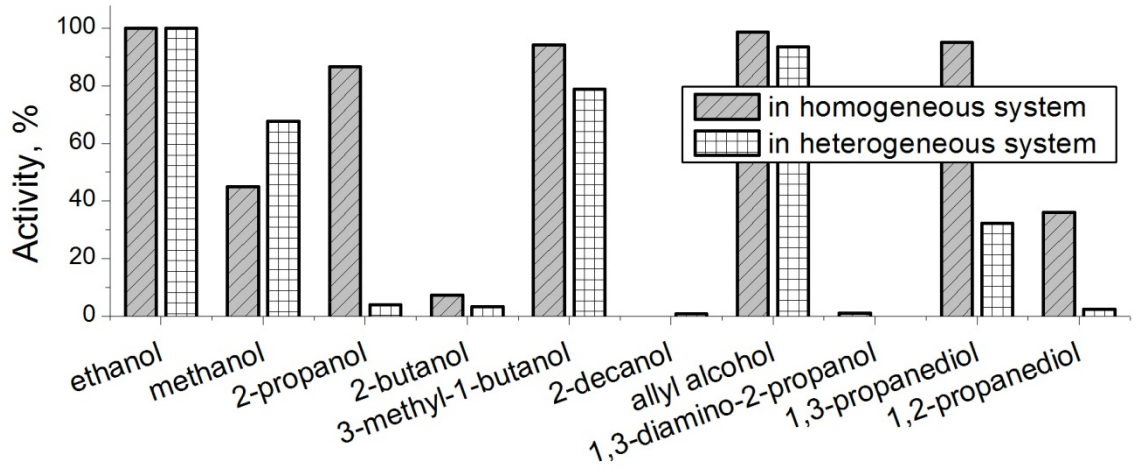

Fig. 9. Substrate specificity of mADH in homogeneous and heterogeneous systems.

3.4 Systems based on FDH

According to previous investigations of bioamperometric systems with FDH, the best results were achieved using a BQ as mediator [27] or modified carbonaceuos materials [28-29]. However, the main disadvantage of BQ in reagentless mediated systems was BQ desorption from electrode surface [27]. The aim of this work was to apply newly synthesized BQ derivates (MN, Ben, BBen, 1M, 2M, 1F, 2F), which possess much lower solubility in water then BQ, to design reagentless mediated bioamperometric systems based on CPE and FDH.

All new quinone-type mediators and BQ were characterised by cyclic voltamperometric measurements. Cyclic voltammograms (CVs) of mediators adsorbed on GE are shown in Fig. 10.

As can be seen in Fig. 10, three oxidation peaks (marked as 1, 3, 5) and four reduction peaks (marked as 2, 4, 6, 7) are found in CVs. The oxidation peak 1 and reduction peak 2 represent quasi-reversible redox processes (in text they are denoted as a process 1 and 2, respectively). Others peaks could be associated to non intensive irreversible and quasi-reversible redox processes of quinones. The potentials of oxidation and reduction peaks (E_{pa1}, E_{pa3}, E_{pa5}, E_{pc2}, E_{pc4}, E_{pc6}, E_{pc7}), peak separation between E_{pa1} and E_{pc2} ($\Delta E_{1\text{-}2}$) and formal redox potential (E_0') of 1 and 2 redox processes are shown in Table 7. E_0' of all studied quinones are quite similar, except for the formal potentials of BQ, Ben and BBen. This confirms that modification of BQ with aniline groups, characterized by strong induction effect, reduces activation energy of electron releasing from atom of quinone oxygen. It was found, that MN possesses good reversibility of 1 and 2 redox processes, which peak potentials are independent from scan rate till 100 mV/s. Linear relationships between anodic peak, cathodic peak currents and square root of scan rate were observed. The heterogeneous rate constant of electron transfer of 1 oxidation process of adsorbed MN on GE (see Fig. 10) found to be 0.78 s^{-1}.

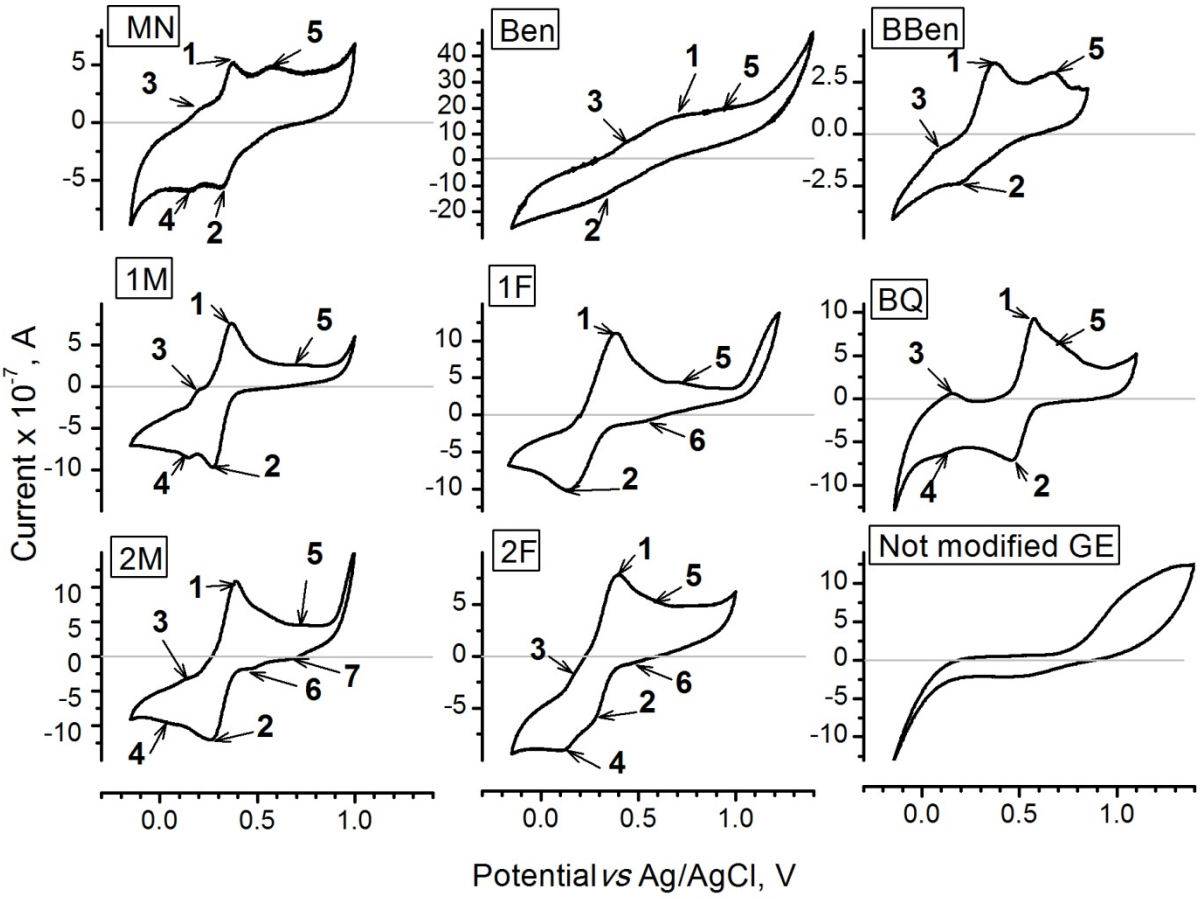

Fig. 10. Cyclic voltammograms of quinone-type mediators, scan rate 10 mV/s, 0.5 M H_2SO_4.

Table 7. Peak potentials (*vs.* Ag/AgCl) and general characteristics of quinone-type mediators obtained from Fig. 10.

	Oxidation potentials, V			***Reduction potentials, V***				ΔE_{1-2}, V	E'_0, V
	E_{pa1}	E_{pa3}	E_{pa5}	E_{pc2}	E_{pc4}	E_{pc6}	E_{pc7}		
BQ	0.57	0.13	0.73	0.46	0.09			0.11	0.522
MN	0.36	0.17	0.59	0.32	0.14			0.05	0.338
1M	0.37	0.19	0.69	0.27	0.13			0.1	0.319
2M	0.38	0.13	0.71	0.26	0.06	0.46	0.69	0.12	0.324
1F	0.46		0.85	0.18		0.63		0.28	0.323
2F	0.39	0.18	0.61	0.26	0.09	0.48		0.14	0.324
Ben	0.58	0.42	0.97	0.28				0.3	0.429
BBen	0.35	0.07	0.69	0.19				0.16	0.271

Bioamperometric systems with FDH were designed on carbon paste electrodes and investigations were performed in McIlvaine's buffer system. In this case the influence of ambience to electrochemical properties of quinones was estimated. It was found, that redox processes on CPE were more effective, than on GE, and occurred at lower potential in buffer solutions.

Aiming to evaluate the new quinone-type compounds as electron transfer mediators, the bioamperometric systems with these compounds and FDH were designed: FDH_MN-Cst, FDH_Ben-Cst, FDH_BBen-Cst, FDH_1M-Cst, FDH_2M-Cst, FDH_1F-Cst and FDH_2F-Cst. The efficiency of new systems was compared with non-mediated reagentless bioamperometric system based on CPE (FDH_Cst). It was observed that the selectivity and stability of new bioamperometric systems are determined by enzyme nature. The conditions were optimized for the most effective bioelectrocatalytic processes: working potential +400 mV (*vs.* Ag/AgCl), McIlvaine's buffer system (pH 5.0), and temperature of 20 ^{o}C. Activation energy of bioelectrocatalysis was similar of all system and found to be 28 ± 2 kJ/mol. The main characteristics of bioamperometric systems based on FDH, obtained on optimal conditions, were summarised in Table 8. It was shown that all bioamperometric systems based on quinone-type mediators possess 2.5-7.5 times higher efficiency comparing with system without mediator (FDH_Cst). An exception is FDH_BBen-Cst, which possessed not efficient re-oxidation of FDH. The efficiency of bioelectrocatalysis of systems with FDH is determined by formal redox potential of mediators. Dependence of I_{max}^{app} on E_0' possesses linear relationship (slope 9 ± 1 $\mu Acm^{-2}V^{-1}$, correlation coefficient 0.9043).

Table 8. The characteristics of bioamperometric systems based on FDH. Inactivation process of FDH_MN-Cst and FDH_Ben-Cst possesses two stages: faster and slower, which were pointed in this table as (1) and (2), respectively.

Bioamperometric system:	***Sensitivity, $\mu Acm^{-2}mM^{-1}$***	***$k_{in} \cdot 10^{-5}$, min^{-1}***	***Linear ranges, mM***	***Apparent maximum current density, μAcm^{-2}***	***K_M^{app}, mM***
FDH_Cst	0.003	12 ± 1	0.001 - 5	0.28 ± 0.004	15 ± 1
FDH_MN-Cst	0.1	(1) 190 ± 50 (2) 2.2 ± 0.8	0.001 - 10	2.8 ± 0.4	30 ± 5
FDH_Ben-Cst	0.05	(1) 270 ± 80 (2) 5.1 ± 0.7	0.001 - 5	1.7 ± 0.3	37 ± 7
FDH_BBen-Cst	0.01	8.7 ± 1.2	0.001 - 15	0.23 ± 0.01	8.5 ± 0.2
FDH_1M-Cst	0.4	12 ± 2	0.001 - 0.25	0.8 ± 0.2	4 ± 2
FDH_2M-Cst	0.4	39 ± 5	0.001 - 0.01	1 ± 0.03	0.6 ± 0.1
FDH_1F-Cst	0.01	11 ± 2	0.001 - 0.5	0.7 ± 0.1	7 ± 2
FDH_2F-Cst	0.01	81 ± 15	0.001 - 0.1	1.1 ±0.2	17 ± 3

It can be concluded, that most effective mediator for bioelectrocatalysis by FDH was MN.

3.4.1 Substrate specificity of FDH

As reported in [30-33], FDH possesses very high selectivity to D-fructose. However for the first time it was determined in this work, that bioamperometric systems based on

quinone-type mediators and FDH are able to oxidised D-(-)-tagatose. Tagatose is one of the rare natural sugars, which is characterized by low caloric value, possesses probiotic, low glycemic, antioxidant and cytoprotective promotions [34]. Thus tagatose becomes very attractive sweetness for food industry. However, determination of tagatose is rather complicated. Several chemical methods and no one biosensor are supposed for determination of tagatose [35].

The ability of FDH to oxidise tagatose was evaluated in different heterogeneous systems, which were obtained by immobilisation of FDH on not modified and modified by MN, Ben, BBen, 1M, 2M, 1F and 2F carbon paste electrodes. The response of each bioamperometric system to addition of tagatose was recorded and presented in Fig. 11 in comparison with response to fructose, which was taken as 100%. It was found, that oxidation of tagatose by FDH is not effective process comparing to oxidation of fructose. The different bioamperometric systems based on FDH possessed different activity by oxidation of tagatose. It could be explained by conformational changes of sADH during interaction with different electrode surfaces that changed different structure of enzyme active site. The most effective oxidation of tagatose was obtained with FDH_MN-Cst system, which can be applied for construction of amperometric biosensor for tagatose determination. However, determination of tagatose by bioamperometric systems based on FDH is possible, if analyzed medium doesn't include better FDH substrate – fructose.

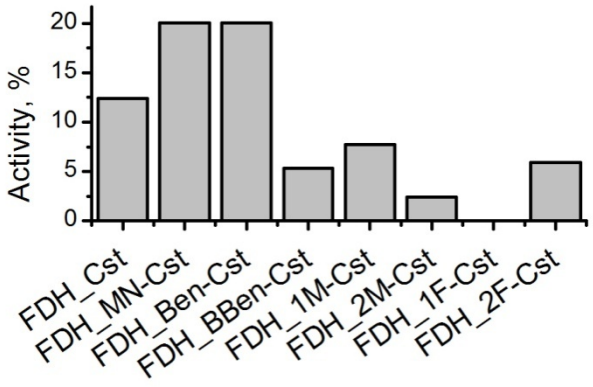

Fig. 11. Substrate specificity of FDH in different heterogeneous systems.

4 Application of bioamperometric systems

4.1 Systems for alcohol determination

The bioamperometric systems sADH_Cst and mADH_ChMA-Cst were tested as alcohol biosensors. Using these systems the concentration of ethanol in various alcoholic beverages was determined. The ethanol concentration was also determined by approbated analyser based on AOx. Results are presented in the Table 9. The ethanol concentration, determined by system with AOx corresponds to the calculated ethanol concentration, which is pointed in the labels of beverages. The concentration of ethanol in beverages determined by the systems with sADH and mADH was slightly higher than those obtained using analyzer. It was shown in previous investigation, that sADH and mADH don't possess high specificity to ethanol and are able to oxidise other alcohols. In this case, the tested alcoholic beverages can include other alcohols (not only ethanol).

These results show that bioamperometric systems using mADH and sADH are suitable for analysis of real medium.

Table 9. Ethanol concentration, which was determined by different bioamperometric systems.

Name of alcoholic beverages, amount of ethanol which is pointed at the label	***Determined ethanol concentration, M***			
	Calculated	***AOx***	***mADH***	***sADH***
"Beauvillon" medium sweet white wine, 11%	1.94	1.85	2.3	2.66
"J. P. Chenet" medium sweet white wine, 11.5%	2.02	1.97	1.97	2.14
Bitter "Palanga", 40%	7.04	7.07	8.19	8.57
Liquor "Mariel", 40%	7.04	7.07	8.53	8.28

4.2 CO indicator system

The principle of mADH inhibition by carbon monoxide was applied to design of CO indicator system. CO was determined by mADH_ChMA-Cst and GDH_Cst systems in cigarettes smoke. It was found, that mADH_ChMA-Cst was inhibited by smoke, while GDH_Cst hasn't lost their activity during treatment by cigarette smoke. These results show, that system based on mADH inhibition by CO could be applied to develop of CO biosensoric systems.

4.3 Systems for D-(-)-tagatose synthesis and determination

System for synthesis of tagatose was designed using newly purified TAI from *Geobacilus lithuanicus 5*, which catalyses galactose bioconvertion to tagatose. The concentration of tagatose during bioconvertion process in reaction medium was determined directly using FDH_MN-Cst bioamperometric system and indirectly – by decreasing of galactose concentration using (PVA)-GDH-MN-Cst bioamperometric system. Tagatose concentration was also evaluated spectrophotometrically by chemical method at the Department of Molecular microbiology and biotechnology (Vilnius University, Institute of Biochemistry). It was found, that tagatose concentration in reaction mixture increased, while galactose concentration proportionally decreased. The obtained results using bioamperometric systems were compared with the ones, obtained by spectrophotometrical methods (see Fig. 12). Good correlation coefficients equalled more up to 0.97 indicating applicability of FDH_MN-Cst and (PVA)-GDH-MN-Cst for evaluated concentration of tagatose in real medium. Evidently, the direct bioamperometric method is more suitable than indirect.

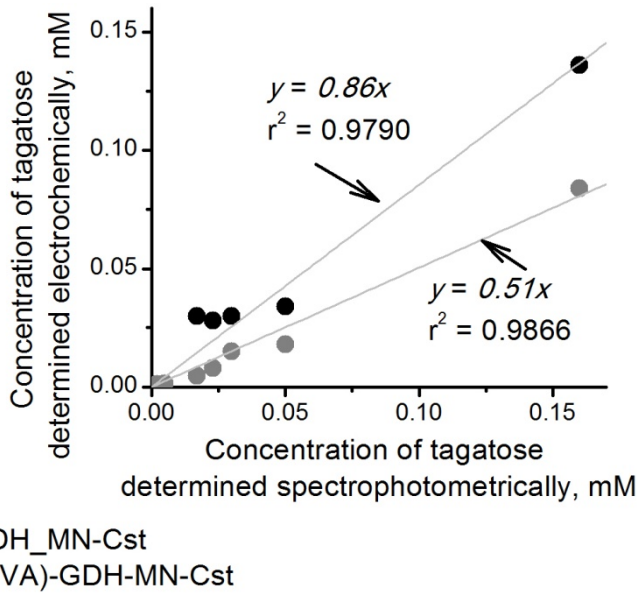

Fig. 12. Concentration of tagatose obtained by electrochemical and spectrophotometric methods using FDH_MN-Cst and (PVA)-GDH-MN-Cst bioamperometric systems.

CONCLUSIONS

1. pK_{a1} and pK_{a2} in aqueous solutions were determined for pyrroloquinoline quinone (PQQ) dependent soluble alcohol dehydrogenase from *Pseudomonas putida* HK5 (sADH) and membrane bound alcohol dehydrogenase from *Gluconobacter sp. 33* (mADH). pK_{a1} and pK_{a2} are 6.1 and 10.3 for sADH; 4.7 and 5.7 for mADH, respectively. Bioelectrochemical systems based on sADH and mADH are able to work in a 50% 1-iso-pentyl-3-methyl imidazole dicyanamide water solution by maintaining up to 50% of enzyme activity.

2. After analysis of sADH and mADH electron transfer pathways in homogeneous and heterogeneous systems it was determined that the direct electron transfer (DET) from reduced PQQ molecule in enzyme active site of sADH and mADH to the electron acceptors may occur in heterogeneous systems.

3. The DET from reduced sADH, mADH and soluble glucose dehydrogenase from *Acinetobacter calcoaceticus* (GDH) active sites to new poly(N-(N',N'-diethyldithiocarbamoylethylamidoethyl)aniline was revealed by impedimetric measurements. The DET was proven by using the chronoamperometric method.

4. The effective GDH immobilisation method on polyvinyl alcohol modified semi-permeable terylene film, which ensures enzyme activity up to 9 months, was developed. A bioamperometric system based on sADH and graphite oxidation product, where DET from enzyme active sites to electrode was realized, was created and characterized. Obtained sensitivity was1 $\mu Acm^{-2}mM^{-1}$. Investigation of bioamperometric systems based on new quinone-type mediators and membrane bound fructose dehydrogenase from *Gluconobacter industruis* (FDH) revealed the 2-(3-nitro(phenyl)amino)- ciclohexa-2,5-dien-1,4-dione (MN) as the most effective electron transfer mediator with the sensitivity of the system equal to 0,08 $\mu Acm^{-2}mM^{-1}$.

5. It was shown that bioamperometric systems based on PQQ dependent enzymes can be applied to determine of alcohols, carbohydrates and CO. For the first time the bioamperometric system based on FDH and MN is proposed for D-(-)-tagatose determination (detection range of tagatose is 0.001-0.1 mM).

ACKNOWLEDGEMENTS

Dear reader, this is the first "big deal" in my scientific life. The main results of my investigations during PhD studies are presented in this short summary of thesis. I could said, that all the measurements, all the results, all the articles were done, written and presented by me and only me. But that would be an outrageous lie! There were a lot of people, who were besides me, who taught me and who helped me during these last 5 years.

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REFERENCES

[1] P. Arora, A. Sindhu, N. Dilbaghi, A. Chaudhury, Biosensors as innovative tools for the detection of food borne pathogens, Biosensors & Bioelectronics, 28 (2011) 1-12.

[2] K.E. Mach, P.K. Wong, J.C. Liao, Biosensor diagnosis of urinary tract infections: a path to better treatment?, Trends in Pharmacological Sciences, 32 (2011) 330-336.

[3] V. Laurinavicius, J. Razumiene, B. Kurtinaitiene, I. Lapenaite, I. Bachmatova, L. Marcinkeviciene, R. Meskys, A. Ramanavicius, Bioelectrochemical application of some PQQ-dependent enzymes, Bioelectrochemistry, 55 (2002) 29-32.

[4] J. Razumiene, J. Barkauskas, V. Kubilius, R. Meskys, V. Laurinavicius, Modified graphitized carbon black as transducing material for reagentless H_2O_2 and enzyme sensors, Talanta, 67 (2005) 783-790.

[5] W.S. Hummers, R.E. Offeman, Preparation of graphitic oxide, Journal of the American Chemical Society, 80 (1958) 1339-1339.

[6] J. Barkauskas, J. Razumienė, I. Šakinytė, E. Voitechovič, Use of carbon nanomaterials for amperometric biosensors, in: Micro- and nanosystems in biochemical diagnosis. Principles and applications: 108th ICB seminar, Polska Akademia Nauk, Warsaw (Poland), 2010, pp. 28-39.

[7] 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.

[8] A. Bratov, J. Ramon-Azcon, N. Abramova, A. Merlos, J. Adrian, F. Sanchez-Baeza, M.P. Marco, C. Dominguez, Three-dimensional interdigitated electrode array as a transducer for label-free biosensors, Biosens. Bioelectron., 24 (2008) 729-735.

[9] M. Sharp, M. Petersson, K. Edström, Preliminary determinations of electron transfer kinetics involving ferrocene covalently attached to a platinum surface, Journal of Electroanalytical Chemistry and Interfacial Electrochemistry, 95 (1979) 123-130.

[10] K.S. Novoselov, D. Jiang, F. Schedin, T.J. Booth, V.V. Khotkevich, S.V. Morozov, A.K. Geim, Two-dimensional atomic crystals, Proceedings of the National Academy of Sciences of the United States of America, 102 (2005) 10451-10453.

[11] Q. Haas, Kovalente Kohlenstoffverbindungen, in: G. Brauer (Ed.) Handbuch der Präparativen anorganischen Chemie, J. F. Enke Verlag, Stuttgart, 1960, pp. 574.

[12] L. Marcinkevičienė, I. Bachmatova, R. Semėnaitė, R. Rudomanskis, G. Bražėnas, R. Meškienė, R. Meškys, Purification and characterization of alcohol dehydrogenase from *Gluconobacter sp. 33*, Biologija, 2 (1999) 24-33.

[13] B.W. Groen, M.A. van Kleef, J.A. Duine, Quinohaemoprotein alcohol dehydrogenase apoenzyme from *Pseudomonas testosteroni*, Biochem. J., 234 (1986) 611-615.

[14] K.F. Tipton, A.G. McDonald, H.B.F. Dixon, Effects of pH on enzymes, in: D.L. Purich (Ed.) Contemporary enzyme kinetics and mechanism, Academic Press, Elsevier, Oxford, 2009, pp. 124-178.

[15] A. Ramanavicius, K. Habermuller, J. Razumiene, R. Meskys, L. Marcinkeviciene, I. Bachmatova, E. Csoregi, V. Laurinavicius, W. Schuhmann, An oxygen-independent ethanol sensor based on quinohemoprotein alcohol dehydrogenase covalently bound to a functionalized polypyrrole film, in: V. Razumas, B. Lindman, T. Nylander (Eds.) Surface and Colloid Science, 2001, pp. 143-148.

[16] A. Vilkanauskyte, Nuo PQQ priklausomų alkoholio ir gliukozės dehidrogenazių veikimas biosensorinėse sistemose, in: Institute of Biochemistry, Vilnius university, Vilnius, 2004. Doctoral thesis.

[17] E. Ferapontova, L. Gorton, Direct electrochemistry of heme multicofactor-containing enzymes on alkanethiol-modified gold electrodes, Bioelectrochemistry, 66 (2005) 55-63.

[18] K. Matsushita, H. Toyama, M. Yamada, O. Adachi, Quinoproteins: structure, functions and biotechnological applications, Applied Microbiology and Biotechnology, 58 (2002) 13-22.

[19] H. Toyama, F.S. Mathews, O. Adachi, K. Matsushita, Quinohemoprotein alcohol dehydrogenases: structure, function, and physiology, Archives of Biochemistry and Biophysics, 428 (2004) 10-21.

[20] H. Toyama, Z.W. Chen, M. Fukumoto, O. Adachi, K. Matsushita, F.S. Mathews, Molecular cloning and structural analysis of quinohemoprotein alcohol dehydrogenase ADH-IIG from *Pseudomonas putida* HK5, Journal of Molecular Biology, 352 (2005) 91-104.

[21] L.J.C. Jeuken, A.K. Jones, S.K. Chapman, G. Cecchini, F.A. Armstrong, Electron-transfer mechanisms through biological redox chains in multicenter enzymes, Journal of the American Chemical Society, 124 (2002) 5702-5713.

[22] M. Stiene, U. Bilitewski, Electrochemical characterization of screen-printed carbonaceous electrodes for the determination of peroxidase activity in novel screen-printed flow-through modules, Analytical and Bioanalytical Chemistry, 372 (2002) 240-247.
[23] D. Li, Y.P. Wen, H.H. He, J.K. Xu, M. Liu, R.R. Yue, Polypyrrole-multiwalled carbon nanotubes composites as immobilizing matrices of ascorbate oxidase for the facile fabrication of an amperometric vitamin C biosensor, J. Appl. Polym. Sci., 126 (2012) 882-893.
[24] P. Bartlett, Bioelectrochemistry. Fundamentals, experimental techniques and applications, in, John Wiley & Sons, Chichester, 2008.
[25] P. Dokter, J. Frank, J.A. Duine, Purification and characterization of quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus* L.M.D. 79.41, Biochem. J., 239 (1986) 163-167.
[26] A.J.J. Olsthoorn, J.A. Duine, On the mechanism and specificity of soluble, quinoprotein glucose dehydrogenase in the oxidation of aldose sugars, Biochemistry, 37 (1998) 13854-13861.
[27] T. Ikeda, F. Matsushita, M. Senda, D-fructose dehydrogenase-modified carbon paste electrode containing *p*-benzoquinone as a mediated amperometric fructose sensor Agricultural and Biological Chemistry, 54 (1990) 2919-2924.
[28] Y. Kamitaka, S. Tsujimura, K. Kano, High current density bioelectrolysis of fructose at fructose dehydrogenase-adsorbed and Ketjen Black-modified electrodes without a mediator, Chemistry Letters, 36 (2007) 218-219.
[29] M. Tominaga, S. Nomura, I. Taniguchi, D-fructose detection based on the direct heterogeneous electron transfer reaction of fructose dehydrogenase adsorbed onto multi-walled carbon nanotubes synthesized on platinum electrode, Biosensors and Bioelectronics, 24 (2009) 1184-1188.
[30] M. Ameyama, E. Shinagawa, K. Matsushita, O. Adachi, D-fructose dehydrogenase of Gluconobacter industrius: purification, characterization, and application to enzymatic microdetermination of D-fructose, Journal of Bacteriology, 145 (1981) 814-823.
[31] J. Biscay, E. Costa Rama, M.B. González García, A. Julio Reviejo, J.M. Pingarrón Carrazón, A.C. García, Amperometric fructose sensor based on ferrocyanide modified screen-printed carbon electrode, Talanta, 88 (2012) 432-438.
[32] K. Damar, D. Odaci Demirkol, Modified gold surfaces by poly(amidoamine) dendrimers and fructose dehydrogenase for mediated fructose sensing, Talanta, 87 (2011) 67-73.
[33] X. Wu, F. Zhao, J.R. Varcoe, A.E. Thumser, C. Avignone-Rossa, R.C.T. Slade, A one-compartment fructose/air biological fuel cell based on direct electron transfer, Biosensors and Bioelectronics, 25 (2009) 326-331.
[34] G.V. Levin, Tagatose, the new GRAS sweetener and health product, J Med Food, 5 (2002) 23-36.
[35] E.C. Bigham, C.E. Gragg, W.R. Hall, J.E. Kelsey, W.R. Mallory, D.C. Richardson, C. Benedict, P.H. Ray, Inhibition of arabinose 5-phosphate isomerase. An approach to the inhibition of bacterial lipopolysaccharide biosynthesis, Journal of Medicinal Chemistry, 27 (1984) 717-726.

OKSIDOREDUKTAZIŲ VEIKIMO BIOELEKTROCHEMINĖSE SISTEMOSE TYRIMAS IR TAIKYMAS

REZIUMĖ

Darbo tikslas:

Ištirti nuo pirolo chinolinchinono (PQQ) priklausomų oksidoreduktazių: periplazminės alkoholio dehidrogenazės, išskirtos iš *Pseudomonas putida* HK5 (tADH), membraninės alkoholio dehidrogenazės, išskirtos iš *Gluconobacter sp. 33* (mADH), periplazminės gliukozės dehidrogenazės, išskirtos ir *Acinetobacter calcoaceticus* (GDH) ir fruktozės dehidrogenazės, išskirtos iš *Gluconobacter industruis* išorinės membranos (FDH) veikimą homogeninėje ir heterogeninėje aplinkose ir jų pagrindu sukurti naujas bioelektrokatalizines sistemas.

Darbe tirtas nuo PQQ priklausomų dehidrogenazių veikimas homogeninėje (kuomet fermentas veikė tirpale) ir heterogeninėje (kuomet imobilizuotas fermentas veikė bioelektrocheminėje sistemoje) aplinkose. Siekiant sukurti efektyviai veikiančias bioelektrokatalizines sistemas buvo ieškoma optimalaus fermento imobilizavimo būdo, tinkamos elektrodinės matricos, mediatorinių medžiagų, aiškinami elektronų pernašos (EP) keliai ir heterogeninės terpės įtaka fermentų savybėms. Buvo išaiškinta, kad tADH ir mADH gali veikti skystųjų druskų tirpaluose, o jų elektronų pernaša (EP) bioelektrocheminėse sistemose gali vykti tiesiai nuo PQQ molekulės, jei nuo hemų c EP nėra galima. Pirmą kartą buvo parodyta, jog fermentai gali tiesiogiai re-oksiduotis ant poli(N-(N',N'-dietilditiokarbamoiletilamidoetil)anilino (poliNDDEAEA), nauju būdu oksiduoto grafito, kad naujos *p*-benzochinono pagrindu susintetintos medžiagos yra tinkami mediatoriai bioemperometrinėms sistemoms su FDH ir sukurta fermentų imobilizavimo metodika, leidžianti išlaikyti GDH aktyvumą iki devynių mėnesių. Taip pat sukurta D-(-)-tagatozės sintezės sistema ir pasiūlyti du tagatozės koncentracijos nustatymo būdai bioamperometriniu metodu.

Šie tyrimai leis prognuozuoti, kokiomis savybėmis turėtų pasižymėti elektrodinė matrica ir kokiomis sąlygomis turi būti imobilizuojamos tADH, mADH, GDH ar FDH, norint sukurti bioelektrochemines sistemas tinkančias biojutiklių ir/ar bioreaktorių kūrimui. Parodyta, jog kai kurios darbe sukurtos bioelektrocheminės sistemos jau dabar gali būti pritaikomos alkoholių ir angliavandenių amperometrinių biojutiklių kūrime.

Darbo išvados:

1. Nustatytos nuo pirolo chinolinchinono (PQQ) priklausomų periplazminės alkoholio dehidrogenazės, išskirtos iš *Pseudomonas putida* HK5 (tADH) ir membraninės alkoholio dehidrogenazės, išskirtos iš *Gluconobacter sp. 33* (mADH) pK_{a1} ir pK_{a2} reikšmės vandeniniuose tirpaluose: tADH yra 6,1 ir 10,25; mADH – 4,66 ir 5,65. Bioelektrocheminės sistemos su PQQ priklausomomis alkoholio dehidrogenazėmis gali veikti 50% 1-iso-pentil-3-metilimidazolo dicianamido vandeniniame tirpale, išlaikant iki 50% fermento aktyvumo.
2. Ištyrus tADH ir mADH elektronų pernašos (EP) kelius homogeninėje ir heterogeninėje sistemose, nustatyta, jog heterogeninėse sistemose yra įmanomas tiesioginis EP kelias nuo redukuotos PQQ molekulės elektronų akceptoriams.
3. Ištyrus impedimetrinės fermentinės sistemos su nauju poli(N-(N',N'-dietilditiokarbamoiletilamidoetil)anilinu (poliNDDEAEA) ir tADH, mADH bei

periplazmine gliukozės dehidrogenaze, išskirta iš *Acinetobacter calcoaceticus* (GDH) ant erdvinių adresuojamojo kontakto elektrodų matricos, nustatyta tiesioginė elektronų pernaša nuo šių fermentų aktyviųjų centrų ant poliNDDEAEA, ir ji patvirtinta chronamperometrijos metodu.

4. Sukurtas efektyvus GDH imobilizavimo būdas ant polivinilalkoholiu modifikuotos pusiau pralaidžios lavsano plėvelės, užtikrinantis fermento stabilų veikimą iki 9-ių mėnesių. Grafito oksidacijos produkto ir tADH pagrindu sukurta bioamperometrinė sistema veikė tiesioginės elektronų pernašos principu ir pasižymėjo iki 1 $\mu Acm^{-2}mM^{-1}$ jautrumu. Ištyrus naujų chinoninių mediatorių, sukurtų *p*-benzochinono ir aromatinių aminų pagrindu, veikimą bioamperometrinėse sistemose su fruktozės dehidrogenaze, išskirta iš *Gluconobacter industruis* išorinės membranos (FDH), nustatytas efektyviausias mediatorius – 2-(3-nitro(fenil)amino)- cikloheksa-2,5-dien-1,4-dionas (MN) (sistemos jautrumas 0,08 $\mu Acm^{-2}mM^{-1}$). Tirtų naujų chinoninių mediatorių elektrono akceptorinės savybės didėja didėjant jų formaliems redokso potencialams.
5. Parodyta, jog efektyviausiai veikiančios bioamperometrinės sistemos su PQQ priklausomais fermentais gali būti naudojamos alkoholių, angliavandenių ir CO nustatymui. Pirmą kartą parodyta, kad bioamperometrinė sistema FDH ir MN pagrindu, gali būti panaudota tagatozės nustatymui (nustatymo ribos 0,001-0,1 mM).

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