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Investigation of bioelectrocatalytic systems with PQQ-dependent GDH and carbonaceous materials

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Different graphite oxidation techniques have been applied aiming to synthesize five different graphene oxides (GOs). Carbonaceous material electrodes (CMEs) were prepared using GOs, amorphous carbon and graphite. Bioelectrocatalytic systems were constructed by formation of pyrroloquinoline quinone (PQQ) dependent glucose-dehydrogenase (PQQ-GDH) isolated from *Acinetobacter calcoaceticus* layers on the CMEs. The fact of direct electron transfer (DET) from the active site of PQQ-GDH to the CMEs has been studied. The proposed bioelectrocatalytic systems have been characterized by kinetic parameters – the apparent Michaelis constant (K_M^{app}) and the apparent maximum current (I_{max}^{app}). It has been concluded that a definite set of the surface functional groups as well as the nano-scale carbon structures are essential for obtained reagentless enzyme-based biosensors. Data of the atomic force microscopy (AFM) analysis revealed the DET efficiency to depend on the content of functional groups in the GOs as well as on its surface morphology.

Key words: carbonaceous materials, PQQ-dependent GDH, bioelectrocatalysis, direct electron transfer

INTRODUCTION

Electron transfer in biological systems is a very important phenomenon for biochemical and biophysical sciences [1, 2]. On the principle of electron transfer between redox enzymes, in the respiratory chain there can be produced various enzymatic bioelectrocatalytic systems that may be used for the further investigation of enzyme-catalyzed reactions in biological systems and as the electrochemical basis for studying the structure of enzymes, the kinetics and thermodynamics of enzymes' molecules, metabolic processes involving redox transformations, and for the production of biosensors and bioreactors [1, 3–5]. Great efforts have been made to develop new mediator-free (reagentless) biosensors, enzymatic bioreactors, and biomedical devices based on direct electron transfer (DET) by immobilizing enzymes on conducting materials [1]. However, the development of an effective and stable reagentless bioelectrcatalytic system is not easy. It is essential to choose a suitable enzyme that can perform DET and the electrode material on which this enzyme can function. Because the redox centre in biomolecules is usually embedded deeply into the large three-dimensional structure of enzyme molecules, many methods and materials, including biopolymers, nanostructures and sol–gel matrices have been studied with the aim to improve the immobilization of enzymes and to promote electron transfer on the surface of electrodes [1, 6, 7]. Carbonaceous materials are promising in this field, because they are friendly to biomolecules and have a large variety of nano-forms. Also, they can be easily chemically modified and possess a high conductivity and suitability for various technologies [8–11]. Recently, a new class of a large surface-to-volume ratio, high conductivity nanostructurized carbonaceous material – graphene – has attracted attention as suitable for optoelectronic devices,

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supercapacitors, gas sensors, pH sensors, chemical sensors, biosensors, and nanocomposite applications [1, 10, 12, 13]. Graphene is a one-atom thick two-dimensional sheet of carbon atoms which can derive various nano-forms of carbon [10, 11]. Graphene is not chemically active, but its activity can be changed depending on structural defects (e. g., vacancies, various dislocations) and different functional groups on graphene surface [14–16].

The aim of this work was to oxidize graphite by different methods with the hope to get various oxidation products – graphene oxides exhibiting different chemical and physical properties, and to create efficient and reagentless bioelectrocatalytic systems. As a biocomponent, the dimeric quinoenzyme PQQ-GDH has been chosen. PQQ-GDH contains a PQQ molecule as a cofactor, and Ca^{2+} ion as well as aspartic and glutamic amino acid residues in the active site [17]. This enzyme is promising for developing bioelectrocatalytic systems, first of all because the atmospheric oxygen doesn't influence its catalytic processes and the electrons can be directly transferred from an enzyme-active site to several electrode materials [18, 19]. In order to discover their effects on the efficiency of bioelectrocatalysis, the morphology of carbon materials was analyzed by atomic force microscopy.

MATERIALS AND METHODS

Enzyme. PQQ-GDH (specific activity 1 286 U/mg) was purified from *Acinetobacter calcoaceticus*. The PQQ-GDH solution of 18 000 U/ml was prepared for the experiments. PQQ-GDH was kindly provided by the Department of Molecular Microbiology and Biotechnology (Institute of Biochemistry of Vilnius University).

Synthesis of graphene oxides. GO synthesis was performed by three techniques: by the Hummers and Offeman method as described elsewhere [20] and using different oxidisers. GOs were synthesized from different dispersity graphite (Merck, Darmstadt, DE). Different dispersity graphite was prepared by sonification of graphite powders (5 g) in water (20 ml) for 10 h using the VCX 130 PB sonificator (Sonycs and Materials Inc., US). Graphite was oxydized with $K_3[Fe(CN)_6]$ (Riedel-de Haen, NL) and H_2O_2 (ChemPur, DE) in an alkaline medium.

Chemical reagents. Sodium acetate, acetic acid and $CaCl_2$ were obtained from J. T. Baker (Holland, NL). KCl and D-glucose were purchased from Riedel-de Haen (DE). Amorphous carbon Raven M was purchased from Columbian Chemicals Co. (Atlanta, US).

Electrochemical measurements. Electrochemical measurements were performed using a PARSTAT 2273 electrochemical system (Princeton Applied Research, US) with a conventional three-electrode system consisting of a platinum plate electrode as an auxiliary, a saturated Ag / AgCl electrode as a reference and CMEs (Ø 2 mm) as working electrodes. The working enzyme electrode was designed by the adsorption on the surface of 2 µl of enzyme solution (1 h, 4 °C). The response of the prepared enzyme electrodes to the addition of enzyme substrate was investigated under potentiostatic conditions at +400 mV (vs. Ag / AgCl) in a stirred 0.05 M acetate buffer solution, pH 6.0, containing 10 mM Ca^{2+}. The enzyme substrates were used as acetate buffer solutions, pH 6.0, containing 100 mM of 1.2-propandiol or 100 mM of D-glucose. The Origin Pro 8.0 program (free trial version from http://www.originlab.com, OriginLab Corporation, US) was used for data analysis.

AFM measurements. The surface of the electrodes was analyzed with an Agilent 5 500 AFM / STM scanning probe microscope (Agilent Technologies Inc, US). A standard AFM method – acoustic AC mode surface scanning – was used for the visualization of surface morphology. Imaging was done in an intermittent contact mode using a rectangular FESP probe (VEECO, 0.01–0.025 Ωcm antimony (n) doped S_i with the frequency f_c = 60–100 kHz, spring constants k = 1–5 N/m, and the nominal tip radius 10 nm). The data and SPM images were processed with the Scanning Probe Image Processor 5.1.2 (free trial version from http://www.imagemet.com, Image Metrology, DK). Samples for AFM imaging were prepared by the standard method of mechanical exfoliation [21] of carbonaceous materials on a freshly cleaved mica surface (SPI Supplies, Division of Structure Probe Inc., US).

RESULTS AND DISCUSSION

We have shown in our previous paper [19] that DET can be achieved from the active site of PQQ-GDH to the carbon electrode surface after modifying carbonaceous materials. This fact encouraged us to study the behaviour of PQQ-GDH on modified graphene electrodes. As a result, a set of different GOs have been synthesized by different graphite

Table 1. **Graphite oxidation methods and notation of the products (GOs)**

Graphite oxidation techniques	Raw material of graphite treatment	GO notation
Hummers and Offeman [20]	Without sonification	**G1**
Oxidation with H_2O_2 in alkaline media	Without sonification	**G2**
	Sonification, treatment time 10 h	**G3(S)**
Oxidation with $K_3[Fe(CN)_6]$	Without sonification	**G4**
	Sonification, treatment time 10 h	**G5(S)**

oxidation techniques and applied for constructing new PQQ-GDH-based biosensors. All newly synthesized GOs were characterized by different morphology and chemical properties. The notation of GOs is presented in Table 1.

The morphology of the electrode as a constituent part of the PQQ-GDH-based bioelectrocatalytic system is characterized by AFM. A three-dimensional representation of AFM topographic data for graphite, amorphous carbon, G2 and G5(S) is shown in Fig. 1. The AFM imaging analysis revealed differences in the synthesized carbonaceous materials. All samples were characterized by AFM image amplitude parameters such as surface roughness and kurtosis (Table 2). In order to understand the influence of the electrode material on the behavior of PQQ-GDH, bioelectrocatalytic systems (biosensors) based on GOs as well as on unmodified graphite and amorphous carbon have been constructed. The principle scheme of the construction of a bioelectrocatalytic system is shown in Fig. 2, and the enzyme DET pathways during bioelectrocatalysis are schematically shown in Fig. 3.

The current generated at the electrodes during the electrocatalytic oxidation of glucose by the enzyme was measured as a function of glucose concentration in the solution. Similar dependences were measured for all types of biosensors manufactured and probed in this work. Kinetic characteristics, namely the apparent Michaelis constant (K_M^{app}) and the maximum current (I_{max}^{app}), calculated for each type of the bioelectrocatalytic systems are summarized in Fig. 4. As shown in Fig. 4, no DET was achieved in PQQ-GDH-based biocatalytic systems using graphite, amorphous carbon or G1 as an electrode material. However, in the case of G2, G3(S), G4 and G5(S), DET can be achieved. Moreover, G3(S) and G5(S), which were synthesized using sonificated raw graphite materials, exhibited increased I_{max}^{app} and lower K_M^{app} values in comparison with G2 and G4, despite the similar synthesis methods (Fig. 4).

Table 2. **Data on amplitude parameters of graphite oxidation products obtained from AFM imagines**

Amplitude parameters	G1	G2	G3(S)	G4	G5(S)
Average roughness, nm	3	4	25	4	12
Surface kurtosis, nm	153	47	4	55	3

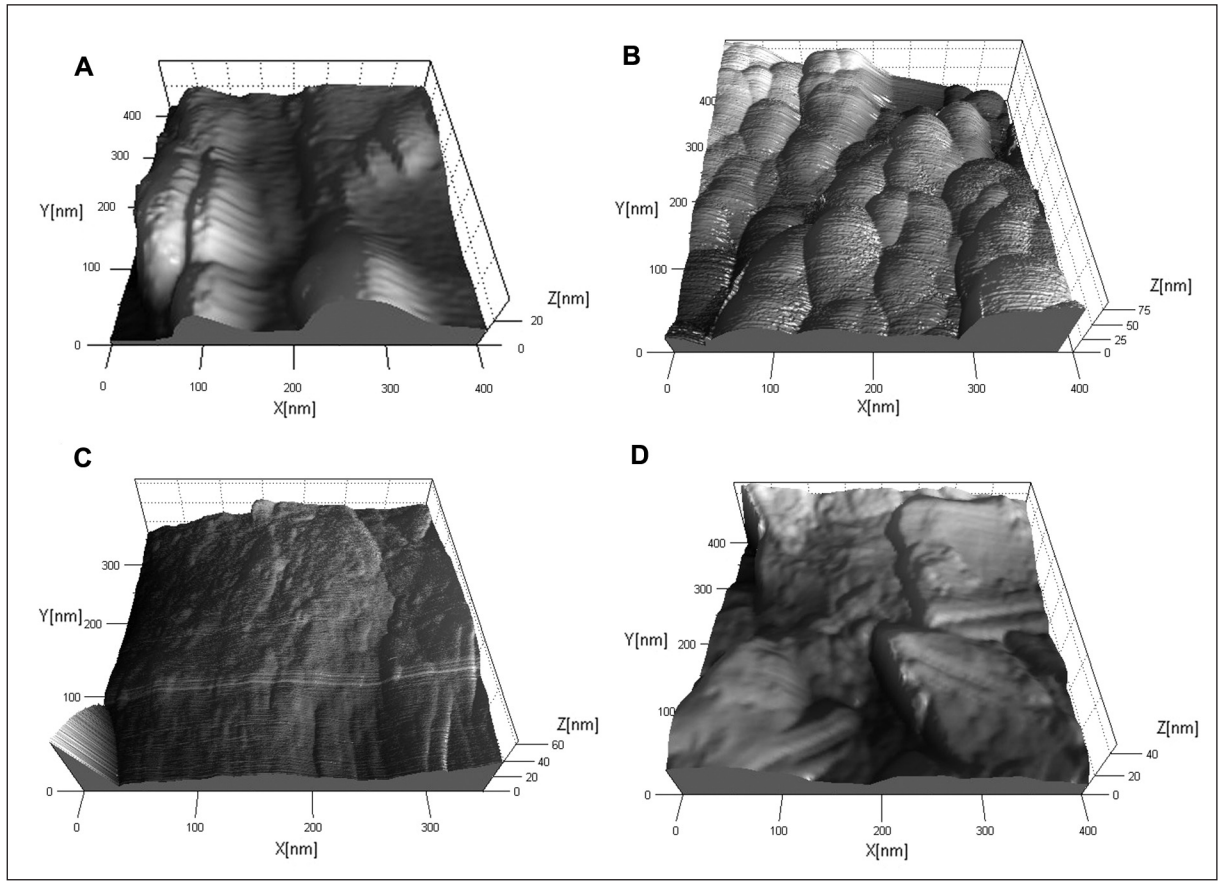

Fig. 1. Three-dimensional representation of AFM topographic data on carbonaceous materials.
A – graphite, B – amorphous carbon, C – G2, D – G5(S)

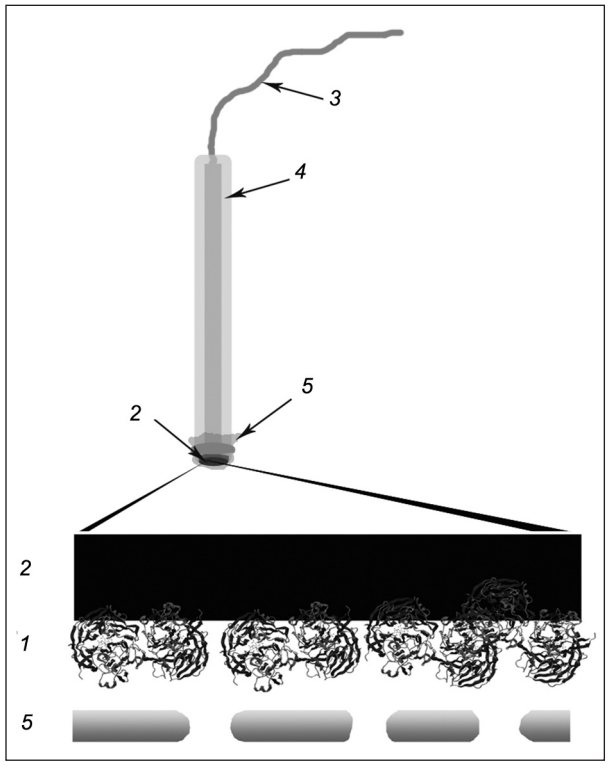

Fig. 2. Scheme of construction of bioelectrocatalytic system based on enzyme and CME. *1* – layer of enzyme, *2* – CME surface, *3* – CME contact area, *4* – insulating layer, *5* – protective semipermeable membrane

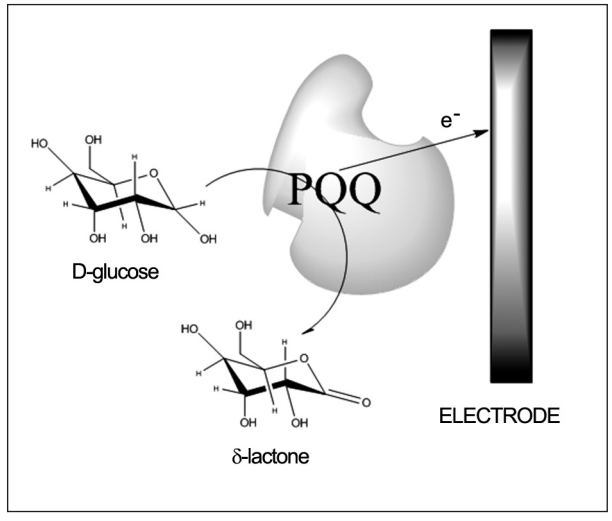

Fig. 3. Proposed mechanism of DET in bioelectrocatalytic systems with PQQ-GDH

I_{max}^{app} describes the efficiency of a bioelectrocatalytic system, whereas K_M^{app} indicates an enzyme's affinity to the electrode surface. Thus, the sonification procedure increased the efficiency of bioelectrocatalysis and improved the PQQ-GDH affinity to CMEs. The AFM data presented in Table 2, namely a higher kurtosis index for unsonificated samples (47 nm for G2 and 55 nm for G4) and a lower roughness of G2 and G4 confirmed the difference between graphene oxidation products and the influence of the sonification procedure on the efficiency of bioelectrocatalysis by PQQ-GDH. Furthermore, using a CME of G1 with the highest surface kurtosis (153 nm) and the lowest average roughness (3 nm) reduced the efficiency to an unsuccessful bioelectrocatalysis (Fig. 4).

It was concluded that DET efficiency not only depends on the content of functional groups of the GOs, but also the surface morphology is substantial for creating reagentless enzyme-based biosensors.

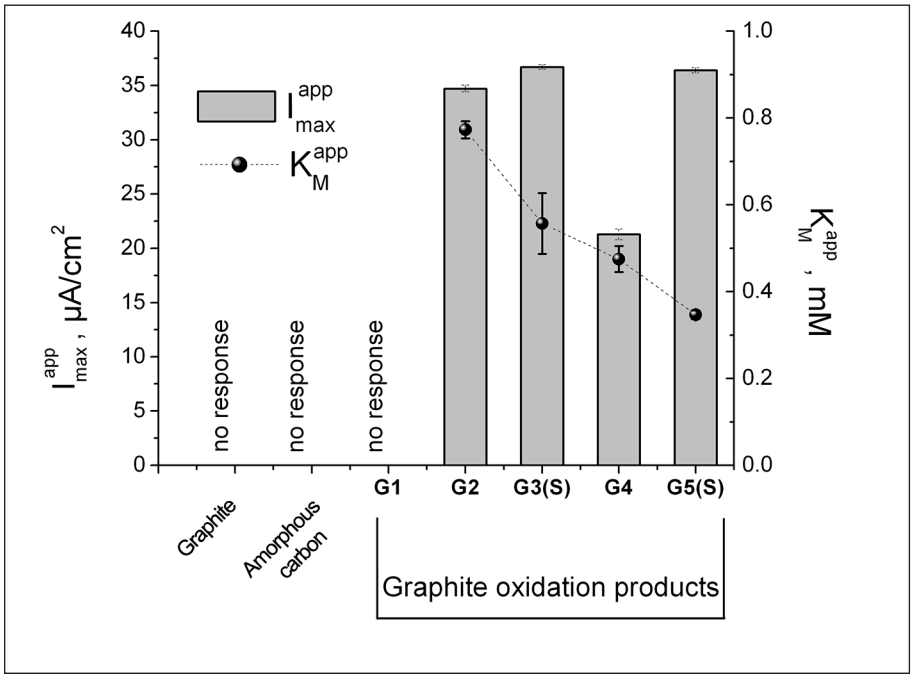

Fig. 4. Kinetic characteristics of bioelectrocatalytic systems with PQQ-GDH and CME materials

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BIOELEKTROKATALIZINIŲ SISTEMŲ SU GDH, PRIKLAUSOMA NUO PQQ, IR ANGLINĖMIS MEDŽIAGOMIS TYRIMAI

Santrauka

Panaudojant skirtingus grafito oksidacijos metodus buvo gauti penki nauji grafeno oksidai (GOs), kurių paviršiuje yra išsidėsčiusios įvairios rūgštinės ir bazinės deguonies turinčios funkcinės grupės. Iš skirtingų GOs pavyzdžių, taip pat iš gryno grafito bei amorfinės anglies buvo pagaminti elektrodai, ant kurių buvo formuojami nuo pirolochinolinchinono (PQQ) priklausomos gliukozės dehidrogenazės (PQQ-GDH, išskirtos iš *Acinetobacter calcoaceticus*) sluoksniai. Taip paruoštose bioelektrokatalizinėse sistemose buvo stebėta tiesioginė elektronų pernaša (DET) nuo fermento aktyvaus centro ant elektrodų ir nustatyti kinetiniai parametrai – tariamoji Michaelio konstanta (K_M^{app}) bei tariamasis maksimalus srovės stipris (I_{max}^{app}). Remiantis kinetiniais parametrais, įvertintas DET efektyvumas ir padaryta išvada, kad jį lemia ne tik anglinių medžiagų funkcinės grupės, bet ir jų paviršiaus morfologija. Tai patvirtino GOs, grafito ir amorfinės anglies paviršių analizė atominės jėgos mikroskopijos (AFM) metodu. Gauti duomenys rodo, kad tikslinga anglinių medžiagų sintezė bei cheminė modifikacija leidžia sudaryti efektyvias bereagentes bioelektrokatalizines sistemas, kurios gali būti pritaikytos kuriant biojutiklius ar bioreaktorius.

Raktažodžiai: anglinės medžiagos, nuo PQQ priklausoma GDH, bioelektrokatalizė, tiesioginė elektronų pernaša