



Communications

In situ modulation of enzyme activity via heterogeneous catalysis utilizing solid electroplated cofactors



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ABSTRACT

During product isolation the received bioreceptors often do not exhibit a sufficient biochemical activity due to multistep dissociation and loss of cofactors. However, for bioelectrochemical applications the presence of cofactors is necessary for a successful oxidative or reductive conversion of the substrates to the products.

Herein, we show how the immobilization of the required electroplated cofactors in a design of amperometric electrodes can *in situ* assist the activity of apo-enzymes. Compared to conventional approaches used in enzyme engineering this tailored nanoengineering methodology is superior from economic point of view, labor and time costs, storage conditions, reduced amount of waste and can fill the gap in the development of tuned bioelectrocatalysts.

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1. Introduction

Rapid degradation of cofactors often leads to low activity of microbial enzymes.[1–3] In some cases, enzymes can be expressed in apo-form and might require several reconstruction steps, *i.e.* the addition of an external co-factor to complete their active holo-form [2–5]. Although, apo-enzymes are known to bind the substrates they cannot oxidase them [2] that makes a rapid search of the high-yielding mutants due to false negative results in particular by electroanalytical sensing tools almost impossible. Therefore, the presence of cofactors (usually noncovalently bonded to the protein to keep its active conformation) is necessary for successful RedOx conversion of the substrates. [6–9]

To solve this problem, a modulation of enzyme activity by *in situ* cofactor introduction could be an efficient strategy for improving the rates of enzyme-driven biochemical reaction in a short period of time. In other words, all expressed amount of microbial or apo-enzymes gets fully active directly on the electrode surface after interactions with the co-immobilized cofactors.

In fact, native enzymes have a very massive structure with a deeply buried active RedOx center causing numerous mass transfer

limitations.[6,10] As a result the obtained electrochemical signals cannot be associated with bonded cofactors but more likely by the free ones.[11,12] Hitherto, several attempts towards immobilization of the exogenous unbound cofactors via entrapment into membranes, physical adsorption from aqueous solutions or recycling on porous surfaces aiming the improvements in enzymes activity and efficiency of biochemical transformations were conducted.[13,14] However, the use of porous materials is limited by (i) the size of cofactors; (ii) their rapid leaching and (iii) significant decrease in activity due to formation of new chemical bonds.[14] Hence, the development of alternative strategies towards the immobilization of cofactors in their intact form with a preservation of function and specificity are strongly needed.

In case of successful electrochemical immobilization on the electrode a direct communication between a target apo-enzyme and RedOx cofactor will allow a minimal diffusion limitation and a maximum surface area per unit mass.[10] In addition, a rapid leaching of the immobilized electroplated cofactors can be avoided.[15]

To this end, we propose a novel concept towards a heterogeneous bioelectrocatalysis and *in situ* modulation of enzyme activity directly on the electrode by reaction with the electroplated cofactors. Electroplating was performed at low currents from the multiple electrolyte solutions containing target cofactors, polymer binding agents and cations of noble metals. This approach results in the formation of cofactors-doped hybrid nanocomposite layer

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with electroactive metal nanoparticles (NPs). The structure of cofactor-containing complexes in the multiple solutions and architecture of the hybrid cofactor-doped electrodes was realized by methods of quantum chemistry.

To test the proposed concept on model systems, first, we studied the impact of cofactors supplement (flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN)) on the activity of conventional glucose oxidase (GOx) and lactate oxidase (LOx) in solutions (1). Afterwards, target cofactors in the required amount were immobilized on the electrodes via electroplating followed by evaluation of their analytical merit in the presence of corresponding enzymes (2). Finally, the concept of heterogeneous biocatalysis utilizing electroplated immobilized cofactors was verified by electrochemical screening of apo-enzymes (apo-GOx as a case study) in a droplet mode (3).

Since the electrodes can be modified by numerous cofactors in the presence of different substrates and enzymes, the proposed bioelectrochemical approach can find a wide utilization for the screening of microbial apo-enzymes requiring the additional reconstitution steps. The proposed methodology allows to avoid the exogenous addition of cofactors in aqueous solutions and also meets all requirements of *Green Chemistry* concept.

2. Experimental part

Used chemicals and reagents are detailed in the [Supplementary Material](#) section.

Electroplating experiment. The synthesis of the hybrid cofactor-doped functional layer on the surface of screen printed electrodes (SPEs) was conducted from Ag- and Pd-salts containing electrolytes mixed with polymer binding agents (0.9 % of alginate (ALG), 0.1 % of pyrrole (Py), or 2.5 % of Nafion (Naf)) and a target cofactor (FAD or FMN) in a ratio of 1:1:1 v/v/v. The immobilization was conducted at pH 7. Concentration of cofactors in the multiple electrolyte solution must not be below 0.4 mM. Electrolysis was performed utilizing a VMP 3 (Biologic SAS, Claix, France) potentiostat. The following synthesis parameters were used: current, I_k , -2.5 mA and, time, t_{el} 90 s to form ALG-cofactor-NPs functional layer; I_k , -2.5 mA and t_{el} 30 s to form Naf-cofactor-NPs. For synthesis of polypyrrole (PPy)-cofactor-doped layers an impulse deposition mode was optimized: -30 mA/cm² for 10 s (*step 1*) followed by 30 mA/cm² for 10 s (*step 2*). This procedure was repeated 5 times. Finally, 0 mA/cm² for 10 s was applied to the electrode.

Oxygen minisensor studies and cyclic experiment. The activity of the intact and modulated by additives oxidases (GOx and LOx) in solutions was controlled by OXR430 retractable needle-type fiber-optic oxygen minisensor (Pyro Science GmbH, Aachen, Germany) according to the previously reported protocol.[16]

The cyclic experiment utilizing optical oxygen minisensor was conducted in the following mode: 100 μL of commercial GOx (0.05 mg/mL) was mixed with 100 μL of 0.05 mg/mL of FAD and added to the mixture of phosphate buffer (10.79 mL) and 110 μL of 100 mM D-glucose (*cycle I*). After the reaction equilibrium was reached, 110 μL of the additional amount of D-glucose were poured into the reactant solution and the reaction equilibrium was evaluated (*cycle II*). This experiment was carried out until the oxygen consumption stopped changing (*cycle III, ... n*).

Scanning Electron Microscopy (SEM). SEM images were taken on a Quanta 400 FEG (Hillsboro, OR, USA) at an accelerating voltage of 10 keV. The image size was 1024x884 pixels.

Electrochemical studies. Electrochemical studies were conducted in both cyclic voltammetry (CV) and multistep chronoamperometric (MAM) mode.[17] CV plots were recorded in a droplet (150 μL) of tested solutions at scan rate of 20 mV/s in the range from - 0.4 V to + 0.4 V vs Ag/AgCl. For quantitative changes MAM mode was

used: *level 1* – polarization at the cathodic range of potentials (-0.08 V unless otherwise specified); *level 2* – read-out at the anodic range of potentials (0.2 V). **RAMAN spectroscopy.** RAMAN studies of the electrode surfaces were conducted on a HORIBA Jobin Yvon (Longmujau, France) Raman microscope equipped with Nd:YAG laser (532 nm).

Laser desorption ionization mass spectrometry (LDI-MS). To control the immobilization of cofactors on the electrodes, LDI-MS approach used in a bioscanner mode according to [18] was utilized. LDI-MS studies were performed on a Bruker Esquire 3000+ ESI-ion trap MS (Bruker Daltonics, Bremen, Germany) operated by Bruker esquire control 5.3 software equipped with atmospheric pressure AP-MALDI ion source and Nd:YAG solid-state laser with a length of 355 nm and frequency of 3 ns.

Quantum-chemical studies. To verify the possibility of stable cofactor-doped complexes formation in the multiple electrolyte solutions as well as to realize the architecture of the hybrid electrodes, methods of quantum chemistry were employed. Quantum-chemical studies were carried out by Gaussian 09 program and density functional theory B3LYP.[19] The calculations were conducted for Pd-based electrolyte as a case study. The following basis set was used: 6-31G(d,p) for O, N, H, C atoms [20] and LANL2DZ ECP with the added f-polarization function for Pd. The polarizable continuum solvation model (PCM) was utilized for evaluation of solvent impact on the results of modeling.[21] During the simulation process the fragment of the (010) plane of a PdO crystal and a layer of palladium atoms under it were used as a model structure. The presence of PdO at 620 nm⁻¹ [22] formed on the surface of Pd-NPs after synthesis was confirmed by RAMAN studies, **ESI, Fig. S1**.

The interaction energies of complex E₁₂ between the consisting fragments of 1 and 2 were calculated as follows:

$$E_{\text{int}} = E_{12} - (E_1 + E_2)$$

where E₁ and E₂ are the energies of fragments 1 and 2, E₁₂ – is the energy of the system obtained by combining of these fragments into a complex. If E₁₂ < 0 complex formation is energetically advantageous, at E₁₂ > 0 – complex is not formed.

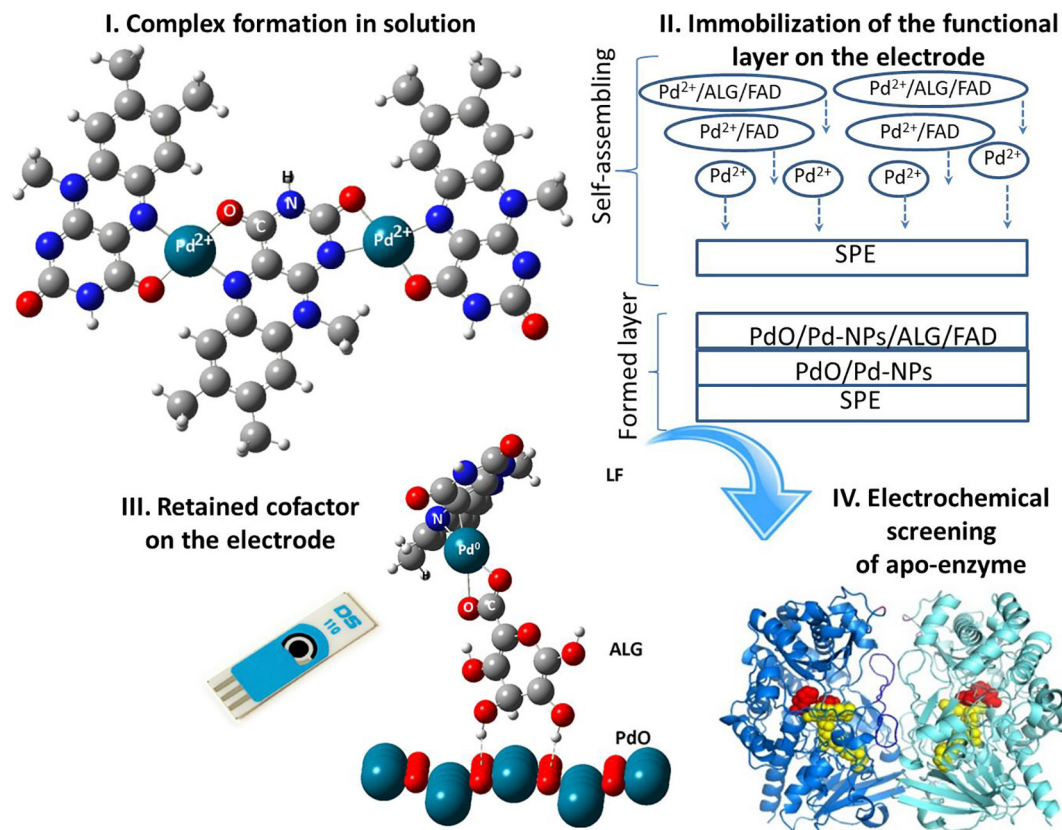
3. Results and discussion

3.1. From molecular interactions in the multiple electrolyte solutions to design of the hybrid electrodes

The proposed concept can be achieved via electroplating of a required cofactor from the multiple electrolyte solutions resulting in the formation of cofactor-doped NPs on a surface of SPE, see **Scheme 1** (shown for Lumiflavin, LF, as a stable and representative fragment of FAD, PdO/Pd-NPs and ALG used as a polymer binding agent).

The immobilization of cofactor from the multiple electrolyte solutions can undergo according to the following routes: deposition of Pd²⁺/cofactor or Pd²⁺/cofactor/polymer. Since Pd²⁺ (d⁸ cation) is a good complexing agent and target cofactors as well as binding polymers (ALG, PPy, Naf) have donor atoms (N, O) in their structure, the probability of complex formation between Pd²⁺/cofactor and Pd²⁺/polymers is quite high. [23–26] Therefore, the formation of complexes between Pd²⁺ and FAD or FMN in solutions can be assumed based on the presence of a free electron pair in nitrogen and oxygen atoms in the molecules of cofactors. During the interaction in solutions this free electron pair occupies free orbital of Pd²⁺ cation resulting in the formation of a donor-acceptor bond.

To validate the mechanism leading to a self-assembling of the functional hybrid cofactor-doped layer, next, quantum-chemical



Scheme 1. Schematic work flow of the *in situ* modulation of enzyme activity directly on the electrode surface. (I) – the structure of $2\text{Pd}^{2+}/3\text{LF}$ complex in solution; (II) – the scheme of the functional layer on the electrode: particles with the same charge and lower mass move faster towards electrode surface at the applied polarization; (III) – the architecture of the hybrid functional layer ($\text{PdO}/\text{Pd-NPs}/\text{ALG}/\text{FAD}$) on the electrode; (IV) – *in situ* reconstruction of enzymes structure and their simultaneous electrochemical screening.

studies were conducted. The optimized structures of complexes between LF and Pd^{2+} cations are summarized in Fig. 1.

The inspection of Fig. 1 indicates that Pd^{2+} directed opposite to $-\text{NCH}_3$ group of LF (marked by the circle in Fig. 1A) and located closer to the atoms O and N of LF is the most energetically advantageous complex between Pd^{2+} and LF. Due to the significant interaction energy between Pd^{2+} and lumiflavin during formation of this structure the appearance of a chain of FAD molecules bound to each other by Pd^{2+} is highly likely. For example, the addition of $2\text{Pd}^{2+}/3\text{LF}$ structure (Scheme 1, I) to Pd^{2+}/LF leads to the appearance of $3\text{Pd}^{2+}/4\text{LF}$ complex. In the course of computer modeling it was found that a fixation of LF on PdO by hydrogen bonding or the orientation of LF rings parallel to the PdO plane is energetically disadvantageous ($E_{\text{int}} > 0$), see ESI, Fig. S2.

In contrast, the deposition of a chain of FAD molecules bonded to each other by Pd^{2+} structure A (see Fig. 2) on PdO accomplished by transformation of Pd^{2+} to Pd^0 (cation reduction at the cathode) is energetically favorable (-13.2 kcal/mol), Fig. 2, structure B. Apart from this attachment model palladium cation can also form a complex with LF and ALG anion (Fig. 2, structure C) which deposition on the electrode is generically possible.

In this case the 1-st functional layer of the electrode after electrolysis is represented by PdO/Pd-NPs/(see ESI, Fig. S1) followed by attachment of ALG (layer 2) and LF (layer 3), Fig. 2, structure D. In other words, two parallel routes, viz. route B and D can underlay the attachment of FAD to the electrode surface. Notably, in both cases the formation of new bonds in the structure of cofactor does not occur.

Further it was important to (i) optimize electroplating conditions allowing co-deposition of cofactors in their intact form with-

out a degradation, conversion and formation of radicals [27–28]; to (ii) provide a sufficient cofactor diffusion rates [29]; to (iii) define a ratio between the cofactor concentration in the electrolyte and its co-deposited amount; as well as to (iv) study the impact of the polymer binding agents (Naf, PPy and ALG) on cofactors attachment, mechanical and electrochemical stability of the formed hybrid electrodes.

Interestingly, that a co-deposition from the multiple electrolytes not supplemented with a polymer binding agent (Naf, PPy or ALG) was accompanied by a rapid leaching of a cofactor from the electrode surface. More importantly, electrochemical behavior of the hybrid electrodes produced in the absence of polymer agents was unstable: analysis of Fig. S3A indicates a dependency of the cathodic peak potential on scan rates with the shifting of the peak potential >100 mV for Pd-NPs immobilized on a SPE with the individual FAD. However, the addition of ALG into the same electrolyte only in the amount of 0.9 % significantly changed the stability of electrochemical responses of the hybrid electrode, see ESI, Fig. S3B. Therefore, the usage of polymer binding agents during synthesis of cofactor-doped hybrid functional layers appears to be essential.

3.2. The addition of non-covalently bonded cofactors significantly impacts the efficiency of bioelectrocatalytic reactions in solutions

At first, the proof of concept towards modulation of enzyme activity via the addition of cofactors was verified in aqueous solutions. To this end, several experiments utilizing oxygen minisensor were conducted. Fig. 3 (shown for electrolyte containing FAD (GOx-based system) and FMN (LOx-based system)) clearly

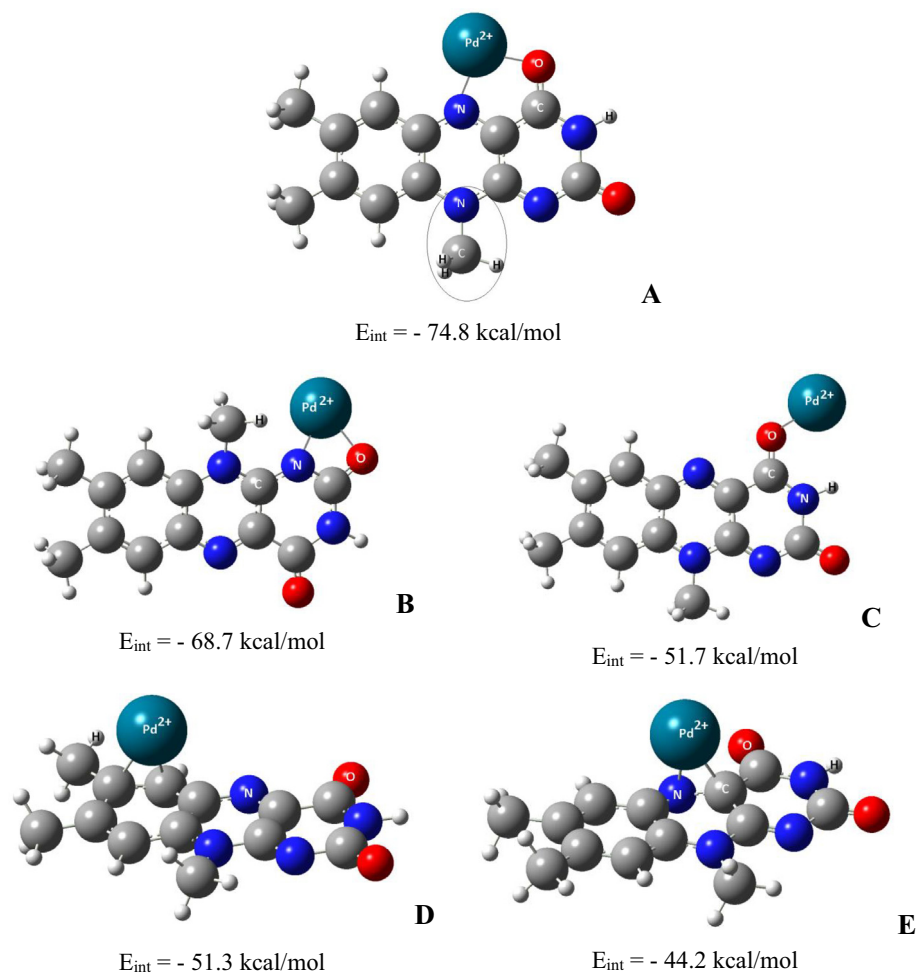
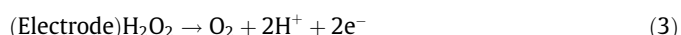
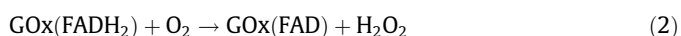
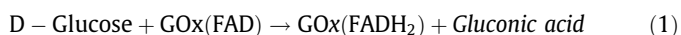


Fig. 1. The optimized structures of complexes between LF and Pd^{2+} cation and calculated energies of interaction between Pd^{2+} with LF (carbon – gray, hydrogen – white, oxygen – red, nitrogen – blue, Pd – turquoise). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

indicates that the addition of a noncovalently bonded cofactor into the multiple Pd-based electrolyte enhances the efficiency of bioelectrocatalytic transformations regardless the composition of the solution. Similar dependencies were obtained for the tested enzymes and cofactors on a basis of Ag-electrolyte, **ESI, Fig. S4**.

Next, the efficiency of bioelectrocatalytic conversion of target substrates after interactions with corresponding enzymes spiked with the extra amount of aqueous cofactors was tested in a droplet mode on the electrodes modified by individual electroplated noble NPs. The efficiency of biocatalytic transformation of glucose on PdO/Pd-NPs electrode immediately increased in the presence of aqueous GOx spiked with a given amount of aqueous cofactor (shown for FAD) in comparison with non-modified commercial system, see **Fig. 4** (see anodic range of potentials).

At the same time, the immobilization of FAD in the absence of GOx did not lead to the current increase (reference system), see **ESI, Fig. S5** (see line *a,b*). In other words, the reduction of FAD does not occur in the absence of a target protein. The reaction sequence for oxidases occurring on the electrode in the range of 0.2 V – 0.4 V can be summarized as follows [30] (shown for GOx(FAD) and glucose):



Due to a common nature of the intermediate product (H_2O_2) formed between the oxidases and a target substrate, similar dependencies were obtained on the electrode surface for LOx supplemented with the additional amount of FMN cofactor, **ESI, Fig. S6**.

Furthermore, the subsequent cyclic experiment performed by optical oxygen minisensor showed the possibility of continuous cascade reactions occurring in the aqueous media after reaching of a thermodynamic equilibrium with glucose without the needs for cofactor refreshments, **Fig. 5**.

This effect observed in solutions can readily be explained in terms of multiple hydrogen bonds formation between the added cofactor, enzyme and a substrate which is enough for several operational cycles in continuous processes to provide the reactions cascade according to the sequences (1) – (2). It means that in case of successful FAD co-immobilization with noble NPs followed by a slow and consecutive release of cofactor already on the electrode can also assist biochemical transformations during several reaction cycles.

The observed dependencies were confirmed by multi-step chronoamperometric studies performed for the electrodes with PdO/Pd-NPs electrocatalytic layer, added 10 μL of aqueous GOx and the additional amount of aqueous FAD, **Fig. S7**. The detected positive effect can be explained by incorporation of FAD into the structure of enzyme, whereby its concentration becomes sufficient for more effective reactions according to (1) – (3).

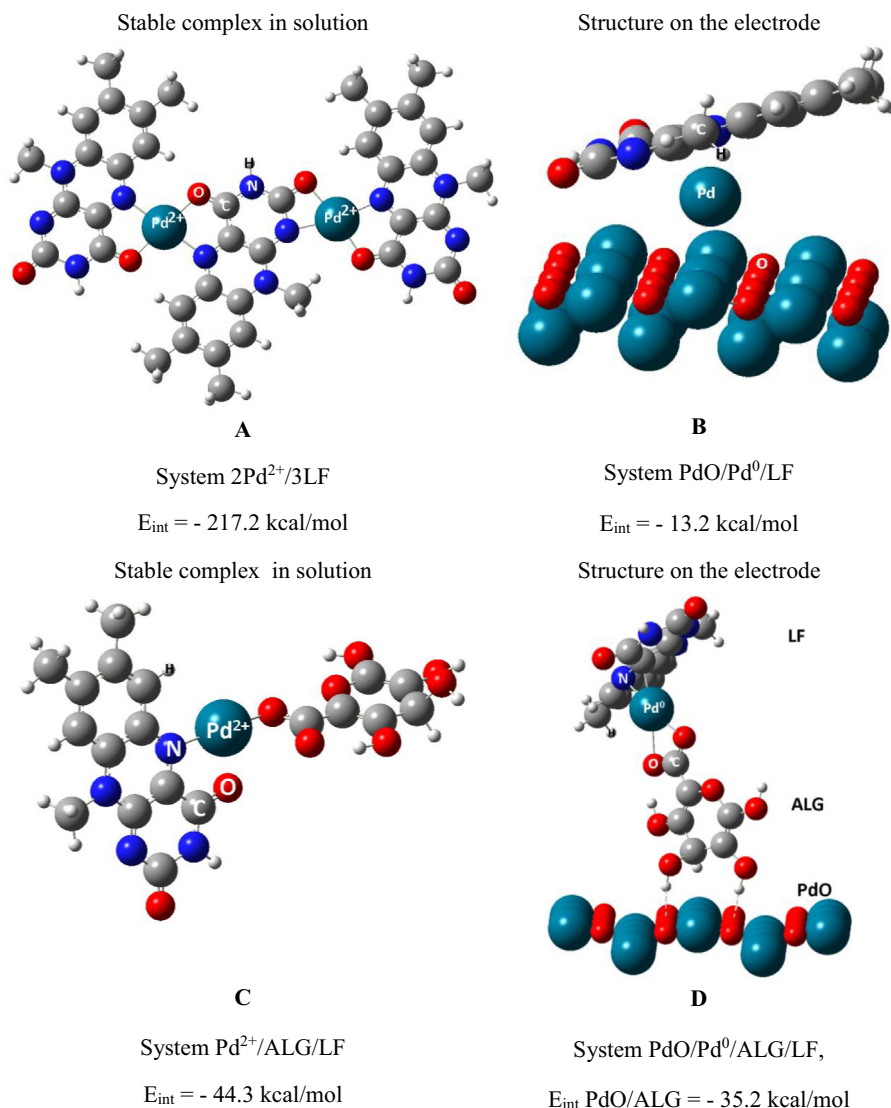


Fig. 2. The optimized structures and interaction energies of the formed complexes in multiple electrolyte solutions and after attachment of these complexes to SPEs: carbon – gray, hydrogen – white, oxygen – red, nitrogen – blue, Pd – turquoise. Note: dash lines – hydrogen bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.3. Cofactor remains retained and functional active after immobilization on the electrode

Next, the dependencies approved in solutions were examined in a solid state after immobilization of cofactors on the electrodes. One of the key challenges on this step was to provide a correct balance between the RedOx forms of a cofactor seen in a solution and after immobilization on the electrode. During electroplating the changing in functionality of cofactors that could dramatically affect the performance of the hybrid systems remains a major issue. We assume, that the presence of a polymer binding agent in the multiple electrolyte solution can guarantee the preserving of the intact structure of the cofactor during electroplating (see below).

The multilayered architecture of the hybrid electrode with co-immobilized cofactor (FAD) and polymer binding agent (ALG) realized by quantum-chemical studies (see section 3.1) was validated by SEM investigations, Fig. 6A. Briefly, a first layer of assemblies was represented by nanoparticles with an average diameter of 30 – 40 nm followed by deposition of the bigger organic–inorganic hybrid nanoparticles (the lighter shade corresponds to the presence of organic component) ranged from 100 nm to 120 nm.

Importantly, that distribution of the hybrid layer on the electrode surface was quite homogeneous.

Furthermore, LDI-MS analysis of the surface of the hybrid PdO/Pd-NPs/ALG/FAD-modified electrode revealed the presence of both bioorganic components (ALG and FAD) in the structure of the functional layer, Fig. 6B. Ionization of FAD in positive mode in addition to the expected $[\text{M} + \text{H}]^+$ at m/z 786 and $[\text{M} + \text{H}]^{2+}$ at m/z 393.8 was accompanied by the formation of abnormal spectral species: for example, $[\text{M} - \text{H} + \text{Na} + 2]^+$ detected at m/z 809. The similar trend during FAD ionization was earlier observed by MALDI-MS.[31] The ionization mechanism of FAD from the surface of the hybrid electrode might be similar to what occurs in the biological RedOx systems.[31] The presence in the mass spectra repeatable units of $\Delta 14$, $\Delta 16$ and $\Delta 18$ is more likely related to ALG ionization. [32,33]

The subsequently performed CV studies (Fig. 7) indicated a successful oxidative conversion of glucose to the product in the presence of a target enzyme (in this model case GOx). The obtained analytical merit for the glucose oxidation after GOx addition on the PdO/Pd-NPs/ALG/FAD-modified electrode was at least two times higher vs electrode with a pure PdO/Pd-NPs/ALG layer (prepared in the absence of FAD). It means that FAD cofactor is present

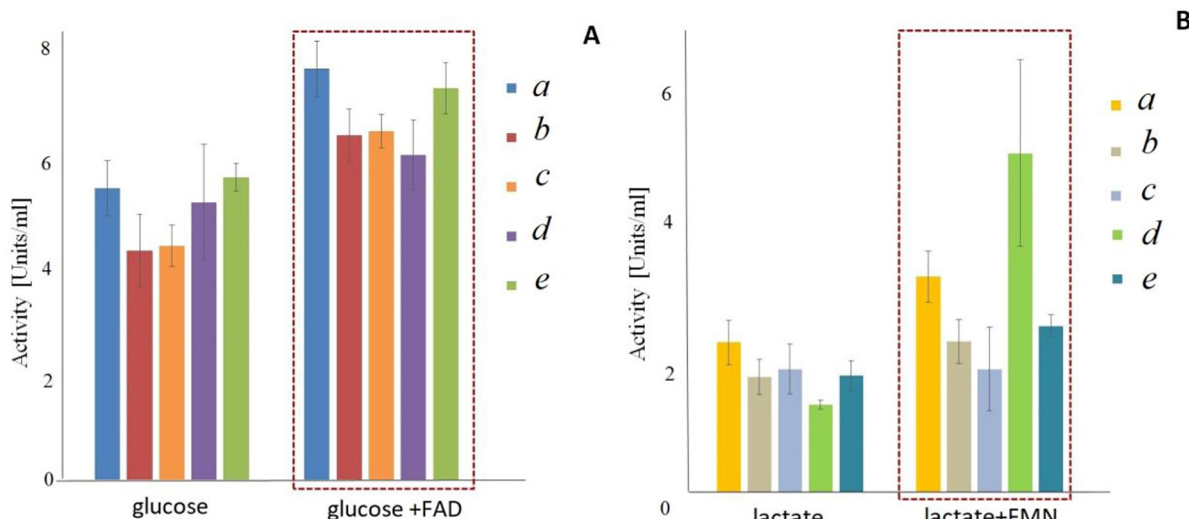


Fig. 3. Tuning of enzymatic activity by composition of the multiple electrolyte solution. The results of optical oxygen minisensor studies (the activity, Units/mL of the intact and modulated oxidases was calculated according to algorithm introduced in [16]) performed for 1 mg/mL of commercial GOx (a) and GOx with different additives spiked with the additional amount of FAD (A): b – Pd-electrolyte, c – Naf, d – Py and e – ALG (shown in the presence of 110 μ L of 100 mM D-glucose). (B) – commercial LOx (a) as well as modulated LOx spiked with FMN and additives: b – Pd-electrolyte, c – Naf, d – Py and e – ALG (evaluated in the presence of 110 μ L of 100 mM L-lactate).

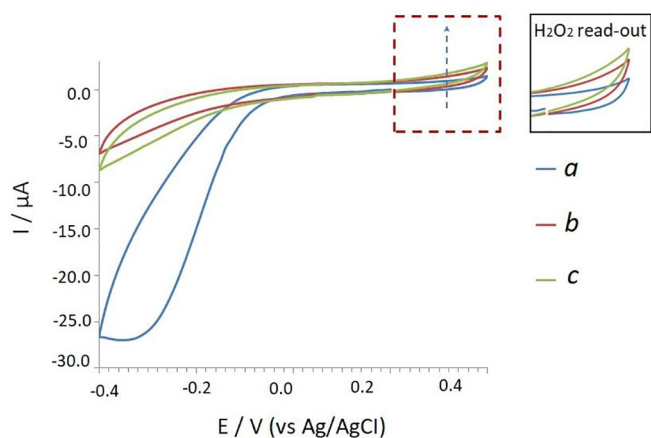


Fig. 4. CV plots obtained at 20 mV/s from PdO/Pd-NPs modified electrode in a droplet of (a) 10 mM glucose, (b) 10 mM glucose spiked with 10 μ L of aqueous GOx (0.05 mg/mL) and (c) 10 mM glucose spiked with 10 μ L of aqueous GOx (0.05 mg/mL) with the additional 0.05 mg/mL amount of FAD cofactor.

in enzymatically active form after immobilization that can support protein function according to a conventional biochemical route.

Notably, the increase of current in the anodic range was accompanied with a current decrease in the cathodic branch of CV (the cathodic peak charge 1.3 times less for the electrode with a hybrid layer), see Fig. 7. The latter effect is due to a decrease of the active area of Pd-NPs which is partially covered by organic components of a hybrid layer. Therefore, the observed result in the anodic range of potentials can only be explained by the impact of FAD cofactor addition on the general electron transfer in this hybrid system.

3.4. Sensing of apo-enzymes by the hybrid electrodes

To demonstrate the possibility for *in situ* modulation of properties of enzymes directly on the electrode, cofactor FAD (as a case study) was removed from the commercial GOx. Briefly, non-covalently bonded FAD was split off from GOx *via* acidic hydrolysis, and the yellow supernatant (depending on the form, *i.e.* FAD/FADH₂ UV-vis peak at 350 and 450 nm) was removed after cen-

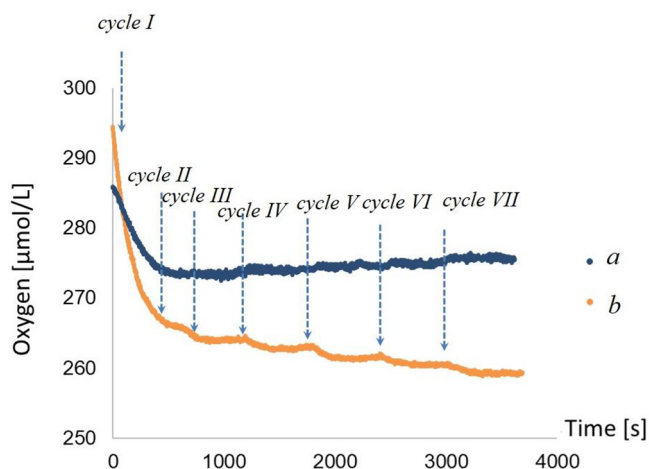


Fig. 5. Cyclic oxygen-time-dependent diagram between GOx and glucose in buffer solution. Oxygen consumption (μ mol/L) is shown for the mixture of commercial GOx (0.05 mg/mL) and 10 mM of glucose (a) in comparison with GOx (0.05 mg/mL) spiked with the additional amount of FAD (0.05 mg/mL) at the beginning of the experiment (b). Note: FAD and glucose concentration were optimized separately for this set of experiments to make the observed trends pronounced. The arrows on the diagram correspond to the time-periods of additional glucose injection.

trifugation at 13000 rpm for 15 min.[34] The obtained precipitate was re-dissolved in a phosphate buffer. Afterwards, GOx in its apo-inactive form (apo-GOx) was received. It should be noted that in the absence of cofactor apo-GOx can still bind the substrate (glucose) but it cannot oxidize the sugar.

Further, the synthesized PdO/Pd-NPs/ALG/FAD-modified electrode was explored in a mixture of apo-GOx and glucose. The electroanalytical performance of the hybrid PdO/Pd-NPs/ALG/FAD-modified electrode was compared with a reference system prepared in the absence of FAD, *i.e.* PdO-NPs/Pd-NPs/ALG.

From Fig. 8A it is obvious that the transition of apo-GOx to holo-GOx occurs directly on the hybrid electrode. The product of biochemical interactions (H₂O₂) formed between holo-GOx and glucose according to the equations (1) – (3) can be used to monitor this process. Notably, the synthesized hybrid PdO/Pd-NPs/ALG/FAD-modified electrode was electrochemically active at least dur-

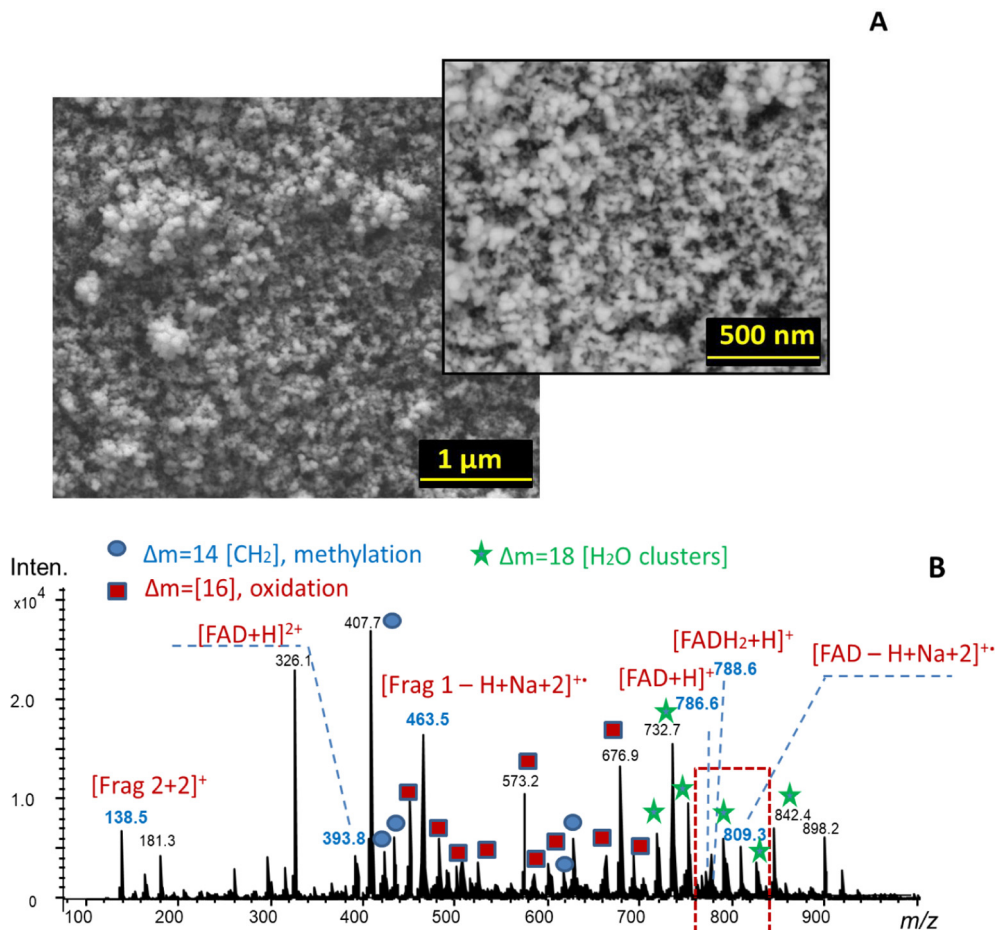


Fig. 6. SEM images (A) and LDI-MS spectra (B) obtained from the hybrid PdO/Pd-NPs/ALG/FAD-modified electrode. Note: Frag. 1 and Frag. 2 correspond to FAD fragments at m/z 439 and 136, respectively. LDI-MS experiments were performed at least in triplicate with the same results, laser fluence was 45%.

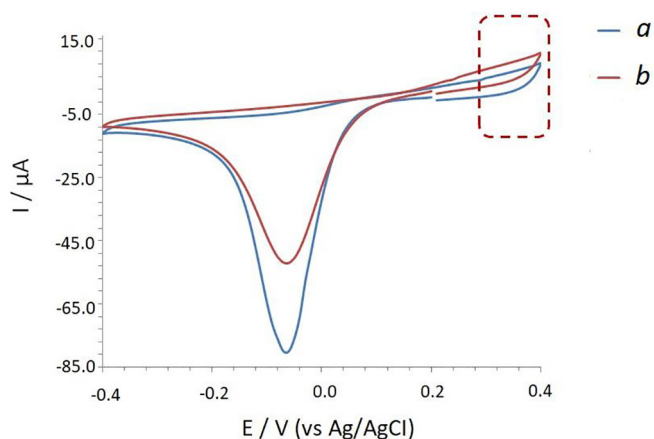


Fig. 7. CV plots obtained from PdO/Pd-NPs/ALG-modified (a) and PdO/Pd-NPs/ALG/FAD- electrodes (b). The CV plots were obtained in a 150 μL droplet of 50 mM of glucose and 0.06 mg/mL GOx; the immobilization of FAD was carried out at pH 7 from the Pd-electrolyte, supplemented with 1 mg/mL FAD and 0.9 % ALG in a ratio of 1:1:1 v/v/v.

ing 30 scans. This trend was in line with results obtained in cyclic experiment by oxygen minisensor for the solutions spiked with the extra amount of FAD (see Fig. 5). At the same time, no electrochemical signal was recorded from the reference PdO/Pd-NPs/ALG electrode in a droplet of apo-GOx and glucose, Fig. 8B. It means that in

the absence of the immobilized FAD the electrochemical screening is not possible.

To sum it up, this set of experiments approves that the immobilized FAD remains biologically active and can readily be used for reconstitution of enzyme structure and activity. Moreover, these data confirmed the successful implementation of heterogeneous biocatalysis occurring directly on the surface of the hybrid electrodes.

4. Conclusions

The proposed concept towards *in situ* modulation of enzymatic activity on the electrodes is potentially useful for the development of bioelectrochemical devices, enzyme nanoengineering, control strategies in biotechnology and drug discovery in pharmacy. The integration of this platform allows skipping several usual routine laborious steps, viz. extraction, chromatographic separation and mass spectrometry analysis conventionally used for monitoring and detection of the expressed in apo-form enzymes. The proposed electrodes with co-immobilized cofactors are very easy to store without a risk of possible degradation of biocomponent typically seen in classical biosensors, for example, with co-immobilized enzymes.

Depending on the type of co-immobilized component, *i.e.* type of cofactors, polymer binding agents, design and operational electrochemical mode the proposed hybrid electrodes can serve as a (i) tailored bioelectrochemical tool for *in situ* modulation of properties of apo-enzymes, (ii) monitoring of natural products of interests or for (iii) real-time screening of overproducers/mutants. In addition,

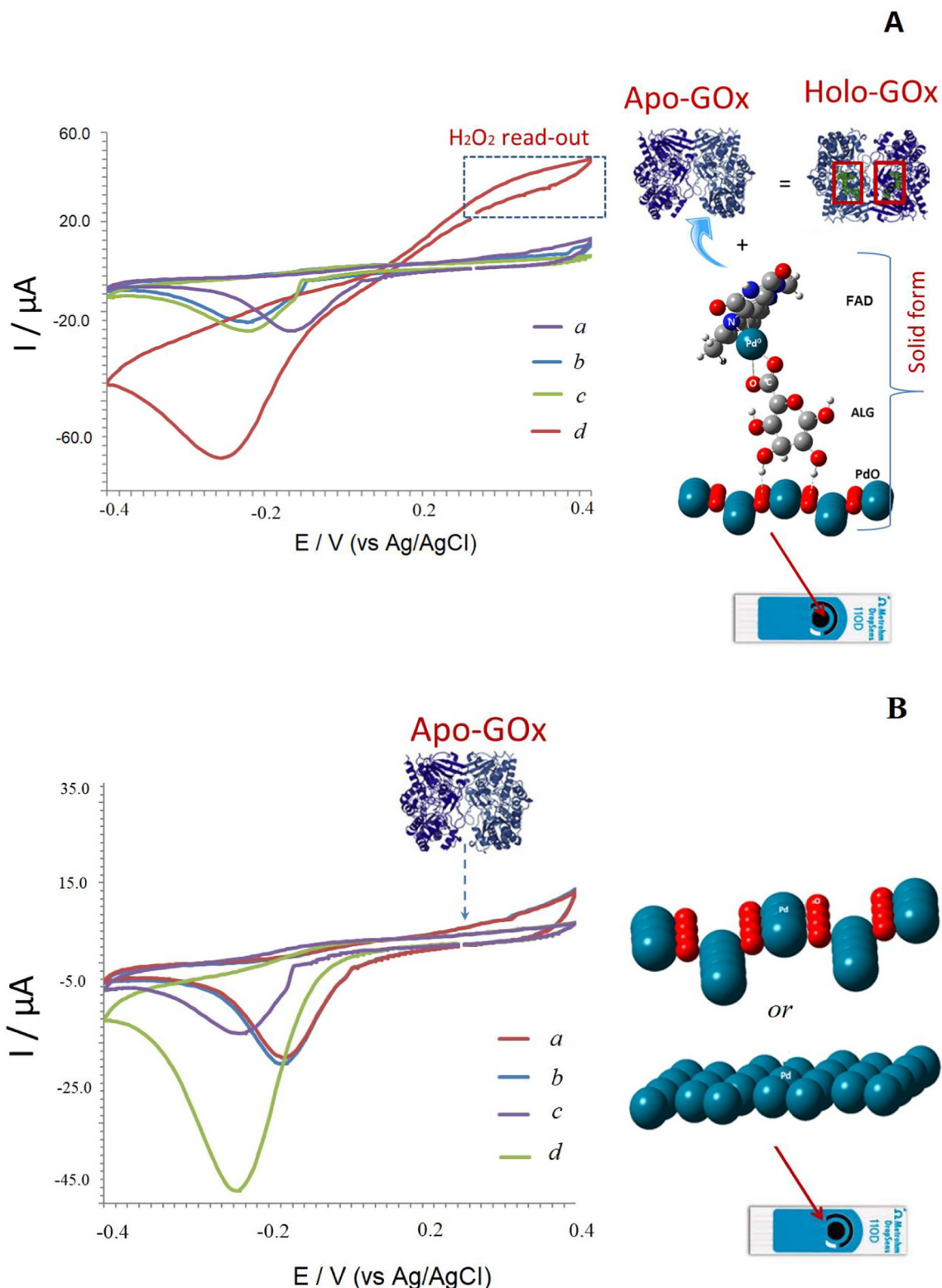


Fig. 8. CV plots obtained at 20 mV/s from the hybrid PdO/Pd-NPs/ALG/FAD (A) and PdO/Pd-NPs/ALG electrodes (B) in the presence of: a – phosphate buffer; b – 10 mM glucose prepared in buffer; c – 0.75 mg/mL apo-GOx in phosphate buffer; d – 0.75 mg/mL apo-GOx + 10 mM glucose. Note: structures shown on the right are the models of the functional layers formed on the electrodes.

this platform can be implemented as a (iv) bio-scanner of appropriate substrates for engineered modified enzymes, (v) as a search tool for the inhibitors/activators of target enzymes or (vi) optimization of the conditions/transporters providing the external expres-

sion of the target enzyme. Moreover, by means of the proposed concept the key factors affecting the environmental stability of engineered enzymes versus wild-type analogues can be established (vii).

CRediT authorship contribution statement

N. Apushkinskaya: Formal analysis, Visualization, Writing. **E.V. Zolotukhina:** Visualization, Validation, review & editing. **E.V. Butyrskaya:** Visualization, Validation, review & editing. **Y.E. Silina:** Conceptualization, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

- Bilyk O, Luzhetskyy A. Metabolic engineering of natural product biosynthesis in actinobacteria. *Curr Opin Biotechnol* 2016;42:98–107. <https://doi.org/10.1016/j.copbio.2016.03.008>.
- Mano N. Engineering glucose oxidase for bioelectrochemical applications: HAL Id: hal-02121438 Engineering Glucose Oxidase for. *Bioelectrochemical Applications* 2019.
- Bankar SB, Bule MV, Singhal RS, Ananthanarayan L. Glucose oxidase—an overview. *Biotechnol Adv* 2009;27:489–501. <https://doi.org/10.1016/j.biotechadv.2009.04.003>.
- Dubey MK, Zehra A, Aamir M, Meena M, Ahirwal L, Singh S, et al. Improvement Strategies, Cost Effective Production, and Potential Applications of Fungal Glucose Oxidase (GOD): Current Updates. *Front Microbiol* 2017;8:1032. <https://doi.org/10.3389/fmicb.2017.01032>.
- Uppada V, Bhaduri S, Noronha SB. Cofactor regeneration - an important aspect of biocatalysis. *Curr Sci* 2014;106:946–57. <https://doi.org/10.18520/cs/v106/i7/946-957>.
- Milton RD, Minteer SD. Direct enzymatic bioelectrocatalysis: Differentiating between myth and reality. *J R Soc Interface* 2017;14. <https://doi.org/10.1098/rsif.2017.0253>.
- Dmitrieva MV, Zolotukhina EV. Data describing the cofactor additives effect on bioelectrocatalytic activity of «crude» extracts. *Data in Brief* 2020;30. <https://doi.org/10.1016/j.dib.2020.105513>.
- E.D. Harris, Cofactors: Organic, in: B.B.T.-E. of H.N. (Third E. Caballero (Ed.), Academic Press, Waltham, 2013: pp. 366–377. <https://doi.org/10.1016/B978-0-12-375083-9.00058-1>.
- Istrate OM, Rotariu L, Bala C. Amperometric L-Lactate biosensor based upon a gold nanoparticles/reduced graphene oxide/polyallylamine hydrochloride modified screen-printed graphite electrode. *Chemosensors* 2021;9. <https://doi.org/10.3390/chemosensors9040074>.
- Arsalan A, Younus H. Enzymes and nanoparticles: Modulation of enzymatic activity via nanoparticles. *Int J Biol Macromol* 2018;118:1833–47. <https://doi.org/10.1016/j.ijbiomac.2018.07.030>.
- Suzuki N, Lee J, Loew N, Takahashi-Inose Y, Okuda-Shimazaki J, Kojima K, et al. Engineered glucose oxidase capable of quasi-direct electron transfer after a quick-and-easy modification with a mediator. *Int J Mol Sci* 2020;21:1–10. <https://doi.org/10.3390/ijms21031137>.
- Bartlett PN, Al-Lolage FA. There is no evidence to support literature claims of direct electron transfer (DET) for native glucose oxidase (GOx) at carbon nanotubes or graphene. *J Electroanal Chem* 2018;819:26–37. <https://doi.org/10.1016/j.jelechem.2017.06.021>.
- Velasco-Lozano S, Benítez-Mateos AI, López-Gallego F. Co-immobilized Phosphorylated Cofactors and Enzymes as Self-Sufficient Heterogeneous Biocatalysts for Chemical Processes. *Angewandte Chemie - International Edition* 2017;56:771–5. <https://doi.org/10.1002/anie.201609758>.
- Liu W, Wang P. Cofactor regeneration for sustainable enzymatic biosynthesis. *Biotechnol Adv* 2007;25:369–84. <https://doi.org/10.1016/j.biotechadv.2007.03.002>.
- Semenova D, Gernaey KV, Morgan B, Silina YE. Towards one-step design of tailored enzymatic nanobiosensors. *Analyst* 2019;145:1014–24. <https://doi.org/10.1039/c9an01745c>.
- Semenova D, Silina YE, Koch M, Micheli L, Zubov A, Gernaey KV. Sensors for biosensors: a novel tandem monitoring in a droplet towards efficient screening of robust design and optimal operating conditions. *Analyst* 2019;144:2511–22. <https://doi.org/10.1039/c8an02261e>.
- Silina YE, Apushkinskaya N, Talagaeva NV, Levchenko MG, Zolotukhina EV. Electrochemical operational principles and analytical performance of Pd-based amperometric nanobiosensors. *The Analyst* 2021;146:4873–82. <https://doi.org/10.1039/d1an00882j>.
- Silina YE, Morgan B. LDI-MS scanner: Laser desorption ionization mass spectrometry-based biosensor standardization. *Talanta* 2021. <https://doi.org/10.1016/j.talanta.2020.121688>.
- Lee C, Yang W, Parr RG. Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density. *Physical Review B* 1988. <https://doi.org/10.1103/PhysRevB.37.785>.
- Franci MM, Pietro WJ, Hehre WJ, Binkley JS, Gordon MS, DeFrees DJ, et al. Self-consistent molecular orbital methods. XXIII. A polarization-type basis set for second-row elements. *J Chem Phys* 1982;77:3654–65. <https://doi.org/10.1063/1.444267>.
- J.B. Foresman, T.A. Keith, K.B. Wiberg, J. Snoonian, M.J. Frisch, Solvent Effects. 5. Influence of Cavity Shape, Truncation of Electrostatics, and Electron Correlation on ab Initio Reaction Field Calculations, *The Journal of Physical Chemistry*. 100 (1996) 16098–16104. <https://doi.org/10.1021/jp960488j>.
- Baylet A, Marécot P, Duprez D, Castellazzi P, Groppi G, Forzatti P. In situ Raman and in situ XRD analysis of PdO reduction and Pd⁰ oxidation supported on γ-Al₂O₃ catalyst under different atmospheres. *PCCP* 2011;13:4607–13. <https://doi.org/10.1039/c0cp01331f>.
- Butyrskaya EV, Korkmaz N, Zolotukhina EV, Krasiukova V, Silina YE. Mechanistic aspects of functional layer formation in hybrid one-step designed GOx/Nafion/Pd-NPs nanobiosensors. *Analyst* 2021;146:2172–85. <https://doi.org/10.1039/d0an02429e>.
- Lawrance GA. *Introduction to Coordination Chemistry*. Wiley; 2013.
- Celedón S, Roisnel T, Artigas V, Fuentealba M, Carrillo D, Ledoux-Rak I, et al. Palladium(ii) complexes of tetradentate donor–acceptor Schiff base ligands: synthesis and spectral, structural, thermal and NLO properties. *New J Chem* 2020;44:9190–201. <https://doi.org/10.1039/D0NJ01982H>.
- Le Bris N, Pineau J, Lima LMP, Tripiet R. Palladium(II) coordination with polyazacycloalkanes. *Coord Chem Rev* 2022;455. <https://doi.org/10.1016/j.ccr.2021.214343>.
- Menegatti T, Žnidarič-Plazl P. Hydrogel-Based Enzyme and Cofactor Co-Immobilization for Efficient Continuous Transamination in a Microbioreactor. *Front Bioeng Biotechnol* 2021;9:1–10. <https://doi.org/10.3389/fbioe.2021.752064>.
- López-Gallego F, Jackson E, Betancor L. Heterogeneous Systems Biocatalysis: The Path to the Fabrication of Self-Sufficient Artificial Metabolic Cells, *Chemistry – A European Journal* 2017;23:17841–9. <https://doi.org/10.1002/chem.201703593>.
- Campbell E, Meredith M, Minteer SD, Banta S. Enzymatic biofuel cells utilizing a biomimetic cofactor. *Chem Commun* 2012;48:1898–900. <https://doi.org/10.1039/c2cc16156c>.
- Koch M, Apushkinskaya N, Zolotukhina EV, Silina YE. Towards hybrid one-pot/one-electrode Pd-NPs-based nanoreactors for modular biocatalysis. *Biochem Eng J* 2021;175. <https://doi.org/10.1016/j.bej.2021.108132>.
- Itoh Y, Ohashi Y, Shibue T, Hayashi A, Maki S, Hirano T, et al. Reduction in Desorption Mass Spectrometry: Multiple Protonation on Flavins without Charge Increment. *Journal of the Mass Spectrometry Society of Japan* 2002;50:52–7. <https://doi.org/10.5702/masspec.50.52>.
- Liu H, Zhang Y, Yin H, Wang W, Zhao X, Du Y. Alginate oligosaccharides enhanced Triticum aestivum L. tolerance to drought stress. *Plant Physiology and Biochemistry: PPB* 2013;62:33–40.
- Zhu B, Ni F, Sun Y, Ning L, Yao Z. Elucidation of degrading pattern and substrate recognition of a novel bifunctional alginate lyase from *Flammeovirga* sp. NJ-04 and its use for preparation alginate oligosaccharides. *Biotechnol Biofuels* 2019;12:13. <https://doi.org/10.1186/s13068-019-1352-8>.
- D'Auria S, Herman P, Rossi M, Lakowicz JR. The fluorescence emission of the apo-glucose oxidase from *Aspergillus niger* as probe to estimate glucose concentrations. *Biochem Biophys Res Commun* 1999;263:550–3. <https://doi.org/10.1006/bbrc.1999.1330>.