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'Artificial peroxidase' nanozyme – enzyme based lactate biosensor

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ABSTRACT

In contrast to bienzyme biosensors, we propose the nanozyme-enzyme based ones substituting the enzyme peroxidase with the more active and stable nanoparticles "artificial peroxidase". The use of catalytically synthesized Prussian Blue based nanozymes simplifies assembling of hydrogen peroxide transducer providing its higher sensitivity. For immobilization of lactate oxidase the composite alkoxysilane – perfluorosulfonated ionomer (PFSI) membranes are proposed achieving the significantly improved operating stability. The resulting nanozyme-enzyme lactate biosensor displays twice higher sensitivity (> 0.2 A M^{-1} cm^{-2}) compared to the Prussian Blue film based one. Nanozymes "artificial peroxidase" are expected to find wide use in elaboration of oxidase based biosensors.

1. Introduction

The great majority of the enzyme-based biosensors and analytical kits use the oxidases as signal generation enzymes. The latter catalyze oxidation of their specific substrate by molecular oxygen, reducing it to hydrogen peroxide (H_2O_2). As shown already in 70-s of the last century [1], the most progressive way to provide operation of the corresponding biosensors was to detect H_2O_2. Up to now in electrochemical clinical analyzers the detection of H_2O_2 is carried out by its oxidation on platinum electrodes due to poor selectivity of the latter to hydrogen peroxide reduction [2]. However, even in 80-s it was found that the analytical signal in this case was suffering from false positive responses due to the presence of easily oxidizable compounds in real biological samples [3].

The discovery of bioelectrocatalysis by peroxidases [4], the enzymes catalyzing reduction of hydrogen peroxide, opened the possibility for selective H_2O_2 detection by its reduction in the presence of oxygen. Soon after this publication the bi-enzyme glucose electrode has been proposed [5]. Particular attention to peroxidase transduction of oxidase-catalyze reaction has been reviewed [6]. However, the enzymes, being inherently unstable, obviously cannot provide the long-term stability. Moreover, the conception of bi-enzyme electrodes presumes the immobilization of oxidase over peroxidase involved in direct electron transfer between the enzyme active cite and the mediator. This has been realized mostly for carbon paste electrodes [7], which not, however, found wide practical applications.

In contrast to enzyme peroxidase bioelectrocatalysis, a variety of modified electrodes active in electrocatalysis of hydrogen peroxide oxidation-reduction have been proposed. Among the most advantageous catalytic materials is Prussian Blue [2]. As compared to the most widely used platinum, this material is characterized by the 1000 times higher electrochemical rate constant in H_2O_2 reduction and 1000 times improved selectivity allowing hydrogen peroxide detection by its reduction in the presence of oxygen. Immobilization of oxidases over Prussian Blue modified electrodes results in biosensors with the most advantageous analytical performance characteristics [2,8–10]. However, electrochemical deposition of Prussian Blue achieving advanced analytical performance characteristics of the resulting modified electrodes is hardly suitable for their mass-production. Despite the reported 10 years ago open-circuit deposition of the electrocatalyst [11] achieving its advantageous performance characteristics over the existing current-free protocols [12–14], the majority of the produced Prussian Blue based hydrogen peroxide transducers are characterized by the rather poor sensitivity and signal-to-noise ratio.

In contrast to bi-enzyme biosensors we propose the nanozyme-enzyme ones. The discovery of nanoparticles with peroxidase-like activity [15], later referred to as nanozymes [16] has opened the new area of chemistry and material science. A particular attention to nanoparticles with enzyme-like activity is due to their dramatically improved stability and reduced cost as compared to natural enzymes. Despite its short history, a search for the term 'nanozyme' through Web of Science returns already > 700 articles. Prussian Blue nanostructures were reported to modify electrode surface [17], as well as to mimic the peroxidase enzyme [18–21]. The best performance characteristics,

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however, are peculiar to catalytically synthesized Prussian Blue based nanozymes with both high activity even defeating the natural enzyme peroxidase and enzymatic selectivity in H_2O_2 reduction, particularly the absence of oxidase-like activity [22]. Simple drop-casting of the nanozyme suspension onto the electrode surface with subsequent drying results in modified electrodes with even higher sensitivity to hydrogen peroxide as compared to Prussian Blue film modified electrodes. Immobilization of lactate oxidase over nanozyme modified electrodes results in the biosensors with improved sensitivity.

2. Materials and methods

2.1. Materials

Experiments were carried out in distilled water. Inorganic salts, potassium L-lactate, γ-aminopropyltriethoxysilane, and 30% hydrogen peroxide solution were obtained from Sigma-Aldrich (USA). γ-Aminopropyltriethoxysilane was distilled before use. Perfluorosulfonated ionomer (Nafion analogue) as 10% solution in isopropanol was purchased from Plastpolymer (St.-Petersburg, Russia). Lactate oxidase (EC 1.1.3.2, 72 IU mg^{-1}) from Pediococcus sp. was from Sorachim (Switzerland). Planar screen-printed three-electrode structures with carbon working electrode (Ø = 1.8 mm) and planar H_2O_2 sensors with Prussian Blue film modified working electrode according to procedure reported in Ref. [11] were produced by Ltd Rusens (Moscow, Russia).

2.2. Instrumentation

Electrochemical experiments were carried out using a PalmSens 3 potentiostat (PalmSens BV, The Netherlands). For Prussian Blue nanoparticles separation Eppendorf MiniSpin centrifuge (Germany) was used. Ultrasonication of nanoparticles suspensions was carried out in PSB-Gals (Russia) ultrasonic bath. Nanoparticles size distribution was estimated by dynamic light scattering performed with Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, UK). Prussian Blue concentration in nanoparticles suspension was evaluated upon UV/Vis measurements in transmission mode using Lambda 35 Spectrophotometer (PerkinElmer, USA). The flow injection system consisted of a Cole Parmer (Vernon Hills, IL) peristaltic pump (7519-10), homemade flow-through wall-jet cell with a 0.5 mm nozzle positioned at 1–2 mm distance from the surface of the disk electrode, homemade injector and PalmSens electrochemical interface. Flow rates used were 0.4–1 mL min^{-1}.

2.3. Methods

Prussian Blue nanoparticles with Ø = 49 nm were used. Synthesis of nanoparticles was carried out in 1:1 mixture of $FeCl_3$ and $K_3[Fe(CN)_6]$ (75 mM) dissolved in 0.1 M KCl/0.1 M HCl solution under ultrasonication. Prussian Blue deposition was initiated with H_2O_2 addition to a final concentration of 50 mM. After 0.5 min the mixture was centrifuged at 13000 r.p.m. during 1 min, dark blue precipitate was redispersed into 0.1 M KCl/0.1 M HCl solution; the procedure of centrifugation-redispersion was repeated 6–7 times. The obtained nanoparticles were stored in 0.1 M KCl/0.1 M HCl solution and ultrasonicated prior to use. Extinction coefficient per Prussian Blue unit cell of $4.85 \cdot 10^4$ M^{-1} cm^{-1} (λ = 700 nm) was used to estimate nanoparticles concentration.

For electrochemical investigations a 2 µl of Prussian Blue nanoparticles suspension (2.1 nM) was drop-cast onto the working electrode surface and dried at a room temperature during 24 h.

The biosensors were made by casting the lactate oxidase containing mixture onto the top surface of Prussian Blue modified electrodes (2 µL) with subsequent drying in a refrigerator (4 °C) for 2 days. The enzyme containing mixture was prepared as follows. First, the lyophilized lactate oxidase samples were dissolved in water to a final concentration of 10 mg mL^{-1}. The stock solution of γ-aminopropyltriethoxysilan was diluted by absolute isopropanol 20 times. PFSI stock solution (10%) was diluted by absolute isopropanol 5 times and neutralized by NH_4OH to a final pH of approximately 6. Then the enzyme casting mixture in 90% isopropanol containing 1.5% γ-aminopropyltriethoxysilan and from 0% to 0.5% of PFSI, was prepared by mixing PFSI and siloxane solutions, their dilution with isopropanol and subsequent mixing with the enzyme aqueous solution.

3. Results and discussion

3.1. Nanozyme-modified electrodes

As mentioned, the use of nanozymes provides the simplicity of electrode modification: the suspension of catalytically synthesized Prussian Blue nanoparticles has been drop-casted onto the electrode surface with subsequent drying. The scanning electron microscopy image of the same amount of nanozyme suspension casted onto the silicon substrate (Fig. S1, Supporting Information) displays dense layer of the uniform nanoparticles. Analytical performance characteristics of the resulting modified electrodes have been investigated in batch mode upon stirring.

Fig. 1 displays the current responses of nanozyme modified electrodes to different hydrogen peroxide concentrations. As seen, the current generated by hydrogen peroxide reduction is stable. Fig. 1 inset displays the corresponding calibration graph. Sensitivity evaluated as its slope when analyte concentration tends to zero, is of 0.85 A M^{-1} cm^{-2}. For comparison, Fig. 1, inset also presents the calibration graph for conventional Prussian Blue film modified electrode. As seen, the slope is lower, and the sensitivity is of 0.65 A M^{-1} cm^{-2}. Hence, in addition to the simplicity of assembling, the use of nanozymes "artificial peroxidase" provides the improved sensitivity of the modified electrodes.

The higher sensitivity of the nanozyme 'artificial peroxidase' modified electrodes can be explained in terms of the obviously higher surface area of Prussian Blue nanostructures as compared to Prussian Blue films grown either in electrochemical or open circuit conditions. Obviously higher surface area can cause lower stability of the modified electrode. We, however, note that the described approach is proposed for biosensors, hence, the operational stability has to be considered in the presence of the membrane of similar composition to the enzyme containing one. As found, after covering with the perfluorosulfonated

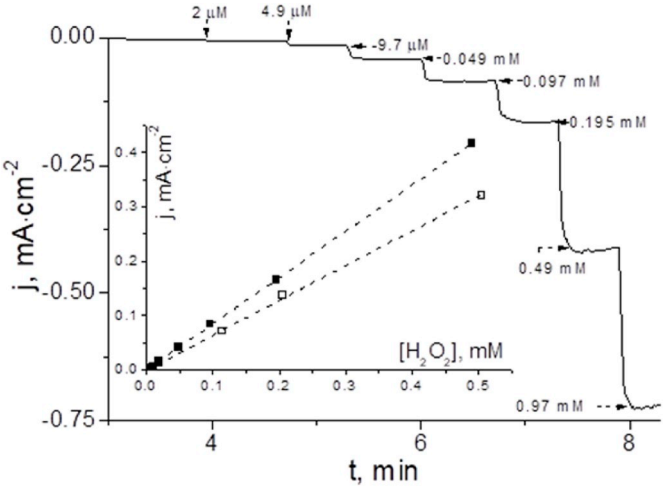

Fig. 1. Current responses towards hydrogen peroxide and calibration curve (■) of the nanozymes modified electrode; (□) – calibration curve of the conventional Prussian Blue modified electrode; batch mode upon stirring; phosphate buffer pH 6.0; E = 0.00 V Ag|AgCl.

ionomer – siloxane membrane the nanozyme-modified electrode exhibits even higher operational stability upon continuous monitoring of hydrogen peroxide (0.1 mM) as compared to Prussian Blue film (Fig. S2, Supporting information). This finding gives promise for elaboration of the nanozyme-enzyme based biosensors.

3.2. Improved lactate oxidase immobilization procedure

For immobilization of lactate oxidase the improved protocol with enzyme exposing to water-organic mixtures with the high content of organic solvent proposed by us 23 years ago has been used [23]. In case of immobilization in sol-gel membranes such protocol provides hydrolysis of siloxanes in optimal conditions without excessive dilution with water and results in the most active and stable enzyme containing membranes [9].

Since lactate oxidase is among the less stable enzymes, further stabilization of the enzyme containing membranes is required for multi-used commercial biosensors. For this aim we've investigated the stabilization of the lactate oxidase containing siloxane membranes [9] with Nafion analogue – perfluorosulfonated ionomer (PFSI). Despite the latter contains negatively charged substituent able to shield the enzyme active site at its high concentrations [8], we believed that it also able to stabilize lactate oxidase similarly to glucose oxidase [10].

The study of lactate oxidase immobilization has been carried out depositing enzyme containing membranes onto the top of Prussian Blue film modified electrodes. As a siloxane the γ-aminopropyltriethoxysilane has been chosen in concentration of 1.5% in the membrane forming mixture according to our previous report [24].

Fig. 2 illustrates that the presence of perfluorosulfonated ionomer in the enzyme containing membrane causes the decrease of sensitivity of the resulting biosensors. However, if one excludes considering of pure siloxane membranes (zero PFSI concentrations), it is possible to observe an absolute maximum of sensitivity together with an absolute minimum of the apparent Michaelis constant reached at the same PFSI concentration in the membrane forming mixture (0.2%). The relatively high standard deviations of the apparent Michaelis constant for not-optimal casting mixtures can be caused by inhomogeneity of the resulting membranes. Accordingly, this particular PFSI content (0.2%), which allows to achieve the highest activity of the immobilized enzyme together with its highest affinity to substrate (lactate), has been used in further study.

Operational stability of lactate biosensors has been evaluated as the number of lactate injections (0.2 mM) in flow-injection mode remaining the response at the level of 95% from its initial value. The highest operational stability has been registered for biosensors made from the mixture of 1.5% γ-aminopropyltriethoxysilane and 0.2% PFSI, which corresponds to the extremes in Fig. 2 considering non-zero PFSI concentrations. Operational stability of the corresponding biosensor in terms of the number of lactate injections remaining 95% activity (81 ± 4 injections) is almost 3 times higher as compared to the biosensor made without perfluorosulfonated ionomer (28 ± 3 injections). Accordingly, the membrane forming mixture containing 0.2% PFSI results in the biosensors, which obey the requirement for application in clinical analyzers. At the same time the decrease in sensitivity compared to the biosensors made without PFSI is only 35%.

Hence, an addition of 0.2% of PFSI to the membrane-forming mixture provides the significantly improved stability of the resulting siloxane based enzyme containing membranes. Thus improved protocol for lactate oxidase immobilization has been used for elaboration of the nanozyme-enzyme biosensor.

3.3. Lactate biosensor powered by nanozymes "artificial peroxidase"

The improved sensitivity of the nanozyme based hydrogen peroxide transducer would obviously provide the improved analytical performance characteristics of the resulting biosensors.

Fig. 3 presents calibration graph for the nanozymes based lactate biosensor in batch mode. As seen, the calibration range is prolonged over 3 orders of magnitude of lactate concentrations. Sensitivity evaluated as the slope of calibration graph at low analyte concentrations is of 210 ± 20 mA M^{-1} cm^{-2}. For comparison, the sensitivity of the Prussian Blue film based biosensor with the same lactate oxidase containing membrane is almost twice lower (see Fig. 2). Hence, the nanozymes "artificial peroxidase" indeed are able to provide a significant improvement in sensitivity of the corresponding biosensors.

Calibration graphs in Fig. 3 are recorded at a solution pH (6.0) seems to be optimal for lactate oxidase. In the range from pH 6.0 to pH 7.0 the biosensor sensitivity is pH independent (Fig. S3, Supporting Information). Despite the sensitivity in pH 7.4 is 4–5 times lower, we note that blood samples taken for analysis are conventionally diluted 50 times, which makes it simple to adjust any required pH value.

For validation of the lactate biosensor the human control serum for clinical chemistry assays (Spinreact, Spain) has been used. As a reference analytical method the clinical analyzer Eco-Basic (Germany) has been chosen. The results for the normal serum are 1.5 ± 0.1 mM and 1.5 ± 0.1 mM for the proposed biosensor based assay and for Eco-Basic, respectively. The results are in an excellent accordance and match the passports value: 1.6 ± 0.3 mM. Hence, the proposed biosensor is valid for analysis of real samples.

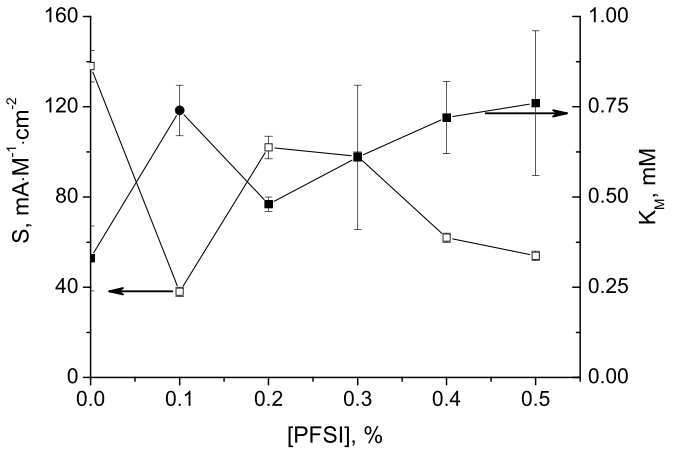

Fig. 2. Sensitivity (□) and an apparent Michaelis constant (■) of Prussian Blue film based lactate biosensors as functions of the perfluorosulfonated ionomer content in the membrane-forming mixture.

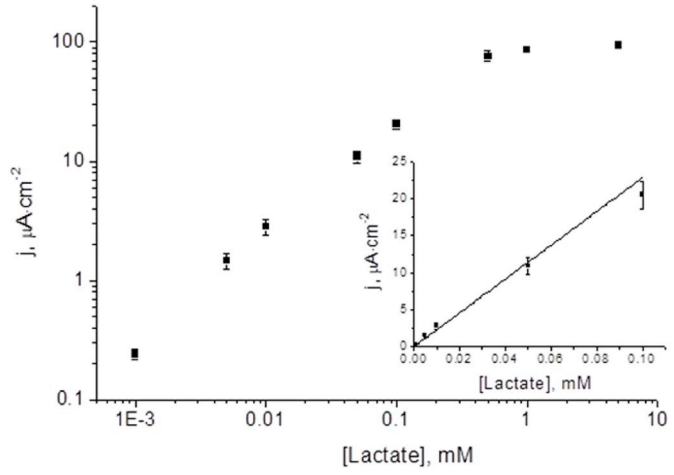

Fig. 3. Calibration graph for nanozymes based lactate biosensor, phosphate buffer pH 6.0, E = 0.00 V Ag|AgCl; error bars represent the deviation of responses for three different biosensors.

4. Conclusions

The term ‘nanozyme’ describing nanoparticles with enzyme-like activity is apparently less than 10 years old [16]. It represents the rapidly growing area with variety of applications including chemical analysis. We, however, note that the use of enzymes for analysis of biological liquids is commonly based on their high specificity. Such specificity towards organic substrate is hardly expectable for nanozymes. On the other hand, the enzymes applied as labels or as transducers have to be highly active and specific only towards the oxidizer, and peroxidase is the most valuable example. Hence, the nanozymes “artificial peroxidase” are, most probably, the only nanozymes suitable and highly required for both oxidase-based biosensors and immuno-(DNA) sensors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.120393.

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