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Novel Electrochemical Lactate Biosensors Based on Prussian Blue Nanoparticles †

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Abstract: We report on the novel electrochemical lactate biosensors based on Prussian blue nanoparticles. The immobilization of lactate oxidase was performed through drop-casting on the sensor surface of mixture containing enzyme, (3-aminopropyl)triethoxysilane and isopropyl alcohol. The apparent Michaelis constant and inactivation constant were determined (0.29±0.03 mM and 0.042±0.002 min^{-1}, respectively) and compared with values obtained for biosensors based on Prussian blue films. The developed lactate biosensors are not inferior in characteristics to those previously known, while the manufacturing process is less laborious. Obtained values also indicate that lactate biosensors based on Prussian blue nanoparticles and lactate oxidase have sufficient sensitivity and operational stability for analytical application in medical and biological researches.

Keywords: biosensors; nanoparticles; Prussian blue; lactate

1. Introduction

Prussian blue is a well-known compound in electrochemical catalysis. It is successfully applied in sensing of hydrogen peroxide [1], amino acids [2] and DNA [3]. Prussian blue nanoparticles are often referred to as "artificial peroxidase" because they have better catalytical properties and operational stability than natural peroxidase enzymes [4]. Artificial peroxidase can be used in oxidase-based biosensors, which are based on oxidation of the substrate accompanied by forming of hydrogen peroxide as a product. Then hydrogen peroxide is reduced/oxidized on an electrode, generating electric current. Prussian blue can catalyze the reduction of hydrogen peroxide, thus decreasing potential of the electrochemical reaction and enhancing selectivity of biosensors. Prussian blue was also used in designing of the glucose biosensors suitable for non-invasive real-time glucose detection in human sweat [5].

Lactate is an important intermediate of many biochemical processes. Accumulation of lactate in human body can be caused by cancer [6], multiple sclerosis [7] and brain injuries [8], so detection of lactate in biological fluids is useful for biological and medical purposes. Lactate oxidase catalyzes oxidation of lactate to pyruvate with forming of hydrogen peroxide, which can be electrochemically detected [9]. Immobilization of the enzyme on a surface of electrode is commonly performed using polymers with ionic-exchanging properties. (3-aminopropyl)triethoxysilane is one of the monomers used for enzyme immobilization. Polycondensation of this compound leads to formation of stable (3-aminopropyl)triethoxysiloxane membrane [10]. Other electrochemical biosensors can be based on different designs, such as using lactate dehydrogenase instead of lactate oxidase, immobilization of the enzyme with another polymer and application of various transducers. Some of the biosensors sensitive to lactate are listed in Table 1.

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Table 1. Examples of described biosensors for lactate detection. Lactate oxidase and lactate dehydrogenase are referred to as LOx and LDH, respectively.

Biosensor Type	Apparent Michaelis Constant	Source
LOx + cobalt phthalocyanine	0.88 ± 0.07 mM	[11]
LDH + cerium oxide nanoparticles	1.54 mM	[12]
LOx + peroxidase + phenazine methosulphate	1.2 mM	[13]
LOx + Prussian blue film	0.30 mM	[14]

Sensitivity of a biosensor depends on the overall rate of electrochemical and catalytic reactions. Hydrogen peroxide reduction and electron transfer rates are higher than enzyme-catalyzed reaction, so kinetics can be described with Michaelis-Menten equation [15]. Moreover, possibility of practical use of the biosensor is determined by its operational stability, limited by inactivation of the enzyme. High operational stability is a prerequisite for continuous real-time detection, which is needed for medical and industrial applications [16]. Inactivation is assumed to be a pseudo-first order reaction of the enzyme transforming from active to inactive form [17].

In this work apparent Michaelis constants and inactivation constants were evaluated for lactate biosensors based on Prussian blue and Prussian blue nanoparticles covered with stabilizing agent–nickel hexacyanoferrate.

2. Materials and Methods

K_2HPO_4 and KH_2PO_4 were purchased from Helicon (Russia). KCl and $NiCl_2{\cdot}6H_2O$ was obtained from Chimmed (Russia). $FeCl_3{\cdot}6H_2O$, $(NaPO_3)_6$ and $K_3[Fe(CN)_6]$ were obtained from Sigma Aldrich (USA). 30% H_2O_2 solution was purchased from Reachim (Russia). Isopropyl alcohol was obtained from Plastopolymer (Russia). Lactate oxidase enzyme (EC 1.1.3.2) from Pediococcus species (lyophilized powder, activity 72 IU) was purchased from Sorachim (Switzerland). 40% sodium L-lactic acid solution in water was obtained from Sigma Aldrich (USA).

The biosensors were produced using planar screen-printed three-electrode structures with carbon working electrode with diameter 1.8 mm, carbon auxiliary electrode and silver reference electrode. These screen-printed electrodes were obtained from Rusens (Russia).

Electrochemical measurements were conducted with potentiostat Palmsens3 (Palm Instruments BV, The Netherlands). Dynamic light scattering particle size distribution analysis was conducted with Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., UK). Spectrophotometrical measurements were conducted with Lambda 950 (Perkin-Elmer, USA).

2.1. Synthesis of Prussian Blue Nanoparticles

Synthesis was carried out as described in the work [4]. The reaction mixture consisted of 75 mM $K_3[Fe(CN)_6]$ and 75 mM $FeCl_3$ in a solution of 100 mM KCl and 100 mM HCl. Sedimentation was initiated with addition of 50 mM H_2O_2 solution. Mixing and dispersing of the mixture was conducted using ultrasound, followed by centrifugation and separation of the sediment. Sizes of nanoparticles were measured with dynamic light scattering method (refraction index 1.56 and absorption coefficient 0.9. Nanoparticles were stored in a solution of 100 mM KCl and 100 mM HCl. Nanoparticles concentration in a suspension was measured using spectrophotometry, ε_{700} nm (per PB unit cell) = $4.85{\cdot}10^4$ $M^{-1}{\cdot}cm^{-1}$.

2.2. Synthesis of Prussian Blue Nanoparticles with Nickel Hexacyanoferrate Shell

Synthesis of stabilizing shell, as described in the paper [18], was carried out in the mixture of 5–10 nM Prussian blue nanoparticles, 0.3–2.1 mM $K_3[Fe(CN)_6]$ and 0.5–3.5 mM

$NiCl_2 \cdot 6H_2O$ in a solution of 100 mM KCl and 100 mM HCl under ultrasonication. Sediment was separated; core-shell nanoparticles were redispersed in a solution of 7 mM $(NaPO_3)_6$, 0.5 M KCl and 0.1 M HCl. Further operations are identical to the synthesis of nanoparticles without the coating.

2.3. Preparation of Lactate Biosensors

Screen-printed electrodes were modified with Prussian blue by drop-casting of 2 μL of 6.5 nM nanoparticles suspension on working surface. Electrodes were air-dried and annealed at temperature 100 °C for 1 h. Nanoparticles layer was activated with cyclic voltammetry in potential range from -0.05 V to 0.35 V vs. Ag/AgCl. 2 μL of 1.0 mg·ml^{-1} lactate oxidase and 1.5% vol. (3-aminopropyl)triethoxysilane solution in isopropyl alcohol was dipped on working surface of the modified electrodes. After this procedure biosensors were air-dried and kept at 4 °C in sealed packaging.

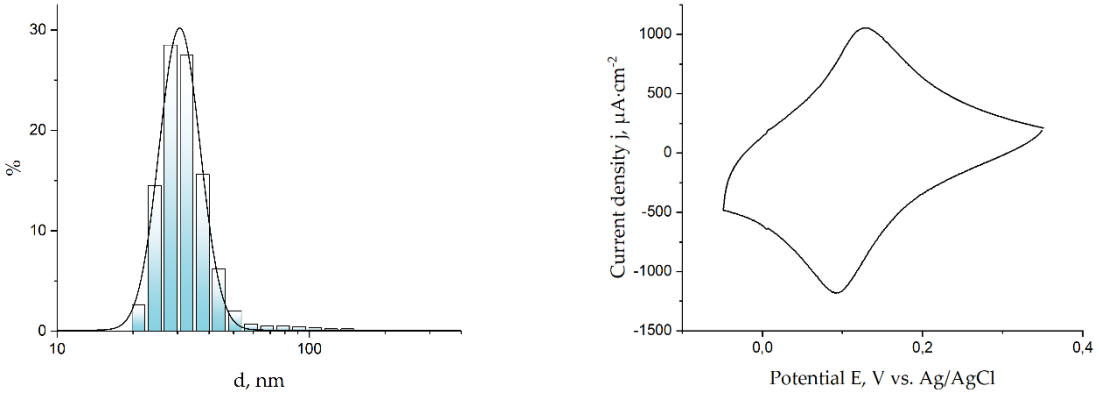
2.4. Electrochemical Detection of Lactate

All electrochemical experiments were conducted in chronoamperometry mode with stirring in a beaker at 25 ± 2 °C. Biosensors were tested in 0.05 M phosphate buffer and 0.1 M KCl solution at potential 0.0 V vs. Ag/AgCl.

3. Results and Discussion

3.1. Preparation of Lactate Biosensors

Prussian Blue nanoparticles were prepared using reduction of $K_3[Fe(CN)_6]$ and $FeCl_3$ mixture with hydrogen peroxide under ultrasonication. The size distribution of the nanoparticles was measured with the dynamic light scattering. For further steps nanoparticles with average diameter 35 nm (Figure 1a) were used, because it is the range in which the electrocatalyst is distributed over the entire surface of the working electrode, accordingly to [19]. Suspension of the nanoparticles was dropped to a surface of screen-printed electrode, then cyclic voltammogram was registered to activate the coating. Cyclic voltammogram (Figure 1b) of electrode modified with nanoparticles exhibits pair of peaks corresponding to the transition from Prussian blue to its reduced form, Prussian White, and vice versa. According to [1], it is needed for the activation of electroactive coating through intercalation of K^+ cations in Prussian blue layer. The voltammogram can also be used for a calculation of Prussian blue concentration on the surface of biosensor and, consequently, control of the amount of Prussian blue deposed on the working electrode. According to the voltammogram, average amount of deposed Prussian blue is 14 nmol·cm^{-2}



(**a**) (**b**)

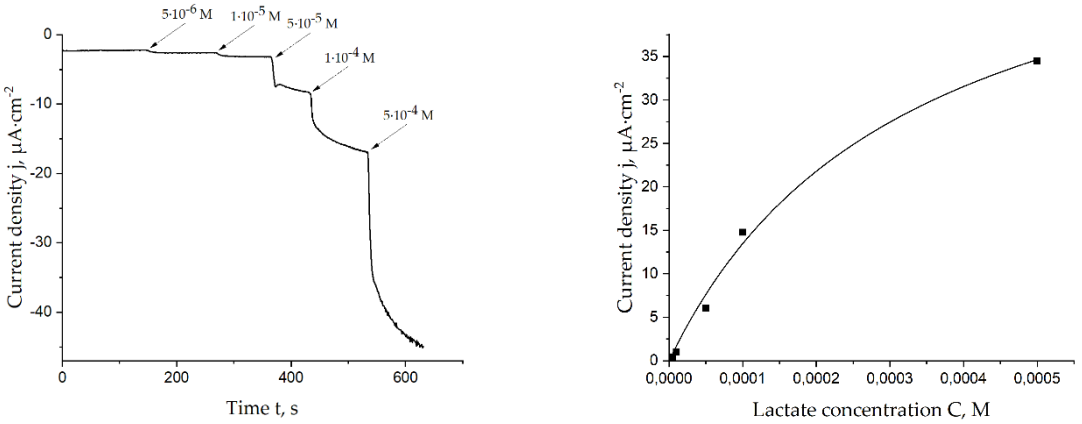
Figure 1. (**a**) Distribution of diameters of Prussian blue nanoparticles in semilogarithmic coordinates; (**b**) Cyclic voltammogram of Prussian blue nanoparticles modified sensor in 0.1 M KCl and 0.1 M HCl solution, 40 mV/s.

Immobilization of lactate oxidase was conducted through deposition of mixture containing enzyme, (3-aminopropyl)triethoxysilane and isopropyl alcohol on the sensor surface. Thus, after formation of (3-aminopropyl)triethoxysiloxane membrane biosensors were ready for electrochemical detection of lactate in chronoamperometric mode.

3.2. Determination of Apparent Michaelis Constant

Apparent Michaelis constant is an important parameter of biosensors, which makes it possible to compare catalytical properties of immobilized enzyme and, consequently, analytical characteristics of biosensors. Its value depends on Michaelis constant of the used enzyme, accessibility of the enzyme in the membrane and effects of transducer, Prussian blue nanoparticles. Thus, calculation of apparent Michaelis constants is useful for the development of biosensors of a certain design.

Different concentrations of lactate were added to the cell with lactate biosensor in a batch mode. Figure 2a demonstrates one of the chronoamperograms which was registered during this procedure. Obtained data was used to plot Michaelis-Menten curves, which was used for apparent Michaelis constants calculation. Corresponding curve is shown in Figure 2b. As seen, linear range of lactate detection can be estimated as $5 \cdot 10^{-6}$–$1 \cdot 10^{-4}$ M; sensitivity in this range is 134 ± 12 mA·cm^{-2}·M^{-1}.



(**a**) (**b**)

Figure 2. (**a**) Chronoamperogram registered on the lactate biosensor during additions of lactate in a 0.05 M PBS, 0.1 M KCl solution, pH = 6.0, E = 0.0 V vs. Ag/AgCl; (**b**) Calibration curve of the lactate biosensor, which was used for calculation of the apparent Michaelis constant.

Mean apparent Michaelis constant is 0.29 ± 0.03 mM. It is equal to constant of Prussian blue film-based biosensors and less than constants of previously known lactate biosensors, which are presented in Table 1. However, this parameter can be optimized by testing other membranes, buffer systems and shapes of Prussian blue nanoparticles.

Thus, manufactured biosensors are not inferior in terms of sensitivity to other lactate biosensors, including Prussian blue film-based ones. Similar values of the apparent Michaelis constants are significant for the simplified approaches of the development of the Prussian blue-based biosensors. Mass production of Prussian blue biosensors is easier with the use of nanoparticles because of the opportunity to prepare large amounts of nanoparticles suspension and apply them by automatic methods.

3.3. Determination of Inactivation Constant

Inactivation constant is important for practical use of the biosensors in on-line chemical analysis. Calculation of this parameter makes it possible to determine if the biosensor has sufficient operational stability for continuous detection of chemical substance, or if further improvement of the sensor design is required.

Stability of the lactate biosensors was evaluated using inactivation constant. Calculation of this parameter was based on the assumption that the inactivation is a pseudo-first order reaction. Use of the semi-logarithmic chronoamperograms registered during 1.0 mM lactate solution addition (Figure 3) makes it possible to calculate inactivation constant as slope of the inactivation curve.

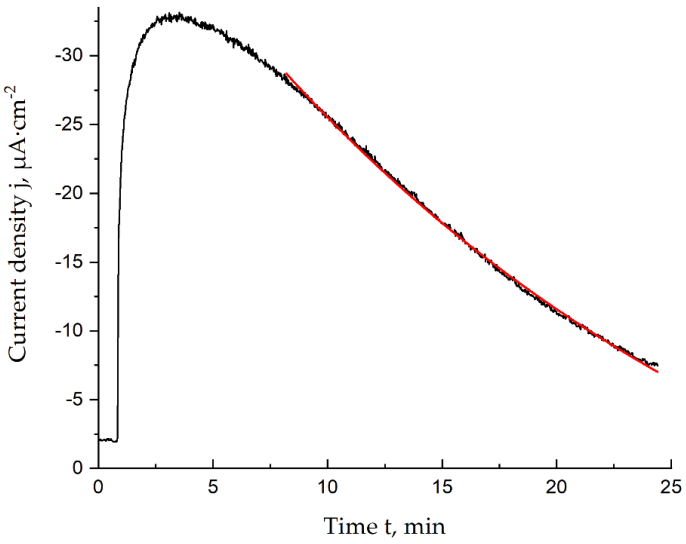


Figure 3. Chronoamperogram (black) and approximation result (red) in the time range corresponding to the inactivation process, 1.0 mM lactate, 0.05 M PBS and 0.1 M KCl, pH = 6.0, E = 0.0 V vs. Ag/AgCl.

Thus, calculated inactivation constant for lactate biosensors modified with Prussian blue nanoparticles was found to be 0.042 ± 0.002 min^{-1}. Operational stability can also be evaluated from the response decrease curve: biosensors retain 90% of their initial response for 6±1 min. This operational stability is relatively small, but it can be enhanced with synthesis of coating on Prussian blue nanoparticles.

Use of Prussian blue nanoparticles coated with nickel hexacyanoferrate for lactate biosensors allowed to achieve 2–10-fold lower values of inactivation constants. This fact indicates that low stability of biosensors based on Prussian blue nanoparticles is due to the transducer degradation rather than enzyme inactivation. As shown in Table 2, inactivation constants for biosensors based on core-shell nanozymes are dependent on size and amount of stabilizing coating. Therefore, operational stability is also better for lactate biosensors with higher amount of stabilizing agent (nickel hexacyanoferrate). It should be noted that biosensors with stabilized nanoparticles have the same linear range as those with Prussian blue nanoparticles.

Table 2. Inactivation constants for biosensors based on core-shell technology.

$K_3[Fe(CN)_6]$ Concentration Used in Synthesis of Shell, mM	Nanoparticle Diameter, nm	Inactivation Constant, min^{-1}
0.3	36	0.0292 ± 0.0003
0.6	47	0.0108 ± 0.0006
0.9	65	0.0047 ± 0.0004
1.2	94	0.0024 ± 0.0004
1.5	122	0.0021 ± 0.0004

2.1	162	0.0017 ± 0.0003

Obtained values indicate that inactivation can be reduced by the use of this type of core-shell nanozymes., but synthetic parameters need optimization to maximize stability of the biosensors.

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References

1. Karyakin, A.A. Prussian blue and its analogues: Electrochemistry and analytical applications. *Electroanal. : Int. J. Devoted Fundam. Pract. Asp. Electroanal.* **2001**, *13*, 813–819.
2. Suprun, E.V.; Karpova, E.V.; Radko, S.P.; Karyakin, A.A. Advanced electrochemical detection of amino acids and proteins through flow injection analysis and catalytic oxidation on Prussian Blue. *Electrochim. Acta* **2020**, *331*, 135289.
3. Suprun, E.V.; Karpova, E.V.; Khmeleva, S.A.; Radko, S.P.; Karyakin, A.A. Advanced electrochemical detection of nitrogenous bases, synthetic oligonucleotides, and single-stranded DNA through flow injection analysis and catalytic oxidation on Prussian Blue. *Electrochim. Acta* **2021**, *378*, 138119.
4. Komkova, M.A.; Karyakina, E.E.; Karyakin, A.A. Catalytically synthesized prussian blue nanoparticles defeating natural enzyme peroxidase. *J. Am. Chem. Soc.* **2018**, *140*, 11302–11307.
5. Karpova, E.V.; Shcherbacheva, E.V.; Galushin, A.A.; Vokhmyanina, D.V.; Karyakina, E.E.; Karyakin, A.A. Noninvasive diabetes monitoring through continuous analysis of sweat using flow-through glucose biosensor. *Anal. Chem.* **2019**, *91*, 3778–3783.
6. Hirschhaeuser, F.; Sattler, U.G.; Mueller-Klieser, W. Lactate: A metabolic key player in cancer. *Cancer Res.* **2011**, *71*, 6921–6925.
7. Amorini, A.M.; Nociti, V.; Petzold, A.; Gasperini, C.; Quartuccio, E.; Lazzarino, G.; Tavazzi, B.; Di Pietro, V.; Belli, A.; Signoretti, S.; et al. Serum lactate as a novel potential biomarker in multiple sclerosis. *Biochim. Et Biophys. Acta (BBA)-Mol. Basis Dis.* **2014**, *1842*, 1137–1143.
8. Kawamata, T.; Katayama, Y.; Hovda, D.A.; Yoshino, A.; Becker, D.P. Lactate accumulation following concussive brain injury: The role of ionic fluxes induced by excitatory amino acids. *Brain Res.* **1995**, *674*, 196–204.
9. Rathee, K.; Dhull, V.; Dhull, R.; Singh, S. Biosensors based on electrochemical lactate detection: A comprehensive review. *Biochem. Biophys. Rep.* **2016**, *5*, 35–54.
10. Vashist, S.K.; Lam, E.; Hrapovic, S.; Male, K.B.; Luong, J.H. Immobilization of antibodies and enzymes on 3-aminopropyltriethoxysilane-functionalized bioanalytical platforms for biosensors and diagnostics. *Chem. Rev.* **2014**, *114*, 11083–11130.
11. Iwuoha, E.I.; Rock, A.; Smyth, M.R. Amperometric l-lactate biosensors: 1. Lactic acid sensing electrode containing lactate oxidase in a composite poly-l-lysine matrix. *Electroanal. Int. J. Devoted Fundam. Pract. Asp. Electroanal.* **1999**, *11*, 367–373.
12. Nesakumar, N.; Sethuraman, S.; Krishnan, U.M.; Rayappan, J.B.B. Fabrication of lactate biosensor based on lactate dehydrogenase immobilized on cerium oxide nanoparticles. *J. Colloid Interface Sci.* **2013**, *410*, 158–164.
13. Liu, H.; Zhanen, Z.; Xiaolin, Z.; Deyao, Q.; Liu, Y.; Yu, T.; Deng, J. A phenazine methosulphate-mediated sensor sensitive to lactate based on entrapment of lactate oxidase and horseradish peroxidase in composite membrane of poly (vinyl alcohol) and regenerated silk fibroin. *Electrochim. Acta* **1997**, *42*, 349–355.
14. Vokhmyanina, D.V.; Andreeva, K.D.; Komkova, M.A.; Karyakina, E.E.; Karyakin, A.A. 'Artificial peroxidase'nanozyme–enzyme based lactate biosensor. *Talanta* **2020**, *208*, 120393.
15. Kirthiga, O.M.; Rajendran, L. Approximate analytical solution for non-linear reaction diffusion equations in a mono-enzymatic biosensor involving Michaelis–Menten kinetics. *J. Electroanal. Chem.* **2015**, *751*, 119–127.
16. Gouda, M.D.; Kumar, M.A.; Thakur, M.S.; Karanth, N.G. Enhancement of operational stability of an enzyme biosensor for glucose and sucrose using protein based stabilizing agents. *Biosens. Bioelectron.* **2002**, *17*, 503–507.
17. Baynton, K.J.; Bewtra, J.K.; Biswas, N.; Taylor, K.E. Inactivation of horseradish peroxidase by phenol and hydrogen peroxide: A kinetic investigation. *Biochim. Et Biophys. Acta (BBA)-Protein Struct. Mol. Enzymol.* **1994**, *1206*, 272–278.
18. Karpova, E.V.; Shcherbacheva, E.V.; Komkova, M.A.; Eliseev, A.A.; Karyakin, A.A. Core–Shell Nanozymes "Artificial Peroxidase": Stability with Superior Catalytic Properties. *J. Phys. Chem. Lett.* **2021**, *12*, 5547–5551.

19. Daboss, E.V.; Shcherbacheva, E.V.; Karyakin, A.A. Simultaneous Noninvasive Monitoring of Diabetes and Hypoxia Using Core-Shell Nanozyme–Oxidase Enzyme Biosensors. *Sens. Actuators B Chem.* **2023**, 133337.