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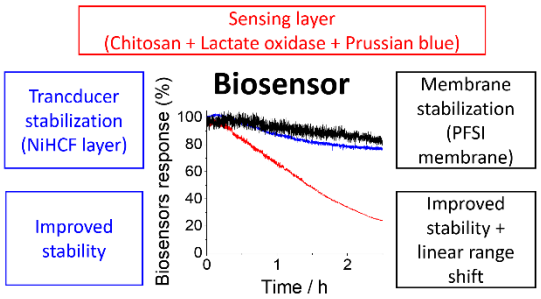
# Stabilized chitosan-based biosensor with a tunable linear range for lactate determination in blood and undiluted sweat

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**In this study the stability of chitosan-based enzymatic biosensors which is limited by the electrocatalyst (Prussian blue) degradation, rather than enzyme inactivation, was improved with additional perfluorosulfonated ionomer (PFSI) or nickel hexacyanoferrate films. In neutral media, up to 6.5 h sensor signal stability was achieved. In addition to stabilization, PFSI coatings shift the linear range of biosensor from micromolar to millimolar lactate concentrations. Enhanced stability, and tunable detection range enables detection of lactate in blood serum and sweat.**




*Keywords: lactate, enzyme, chitosan, biosensor, amperometry, flow injection, sweat analysis, linear range, Prussian blue.*

Lactate detection is an important task in biomedical research. Lactate is a marker for various diseases, including type 2 diabetes and obesity. It is also used in sports medicine as index of exercise intensity or clinical hyperlactatemia[1]. It can be detected in various biological fluids, such as blood and sweat. Biosensors based on lactate oxidase enzyme (LOx) are widely used for this task because of their exceptional selectivity[2-5].

Chitosan is prominent biologically derived material for enzyme-based biosensors because of its biocompatibility, solubility in water, safety and low cost[6]. It was successfully applied for glucose sensors, and it was noted that chitosan membrane provides enzyme stability in biological fluids (e.g. blood serum)[7]. Water solubility is important for organic solvents-free enzyme immobilization and biodegradability allows lesser ecological effect from discarding of biosensors, thus applying principles of green analytical chemistry[8]. It was shown that chitosan membranes can be used to immobilize lactate oxidase for electrochemical biosensors preparation[9]. Chitosan-based lactate biosensors can be used for biological fluids, such as saliva and blood serum, and it is important to investigate possibilities of linear range adjustment[10]. It should be noted that in literature there is no information on chitosan concentration dependence of analytical performance of lactate biosensors. It means that sensitivity as well as stability of chitosan-based lactate biosensors, can be enhanced.

Another important component of first-generation lactate biosensor is an electrocatalyst. Prussian blue (PB) is an electrocatalyst widely used in hydrogen peroxide sensors[11]. $H_2O_2$ is produced during enzymatic oxidation of lactate, so Prussian blue generates amperometric response from electrochemical reduction of hydrogen peroxide. Prussian blue-chitosan composites are applied in enzymatic electrochemical biosensors for glucose and lactate detection[9,12], but they have insufficient stability. Enhancement of stability is important for continuous monitoring and noninvasive diagnostics. Furthermore, in various biological fluids concentrations of lactate are different. Thus, in blood it is approximately 1 mM, but in sweat it is about 10 mM or higher[13]. It means that there is need for lactate biosensors with various linear ranges, which can be used for different biofluids.

Hydrogen peroxide sensors were manufactured using interfacial synthesis of Prussian blue, which included casting a droplet mixture of $K_3[Fe(CN)_6]$ and $FeCl_3$ in 0.1 M KCl, 0.1 M HCl with further reduction of $FeFe(CN)_6$ formed with $H_2O_2$. Lactate biosensors were made with drop casting lactate oxidase (1.5 mg/mL) dissolved in an aqueous solution of chitosan (0.01-0.3 wt %) on PB-modified electrode.

Consequently, the analytical performance of biosensors with different mass fractions of chitosan was compared using flow-injection analysis (FIA), which is preferable for multiple samples analysis[14]. In this mode biosensor response appears as a distinct peak (Figure 1 Inset). The best sensitivity, 235±28 $mA \cdot M^{-1} \cdot cm^{-2}$ (n = 3), was achieved with a chitosan content of 0.05%, within the linear range from $1 \cdot 10^{-6}$ to $1 \cdot 10^{-4}$ M, LOD is $5 \cdot 10^{-7}$ (Figure S1).

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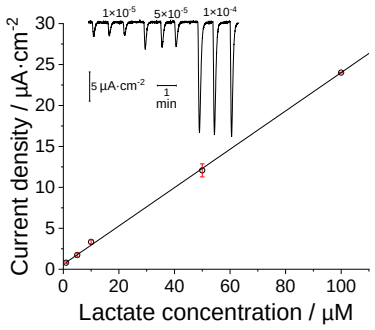



**Figure 1** Calibration graph of lactate biosensor based on Prussian blue and lactate oxidase immobilized in 0.05% chitosan aqueous solution, flow injection mode, 0.05 M PBS, 0.1 M KCl, pH 6.0, E=0.0 V Ag/AgCl. Inset: corresponding chronoamperogram of lactate biosensor.

For biosensors, stability may depend on the transducer (PB) or membrane degradation, and enzyme inactivation. Effect of chitosan concentration in the membrane on the degradation of biosensors was investigated. Stability was estimated as the time of maintaining of 95% of the initial biosensor response. The obtained dependence is not trivial. In the range of chitosan concentrations from 0.01 to 0.3% stability of biosensors is a curve with minimum at 0.05 - 0.1%. In order to check if the observed destabilizing and stabilizing effects of chitosan refers to transducer or enzyme the same experiments were carried out with enzyme-free sensors. Degradation rate of the electrocatalyst was tested in $H_2O_2$ solution using PB-based sensors covered with membranes with the same chitosan content. It was shown that stability profiles of biosensors and hydrogen peroxide sensors on chitosan concentration are similar (Figure 2). Increase of chitosan concentration from 0.01 to 0.1% leads to decrease of stability, but after that stability increases up to original value at 0.3%. Therefore, it may be assumed that total stability of the lactate biosensor is limited with the degradation of electrocatalyst, rather than enzyme inactivation.

Two approaches were employed to stabilize the biosensor, including modification of PB with analogous compounds (e.g. nickel hexacyanoferrate[15]) or application of additional polymer membrane – perfluorosulfonated ionomer (e.g. Nafion)[16].

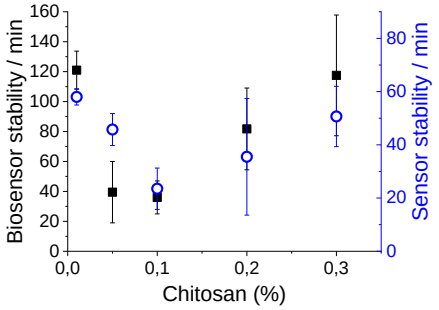



**Figure 2** Dependencies of 95% chronoamperometric signal stability period durations in 1 mM lactate solution (■) of biosensor based on Prussian blue and lactate oxidase in chitosan membrane and in 0.1 mM $H_2O_2$ solution of sensor based on Prussian blue and chitosan membrane (○) on chitosan concentration in membrane-forming mixture, batch mode upon stirring, 0.05 M PBS, 0.1 M KCl, 0.0 V Ag/AgCl.

Prussian blue is notably unstable in non-acidic pH solutions[17], but it can be coated with stabilizing layer of nickel hexacyanoferrate (Ni-HCF)[16]. Ni-HCF-stabilized sensors were prepared using interfacial synthesis of nickel hexacyanoferrate over PB layer from mixture of $K_3[Fe(CN)_6]$ and $NiCl_2$ in 0.1 M KCl, 0.1 M HCl. This method increased stability of chitosan-based lactate biosensors from 40 ± 6 (n = 4) up to 260 ± 28 (n = 3) min, though sensitivity decreased down to 85 ± 7 $mA \cdot M^{-1} \cdot cm^{-2}$.

Application of additional membrane of perfluorosulfonated ionomer (PFSI) is widely used in electrochemical biosensors[18]. In contrast to chitosan, PFSI is negatively charged in neutral media, and is known to stabilize Prussian blue[19]. Moreover, this polymer is less soluble in water and may impact in stabilization of the chitosan membrane. For PFSI-stabilized lactate biosensors preparation isopropanol droplet containing perfluorosulfonated ionomer (PFSI) was applied to chitosan-based biosensor. In Figure S2, profiles of sensitivity loss are similar for both mentioned techniques if PFSI 0.02% is used. Working time of biosensor, corresponding to the response stability within 95% of its initial value, is 2-3-fold improved.

The stability of the resulting biosensors depended on the PFSI fraction in the mixture (Figure S3), though sensitivity decreased as the PFSI fraction increased (Figure S4). Maximal stability of 9 ± 0.2 h (n = 3) was achieved using 5% PFSI solution.

For analysis of undiluted samples linear range of a biosensor should encompass both normal and pathological metabolite content in this specific fluid. Notably, the additional PFSI membrane shifts the linear range toward higher concentrations. This shift also depends on PFSI fraction. Biosensors covered with 5% PFSI can be used for detection of 3 orders of magnitude higher concentrations of lactate ($1 \cdot 10^{-4}$ to $1 \cdot 10^{-1}$ M, LOD is $5 \cdot 10^{-5}$). Therefore, the developed biosensor features a tunable linear range (Figure 3). This is likely to be due to the changes in the surface charge, which increases with PFSI content on the electrode surface. This assumption was confirmed by evaluation of ion exchange properties of the clean screen-printed carbon electrodes covered with the same membranes. In constant potential flow-injection amperometry, anodic or cathodic current was generated upon injection of KCl depending on the charge of polymer (Figure S5). Cathodic current peaks were observed for electrodes modified with chitosan only. Current sign changed from negative to positive, when 0.05% chitosan membrane was covered with 0.1% PFSI. This indicates that this amount of PFSI is enough for compensation of chitosan positive charge. Further increase of PFSI concentration resulted in gradual increase of anodic current due to higher charge density of polymer.

Therefore, an increase in the PFSI fraction imparts a negative surface charge that repels negative lactate ions, thereby decreasing sensitivity and shifting the linear range to higher lactate concentrations. Given that average lactate concentrations are 1 mM in blood and 10 mM in sweat, a PFSI fraction of 4% in the membrane-forming mixture is optimal for effective analysis of these fluids.

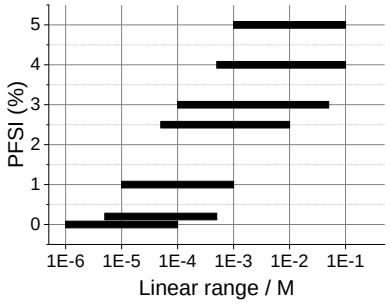



**Figure 3** Dependence of the linear range of lactate biosensors based on Prussian blue and lactate oxidase immobilized in 0.05% chitosan aqueous solution on proportion of PFSI in isopropanol solution deposited over

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chitosan membrane; flow injection mode, 0.05 M PBS, 0.1 M KCl, pH 6.0, E= 0.0 V vs. Ag/AgCl.

Fluid properties (pH, interferents, viscosity) can differ from model solutions. For instance, degradation of Prussian blue fastens in alkaline solutions, making these biosensors better suited for acidic environments (Figure S6). However, in neutral media (pH 6-7) the stability of biosensors covered with PFSI is constant (~ 6.5 ± 0.2 h, n = 3).

Validity of the proposed biosensors with 4% PFSI coating was confirmed using commercial blood serum samples with known lactate level (Figure S7), values are correlated with R = 0.9811 and regression slope 0.8 ± 0.1. It was shown in previous studies that blood and sweat lactate concentrations are correlated[20], so detection of lactate in sweat has value for noninvasive diagnostics and continuous monitoring. While FIA is effective, it is limited to laboratory settings and is unsuitable for wearable devices. The test strip method, based on addition and removal of analyte on biosensor surface with capillary effect, is more practical for such applications, providing rapid results. To prepare test strips, biosensors were covered with plastic except for a 1 cm wide strip, thus enabling capillary effect. Analytical signal recording takes 10 s without a complex system (Figure S8). This method requires small amount of the analyte, but exhibits lower sensitivity than FIA (Figure S9), specifically 5.3 ± 0.4 $\mu A \cdot M^{-1} \cdot cm^{-2}$ (n = 6). For sweat samples, results were compared against lactate biosensors based on siloxane validated earlier in[19], and the developed biosensors demonstrated sufficient accuracy with R = 0.9769 and regression slope 1.0 ± 0.1 (Figure 4).

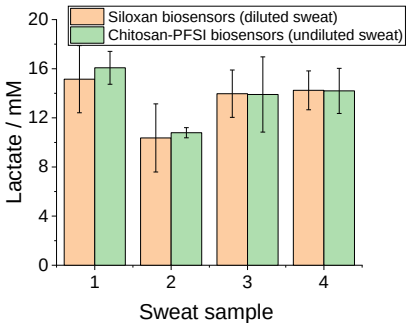



**Figure 4** Comparison of lactate detection in human sweat samples in capillary mode using biosensor based on Prussian blue, lactate oxidase immobilized in 0.05% chitosan water solution and 4% perfluorosulfonated ionomer isopropanol solution and in flow injection mode with 100 times dilution using biosensor based on Prussian blue and lactate oxidase immobilized in siloxane, 0.0 V Ag/AgCl.

Thus, electrochemical lactate biosensor with tunable linear range was developed. It was shown that it is suitable for biological fluids analysis, including flow injection analysis, batch mode with constant stirring and capillary mode. It opens the prospects for integration of the biosensor in wearable systems.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.71267/mencom.8150.

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