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Noninvasive Hypoxia Monitor Based on Gene-Free Engineering of Lactate Oxidase for Analysis of Undiluted Sweat

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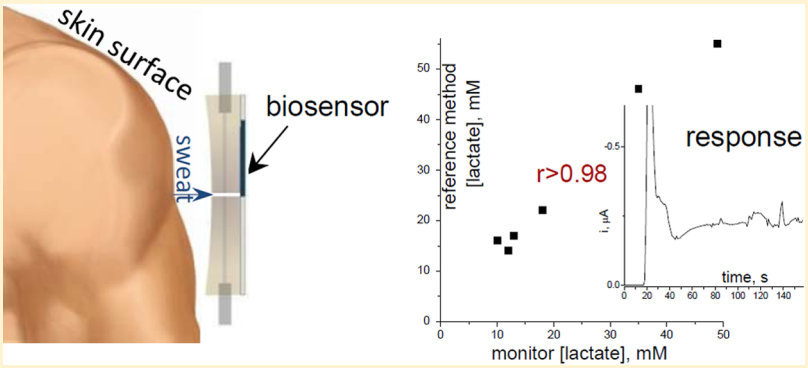
Supporting Information



ABSTRACT: We report on the Prussian Blue based lactate biosensor with the remarkably increased upper detection limit suitable for analysis of undiluted sweat. Engineering of the enzyme lactate oxidase has been carried out upon its immobilization from water−isopropanol mixtures with the high (90%) content of organic solvent. To decrease the enzyme binding constant, we propose to shield the substrate binding sites in its active center with negatively charged polyelectrolyte. The biosensor made from the optimal mixture (3% γ- aminopropyltriethoxysilane and 5% perfluorosulfonated ionomer) is characterized by the calibration graph, which even in batch mode is shifted for 2 orders of magnitude toward high analyte concentrations as compared to it of lactate sensitive electrode made without Nafion analogue. In flow-injection mode, the biosensor allows lactate detection up to 0.5 M. The biosensor displays stable response for 4 h of continuous operation. The achieved analytical performance characteristics allow the monitoring of lactate content in undiluted sweat. A successful validation of the elaborated flow-through monitor with the integrated biosensor opens new horizons for noninvasive diagnostics of hypoxia-related conditions.

Among the most important metabolites in clinical analysis is L-lactate. It is controlled in blood for patients with lactic acidosis,[1,2] in sport medicine for calculating lactate anaerobic thresholds.[3−5] Intensive physical activity can cause an increase in blood lactate concentrations up to 10−15 times.[3,6,7] However, collection of blood samples during sport training is not convenient and safe. That is why lactate monitoring is not involved in everyday sport training up until now.

Accordingly, an interest to low-invasive and particularly noninvasive lactate monitoring is increased. Among excreted liquids, sweat seems to be the most promising one. Sweat lactate is a function of eccrine gland energy metabolism; an increase in the exercise intensity causes increased production of sweat lactate.[8] Lactate in sweat was found to be effective noninvasive method for estimation of physical condition.[9] We also have recently reported on the correlation of lactate increase in sweat and blood for sportsmen before and after physical loading test.[10]

The main problem with analysis of undiluted sweat is high lactate concentration, which is on average 10 times higher compared to blood. Lactate content in sweat of a healthy human is in the range from 4 to 25 mM,[9−12] whereas after loading test it is increasing up to 50−80 mM.[9−11] Hence, the upper detection limit of lactate biosensors has to be at least 80−100 mM.

However, the upper detection limits for the first lactate-sensitive electrode,[13] old systems,[14−17] and for recently reported ones[18−21] are of 5−10 mM, which are 10−20 times lower than it required for analysis of the undiluted sweat (above). Even the "real-time noninvasive lactate monitoring" biosensor[22] with the upper detection limit of 20 mM is hardly suitable for this aim.

To increase the upper detection limit required for analysis of undiluted sweat, it is necessary to increase an apparent Michaelis constant of lactate oxidase. As known, two positively charged arginine (Arg) residues located in the active site of lactate oxidase, Arg181 and Arg268,[23−25] form a part of the substrate-binding site. Hence, by shielding positive charges in the enzyme active site, it is possible to decrease the affinity of lactate oxidase to its substrate.

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For coupling oxidase-catalyzed and electrochemical reactions, the most progressive way described already in the 70s, is the detection of hydrogen peroxide.[26] The most advantageous transducer for hydrogen peroxide is Prussian Blue.[27−30] Its electroactivity is known to be pH independent.[30] Compared with the most widely used platinum, Prussian Blue modified electrodes are (i) 3 orders of magnitude more active in H_2O_2 reduction and oxidation in neutral media and (ii) 3 orders of magnitude more selective for hydrogen peroxide reduction in the presence of oxygen.[28,30] On the basis of nanostructured Prussian Blue, an electrochemical sensor with the record performance characteristics has been elaborated.[31,32]

To obtain advanced membranes displaying high enzyme activity and stability, we proposed immobilization protocol involving exposing of proteins to water−organic mixtures with the high content of organic solvent.[33,34] This protocol elaborated for perfluorinated ionomer (Nafion or its analogue) membranes was successfully used for immobilization of lactate oxidase in siloxane gels resulting in advanced lactate biosensor.[19]

We report here on engineering of lactate oxidase affinity to its substrate by addition of negatively charged polyelectrolyte (Nafion analogue) in siloxane gel. The resulting Prussian Blue based biosensor allowing continuous lactate detection up to 0.07 M (up to 0.5 M in flow injection analysis (FIA) mode) was integrated in a flow-through sweat collector for successful monitoring of lactate content in whole sweat.

EXPERIMENTAL SECTION

Materials. Experiments were carried out with Millipore Milli-Q water. All inorganic salts, γ-aminopropyltriethoxysilane (γ-$NH_2PrSi(OEt)_3$), perfluorosulfonated ionomer MF4 SK (Nafion analogue), organic solvents, and hydrogen peroxide (30% solution) were obtained at the highest purity from Reachim (Moscow, Russia) and used as received. Sodium lactate, 40% solution, was purchased from ICN. Lactate oxidase (EC 1.1.3.2) from Pediococcus sp. (lyophilized powder, activity 72 IU) was purchased from Sorachim, Switzerland.

Instrumentation. Planar hydrogen peroxide sensors (Rusens, Russia) were made on the basis of three electrode screen printed structures. Working electrode (diameter 1.9 mm) was modified with Prussian Blue. The sensitivity of the sensors in batch mode was of 1 ± 0.1 A M^{-1} cm^{-2} (0.00 V, AgI AgCl). The electrochemical experiments were carried out using a PalmSens potentiostat interfaced to a PC. The flow-injection setup consisted of a Cole Parmer (Vernon Hills, IL) peristaltic pump (7519-10), homemade flow-through wall-jet cell with 0.5 mm nozzle, and a homemade injector. The flow rate used was of 0.8 mL min^{-1}.

Methods. Biosensors were made suspending lactate oxidase aqueous solution (10 mg/mL) in isopropanol containing γ-aminopropyltriethoxysilane (0.1−5%) and Nafion analogue (0.1−8.5%). The final concentration of the enzyme was 0.1 mg/mL, of water 10%. The resulting mixture (2 μL) was deposited with a syringe onto the top of the Prussian Blue modified working electrode covering its entire surface and dried at room temperature for 1 h.

To collect sweat sample, a skin spot was stimulated with 1% pilocarpine chloride (Ferein, Russia) electrophoresis (Potok-1, Russia). After stimulation, sweat was collected with a sweat collector (Macroduct) during 20−30 min. Sweat samples diluted 1000 times were analyzed using a FIA system equipped with lactate biosensor as reported in ref 19.

The principal scheme of the flow-through sweat monitor is shown in Figure 1. The bottom side (contacted with skin) of the base (1) is made similarly to the Macroduct sweat collector. The nozzle (2) of 0.7 mm in diameter serves for sweat delivery to the flat top side of the base (1). A double side adhesive (3) with the capillary formed 1.3 mm wide slot is glued on the top side of the base. The final layer of the monitor is the three electrode lactate biosensor (4) and polyethylenethereftalate covering (5) of the rest of the adhesive top surface (3).

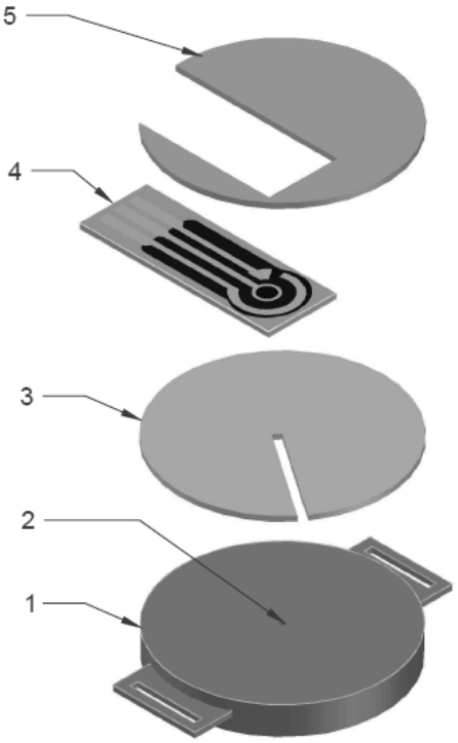

Figure 1. Principal scheme of the flow-through sweat monitor: (1) base, (2) nozzle, (3) double side adhesive, (4) three electrode lactate biosensor, (5) polyethylenethereftalate covering.

RESULTS AND DISCUSSION

Engineering of Immobilized Lactate Oxidase. Lactate oxidase containing membranes were formed onto the top of the Prussian Blue modified working electrodes by condensation of γ-aminopropyltriethoxysilane according to our previous study.[19] To decrease enzyme affinity to its substrate we propose to shield positively charged groups in its active site. Previously we have shown that negatively charged perfluorosulfonated ionomer (Nafion analogue) upon immobilization from water−organic media with the high content of organic solvent forms enzyme−polyelectrolyte complexes at the molecular level.[34]

Accordingly, enzyme containing membranes were formed by γ-aminopropyltriethoxysilane condensation in the presence of a certain content of perfluorosulfonated ionomer. The calibration graphs of the resulting biosensors were shifted toward a higher lactate concentration range. In order to characterize an ability of the biosensor to detect high analyte concentrations, it is convenient to use an apparent Michaelis constant of the enzyme electrode according to the equation:

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$$i = \frac{I[S]}{K_m + [S]}$$

where K_m is an apparent Michaelis constant, I is maximum current response, and [S] is analyte concentration.

As seen in Figure 2, the extremes in the apparent Michaelis constant value form an archipelago corresponding to the certain

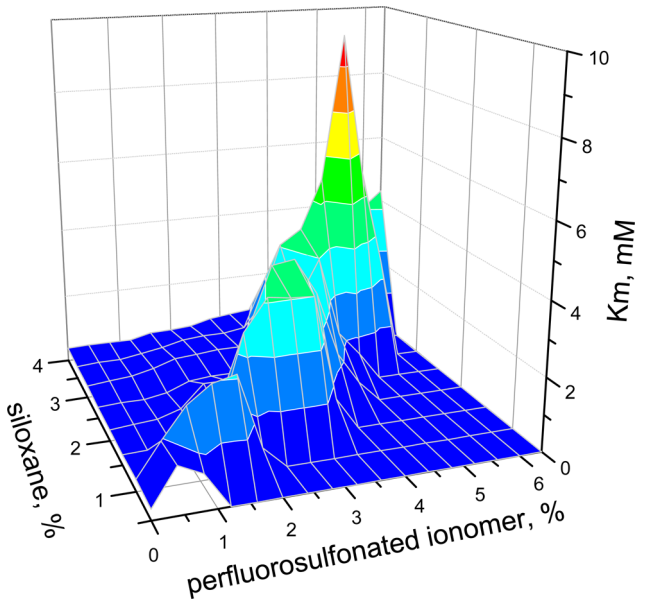

Figure 2. Apparent Michaelis constant of the lactate biosensor in batch mode upon stirring as a function of γ-aminopropyltriethoxysilane and perfluorosulfonated ionomer contents in the casting mixture: 0 mV vs Ag/AgCl; 0.05 M phosphate, pH 6.0 with 0.1 M KCl.

Nafion analogue-to-siloxane ratio of approximately 1.5. If lactate oxidase is immobilized only in siloxane gel, the K_m value is at the level of 0.4 ± 0.1 mM. With the use of perfluorosulfonated ionomer for immobilization, an apparent Michaelis constant is increased 25 times up to 10 ± 1 mM. The highest value of K_m corresponding to the lowest lactate oxidase affinity is reached at 3% of siloxane and 5% of perfluorosulfonated ionomer concentrations in the casting solution.

We note that despite lactate oxidase is unstable, the proposed immobilization protocol preserves its activity in a wide concentration range of membrane forming components. Moreover, the extremes in Figure 2 at similar ratios of independent variables point to formation of the enzyme–polymer complexes at the molecular level, similar to ref 33.

Figure 3 displays the calibration graph of the lactate biosensor made on the basis of the optimal membrane forming mixture (3% γ-aminopropyltriethoxysilane and 5% perfluorosulfonated ionomer) in batch mode. As seen, compared to the calibration plot of the lactate sensitive electrode has made in the absence of the Nafion analogue, the calibration graph of the resulting biosensor is shifted for 2 orders of magnitude toward high analyte concentrations. Accordingly, even in batch mode the upper limit of the calibration graph reaches 7×10^{-2} M, which makes possible analysis of undiluted sweat. The linear range of the calibration graph is prolonged over two orders of lactate concentrations: from 1×10^{-4} to 1×10^{-2} M. The sensitivity determined as a slope of the calibration graph at the lower concentration limit is of 1.2 mA M^{-1} cm^{-2}.

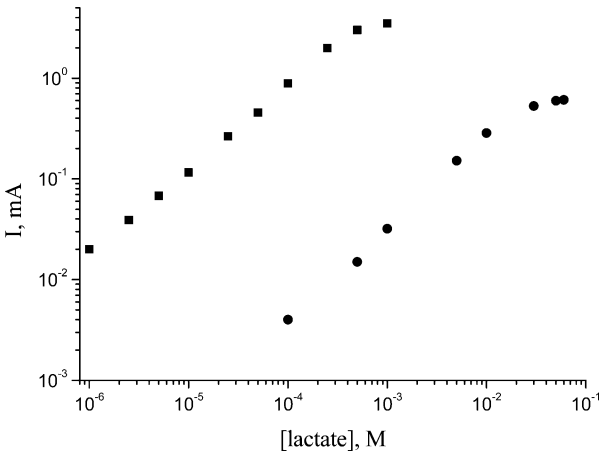

Figure 3. Calibration plots in batch mode for lactate biosensors made without (■) and with (●) perfluorosulfonated ionomer; 0.05 M phosphate buffer, pH 6.0 with 0.1 M KCl.

Since operation under high lactate concentrations can be limited by oxygen (its concentration is of 0.2 mM in air saturated buffer), it is interesting to investigate the biosensor performance in flow-injection mode. Indeed, despite the slightly decreased sensitivity (0.8 mA M^{-1} cm^{-2}), which is in accordance with the dispersion coefficient[35] of 1.5 for the flow-injection system used, the elaborated biosensor allows the detection up to 0.5 M (!) of lactate (data not shown). Hence, we are able to conclude that the proposed enzyme engineering approach results in the biosensor sensitive to extra-high analyte concentrations.

Operational stability of the elaborated lactate biosensor has been investigated in 2.5 mM of lactate in batch mode upon stirring. During the first 4 h of continuous monitoring, there is no decrease of the signal. Afterward, the sensor response decreases for 10% during each hour reaching after 9 h 50% value of the initial response (data not shown). The stability of the signal during the first 4 h of continuous operation proves the applicability of the elaborated biosensor for continuous monitoring.

Both high operational stability and the decreased affinity causing the shift of the calibration graph toward high lactate concentrations provide an ability of the resulting biosensor to analyze undiluted sweat.

Flow-Through Sweat Monitor. Prior to *in vivo* analysis, the flow-through sweat monitor with the integrated lactate biosensor has been calibrated. For this aim, a droplet of model solution was deposited with a syringe onto the sweat collecting part of the monitor. The model solution was sucked due to capillary forces and delivered to the biosensor surface. *In vivo* analysis was carried out applying the elaborated monitor on the arms or legs of healthy human volunteers before and after the loading test.

The current response of the flow-through sweat monitor attached to the leg of a human volunteer is displayed in the inset of Figure 4. In the absence of liquid, only zero current is observed. When all electrodes are connected by electrolyte, the current pulse (overscaling in the inset) required for working electrode polarization appears. After 20–25 s, the current tends to bring the monitor back in measurement-ready state.

Figure 4 presents the correlation between lactate concentration data recorded by the flow-through sweat monitor attached to legs or arms of human volunteers and the results of

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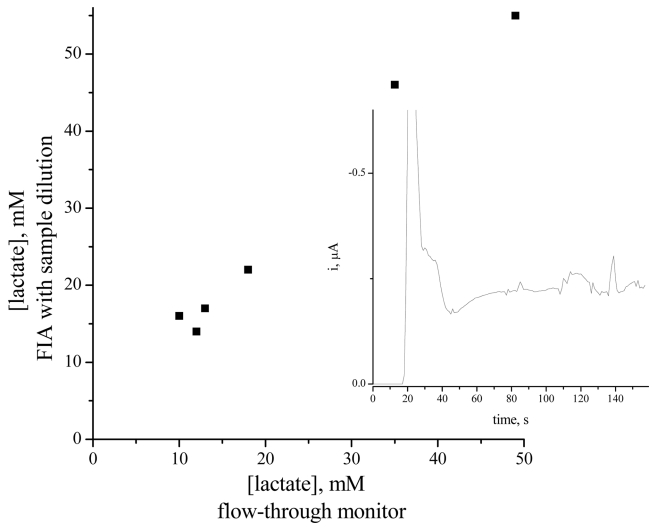

Figure 4. Correlation in sweat lactate concentrations measured by the flow-through monitor (abscissa) and using the FIA system with 1000-fold sample dilution (ordinate). Inset: current response of the sweat monitor.

analysis of the same samples (collected from the monitor outlet) using the FIA system equipped with lactate biosensor presented in ref 19. The readings of the flow-through monitor are slightly lower due to the matrix effect, since for the FIA system the sweat samples have been diluted 1000 times. Despite this fact, the high correlation coefficient achieved (r = 0.989) points to validation of the flow-through sweat monitor based on the elaborate biosensor.

CONCLUSIONS

Noninvasive diagnostics requires excreted liquid, which content correlates with metabolite concentrations in blood. Among possible candidates are sweat and tears. Noninvasive collection of the former is already adopted in clinics for prescreening of inherited pathologies; the tested parameter is sweat conductivity. In this respect it is hard to overestimate the proposed continuous sweat monitor, which provides a possibility for noninvasive personification of sport training, as well as of hypoxia-related therapy.

We expand here the application areas of our immobilization protocol from water−organic mixtures with the high content of organic solvent.[33,34] Since this protocol results in formation of enzyme−polymer complexes at a molecular level,[34] it is possible to use it for enzyme engineering, particularly for shielding terminal groups in active centers affecting enzyme affinity.

ASSOCIATED CONTENT

Supporting Information

Calibration plot for the lactate biosensor in flow-injection mode. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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