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Sol–Gel Immobilization of Lactate Oxidase from Organic Solvent: Toward the Advanced Lactate Biosensor

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We report on the novel protocol for enzyme immobilization into gel of siloxanes using water–organic mixtures with the high content of organic solvent as a reaction medium. Hydrolysis of alkoxysilanes carried out without excessive dilution with water resulted in more active and stable enzyme containing membranes. Immobilization of an inherently labile lactate oxidase according to the proposed sol–gel protocol over Prussian Blue modified electrode resulted in an advanced lactate biosensor characterized with a sensitivity of 0.18 A M^{-1} cm^{-2} in the flow injection analysis (FIA) mode over a wide dynamic range. A comparison with the known sensors has shown that analytical performances of the elaborated lactate biosensor are advantageous over both published systems and commercialized devices. The biosensor shows an appropriate stability and is suitable for clinical analysis (including noninvasive diagnostics) and food quality control.

Clinical diagnostics consider lactate as a marker for glycolysis, anaerobic metabolism, which causes death of tissues. Sports medicine requires monitoring of lactate for training as well as for evaluating a so-called "lactate threshold" indicating the physical training level of a sportsman. Lactate as a product of fermentation can be used as a marker for naturalness of food products.

Apparently the first lactate biosensor on the basis of lactate oxidase was made by retention of the enzyme under the dialysis membrane near the electrode surface.[1] Coupling the immobilized lactate oxidase and oxygen sensor was reported in refs 2–5. The most progressive way of providing the best analytical performance for biosensors (sensitivity, detection limit, dynamic range) is the coupling of the oxidase enzyme and the hydrogen peroxide transducer. The lactate biosensor made on the basis of the platinum probe polarized to +650 mV to fulfill that H_2O_2 oxidation was reported in 1986[6] showing a sensitivity of 0.016 A M^{-1} cm^{-2} and practical dynamic range from 1×10^{-6} to 1×10^{-4} M. The avoidance of the use of a separate mechanically attached enzyme-containing membrane and immobilization of lactate oxidase directly onto the transducer surface was reported in ref 7, achieving almost similar sensitivity of the biosensor. Immobilization of lactate oxidase in an Os-containing hydrogel provided an oxygen-independent electron exchange between the enzyme active site and the electrode[8] and caused only a slight increase in the sensor sensitivity. The biosensor based on H_2O_2 detection but with the use of the most advantageous transducer (Prussian Blue[9,10]), which allows hydrogen peroxide detection by its reduction in the presence of oxygen, showed similar sensitivity despite the rather doubtful immobilization protocol (the enzyme was dried on the transducer surface and covered by polymer membrane).[11] More recent articles on lactate biosensors[12–14] reported only units of milliampere Molarity^{-1} centimeter^{-2} of sensitivity. Even the so-called "highly sensitive" sensor based on carbon nanotubes[15] was limited by 0.02 A M^{-1}

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cm^{-2}. Lactate biosensor recently made according to the sol−gel immobilization procedure[16] was characterized also by low sensitivity <0.01 A M^{-1} cm^{-2}.

We believed that the procedure of enzyme immobilization can play a key role in achieving improved characteristics of the biosensor. The novel immobilization protocol we proposed some years ago[17,18] was based on enzyme exposure to water−organic mixtures with a high content of organic solvent. The resulting immobilized enzymes exhibited both improved activity and stability because the membrane forming polymer was deposited from its real solution without an excessive dilution with water.

Looking for the suitable matrix for lactate oxidase immobilization, we considered a gel of siloxanes. The use of charged matrixes like Nafion obviously dramatically reduces the dynamic range of the resulting biosensor because the analyte (lactate) is negatively charged. Uniform gel membranes can be deposited from diluted solutions of alkoxysilanes (<3−5%). Since the optimal amount of water is equal to what is required for hydrolysis of alkoxysilane, H_2O content in the casting solution should be less than 10−15%. This provides the requirements to use water−organic mixtures with the high content of organic solvent for enzyme immobilization in gel siloxane membranes.

As a transducer for hydrogen peroxide, we have chosen Prussian Blue known to be the most advantageous electrocatalyst for H_2O_2 reduction. Compared to 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.[10] The latter allows low-potential H_2O_2 detection by its reduction with advantageous analytical characteristics: (i) the sensitivity of 1−1.5 A M^{-1} cm^{-2}, which is at the upper limit of the sensitivity level, (ii) the detection limit of 1×10^{-7} M in the flow injection analysis (FIA) mode, and (iii) linear calibration range starting from the detection limit and extending over more than 3 orders of magnitude of hydrogen peroxide concentration.[9,10,19] Moreover, on the basis of Prussian Blue based nanoelectrode arrays,[20] the electrochemical sensor characterized by the linear calibration range prolonged over 7 orders of magnitude of analyte concentration, denoted in ref 21 as a sensor with record performance characteristics, has been elaborated.

Here we report on a lactate biosensor based on lactate oxidase immobilized in gel membranes formed from alkoxysilanes on the top of Prussian Blue modified electrodes. The novel immobilization protocol of gel formation from water−organic mixtures with the high content of organic solvents resulted in the highest sensitivity of the biosensor (0.18 A M^{-1} cm^{-2} in the FIA mode) over a wide dynamic range. The attractive performance characteristics of the elaborated biosensor are advantageous over known lactate-sensitive electrodes.

EXPERIMENTAL SECTION

Materials. Experiments were carried out with Milli-Q water from a Millipore Milli-Q system. All inorganic salts, siloxans, 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, France.

Instrumentation. Planar three electrode hydrogen peroxide sensors were purchased from Rusens LTD (Moscow, Russia). Planar three-electrode structures were made by screen-printing. The outer surface of both the working and auxiliary electrodes was made by printing carbon ink (usually, C10903P14 or C2030519D4, Gwent, U.K.). Working electrode of the sensor modified with Prussian Blue was of 1.9 mm in diameter. Sensor performance characteristics in flow-injection mode were sensitivity of 0.45 ± 0.1 A M^{-1} cm^{-2} and the lower detection limit of 1×10^{-7} M.

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 interfaced to an IBM PC. Flow rates used were in the range 0.5−1 mL min^{-1}. In FIA experiments, the peak current density values were taken for data treatment, the sample volume was 50 μL, and the working electrode potential was 0.00 V (vs Ag|AgCl|0.1 M KCl reference), allowing hydrogen peroxide reduction on Prussian Blue-modified electrodes. The carrier solution in the FIA experiments was 0.05 M phosphate buffer pH 6.0−7.0 containing 0.1 M KCl as the supporting electrolyte.

Methods. Lactate oxidase activity was measured with a Clark-type oxygen electrode according to the rate of oxygen consumption. The Clark-type electrode cell had a volume of 1.15 mL.

The remaining activity of lactate oxidase after exposing to water−isopropanol mixtures was measured as follows. Lactate oxidase aqueous solution (1 mg/mL) was injected into a water−isopropanol mixture to its final concentration of 0.1 mg/mL. After incubation for 10 min at a room temperature, 10−15 μL of the enzyme containing the water−isopropanol mixture was injected into a Clark-type electrode cell filled with an aqueous solution of lactate (1×10^{-4} M) and phosphate buffer (0.05 M, pH 6.0−6.5). Because of dilution of the enzyme containing the water−isopropanol mixture for an average of 1000 times, lactate oxidase activity was measured in the aqueous buffer containing a negligible amount of organic solvent. In independent experiments, it was shown that such an amount of isopropanol does not affect the lactate oxidase activity. The concentration of hydrogen peroxide in the stock solutions was measured recording the optical density at 230 nm with an LKB-Ultraspec UII spectrophotometer (Broma, Sweden).

Lactate biosensors were made as follows. Lactate oxidase aqueous solution (10−15 mg mL^{-1}) was suspended in an isopropanol solution (0.1−1%) of triethoxysiloxane (γ-aminopropyl-, vinyl-, or phenyl-triethoxysiloxane). The resulting

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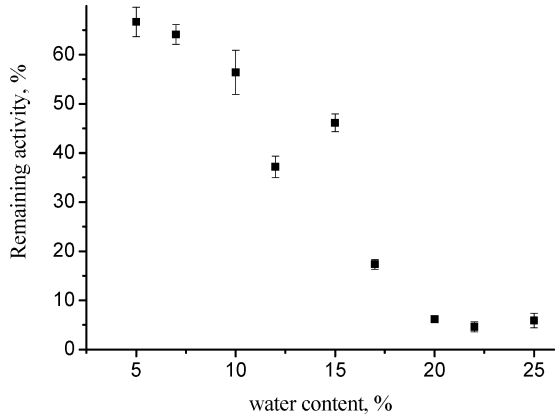

Figure 1. Remaining activity of lactate oxidase after 10 min of exposure to water−isopropanol mixtures.

mixture (2−3 μL) was syringed onto the of Prussian Blue modified working electrode covering its entire surface and dried at room temperature. Rough estimation of the membrane thickness using profilometer gives a value of 200−300 nm.

The reference method for lactate detection on whole blood was according to ref 22. The enzyme kit used was Randox LC 2389. Measurements were carried out using a HumaStar 3000 (Human Gmbh, Germany).

Blood samples were collected from athletes before and after a loading test on an ah/p Cosmos treadmill, LE 580 CE (h/p/cosmos sports and medical GmbH, Germany) with stepwise increasing working power before noon (initial tape speed 2.5 m/s, slope 1°, duration of each stage 3 min, speed increment 0.5 m/s). Venous blood samples were taken with the subjects in a sitting position with heparin.

RESULTS AND DISCUSSION

Tolerance of Lactate Oxidase to Isopropanol. The enzyme containing membrane of the biosensors has to be solid enough to successively immobilize the enzyme, but also has to be rather permeable for low-molecular weight organic compounds (analytes and products of their conversion) to provide high sensitivity and low detection limits. As mentioned, the membranes formed from sols due to hydrolysis of alkoxysilanes require low concentrations of the monomer to meet such properties. Hence, in order to avoid excessive dilution of the reaction mixture with water, which deteriorate the properties of the resulting membranes, enzyme immobilization should be carried out from concentrated organic solvents.

Immobilization from water−organic mixtures with the high content of organic solvent requires enzyme stability in such media. Considering the fact that lactate oxidase is a rather labile enzyme compared to other oxidases and in particular to glucose oxidase, we first examined it's tolerance to water−isopropanol mixtures with the high isopropanol content.

As seen in Figure 1, despite it's labile nature, lactate oxidase remained with more than 60% of it is initial activity after exposing to water−isopropanol mixtures with the high (>90% v/v) content of organic solvent. As the water content in the mixture is increased, a dramatic decrease of remaining lactate oxidase activity is observed. Inactivation of enzymes in water−organic mixtures with moderate concentrations of organic solvent is known[23,24] and is explained in terms of destabilization of hydrophobic interactions responsible for tertiary protein structure. Only certain proteins containing inorganic bridges, which preserve their tertiary structure, can be stable in such water−organic mixtures. For example, for glucose oxidase we have not observed such inactivation with the raise of water content in the organic solvent.[17]

Similarly to our previous observations, the remaining activity reached a local maximum when the enzyme was exposed to an azeotropic water−organic mixture (15% v/v water, Figure 1). In azeotropic conditions, on one hand, there is no excessive amount of organic solvent to capture water tightly connected to enzyme active site. On the other hand, in an azeotrope there is no excessive amount of organic solvent to deteriorate the tertiary protein structure. However, in the case of lactate oxidase, this extreme is reached on the decay of remaining enzyme activity (Figure 1). Accordingly, for further experiments we have chosen water−isopropanol mixtures with 10% water. In such mixtures, lactate oxidase remained at 55−60% of it is initial activity. In addition, 10% of water provides involvement of an enough amount of enzyme for immobilization.

Lactate Biosensor. Lactate biosensor was made by immobilization of lactate oxidase into the sol of alkoxysilanes on the top surface of the Prussian Blue modified working electrode of the screen-printed three-electrode sensor structures. To obtain uniform and stable membranes, the sol−gel procedure occurred without excessive dilution with water in the water−isopropanol mixture with a water content of 10%. The latter was chosen in accordance to the optimum of lactate oxidase surviving in the water−organic mixture (above).

Various alkoxysilanes were investigated as matrixes for immobilization of lactate oxidase. The most active enzyme containing membranes were obtained using triethoxysiloxanes: γ-aminopropyl-, vinyl-, or phenyl-triethoxysiloxane. The content of the alkoxysilanes in the water−organic mixture used for enzyme immobilization was optimized in order to achieve the highest sensitivity and stability of the resulting lactate-sensitive electrode.

Figure 2 displays responses of the FIA system equipped with the elaborated biosensor to various lactate injections. The biosensor was made by lactate oxidase immobilization in the sol of γ-aminopropyl triethoxysiloxane (0.1% content in the mixture) over the Prussian Blue modified electrode. It is seen that despite the manual injector used, the peaks are sharp and the time for one analysis takes less than 1 min. Variations of peak current are rather low for all concentrations (Figure 2) and seem to be determined by properties of the manual flow-injection system used. Peak current values are proportional to lactate concentration.

Figure 3 displays the calibration graph for the lactate recorded flow-injection system equipped with a lactate biosensor. As seen (Figure 3), the linear calibration range is prolonged over more than 3 orders of magnitude of lactate concentration from 5×10^{-7} to 1×10^{-3} M. The sensitivity evaluated as a slope of the initial part of the calibration graph (Figure 3) is of 0.18 A M cm^{-2}. Taking into account the dispersion coefficient of the flow-injection mode (1.5), we conclude that the sensitivity of the

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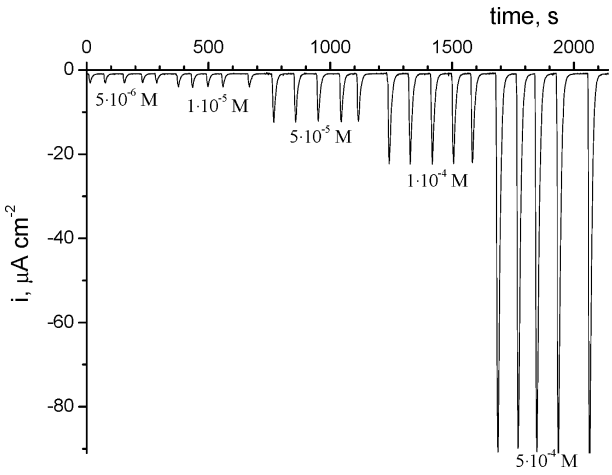

Figure 2. Response of the FIA system equipped with the biosensor to injections of lactate; 0.05 M phosphate buffer, pH 7.0 with 0.1 M KCl; flow rate 0.8 mL min^{-1}, operating potential 0.00 V (vs Ag|AgCl|0.1 M KCl reference).

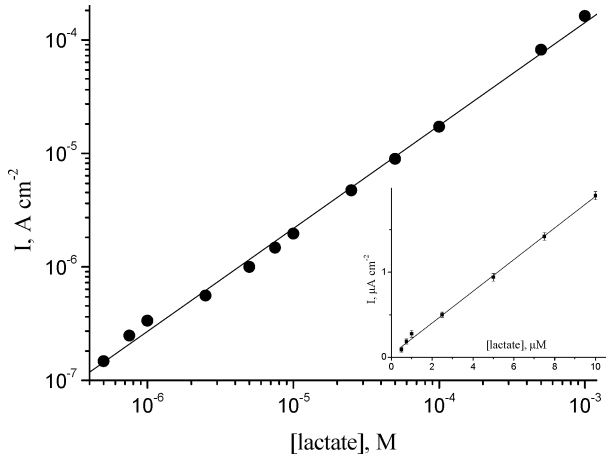

Figure 3. Calibration graph for lactate detection in the FIA system equipped with lactate biosensor; 0.05 M phosphate buffer, pH 7.0 with 0.1 M KCl; flow rate 0.8 mL min^{-1}. Error bars in double logarithmic plot are masked by the symbols. In the inset, the slope is 0.18 A M^{-1} cm^{-2}, intercept is 0.03 μA cm^{-2}.

elaborated lactate biosensor is 1 order of magnitude higher than for known lactate sensitive electrodes. The dynamic range of the biosensor is also the widest compared to the reported systems.

It is interesting to compare analytical performances of our biosensor with commercial lactate analyzers despite the latter are oriented to blood analysis. Anyway, compared to the Biosen C_Line glucose-lactate analyzer (EKF Diagnostic, Germany), a manual FIA system equipped with the elaborated biosensor has displayed 100 times higher sensitivity and more than the 1 order of magnitude decreased lower calibration limit. Hence, analytical performances of the elaborated lactate biosensor are advantageous over both published systems and commercialized devices.

Operational stability of the elaborated lactate biosensor was tested in flow-injection mode by injecting 0.1 mM of lactate. It was found that after 500 injections the height of the peak current response remained at a 85% level of it is initial value (data not shown). The biosensor remained not less than 90% of its initial activity after 6 months of storage in a waterproof package at 4 °C.

The relative selectivity of the elaborated biosensor to the so-called reductants (ascorbate, urate, paracetamol) present in real samples was investigated. In general, selectivity of the Prussian Blue based biosensors operated due to hydrogen peroxide reduction is provided by the low operation potential. Accordingly, no recognizable response to physiological concentrations of these tested reductants (including ascorbate, which is oxidized irreversibly) was observed (data not shown).

The biosensor was validated in the course of analysis of whole vein blood. As a reference, a standard spectrophotometric test was chosen. To achieve a remarkable variation in blood lactate, we tested blood samples of sportsmen before and after physical exercise. The variation of lactate concentration in blood samples was in the range from 2 to 8 mM. The data obtained using the FIA system equipped with the elaborated lactate biosensor are in good agreement with the levels of blood lactate evaluated through a standard blood analyzer test. The corresponding correlation coefficient was above 0.9987. Hence, the elaborated biosensor is valid for lactate detection in whole blood. Except for whole blood, the biosensor was used for analysis of sweat as well as for analysis of some food products (beverages) (data not shown).

CONCLUSIONS

The use of an appropriate medium for hydrolysis of alkoxysilanes avoiding excessive dilution with water resulted in improved activity of the immobilized lactate oxidase. The combination of the improved immobilization protocol and the most advantageous hydrogen peroxide transducer (Prussian Blue) leads to elaboration of the most sensitive lactate biosensor. Indeed, the sensitivity of the latter encountering 0.18 A M^{-1} cm^{-2} in flow injection mode is taking into account the dispersion coefficient at least 1 order of magnitude higher than it for reported lactate sensitive electrodes. Moreover, the sensitivity of the elaborated lactate biosensor is just 3 times less than the sensitivity of the H_2O_2 transducer used. This ratio seems to be the lowest among published systems indicating the immobilization protocol as an advantageous one.

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