Prototyping of lactate biosensor for non-invasive biomarker monitoring

Thesis for M.Sc. in Chemical and Process Engineering

by

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Abstract

Title of the thesis:	Prototyping of lactate biosensor for non-invasive biomarker monitoring
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Biosensors are widely used in diagnostics and health monitoring in different applications. They are simple, and cost-effective tools to monitor health and diseases and, therefore, they have attracted significant interest especially during the recent years. Lactate biosensors have got into focus since lactate is an important parameter of health and fitness.

The objective of this thesis was to prepare prototypes of lactate biosensors for non-invasive biomarker monitoring. Lactate oxidase was used as a biorecognition element, which produces H_2O_2 during the conversion of lactate and oxygen that can be measured amperometrically. Different immobilization methods were used to investigate the possibilities of increasing the long-term stability and sensitivity of biosensors. For that, the enzyme was entrapped in a chitosan and in a Nafion matrix, it was immobilized with PEGDE and cross-linked with GA.

Three of the different methods gave promising results and with further optimizations, it is possible to increase the stability and sensitivity even further.

Keywords: Biosensor, lactate, lactate oxidase, amperometry, Prussian Blue

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Abbreviations

AA	ascorbic acid
BSA	bovine serum albumin
CE	counter electrode
D	dopamine
DI	deionized
GA	glutaraldehyde
GOx	glucose oxidase
ISF	interstitial fluid
LDH	lactate dehydrogenase
LOD	limit of detection
LOQ	limit of quantitation
LOx	lactate oxidase
MHD	magnetohydrodynamic
NAD	nicotinamide adenine dinucleotide
o-PD	o-phenylenediamine
PB	Prussian Blue
PBS	phosphate buffered saline
PEGDE	poly(ethylene glycol) diglycidyl ether
POD	peroxidase
RE	reference electrode
RI	reverse iontophoresis
RMS	root mean square
SPE	screen-printed electrode
UA	uric acid
WE	working electrode

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1 Introduction

Health monitoring has gained significant attention during the last few years, because the number of diagnosed disease cases has increased considerably. Besides, diseases, such as diabetes have become more widespread within the population. Therefore, the demand for rapid and easy health monitoring devices has increased. One way to monitor the state of health in a simple and cost-effective way is with biosensors, which have attracted interest especially during the recent years.

Biosensors can be tools of diagnostics and monitoring both in private and hospital use. In addition to clinical diagnostics, they are widely used in food and chemical industry, as well as for environmental monitoring. New biosensors are being developed constantly, especially for clinical diagnostics, where the non-invasive techniques are also of interest due to their user-friendliness. Enzymatic biosensors are a commonly used type of biosensors. Here, an enzyme, *e.g.*, lactate oxidase or glucose oxidase, is used as a biorecognition element.

One of the largest challenges in biosensor development is maintaining the stability and sensitivity of the device. It is important that the biosensor can maintain its stability during storage, and it can give reliable results throughout the usage. Lactate and glucose are analytes that are often detected with the help of biosensors since both are important indicators of health. The challenges of biosensor stability can be addressed with different enzyme immobilization methods.

Much of the previous research has been focusing on glucose oxidase and on the different immobilization methods for glucose biosensors. However, in this work the focus is on lactate biosensors. Therefore, lactate oxidase was used as a biocatalyst, which produces H_2O_2 during the conversion of lactate and oxygen that can be measured electrochemically. Different immobilization methods were used to investigate the possibilities of increasing the stability and sensitivity of the prepared lactate biosensors.

2 Literature review

2.1 Biosensors

Biosensors have gained significant attention due to their simple operation, high sensitivity, high selectivity, and low cost. They typically consist of two main parts: a biorecognition element and a transducer. The biorecognition element is a component of biological origin that can specifically detect the analyte, while the transducer converts the signal of the biorecognition event to a physical signal (electric, optical, thermal, *etc.*), which can be measured.[1, 2] From this signal, the concentration of the analyte in the sample can be determined.[3] Figure 1 illustrates the structure of a biosensor.

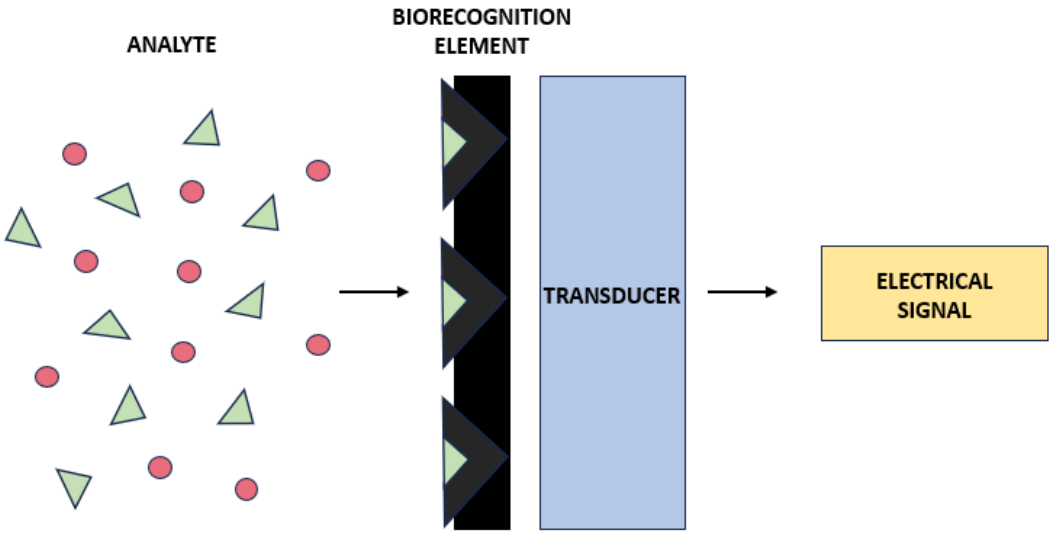

Figure 1. *Schematic structure of a biosensor.*

2.1.1 Electrochemical biosensors

The electrochemical biosensors are one of the most frequently used type of biosensors, since they enable the fast and highly sensitive detection of different analytes in a simple, compact, and inexpensive way. An electrochemical biosensor is typically a three-electrode system, which consists of a working electrode (WE), a reference electrode (RE), and a counter electrode (CE) (Figure 2).[4] The biorecognition event and the electron transfer take place at the surface of the WE. The stable potential of the RE is used to control the potential of the WE. Finally, the CE makes the electrical circuit complete since the current derived from the WE passes through the CE and, therefore, the potential of the RE stays constant.[3, 5] During the operation of the biosensor, the three-electrode system is immersed in a solution (electrolyte), which contains the analyte.

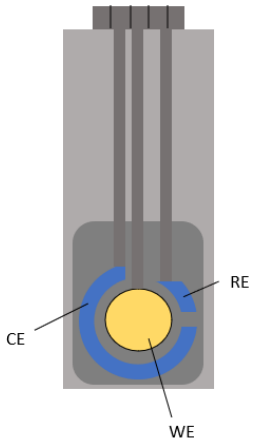

Figure 2. *A schematic drawing of a screen-printed electrode (SPE), which is a popular choice in developing electrochemical biosensors. The SPE typically consists of a working electrode (WE), a counter electrode (CE), a reference electrode (RE) and the electrolyte containing the analyte is drop-cast on the electrodes.*

As biosensors in general, an electrochemical biosensor consists of a biorecognition element and a transducer. The biorecognition element can be an enzyme, an antibody, or an aptamer, which can specifically and sensitively detect the target analyte. The transducer of the biosensor is the WE, which converts the signal from the biorecognition event to an electrically measurable signal.[2, 6]

The electrochemical biosensors can be classified based on their operation principle into voltammetric, amperometric, potentiometric, and conductometric types.[1] In voltammetry, a potential applied to the WE is swept across a number of values linearly or in staircase manner, and the resulting current is recorded at each potential and is used to infer *e.g.* the mechanism of an electrochemical reaction. Amperometry is a subtype of voltammetry, where a constant potential is applied at the WE and the current is measured for a certain time. If the applied potential is the potential of an electrochemical conversion of the analyte at the WE, the measured current is proportional to the analyte's concentration. In potentiometry, spontaneous reduction and oxidation reactions occur at the WE and RE, correspondingly, and the difference of the potential between these two electrodes is measured in the absence of current flowing through the electrochemical cell (so-called open circuit potential). The difference of potentials depends on the concentration of oxidized and reduced species, one of which can be an analyte, and obeys the Nernst equation.[6–8] Finally, in conductometry, the change in the conductivity is measured upon the chemical reaction which causes alterations in the sample solution.[7]

2.1.1.1 Amperometric biosensors

The amperometric biosensors are typically three-electrode systems consisting of a WE, a RE, and a CE. During an amperometric measurement, a fixed or a pulsed potential is applied on the WE and the resulted current is measured as a function of time or potential. The applied potential is chosen to be sufficient to start a chemical reaction at the surface of the WE, which results in a measurable current.[3]

Chronoamperometry is one type of amperometric methods, where a fixed potential is applied to the WE and the current response is measured as a function of time.[7] The concentration of the analyte can be calculated by using the Cottrell equation:

$$I = \frac{nFA\sqrt{D}}{\sqrt{\pi t}} C, \qquad (1)$$

where A [cm^2] is the surface area of the WE, n [-] is the number of electrons that are necessary to oxidize or reduce one molecule of the analyte, F (96485 C/mol) is the Faraday constant, C [mol/cm^3] is the concentration of the analyte, D [cm^2/s] is the diffusion coefficient of the analyte and t [s] is the time.[9]

2.2 Enzymatic biosensors

2.2.1 Introduction to enzymatic biosensors

Enzymatic biosensors utilize an enzyme as biorecognition element, which catalyze a biochemical reaction of the analyte and, therefore, enables its specific detection.[10] In the electrochemical biosensors, which utilize enzyme as a biorecognition element, the enzyme is immobilized on the WE, so the detection of the product or the transfer of the electrons originated from the biochemical reaction results in a measurable electric signal.[10, 11]

2.2.1.1 Operation principle of the enzymatic biosensors

During the operation of enzymatic biosensors, electrons are transferred from the active site of the immobilized enzyme to the surface of the WE and the resulting current is measured.[3, 12] There are three different generations of enzymatic biosensors based on the different types of electron transfer mechanisms (Figure 3). A sufficient electron transfer to the electrode and, therefore, satisfactory sensitivity can be provided by choosing an appropriate setup.

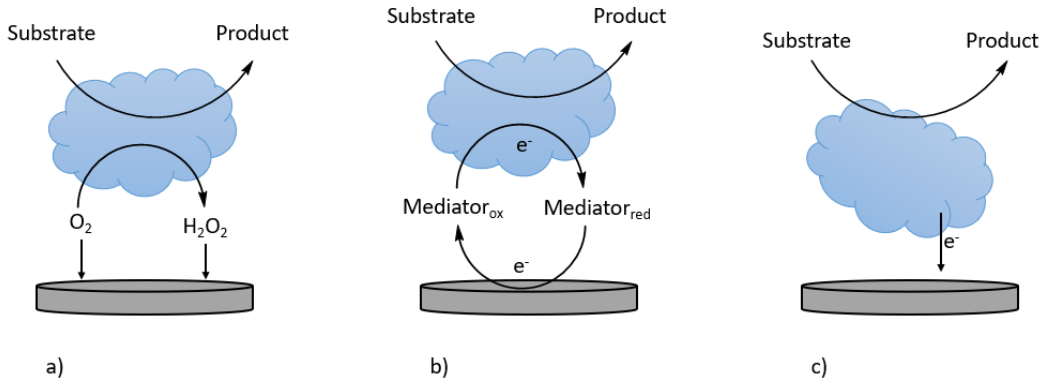

Figure 3. *The three generations of enzymatic biosensors according to the electron transfer mechanism. a) In first-generation enzymatic biosensors, the product of the enzymatic reaction (e.g. H_2O_2) is directly oxidized or reduced at the surface of WE, b) in second-generation enzymatic biosensors, the electron originated from the biochemical reaction is transferred to a mediator, which is then oxidized or reduced at the WE, c) while in third-generation enzymatic biosensors, the electron originated from the biochemical reaction is directly transferred to the WE.* [14]

The operation principle of the first-generation enzymatic biosensors is based on the direct electrochemical detection of the substrate or product of the enzymatic reaction on the WE (Figure 3.a). The substrate and product can be oxygen or hydrogen peroxide, which can be detected *e.g.* amperometrically.[14, 15]

The second-generation enzymatic biosensors need redox mediators (*e.g.* ferrocene derivatives, Prussian Blue) during their operation.[16] These redox mediators are utilized as electron transferring agents to connect the cofactor that is located at the enzyme's active site, or the product of the enzymatic reaction, and the WE surface. It is important to immobilize the mediator near the electrode surface, so it can enable the electron transfer through its conversion between its oxidized and reduced forms without significant leaching.[3]

Prussian Blue (PB) or ferric hexacyanoferrate can be used as redox mediator in second-generation enzymatic biosensors, since it has a peroxidase activity, *i.e.*, it can catalyze the decomposition of H_2O_2 and transfer its electrons to the WE. PB consists of iron(II) and iron(III) ions surrounded by carbon and nitrogen atoms, forming a cubic structure.[17] It is possible to selectively detect H_2O_2 by electrooxidation or electroreduction on PB-modified electrodes. Its entirely reduced form is Prussian

White, which can reduce oxygen and H_2O_2, while Berlin Green is the completely oxidized form capable of oxidizing H_2O_2.[15] Figure 4 describes the mechanism of a PB-based lactate biosensor.

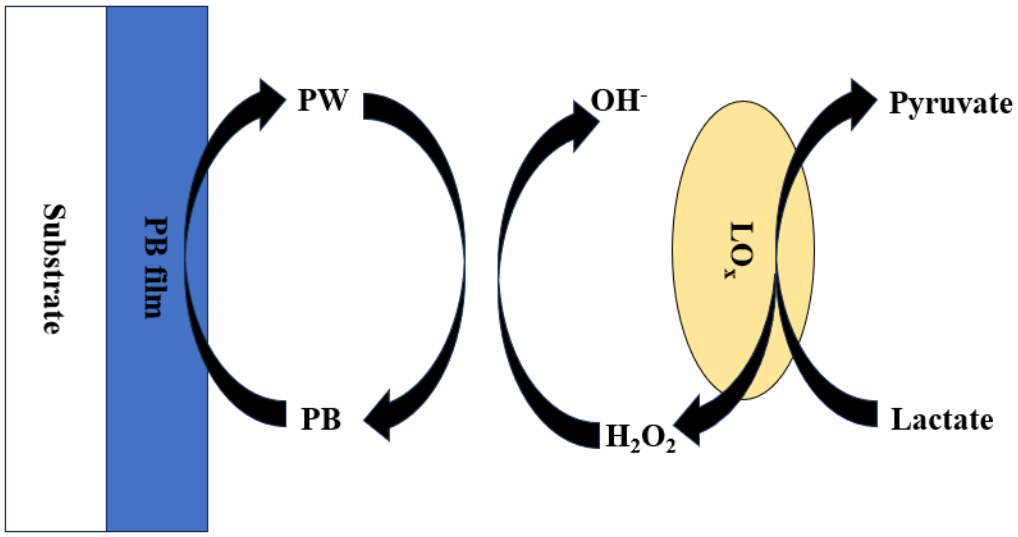

Figure 4. *Mechanism of a Prussian Blue-based lactate biosensor, incorporating lactate oxidase as a biorecognition element, which catalyzes the oxidation of L-lactate to pyruvate.* [18]

PB-modified electrodes were extensively studied by Karyakin *et al*.[19] As described in their studies, the reduction of H_2O_2 is possible in a neutral media with a Prussian White catalyst (2).

$$H_2O_2 + 2e^- \xrightarrow{k_{cat}} 2OH^- \qquad (2)$$

Due to its advantageous properties, such as low cost, inorganic nature and the capability of its reduced form, Prussian White, to act as a catalyst for the reduction of H_2O_2,[20] PB has gained significant attention during the last few years. PB-modified electrodes can provide higher specific activity, sensitivity and selectivity compared to other electrode materials. These specially deposited electrodes have the potential to retain long-term operational stability after a certain post-treatment and, therefore, they are of great interest in different fields of electrochemistry.[17] During the sensor-preparation, PB can be electrodeposited on the electrode surface and thereby form a compact redox active layer.[15] It is also possible to chemically deposit PB on a desired

substrate, as is done in carbon-PB ink for screen printing. The resulting composite or hybrid material can then be printed as the WE. Screen printing is a technique where ink is deposited layer by layer on a solid substrate with the help of a mesh stencil to form the sensor. This is an easy and fast technique that suits well for mass production and, therefore, it is widely used.[21]

The operation principle of third-generation enzymatic biosensors is based on the direct electron transfer from the cofactor at the active site of the enzyme to the WE surface. It has gained significant attention since it does not require any oxygen or mediators. In this approach, specific dehydrogenases, such as glucose dehydrogenase and fructose dehydrogenase,[22] are utilized as biorecognition elements. However, not all the enzymes are capable of direct electron transfer without their modification, since the technique requires specific conformation of the enzyme's redox centre allowing for electron to tunnel. These biosensors are considered to be suitable for continuous monitoring as well, since they can be operated at low oxidation potential, which can mitigate selectivity problems.[16]

2.2.1.2 Basics of enzyme kinetics

The reaction of an enzyme and its substrate can be described with enzyme kinetic models.[23, 24] To illustrate the formation of a product (P) from a substrate (S) by an enzyme (E), the following scheme can be used:

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \overset{k_2}{\rightarrow} E + P \qquad (3)$$

Here, the enzyme reacts with the substrate in a reversible way and forms an enzyme-substrate complex (ES), which can be described with the second-order association rate constant, k_1. The dissociation reaction of ES complex to enzyme and substrate can be described with the first-order dissociation rate constant, k_{-1}. The enzyme-substrate complex is then irreversibly converts into enzyme and product with the first-order catalytic rate constant, k_2.[23, 24]

The reaction (3) in the simplest terms can be described with the Michaelis-Menten model, one of the most widely used models in enzyme kinetics. Its fundamental equation describes the relation of the reaction velocity (v) and the substrate concentration ([S]):

$$v = \frac{V_{max} \cdot [S]}{K_M + [S]}, \quad (4)$$

where V_{max} is the maximum velocity and K_M is the Michaelis-Menten constant.
This equation can also be illustrated as a Michaelis-Menten curve, as presented in Figure 5.

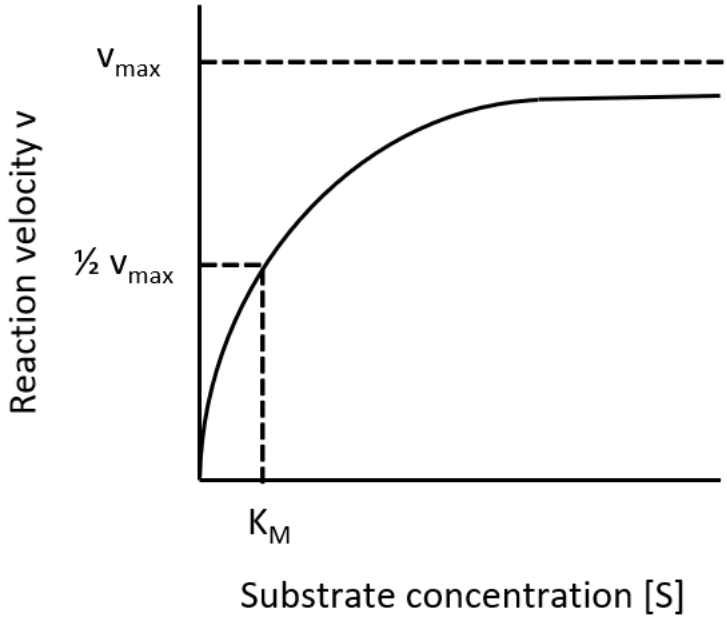

Figure 5. *Michaelis-Menten curve, where v is the reaction velocity, [S] is the concentration of the substrate, V_{max} is the maximum reaction velocity, and K_M is the Michaelis-Menten constant, which represents the substrate concentration at half of the V_{max}.*

According to the model, the reaction velocity can be estimated from the concentration of the ES complex:

$$v = k_2[ES]. \quad (5)$$

V_{max} represents the maximum velocity, where all enzyme molecules are saturated with substrate, and which can be calculated as:

$$V_{max} = k_2[E]. \quad (6)$$

The Michaelis-Menten constant K_M represents the substrate concentration, where the velocity is half of the maximum. It can be defined with the different rate constants: [23,24]

$$K_M = \frac{k_{-1}+k_2}{k_1}. \quad (7)$$

2.2.1.3 Significance of the enzymatic biosensors

Enzymatic biosensors can detect different molecules that are present in the human body. In addition, these biosensors can be used in the chemical and food industry. They have very high specificity to one analyte due to specifically shaped active centre of the enzyme for binding one substrate with a very few interferences from other molecules with similar structure. Besides, they are small, easy to operate, have low-cost, high sensitivity, and they have great potential in mass production. These advantages make enzymatic biosensors attractive for both qualitative and quantitative analysis of different analytes, such as glucose and lactate. Oxidoreductases, such as glucose oxidase, lactate oxidase or lactate dehydrogenase,[25] are the most typically used enzymes in electrochemical enzymatic biosensors.[26]

2.2.2 Lactate biosensors

2.2.2.1 Significance of lactate sensing

Lactate is an intermediate, which is formed during the anaerobic metabolism of glucose. It is an important marker of health since lactate levels can indicate different pathological conditions. Increased lactate levels can be present *e.g.* in diabetes and cardiac diseases.[27]

Lactate is a deprotonated, conjugate base form of lactic acid.[28] L-(+)-lactate and D-(-)-lactate are the two different enantiomers of lactic acid, from which L-(+)-lactate has

significance in the human metabolism and, therefore, in clinical diagnostics.[29] In healthy individuals, the lactate concentrations in blood and in interstitial fluid (ISF) is 0.5-2.0 mM at rest and it can increase up to 15 mM during exercise. In intense physical exercise, the concentration in human sweat can be as high as 25 mM. In septic shock, the blood lactate concentration can reach even 7-8 mM in healthy individuals. This can cause lactic acidosis or hyperlactatemia.[30] Lactate levels is an interesting parameter to follow when evaluating of an athlete's maximum performance and, therefore, lactate is also an important indicator of fitness.[27]

2.2.2.2 Enzymes of lactate biosensing

The concentration of lactate in different samples can be determined with enzymatic biosensors. Lactate oxidase (LOx) and lactate dehydrogenase (LDH) are lactate-specific enzymes, which are widely used for this purpose.[29]

LOx is a flavoenzyme, typically obtained from *Mycobacterium smegmatis* or *Streptococcus species.* It catalyzes the oxidation of L-lactate to pyruvate at pH~7.5, meanwhile producing H_2O_2.[31]

$$L - lactate + O_2 \overset{LOx}{\longleftrightarrow} pyruvate + H_2O_2 \qquad (8)$$

The reaction product, H_2O_2 can be reduced or oxidized on the surface of the WE during an amperometric detection and the produced current is directly proportional to the lactate concentration according to the Cottrell equation (1).[32] A redox mediator (*e.g.* PB) can be used in the amperometric detection to lower the necessary potential and to avoid interference from different species.[33] LOx is widely used in lactate biosensors, [34] although due to its instability, it needs to be immobilized on the surface of the WE. For that purpose, different enzyme immobilization techniques (covalent binding, cross-linking, physical entrapment, *etc.*) can be used.[35]

LDH is an oxidoreductase, which can be used in lactate biosensors. LDH catalyzes the conversion of L-lactate to pyruvate at its optimal pH of 6.0, meanwhile reducing the nicotinamide adenine dinucleotide (NAD^+) cofactor (9).[31]

$$L - lactate + NAD^+ \overset{LDH}{\longleftrightarrow} pyruvate + NADH + H^+ \quad (9)$$

The reaction product, NADH can be detected amperometrically. The main advantage of LDH-based biosensors is that they are independent of oxygen, in contrast to LOx-based biosensors.[36] However, the high potential, needed for the electrochemical oxidation of NADH can lead to electrode fouling and stability problems. Furthermore, at this high potential, other oxidizable components can cause interference. To overcome the difficulties originated from the necessary overpotential, various organic and inorganic redox mediators, such as PB or Meldola Blue have been used in the electrode setups.[36]

2.2.2.3 Enzymatic biosensors for lactate sensing

Several lactate sensing devices are available on the market, which typically measure lactate from serum, plasma, or capillary blood. StatStrip® Xpress™ [i], Lactate Scout 4 [ii] and Lactate Pro 2 [iii] are commercially available lactate meters that measure lactate amperometrically from a small volume of capillary blood, typically 0.3 - 0.6 µl. These devices offer an effective and simple way to measure lactate in a point-of-care way, but they can cause stress by requiring multiple finger-pricking within a day.[37]

Besides the above mentioned, other body fluids, such as ISF, sweat, saliva, and tears can be used as a sample to measure lactate.[5] Recently, there is extensive research on the development of non-invasive and wearable devices for lactate detection in different body fluids since they would offer an easier and more comfortable option for lactate monitoring.[26, 29] ISF, the fluid which surrounds cells to supply nutrients and to remove excess products, can provide important health information. Therefore, ISF has been utilized as a sample for the development of non-invasive biosensors coupled with reverse iontophoresis (RI)[38] or magnetohydrodynamic (MHD)[39] sampling techniques.[30]

[ii] https://www.novabiomedical.com/statstrip-lac-hb-hct/
[ii] https://www.ekfdiagnostics.com/lactate-scout.html
[iii] https://www.arkray.eu/english/products/lt-1730.html

2.3 Enzyme immobilization techniques

To obtain and improve the long-term stability and response of the biosensor, the immobilization of the enzyme molecules is one of the most important steps. The immobilization technique must be chosen according to the components of the enzymatic biosensor (*e.g.* type of enzyme, transducer), because it has an effect on the stability and operation of the biosensor. It is important to maintain the native structure and biological activity of the immobilized enzyme too.[40] The immobilization technique can enhance the electron transfer as well, by keeping the enzyme in the close proximity of the electrode surface.[5] Three different types of enzyme immobilization techniques, physical entrapment, cross-linking, and covalent immobilization are discussed here (Figure 6).

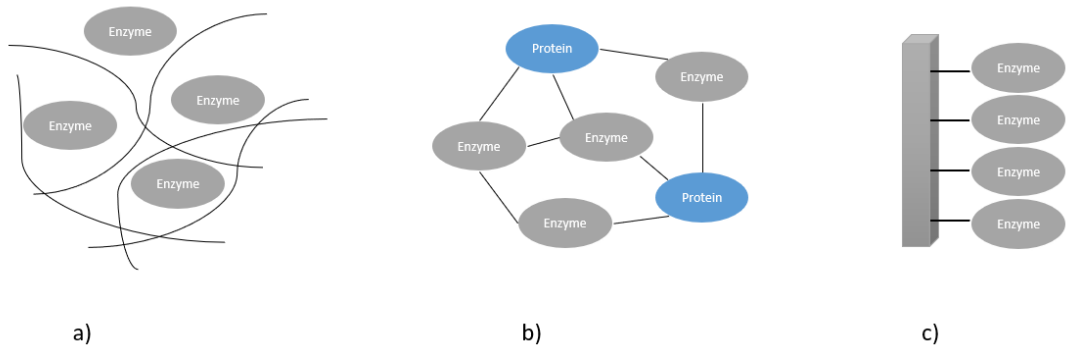

Figure 6. *Enzyme immobilization techniques for improving the stability of enzymatic biosensors. a) Physical entrapment of the enzyme, b) cross-linking of the enzyme molecules, and c) covalent immobilization of the enzyme on a solid support (e.g. WE).* [40]

2.3.1 Physical entrapment of the enzyme

Physical entrapment means enzyme immobilization methods, where the enzyme molecules are entrapped in a sol-gel or in a polymer matrix. These techniques increase the storage and operational stability of enzymatic biosensors. Since is not modified during the process, the enzyme can maintain its activity during the entrapment. However, these methods have some drawbacks too: the performance of the biosensors can be restricted by leaching of the enzymes or the diffusion barriers within the matrix.[40]

One type, the sol-gel process consists of two steps in general. First, an alkoxide precursor (*e.g.* tetramethoxysilane, tetraethoxysilane) is hydrolysed under acidic or alkaline conditions, then a porous gel is formed when the hydroxylated units join in a condensation reaction.[41] Then the enzyme can be entrapped in the matrix formed during the second step. Another way to entrap the enzyme is by mixing it with monomers which are then cross-linked via photo- or electropolymerization.[3, 39] An easy way to implement physical entrapment is drop-casting, where a drop of the solution containing the enzyme and other reagents, is dropped on the electrode.[42]

Chitosan and Nafion are polymers that are often used for the physical entrapment of enzyme molecules. These compounds form a polymer membrane, which prevents enzyme-leaching by working as a physical barrier.[14, 37] Chitosan is a natural polysaccharide derived from chitin. Mechanical strength, low cost, and non-toxicity are some of the key advantages of chitosan. Its reactive hydroxyl and amino groups make it also possible to easily adjust the polymer both chemically and biologically to the intended usage.[43] During the entrapment process, chitosan forms a complex with the negatively charged enzyme molecules, which is a mild way to bind and entrap them. Nafion is another polymer with excellent properties and, therefore, it is widely used in different immobilization processes. The chemical stability of the biosensor can be increased by using Nafion. It is often utilized since it has a very tight pore structure allowing only small molecules, such as H^+, H_2O and H_2O_2 to penetrate into the matrix. Moreover, Nafion is negatively charged and, therefore, repels most of the interfering molecules.[44] Chitosan and Nafion are suitable to be used in drop-casting to physically entrap the enzymes on the electrode.[14, 44]

2.3.2 Cross-linking of the enzyme molecules

Cross-linking is a simple and widely used immobilization technique, where a connection is formed by a bifunctional cross-linking agent between different components of the system.[3] This cross-linking agent works as a ligand to incorporate enzyme molecules in three-dimensional networks. The enzyme-loss is minimal in this case, since the enzyme molecules are connected by strong covalent bonds during cross-linking.[46]

One of the most widely used reagent of this technique is glutaraldehyde (GA), which can be used to cross-link enzyme molecules through imine bonds by reacting with those amino groups.[46, 47] GA is a cheap, commercially available, highly reactive, but toxic reagent, which can produce stable networks that can increase the stability of enzymatic biosensors.[3, 48]

2.3.3 Covalent immobilization of the enzyme

In this method, functional groups (*e.g.* primary amines, carboxyl groups) of the enzyme molecules are utilized to tether them to the surface of the WE through covalent interactions. With this technique, enzyme molecules can be immobilized directly on the WE surface or to a thin membrane that is then attached to the electrode surface. This type immobilization method is widely used in enzymatic biosensors.[39, 49]

Poly(ethylene glycol) diglycidyl ether (PEGDE) is a non-toxic compound with two highly reactive epoxy groups, capable of cross-linking and covalent immobilizing enzyme molecules by reacting with those amino or carboxyl groups.[51] The temperature plays an important role in this case. At room temperature, the reaction between the epoxy groups and proteins is slow, but it can be significantly enhanced by increasing the temperature.[50, 51]

Many of the previous projects have been focusing on glucose oxidase (GOx) and on the different immobilization methods for glucose biosensors. However, in this work the focus is on lactate biosensors. Therefore, here lactate oxidase is used as a biocatalyst, which produces H_2O_2 during the conversion of lactate and oxygen. Different immobilization methods were used to investigate the possibilities of increasing the stability and sensitivity of lactate biosensors.

3 Materials and methods

3.1 Materials

4-acetamidophenol (98%), bovine serum albumin (BSA), chitosan, D-(+)-glucose (> 99.5%), dopamine hydrochloride, glutaraldehyde (GA) solution, (Grade II, 25% in H_2O), glycerol, L(+)-lactic acid, L-ascorbic acid, Nafion® 117 solution, o-phenylenediamine dihydrochloride (o-PD), poly(ethylene glycol) diglycidyl ether (PEGDE), sodium chloride, sodium hydroxide, sodium phosphate monobasic, uric acid was purchased from Sigma-Aldrich (St. Louis, USA). Lactate oxidase (LOx) from microorganism (Lot number: 3162116000, EC 1.1.3.2) was purchased from Sorachim (Lausanne, Switzerland). Peroxidase from horseradish was purchased from abcr GmbH & Co. KG (Karlsruhe, Germany). Ethanol (94.0 %) was purchased from ALTIA Oyj (Rajamäki, Finland). 2-Propanol was purchased from Honeywell. Acetic acid glacial (100%) was purchased from Merck (Darmstadt, Germany). The deionized (DI) water used in the work was obtained from ELGA Purelab Chorus 1 (18.2 MΩ.cm) (Veolia Water Technologies).

The sensors used for the fabrication of lactate biosensors were custom made by Screentec Oy (Oulu, Finland) by screen-printing a three-electrode system, consisting of a Ag/AgCl RE, a Ag/AgCl CE and Prussian blue-modified SunChemical Gwent C2070424P2 Carbon Graphite Ink WE. Ag/AgCl is 50:50 by weight.

3.2 Instruments, tools, and software

IviumStat.h potentiostat (Ivium Technologies B.V., The Netherlands) was used to perform all the amperometric measurements and its IviumSoft software was used for the data analysis.

The pH of the buffer solutions was adjusted with SevenEasy and SevenCompact pH meters (Mettler Toledo, USA).

The weight measurements were performed with XS205 DualRange Analytical (Mettler Toledo, USA) and Mettler AT261 DeltaRange® (Mettler Toledo, USA) balances.

To mix the different casting solutions for drop-casting on the electrodes, Nutating Mixer (VWR International, USA) was used. Mini Star Silverline microcentrifuge (VWR International, USA) was used to reduce the possible foaming of the enzyme-casting solutions.

Eppendorf Research® plus (Eppendorf, Germany) and Finnpipette F1 (Thermo Fisher Scientific, USA) automatic pipettes were used during the work.

Termaks drying oven, type T 1119 UV (Termaks, Norway) was used to dry the PEGDE sensors.

NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was used in the enzymatic assays to determine the activity of LOx and the data was processed with the instrument's software.

Origin 2020b software (OriginLab Corporation, USA), was used to process the data and to prepare calibration curves and figures.

3.3 Solutions

Sodium hydroxide solution (~1M), V = 50 ml

Ca. 2.0 g of NaOH was dissolved in 50 ml of DI water in a volumetric flask.

Phosphate buffered saline (PBS) (50mM, pH 7.4, I = 0.154 M), V = 2000 ml

12.0 g of $Na_2H_2PO_4$ was dissolved in approximately 1800 ml of DI water, then, 2.879 g of NaCl was added. This solution was titrated with ~1 M NaOH solution to adjust the pH to 7.4. Finally, the volume was made up to 2000 ml with DI water.

Solutions used in the enzymatic assay for LOx activity determination

O-phenylenediamine dihydrochloride solution (o-PD) (0.21 mM), V = 100 ml

One 10 mg tablet of o-PD was dissolved in 7.0 ml of DI water in a vial protected from light. Then, 2.675 ml of the solution was diluted to 100 ml with PBS.

Lactate substrate solution (10 w/v%), V = 60 ml

6.0g of L-(+)-lactic acid was dissolved in 60 ml of DI water.

Peroxidase enzyme solution (POD) (60 U/ml)

This solution was prepared freshly right before use. 0.12 mg of POD was dissolved in 1.09 ml of cold DI water. The prepared solution was kept in an ice bath.

Reaction cocktail

This solution was prepared freshly before use. 9.6 ml of the o-PD solution and 2.0 ml of the lactate substrate solution were mixed. The solution was then kept in a water bath at 35˚C, protected from light.

LOx enzyme solution (30 U/ml)

0.41 mg of LOx was dissolved in 1.46 ml of cold PBS to prepare a 30 U/ml stock solution. Right before use, 20 µl of the stock solution was diluted with 98 µl of cold PBS to prepare a 0.6 U/ml solution.

Solutions used for the enzyme immobilization methods

Chitosan solution in 0.1M acetic acid (0.5 wt %), V = 5 ml

25.00 mg of chitosan was dissolved in 28.7 µl of 17.4 M acetic acid. Then it was diluted with DI water to 5.0 ml in a volumetric flask, so the concentration of the acetic acid was 0.1 M in the final solution.

Nafion solution in isopropanol (0.5 wt%), V = 5 ml

0.5 ml of 5% Nafion was diluted with 4.5 ml of isopropanol to the final concentration of 0.5 wt %.

Bovine serum albumin (BSA) solution (10 mg/ml), V ~ 60 µl

0.87 mg of BSA was dissolved in 87 µl of PBS in a microtube. This solution was made separately for every sensor, so the solutions may have slightly different amounts of materials.

LOx solution in 0.25 wt% chitosan (17 mg/ml LOx containing 5 mg/ml BSA as stabilizer), V = 100 µl for four sensors

To make 50 µl of 34 mg/ml enzyme solution with BSA, 1.70 mg of LOx was dissolved in 50 µl of 10 mg/ml BSA solution in a microtube. Then, 50 µl of 0.5 wt% chitosan solution (in 0.1 M acetic acid) was added to the LOx-BSA solution in 1:1 volume-to-volume ratio. The lid of the tube was sealed with parafilm, and it was placed on an orbital shaker for 30 minutes to dissolve the solid material and homogenize the mixture.

LOx solution in 0.25 wt% Nafion (17mg/ml LOx containing 5 mg/ml BSA as stabilizer), V = 60 µl for two sensors

This solution was made in the same way as the previously described LOx-BSA-chitosan, but the chitosan was replaced with Nafion. First, 1.13 mg of BSA was dissolved in 113 µl of PBS in a microtube to obtain a 10 mg/ml solution. Then, 1.02 mg of LOx was dissolved in 30 µl of this BSA solution in a separate tube. Finally, 30 µl of 0.5 wt% Nafion solution was added to the LOx-BSA in 1:1 volume-to-volume ratio. The lid of the microtube was sealed with parafilm and it was placed on an orbital shaker for 30 minutes to dissolve the solid material and homogenize the mixture.

LOx solution in 20 mg/ml PEGDE (60 mg/ml LOx, containing 30 mg/ml BSA as stabilizer and 1 w/v% glycerol), V = 250 µl for two sensors

14.56 mg of LOx was dissolved in 121 µl PBS, while 7.28 mg of BSA in 122 µl PBS in two separate microtubes. These two solutions were mixed, then 1.94 µl of glycerol and 4.30 µl of PEGDE was added.

LOx solution in 0.6 wt% GA (20mg/ml LOx, containing 40 mg/ml BSA as stabilizer), V = 60 µl for two electrodes

2.39 mg of BSA was dissolved in 30 µl PBS in a microtube and 1.19 mg of LOx was dissolved in 29.7 µl PBS in another tube. Then, the two solutions were mixed and 0.33 µl of 25% GA solution was added. The lid of the plastic tube was sealed with parafilm, and it was placed on an orbital shaker for 30 minutes to dissolve the solid material and homogenize the mixture.

Solution used in the sensor calibrations

Lactate solution (1.0 M)

10.63 mg L-(+)-lactic acid was dissolved in 111 µl PBS. This solution was prepared freshly before every calibration. The other calibration solutions with concentrations $1.0 \cdot 10^{-2}$ M, $1.0 \cdot 10^{-3}$ M and $1.0 \cdot 10^{-4}$ M were made from this 1.0 M lactate solution by serial dilution with PBS.

Solutions for the interference tests

Lactate solution ($1.0 \cdot 10^{-3}$ M)

First, 13.15 mg of L-(+)-lactic acid was dissolved in 137 µl of PBS to make a 1.0 M solution. Then the $1.0 \cdot 10^{-3}$ M solution was prepared by serial dilution of the 1.0 M stock solution with PBS.

Glucose solution ($1.0 \cdot 10^{-3}$ M)

First, 20.39 mg of glucose was dissolved in 113 µl PBS to make a 1.0 M stock solution. Then the $1.0 \cdot 10^{-3}$ M solution was prepared by the serial dilution of the 1.0 M stock solution with PBS.

Paracetamol (acetaminophen) solution ($1.0 \cdot 10^{-3}$ M)

The solubility of Paracetamol is ~19 mg/ml in water. Therefore, a 0.1 M solution was prepared first by dissolving 15.00 mg of paracetamol in 1.0 ml PBS in a microtube. The tube was kept in water bath at ~40 ˚C to dissolve the solid material. The $1.0 \cdot 10^{-3}$ M solution was obtained from it by serial dilution with PBS.

Uric acid solution ($1.0 \cdot 10^{-4}$ M)

The $1.0 \cdot 10^{-4}$ M uric acid solution was prepared with the serial dilution of the $1.0 \cdot 10^{-3}$ M stock solution with PBS.

Ascorbic acid solution ($1.0 \cdot 10^{-4}$ M)

34.30 mg of ascorbic acid was dissolved in 195 µl PBS to prepare a 1.0 M stock solution. Then, the $1.0 \cdot 10^{-4}$ M solution was obtained by the serial dilution of the stock solution with PBS.

Dopamine solution ($1.0 \cdot 10^{10}$ M)

22.80 mg of dopamine was dissolved in 121 µl PBS in a microtube to make a 1.0 M stock solution. It was protected from light, since dopamine is light-sensitive. The $1.0 \cdot 10^{10}$ M solution was made by serial dilution of the stock solution with PBS.

3.4 Enzymatic assay for determining the activity of LOx

The enzymatic assay for the determination of the LOx enzymatic activity was performed according to the protocol of Merck, Enzymatic assay of glucose oxidase.[4] However, instead of β-D-(+)-glucose, glucose oxidase and o-dianisidine, L-(+)-lactic acid, LOx and o-PD were used. The solutions were made as described in Chapter 3.3., then from these the test and blank solutions were prepared according to Table 1.

[4] https://www.sigmaaldrich.com/FI/en/technical-documents/protocol/protein-biology/enzyme-activity-assays/enzymatic-assay-of-glucose-oxidase

Table 1. *Composition of the test and blank solutions for the LOx activity determination.*

	Test solution (ml)	**Blank solution (ml)**
Reaction cocktail	2.9	2.9
POD	0.1	0.1
LOx	0.1	-
PBS	-	0.1

The components of the test and blank solutions were pipetted in cuvettes, were mixed, and were immediately analyzed with NanoDrop 2000 spectrophotometer. The change of absorbance was monitored for 10.5 minutes at 417 nm and finally the activity of LOx was determined.

3.5 Enzyme immobilization methods

Four different types of lactate biosensors were prepared by utilizing four different types of immobilization methods and reagents (chitosan, Nafion, PEGDE and GA).

3.5.1 Enzyme entrapment in a chitosan matrix

These lactate biosensors were prepared as described previously.[38] Briefly, a drop-casting solution containing 17 mg/ml LOx, 5 mg/ml BSA and 0.25 w/w% chitosan was prepared, and a 20 µl droplet of it was drop-cast on each WE. The biosensors were incubated at 4˚C overnight, then the analytical performance was tested as described in Chapter 3.6.1.

3.5.2 Enzyme entrapment in a Nafion matrix

These lactate biosensors were prepared similarly as the LOx-BSA-chitosan biosensors, as described previously, but chitosan was replaced with Nafion.[38] Briefly, a drop-casting solution was prepared, containing 17 mg/ml LOx, 5 mg/ml BSA and 0.25-% Nafion, then a 20 µl droplet was drop-cast on each WE, and the biosensors were incubated 4˚ C overnight. Afterwards, the analytical performance was tested as described in Chapter 3.6.1.

3.5.3 Enzyme immobilization with PEGDE

These lactate biosensors were made as described previously in the literature.[51] Shortly, first a drop-casting solution was made, which contained 60 mg/ml LOx, 30 mg/ml BSA, 20 mg/ml PEGDE and 1 w/v% glycerol. Then, a 103 µl droplet was drop-casted on each WE. The volume of the droplet was calculated so that the final enzyme load was 0.34 mg on the WE as in the case of the other types of biosensors. The drop-cast biosensors were incubated in an oven at 55˚C for 2 hours. The analytical performance was tested afterwards as described in Chapter 3.6.1. Since the biosensors did not give any response in this case, a lower temperature (40˚C) and a longer incubation time (18 h) was also tested for drying.

3.5.4 Enzyme immobilization by cross-linking with GA

These lactate biosensors were prepared as described previously.[47,53] First, a drop-casting solution was prepared, containing 20 mg/ml LOx, 40 mg/ml BSA and 0.6 wt% GA. A 20.1 µl droplet was drop-cast on each WE to obtain a final enzyme load of 0.34 mg. Then the biosensors were incubated at room temperature for ~3 h. The analytical performance of the biosensors was determined as described in Chapter 3.6.1.

3.6 Investigation of the analytical performance of the biosensors

3.6.1 Investigation of the sensitivity of biosensors towards lactate

To determine the sensitivity of the different biosensors towards lactate as the slope of the calibration curve, first the biosensor was placed in a custom-made chamber, which contained DI to provide the appropriate relative humidity. Then, a 50 µl droplet of PBS was drop-cast on the electrodes to attain a stable baseline at the applied 0.0 V potential. The current response of the biosensor was recorded afterwards at this 0.0 V potential during the sequential addition of the lactate solutions indicated in Table 2.

Table 2. *Calibration solutions for the lactate biosensors to determine the sensitivity towards lactate.*

$C_{spiked\ solution}$ (M)	$V_{spiked\ solution}$ (µl)	$C_{final\ solution}$ (µM)
$1.0 \cdot 10^{-4}$ M	0.5	1.0
	1	1.9
	2	3.7
	4	7.0
$1.0 \cdot 10^{-3}$ M	0.5	8.6
	1	17.0
	2	33.0
	4	62.0
$1.0 \cdot 10^{-2}$ M	0.5	76.0
	1	150.0
	2	290.0
	4	550.0

The sensitivity to lactate was determined four times for each biosensor: first when the sensor was prepared, then after one, two and four weeks of storage in the refrigerator. By performing these measurements, it was possible to monitor the storage stability, *i.e.*, the change in the sensitivity of the biosensors.

3.6.2 Investigation of the selectivity of the biosensors

The analytical performance of a biosensor is affected by the different interfering species that can be found in human body fluids. Therefore, two new biosensors were prepared by using three different immobilization reagents (chitosan, Nafion and GA) to test the effect of possible interference. After recording a stable baseline in 50 µl PBS, the measurements were performed as described in Chapter 3.6.1., *i.e.*, by recording the current response at 0.0 V during the sequential addition of lactate and the different interfering components (Table 3).

Table 3. *Interfering components used to determine the selectivity of the lactate biosensors.*

Component	**Volume of the droplet (µl)**	**Final concentration on the WE (µM)**
lactate	1.95	37.50
glucose	1.33	25.00
acetaminophen	0.27	5.00
Uric acid	0.82	1.50
ascorbic acid	0.69	1.25
dopamine	0.54	$9.79 \cdot 10^{-7}$

3.6.3 Investigation of the operational stability of the biosensors

The operational stability tests were performed in a custom-made electrochemical cell. First, the biosensor was immersed in 60 ml PBS to record the baseline at 0.0 V. Then 6.0 ml of 1.0 M lactate solution was added, and the current signal was recorded at 0.0 V for 8 hours. The solution was stirred during the entire operational stability test to ensure the sufficient supply of lactate to the surface of the working electrode.

3.7 Analysis of the data

Six biosensors were prepared with each immobilization method to investigate their analytical performance. Those sensors, which gave poor response during the calibration and where an experimental error was suspected, were excluded from the data analysis and further tests. Therefore, some of the data originates only from five biosensors within a batch. Each biosensor was calibrated four times: immediately after the preparation (as prepared) and after 1, 2, and 4 weeks of storage in the refrigerator at 4 °C. After the calibration, the measurement data for each biosensor were plotted with OriginLab 2020b software to obtain the sensitivity values from the slope of the calibration curves. Finally, a mean sensitivity value and its standard deviation was calculated with Microsoft Excel (for Windows 10) separately for every biosensor batch of different methods and for every week.

The calibration data were processed with OriginLab 2020b software. Calibration curves were plotted to determine the sensitivity of the biosensors towards lactate as the slope. The measurement data were plotted by using current density values for the y-axis, which is the current value recorded at the drop in the signal upon the addition of lactate divided by the area of the WE (0.324 cm^2). The values for the x-axis were taken from the final concentration of the lactate solution on the WE (Table 2). Finally, a linear model was fitted on the points by setting instrumental error as weight to obtain the calibration curve and the sensitivity as the slope.

The limit of detection (LOD) and limit of quantitation (LOQ) values were determined separately for each biosensor. Then, a mean value and standard deviation were calculated for every batch regarding the different immobilization methods and storage times. LOD indicates the lowest concentration of the analyte that can be detected,[1] while LOQ indicates the lowest level of an analyte that can with any level of certainty be quantitated.[54] The LOD and LOQ values for the biosensors were calculated by using formulas (10-12).

$$RMS = \sigma = \sqrt{\frac{\sum(i-\bar{i})^2}{n}} \quad (10)$$

$$LOD = \frac{3\sigma}{S} \quad (11)$$

$$LOQ = \frac{10\sigma}{S} \quad (12)$$

In (11) and (12), S [A/M·cm^2] is the sensitivity value and σ [A] is the root mean square, which is calculated with formula (10). In (10), $\bar{i}$ [A] is the mean value of the current signal of the biosensor batch, i [A] is the current signal value of one biosensor and n [-] is the number of biosensors within a batch. For these calculations, the baseline was taken from 100 data points before the addition of the lactate solution.

4 Results and discussion

4.1 Determination of the enzymatic activity of LOx

To estimate the enzymatic activity of LOx, a protocol of Merck "Enzymatic assay of glucose oxidase" [5] was utilized, but using L-(+)-lactic acid, LOx and o-PD instead of β-D-(+)-glucose, glucose oxidase and o-dianisidine.

$$L - lactate + O_2 \xrightarrow{LOx} pyruvate + H_2O_2 \quad (13)$$

$$H_2O_2 + o - PD \xrightarrow{\text{POD}} 2{,}3 - diaminophenazine \quad (14)$$

The assay is based on reactions (13-14), where LOx oxidizes L-lactate to pyruvate, meanwhile producing H_2O_2. By using H_2O_2, produced in the first reaction, the peroxidase enzyme (POD) converts its chromogen substrate, o-PD to an optically measurable product, 2,3-diaminophenazine. By definition, one unit of an enzyme that converts 1.0 µM of the substrate per minute. The specific activity can be calculated by dividing the enzyme unit with the mass of the solid enzyme product.

For the assay, first a test solution containing the LOx, its substrate (lactate), the POD, its chromogen substrate (o-PD) and a blank solution containing all the components except the LOx were prepared. Then the change of the absorbance at 417 nm as the result of the consecutive enzymatic reactions was followed for 10.5 min (Figure 7).

[5] https://www.sigmaaldrich.com/FI/en/technical-documents/protocol/protein-biology/enzyme-activity-assays/enzymatic-assay-of-glucose-oxidase

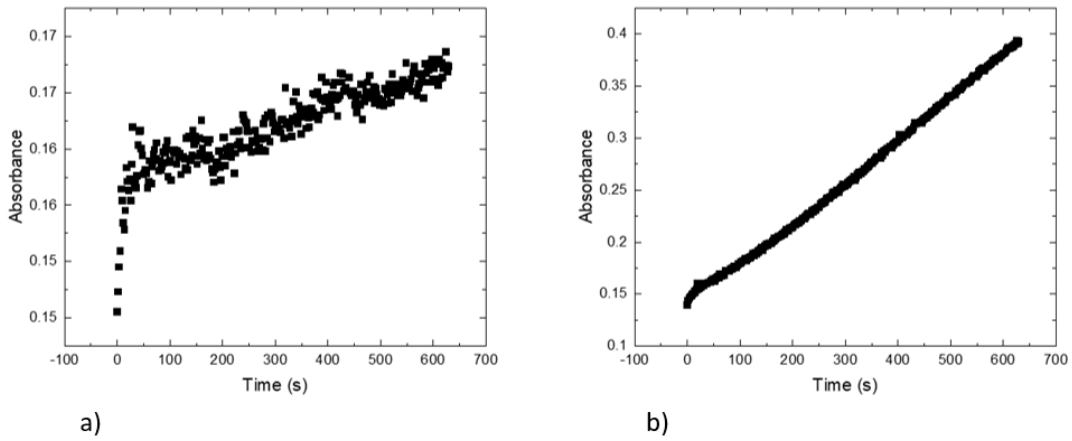

Figure 7. *Change in the absorbance upon the progress of the reactions of the enzymatic assay: a) for the blank solution containing lactate, o-PD, POD, and b) for the test solution, which contained those three and LOx. The curves were recorded at 417nm, with 10 mm path length.*

When comparing the curves of the test and blank solutions, the change in absorbance is significantly smaller in the case of the blank solution. This comes from the fact that the blank solution did not contain the enzyme LOx and, therefore, the reactions (13-14) could not happen. The minor change in the absorbance is probably due to the oxidation of o-PD by the oxygen-content of the surrounding air. Since the test solution contained LOx, the reactions (13-14) could proceed and result in the coloured product. The accumulation of this could be followed by the change in the absorbance.

By using the absorbance values at the beginning and the end of the measurements, the activity of LOx (in enzyme units (U), *i.e.*, µmol/min) in 1.0 ml of buffer was calculated (15).

$$\frac{\frac{\Delta A_{417nm}\,(test)}{\min} - \frac{\Delta A_{417nm(blank)}}{\min} \cdot V_{solution} \cdot df}{\varepsilon \cdot V_{LOx}} \qquad (15)$$

$$= \frac{\left(\frac{0.3923-0.1393}{10.5} - \frac{0.1673-0.1455}{10.5}\right) \cdot 3.1 \cdot 50}{16.7 \cdot 0.1} \; \frac{U}{ml}$$

$$\text{activity (LOx): } 2.044 \; \frac{U}{ml}$$

In (15), ΔA [-] is the change in the absorbance at 417 nm for the test or blank solution; $V_{solution}$ [ml] is the total volume of the test or blank solution; df [-] is the dilution factor,

coming from the dilution of the 30.0 U/ml LOx stock solution to 0.6 U/ml; ε [1/mM^{-1}·cm] is the millimolar extinction coefficient of o-PD at 417 nm and V_{LOx} [ml] is the volume of LOx solution. To calculate the specific activity of LOx, the following formula was used:

$$\text{specific activity: } \frac{activity\ (LOx)}{\frac{mg\ solid}{ml\ LOx}} = \frac{2.044}{0.28}\ \frac{U}{mg} \tag{16}$$

specific activity: 7.30 U/mg

In (16), the denominator (mg solid/ml LOx) is the mass of the LOx in 1.0 ml solution.

The specific activity for LOx was marked 108 U/mg on the original product, which is significantly higher than the one calculated above. It is possible that Sorachim, the company where the enzyme has been purchased from, has used a different protocol for LOx activity determination than the protocol utilized here. Another reason for the difference can be that a different substrate of the POD was used here than the one, o-dianisidine in the original protocol. It is possible that POD has different reactivity towards these substrates, which can result in differences in the calculated specific activity. Nevertheless, the specific activity determined in this work was used as a reference point to determine the enzyme degradation during its storage and use.

4.2 Investigation of enzyme immobilization methods

4.2.1 Calibration of the lactate biosensors

In this work, different immobilization methods for LOx were utilized to prepare lactate biosensors and the stability and degradation of the biosensors were investigated. For the enzyme immobilization, the methods of physical entrapment of the enzyme in a chitosan or a Nafion matrix, the cross-linking of the enzyme molecules with GA and the immobilization with PEGDE were utilized.

Six biosensors were prepared with each immobilization methods and their current signal was recorded upon addition of lactate in concentrations indicated in Table 2. As a result of these chronoamperometric measurements at 0.0 V, trace calibration curves were attained (Figure 8).

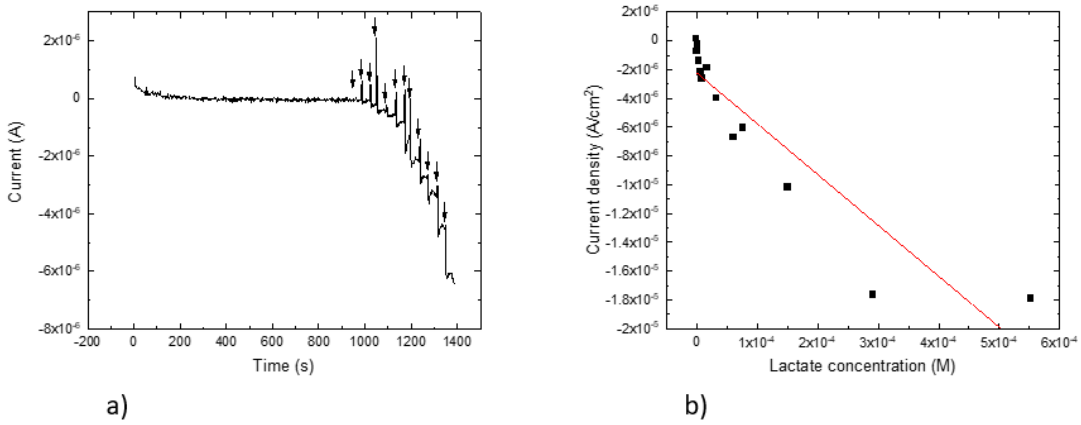

Figure 8. *a) A trace calibration curve of a biosensor prepared by immobilizing LOx with Nafion on a PB-carbon WE. The chronoamperometric measurement was performed at 0.0 V in PBS (pH 7.4, I = 154 mM, c = 50 mM, T = 25 ˚C) and the arrows indicate the addition of lactate solution droplets. b) Point calibration curve obtained from the trace calibration curve with linear fitting.*

The addition of the lactate solutions, which are indicated with arrows in the figure, resulted in immediate decrease in the current signal. These current signals after each drop were used to prepare the point calibration curves with OriginLab software. To gain the current density values for the y-axis of the curve, the current values recorded at the drop in the signal were divided by the area of the WE (0.324 cm^2). The values of the x-axis values were taken from the final concentration of the lactate solution on the WE. After performing a linear fit of the data points within the linear range of the curve, the sensitivity was obtained from the slope of the calibration curve, goodness of fitting was expressed as R^2. The LOD and LOQ values were calculated as described in Chapter 3.7. Then, finally, the mean and standard deviation of sensitivity, LOD and LOQ values were determined for each biosensor batch to compare the different immobilization methods and to follow the degradation of the biosensors during the storage at 4 °C.

4.2.2 Entrapment of LOx in a chitosan matrix

A batch of six biosensors was made by entrapping LOx in a chitosan matrix (LOx-chitosan biosensors). For that, a solution containing 17 mg/ml LOx, 0.25 w/w% chitosan, and 5 mg/ml BSA stabilizer was drop-cast on PB-modified carbon WEs and let dry. As a result of the drop-casting process, the final enzyme load was 0.34 mg on each WE.

Each biosensor was calibrated as described in Chapter 4.2.1. right after the biosensor was prepared, then after 1, 2 and 4 weeks of storage in the refrigerator. The parameters of the analytical performance (Table 4) were determined from the point calibration curves (Figure 9 and Figure 10) in case of each biosensor.

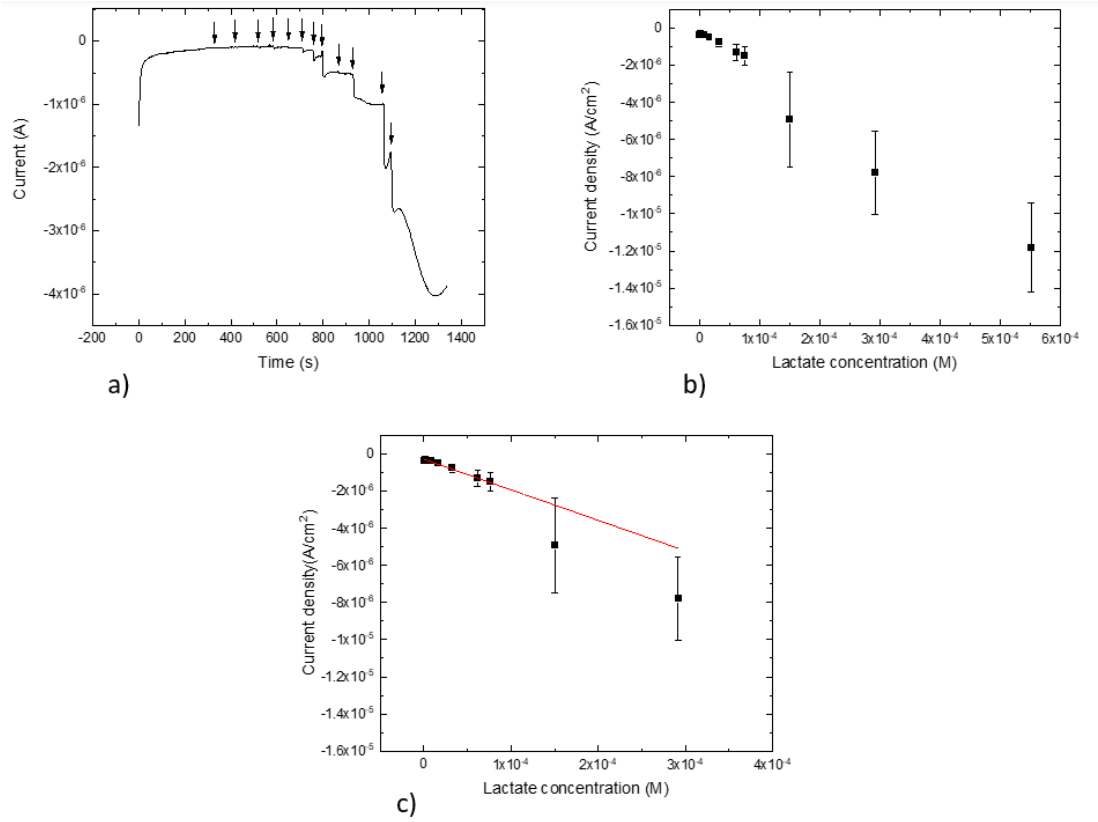

Figure 9. *Calibration of the as prepared LOx-chitosan biosensors (N=6) to determine their sensitivity towards lactate. a) Trace calibration curve recorded at 0.0 V in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 °C). The arrows indicate the additions of lactate solution droplets. b) Point calibration curve obtained from the trace calibration curve. c) Point calibration curve with linear fitting (the last point is excluded).*

On the trace calibration curve (Figure 9.a), the arrows indicate the additions of lactate solution droplets. Immediately after these additions, the current decreased significantly, since the LOx converted lactate meanwhile producing H_2O_2 (13). The produced H_2O_2 is then reduced by PB on the surface of the WE during the amperometric detection at 0.0 V (17).

$$H_2O_2 + 2e^- \overset{PB}{\rightarrow} 2OH^- \qquad (17)$$

The current of H_2O_2 reduction is assumed to be directly proportional to the lactate concentration based on equations (13), (17), and the Cottrell equation. As can be seen in the figure, the concentration range used for this calibration is not entirely the same as the range where the biosensor gives response, since not every lactate solution droplet results in change in the current signal. The concentrations of the calibration solutions were chosen according to the concentration levels of lactate in serum in different health conditions, considering the dilution effect of the extraction as well. Therefore, it is important to further optimize the performance of these biosensors primarily by improving the sensing in the lower concentration range.

The mean current density values and standard deviations of six biosensors were used to prepare the point calibration curves of the as prepared biosensors (Figure 9.b-c). The current density decreases with increasing lactate concentrations due to the reduction of H_2O_2 on the WE. The standard deviations are significantly higher at higher concentrations, which may originate from the fact that the individual biosensors within the batch have different upper limits of the linear region. The concentrations of the calibration solutions did not entirely match with the linear range of the biosensor, which can be seen as the saturation of the curve at higher concentrations. Therefore, to fit the linear model for the sensitivity determination, the goodness of the fit, *i.e.*, the R^2 value of the fitting, was investigated by including and excluding different points. As a result of the investigations, the last data point of the calibration was excluded to improve the linear fitting (Figure 9.c). By fitting the linear model, the sensitivity could be also determined as the slope.

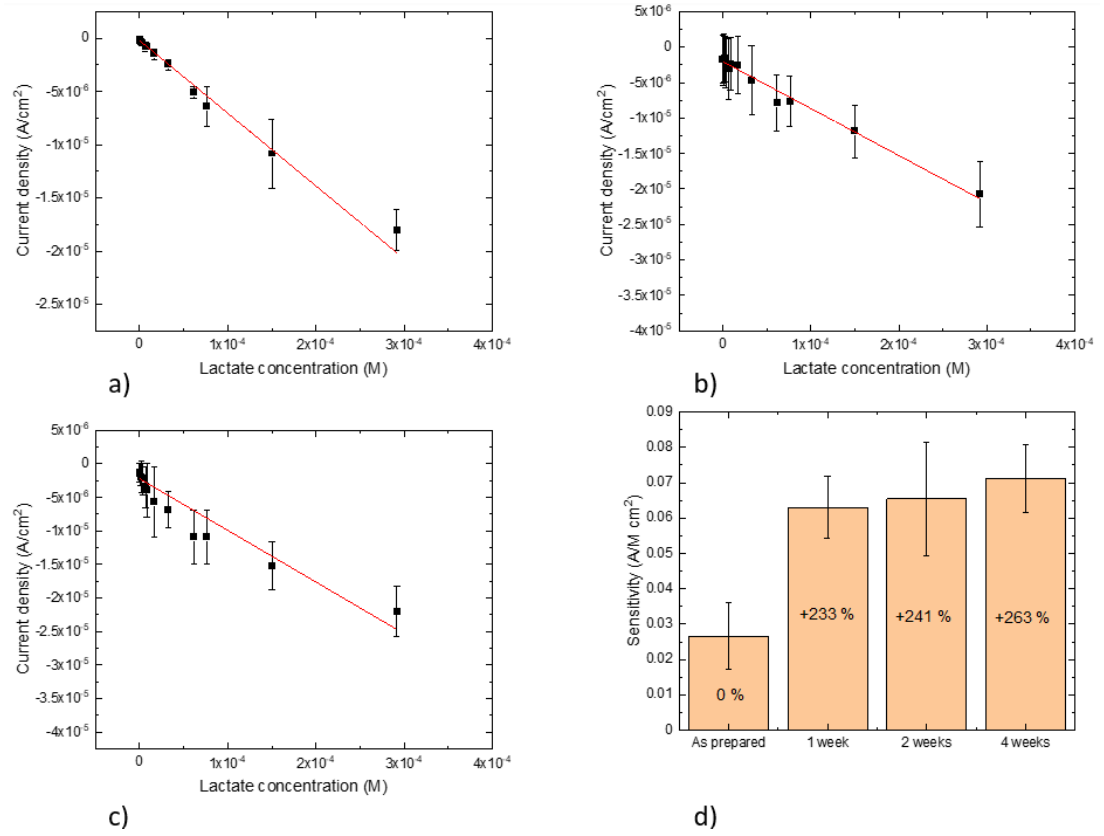

Figure 10. *Point calibration curves of the LOx-chitosan biosensors (N=6) with linear fitting after a) 1, b) 2, and c) 4 weeks of storage in the refrigerator. The last point of each calibration was excluded from the fitting. d) Investigation of the degradation of the biosensors during the storage in the refrigerator. The change in the percentage value indicates the change in sensitivity compared to the as prepared biosensors. The calibrations were performed by addition of lactate solution droplets in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 ˚C), while performing amperometry at 0.0 V.*

The calibrations were performed for the same biosensors after 1, 2 and 4 weeks of storage. The point calibration curves utilizing the mean values and standard deviations of the six biosensors with linear fitting can be seen in Figure 10.a-c. As in the case of the calibration of the as prepared biosensors, the last points were excluded from the fitting.

It can be seen in Figure 10.a and b and the R^2 data presented in Table 4 that the goodness of fit has clearly improved after 1 and 2 weeks of storage compared to the as prepared sensors (Figure 9.c), while after 4 weeks (Figure 10.c), R^2 starts to decrease. The R^2=0.8358 value of the as prepared biosensors is significantly lower than the other values, which are above 0.9. The latter is acceptable for biosensors prepared with an

unoptimized protocol, however, in case of an ideal performance R^2 should preferably be above 0.99.

Figure 10.d presents change in the mean sensitivities and standard deviations of the calibrations from the different weeks. The sensitivity of the biosensors increased significantly after the 1-week storage, compared to the as prepared biosensors (Table 4), while the difference between weeks 1-4 is not significant. The higher sensitivity means that the biosensor can give a signal even in case of a small change in the analyte concentration in the sample. A reason for this increase can be the change in the porosity of the chitosan matrix. It is possible that the structure of the matrix has changed during the storage, which resulted in better access of the enzyme to the analyte inside the pores of the nanoporous material, which finally led to the increased sensitivity.[55] As the results show, there is no sign of enzyme degradation, *i.e.*, the chitosan matrix helped to preserve the stability of the LOx. The standard deviations are relatively high in each case, which can be the result of the different performance of the individual sensors within the batch. This indicates that the sensor preparation method still needs further optimization.

Zanini *et al.*[56] have prepared an amperometric lactate biosensor. They immobilized LOx on a glassy carbon electrode with laponite-chitosan hydrogel. The sensitivity value was 0.326 ± 0.003 A/M·cm^2 in that case, which is significantly higher than the sensitivity of the LOx-chitosan biosensors described here (Table 4). However, a different electrode material was used there, and the biosensor was aimed for a different application, *i.e.*, it was developed for the analysis of L-lactate in food samples, which requires a different concentration range. Nevertheless, the LOx-chitosan biosensors described here need further optimization, which can result in higher sensitivities as well.

Table 4. *Analytical performance parameters (sensitivity, R^2, LOD, LOQ) of the LOx-chitosan biosensors (N=6).*

	As prepared	Week 1	Week 2	Week 4
Sensitivity (A/M·cm²)	0.027 ± 0.009	0.063 ± 0.009	0.065 ± 0.016	0.071 ± 0.009
R^2 (-)	0.8358	0.9831	0.9833	0.9218
LOD (µM)	2.79 ± 1.05	1.26 ± 1.60	3.17 ± 5.01	2.67 ± 2.24
LOQ (µM)	9.31± 3.50	4.20 ± 5.33	10.56 ± 16.71	8.89 ± 7.48

The LOD and LOQ values of the LOx-chitosan biosensor batches were also determined (Table 4). The LOD values for the four different weeks are in the range of 1.26-3.17 µM and there is no trend in the change of the values during the storage The above-mentioned lactate biosensors by Zanini *et al.*[56] had a LOD of 3.8 ± 0.2 µM, which is in the same range as the values of this work's LOx-chitosan biosensors. The LOQ values were also determined for the four different weeks, and these are in the range of 4.20-10.56 µM.

The entrapment of LOx in the chitosan matrix appears to be a suitable method to immobilize the enzyme since the biosensors could be successfully prepared, and the analytical performance parameters could be determined. The results showed that the enzyme molecules probably did not degrade during the four weeks of storage, and the sensitivity even increased during this period. This can be explained with the change in the structure of the matrix, which enabled the better access of the enzyme to the analyte. The results prove that this method can be a suitable way to prolong the enzyme stability. However, it still needs further optimization to decrease the difference between the sensors within one batch and to improve the analytical performance.

4.2.3 Entrapment of LOx in a Nafion matrix

Another type of immobilization was implemented by entrapping LOx in a Nafion matrix. For that, a solution containing 17 mg/ml LOx, 5 mg/ml BSA, and 0.25% Nafion, was drop-cast on PB-modified carbon WEs and let dry. As the results of the drop-casting process, the final enzyme load was 0.34 mg on each WE. Totally, six

biosensors were prepared, of which five gave acceptable response and were used in the data analysis. When calibrating these biosensors, one of the five biosensors had almost eight times higher sensitivity value compared to the other biosensors during the calibration performed right after the preparation. This sensor was also excluded from the following data analysis as an outlier. The suspected reason for such behaviour is a pinhole in the carbon layer, exposing silver trace underneath.

Each biosensor was calibrated as described in Chapter 4.2.1., right after the biosensor was prepared, as well as after one, two, and four weeks of storage in the refrigerator. The parameters of analytical performance (Table 5) were determined from the point calibration curves (Figure 11 and Figure 12) in case of each biosensor and each batch.

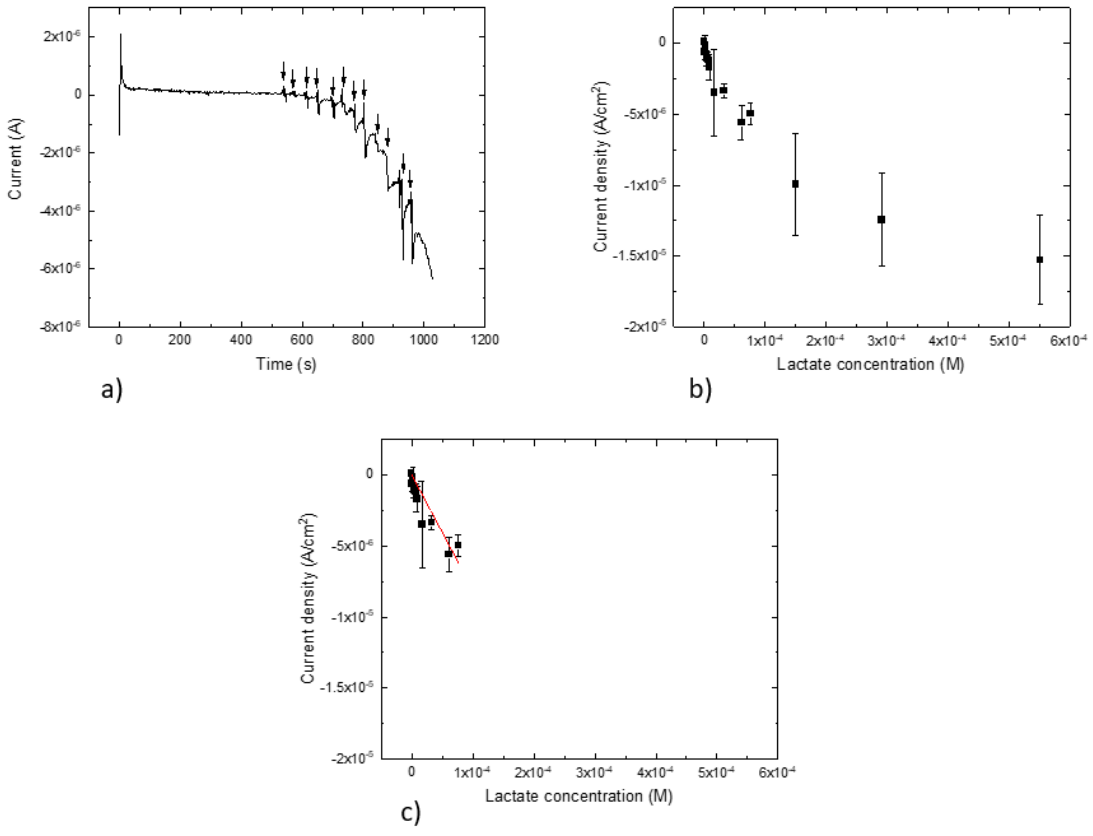

Figure 11. *Calibration of the as prepared LOx-Nafion biosensors (N=4) to determine their sensitivity towards lactate. a) Trace calibration curve recorded at 0.0 V in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 ˚C). The arrows indicate the additions of lactate solution droplets. b) Point calibration curve obtained from the trace calibration curve. c) Point calibration curve with linear fitting (the last three points are excluded).*

On the trace calibration curve of a LOx-Nafion biosensor, the times of the lactate solution-additions can be clearly visible as drops in the current signal, due to the reduction of H_2O_2 by PB (Figure 11.a). The current density decreases with increasing lactate concentration, as can be seen at the point calibration curve, representing the mean sensitivity values of four biosensors (Figure 11.b). The standard deviations in this case are relatively high in the entire concentration range, which can originate from the difference between the individual biosensors within the batch. This indicates that this method would need further optimization. The concentrations used in the calibrations were based on the concentration range of lactate in body fluids, considering the dilution due to the extraction of fluid as well. However, the biosensors did not respond in the lower range and the signal saturated in the higher range. Therefore, not every point was used for the linear fitting, the three last points were excluded from it (Figure 11.c). From the fitting, sensitivity values could also be acquired as the slope of the linear regression, while the R^2 values indicated the goodness of fit.

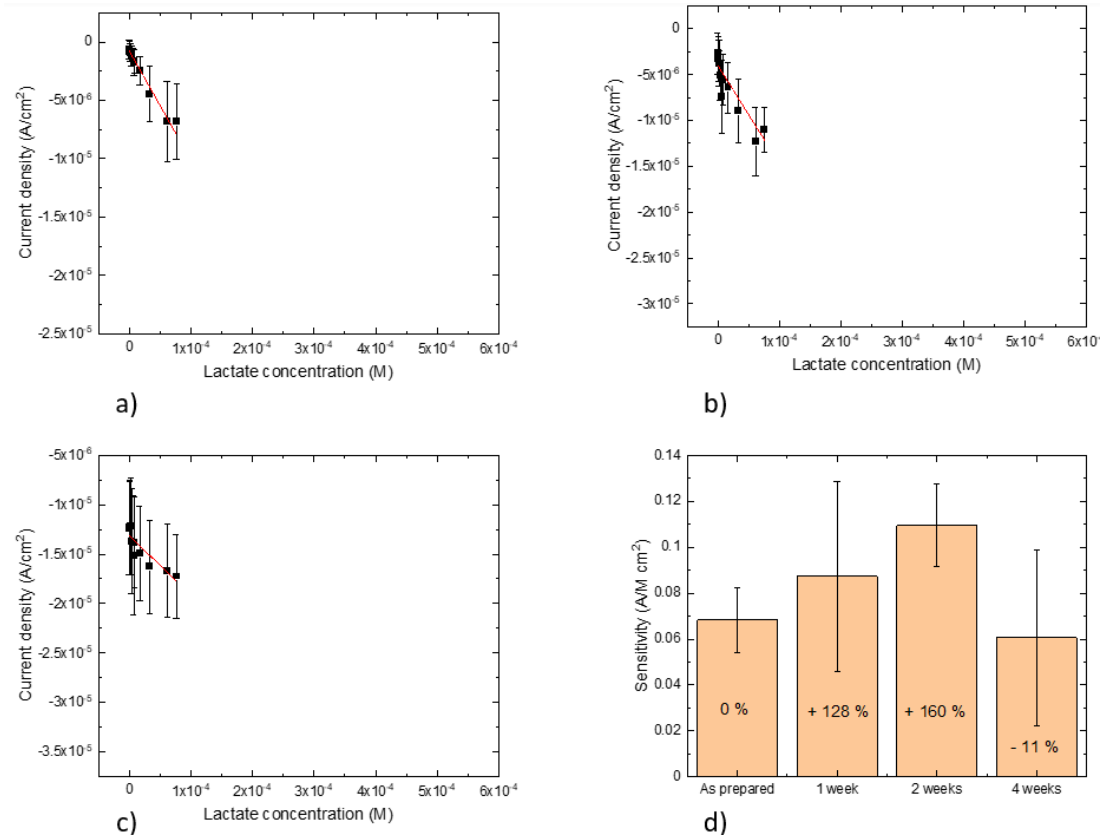

Figure 12. *Point calibration curves of the LOx-Nafion biosensors (N=4) with linear fitting after a) 1, b) 2, and c) 4 weeks of storage in the refrigerator. The last three points of each original curve were excluded from the fitting. d) Investigation of the degradation of the biosensors during the storage in the refrigerator. The change in the percentage value indicates the change in sensitivity compared to the as prepared biosensors. The calibrations were performed by addition of lactate in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 ˚C) while recording the current at 0.0 V.*

The calibrations were performed for the same biosensors after 1, 2, and 4 weeks of storage. The point calibration curves with linear fitting can be seen in Figure 12.a-c. In these curves, the last three points were excluded, as in the case of the as prepared curve to improve the fitting. The goodness of fit has improved after 1 week compared to the as prepared biosensors (Figure 11.c), while it was slightly worse after 2 and 4 weeks. The R^2 values indicate the same and all of the values are relatively acceptable (Table 5).

In Figure 12.d, the mean sensitivity values and standard deviations can be observed for the biosensors calibrated during four weeks. The sensitivity of the biosensors

increased gradually during the first two weeks of storage and the sensitivity values are higher, compared to the LOx-chitosan biosensors. During week 4, the sensitivity decreased significantly compared to week 2 (Table 5). The decrease of the sensitivity during week 4 is most probably the result of enzyme degradation and it is possible that the activity of the enzyme has declined. The relatively high standard deviations, especially in case of the week 1 and 4 biosensors, indicate that the reproducibility within the batches is not ideal. For these reasons, further optimization of the methods is needed.

Shimomura *et al.* have developed an amperometric lactate biosensor by immobilizing LOx in a mesoporous silica with a Nafion layer on a screen-printed carbon electrode. [57] The sensitivity of these biosensors was 0.00454 A/M·cm^2, which is significantly lower than the values of LOx-Nafion biosensors described here (Table 5). However, it is important to note that the enzyme molecules were immobilized in a different way and the electrodes were not PB-modified carbon electrodes in the other work. Therefore, the differences in the experimental setups may explain the difference in the sensitivity values. The results presented here indicate that the immobilization method utilizing Nafion and PB-modified electrodes, are promising.

Table 5. *Analytical performance parameters (sensitivity, R^2, LOD, LOQ) of the LOx-Nafion biosensors (N=4).*

	As prepared	**Week 1**	**Week 2**	**Week 4**
Sensitivity (A/M·cm^2)	0.068 ± 0.014	0.087 ± 0.041	0.110 ± 0.018	0.061 ± 0.038
R^2 (-)	0.8981	0.96914	0.8614	0.81223
LOD (µM)	3.47 ± 3.13	2.08 ± 1.46	4.42 ± 2.36	77.05 ± 71.84
LOQ (µM)	11.57 ± 10.43	6.93 ± 4.85	14.72 ± 7.88	256.83 ± 239.45

The LOD values of the investigated four weeks are in the 2.08-77.05 µM range (Table 5). The LOD values determined after preparation and after 1 and 2 weeks of storage are roughly in the same range. In contrast, the value after 4 weeks is significantly higher. It means that the biosensors could only measure significantly higher concentrations of lactate. This result agrees with the ones presented in Figure 12.d, which also show signs of the enzyme degradation. The above mentioned lactate

biosensors by Shimomura *et al.* had a LOD value of 18.3 μM, which is in the same range with the LOx-Nafion biosensors described here. [57] The LOQ values are in the 6.93-256.83 μM range for the 4-week storage time. The value after four weeks is significantly higher than the other three ones, indicating the decreased performance.

This immobilization method utilizing Nafion is promising from the point of view that the sensitivity values are quite high during the 2-week storage period. However, it is clear that the long-term stability of these biosensors is not satisfactory, since the Nafion membrane and the enzyme most probably degrade during the storage, as can be seen from the decreasing sensitivity and increasing LOD and LOQ values. Therefore, further optimization of the method is needed, together with investigation of the effects of the environmental parameters on the analytical performance.

4.2.4 Immobilization of LOx with PEGDE

The immobilization of LOx by covalently binding it in a PEDGE matrix at ~55˚C was investigated. To test the response of the biosensors, chronoamperometric measurements were run at 0.0 V. The prepared two biosensors did not respond at all, therefore, a lower temperature was tested in the next step to dry them. The second set of two biosensors was produced exactly the same way as the first one, but the drying was implemented in an oven for 18 hours at ~40˚C in this case. The parameters of analytical performance (Table 6) were determined from the point calibration curve (Figure 13).

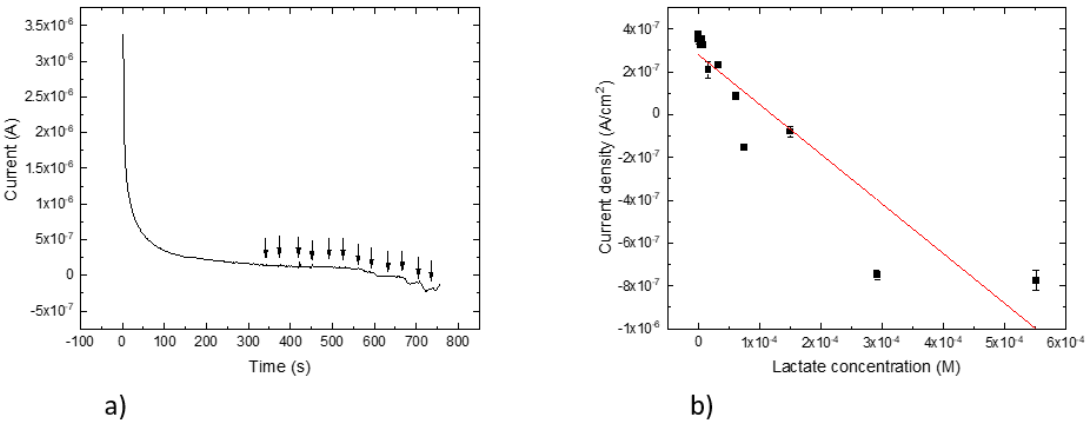

Figure 13. *Calibration of the as prepared LOx-PEGDE biosensors (N=2) to determine their sensitivity towards lactate. a) Trace calibration curve, recorded at 0.0 V in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 °C). The arrows indicate the additions of lactate solution droplets. b) Point calibration curve obtained from the trace calibration curve with linear fitting (all data points are included).*

The calibration results of the two biosensors right after their preparation are presented in Figure 13. The signal drop upon additions of the lactate solutions is almost invisible on the trace calibration curve (Figure 13.a), which indicates that the biosensor did not respond to lactate. In Figure 13.b the mean sensitivity values and standard deviation of the two biosensors can be seen after performing a linear fit. In this case all data points were included in the point calibration curve.

As can be seen from the results, the biosensors did not give any response even after the temperature was decreased for the curing (Figure 13.a). At higher concentrations, a minor decrease can be seen in the current, but it is negligible compared to the previously described LOx-Chitosan and LOx-Nafion biosensors. According to these results, the temperature has a great impact on the LOx enzyme molecules. Since enzymes are proteins, higher temperature can lead to their denaturation. When it is denatured, the protein loses its tertiary structure and native enzymatic activity. [58] It is highly probably that LOx was denatured during the curing process, which makes this method unsuitable for use.

Table 6. *Analytical performance parameters (sensitivity, R^2, LOD, LOQ) of the LOx-PEGDE biosensors (N=2).*

	As prepared
Sensitivity (A/M·cm^2)	$0.002 \pm 1.3 \cdot 10^{-4}$
R^2 (-)	0.8574
LOD (µM)	30.12 ± 19.48
LOQ (µM)	100.41 ± 64.95

The results presented in Table 6 show that the sensitivity of the calibration curve is significantly lower than the values provided by the other methods. This sensitivity of the LOx-PEGDE biosensors means that the biosensors cannot give an acceptable response upon a small change in the concentration of lactate. The LOD and LOQ values are relatively high as well, which means that the biosensors can only detect and quantify higher concentrations. In conclusion, PEGDE can be a good immobilization reagent for other enzymes, but it is not suitable for LOx.

4.2.5 Immobilization of LOx by cross-linking with glutaraldehyde

The immobilization of LOx by cross-linking with GA was also investigated. In this case a batch of six biosensors were prepared. For that, a solution containing 17 mg/ml LOx, 40 mg/ml BSA and 0.6 wt% GA was drop-cast on PB-modified WEs and let dry in room temperature. As a result of the drop-casting process the final enzyme load was 0.34 mg on each WE.

Each biosensor was calibrated as described in Chapter 4.2.1. right after the biosensor was prepared, then after one, two and four weeks of storage in the refrigerator. The parameters of analytical performance (Table 7) were determined from the point calibration curves (Figure 14 and Figure 15) in case of each biosensor and each batch.

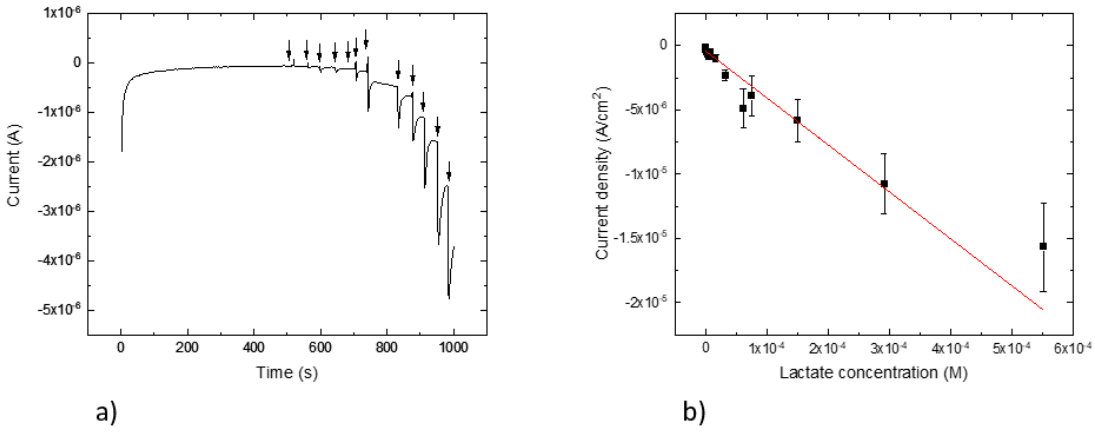

Figure 14. *Calibration of the as prepared LOx-GA biosensors (N=6) to determine their sensitivity towards lactate. a) Trace calibration curve, recorded at 0.0 V in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 ˚C). The arrows indicate the additions of lactate solution droplets. b) Point calibration curve obtained from the trace calibration curve with linear fitting (all data points are included).*

In Figure 14, the trace calibration curve of an as prepared biosensor can be seen. In this case, the lactate additions result in distinct drops in the current signal. The response of the biosensors is quite poor in the lowest concentration range The point calibration curve was prepared by using the mean sensitivity values and standard deviations of six biosensors (Figure 14.b) and the data points were fitted with a linear regression. For the fitting, all data points were utilized since any exclusion of points did not improve the fitting considerably. The current density decreases in this case too with increasing lactate concentrations due to the reduction of H_2O_2 on the WE (Figure 14.b). The standard deviations are higher in the higher concentration range probably due to the differences in the upper limit of the linear range between the individual biosensors within the batch.

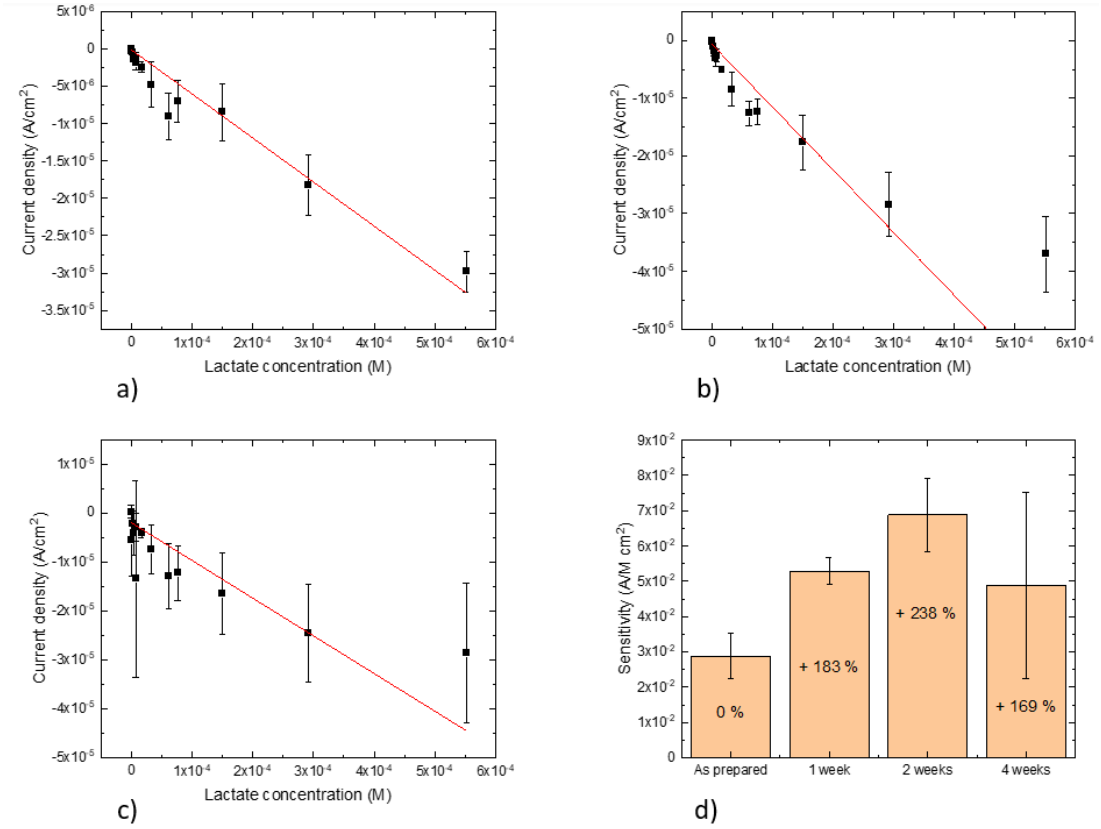

Figure 15. *Point calibration curves of the LOx-GA biosensors (N=6) with linear fitting after a) 1, b) 2, and c) 4 weeks of storage in the refrigerator. All data points included. d) Investigation of the degradation of the biosensors during the storage in the refrigerator. The change in the percentage value indicates the change in sensitivity compared to the as prepared biosensors. The calibrations were performed by addition of lactate in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 ˚C) at 0.0 V.*

The calibrations were performed for the same six biosensors after 1, 2, and 4 weeks of storage (Figure 15.a-c). The goodness of fit of the linear model on the calibration curves is best in case of the as prepared biosensors (Figure 14.b) and after 1 week storage (Figure 15.a) as can be seen in Table 7 as well. The R^2 values are relatively high, but they are quite far from the ideal, where it is at least over 0.9. After 2 and 4 weeks of storage, the goodness of fit decreases significantly, indicated by the lower R^2 values. It can be seen also in Figure 15.b and c that the last points of the calibration curves are not any more in the linear range, *i.e.*, the biosensors are unable to respond the same way as previously. It is possible that the GA matrix and some of the enzyme molecules have degraded during this storage period and, therefore, the biosensors cannot give a proportional response to the highest concentrations anymore. This results in the reduced linear range of the biosensor that may have an impact on the performance of the wearable device using this type of the biosensor.

The mean sensitivity values and standard deviations for the same calibrations are presented in Figure 15.d. The biosensor sensitivity increased significantly during the first 2 weeks of storage compared to the as prepared biosensors. The sensitivity is the highest in case of the calibration after 2 weeks then it starts to decrease. This can be the sign of degradation of the enzyme or the GA matrix. It is possible that the V_{max} of the biosensors decreased, since the number of the enzyme molecules or their activity has declined.[59] Besides, the standard deviation has increased significantly after 4 weeks.

Suman *et al.* has developed an amperometric lactate biosensor, where LOx was immobilized through GA to a polyanaline-co-fluoroaniline film on a glass plate coated with indium tin oxide.[60] This biosensor was designed to determine lactate in serum. Its sensitivity was 0.00118 A/M·cm^2, which is significantly lower than the biosensors' described here (Table 7). This difference can originate from the differences in the immobilization protocols and biosensor construction. Based on these, the results presented here are promising and show potential for these types of biosensors after further optimization.

Table 7. *Analytical performance parameters (sensitivity, R^2, LOD, LOQ) of the LOx-GA biosensors (N=6).*

	As prepared	**Week 1**	**Week 2**	**Week 4**
Sensitivity (A/M·cm^2)	0.029 ± 0.006	0.053 ± 0.004	0.069 ± 0.010	0.049 ± 0.026
R^2 (-)	0.8608	0.8433	0.7182	0.6923
LOD (µM)	2.76 ± 2.63	0.93 ± 0.26	1.59 ± 0.97	122.93 ± 170.99
LOQ (µM)	9.19 ± 8.77	3.09 ± 0.86	5.31 ± 3.23	409.78 ± 569.96

The LOD values are in the 0.93-122.93 µM range of the 4-week investigation (Table 7). For 3 weeks the LOD value does not change significantly, but after the fourth week there is a *ca*. hundred-fold increase. This result agrees with the ones presented on the point calibration curves, and it probably indicates the onset of the degradation processes. The above-mentioned lactate biosensors by Suman *et al.*[60] had a LOD value of 0.1 mM, which is similar to the value after 4 weeks of storage of these LOx-GA biosensors. This means that these freshly prepared LOx-GA biosensors can detect

lower analyte concentrations. The LOQ values are in the 3.09-409.78 μM range during the investigation. Here again the value is significantly higher after 4 weeks.

This method of enzyme immobilization is promising from the point of view, that the biosensors have relatively high sensitivity, which is well maintained throughout most of the storage period. However, there are signs of enzyme degradation after 4 weeks of storage, which can be seen from decrease of the sensitivity. The method still needs further optimization to minimize the enzyme degradation and improve the analytical performance.

4.2.6 Comparison of the enzyme immobilization methods

The sensitivity is one of the most important analytical performance parameters of a biosensor. A high sensitivity means that a biosensor can detect a small change in the analyte concentration in the sample. A biosensor with low sensitivity, however, can only give signal in case of a significant change in the analyte concentration. This concept has serious impact *e.g.* in the field of medical diagnostics, where it is crucial to be able to monitor even the small changes in the analyte concentration in body fluids in order to manage diseases. Therefore, during the development of a new biosensor, one of the most important aims is to reach the highest sensitivity, which is possible and maintain it during the storage and usage, while also maintaining the stability of the biosensor. From the analytical chemistry point of view, the standard deviation of sensitivity has also a great importance. Its high value shows that the reproducibility within the same batch is low, which calls for further optimization of the preparation method.

In order to find the most suitable immobilization method to improve the stability and long-term storage of the LOx and the lactate biosensor, the results of the investigated immobilization methods were compared (Figure 16). For that, the mean sensitivity values of the batches, consisting of six biosensors, were determined for the as prepared biosensors by performing calibration with lactate solutions as described in Chapters 4.2.1-5. In case of the method with Nafion, one of the biosensors was treated as outlier due to possible experimental error, therefore, this batch only included five biosensors. When calibrating these biosensors, one of the five biosensors had almost eight times

higher sensitivity value compared to the other biosensors during the calibration performed right after the preparation. This sensor was also excluded from the following data analysis.

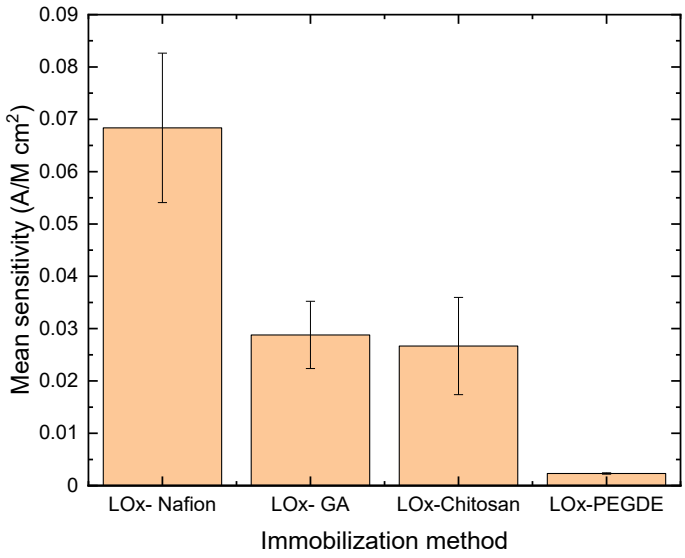

Figure 16. *Mean sensitivity of batches (N=6) of as prepared lactate biosensors prepared with the different immobilization methods (for LOx-Nafion N=4 and for LOx-PEGDE N=2). The biosensors were calibrated with lactate (in PBS) to determine the sensitivity as the slope of the point calibration curves for each biosensor within a batch, then the mean sensitivity and its standard deviation was calculated for every batch.*

The mean sensitivity of the LOx-Nafion biosensor batch significantly exceeds the values of the other immobilization methods. Namely, the value of LOx-Nafion is more than two times higher than the values of the LOx-GA and LOx-chitosan biosensor batches, which latter are nearly equal. In contrast, the PEGDE biosensor batch has the lowest mean sensitivity, not significantly higher than zero.

The relatively high sensitivity in case of the investigated three methods, can be supported by different explanations. These immobilization methods can possibly keep the enzyme near the WE and thereby minimize their leaching.[55] These methods can also help the enzyme molecules to preserve their native structure and activity and prevent their denaturation by providing an environment similar to the native one within the cells.[40] The immobilization method utilizing PEGDE requires an elevated temperature for the biosensor curing. However, this most probably denatures the

enzyme. Therefore, this immobilization method is not suitable for LOx, although it can be used for other enzymes.

The results of the sensitivity investigation show that the immobilization methods using chitosan, Nafion, and GA are suitable for the immobilization of LOx on the WE and, therefore, for improving the long-term stability of the biosensors. From the three methods, the one with Nafion seems to be the most promising with the current version of the protocols. However, the relatively high standard deviations indicate that the reproducibility within the batches is not ideal. This means that further optimization of the methods is needed to reach even higher sensitivity, reproducibility, and stability. In case of the method, utilizing PEGDE, the results show that it is not suitable for the immobilization of LOx, since the relatively high temperature needed for the biosensor preparation probably denature the enzyme. Therefore, these LOx-PEGDE biosensors were not included in the following tests.

4.3 Investigation of the selectivity of the lactate biosensors

Different interfering components can affect the analytical performance of the biosensors by falsely changing the analytical signal and hiding the true specificity of the biosensor. Therefore, the selectivity of the lactate biosensors was tested for five different interfering agents, glucose, paracetamol, uric acid (UA), ascorbic acid (AA) and dopamine (D). These can be found in the human body fluids and can cause interference by being oxidized at the WE in the same potential range as H_2O_2.

For the interference tests, the concentrations of the given component in interstitial fluid (ISF), in serum or in plasma were used based on the data found in scientific literature (Table 8). The concentrations, found in the literature were divided by 200, which represents the dilution of the analyte due to the extraction through the skin. The concentration of lactate is 1.0-2.0 mM in blood and in ISF at rest.[30] However, the five-times multiplied value of the mean, *i.e.*, 7.5 mM was used instead, to acquire a reliable response for lactate. For glucose, the typical plasma value of 5.0 mM was used.[61] A typical level for ascorbic acid (AA), is 250 μM in plasma according to *Mishra et al.*[62] and 300 μM for uric acid (UA).[63] In case of paracetamol, the concentration was chosen to be 1.0 mM. According to *Freeman et al. a* typical concentration in serum after an

oral dose is less than 1.0 mM.[64] Therefore, this value was chosen, although this high level is already toxic for humans: if the concentration is over 100 µg/ml six hours after administration, it is considered as a risk for liver dysfunction.[65] The high concentration was picked, since it was thought that if the biosensors do not show any response or only a small one for this, the smaller concentrations would be acceptable too. Finally, for dopamine, a typical concentration in blood is 195.8 pM.[66]

Table 8. *Concentration of lactate and the interfering components, used in the interference tests, in ISF (*), in serum (‡) or in plasma (†). The concentration which was chosen for the test was divided by the dilution factor 200 to obtain the concentration of the solution on the WE.*

Component	Concentration in body fluid	Picked concentration	Final concentration on the WE (µM)
lactate	1.0-2.0 mM * †	7.5 mM	37.50
glucose	5 mM †	5 mM	25.00
paracetamol	< 1 mM ‡	1 mM	5.00
AA	250 µM †	250 µM	1.25
UA	300 µM †	300 µM	1.50
D	195.8 pm †	195.8 pm	$9.79 \cdot 10^{-7}$

For the interference tests, two new biosensors were prepared as described in Chapter 3.5 by using chitosan, Nafion, and GA for the immobilization. During the test of each biosensor, the current was recorded at 0.0 V: first to acquire a stable baseline in PBS, then to test lactate and the interfering components indicated in Table 8 (Figure A 4 - Figure A 6 in Appendix).

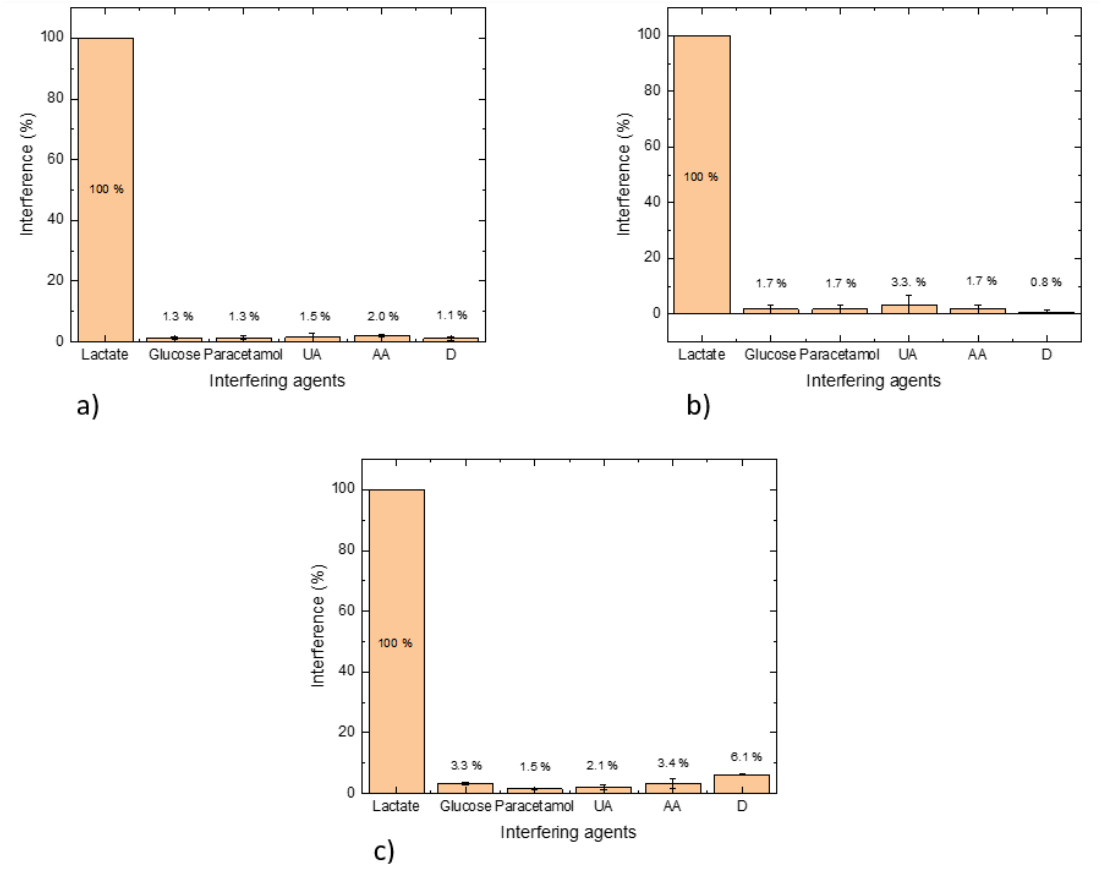

Figure 17. *Interference tests of the biosensors with glucose, paracetamol, uric acid (UA), ascorbic acid (AA), and dopamine (D) with concentrations indicated in Table 8. a) Interference test of LOx-chitosan biosensors (N=2), b) LOx-GA biosensors (N=2), and c) LOx-Nafion biosensors (N=2). The original chronoamperometric curves were recorded at 0.0. V in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 ˚C).*

The signals for lactate and the interfering components were taken as the difference between the current before the addition of the solution on the WE and the drop in the current after the same addition. To compare the signal of the different components, the signal of lactate was set as reference value and the signals of the interfering agents have been normalized with it (Figure 17).

As the results show, each type of biosensor gave some response to the interfering components (Figure 17). However, it can be said that the interference was minor in case of all the immobilization methods. The LOx-chitosan biosensors were the most selective among the three investigated types (Figure 17.a), while the LOx-GA (Figure 17.b) and LOx-Nafion (Figure 17.c) biosensors gave slightly higher response to the interfering agents. The LOx-Nafion biosensors had the lowest selectivity, and these

biosensors gave the highest signal in case of glucose, AA and D. The LOx-GA biosensors were the less selective toward UA, but the standard deviation was also rather high in this case. The results show that these biosensors are not entirely selective for only one component as it would be desirable in case of an ideal biosensor. However, a big difference can be seen between lactate and the other interfering agents, which originates from the high selectivity of LOx towards lactate. Therefore, the source of the minor interferences is probably not the poor specificity of the biorecognition element, but it can possibly come from the direct oxidation of the components at the WE.[67]

The results show that the different interfering agents caused some interference in each type of lactate biosensors. A desirable biosensor is interference-free.[68] However, this is an unrealistic aim and impossible to reach. Instead, a realistic aim is to keep the interference as low as possible. From this point of view, these results are promising since the interference was relatively low, and with some further optimization, this parameter can probably be improved.

4.4 Investigation of the operational stability of the lactate biosensors

To test the operational stability, two new biosensors were prepared by using three different immobilization protocols utilizing chitosan, Nafion and GA. The biosensors were tested by recording the current signal at 0.0 V for 8 hours in a solution containing 91 mM lactate (Figure A 7-Figure A 9 in Appendix). The stability tests were run for 8 hours, the length of a workday since ideally a biosensor, intended to be a wearable device, must maintain its stability for a long period of time.

The operational stability diagrams were calculated from the chronoamperometric curves (Figure 18). The first point was taken after the current response had stabilized upon the addition of lactate solution, the second point was around half-time of the test, while the last point at the end of the 8-hour measurement. The percentage values of the diagram were calculated by normalizing the current values of the chronoamperometric curves (I) with the reference point, *i.e.*, the first stable point after the addition of lactate (I_0).

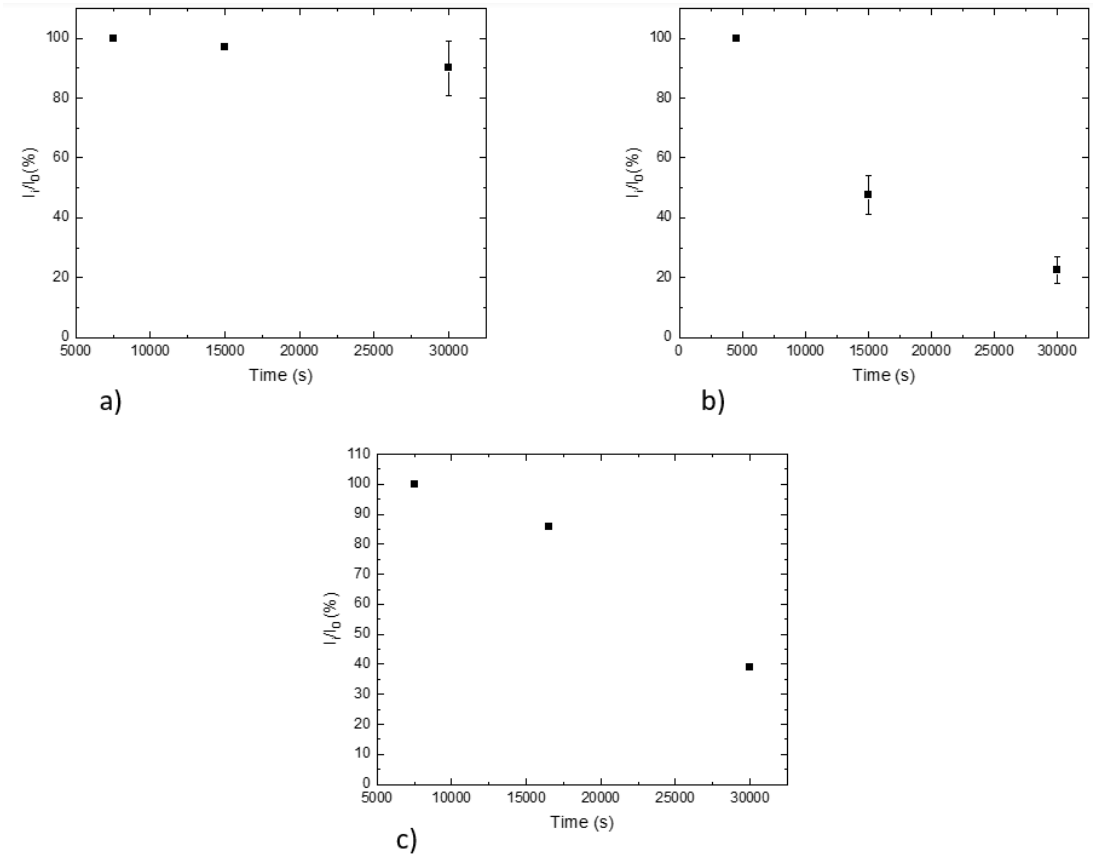

Figure 18. *Operational stability tests of lactate biosensors prepared with different immobilization methods. a) LOx-chitosan biosensors (N=2), b LOx-Nafion biosensors, (N=2) c) LOx-GA biosensor (N=1). The diagrams were calculated from the chronoamperometric curves by normalizing the current signal (I) with the first point, i.e., the reference point (I_0). The chronoamperometric curves were recorded in 91 mM lactate solution (in PBS (pH: 7.4, I= 154 mM, c= 50 mM, T = 25˚C)) at 0.0 V for 8 hours.*

The first point is of the diagram is 100% for each sensor since it was taken as the reference point. The value of LOx-chitosan biosensors has decreased to ~97 % and ~90% of the original value after 4 and 8 hours of run, correspondingly (Figure 18.a). The LOx-Nafion biosensors were quite unstable already at the beginning of the test, and after 4 and 8 hours of run, the value has decreased to ~48 % and ~23 % (Figure 18.b). Finally, the value of the LOx-GA biosensor has decreased to ~86% and ~39% after 4 and 8 hours (Figure 18.c). In case of the LOx-GA method, the result is from only one biosensor since the other one was excluded due to possible experimental error (Figure A 9.a in Appendix).

The percentage values originated from the operational stability tests give valuable information on the biosensors since the less the decrease in the signal in the investigated time range, the more stable the biosensor is. The LOx-chitosan biosensors had the best operational stability since they maintained their stability well under the 8-hour period. In contrast, the LOx-Nafion biosensors had the lowest operational stability: the normalized current signal decreased to the quarter of the original value during the 8 hours. The LOx-GA biosensors maintained their stability relatively well in the first half of the measurement, but at the end of the 8-hour period the signal was quite low.

The operational stability of the biosensors can be affected by many factors, such as the change in the pH. During the operation of the biosensor, the H_2O_2 is reduced to hydroxyl ion by PB at the WE, which can possibly result in a significant change in the pH during 8 hours of measurement. However, this should not occur in buffer, if the buffer concentration is higher than the concentration of H_2O_2. LOx is an enzyme and, therefore, it is sensitive to the changes of pH, which can lead to its denaturation and decrease of the enzymatic activity. In case of LOx-Nafion biosensors, it is possible that the Nafion matrix was not stable over time either since the Nafion membrane may have shrunk due to the change in the pH. This could cause changes in biosensor response and lead to lower operational stability.[59] It is also possible that the local pH of the Nafion film was too low, since it was in acidic form. In case of LOx-GA biosensors, it is possible that the cross-linking process has not had enough time to reach completion. This could lead to incomplete enzyme-immobilization, then degradation and leaching and finally decreased operational stability.[47]

In conclusion, the LOx-chitosan biosensors maintained their stability the best during the 8-hour measurement period. From this point of view this enzyme immobilization method almost meets the requirements of operational stability performance. However, the biosensors prepared with Nafion and GA would need further optimization to be more stable. This problem can be addressed by the investigation of different environmental factors (*e.g.* pH) on the operational stability.

5 Conclusions

Four different methods of enzyme immobilization have been studied in this work to prepare lactate biosensors. The calibration results showed that one of the four reagents, PEGDE was not suitable for LOx immobilization since the enzyme probably denatured by the elevated temperature used during the curing process of the biosensors. The other three methods, utilizing chitosan, Nafion, and glutaraldehyde for the immobilization, were examined further with operational stability and interference tests, since they gave promising results when investigating the sensitivity of biosensors towards lactate.

The LOx-Nafion biosensors had the highest sensitivity among the methods with the value of 0.110 ± 0.018 A/M·cm^2 during the week 2 calibration. However, some of the sensitivity values in these biosensors had high standard deviation, which means that the reproducibility was relatively low for these biosensors. This immobilization method is promising since the sensitivity values are high, even higher than the ones for similar biosensors in the literature. However, the storage stability of these biosensors should be improved, since during 4 weeks of storage, the sensitivity value decreased significantly. The sensitivity of the LOx-chitosan biosensors increased from 0.027 ± 0.009 A/M·cm^2 to 0.071 ± 0.009 A/M·cm^2 during the 4-week storage period. The increase in the sensitivity can possibly be explained with the change in structure of the chitosan matrix, which enabled the better access of the enzyme to the lactate. The operational stability of these was the best of among the three methods: the signal decreased only with ~10% during the 8-hour measurement period. The selectivity was also the highest for these biosensors from the three methods. The sensitivity of the LOx-GA biosensors increased from 0.029 ± 0.006 A/M·cm^2 to 0.069 ± 0.010 A/M·cm^2 during the 2 weeks of storage. However, there were signs of enzyme degradation afterwards since the sensitivity decreased to 0.049 ± 0.026 A/M·cm^2 for the end of the 4-week investigation. The signal during the operational stability test of the LOx-GA biosensors decreased to ~61% during the 8-hour measurement period. Furthermore, these biosensors were quite selective towards lactate too. However, this method possibly needs further optimization to minimize the enzyme degradation and to improve the analytical performance.

Biosensors should be designed as highly sensitive devices to detect even the smallest changes in the analyte concentration. An ideal biosensor would also be interference-free. However, this is an unrealistic aim and impossible to reach. The aim is to keep the interference as low as possible, as it is in these biosensors. From this point of view, these results are quite promising and with some further optimization, the interference parameters may be improved further. The biosensor should also keep its operational stability during at least a workday, which is about 8 hours. It is also important that it maintains its stability during storage. In conclusion, three biosensors prepared with immobilization reagents, chitosan, Nafion, and GA, provided promising results regarding these criteria. However, the methods need further optimization to improve the analytical performance of the biosensors.

6 Swedish summary – Svensk sammanfattning

Prototypsframställning av laktatbiosensorer för icke-invasiv biomarköruppföljning

Diabetes och flera andra sjukdomar sprider sig alltmer aktivt bland populationen i dagens läge, och diagnostiseringen av sjukdomar har därmed ökat. Det ökade diagnostiseringsbehovet har lett till en ökad efterfrågan av hälsouppföljning. Att undersöka hälsan med hjälp av biosensorer är ett enkelt och förmånligt sätt som har blivit alltmer aktuellt under de senaste åren. Biosensorer är små, känsliga och förmånliga apparater som lätt kan användas för att upptäcka olika analyter. [1] Dessa verktyg har mycket potential inom klinisk användning, även i industrier som fokuserar på livsmedel, kemi och miljöövervakning. Nya biosensorer, speciellt icke-invasiva är av stort intresse inom klinisk diagnostik. Icke-invasiva biosensorer är ett mera användarvänligt alternativ till invasiva lösningar. Med hjälp av biosensorerna kan man undersöka viktiga analyter i människokroppen som är essentiella för människans hälsa.

Biosensorer består av två delar: ett bioigenkänningselement och en transduktor. Bioigenkänningselementet har oftast biologiskt ursprung och fungerar som den upptäckande delen för den analyt som söks. Till exempel enzymer såsom laktatoxidas kan användas som ett bioigenkänningselement i biosensorer, och då kallas biosensorn för en enzymatisk biosensor. Transduktorn omvandlar signalen från en bioigenkänningshändelse till en fysisk signal som i sin tur kan avläsas. Utifrån den fysiska signalen kan koncentrationen av analyten inom provet bestämmas.[1–3]

Några av de största utmaningarna med biosensorer är att de ska hålla sin stabilitet och sin känslighet under lagringstiden. Detta är viktigt för att biosensorerna ska ge tillförlitliga resultat under sin livstid och efter lagring. Utmaningarna kan överkommas med hjälp av olika immobiliseringsmetoder. I den här avhandlingen behandlas olika immobiliseringsmetoder som kan användas för att förbättra stabiliteten och känsligheten hos laktatbiosensorer. Laktatoxidas används som ett biologiskt element i undersökningens biosensorer.

Elektrokemiska biosensorer är en av de mest använda typerna av biosensorer, i och med att de erbjuder ett snabbt och känsligt sätt att upptäcka olika analyter. Elektrokemiska biosensorer består oftast av tre delar: en arbetselektrod, en motelektrod och en referenselektrod. Bioigenkänningshändelserna förekommer på ytan av arbetselektroden. Under processen sänks elektroderna ner i en lösning som heter elektrolyt som fäster sig på ytan av elektroderna. Elektrolyten innehåller den sökta analyten.[4]

Laktat är en metabolit som bildas i musklerna under anaerob metabolism av glukos. Laktat är en viktig parameter för hälsan och förhöjda laktatnivåer kan vara ett tecken på sjukdom. Ifall laktatkoncentrationen i kroppen har ökat, kan detta vara ett tecken på diabetes eller hjärtsjukdom. [27] Därför har behovet för laktatbiosensorer ökat och undersökningen av nya lösningar fortsätter. Laktatkoncentrationen inom olika prov kan bestämmas med hjälp av enzymatiska biosensorer. Som tidigare nämnts är laktatoxidas ett enzym som kan användas i enzymatiska biosensorer. Laktatoxidas fungerar som en katalysator i reaktionen där L-laktat omvandlas till pyruvat och väteperoxid (H_2O_2). Den andra reaktionsprodukten, H_2O_2, kan sedan reduceras eller oxideras på ytan av arbetselektroden. Den bildade strömmen från reaktionen är direkt proportionell till laktatkoncentrationen i provet. Detta kan undersökas med hjälp av amperometri, som är en elektrokemisk mätningsteknik.[31, 32] För att stabilisera laktatoxidas på ytan av arbetselektroden, måste den immobiliseras.

För att förbättra biosensorernas långtidsstabilitet och respons, kan olika slags immobiliseringsmetoder användas. Enzymer kan immobiliseras på elektroden med hjälp av olika metoder. Metoderna kan exempelvis vara fysisk immobilisering på elektroden med hjälp av polymerer såsom chitosan och Nafion. En annan metod är tvärbindning av enzymmolekyler med hjälp av olika reagenser.[40] Den här avhandlingen behandlar olika immobiliseringsmetoder för laktatoxidas. Målet är att förbättra laktatbiosensorernas stabilitet och känslighet, med fokus på olika immobiliseringsmetoder. De metoder som har undersökts är: immobilisering med polyetylenglykoldiglycidyleter (PEGDE), tvärbindning av enzymmolekylerna med glutaraldehyd, chitosanmatriser och Nafionmatriser. Både chitosanmatriser och Nafionmatriser har använts för att fånga enzymet fysiskt på elektroden.

Tre av de fyra immobiliseringsmetoderna gav lovande resultat. Metoden med PEGDE var inte en passande metod för immobilisering av laktatoxidas. Detta kan bero på enzymets denaturering under elektrodernas torkning, som utfördes i cirka 40 grader Celcius. De andra tre metoderna med chitosan, Nafion och glutaraldehyd gav lovande resultat efter undersökning av biosensorernas känslighet för laktat. Biosensorerna gjorda med Nafion och laktatoxidas hade den högsta känsligheten. Detta betyder att även de minsta ändringarna i laktatkoncentrationen är möjliga att upptäcka. Även biosensorernas selektivitet undersöktes och resultaten visade att de tre biosensorerna var väldigt selektiva för laktat. Däremot var dessa biosensorer inte lika selektiva mot andra vanliga ämnen i kroppen, såsom glukos och dopamin. I en ideal laktatbiosensor skulle det inte finnas någon interferens för andra ämnen än laktat, men detta är omöjligt att nå i praktiken. Däremot är den låga selektiviteten för glukos och dopamin ett lovande resultat. Till sist testades biosensorernas stabilitet under en åtta timmars period. Resultaten visade att biosensorerna gjorda med chitosan och laktatoxidas hölls mest stabila under tidperioden på åtta timmar.

Även om resultaten var lovande hos de tre fungerande metoderna, syntes det också tecken på biosensorernas försämring under lagringstiden, som var fyra veckor. Detta kan exempelvis bero på att enzymet har förlorat sin aktivitet. Med optimering av dessa immobiliseringsmetoder är det eventuellt möjligt att förbättra känsligheten, selektiviteten och stabiliteten av laktatbiosensorer i framtiden.

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8 Appendix

Trace and point calibration curves after 1, 2 and 4 weeks storage

LOx-chitosan biosensors

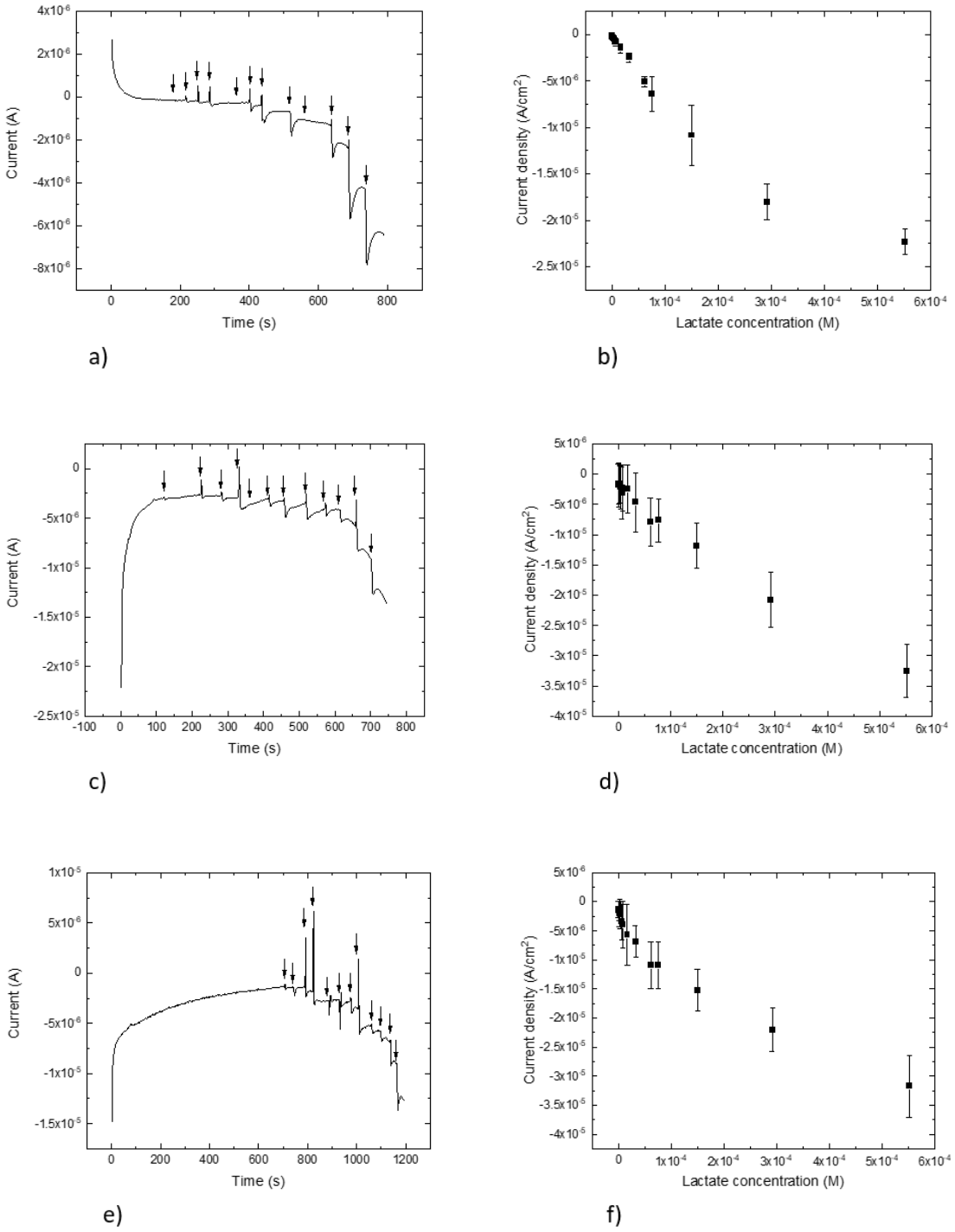

***Figure A 1**. Calibration of the LOx-chitosan biosensors (N=6) after 1, 2 and 4 weeks of storage in the refrigerator to determine their sensitivity towards lactate. Trace calibration curve recorded at 0.0 V in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 ˚C) a) after 1 week, c) 2 weeks, and e) 4 weeks storage., Point calibration curves obtained from the trace calibration curves after b) 1 week, d) 2 weeks, and f) 4 weeks storage.*

LOx-Nafion biosensors

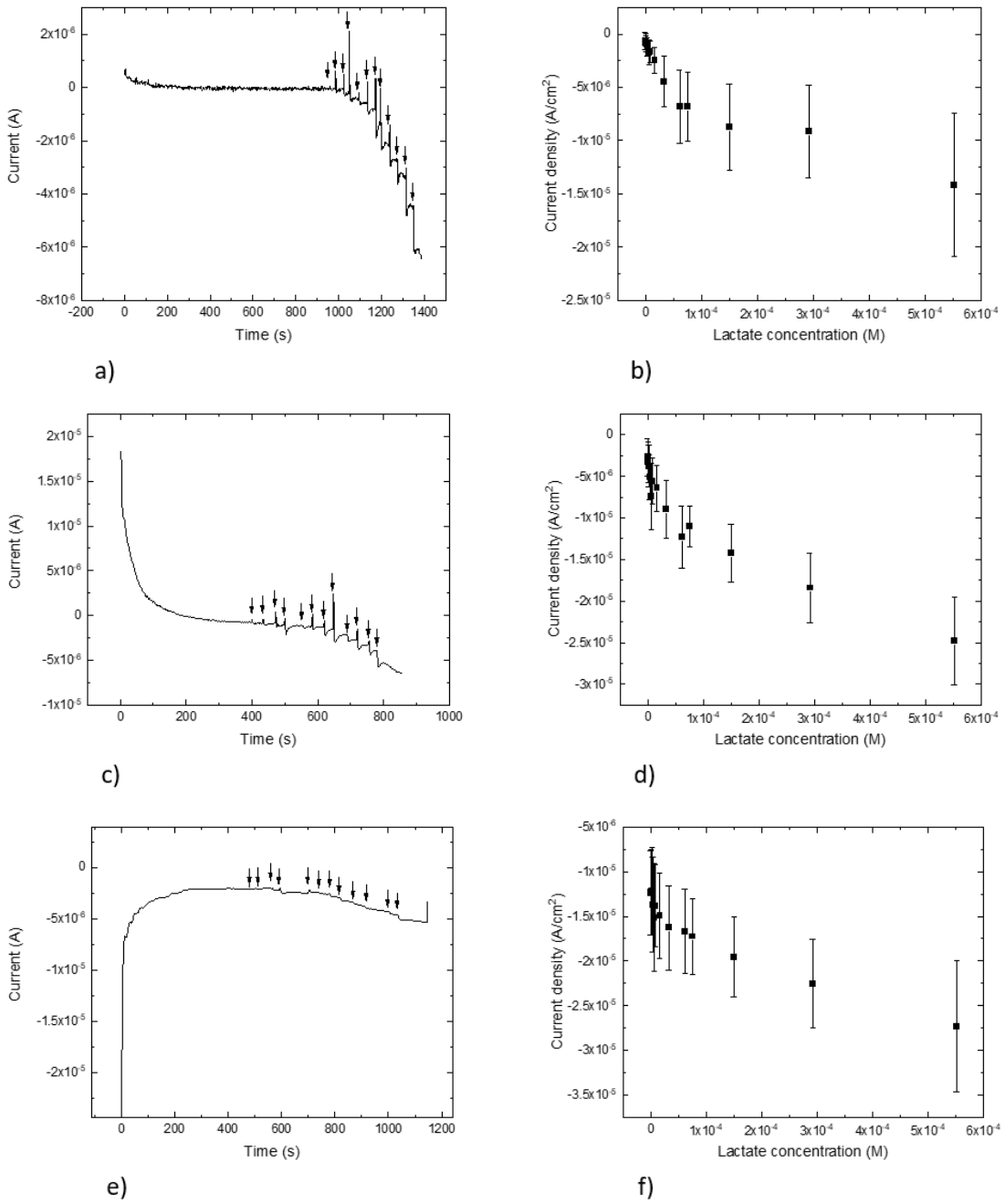

Figure A 2. *Calibration of the LOx-Nafion biosensors (N=5) after 1, 2 and 4 weeks of storage in the refrigerator to determine their sensitivity towards lactate. Trace calibration curve recorded at 0.0 V in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 ˚C) a) after 1 week, c) 2 weeks, and e) 4 weeks storage., Point calibration curves obtained from the trace calibration curves after b) 1 week, d) 2 weeks, and f) 4 weeks storage.*

LOx-GA biosensors

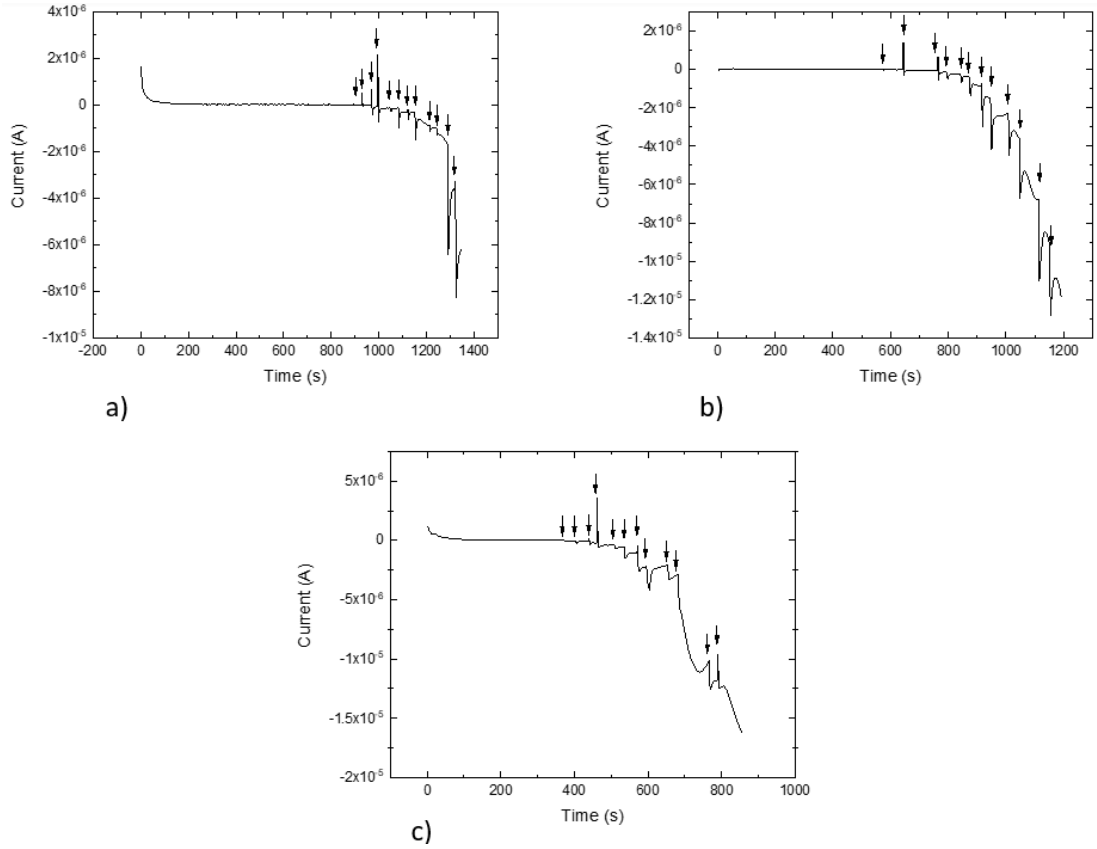

Figure A 3. *Calibration of the LOx-GA biosensors (N=6) after 1, 2 and 4 weeks of storage in the refrigerator to determine their sensitivity towards lactate. Trace calibration curve recorded at 0.0 V in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25˚C) a) after 1 week, c) 2 weeks, and e) 4 weeks storage., Point calibration curves obtained from the trace calibration curves after b) 1 week, d) 2 weeks, and f) 4 weeks storage.*

Interference tests

LOx-chitosan biosensors

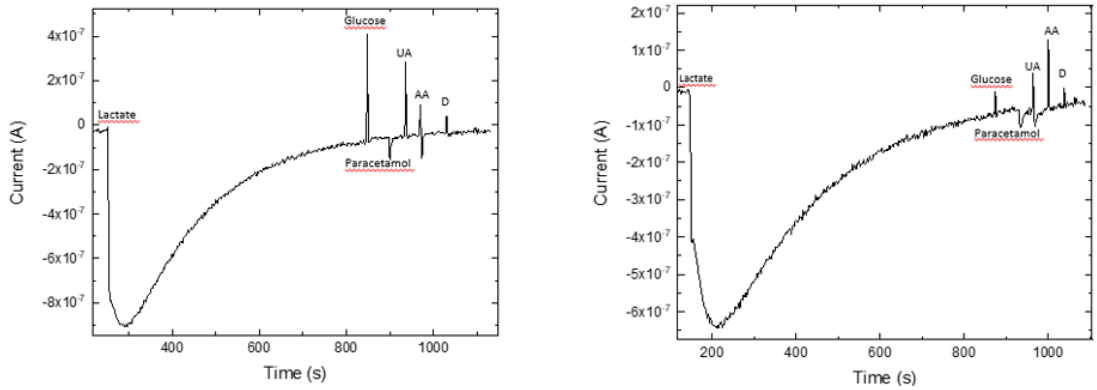

Figure A 4. *Interference test of LOx-chitosan biosensors with glucose, paracetamol, uric acid (UA), ascorbic acid (AA) and dopamine (D) interfering agents. The spikes with the names above on the curves indicate the addition times of the interfering agents. The curves are from recording the current at 0.0 V upon the addition of the interfering components (in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 ˚C)).*

LOx-Nafion biosensors

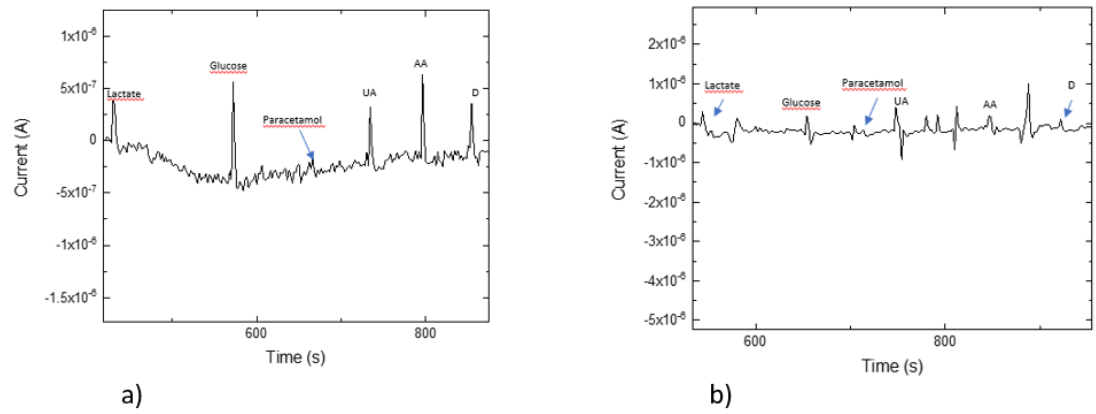

Figure A 5. *Interference test of LOx-Nafion biosensors with glucose, paracetamol, uric acid (UA), ascorbic acid (AA) and dopamine (D) interfering agents. The spikes with the names above on the curves indicate the addition times of the interfering agents. The curves are from recording the current at 0.0 V upon the addition of the interfering components (in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 ˚C)).*

LOx-GA biosensors

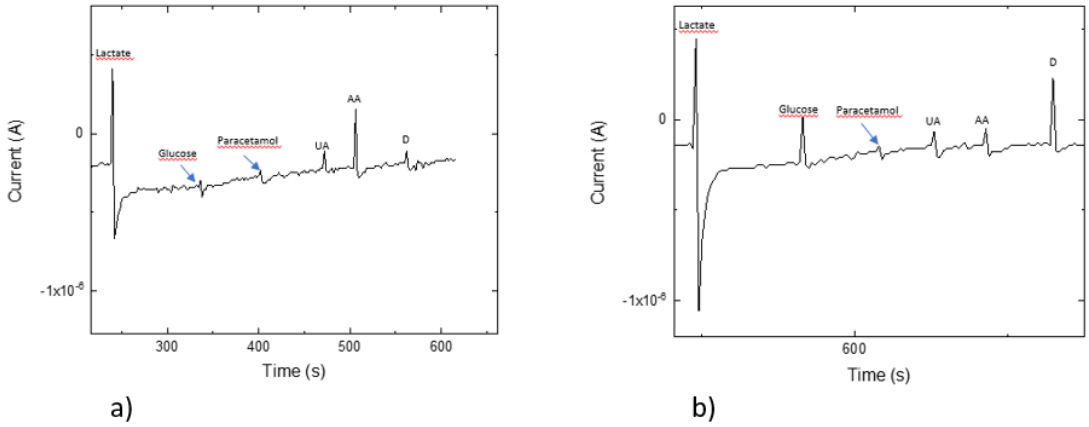

Figure A 6. *Interference test of LOx-GA biosensors with glucose, paracetamol, uric acid (UA), ascorbic acid (AA) and dopamine (D) interfering agents. The spikes with the names above on the curves indicate the addition times of the interfering agents. The curves are from recording the current at 0.0 V upon the addition of the interfering components (in PBS (pH: 7.4, I = 154 mM, c= 50 mM, T = 25 ˚C)).*

Operational stability tests

LOx-chitosan biosensors

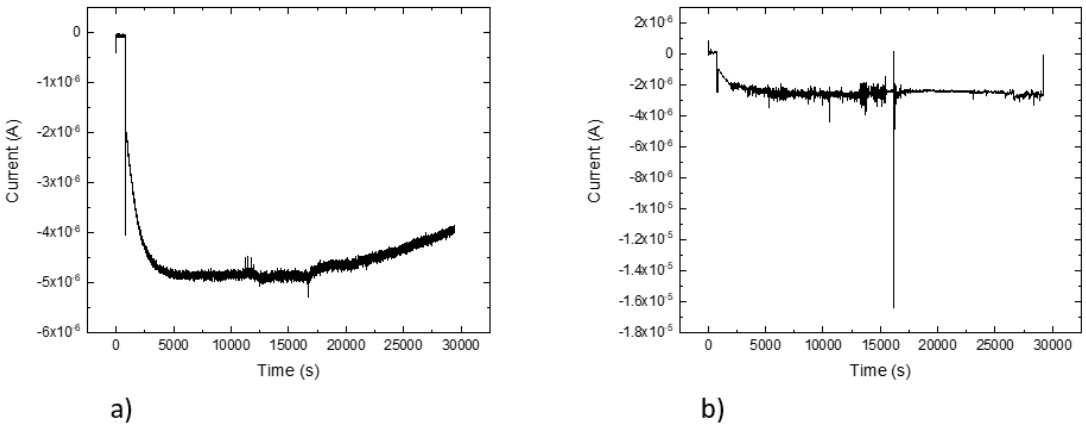

Figure A 7. *Operational stability test of LOx-chitosan biosensors. The curves were recorded at 0.0 V for 8 hours in PBS (pH: 7.4, I= 154 mM, c= 50 mM, T = 25 ˚C). The drop in the current at the beginning of the measurement indicate the addition of 91 mM lactate solution.*

LOx-Nafion biosensors

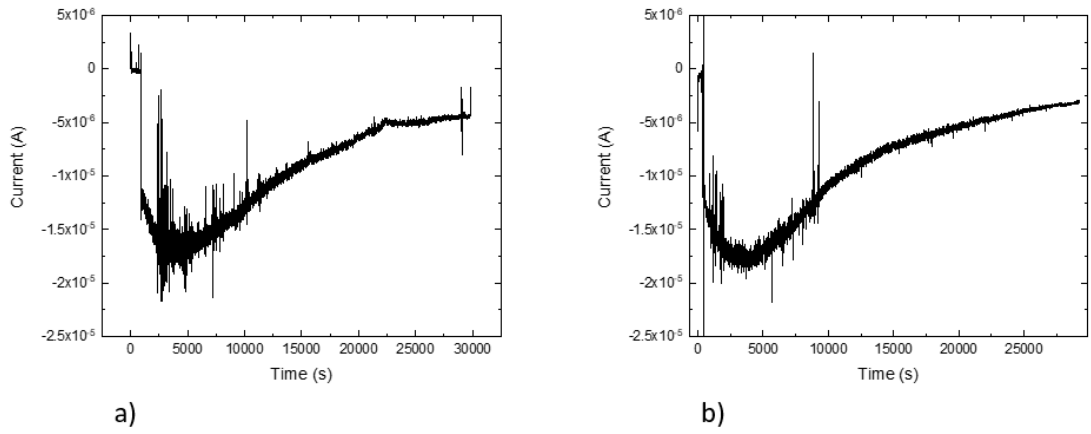

Figure A 8. *Operational stability test of LOx-Nafion biosensors. The curves were recorded at 0.0 V for 8 hours in PBS (pH: 7.4, I= 154 mM, c= 50 mM, T = 25 ˚C). The drop in the current at the beginning of the measurement indicate the addition of 91 mM lactate solution.*

LOx-GA biosensors

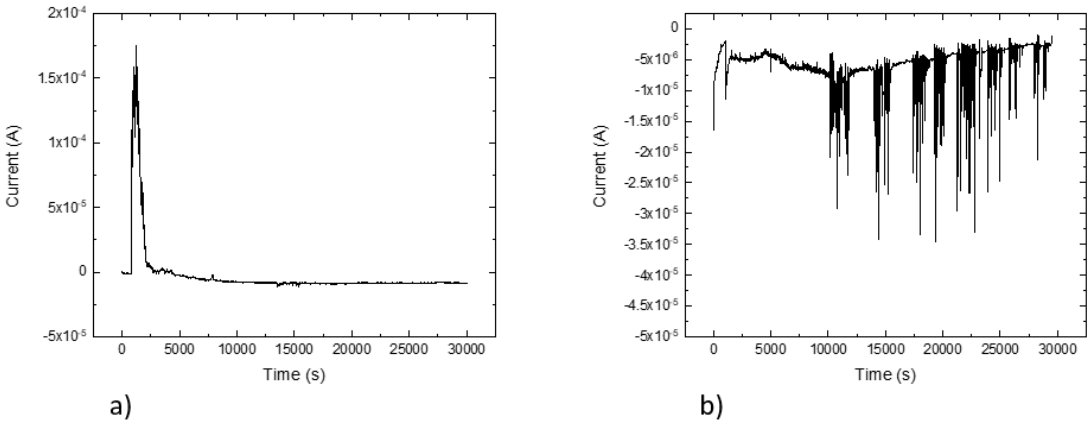

Figure A 9. *Operational stability test of LOx-GA biosensors. The curves were recorded at 0.0 V for 8 hours in PBS (pH: 7.4, I= 154 mM, c= 50 mM, T = 25 ˚C). The drop in the current at the beginning of the measurement indicate the addition of 91 mM lactate solution.*