Single-Use Printed Biosensor for L-Lactate and Its Application in Bioprocess Monitoring

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Keywords: in-line monitoring, at-line measurement, screen-printing, off-line monitoring, enzyme electrode, lactate biosensor

Abstract:

There is a profound need in bioprocess manufacturing for low-cost single-use sensors that allow timely monitoring of critical product and production attributes. One such opportunity is screen-printed enzyme-based electrochemical sensors, which have the potential to enable low-cost online and/or off-line monitoring of specific parameters in bioprocesses. In this study, such a single-use electrochemical biosensor for lactate monitoring is designed and evaluated. Several aspects of its fabrication and use are addressed, including enzyme immobilization, stability, shelf-life and reproducibility. Applicability of the biosensor to off-line monitoring of bioprocesses was shown by testing in two common industrial bioprocesses in which lactate is a critical quality attribute (Corynebacterium fermentation and mammalian Chinese hamster ovary (CHO) cell cultivation). The specific response to lactate of the screen-printed biosensor was characterized by amperometric measurements. The usability of the sensor at typical industrial culture conditions was favorably evaluated and benchmarked with commonly used standard methods (HPLC and enzymatic kits). The single-use biosensor allowed fast and accurate detection of lactate in prediluted culture media used in industrial practice. The design and fabrication of the biosensor could most likely be adapted to several other critical bioprocess analytes using other specific enzymes. This makes this single-use screen-printed biosensor concept a potentially interesting and versatile tool for further applications in bioprocess monitoring.

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Single-Use Printed Biosensor for L-Lactate and Its Application in Bioprocess Monitoring

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Abstract: There is a profound need in bioprocess manufacturing for low-cost single-use sensors that allow timely monitoring of critical product and production attributes. One such opportunity is screen-printed enzyme-based electrochemical sensors, which have the potential to enable low-cost online and/or off-line monitoring of specific parameters in bioprocesses. In this study, such a single-use electrochemical biosensor for lactate monitoring is designed and evaluated. Several aspects of its fabrication and use are addressed, including enzyme immobilization, stability, shelf-life and reproducibility. Applicability of the biosensor to off-line monitoring of bioprocesses was shown by testing in two common industrial bioprocesses in which lactate is a critical quality attribute (*Corynebacterium* fermentation and mammalian Chinese hamster ovary (CHO) cell cultivation). The specific response to lactate of the screen-printed biosensor was characterized by amperometric measurements. The usability of the sensor at typical industrial culture conditions was favorably evaluated and benchmarked with commonly used standard methods (HPLC and enzymatic kits). The single-use biosensor allowed fast and accurate detection of lactate in prediluted culture media used in industrial practice. The design and fabrication of the biosensor could most likely be adapted to several other critical bioprocess analytes using other specific enzymes. This makes this single-use screen-printed biosensor concept a potentially interesting and versatile tool for further applications in bioprocess monitoring.

Keywords: lactate biosensor; enzyme electrode; off-line monitoring; screen-printing; at-line measurement; in-line monitoring

1. Introduction

The need for sensors that can contribute to make biological production more efficient and better controlled is profound [1–3]. Sensors able to monitor critical process events in close to real time are especially needed. This includes changes in concentrations of critical process parameters (CPPs) and critical quality attributes (CQAs) that enable active control to ensure optimal production conditions of the bioprocess [2]. Examples are (i) metabolites that control metabolic flow rates of the target end-product or (ii) overflow metabolites of the central pathways, such as acetate, ethanol and lactate [4]. If these analytes can be monitored in time, cellular growth and expression of target products can be enhanced [5].

As industrial microbial and cell cultures are slow growing processes, it is sufficient to measure these analytes intermittently at the process line and to take appropriate control actions based on the measurement results [6]. However, the procedures require sensors that are easy to handle, cost-effective and accurate enough for the purpose of the corrective control action. Examples of efforts in applying such sensors for monitoring of bioprocesses and cell cultures have recently been reported [7–9].

Currently, commercial alternatives are available, such as high-pressure liquid chromatography (HPLC), enzyme kits and sensors for medical care [10], some with excellent sensitivity, which is important in many medical applications, but less essential in bioprocesses. Although, these alternatives require either high investments or extensive maintenance or time-consuming analytical procedures; still, their analytical performances may be insufficient.

Biosensors, with their inherent enzymatic specificity towards certain metabolites and their favorable analytical performance in biological fluids, have slowly gained acceptance in a variety of fields including clinical, food and environmental analysis [10–12]. Among the signal transduction methods applied with biosensors, the electrochemical biosensors are highly attractive due to their suitability for miniaturization, mass production and low-cost manufacturability (e.g., by printing techniques) [13,14].

The interest in low-cost sensors has significantly boosted the interest in printing; for example, roll-to-roll and gravure printing have been increasingly reported for the manufacturing of electrochemical biosensors [15]. Together with an enhanced importance of the single-use approach, these techniques present regulatory advantages, including transparent conditions for implementation, distribution, storage and reproducible validation [16].

A plethora of electrochemical lactate biosensor designs has previously been presented for applications in medicine and biotechnology [17–20]. The majority of these rely on the use of enzymes such as lactate oxidase or lactate dehydrogenase incorporated in a supporting membrane [21].

Despite clinical analysis having been the most explored application for lactate biosensors, reports on this technology for cell culture monitoring have also been presented. For example, Boero and colleagues [7] reported on fabrication via thin film technology of a dual sensing system for on-line monitoring of glucose and lactate in pharmaceutical processes. The same authors reported on a screen-printed electrochemical biosensor based on carbon-nanotube-modified carbon electrodes for the detection of glucose and lactate in an SN56 cell culture [22]. Later, Li and colleagues [8] reported on continuous monitoring of extracellular lactate in cardiac cell culture using an electrochemical biosensor based on lactate dehydrogenase with methylene green on nanotubes.

The requirements on lactate measurements vary considerably in medical applications, drug manufacturing and bioproduction regarding validation, clinical safety and reliability. For industrial bioprocess applications, as addressed here, lactate measurement at higher levels (e.g., 5–35 μM) are of decisive value for the process performance [23]. Furthermore, compliance of these sensors with good manufacturing practice (GMP) guidelines, validation criteria and specific needs at the production plant are important requirements which also must be met.

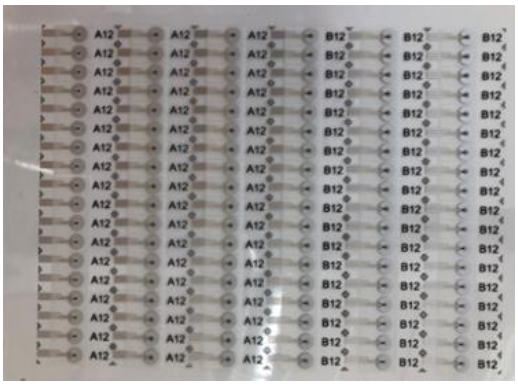
In this article we present a novel design for a single-use screen-printed lactate biosensor based on a fabrication method easy to mass produce. The sensor is intended for use at-line in bioprocesses, such as mammalian cell cultivations for biopharmaceuticals and bacterial cultivations for primary metabolites and heterologous protein production. The specific enzyme used in the sensor, lactate oxidase, was immobilized onto a polyethylene terephthalate foil, creating a recognition element of high specificity, acceptable stability and low enzyme cost. This lactate biosensor design exhibited promising and reliable data, both in bacterial and mammalian cell cultures, when used in typical cell cultivation media.

2. Materials and Methods

2.1. Single-Use Biosensor Fabrication

2.1.1. Printing

The electrochemical strips, in the two-electrode configuration, used for the assembly of the single-use biosensor were fabricated by screen-printing onto a 125 µm thick polyethylene terephthalate (PET) substrate foil (Polyfoil Bias). To minimize deformation of the substrate foil during the printing process, the PET substrate underwent a thermal pretreatment (140 °C for 45 min). The printing procedure consisted of four consecutive steps: (1) printing of the conductive tracks using a commercial silver ink (Ag5000, DuPont, UK), followed by its thermal curing (180 s at 130 °C); (2) printing and thermal curing (180 s at 130 °C) of the Ag/AgCl reference/counter electrode (C61003P7, Gwent, UK); (3) printing and curing (180 s at 120 °C) of the carbon working electrode (7102, DuPont, UK); (4) coating the sensor electrodes with two layers of a UV-curable dielectric layer (5018, DuPont, UK) in order to ensure that only the sensing area (working electrode and reference/counter electrode) and the connecting pads at the far end of the Ag conductive tracks were exposed (see Figure 1b). A semiautomatic screen printer (DEK Horizon 03i printer, ASM Assembly Systems GmbH, Germany) was used to fabricate the strips. The final electrochemical strips had a working electrode with an area of 0.0177 cm^2 (geometrical) and a counter/reference electrode with an area of 0.212 cm^2 (about 12 times that of the working electrode area). Each sheet (A4 area) contained 108 strips.



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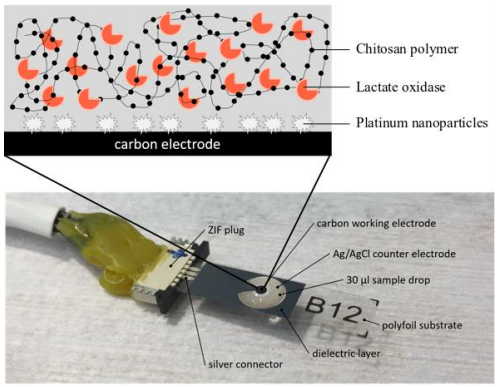


(**b**)

Figure 1. (**a**) An A4-sheet containing 108 screen-printed electrochemical sensors for biosensor development; (**b**) the fabricated electrochemical biosensor. The insert shows the architecture of the enzyme layer with the Pt-nanoparticle-modified carbon electrode surface covered by the enzyme/chitosan membrane.

2.1.2. Biosensor Assembling

Prior to the immobilization of the enzyme, the working electrode, where sensing takes place, underwent an activation step and a metallization step. Activation of the electrode was carried out by potential cycling (0 to 1.5 V, scan rate 50 mV s^{-1}) applied to it 25 times, with the strips immersed in a 10 mM PBS buffer. The working electrode was subsequently modified by platinum nanoparticles (Pt-NPs) that performed catalytic oxidation of H_2O_2 formed at enzymatic oxidation of lactate. The Pt-NPs were electrodeposited onto the working electrode following a two-step process inspired by the protocol previously reported by Diacci et al. [24] where Pt-NPs were deposited by applying one drop (ca. 30 µL) of a 1 mM solution of K_2PtCl_6 in 0.1 M KCl and by sequential pulsing a potential of 0.5 V for 0.01 s followed by −0.7 V for 10 s for 25 cycles.

Subsequently, the sensors with the deposited Pt-NPs were coated with the enzyme-containing membrane. Chitosan membrane was prepared adapting previously reported protocols [25,26]. The membrane was prepared by dissolving 0.1 wt% of chitosan in 0.1 M HCl at 80–90 °C for 2 h. The pH of the obtained chitosan solution was adjusted to 4.5 before the solution was filtered through a 0.45 µm syringe filter and stored at 4 °C.

Dry lactate oxidase (Sigma Aldrich) was subsequently dissolved in PBS at a concentration of 200 U/mL. Different dilutions of the enzyme stock solution were prepared and mixed with the chitosan solution in 3:4 (enzyme:chitosan) proportion to obtain the desired enzyme concentration on the sensor surface. A drop of 2 µL of the mixture was cast onto the working electrode and left to dry for 2 h at room temperature. These enzyme sensor strips were stored at 4 °C until use.

2.1.3. Characterization of the Single-Use Biosensor

Cyclic voltammetry was used to characterize the sensors. Sensor strips were recorded (−0.4 to 0.6 V at a scan rate 50 mV s^{-1}) before and after modification of the Pt nanoparticles to evaluate the electroactivity of different culture media.

Chronoamperometric measurements (0.4 V for 180 s) were used for lactate detection in both laboratory solutions and culture samples. The current was recorded after 180 s in all of the chronoamperometric measurements.

Electrochemical measurements of culture samples were performed by dropping 35 µL of the sample onto the electrode.

When the sensors were recorded for 24-h periods, the sensors were immersed in a beaker with PBS containing L-lactate at different concentrations. This procedure was used to (i) minimize the effect of evaporation and (ii) reduce influence of substrate depletion and/or product accumulation.

Prior to measurement of lactate in culture samples a precalibration of the sensor was carried out either in culture media or diluted PBS.

2.2. Cell Cultivation Processes

2.2.1. Corynebacterium Cultivation

A *Corynebacterium* strain DM1945 Δ*act3*:P_{tuf}-*ldcC*$_{OPT}$ [27,28] was precultivated in two consecutive steps (I and II) before running the batch culture in which the sensors were evaluated.

Preculture I was carried out in an LB medium (50 mL supplemented with 22 g/L glucose) with 200 µL of the cryo-preserved *C. glutamicum* added. After 4 h at 30 °C of cultivation, using an orbital shaker with an amplitude of 70 mm at 200 rpm, the cells were transferred to Preculture II.

Preculture II was carried out in CgXII medium (85 mL supplemented with 24 g/L glucose) seeded with 15 mL of Preculture I using a procedure described by Keilhauer et al. [29]. Preculture II was cultured for 20 h at 30 °C in a rotary incubator at 200 rpm.

Subsequently, 10 mL of Preculture II was transferred to a shake-flask (batch) with the same CgXII medium (50 mL supplemented with 24 g/L glucose). The batch culture was run for 7 h at 30 °C in a rotary incubator using intermittent shaking with 15 min off/on intervals.

Samples were collected once per hour and centrifuged; the supernatant was frozen at −20 °C prior to lactate analysis. Dry cell mass was determined by duplicates gravimetrically.

2.2.2. Chinese Hamster Ovary (CHO) Cell Cultivation with RPMI Medium

CHO cells (CHO-K1 (ACC 110, DSMZ)) were cultured for seven days in 500 mL shake-flasks (batch) containing 100 mL of RPMI medium with an initial glucose concentration of 20 g/L glucose. The cultivations were run in a CO_2-incubator on a shaker at 50 rpm, at 37 °C and 5% CO_2.

Samples were taken once per day, centrifuged, sterile filtered and stored in a freezer at –20 °C until measurement. The collected samples were thawed and analyzed using the single-use biosensor and, in parallel, measured by HPLC for correlation of the single-use biosensor readings.

Cell counts were carried out by microscopy using a Neubauer chamber. Between 300 and 1000 cells were counted in each sample. To assess cell viability, trypan-blue staining was applied.

2.2.3. Chinese Ovary Hamster (CHO) Cell Cultivation with Proprietary Medium

Another Chinese hamster ovary (CHO) cell line was provided by Fujifilm Diosynth Biotechnologies (Billingham, UK). The cells were cultured in 500 mL spinner-flasks for 13 days by intermittent addition of fresh culture medium supplemented with glutamine and antibiotic specially designed for the CHO cell line (proprietary medium provided by Fujifilm Diosynth Biotechnologies). Cultivations were run in a CO_2-incubator at 5% CO_2 and 36.5 °C and using a stirrer speed of 50 rpm. The initial starting concentration of the cells was 200,000 cells/mL in a volume of 350 mL.

Samples were taken once per day and centrifuged; the supernatant was stored at –20 °C until measurement. The collected samples were thawed and analyzed using the single-use biosensor and, in parallel, measured by a Megazyme lactate kit for determining the correlation of the single-use biosensor readings. Cell number was determined by staining with trypan-blue and manually counting in a light microscope using a Bürker chamber.

2.3. Measurement of Lactate with the Single-Use Biosensor

In order to evaluate the applicability of the single-use printed biosensor, the following measurement/correlation protocol was used.

The L-lactate content in the diluted sample was measured by using three different biosensors. Concentration of the L-lactate in the sample was then calculated by fitting the obtained sensor's reading in a calibration curve, which was made from five single-use biosensors (calibration points: 50, 275 and 500 µM). The samples (35 µL) were pipetted on the measurement area of the sensor.

The proposed biosensor is based on the FAD-mediated enzymatic oxidation of L-lactate (1 and 2) followed by electrocatalytic detection of hydrogen peroxide (3) formed in stoichiometry proportions to lactate. The sensitive and stable detection of H_2O_2 at the sensor surface is crucial for proper function of the sensor.

$$\text{L-lactate} + \text{FAD}_{(\text{LOx})} \rightarrow \text{Pyruvate} + \text{FADH}_{2(\text{LOx})} \quad (1)$$

$$\text{FADH}_{2(\text{LOx})} + \text{O}_2 \rightarrow \text{H}_2\text{O}_2 + \text{FAD}_{(\text{LOx})} \quad (2)$$

$$\text{H}_2\text{O}_2 \rightarrow 2\text{H}^+ + \text{O}_2 + 2\text{e}^- \quad (3)$$

2.4. Measurement of Lactate with Reference Methods

2.4.1. High-Performance Liquid Chromatography

Samples were filtered through a membrane filter with a pore size of 0.8 µm (Carl Roth, Karlsruhe, Germany) directly after sampling. The supernatant was transferred to 1.5 mL tubes, immediately stored at −80 °C and thawed before measurements.

The concentration of L-lactate in culture's samples (supernatant) was obtained by HPLC with an Agilent 1200 system (Waldbronn, Germany) equipped with a refractive index detector and a

HyperRezTM XP Carbohydrate H+ column (300 × 7.7 mm, 8 µm) (Fisher Scientific, Schwerte, Germany) using 0.1 M H_2SO_4 as carrier solution at a flow rate of 0.5 mL min^{-1} and a temperature of 15 °C [28,30]. The HPLC method had an LOD of 1 µM. Measurement data were analyzed with the Agilent Chemstation for LC 3D systems software Rev. B.04.01.

2.4.2. Ultraviolet Spectroscopy

Samples were thawed and diluted 1:60 with PBS. The L-lactate concentration was obtained using a lactate kit (Megazyme, Ireland) according to product instructions. The assay had a linear range between 34 and 225 µM and an LOD of 2.36 µM. UV spectra (340 nm) were recorded with an Ultraspec 1000 UV/VIS spectrophotometer (Pharmacia Biotech, UK).

3. Results and Discussion

The optimization of the fabrication procedure of the single-use lactate biosensor, the evaluation of its storage and operation stabilities and its analytical performances in a variety of relevant crude media are detailed below. Finally, data are presented from two types of cultivations that are commonly applied in industry.

3.1. Sensor Fabrication and Functionalization

In order to evaluate the feasibility of the screen-printing method for the manufacturing of the electrochemical sensor suitable as a platform for enzyme immobilization, and for demonstrating the possibility of its mass-production, ten sheets with 108 sensor strips on each were fabricated (Figure 1a). The fabrication yield (defined as the percent of sensors with individually addressable electrodes) was estimated by optical inspection and electrical verification to be 92%. This number was not considered an optimal value of the method per se but was an indicator that the sensors had the necessary prerequisites for being developed into a reliable single-use enzyme sensor.

For the purpose of the study, it was an important indication that the manufacturing process had enough robustness for further development.

In order to enable this catalytic oxidation with high efficiency, the carbon surface of the sensor electrode was functionalized by platinum nanoparticles (Pt-NPs) using the procedure described in Section 2.1.2.

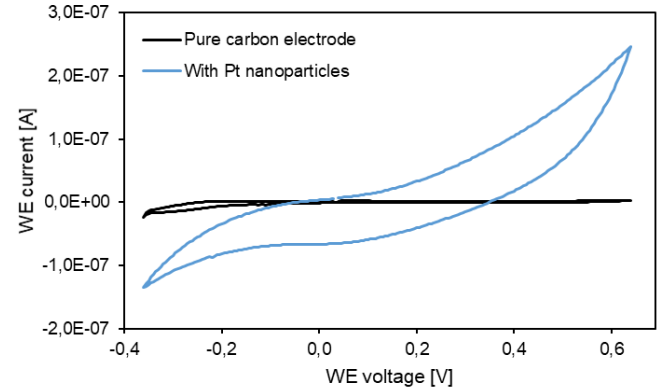
Importantly, as can be seen in Figure 2a, no electrochemical response was recorded at the bare carbon electrode (black line); while when Pt-NPs (blue-colored line) (without enzyme) were electrodeposited onto the electrode surface, distinct and significant redox reactions related to the oxidation and reduction of H_2O_2 were recorded. Based on the transient shape of the CV-curve, an applied potential of 0.4 V (vs. internal Ag/AgCl reference electrode) was chosen for the subsequent amperometric measurements.

As demonstrated above, the electrocatalytic ability of the Pt-NP-modified carbon electrode to detect H_2O_2 makes it feasible to assemble the biosensor by further modifying its composite Pt-NP surface with an Lox-containing chitosan membrane. As a first step, the biosensor surface was provided with increasing loading of LOx (5, 10, 20 and 40 U) in the membrane. These membranes were prepared to identify a favorable and optimal LOx concentration.

The optimal concentration was considered to be the one that would allow to widen the linear range of the sensor (towards high concentration) and to provide an easily recordable electrochemical response. As expected, the increase of LOx loading in the membrane resulted in an increase of the biosensor response (Appendix A Figure A1). However, this did not result in any significant improvement in the linear dynamic range of the sensor. Subsequently, in a compromise between the sensor response and its costs, an LOx loading of 20 U in the membrane was selected for the measurements.

Based on the electrocatalytic effect of the Pt-NPs, which was observed as described above by cyclic voltammetry (Figure 2a), and the selected enzyme composition of the membrane, a set of biosensors (ca. 20 units) were fabricated and their reproducibility and stability during storage and at operation were

both evaluated. As can be seen in Figure 2b, good reproducibility of these biosensors was achieved, as proven by the low standard deviation obtained at calibration in pure laboratory solutions (PBS) (at 0 to 500 µM L-lactate using 10 sensors).



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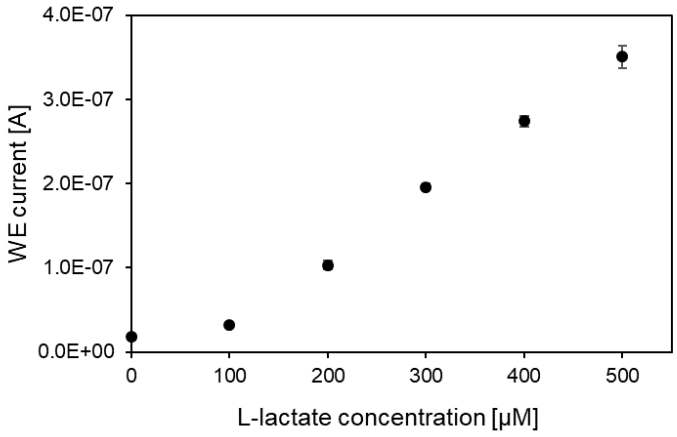

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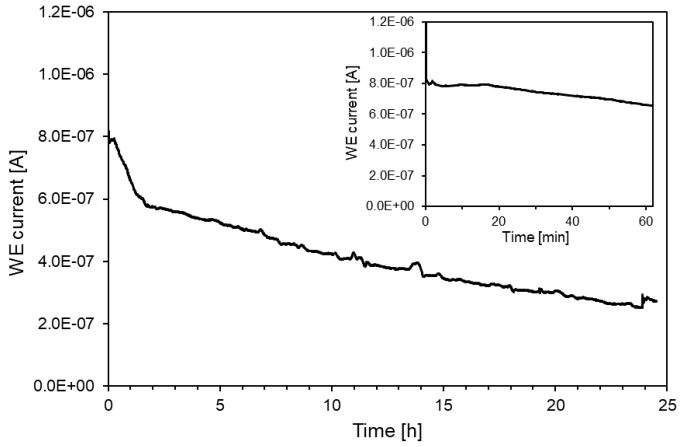
Figure 2. (**a**) Cyclic voltammetry in 50 µM H_2O_2, dissolved in PBS at a 25 mV/s scanning rate with a bare carbon electrode and a carbon electrode with electrodeposited Pt-NPs; (**b**) calibration curve of a L-lactate biosensor in PBS. The standard deviation was calculated using the response of ten biosensors.

The operational and storage stability of the biosensors were evaluated with the purpose of gaining better understanding of their applicability for off-line or at-line bioreactor monitoring. As seen in Figure 3a, the electrochemical response of the biosensor was rapidly degrading when applied in continuous measurement mode with a fast decrease, especially after the first hour, which was followed by a continuous drift of the signal amplitude. This result clearly highlights the inappropriateness of using the developed biosensors for continuous real-time monitoring in bioreactors. However, the observed stability is still adequate for the application of the sensor in off-line single-use modality.

From Figure 2b, it is evident that the high level of reproducibility of freshly prepared sensors allows their off-line single-use utilization. A further aspect to be evaluated to ensure the biosensor applicability during cultivation (for two weeks) is its stability upon storage. To test this, a batch of biosensors (44 units) were fabricated the same day, using the same stock solution of enzyme membrane, and stored either dry at room temperature (21 °C) or at 4 °C in closed petri dishes. Prior to testing, biosensors where transferred to room temperature and washed with PBS (to hydrate the membrane). Then, these sensors were used for measuring amperometrically a 500 µM solution of L-lactate in PBS.



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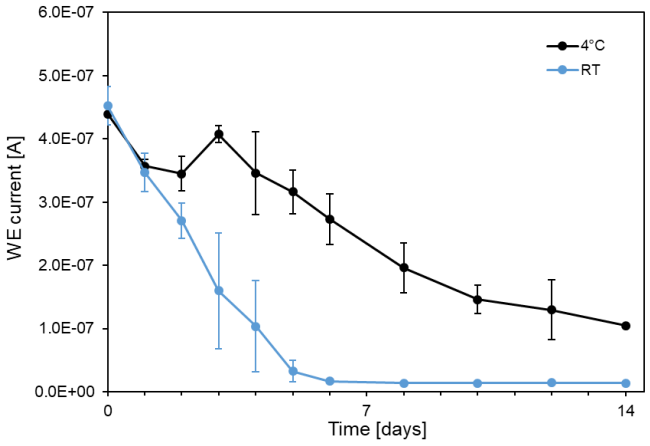

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Figure 3. (**a**) Continuous reading of the biosensor for 24 h (the first hour inserted) in 500 µM L-lactate PBS solution; (**b**) stability of the biosensors upon storage (14 days) in dry conditions at 4 °C and at room temperature (21 °C). All readings were performed in PBS solutions containing 500 µM of L-lactate.

As seen in Figure 3b, sensor units stored at room temperature quickly lost their response, and no activity could be recorded after 6 days. Similarly, the sensor units stored at 4 °C lost their activity upon storage, even if the loss was partial (after 14 days, about 30% of the performance at day 1 was recorded). No significant improvements in stability resulted from other storage conditions: (i) in dry state at −20 °C; (ii) in liquid state in PBS at 4 °C; (iii) in PBS containing 250 µM lactate at 4 °C (Appendix A Figure A2). Thus, stability of the sensor is short, suggesting that the immobilization technique should be further refined. Previous experiences of enzyme immobilization in hydrogels may inform such procedures [31].

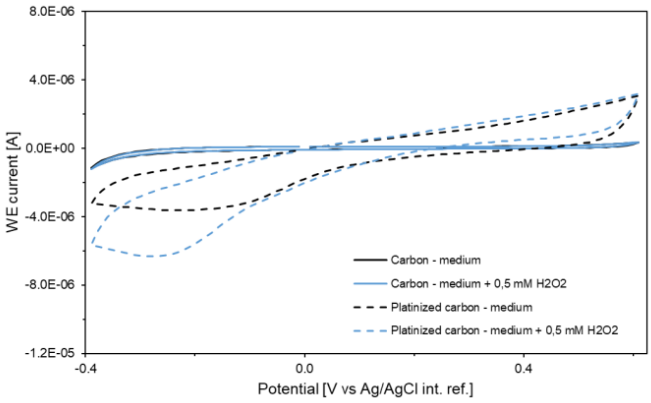
3.2. Characterization of the Electrochemical Behaviour of Culture Media

Prior to applying the developed single-use biosensors to monitor bacterial and cell cultures, electrochemical characterization of the biosensors in typical culture media was performed; this evaluation was needed to identify potential interferences from media components in the electrochemical surface process (e.g., redox processes overlapping those of H_2O_2).

Electrochemical responses in the culture media to be used in the subsequent off-line testing of the sensors were systematically recorded in presence or absence of H_2O_2 and using bare carbon electrodes or Pt-NP-covered electrodes. The media investigated were as follows: (i) an industrial culture medium

(proprietary medium from Fujifilm Diosynth Biotechnologies; in dilution 1:60 with PBS); (ii) RPMI media; (iii) a CGXII medium.

As seen in Figure 4, no significant electrochemical interferences were recorded for the industrial cell culture medium (Figure 4a) and the RPMI medium (Figure 4b); on the other end, in these media the catalytic oxidation of the H_2O_2 seems to be significantly inhibited.



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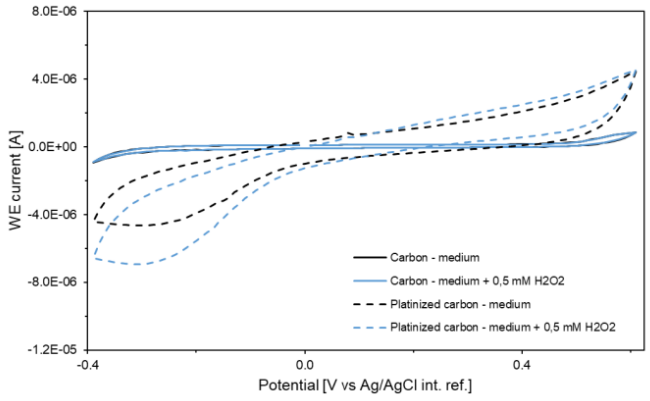

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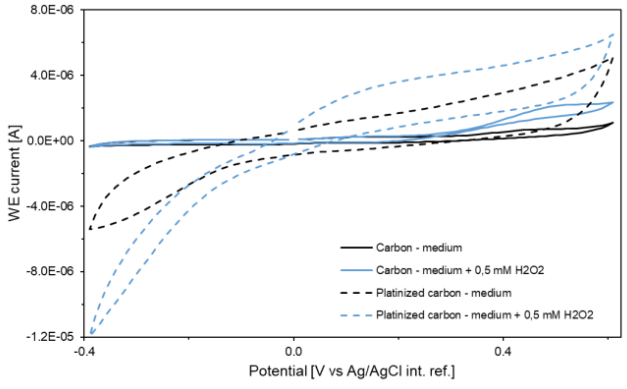


(**c**)

Figure 4. Cyclic voltammetry measurements in three cell culture media using bare and Pt-NP-functionalized carbon electrodes in the absence or presence of 500 µM H_2O_2. (**a**) Fujifilm media diluted 1 to 60 in PBS; (**b**) RPMI medium; (**c**) CGXII medium. Voltage is cycled between −0.4 and 0.6 V at a rate of 50 mV/s with a voltage step of 10 mV.

In the case of the CGXII medium (Figure 4c), an intrinsic electrochemical activity was recorded at the bare carbon electrode (full black line); however, this response was significantly smaller than those recorded for H_2O_2 at both bare carbon electrode (full blue line) and Pt-NP-covered electrode (dotted blue line). The results shown in Figure 4 confirm that electrochemical measurements are feasible in the investigated media.

It should be noted however, that the measurement of L-lactate in the culture medium was significantly affected by poor reproducibility, as seen in data in Appendix A Figure A3, in which the calibration curves carried out in RPMI medium and PBS are also shown for comparison. The analytical performance of the biosensor in the investigated media is summarized in Table 1.

Table 1. Analytical performance of the developed biosensor in the different media.

	PBS Diluted Fujifilm Media	**RPMI**	**DGXII**
Sensitivity (A/μM)	7.64×10^{-10}	6.27×10^{-10}	5.3×10^{-10}
LOD (μM)	10	67	101

3.3. Application of the Printed Single-Use Biosensor in Microbial and Mammlian Cell Cultivation Monitoring

Based on the findings in Sections 3.1 and 3.2, it is concluded that application of printed single-use biosensors in cell culture analysis is possible, but only by applying an off-line single-use approach. It was also obvious from the results that careful precalibration is needed in order to compensate for storage-related losses and for effects of the media composition. Furthermore, the limited linear range of the biosensors requires dilution of cultivation samples to bring the concentrations of L-lactate within the detectable sensitivity window of the biosensor. This need for dilution of cell culture samples prior to measurement has been previously been discussed by other authors [22].

3.3.1. *Corynebacterium glutamicum* Cultivation

Prior to lactate analysis of samples from *C. glutamicum* cultures, five freshly fabricated biosensor units were used carry out a three-point calibration in the range 0 to 500 μM of L-lactate in CGXII media. Each biosensor was used three times from lower to higher concentrations. The L-lactate concentrations in the sample were calculated by fitting the off-line biosensor response to this calibration.

In order to reach the dynamic L-lactate concentration range of the biosensor, the samples were diluted 1 to 42 (decided according to data available from previous cultivations) in CGXII media. Eight biosensors were used (each of them three times) in alternating order to determine the lactate concentration of the samples. The first readings of each biosensor have been recorded separately (1× sensor) to be able to study the effect of multiple use on accuracy.

As seen in Figure 5, the values calculated using the single-use biosensor measurements coincided well with those recorded by HPLC.

At low L-lactate concentrations (<5 mM), the measurements of the single-use biosensors underestimate the concentrations; at higher concentrations (>5 mM) the measurements have high standard deviation.

The negative L-lactate concentrations can be explained by the fact that the measurements, as a result of the dilution, are very close to the limit of detection (0.101 mM) of the biosensor in the CGXII media. This is not a significant problem from a process-monitoring perspective, since the relevant information is in the higher concentrations of L-lactate in the cell culture.

The high standard deviation is a result of two interconnected effects: (1) being close to the limit of linearity (above 0.6 mM) and (2) the calibration method. These, together with the multiple usage of the biosensor, lead to saturation of the enzymes on the sensor surface, increasing the signal after each measurement and making multiple readings impossible. This explanation is also supported by the graph of the single-fold (1x) biosensor measurements (Figure 5), where the fresh biosensors show

slightly higher concentrations than HPLC, while the threefold-measured mean biosensor values show slightly less response.

In order to achieve a better monitoring performance and accuracy, different calibration methods were compared in the upcoming cultivations.

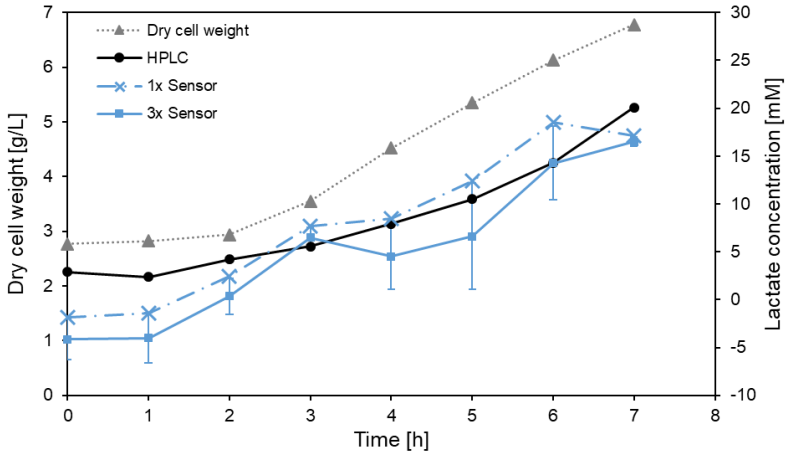


Figure 5. Monitoring of lactate off-line for seven hours of *C. glutamicum* cultivation in CGXII medium using the single-use biosensor, and its comparison with off-line HPLC lactate measurements. The 1× graph shows the first recordings with individual biosensors. The 3× graph shows the mean value of recordings of three individual thrice-used biosensors. The cultivation chart also shows the growth of the bacteria as dry cell weight concentrations.

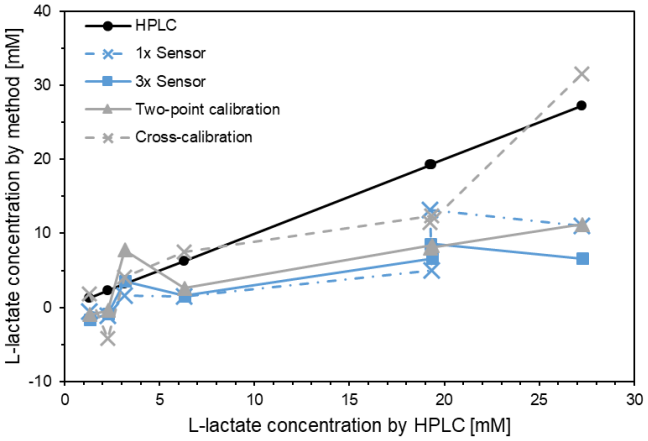
3.3.2. Mammalian Cell (CHO) Culture

In order to demonstrate the versatility of the single-use biosensor, CHO cells were cultured according to two different procedures: in one procedure the cells were cultured for seven days in shake-flasks as batch cultures using RPMI medium (see procedure described in Section 2.2.2); in another procedure the cells were cultured for 13 days in larger spinner-flasks as fed-batch cultures using an industrial culture medium (see procedure in Section 2.2.3). The collected frozen samples were thawed and analyzed by the single-use biosensor and, in parallel, either analyzed by HPLC (first procedure) or by a colorimetric lactate enzyme assay (second procedure) for correlation of the biosensor readings.

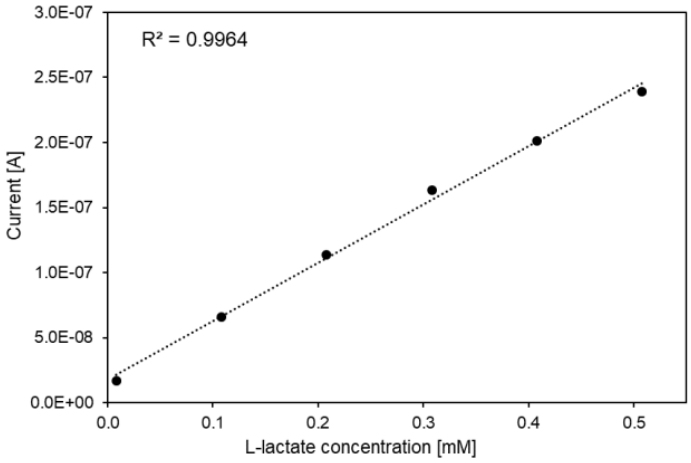
Prior to analysis, all samples were diluted 1 to 55 (dilution factor defined according to pre-existing data) in the RPMI medium, or 1 to 60 in PBS for the industrial medium (as shown feasible in preliminary tests), to reach the dynamic range for lactate of the single-use biosensor. With the aim to improve the accuracy of the biosensor measurement, two other calibration methods, a two-point calibration and a cross-calibration, were compared with linear calibration when used in RPMI medium.

In the cross-calibration approach, each biosensor was used to measure the start sample, the end point sample and a third sample in between these two samples. The results of the HPLC measurements (start and end points) were used to cross-calibrate each biosensor separately. L-lactate values of the third sample were calculated based on a linear regression of the two-point cross-calibration. As seen in Figure 6a, linear calibration, either with the single or multiple use of the biosensors, shows lower responses than HPLC in the previous bacterial cultivations.

The two-point calibration using two biosensors proved inaccurate, while cross-calibration using the HPLC measurements showed better accuracy, both at lower and higher concentrations. Thus, the cross-calibration method was used to calibrate the single-use biosensors in the first CHO procedure.



(**a**)

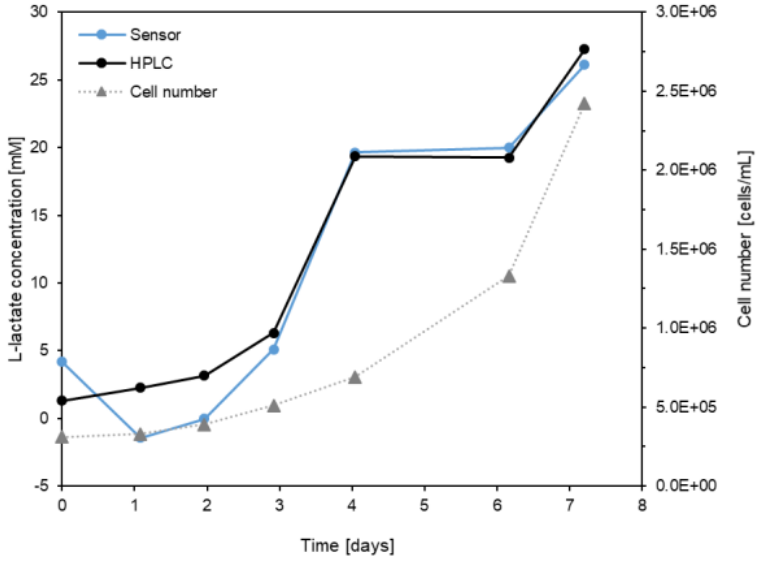


(**b**)

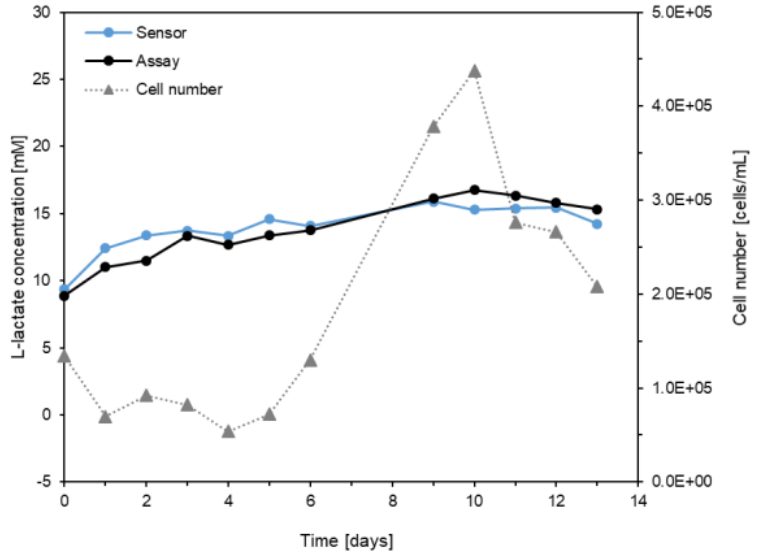
Figure 6. (**a**) The correlation of the different calibration methods with the HPLC measurements. The lactate measurements of the HPLC have been compared with the measurements of single and multiple use of the biosensors, two-point calibration and cross-calibration. (**b**) Five-point calibration in the industrial medium.

With lactate samples collected from the CHO culture performed in the industrial medium, a five-point calibration was carried out (Figure 6b). The biosensor response showed good linearity between 0.008 and 0.5 mM. Thus, this calibration method was used in the spinner-flask fed-batch CHO cell cultivation.

In Figure 7a, the correlation between the biosensor and HPLC measurements in the shake-flask batch CHO cell culture is shown. Each datapoint was measured by a separate sensor in a single-use way, as it would be in industrial use. As seen, the correlation is high during this CHO cell cultivation, except in the early phase, where the cell concentration and viability is low and where L-lactate concentrations are close to the limit of detection of the biosensor after sample dilution. Clearly, the biosensors showed an improved accuracy with the cross-calibration method, being able to follow the increasing lactate concentration in a wide range (0–25 mM) shown in the fed-batch culture. This variation of L-lactate concentration also mirrors the expected lactate profile during growth and metabolic overflow in a batch CHO cell culture.



(**a**)



(**b**)

Figure 7. Mammalian cell culture measurements at line using the single-use biosensor. (**a**) Seven-day batch cultivation showing lactate measurements with the single-use biosensor in comparison with off-line HPLC data; (**b**) Thirteen-day fed-batch cultivation showing lactate measurements with the single-use biosensor in comparison with off-line spectroscopic lactate assay data. The growth profiles of CHO cell cultures are shown in (**a**) and (**b**) from viability assays.

In the fed-batch CHO cell cultivation (Figure 7b), higher dilution of the samples than for those from the RPMI medium was necessary, because L-lactate was included as a growth component in the industrial medium.

As seen in Figure 7b, good correlation was also obtained between data from the single-use biosensor and the standard lactate assay. Similarly to the batch CHO cultivation, each datapoint was obtained by a separate sensor. The addition of a higher amount of feed solution to the culture resulted

in a drop in cell concentration at the end of the cultivation (day 11 and forward), which is due to the dilution of the culture and not due to cell death (Figure 7b).

The lactate component in the cultivation media is, as expected, produced in a higher amount when the culture is growing (between days 0 and 10), and it stagnates when the cell proliferation retarded (day 11 and onwards). As can be seen in Figure 7b, the biosensor was able to accurately monitor this event in the cultivation.

Evidently, the single-use screen-printed biosensor calibrated with the appropriate method (either five-point or cross-calibration) is able to detect L-lactate in a wide concentration range in various culture media. Its measurement range also covers the typical lactate concentration range (5–35 mM) expected during mammalian processes [23]. Thus, the sensor seems to be a realistic alternative for at-line bioreactor monitoring, providing a convenient and user-friendly low-cost alternative to other sensor methods applied today [32,33].

4. Conclusions

The screen-printed single-use biosensor for lactate bioprocess monitoring developed and evaluated here is convenient to mass-produce at a low batch production cost. The fabrication procedure results in a device unit that shows the necessary sensitivity, reproducibility and, partly, the stability needed for the monitoring of certain bioprocess applications. The developed enzyme sensor with the right calibration method proved to be suitable for the fast screening of different cultivation processes.

Certain needs for improvement can also be mentioned, such as (1) a more extended long-term stability and higher reproducibility of the sensor; (2) access to automated dispensing tools for deposition of the enzyme membrane; and (3) aluminum pouches for sensor storage in controlled atmosphere. These improvements would significantly enhance the performance and shelf-life of the sensor as well as contribute to satisfying regulatory demands. Even without these improvements, however, the sensor can be suitable for internal in-process use as a complement to analytical methods and devices in use today.

The devised fabrication method is expandable to other enzymes. The number of potential applications for biotechnology is vast, e.g., other metabolites prevalent the culture media such as amino acids, other organic acids and alcohols, some main products, other side-products.

The impact of at-line monitoring using single-use biosensors on production efficiency and economy is potentially profound. Total production time and product titer can be optimized under conditions of the chosen process. The printing-based fabrication of the biosensor has the flexibility of a variety/assortment of enzyme variants. Thus, these initial findings may have significant impact and motivate further development.

Author Contributions: L.T. performed development of the fabrication of the printed biosensor. J.R. and L.T. conceived and performed the bioprocess application study. B.V., D.S., M.G. and L.T. conceived the design of the printed device. J.R., L.T., S.J., P.N. and C.-F.M. conceived the study of the application in the two bioprocesses. L.T. and J.R. evaluated the results. J.R., L.T., V.B. and C.-F.M. drafted the manuscript and all co-authors reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

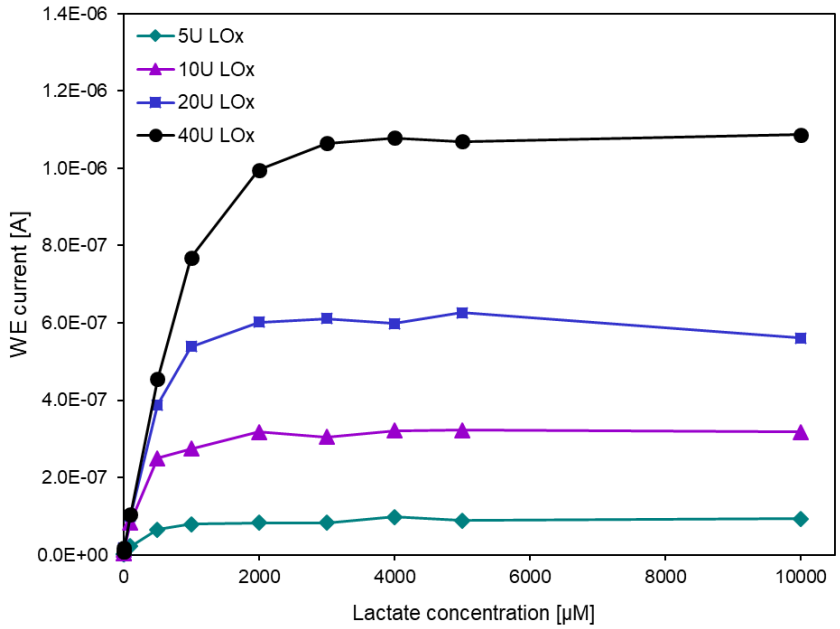

Figure A1. Amperometric L-lactate biosensor calibration curves (at potential 0.4 V and 180 s) in PBS with different enzyme loads on the sensor.

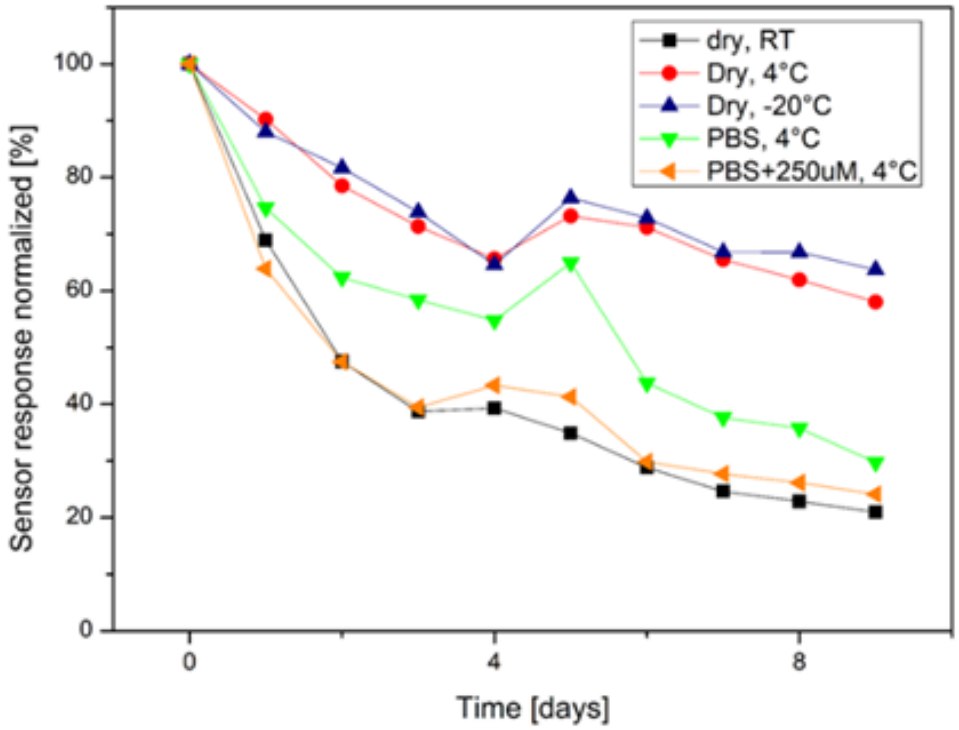

Figure A2. Preliminary comparison of different storing conditions for the biosensor. Response obtained daily in a 500 µM solution of L-lactate in PBS and normalized against response obtained on day 0.

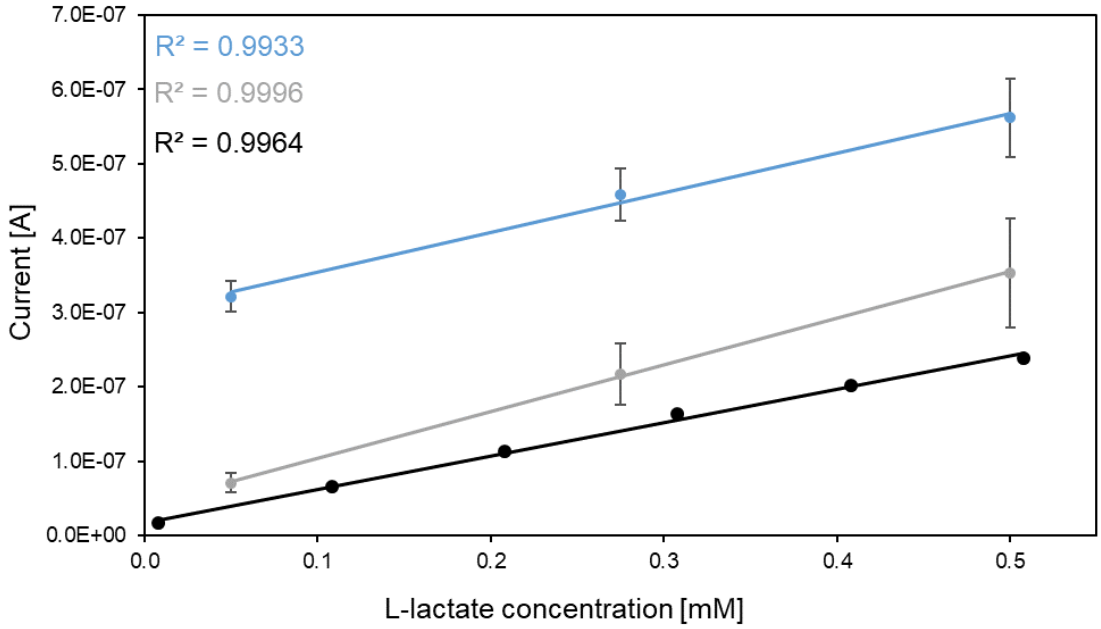

Figure A3. Example of biosensor calibration curve in CGXII medium diluted in PBS (blue line, $n = 5$), RPMI media (grey line, $n = 5$) and Fujifilm media (black line, $n = 1$).

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