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Study of enzyme sensors with wide, adjustable measurement ranges for in-situ monitoring of biotechnological processes

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ABSTRACT

We report on a study of enzymatic glucose and lactate sensors for measurements of wide concentration ranges from 1 mM up to 600 mM and 900 mM, respectively, in biotechnological processes. Diffusion-limiting polyurethane membranes were used to extend the linear measurement range and the influence of two fabrication parameters on membrane properties and sensor performance evaluated. The polymer concentration was varied between 4% and 10% and the ratio of the solvents tetrahydrofuran and dimethylformamide between 9:1 and 1:9. Surface morphology and permeability of the membranes and the linear measurement range of membrane-covered enzyme sensors were determined. A distinct relationship between the different results was found. The sensors retained their functionality after being sterilized using gamma and electron beam irradiation. The long-term performance of the sensors was evaluated and good performance in the glucose monitoring of a culture of *Saccharomyces cerevisiae* over 13 days was shown.

1. Introduction

The demand for products with high expression rates, including monoclonal antibodies, therapeutic proteins and vaccines, is rapidly growing [1,2]. As a consequence, biotechnological processes in production and research become increasingly complex and sophisticated. At the same time, high efficiency and product quality are key requirements for various processes, be they new or well-established [3]. The monitoring of glucose and lactate is of major importance for good process control, high product quality and maximum yield. The precise control of glucose allows increasing antibody yield, reducing applied nutrients, ensuring reproducible glycosylation and preventing stuck fermentations [4]. Similarly, lactate is one of the main metabolites in mammalian cell cultures [5] and gives information about the health of the cells and their metabolic activity [3].

Today, glucose and lactate in bioreactors are typically measured after sampling in external electrochemical analyzers [3]. However, sampling requires additional ports, bears the risk of contamination and delays the measurement results. In contrast, continuous in-situ glucose and lactate measurements could provide real-time data and thus be of great benefit for many applications.

When designing in-situ sensors for glucose and lactate, the wide concentration ranges of these analytes in bioprocesses have to be considered. Concentrations go up to around 50 mM for mammalian cell cultures but can reach values up to 1500 mM glucose in alcoholic fermentation [6] and 1000 mM lactate in the production of biodegradable polymers [7]. Sensors for such wide concentration ranges have rarely been reported since most of the research in glucose and lactate sensing is directed towards applications in health care. Demonstrated sensors for in-situ measurements in bioreactors are also limited to concentration ranges up to 50 mM [8,9].

Diffusion-limiting membranes are a way to extend the linear range of enzyme sensors [8–12]. Since most of the research in this field is limited to low concentrations, membranes have been optimized for this range, and there are only few studies aimed at higher ranges [13–15]. In this contribution, we develop glucose and lactate sensors with polyurethane (PU) membranes for wide concentration ranges. PU has often been used as a material for diffusion-limiting membranes for enzyme sensors [11,16,17], however, the influence of fabrication parameters such as polymer concentration and solvent composition on the sensor performance has rarely been studied. These parameters have, however, been found to have significant impact on the membrane structure in the context of tissue engineering [18]. Hence, we decided to evaluate the influence of polymer concentration and solvent composition on membrane morphology, permeability and the linear range of fabricated enzyme sensors.

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2. Experimental method

The glucose and lactate sensors were based on enzymatic conversion of the target molecules using glucose oxidase or lactate oxidase, followed by oxidation of the generated H_2O_2 and amperometric detection of the current by a potentiostat [4]. Enzymatic reactions can be described by the Michaelis-Menten model, in which the general form of an enzymatic reaction is written as [19]

$$E + S \underset{k-1}{\overset{k1}{\rightleftharpoons}} ES \overset{k2}{\rightarrow} E + P, \quad (1)$$

where E denotes the enzyme, S its substrate, P the product of the enzymatic reaction and k_1, k_{-1} and k_2 are the rate constants for the individual steps of the reaction.

A helpful quantity in the description of enzyme kinetics is the Michaelis constant K_M. It can be seen as a measure for the affinity of the enzyme for its substrate. The lower K_M, the higher the affinity. Under the assumption that the product formation is much faster than dissociation of the enzyme-substrate complex, i.e. $k_2 \ll k_{-1}$, K_M is calculated as

$$K_M \approx \frac{k_{-1}}{k_1}. \quad (2)$$

Up to K_M the relationship between the substrate concentration and the velocity of the enzymatic reaction can be considered linear. The Michaelis constants for glucose oxidase and lactate oxidase are 33 mM and 0.7 mM, respectively [20].

For glucose oxidase in particular, the enzymatic reaction mechanism is as follows [21]:

$$\beta\text{-d-Glucose} + \text{GOx-FAD} \rightarrow \text{GOx-FADH}_2 + \delta\text{-d-gluconolactone} \quad (3)$$

$$\text{GOx-FADH}_2 + O_2 \rightarrow \text{GOx-FAD} + H_2O_2. \quad (4)$$

In electrochemical measurements typically a three-electrode setup is employed, which includes a working electrode (WE) and a counter electrode (CE) made from noble metals, and a silver/silver chloride reference electrode (RE). A potentiostat keeps WE at a constant potential in respect to RE. The H_2O_2 generated in the enzymatic reaction is oxidized at the working electrode, yielding two electrons:

$$H_2O_2 \rightarrow 2e^- + O_2 + 2H^+. \quad (5)$$

The generated current is then detected by the potentiostat.

To achieve wide measurement ranges in enzyme sensors, diffusion-limiting membranes can be fabricated on top of the enzyme layer. For such sensors the upper limit S of the linear measurement range can be expressed as [23]

$$S = 1 + 0.5 k_a l_m / D_{S,m}. \quad (6)$$

In Eq. (6), k_a includes several parameters of the enzyme layer, such as the Michaelis constant, enzyme concentration and enzyme layer thickness [22]. $D_{S,m}$ is the diffusion coefficient of substrate in the membrane and l_m is the membrane thickness. Since the response time of the sensor rises with the square of l_m but scales only linearly with the inverse of $D_{S,m}$ [23], we focused on adjusting the diffusion coefficient to extend the linear measurement range in this study.

The enzyme electrodes were designed with concentric working (WE), counter (CE) and reference (RE) electrodes. Thanks to the concentric arrangement an even distribution of the electric field lines between the electrodes was ensured and the sensor was made independent of the direction of flow in stirred solutions in comparison to finger shaped electrodes. The WE had a diameter of 400 μm, CE and RE had a width of 100 μm and the spacing was 50 μm, giving a total diameter of 1 mm. This diameter matched the droplet size of 0.1 μl of enzyme solution that was used during the functionalization of the electrodes. The electrodes were fabricated on oxidized silicon substrates by evaporation of gold or platinum with titanium as an adhesion layer, followed by a lift-off process. The design of the sensor chip is shown in Fig. 1. Together with the enzyme sensors other sensor elements were integrated, forming a multi-sensor system for the monitoring of biotechnological processes. A description of the other sensors can be found elsewhere [24,25].

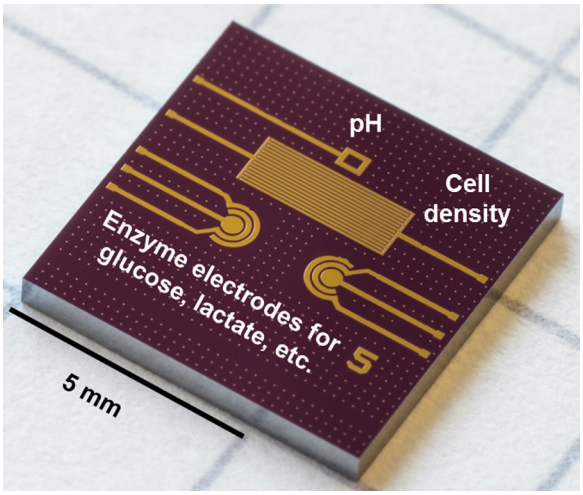

Fig. 1. Photograph of silicon multi-sensor chip with concentric three-electrode configurations for amperometric enzyme sensors; chip size was 7.16 mm × 7.16 mm; a description of the other sensors can be found elsewhere [24,25].

To functionalize the electrodes a solution of 10 mg glucose oxidase (GOx) or lactate oxidase (LOx), 10 mg bovine serum albumin and 25 μl glutaraldehyde was prepared in 500 μl of 10 mM phosphate buffered saline (PBS). 0.1 μl of the solution were dispensed on the three-electrode configuration and allowed to dry in air. The diffusion-limiting membrane was made from polyurethane (Selectophore®, Sigma Aldrich, USA), which is biocompatible and permeable for oxygen, glucose and lactate. In order to evaluate the influence of polymer concentration and solvent composition on the linear range, a series of membranes was produced. PU solutions in concentrations of 4%, 6%, 8% and 10% (w/w) were prepared with a mixture of the solvents tetrahydrofuran (THF) and dimethylformamide (DMF). Higher concentrations were too viscous to be dispensed using microliter pipettes. The THF-DMF ratio was adjusted to 9:1, 7:3, 5:5, 3:7 or 1:9, respectively, giving five series of solutions. The PU used in this study was found to be indissoluble in the mixture THF:DMF 1:9 even after several days, so this series was not employed in the further experiments. 0.15 μl of the PU solution were deposited on the enzyme layer and dried in air.

The diffusion coefficient for glucose in the membranes was determined using a custom-made diffusion cell connected to a stirred reservoir containing a 2 M glucose solution and to a second stirred reservoir containing deionized water. Samples were drawn regularly from the reservoirs, the glucose concentrations measured and the diffusion coefficient D determined according to the formula [26]

$$D = \frac{1}{\beta t} \ln\left(\frac{c_I^0 - c_{II}^0}{c_I - c_{II}}\right), \quad (7)$$

with

$$\beta = \frac{A}{l}\left(\frac{1}{V_I} + \frac{1}{V_{II}}\right). \quad (8)$$

In Eqs. (7) and (8), c_I and c_{II} are the glucose concentrations in the first and second reservoir at time t, $c_I{}^0$ and $c_{II}{}^0$ are the respective glucose