

Optical biosensor optimized for continuous in-line glucose monitoring in animal cell culture

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Abstract Biosensors for continuous glucose monitoring in bioreactors could provide a valuable tool for optimizing culture conditions in biotechnological applications. We have developed an optical biosensor for long-term continuous glucose monitoring and demonstrated a tight glucose level control during cell culture in disposable bioreactors. The in-line sensor is based on a commercially available oxygen sensor that is coated with cross-linked glucose oxidase (GOD). The dynamic range of the sensor was tuned by a hydrophilic perforated diffusion membrane with an optimized permeability for glucose and oxygen. The biosensor was thoroughly characterized by experimental data and numerical simulations, which enabled insights into the internal concentration profile of the deactivating by-product hydrogen peroxide. The simulations were carried out with a one-dimensional biosensor model and revealed that, in addition to the internal hydrogen peroxide concentration, the turnover rate of the enzyme GOD plays a crucial role for

biosensor stability. In the light of this finding, the glucose sensor was optimized to reach a long functional stability (>52 days) under continuous glucose monitoring conditions with a dynamic range of 0–20 mM and a response time of $t_{90} \leq 10$ min. In addition, we demonstrated that the sensor was sterilizable with beta and UV irradiation and only subjected to minor cross sensitivity to oxygen, when an oxygen reference sensor was applied.

Keywords Optical biosensor · Glucose monitoring · Bioreactor · Cell culture

Introduction

In biotechnological processes, the supervision and control of many important parameters, such as temperature, pH, dissolved oxygen and metabolite concentrations, play a key role [1]. Reliable in situ sensors for temperature, pH and oxygen are already established, whereas continuous monitoring of other important parameters like glucose remains a challenge [2]. The accurate monitoring of such parameters is a critical requirement for cell culture processes in bioreactors, in order to reach high protein yields with a consistent product quality [1]. Thus, the cultivation process must be maintained close to optimal parameter values and at best involves automatable control that relies on real-time sensors. For single-use bioreactors, disposable in-line glucose biosensors are desired that are not only stable over the typical cell culture durations but can be integrated during the bioreactor manufacturing process and tolerate gamma irradiation [2].

Much work has been devoted in the past to develop stable biosensors for continuous glucose monitoring that

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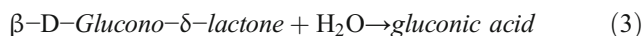
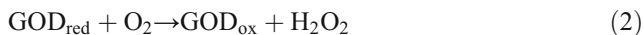
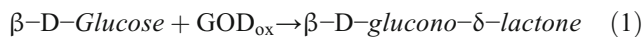
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rely on the consumption or formation of metabolites from the glucose oxidase reaction according to the following equations [3]:



where GOD_{ox} and GOD_{red} refer to the oxidized and reduced glucose oxidase, respectively. Gluconic acid and H_2O_2 are by-products of the reaction, of which hydrogen peroxide is an enzyme-deactivating agent [4–7]. The glucose concentration can be determined indirectly by measuring the hydrogen peroxide production or the oxygen consumption due to the enzymatic reaction via amperometric [8] or optical transducers [9]. Although commercially available amperometric glucose biosensors for single-use bioreactors have been developed [10], optical in-line sensors are more advantageous, since they cannot only be pre-integrated into bioreactors, but also allow contamination-free measurements through optical fibres via the transparent bioreactor surface. Others have presented a fibre optic sensor system optimized for healthcare applications [11, 12] but did not show functional stability over typical cell culture durations, which is of major importance regarding continuous glucose measurement in the biotechnological industry.

This paper presents a disposable optical glucose biosensor with an optimized lifetime for continuous glucose measurement in suspension cell culture processes. The biosensor is based on a commercially available oxygen sensor, coated with a glucose oxidase-containing enzyme layer. The oxygen partial pressure inside the enzyme layer is monitored non-invasively via the oxygen sensor and is inversely proportional to the glucose concentration in the sample. A tuneable dynamic range of the biosensor was obtained by covering the enzyme layer with different hydrophilic perforated membranes. The biosensor was optimized in terms of lifetime and applied for glucose monitoring and control in cell culture experiments. In addition, we developed a COMSOL computer model based on Michaelis-Menten kinetics to gain insight into the concentration profile of the relevant reactants and products within the biosensor such as the enzyme-deactivating agent hydrogen peroxide. The diffusion coefficients of the diffusion membrane and the enzyme layer were experimentally determined and incorporated into the COMSOL Multiphysics software to obtain an accurate biosensor model. The quality of

the model was validated by comparing the simulated accumulation of gluconic acid inside the enzyme layer to experimental data.

Material and methods

Materials

Glucose oxidase (EC 1.1.3.4 from *Aspergillus niger*, 128,200 U/mg), catalase (from bovine liver, 3809 U/mg), bovine serum albumin (BSA), peroxidase (from horseradish, 150 U/mg), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), $\beta\text{-D}(+)\text{glucose}$, glutaraldehyde (50% aqueous solution) and phosphate-buffered saline (PBS) buffer were obtained from Sigma-Aldrich (Taufkirchen, Germany). Silicone glue (RS 232), HydroDish[®] plates for pH measurements, and optically isolated oxygen sensors were obtained from PreSens GmbH (Regensburg, Germany). Hydrophilic perforated membranes that are biologically inert and satisfy USP class IV biocompatibility with negligible cytotoxicity were used as a diffusion barrier for glucose and were attained from PreSens GmbH (Regensburg, Germany). PowerCHO-2TM chemically defined, serum-free medium without glucose from Lonza (Basel, Switzerland) was used for cell cultivation in shake flasks with integrated pH and oxygen sensors from PreSens GmbH (Regensburg, Germany). Disposable 2-L cell bags were purchased from GE Healthcare (Munich, Germany). Steinbeis Transferzentrum für Angewandte Biologische Chemie (Mannheim, Germany) kindly provided a recombinant darbepoetin alfa-producing cell line.

Instrumentation

A 24-channel prototype and a commercially available SensorDish[®] Reader (SDR) from PreSens GmbH (Regensburg, Germany) were applied for glucose and pH measurements, respectively, in 24-well plates. The data acquired with the SDR was analysed with the analysis freeware TReCCA Analyser [13]. A four-channel oxygen meter (OXY-4 mini[®]) with polymeric optical fibres (POFs) and a Shake Flask Reader[®] (SFR) from PreSens GmbH (Regensburg, Germany) were utilized to measure the glucose response and the oxygen level, respectively. The glucose concentration in the 125-mL flasks was controlled through glucose addition by a custom-made pump via LabVIEWTM, a system design software from National Instruments (Texas, USA). A coating thickness gauge Elcometer 345 from Elcometer GmbH (Aalen, Germany) was utilized to determine the layer thickness of the biosensor components. Flow-through experiments were carried out with a peristaltic pump (Minipuls 3, Gilson, Villiers-le-Bel, France) in combination with the oxygen meter Fibox 3 from PreSens GmbH (Regensburg, Germany). Cell bag experiments were carried out

on a WAVE Bioreactor system 20/50 from GE Healthcare (Munich, Germany). Reference glucose measurements were performed with a hexokinase assay in part manually using a HT3 photometer (Anthos, Friesoythe, Germany) and in part automatically using the Cedex Bio (Roche, Mannheim, Germany).

Methods

Glucose biosensor fabrication and sterilization

An optically isolated oxygen sensor was used for glucose biosensor fabrication, to prevent an increased fluorescence background, caused by the enzyme layer. The oxygen sensor was pre-treated with 20% 3-aminopropyltriethoxysilane (APTES) for 2 h at 40 °C to improve the enzyme layer adhesion to the hydrophobic oxygen sensor surface [14]. The optically isolated side of the oxygen sensor was covered with a polymerizing enzyme solution that was prepared as follows: 8 mg GOD, 50 mg BSA and 10 µL glycerol were dissolved in 250 µL distilled water. The immobilization process was initiated by adding 50 µL glutaraldehyde (2.5% aqueous solution) to the enzyme solution. An adequate proportion of the polymerizing enzyme solution was coated onto the oxygen sensor surface by means of a micro pipette. Subsequently, a perforated hydrophilic membrane with either a low permeability membrane (LPM) or a high permeability membrane (HPM) was placed on top to achieve an evenly distributed enzyme layer due to capillary forces. The biosensor rim was sealed after the polymerization process (15 min) with silicone glue. Finally, the biosensor was sterilized with β -irradiation (18 kGy) or UV (15 W) for 1 h and then stored in sterile PBS buffer at 4 °C until further usage. Gamma irradiation, although possible (data not shown), was not applied in this work.

Shake flask and cell bag preparation for glucose monitoring

Glucose biosensors in combination with reference oxygen sensors were used to monitor the glucose concentration in 125-mL shake flasks and a 2-L cell bag. In the case of the shake flask experiment, the reference oxygen sensor was already an integral part of the shake flask which was measured via the Shake Flask Reader[®] through the transparent bottom of the flask. The glucose biosensor was aseptically mounted onto an optical fibre and inserted through the lid of the flask (Fig. 1). For the cell bag experiment, a glucose biosensor was fabricated in a laminar flow hood and placed together with an oxygen reference sensor on a transparent foil with silicone glue. After the sensors were sterilized with UV (15 W, 253.7 nm) irradiation for 1 h, the foil was inserted aseptically through a screw cap port of the cell bag and fixed with sterile neodymium magnets from the inside and non-sterile counter magnets from the outside of the cell bag. The measurement

was performed with external optic fibres in a mounting via the transparent surface of the cell bag (see Electronic Supplementary Material (ESM) Fig. S1).

The oxygen transducer inside the glucose biosensor and the oxygen reference sensor were initially calibrated according to the calibration data sheet of the manufacturer. The measurement signal of both sensors is expressed as oxygen partial pressure in percent air saturation and results from an oxygen concentration-dependent dynamic fluorescence quenching of the excited luminophores within the oxygen sensitive layer.

Cell culture experiments

Cell culture experiments were carried out in 125-mL shake flasks according to section “[Shake flask and cell bag preparation for glucose monitoring](#)” with a working volume of 50-mL CHO-2 medium including antibiotics. The inoculation density was 2.5×10^5 cells/mL. All flasks were incubated at 37 °C, 130 RPM, 50% air saturation and 5% CO₂ in a Heracell 240i incubator (Thermo Fischer Scientific, USA). A 2-L cell bag was filled with 0.5 L of CHO-2 medium and supplemented with antibiotics. The cell bag was operated at 37 °C, 3.5% CO₂ and 80% air saturation and inoculated with 5×10^5 cells/mL.

Concentrated glucose solution (0.5 M) was added via a custom-made pump, connected with tubes to the shake flask lid, or added manually in the case of the cell bag. Samples were taken periodically and centrifuged at 400g for 5 min to determine the glucose concentration in the supernatant offline, by means of a hexokinase assay kit from Sigma-Aldrich (Taufkirchen, Germany) or the Cedex Bio, respectively.

Determination of hydrogen peroxide

The hydrogen peroxide concentration was measured enzymatically via horseradish peroxidase (HRP) and ABTS in PBS buffer [15]. The increase in absorbance of the chromogenic dye ABTS was followed photometrically at 405 nm.

Determination of the diffusion coefficients for the applied biosensor materials

The apparent diffusion coefficients of the applied biosensor materials were determined for glucose and hydrogen peroxide in custom-made cylinder tubes, placed inside a 24-well plate. The tube volume was separated from the well content by the investigated material. Each tube was placed individually into a well with GOD, HRP and ABTS dissolved in PBS buffer. The investigated material divided the system into two compartments. One compartment was confined by the tube, which was filled with a defined concentration of either glucose or hydrogen peroxide, while the other compartment was represented by the well. The

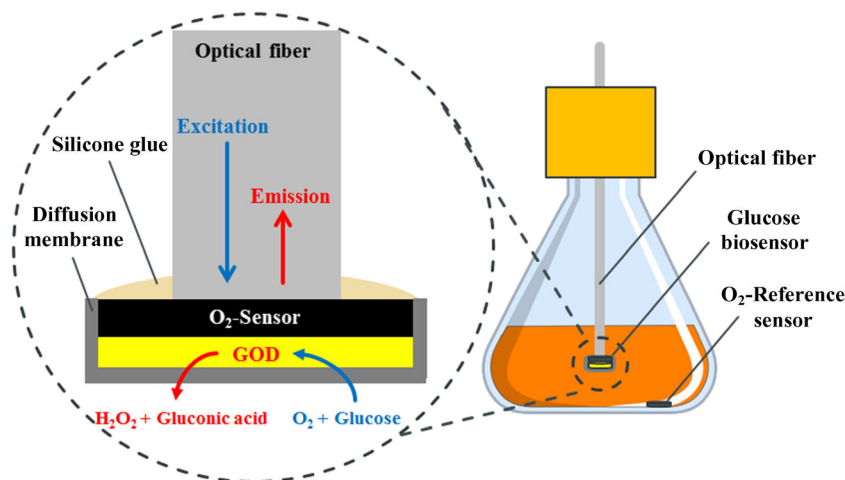


Fig. 1 Measuring principle of a glucose biosensor in a 125-mL shake flask. The glucose biosensor consists of an optical oxygen transducer that measures the oxygen consumption inside the enzyme layer due to the enzymatic conversion of glucose and oxygen, which enter the enzyme layer by diffusion through a diffusion membrane. An additional oxygen

reference sensor at the *bottom* of the shake flask is used to compensate for oxygen fluctuations in the medium. The glucose response (ΔpO_2) is attained by subtracting the oxygen partial pressure within the biosensor from the oxygen partial pressure in the medium

diffusion of glucose or hydrogen peroxide through the material triggered a colorimetric reaction in the well, which was measured according to section “[Determination of hydrogen peroxide](#).” Since the diffusion rate was very small, the concentration in both compartments was assumed to be constant. The substance concentration inside the tube was practically unchanged, while the glucose or hydrogen peroxide concentration inside the well was assumed to be virtually zero, due to the instant conversion of the diffusing substance. Under the assumed steady-state conditions, the effective diffusion coefficient was calculated according to Fick’s first law by measuring the molar flux ($dn_i/dt/A$) of the substance as an increase in absorbance over time (t)

$$\frac{dn_i}{dt \times A} = -D_i \frac{dc_i}{dx} \quad (4)$$

n_i denotes the amount of the substance of interest, while D_i , A and dx denote the diffusion coefficient, the area and the thickness of the applied material, respectively. The concentration difference of the substance of interest between the two compartments (well and tube) is given as dc_i and is assumed to be equal to the concentration of the substance in the tube.

The diffusion coefficient for oxygen was determined in a flow-through cell by placing the biosensor material in front of an oxygen sensor. The oxygen sensor within the flow-through cell was equilibrated with nitrogen-saturated PBS buffer and subsequently flushed with air-saturated PBS buffer. The oxygen diffusion through the tested sensor material was monitored by the underlying oxygen sensor. As an approximation,

the initial slope of the oxygen response was assumed to be equal to the oxygen flux. The oxygen gradient was obtained as the difference in oxygen partial pressure between the nitrogen and the air-saturated buffer divided by the thickness of the investigated material.

Biosensor model

A time-dependent simulation of the biosensor response under various conditions was performed using the COMSOL Multiphysics 4.4 (COMSOL, Inc., Burlington, USA) finite element method by coupling Fick’s second law to the enzyme reaction rate (R_i)

$$\frac{dc_i}{dt} + \nabla(-D_i \nabla c_i) = R_i \quad (5)$$

where c_i denotes the concentration of the substance of interest and ∇ the nabla operator.

A simple one-dimensional model of the biosensor was created to simulate the biosensor characteristics and the concentration profiles associated. The simulation of the glucose oxidase reaction is based on the ping-pong mechanism [16] characterized by the following rate equation [17]:

$$R_i = \frac{V_{\max}}{\left(1 + \frac{K_G}{G} + \frac{K_O}{O}\right)} \quad (6)$$

where G and O are the concentration of glucose and oxygen, respectively. K_G and K_O represent the Michaelis-Menten

constants for glucose and oxygen. V_{\max} denotes the maximum reaction velocity.

We assumed the enzymatic reaction of glucose oxidase and catalase to be described by Michaelis-Menten kinetics [18, 19] with Michaelis constants from free enzymes [5, 18, 20]. K_G was determined spectrophotometrically by monitoring the hydrogen peroxide concentration generated by free glucose oxidase according to section “[Determination of hydrogen peroxide.](#)” The Michaelis-Menten constants for oxygen and hydrogen peroxide were attained from the literature. As described in section “[Determination of the diffusion coefficients for the applied biosensor materials,](#)” the diffusion coefficients for a low permeability and a high permeability diffusion membrane as well as the enzyme layer were obtained through measurements. The material property of the oxygen sensor layer was assumed to be similar to silicone, while the diffusion coefficients for the sample medium were approximated to be similar to water. A coating thickness gauge (Elcometer 345) was used to determine the height of the enzyme layer, while the thickness of the oxygen sensor and the diffusion membranes was specified by the manufacturer. The maximum enzyme velocity of the immobilized enzymes was fitted with COMSOL.

In this study, the partitioning effects were neglected. The oxygen concentration in the medium and the biosensor was considered to be initially around 0.296 mM, while the gluconic acid and hydrogen peroxide concentration were set to virtually zero.

The model was based on the following assumptions:

- (1) The enzymes (GOD and Cat) are evenly distributed inside the enzyme layer where the reaction occurs
- (2) The mass-transfer resistance between the sample and the diffusion membrane of the biosensor is negligible
- (3) The dissolved oxygen concentration in the sample remains constant
- (4) The diffusion of substrates and products are represented by Fick’s law

(5) No substrate or product in-

hibition occurs

(6) Isothermal and isobaric reaction conditions

(7) The biosensor signal corresponds to the simulated oxygen level within the oxygen sensitive layer of the biosensor

Results and discussion

Biosensor characteristics

The glucose level in the PBS buffer solution was indirectly measured via the oxygen partial pressure in the enzyme layer of the biosensor, which depends on the diffusion and the enzymatic consumption of glucose and oxygen according to the reaction Eqs. 1, 2 and 3. Figure 2a shows the glucose response to incremental analyte increase in the presence of an oxygen reference sensor. The resulting signal (ΔpO_2) corresponds to the difference between the partial pressure in the aqueous sample solution, measured by the oxygen reference sensor, and the oxygen level within the enzyme layer of the biosensor. In this way, oxygen fluctuations in the sample during the experiment were compensated.

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the sensitivity of the resulting calibration curve from Fig. 2a and the standard deviation of the glucose response according to IUPAC [21]. The results of the LOD and LOQ calculation are summarized in Table 1. Apart from glucose, the biosensor response depends on the temperature, the flow velocity and the oxygenation of the sample. In agreement with Pasic et al., we found that the pH does not affect the biosensor signal in the pH range between 5 and 8 [22] (data not shown), because the diffusion of glucose through the diffusion membrane and not the enzyme activity is the rate-limiting step. Under constant flow velocity and temperature, the sensitivity and the dynamic range of the

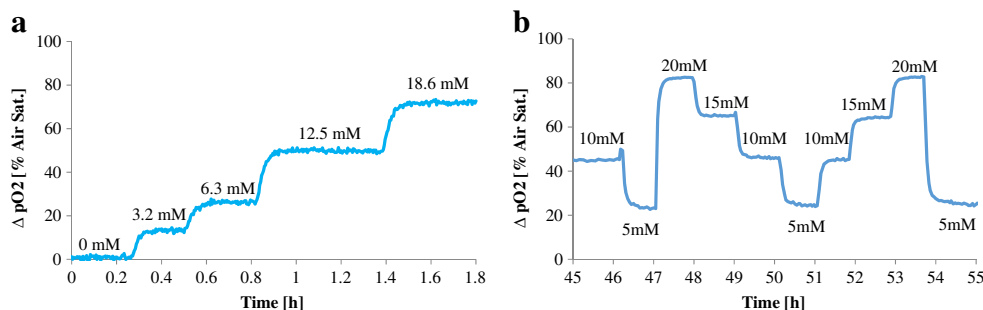


Fig. 2 **a** Glucose response at 0, 3.2, 6.3, 12.5 and 18.6 mM glucose. The measurement was performed in a 24-well plate with 2 mL PBS at 25 °C, 230 RPM and 100% air saturation. **b** Signal reversibility for increasing and decreasing glucose concentrations. The glucose concentration was

changed by shifting the glucose biosensor between four shake flasks with 5, 10, 15 and 20 mM glucose solution in PBS buffer at 25 °C, 230 RPM and 100% air saturation

Table 1 Figure of merit

Parameter	Value
Dynamic range (at air saturation, room temperature and 230 RPM)	0–20 mM
LOD (at 230 RPM and room temperature)	0.45 mM
LOQ (at 230 RPM and room temperature)	1.5 mM
Response time (at 230 RPM and room temperature)	$t_{90} \leq 10$ min
Functional stability (at 17.5 mM glucose and room temperature)	>52 days
Cross sensitivity to oxygen (measured at 5 mM glucose)	$\leq 1.4\%$

glucose sensor depend on the permeability of the diffusion membrane for glucose and oxygen. The dynamic range of the biosensor can therefore be customized via different diffusion membranes.

The response time (t_{90} , the time until 90% of the steady-state signal was reached) and the reversibility of the biosensor response was measured after a 45-h pre-equilibration by stepwise decrease and increase of the glucose concentration (Fig. 2b). The biosensor signal was fully reversible with a response time (t_{90}) of 6 min for increasing and 10 min for decreasing glucose concentrations.

Biosensor stability

The functional stability of glucose biosensors with different GOD loadings and two different diffusion membranes was investigated under continuous glucose monitoring over several weeks. A glucose level at the upper end of the dynamic range was chosen to test the stability under a considerable production and accumulation of hydrogen peroxide inside the enzyme layer. Because the inactivation rate of GOD depends on the hydrogen peroxide concentration [23], the enzyme catalase (Cat) was co-immobilized in one biosensor set-up to decompose hydrogen peroxide inside the enzyme layer according to the following equation:



Three glucose biosensors for each type of sensor were placed separately in a 24-well plate with 2 mL PBS buffer solution per well. The sensitivity of the biosensor is equal to the slope of its calibration curve, obtained by stepwise glucose addition as illustrated in Fig. 2a. The sensitivity was measured repeatedly during the continuous glucose monitoring experiment and compared to the initial sensitivity on day zero (Fig. 3a).

Two opposing trends were encountered during the functional stability experiment illustrated in Fig. 3a. First, a sensitivity increase was observed for all biosensor

types, which was followed by a sensitivity drop at a later time point. In most cases, the sensitivity increase was complete until the second measurement of the calibration curve on day 4. But, for catalase-containing biosensors, the sensitivity increased significantly even after 4 days of continuous glucose monitoring. The sensitivity increase is caused by a continuous loss of catalase activity, presumably due to hydrogen peroxide deactivation [7, 24]. As a consequence, the sensor signal (pO_2) at a given glucose concentration is lower, because the enzymatic decomposition of hydrogen peroxide into oxygen (Eq. 7) is impaired. The same rationale applies for all biosensors, since all enzyme layers contain catalase as an impurity due to the manufacturing process of GOD (≤ 5 units/mg protein according to the manufacturer). Figure S2 in the ESM illustrates the influence of the biosensor signal with and without catalase.

Biosensors that were generated from an enzyme solution with 2 and 8 mg GOD with a diffusion LPM showed a relatively stable signal phase until day 17, as opposed to 1-mg GOD biosensors that experienced a sensitivity drop much earlier. Generally, the enzyme activity of GOD decays naturally with time. However, a major portion of the sensitivity drop is caused by hydrogen peroxide [4–7]. This was particularly a problem for biosensors with a low GOD loading. Thus, sensors with excess GOD concentrations suffered less from decreasing sensitivity and are more suitable for long-term measurements. Biosensors with 8 mg GOD but a diffusion HPM exhibited, however, a lower functional stability compared to their counterpart with a LPM. The reason for this phenomenon is discussed in detail in section “Numerical simulations of biosensor characteristics”.

Figure 3b illustrates the mean value for the accumulated hydrogen peroxide concentration of different biosensor types during the initial phase of the experiment (days 1–3) and a later phase (days 21–23). As expected, for biosensors with additional catalase, the emitted hydrogen peroxide concentration in the well was significantly lower during the early phase (days 1–3). However, after 3 weeks of continuous glucose monitoring, the hydrogen peroxide emission was comparable to those without additional catalase (days 21–23). This confirms that the enzyme catalase was initially more active and was inactivated with time. Although catalase has been reported to have a protective effect on GOD [7, 25–28], the catalase biosensors experienced a higher sensitivity drop once catalase was inactivated and thus had no relevant increase in the overall functional stability. Instead, additional catalase led to a high signal drift caused by its deactivation.

The specifications of the glucose biosensor depend strongly on the measurement conditions. Summarized in Table 1 are the specifications attained during this work.

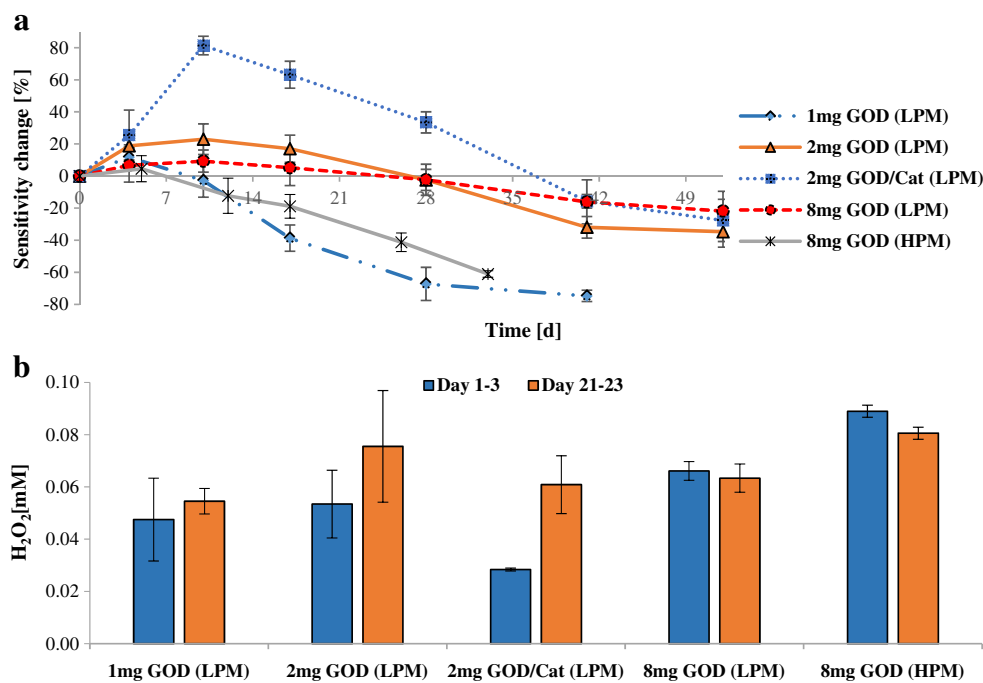


Fig. 3 **a** Functional stability of biosensors with different enzyme layer compositions and diffusion membranes under continuous glucose monitoring conditions at $T = 25\text{ }^{\circ}\text{C}$, 230 RPM and 100% air saturation over time. The glucose level for biosensors with a low permeability membrane (LPM) was 17.5 mM, whereas the glucose concentration for biosensors with a high permeability membrane (HPM) was 35 mM. The

error bars correspond to the standard deviation of three biosensors for each type of biosensor. **b** Hydrogen peroxide accumulation during an early phase (days 1–3) and later phase (days 21–23) of the continuous glucose monitoring experiment (Fig. 3a). The error bars correspond to the standard deviation of three biosensors for each type of biosensor

Numerical simulations of biosensor characteristics

The geometry of the biosensor and the apparent diffusion coefficients for the substrates and products involved are crucial parameters for the simulation of the sensor characteristics and were obtained according to sections “Determination of hydrogen peroxide,” “Determination of the diffusion coefficients for the applied biosensor materials” and “Biosensor model.” All simulation parameters are summarized in Table 2.

Numerical simulations were performed for varying glucose concentrations and two diffusion membranes with different permeability. The characteristic line of the biosensors was simulated with the parameters stated in Table 2 and compared to experimental data (Fig. 4a, b). The simulated and the actual biosensor signal showed a good correlation and a broad linear region, in which the diffusion of glucose is rate limiting [23, 34].

Illustrated in Fig. 4c is the simulated accumulation of H_2O_2 within the enzyme layer at various biosensor signals for the applied diffusion membranes. The diffusion coefficients for the HPM were in part 1 order of magnitude higher for the involved reaction species. This means that more glucose and oxygen enter the enzyme layer and increase the enzymatic turnover rate, but also more gluconic acid

and hydrogen peroxide is emitted (Fig. 3b). As a consequence, the hydrogen peroxide build-up inside the biosensor is comparable for both diffusion membranes (Fig. 4c). This finding was indirectly confirmed by the correspondence of the simulated gluconic acid accumulation inside the biosensor to the experimental data (Fig. 4d). This approach is valid, because the formation of hydrogen peroxide and gluconic acid are stoichiometrically linked to each other. The gluconic acid formation at various glucose concentrations was measured with optical pH sensors, coated with an enzyme solution and covered by the respective diffusion membrane in analogy to section “Glucose biosensor fabrication and sterilization”. The pH change caused by the gluconic acid accumulation inside the enzyme layer was compared to a calibration curve attained by pure gluconic acid addition without glucose, in order to correlate the gluconic acid concentration to the measured pH change.

Despite the comparable hydrogen peroxide burden for GOD, the diffusion membrane with the higher permeability provided a much shorter biosensor lifetime (Fig. 3a). Consequently, the hydrogen peroxide concentration inside the enzyme layer is not the only factor that influences the enzyme activity. It is the amount of GOD that is in its reduced state (Eq. 1) that matters too, since the reduced form of GOD is 100-fold more susceptible to hydrogen

Table 2 Diffusion coefficients and kinetic parameters used for the biosensor model

Parameter	Value
Aqueous solution (boundary layer)	
Diffusion coefficients for glucose, D_G^a	$6.75 \times 10^{-10} \text{ m}^2/\text{s}$
Diffusion coefficients for gluconic acid, D_{GA}	$6.75 \times 10^{-10} \text{ m}^2/\text{s}^b$
Diffusion coefficients for oxygen, $D_{O_2}^a$	$2.3 \times 10^{-9} \text{ m}^2/\text{s}$
Diffusion coefficients for hydrogen peroxide, D_H^c	$1.0 \times 10^{-9} \text{ m}^2/\text{s}$
Layer thickness ^b	7.5 μm
Diffusion membrane with a low permeability membrane (LPM)	
Diffusion coefficients for glucose, D_G	$3.0 \times 10^{-13} \pm 2.0 \times 10^{-13} \text{ m}^2/\text{s}^d$
Diffusion coefficients for gluconic acid, D_{GA}	$3.0 \times 10^{-13} \text{ m}^2/\text{s}^b$
Diffusion coefficients for oxygen, D_{O_2}	$10.5 \times 10^{-12} \pm 0.9 \times 10^{-12} \text{ m}^2/\text{s}^d$
Diffusion coefficients for hydrogen peroxide, D_H	$3.4 \times 10^{-13} \pm 1.8 \times 10^{-13} \text{ m}^2/\text{s}^d$
Layer thickness (manufacturer specification)	6 μm
Diffusion membrane with a high permeability membrane (HPM)	
Diffusion coefficients for glucose, D_G	$1.0 \times 10^{-12} \pm 3.9 \times 10^{-13} \text{ m}^2/\text{s}^d$
Diffusion coefficients for gluconic acid, D_{GA}	$1.0 \times 10^{-12} \text{ m}^2/\text{s}^b$
Diffusion coefficients for oxygen, D_{O_2}	$10.5 \times 10^{-11} \text{ m}^2/\text{s}^e$
Diffusion coefficients for hydrogen peroxide, D_H	$3.5 \times 10^{-12} \pm 4.6 \times 10^{-13} \text{ m}^2/\text{s}^d$
Layer thickness (manufacturer specification)	25 μm
Enzyme layer	
Diffusion coefficients for glucose, D_G	$2.2 \times 10^{-10} \pm 0.74 \times 10^{-10} \text{ m}^2/\text{s}^d$
Diffusion coefficients for gluconic acid, D_{GA}	$2.2 \times 10^{-10} \text{ m}^2/\text{s}^b$
Diffusion coefficients for oxygen, D_{O_2}	$8.2 \times 10^{-10} \pm 0.87 \times 10^{-10} \text{ m}^2/\text{s}^d$
Diffusion coefficients for hydrogen peroxide, D_H	$6.3 \times 10^{-10} \pm 1.07 \times 10^{-10} \text{ m}^2/\text{s}^d$
Layer thickness	$20 \pm 2.5 \mu\text{m}^d$
Oxygen sensor layer	
Diffusion coefficients for glucose, D_G	$1 \times 10^{-15} \text{ m}^2/\text{s}^b$
Diffusion coefficients for gluconic acid, D_{GA}	$1 \times 10^{-15} \text{ m}^2/\text{s}^b$
Diffusion coefficients for oxygen, $D_{O_2}^f$	$1.7 \times 10^{-9} \text{ m}^2/\text{s}$
Diffusion coefficients for hydrogen peroxide, D_H	$1 \times 10^{-15} \text{ m}^2/\text{s}^b$
Layer thickness	$120 \pm 2.5 \mu\text{m}^d$
Reaction kinetics	
Maximum velocity, $V_{\max}(\text{GOD})$	0.31 mM/s ^e
Maximum velocity, $V_{\max}(\text{Cat})$	0.041 mM/s ^e
Michaelis-Menten constant for glucose, $K_G(\text{GOD})$	$21.1 \pm 1.8 \text{ mM}^d$
Michaelis-Menten constant for H_2O_2 , $K_{\text{H}_2\text{O}_2}(\text{catalase})^g$	60 mM
Michaelis-Menten constant for oxygen, $K_{O_2}(\text{GOD})^h$	61.2 μM

^a Diffusion coefficient values for glucose and oxygen in aqueous solution was obtained from Albin et al. [29]

^b Assumed

^c Diffusion coefficient for hydrogen peroxide in aqueous solution was obtained from Dayal and Godjevargova [30]

^d Measured

^e Fitted

^f Diffusion coefficient for oxygen in silicone was obtained from Brandrup and Edmund [31]

^g The Michaelis-Menten constant for the enzyme catalase was obtained from Mafra et al. [32]

^h The Michaelis-Menten constant regarding oxygen for the enzyme glucose oxidase was obtained from Hsieh et al. [33]

peroxide deactivation than the oxidized form [4]. Hence, besides a high GOD loading, a diffusion membrane with a

low permeability for glucose is a key to an increased biosensor lifetime. In addition, this also explains why

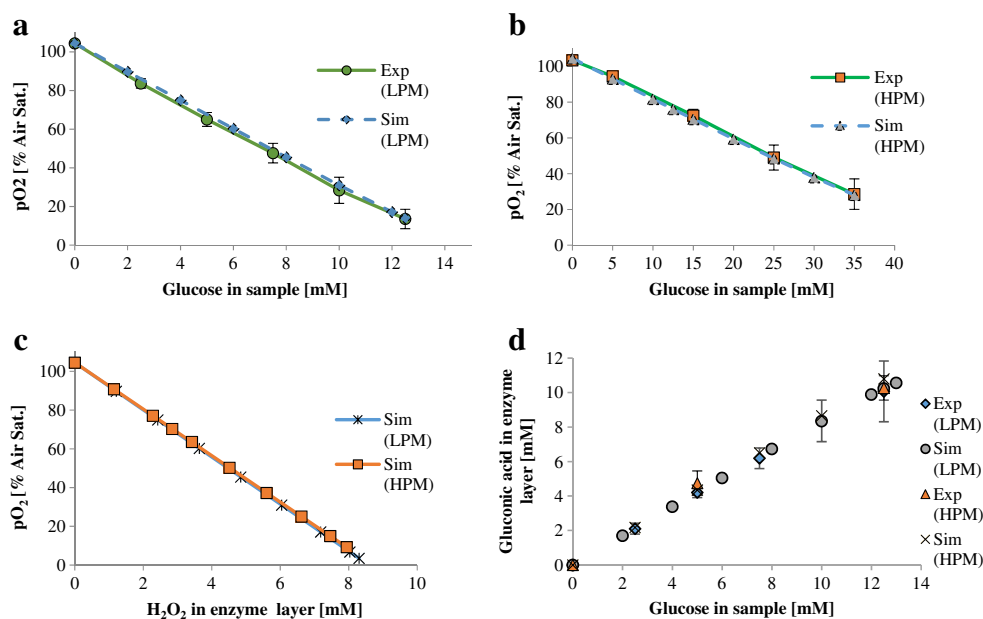


Fig. 4 a, b Experimental measurements (solid line) compared to the simulated biosensor response (dashed line) for biosensors with a low permeability membrane (LPM) or a high permeability membrane (HPM). The experimental data are illustrated as a mean of three biosensor measurements in a 24-well plate under the following conditions: 25 °C, 2 mL air-saturated sample solution and 130 RPM. The error bars correspond to the standard deviation calculated from the

experimental data. c Simulated hydrogen peroxide concentration inside the enzyme layer at various glucose levels for biosensors with LPMs or HPMs. d Measured gluconic acid accumulation inside the enzyme layer compared to computer simulations for biosensors with HPM and LPM. The experimental data are illustrated as a mean of three measurements in a 24-well plate under the following conditions: 25 °C, 2 mL air-saturated sample solution and 130 RPM

the biosensor lifetime is dramatically reduced, if the sensor is operated outside the dynamic range, where oxygen is scarce and thus the reoxidation of the reduced GOD (Eq. 2) is impaired.

One additional benefit of a biosensor with a low permeability diffusion membrane is a lower cross sensitivity to oxygen. This is illustrated in Fig. S3 (see ESM), where a decrease in the dissolved oxygen concentration at a constant glucose level in the medium leads to a minor

influence on the sensor signal ($\Delta pO_2 \leq 1.4\%$) within the dynamic range. For biosensors with a high permeability diffusion membrane, the cross sensitivity to the dissolved oxygen concentration in the medium is increased.

Glucose monitoring during cell culture

All glucose biosensors were pre-equilibrated for 2 days in a glucose solution to prevent an initial sensitivity increase

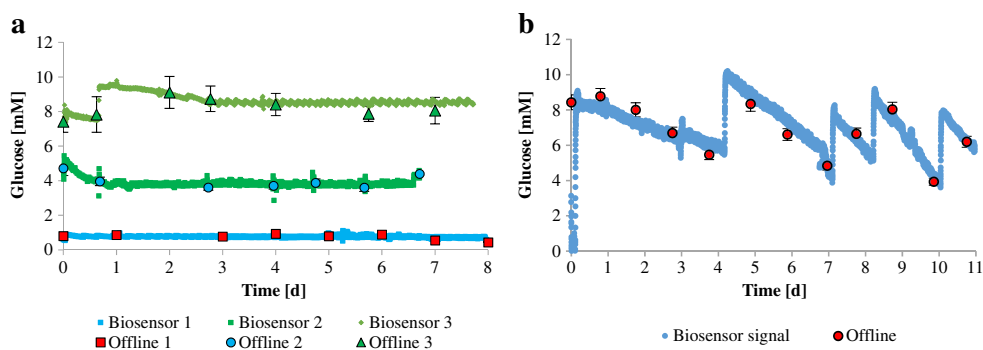


Fig. 5 a Glucose level monitoring and control in three shake flasks with CHO cells. The experiment was carried out in 50 mL at 37 °C, 130 RPM, 5% CO_2 and 50% air saturation. The CHO medium with Biosensor 3 exhibited a minimal glucose overshoot after 24 h, due to a pressure build-up caused by the 0.2 μm sterile filter resistance in the tubing of the glucose feed. b Glucose level monitoring in a cell bag bioreactor with

CHO cells (500 mL). The experiment was carried out at 37 °C, 3.5% CO_2 and 80% air saturation. Glucose was added manually on days 4, 7, 8 and 10. In all cell culture experiments, samples were drawn periodically and analysed as triplicates offline (error bars correspond to the standard deviation)

during the cell culture experiments. Three biosensors were subsequently mounted separately onto three optical fibres. Each fibre was placed in a 125-mL shake flask (Fig. 1), equipped with a reference oxygen sensor. A one-point calibration of the biosensor was carried out by measuring the initial glucose response of a pre-defined glucose level in the cell culture medium after inoculation with Chinese hamster ovarian (CHO) cells. The glucose levels were monitored and controlled in 2-min intervals at 0.8, 4 and 9 mM during three different cell culture experiments in a shake flask (Fig. 5a). Glucose was added via LabVIEW[®] through automated injection of glucose, as soon as the glucose concentration dropped below the respective set point.

A similar experiment was performed in a 2-L single-use bioreactor for 11 days with the manual addition of glucose on days 4, 7, 8 and 10. In this case, a recalibration of the biosensor was performed on day 7 (Fig. 4b).

In order to confirm the glucose concentrations, several samples were drawn during the cell culture experiments at different time points and measured offline via a hexokinase assay. In all experiments, the biosensors did not experience biofouling due to the hydrophilic membrane and had no obvious influence on the cell viability and cell growth (data not shown).

Conclusion

In this study, we developed a disposable inline biosensor, suitable for long-term continuous glucose monitoring in cell culture. The measurement signal was reversible without hysteresis and linear up to 20 mM glucose with a response time of $t_{90} \leq 10$ min. Once the glucose sensor was mounted into a bioreactor, a one-point calibration was performed for cell culture experiments of up to 8 days without recalibration. The overall functional stability of the presented glucose sensor was sustained over at least 52 days under continuous monitoring conditions. The biosensor was sterilizable with beta and UV irradiation and experienced no biofouling during the cell culture experiments.

In addition to the biosensor development, a detailed investigation of the diffusion properties of the biosensor materials was performed for the development of a numerical model, which describes the biosensor characteristics adequately and revealed the hydrogen peroxide accumulation inside the biosensor for different glucose levels. The quality of the model was validated by comparing the simulated accumulation of the by-product gluconic acid to actual data, which were in good agreement. Our simulations suggest that the enzymatic turnover rate affects the enzyme activity, which is determined by the slowest step, the diffusion of glucose through the diffusion membrane. We therefore conclude that a diffusion membrane with a low permeability for glucose, in combination with a high GOD loading, is optimal for a long biosensor lifetime.

In conclusion, we established a disposable biosensor for real-time continuous glucose monitoring with a long-term functional stability and, by means of a one-dimensional biosensor model, offer an insight into the associated inactivation process which occurs through accumulation of hydrogen peroxide.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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