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Biosensors

Catalytic Biosensors Operating under Quasi-Equilibrium Conditions for Mitigating the Changes in Substrate Diffusion

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Abstract: Despite the success of continuous glucose measuring systems operating through the skin for about 14 days, long-term implantable biosensors are facing challenges caused by the foreign-body reaction. We present a conceptually new strategy using catalytic enzyme-based biosensors based on a measuring sequence leading to minimum disturbance of the substrate equilibrium concentration by controlling the sensor between "on" and "off" state combined with short potentiometric data acquisition. It is required that the enzyme activity can be completely switched off and no parasitic side reactions allow substrate turnover. This is achieved by using an O_2-independent FAD-dependent glucose dehydrogenase embedded within a crosslinked redox polymer. A short measuring interval allows the glucose concentration equilibrium to be restored quickly which enables the biosensor to operate under quasi-equilibrium conditions.

Introduction

Long-term implantable biosensors play a key role in the monitoring and treatment of metabolic diseases, particularly for applications in continuous glucose monitoring (CGM).[1–4] To date, there are several commercial CGM systems available that mainly rely on two different detection principles. Most of the through-skin operating CGM systems are based on amperometric catalytic biosensors detecting current responses proportional to the conversion of the glucose concentration at the active site of an enzyme which in most cases is immobilised on the electrode surface.[3,5] Besides their simple detection principle, the specific substrate affinity is another advantageous feature of enzyme-based sensors, which allows highly selective and reliable substrate determination in complex body fluids. Importantly, the enzyme-catalysed conversion of the substrate followed by product release is a self-cleaning process which makes the active site of the enzyme again available for the conversion of the substrate. However, the continuous conversion of the substrate leads to the most significant disadvantage of these types of sensors for their application in long-term implantable systems. In principle, a catalytic biosensor can be seen as a technical chemical reactor with a reaction volume (the sensing layer) which is separated from the external solution by a permeable barrier through which the substrate can enter the reaction volume and the product can leave it. Within the sensing layer, the substrate is consumed at a constant reaction rate if sufficient enzyme activity is available, leading to a difference in substrate concentration between the enzyme layer and the external environment and, thus, to the formation of a diffusion gradient. Consequently, the apparent concentration of the substrate (c_{app}) within the sensing layer and therefore the substrate turnover depends on the diffusional flux into the reaction volume. Under continuous sensor operation of an assumed long-term implantable sensor, the so-called foreign-body reaction is adding encapsulation layers around the sensor and lowers by this the diffusional flux of the substrate into the sensor. This decrease in diffusional flux in turn decreases c_{app} within the sensor layer and lowers the recorded signal (Scheme 1). Importantly, the signal varies even at the same external substrate concentration and the same reaction rate of the enzymatic conversion (Scheme 1).

Since it is impossible to prevent the formation of additional diffusion barriers by fibrous encapsulation of implanted sensors,[6–8] it is unfeasible to obtain a long-term stable sensor output in the case of implanted catalytic biosensors with implantation times above several months.

To circumvent this issue, there are basically two opportunities. On the one hand, there is the possibility of frequent recalibration using a direct measurement of the external substrate concentration to readjust the calibration graph using a one-point calibration. Alternatively, one would have to avoid the building up of a concentration gradient between the external substrate reservoir and the sensor layer irrespective of the permeability of the layers formed by the foreign-body reaction, i.e., $c_{ext} = c_{app}$.

One commercial CGM system is available on the market which follows an affinity interaction principle on modified hydrogels which is governed by an equilibrium concentration of the substrate inside and external of the sensor layer, however, in this case, no substrate conversion occurs. The operational lifetime of this sensor amounts to 90 days

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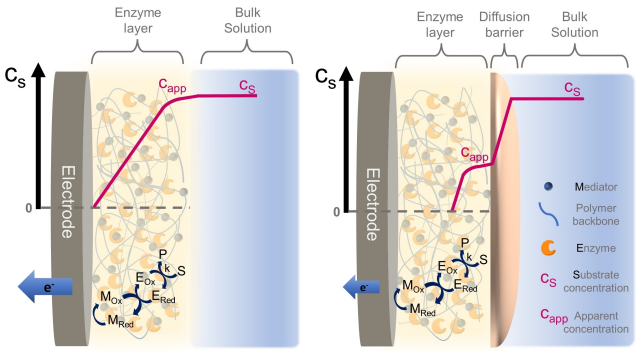


Scheme 1. Schematic representation of a typical concentration profile of a catalytic enzymatic sensor under continuous operation without (left) and with an additional layer as formed after implantation due to the foreign-body reaction (right). The substrate conversion depends on its local concentration at the enzyme which is determined by the reaction rate and the diffusional flux. The gradient changes with time with the increase of the diffusion layer, which leads to a more pronounced difference between c_{app} inside the sensor layer and the external substrate concentration which leads to a signal change at unchanged external substrate concentration.

whereby recent studies also show a possible usability of up to 180 days.[9] The sensor concept is based on affinity interactions between substrate and recognition matrix using an optical detection system coated with a boronic-acid modified hydrogel. In contrast to catalytic biosensors, these affinity-based sensors determine the glucose concentration level without chemical conversion of the glucose. Instead, glucose is reversibly bound to the recognition matrix followed by a modulation in the detected fluorescence signal. Since affinity sensors are less dependent on substrate diffusion, encapsulation by the foreign-body reaction has a minor effect on the functionality of such sensors. While recalibration is still required, the changes in the diffusional properties due to the foreign-body reaction have less impact on the sensor signal. However, non-catalytic sensors face a dilemma of binding constant versus response time and accuracy, while they have the advantage of a non-enzymatic and hence possibly more robust recognition site. The binding constant of the sensor matrix must be precisely adjusted for the prevailing conditions. If it is too strong, the detachment of the substrate from the recognition matrix will be very slow and changes in glucose concentration in the sensor environment can be determined only very slowly. If the binding constant is too weak, the accuracy of the detection will be compromised. Additionally, not only glucose molecules but also other sugars and diols bind to the boronate groups resulting in decreased measurement accuracy or false results.[10]

Some advances to overcome the limitations of dynamic electrochemistry have been made in the field of potentiometry. Bakker's group showed a triple-pulse measurement sequence for ion selective electrodes that provides a reference electrode-independent signal with higher sensitivity than predicted by Nernst equation.[11] Ishige et al. developed a field-effect transistor (FET)-based enzyme sensor that measures the changes in the interfacial potential depending on the ratio of a redox compound participating in an enzymatic reaction.[12] First,[13] second[14] and third[15,16] generation open-circuit potential (OCP) biosensors have been demonstrated for the detection of glucose[13–16] or other analytes,[14,15] and the performance of the sensors demonstrate the size- and interference-independence advantage of potentiometry. However, continuous monitoring of the OCP can still result in a concentration gradient because the enzyme may not be completely switched-off, and third-generation sensors with the enzymes in the direct-electron transfer regime show disadvantages at low substrate concentrations due to undefined mixed potentials.

The question therefore arises if it is possible to conceive a self-cleaning catalytic biosensor operating under substrate concentration equilibrium conditions, i.e., with $c_{ext}=c_{app}$? We propose three prerequisites to design such a sensor: 1. The catalytic turnover reaction can be switched off. 2. Uncontrollable parasitic reactions, such as oxygen reduction at the enzyme or the electrode, need to be avoided. 3. During the measurements, the disturbance of the substrate concentration equilibrium must be minor to guarantee a fast reestablishment of the equilibrium after enzymatic substrate conversion.

We introduce a redox hydrogel-based catalytic microbiosensor concept for the detection and monitoring of glucose with minimal disturbance of the substrate equilibrium concentration which overcomes the issues imposed by sensor encapsulation as expected by the foreign-body reaction upon implantation. Specifically, the signal becomes independent of any diffusional flux into the sensing layer provided a sufficient waiting time to fully establish concentration equilibrium. The proposed strategy is the first demonstration of a catalytic substrate equilibrium biosensor for the reliable determination of glucose based on an enzyme embedded in a redox polymer.

Results and Discussion

The proposed sensor concept is based on a switching mechanism which allows the control of the enzymatic substrate conversion by applying predefined potentials (Scheme 1). In the "off" state, the enzymatic conversion must be fully stopped which requires an enzyme which can be on the one hand wired to the electrode by means of a redox polymer but is completely insensitive to accept molecular oxygen as an alternative electron acceptor.[17] This ability to completely switch-off the enzymatic turnover allows the glucose concentration in the sensor layer to equilibrate with the external glucose concentration. The time of applying the "off"-state potential depends on the thickness and permeability of encapsulating layers. For switching the enzyme to the "off"-state a potential must be applied which is at least 250 mV cathodic of the formal potential of the redox mediator tethered to the redox polymer, which will make the oxidised mediator species negligible according to the Nernst equation. We utilised a FAD-GDH which is known to have a low sensitivity towards oxygen.[18] *Glomerella cingulata* FAD-dependent glucose

dehydrogenase (FAD-GDH) expressed in *Pichia pastoris* was used as oxygen-insensitive FAD-GDH[19] embedded in an Os-complex modified redox hydrogel (see Figure S1 left), namely P(VI-AA_{NH_2})-Os (poly(1-vinylimidazole-*co*-acryl hydrazide)-[$Os(bpy)_2Cl]^+$, with bpy = 2,2′-bipyridine).[20,21] Besides its function as an immobilisation matrix, Os-complex modified redox polymers are known to serve as efficient relays for the mediated electron transfer between enzymes and electrode.[17,22]

Potentiostatic control enables to adjust the redox state of the Os-complexes within the redox hydrogel correlating to a specific concentration ratio of oxidised and reduced Os-complexes according to the Nernst equation.

Additionally, the proximity between the Os-complexes and the enzymes within the recognition layer together with the only accessible electron-transfer pathway for the enzyme enables to control the catalytic activity of the enzyme by the applied potential.

A potential pulse from the "off"-state (-250 mV of E^0 (Os^{3+}/Os^{2+})) to the "on"-state (+250 mV of E^0 (Os^{3+}/Os^{2+})) allows charging of the redox polymer film and enables electron transfer from the enzyme via the polymer-bound Os-complexes by means of electron hopping to the electrode (Figure S2). To reliably turn the enzymatic turnover activity off, parasitic side reactions by oxygen reduction must be prevented. Otherwise, reoxidation of the Os-complexes or the enzyme itself may occur resulting in substrate consumption and, thus, in the formation of a glucose diffusion gradient. It was shown that O_2 can be reduced to H_2O_2 by Os-complexes with a redox potential more negative than +0.07 V versus Ag/AgCl/3 M KCl (pH 7).[23] Therefore, the redox potential of the redox polymer should be sufficiently positive to prevent catalytic reduction of O_2 but concomitantly low enough to avoid co-oxidation of interfering compounds, such as ascorbic acid or uric acid directly at the electrode surface.[24] Furthermore, as already pointed out above, the enzyme itself must be insensitive to oxygen.

Since the substrate is consumed under enzymatic turnover leading to a concentration gradient between the sensing layer and the external volume evoking diffusion, it is essential to keep the "on"-time as short as possible, however long enough, that the $Os^{3+/2+}$ ratio is sufficiently changed to allow wiring of the embedded enzyme. The disturbance of concentration equilibrium must be kept to a minimum. For this, electrodes with dimensions at the micro- to nanometre scale are utilised because their intrinsic properties, such as the low capacitive charging current, make them ideal for measurements at short timescales.[21,25] Since the redox polymer acts as a pseudo-capacitive element, the time to charge the film depends on the film thickness. Hence, homogeneous and thin polymer/enzyme films have to be immobilised on the microelectrode surface.

We utilised carbon microelectrodes for the sensor fabrication (Figure 1). The immobilisation of the enzyme embedded in the P(VI-AA_{NH_2})-Os matrix was performed by a layer-by-layer dipping approach which enables the total thickness of the active layer to be controlled by the number of immersion processes (for details see Supporting Information and Table S1). Following, the measurement sequence in Scheme 2, the enzymatic turnover is switched between the "off" and "on" state according to a predefined time regime, and after a short period in the "on" state data acquisition is invoked by opening the electrochemical circuit and following the change of the sensor potential by measuring the OCP. In a typical measurement sequence, the sensor is switched "off" for 90 s by applying a potential 250 mV more negative than the E^0 of P(VI-AA_{NH_2})-Os which is corresponding to a potential of −50 mV vs. Ag/AgCl/3 M KCl. Subsequently, a short oxidative pulse to a potential of +450 mV vs. Ag/AgCl/3 M KCl was applied for 1 s. While applying the short potential pulse, the redox hydrogel is charged by the formation of an excess of Os^{3+} as defined by the Nernst equation. It is essential that the duration of the "on" phase lasts long enough to sufficiently charge the polymer film to be able to wire the enzyme productively allowing the enzyme to oxidize the glucose present at equilibrium concentration in the sensing layer. However, the charging process must be short enough to avoid the generation of a diffusion gradient due to substrate depletion within the film. This charging pulse is followed by switching off the electrical circuit and measuring the course of the

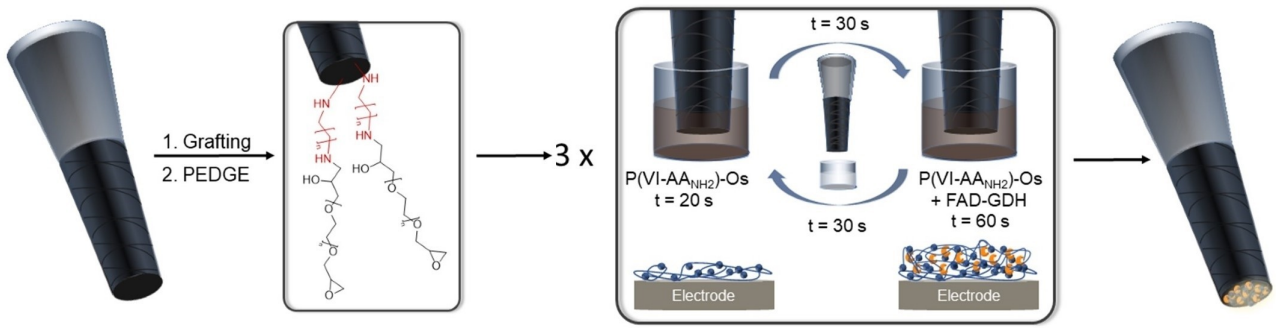
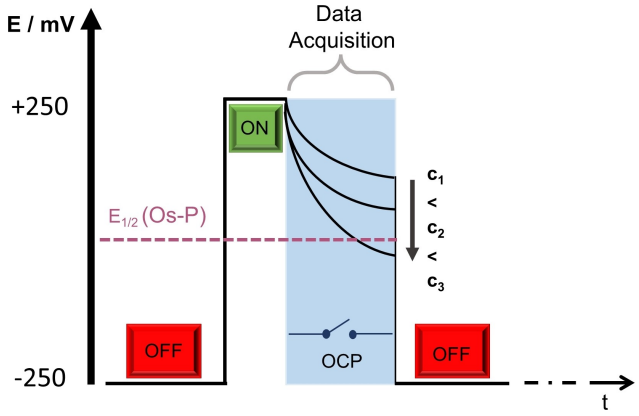


Figure 1. Schematic representation of the sensor fabrication. The surface of a freshly prepared carbon electrode is initially functionalised with amino groups by means of grafting followed by the immobilisation of PEGDGE crosslinker via dipping the electrode into a 5 mg mL^{-1} PEGDGE solution for 5 minutes. Subsequently, the electrode is coated with the active enzyme layer using a layer-by-layer approach. Herein, the electrode was alternately immersed in a polymer solution and in a mixture of polymer and enzyme solution. In between, the electrode was kept in the air to dry. This coating step was carried out a total of 3 times but can be varied in dependence on the desired total film thickness.



Scheme 2. Schematic representation of the proposed measuring principle. According to the Nernst equation, the redox polymer and with it the embedded enzyme is either switched "on" or "off" depending on the applied potential with respect to $E_{1/2}$ of the Os-complex tethered to the redox polymer. After switching to a short "on"-phase to charge the redox polymer and with this to invoke enzymatic turnover, the circuit is interrupted by switching to open-circuit potential (OCP) and following the decay of the potential which is governed by the glucose concentration in the sensor layer.

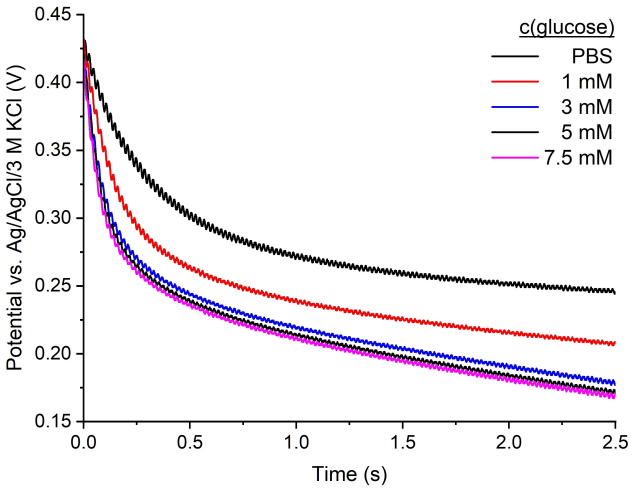


Figure 2. Potential transients recorded during the OCP measurement for different glucose concentrations in the range of 0 mM (PBS) and 7.5 mM. Before the potentiometric data acquisition, the sensor was kept at −50 mV vs. Ag/AgCl/3 M KCl for 90 s followed by an oxidative potential pulse to 450 mV vs. Ag/AgCl/3 M KCl for 1 s.

OCP potentiometrically. The enzymatic turnover will consume Os^{3+} and discharge the redox polymer in dependence of the available glucose concentration in the sensing layer. Hence, the measured change in potential relies solely on the enzymatic glucose oxidation causing reduction of Os^{3+} to Os^{2+} and the discharge of the Os-complex modified redox polymer film, respectively.

Depending on the time for the charging pulse and the potentiometric measurement the decay of the potential is solely governed by the glucose concentration in the sensing layer and only to a minor extent modulated by a diffusional flux of glucose into the sensing layer. It seems to be advantageous to make the potentiometric data acquisition as short as possible before the enzymatic turnover is again disabled by applying the "off" state potential.

During this phase, the glucose concentration inside the sensor layer (c_{app}) is regenerating to c_{ext} and this regeneration time must be sufficiently long to allow the concentration equilibrium to be fully attained. Figure 2 shows individual potential transients recorded after a 1 s charging of the redox polymer film in the presence of different concentrations of glucose in the range of 0 mM and 7.5 mM.

Figure S3 shows the results of a similar experiment in which each potential discharge curve was measured 4 times at each glucose concentration demonstrating the reproducibility of subsequent measurements with the same sensor. As suggested by the Nernst equation, the slope of the potential decay decreased significantly within the first 500 ms. After this, it becomes quasi stationary, and in this time regime the glucose within the sensing layer was mainly consumed and is replenished by diffusion from the external reservoir. These measurements not only confirm the proposed sensing strategy, but show that the charging pulse and the potentiometric data acquisition has to be performed during a very short time to avoid complete depletion of glucose in the sensing layer.

One of the key aspects for a substrate-consuming implantable sensor that functions reliably over a long period of time is its behaviour in the presence of an increased diffusion barrier (such as the encapsulation imposed by the foreign-bode reaction).[1,6–8] Ideally, the implanted sensor is unaffected by the continuous increase of the diffusion barrier. We conducted a control experiment and varied the regeneration time ("off" state) using a sensor modified with an additional thick layer of a redox-inactive polymer, P(SS-GMA-BA) (see Figure S1, right) to simulate encapsulation by the foreign-body reaction. This additional layer decreases the diffusion of glucose into the sensing layer. For short regeneration times, we observed a depletion of the glucose concentration within the catalytic recognition layer indicated by the shift of the diffusion-dependent potentials towards higher values compared to the first potentiometric transient (Figure 3a). However, the depletion of glucose within the sensing layer is decreasing with increasing regeneration time. For a regeneration time of above 30 s, the obtained transients coincided again (Figure 3b, c) and make the sensor response independent from the additional polymer layer thus completely mitigating the effect of the encapsulation.

A series of measurements, using a sensor without an additional diffusion barrier, was carried out yielding similar results (Figure S4). The glucose depletion is though less distinct compared to the results for a sensor with additional diffusion layer and it almost vanished for a regeneration time of 10 s. In the case of the sensor without the additional layer the in-diffusion of glucose during the regeneration time is substantially faster and glucose concentration equilibrium is faster attained. Based on these results, we suggest operating such a catalytic equilibrium sensor with a typical measurement frequency of ca. 35 s. The measurement

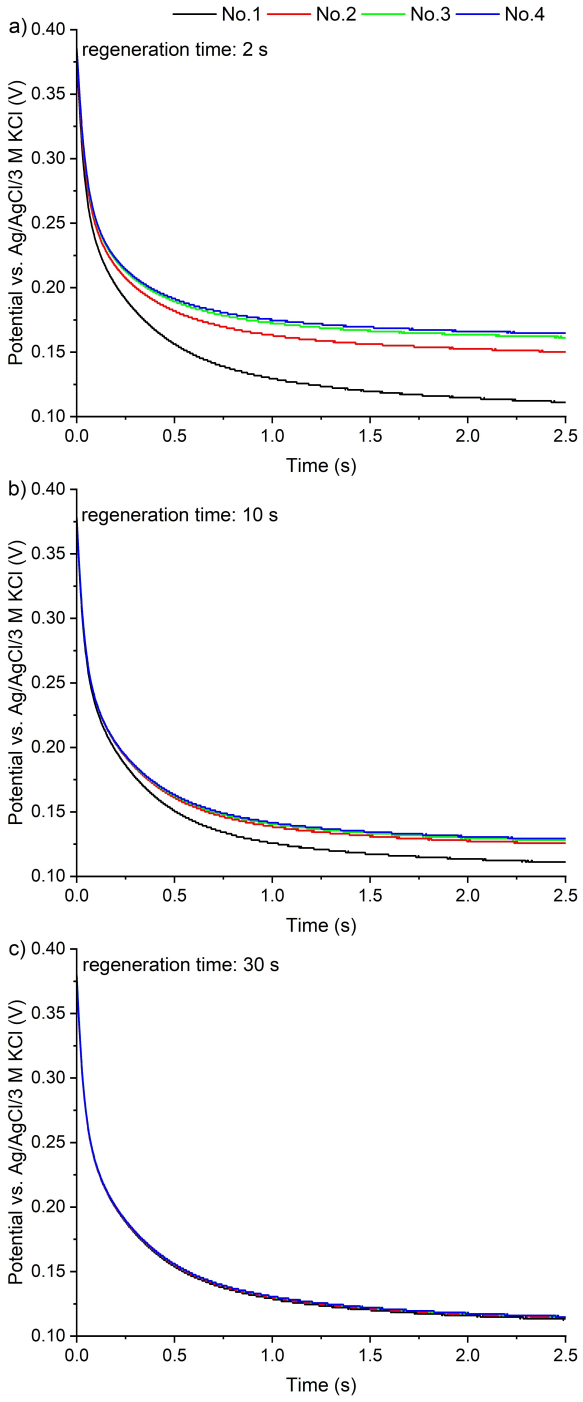


Figure 3. Comparative measurements of a sensor modified with a high diffusion barrier to affect the mass transfer of glucose into the catalytic sensing layer. The duration of the charging pulse was set to 2 s. For short regeneration times of 2 s between the individual measurements (a), a depletion of glucose in the film is observed between the first measurement (black trace) and the last measurement (blue trace). The depletion of glucose in the film is decreasing with increasing regeneration times (b). From a regeneration time of 30 s (c), no differences between the individual measurements can be detected .

frequency, which is mainly determined by the "off" phase, may be adjusted if needed.

The procedure was further optimised by reducing the duration of the potentiometric data acquisition. The depletion of glucose in the sensing layer is substantially reduced, the overall measurement interval is shortened, and the replenishment of the glucose within the sensing layer by diffusion is facilitated. To detect the substrate concentration present in the sensing layer without any influence of substrate diffusion, we evaluated the slope in the linear range of the potential transient recorded directly after charging the sensing layer (Figure 4a).

The slopes of the potential transients clearly show differences in dependence from the glucose concentration in the range of 20–45 ms with an increased negative slope at higher substrate concentrations which is in accordance with our predictions. To validate the dependence of the potential slopes (Figure 3a) on the glucose concentration, we compared the results with steady-state amperometric data for glucose concentrations in the range of 0 mM and 7.5 mM (Figure 4b). Corresponding potentiometric and amperometric calibration curves are shown in Figure 4b and Figure S5, respectively. The correlation confirms that the newly suggested measuring sequence leads to robust data within only a hundred of milliseconds. The short data acquisition time and with this the short time during which the enzyme is switched on and depletes the glucose concentration within the sensing layer accelerates the re-adjustment of the substrate equilibrium within the sensing layer after deriving a glucose concentration value. This provides the basis for a substantial decrease of the total measurement interval between two measured values. The potentiometric and amperometric responses measured for different individual sensors differ substantially (Figure S6), which is due to differences in the electrode geometry and variations in the manual sensor fabrication. We are confident that this can be mitigated using automatic non-manual sensor fabrication procedures in the future.

A dynamically changing glucose concentration profile was recorded over 4 h to simulate the rapid response of the proposed sensor with respect to changes in c_{ext} (Figure 5). We frequently measured the change in the OCP values after short oxidative pulses during controlled exchange of the solution via an in- and outlet. In addition, chronoamperometry was performed at a constant potential of 450 mV vs. Ag/AgCl/3 M KCl for 180 s after applying each 20 potentiometric measuring pulses to validate the measured glucose concentrations which varied between 1 mM and 7.5 mM (Figure S6). The amperometric measurements fit almost seamlessly into the course of the determined slopes of the potentiometric measurements demonstrating that the sensor concept is capable to rapidly detect fluctuations in the external glucose level over time.

Conclusion

In summary, we proposed a proof-of-principle procedure of a catalytic biosensor operating under quasi-equilibrium conditions. Its feasibility was demonstrated for an example of a glucose micro-biosensor employing completely oxygen-

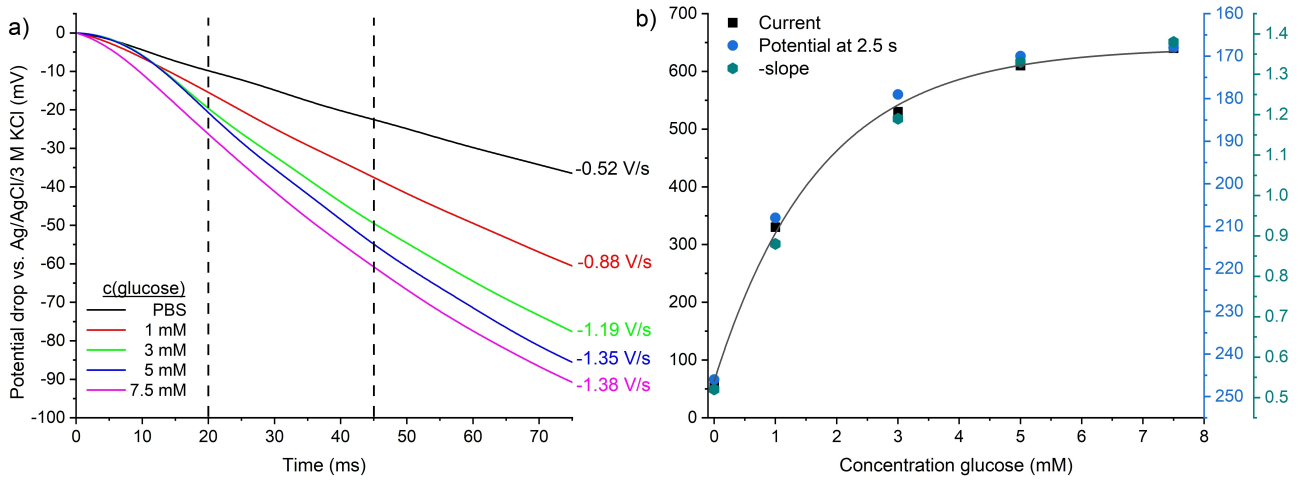


Figure 4. a) Slopes of individual potential transients vs. their initial potential for increasing glucose concentration with determination of the respective straight line slope in the range between 20 ms and 45 ms. b) Calibration curve consisting of the current, potential and slope of potential transient values for the same sensor in dependence on the glucose concentration.

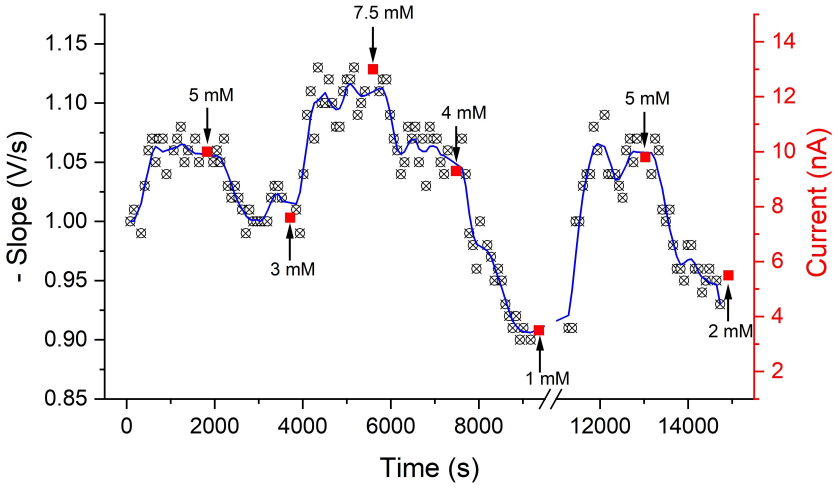


Figure 5. Series of multiple measurements while dynamically changing the external glucose concentration in the range between 1 mM and 7.5 mM. The individual measurement values (black crosses) were determined from the slope of the corresponding potential transients between 10 ms and 20 ms after charging the film for 1 s. The red squares show independently performed amperometric measurements correlated to the pulse potential transients. The plotted blue trace was obtained from the moving average of 4 individual pulse measurements.

independent FAD-GDH immobilised within an Os-complex modified redox polymer on the electrode surface. By means of potentiostatic control, the sensor was repetitively switched between catalytic conversion and regeneration of the substrate's concentration equilibrium. To ensure the reliable control over the enzymatic turnover state, parasitic side reactions were prevented by choosing a redox polymer with a specifically tailored potential and by employing an oxygen-independent highly selective enzyme. To invoke just a minimal disturbance of the concentration equilibrium between sensor and analyte solution, the actual potentiometric measurement after charging the sensor film was kept extremely short to a few milliseconds. The sensors could reliably detect glucose concentrations regardless of the microelectrode size. The evaluation of the slopes of the potential transients at different glucose concentrations showed a good correlation with steady state control measurements using the same sensors. Even in the presence of an artificially enlarged diffusion barrier for simulation of the effect of the foreign-body reaction the same quality of measurement has been achieved. The short measurement intervals could be beneficial to identify trends during fast changing glucose concentrations in the human body. The sensor concept offers the opportunity to combine the advantages of the competing glucose sensor technologies currently dominating the market and concomitantly to overcome the previous disadvantages, mainly the dependence of the signal from diffusional fluxes of the substrate towards the enzyme under non-equilibrium conditions. Moreover, the presented strategy is applicable for other enzyme classes and can be extended for applications detecting other important analytes, such as lactate or

glutamate,[21,27] by exchanging the biorecognition element within the redox hydrogel.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Catalytic Biosensors · Enzymatic Turnover Control · Layer-by-Layer · Measuring Principle · Substrate Equilibrium

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