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ORIGINAL ARTICLE

Substrate Specificity and Interferences of a Direct-Electron-Transfer-Based Glucose Biosensor

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Abstract

Objective:

Electrochemical sensors for glucose monitoring employ different signal transduction strategies for electron transfer from the biorecognition element to the electrode surface. We present a biosensor that employs direct electron transfer and evaluate its response to various interfering substances known to affect glucose biosensors.

Methods:

The enzyme cellobiose dehydrogenase (CDH) was adsorbed on the surface of a carbon working electrode and covalently bound by cross linking. The response of CDH-modified electrodes to glucose and possible interfering compounds was measured by flow-injection analysis, linear sweep, and chronoamperometry.

Results:

Chronoamperometry showed initial swelling/wetting of the electrode. After stabilization, the signal was stable and a sensitivity of 0.21 μA mM^{-1} cm^{-2} was obtained. To investigate the influence of the interfering substances on the biorecognition element, the simplest possible sensor architecture was used. The biosensor showed little (<5% signal deviation) or no response to various reported electroactive or otherwise interfering substances.

Conclusions:

Direct electron transfer from the biorecognition element to the electrode is a new principle applied to glucose biosensors, which can be operated at a low polarization potential of -100 mV versus silver/silver chloride. The reduction of interferences by electrochemically active substances is an attractive feature of this promising technology for the development of continuous glucose biosensors.

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Abbreviations: (Ag/AgCl) silver/silver chloride, (CDH) cellobiose dehydrogenase, (CGMS) continuous glucose monitoring system, (CLSI) Clinical and Laboratory Standards Institute, (DET) direct electron transfer, (FAD) flavin adenine dinucleotide, (GDH) glucose dehydrogenase, (GOx) glucose oxidase, (PBS) phosphate-buffered saline, (PQQ) pyrroloquinoline quinone

Keywords: biosensors, cellobiose dehydrogenase, continuous glucose measurement, direct electron transfer, interference, selectivity

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Introduction

An essential element in many medical treatments provided today is determination of blood glucose concentration.[1] Technological development of glucose biosensors has been driven by the need for high selectivity and sensitivity, which are necessary for tight glucose monitoring of diabetes mellitus patients.[2] Much effort has been put into the development of interstitial continuous glucose monitoring systems (CGMSs), which have a growing impact in therapy[3,4] and will probably be employed in consumer-based closed-loop artificial pancreas systems, a long desired element in diabetes therapy.[5]

Since the first patent of an enzyme-based glucose electrode in 1970,[6] a series of improvements was required to increase the accuracy, selectivity, and stability of glucose biosensors, using enzymes as biorecognition elements. The crucial points defining these properties are the choice of the sensing enzyme and its connection to the electrode.[7] Sensors that measure oxygen consumption to infer glucose concentration (first-generation biosensors) are affected by fluctuating oxygen concentrations.[8] Additionally, the reaction product hydrogen peroxide contributes to the deactivation of glucose oxidase (GOx) and reduces sensor stability.[9] Multi-electrode setups including an internal correction reference and diffusion limiting membranes[10,11] have been identified as suitable strategies to overcome oxygen fluctuations, and catalase can be used to remove hydrogen peroxide.[12]

Such problems are minimized by the use of redox mediators[13] for signal transduction (second-generation biosensors), which additionally offer the possibility to use, aside from GOx, enzymes with diverse molecular and catalytic properties such as pyrroloquinolone quinone (PQQ)-dependent glucose dehydrogenase (GDH) or flavin adenine dinucleotide (FAD)-dependent GDH. Mediator-based architectures increase accuracy and are successfully applied in glucose test strips; however, leaking mediators, such as ferrocene,[14] limit the technology to *in vitro* devices. The possibility of using new enzymes as an alternative to the established GOx in second-generation biosensors has some benefits, such as the independence of measurements from the oxygen concentration, but also stresses the importance of substrate specificity. In one case, detection of maltose by the applied PQQ-dependent GDH led to 13 reported deaths despite immediate communication.[15,16] For FAD-dependent GDH, protein engineering was used to reduce maltose turnover.

A major problem in designing accurate *in vivo* biosensors is the interference by endogenous and exogenous substances. Some of them, such as ascorbic acid or acetaminophen, are electroactive species, which can be directly oxidized at the working electrode and alter the signal.[17] To avoid these interferences, selectivity-enhancing membranes have been applied.[1] Outer sensor layers such as polyvinylpyridine derived hydrogels,[18] swellable polyurethanes,[19] and various modified hydrogels are heavily researched.[20] A complementary approach is to lower the polarization potential close to the midpoint potential of the enzyme's cofactor to reduce unspecific interference by electroactive compounds. One approach uses hydrogels consisting of hydrophilic cross-linked polymer networks and connected tethered redox mediators.[21] This "wiring" of entrapped enzymes became the technological base of a commercial product line.[7]

The third-generation biosensor approach uses enzymes, which are capable of establishing direct electron transfer (DET) between their cofactor and the electrode surface without any mediating substances. This can be facilitated by nanostructured electrode surface designs.[22] A limited number of enzymes are capable of directly transferring electrons without any electrode modifications. This feature allows the application of low polarization potentials close to the midpoint potential of the enzyme's cofactor.

One of these enzymes is cellobiose dehydrogenase (CDH; EC 1.1.99.11). The large (~65 kDa), catalytically active, glucose oxidizing flavodehydrogenase domain (**Figure 1A**) shares the protein fold and cofactor with GOx and FAD-dependent GDH. All three enzymes are members of the glucose-methanol-choline oxidoreductase superfamily. The difference between CDH on the one side and GOx/GDH on the other is its additional small (25 kDa) N-terminal cytochrome domain, which features a heme *b* cofactor. This mobile domain can act as "built-in mediator" and transfers electrons from the reduced $FADH_2$ cofactor to macromolecular electron acceptors.[23] The natural electron acceptor of CDH is lytic polysaccharide monooxygenase, which participates in oxidative cellulose degradation. The cytochrome domain can

also transfer electrons directly to various electrode surfaces (**Figure 1B**). Cellobiose dehydrogenase therefore belongs to the limited number of enzymes that, in their native form, show efficient DET between the enzyme's active site and an electrode surface.

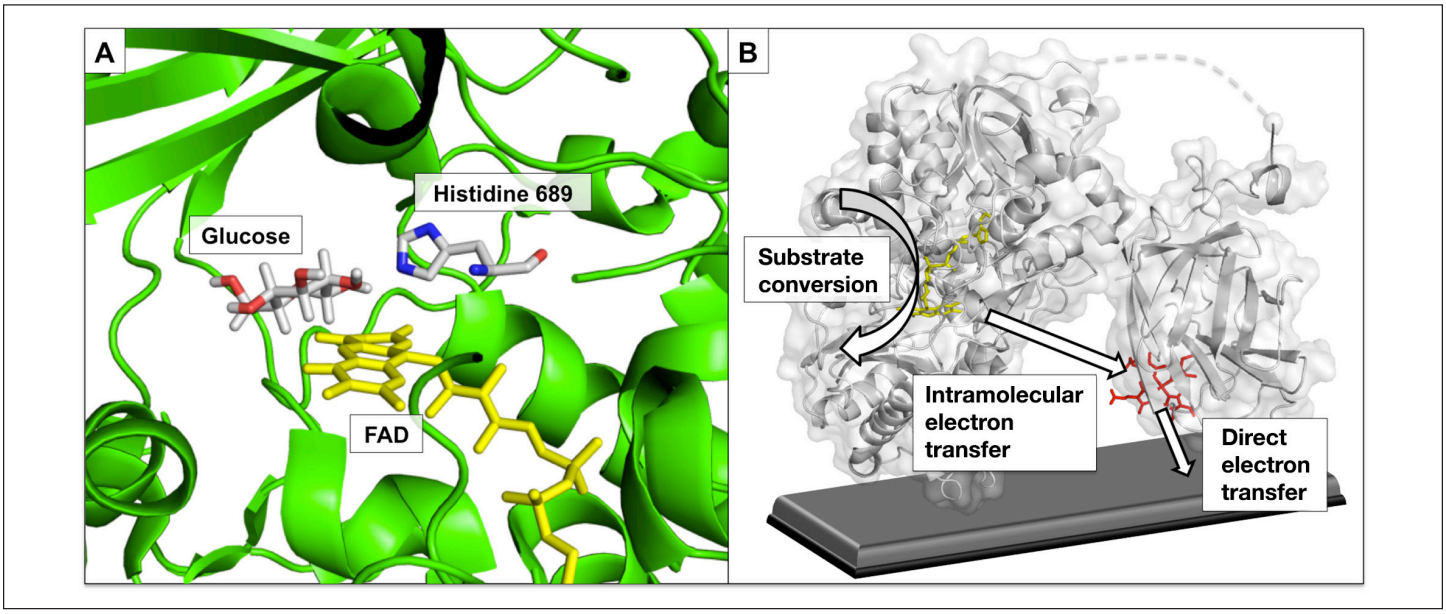

Figure 1. (A) Model of the active site in CDH's flavodehydrogenase domain with glucose bound closely to the catalytic base (Histidine 689) and the FAD cofactor (yellow). **(B)** Schematic presentation of a single CDH molecule immobilized on an electrode surface. The electrons obtained from glucose oxidation are subsequently transferred from the FAD (yellow) via the heme *b* (red) of the cytochrome domain to the electrode in two electron transfer steps.

Cellobiose dehydrogenase is produced by many fungi and forms a diverse family within the glucose-methanol-choline oxidoreductases with differences in substrate specificity, pH optimum, stability, and DET efficiency.[23–25] The heterologous expression of CDH in *Pichia pastoris* allows protein engineering and a fast and reliable enzyme production. Cellobiose dehydrogenase has been tested in combination with various electrode materials to utilize and enhance DET and increase the current density of CDH bioelectrodes.[26–30]

Previous studies applying CDH as the biorecognition element showed promising results, including high current densities (6.84 μA cm^{-2}), a good linear range (0.1–30 mM), and a low detection limit (0.05 mM).[31,32] However, until now, no study has investigated the effects of known interfering compounds possibly encountered *in vivo*.

The aim of this work was to test a CDH-based third-generation biosensor with regard to potential interfering substances as indicated by the Clinical and Laboratory Standards Institute (CLSI) to assess its applicability in CGMSs. The simplest electrode architecture possible was used to focus on their immediate influence on the biorecognition element.

Experimental Details

Materials

Chemicals were of highest grade available and purchased from Sigma-Aldrich. Cellobiose dehydrogenase from *Corynascus thermophilus* with engineered substrate specificity[33] was recombinantly produced and purified as described previously for the wild-type enzyme.[34] Measurements were performed in phosphate-buffered saline (PBS) pH 7.4 at 25 °C.

Preparation of Cellobiose-Dehydrogenase-Modified Graphite Electrodes

Working electrodes were prepared from spectroscopic graphite rods (FP-254, OD 3.05 mm, Schunk Materials, Heuchelheim, Germany), which were cut and polished on wet emery paper. After sonication (10 min) and rinsing with high-quality water, the electrodes were dried under a nitrogen stream, covered with 4 μl of an 11.9 mg/ml CDH

solution (54.3 U/ml) and 1 µl of a poly(ethylene glycol) diglycidyl ether solution (10 mg/ml), and stored overnight at 4 °C. The immobilized amount and activity of CDH per electrode was 0.048 mg and 0.22 U, respectively. Before use, each electrode was rinsed carefully with PBS buffer.

Flow Injection Analysis

Flow injection analysis was carried out in a three-electrode flow-through amperometric wall-jet cell (BASi, West Lafayette, IN). The electrochemical cell (dispersion factor 1.717) was connected to a single line flow injection system. A constant carrier flow (0.5 ml/min) was maintained by a peristaltic pump. Samples (injection volume 80 µl) were automatically injected by a Kontron 460 autosampler (Kontron AG, Eching, Germany). The cell was equipped with a reference electrode [silver/silver chloride (Ag|AgCl) versus 3M sodium chloride, RE-6, BASi] and a counter electrode block (BASi) connected to a Gamry Reference 600 potentiostat (C3 Analysentechnik, Munich, Germany). The dispersion factor of the flow system was determined according to the Ruzicka and Hansen relationship.[35] A potential of -100 mV versus Ag|AgCl was applied until a stable background current was obtained before injections started. Data presented in this study are the average of three consecutive glucose injections for each one of three independently prepared electrodes. Interferences were measured by comparing the currents of alternate injections of glucose (90 mg/dl) and the possibly interfering substance dissolved in the same glucose solution with the indicated concentration (CLSI document EP7-P). Glucose calibration data were fitted to a parabolic function (the Michaelis–Menten equation) by a nonlinear curve-fit and least-squares regression by SigmaPlot (Systat Software, San Jose, CA) to evaluate the results by means of apparent $K_{M,app}$ and maximum current (I_{max}).

Amperometry in Static Solution (Quiescent Conditions)

Measurements were done in a Dr. Bob Cell (C3 Analysentechnik) equipped with a reference electrode (Ag|AgCl, saturated potassium chloride) and a platinum wire counter electrode. The CDH-modified working electrode was submersed in 30 ml PBS buffer and a potential of -100 mV versus Ag|AgCl was applied until a stable background current was obtained before a stock solution containing glucose or an interfering substance was added stepwise. All solutions were degassed and covered with argon atmosphere during the measurements. From all data, a blank obtained with a CDH-modified electrode in the absence of substrate or interfering substance in the buffer solution was subtracted.

Voltammetric Sweep Experiments

The described Dr. Bob Cell in the same three-electrode setup was used for linear sweep experiments. The cell was filled with PBS buffer, and an initial potential of -200 mV versus Ag|AgCl was applied until a stable background current was obtained. After adding glucose and/or electroactive substances, a potential sweep (10 mV/s) was started. All solutions were carefully degassed under vacuum and purged with argon prior to experiments. To maintain the inert atmosphere, argon was blown over the solution during the measurements.

Structural Data

Protein structures were derived by comparative modeling performed with the SWISS model[36,37] based on the crystal structure of *Phanerochaete chrysosporium* CDH (Protein Data Bank identifier 1KDG)[38] as template. Figures were created using the pyMOL Molecular Graphic System (DeLano Scientific, San Carlos, CA).

Electrode Performance

The time-dependent amperometric response of CDH-modified electrodes was evaluated in flow injection experiments. **Figure 2A** shows that the graphite rod needs a period of 6 h before reaching stable operation conditions. During the first 6 h, the wetting and swelling of the electrode cavities result in an increase in the contacted surface area and, proportionally, the amperometric response. After 6 h, the signal was stable, and no further deterioration of signal was observed.

Amperometric response to various glucose concentrations was measured with well-conditioned electrodes in the flow-injection cell. Data shown are the mean values from three consecutive glucose injections to each one of three separately prepared electrodes and their standard deviation (**Figure 2B**). Measurements were performed at a low potential of

-100 mV versus Ag|AgCl, which was used in the following interference studies. The data can very well be fitted to a parabolic function, and the apparent Michaelis constant for glucose ($K_{M,app}$ = 33 mg/dl) and the maximum current ($I_{max,app}$ = 1.53 μA/cm^2) were calculated. Sensitivity was determined from the linear slope of the curve at the lowest concentrations and was 0.21 μA cm^{-2} mM^{-1}. The linear range of the chosen electrode starts at a very low concentration of 1.8 mg/dl but ends already at an upper concentration of 18 mg/dl.

Specificity and Liability to Interferences

In voltammetric sweep experiments, the operating potential/polarization of the CDH-modified electrode was determined. It is obvious that, with an increasing polarization potential, the amperometric response also increases (**Figure 3A**). This can be observed for the potential range from -150 to +300 mV versus Ag|AgCl. Sweeps were also performed in the absence of glucose with two well-known interfering electroactive substances: ascorbic acid and acetaminophen. While oxidation of ascorbic acid and acetaminophen was observed at -20 mV and +280 mV, respectively, the CDH-catalyzed oxidation of glucose starts already at -130 mV versus Ag|AgCl.

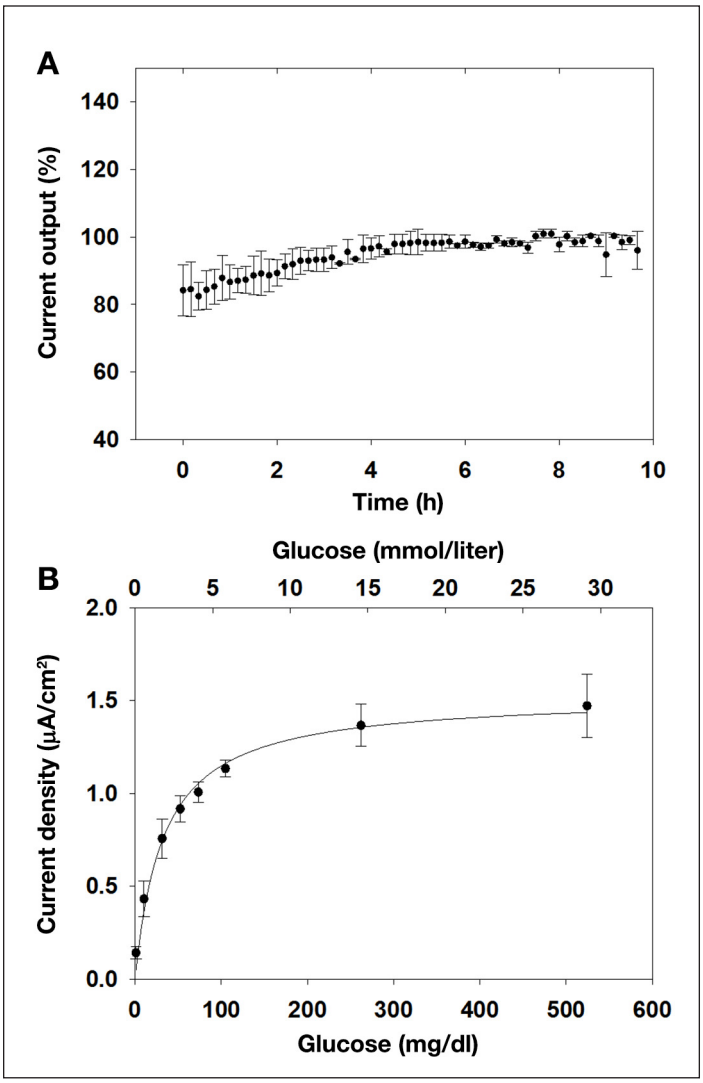

Figure 2. (A) Amperometric response of CDH-modified electrodes in the flow injection cell to 5 mM glucose in PBS buffer at -100 mV versus Ag|AgCl over 10 h. **(B)** Amperometric response of CDH-modified graphite electrodes in the flow injection cell to various glucose concentrations in PBS at -100 mV versus Ag|AgCl.

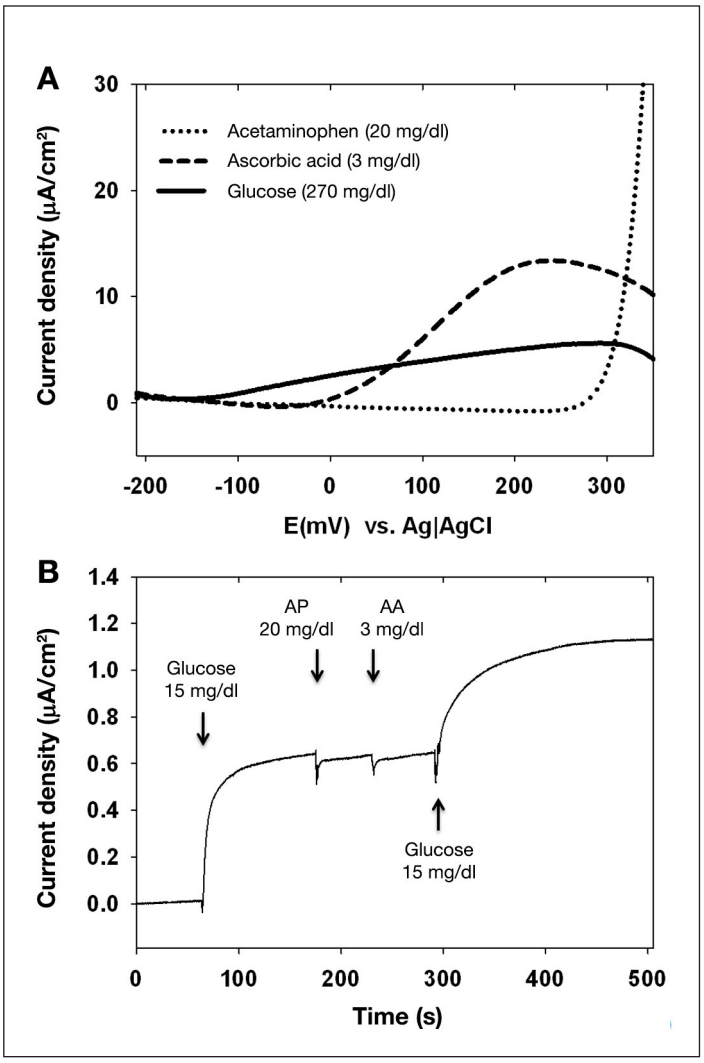

Figure 3. (A) Polarization of a CDH-modified electrode at a sweep rate of 10 mV/s in PBS buffer in the presence of glucose, acetaminophen, and ascorbic acid. **(B)** Amperometric response of a biosensor polarized at -100 mV versus Ag|AgCl to the stepwise addition of glucose, acetaminophen, ascorbic acid, and glucose. AP, acetaminophen; AA, ascorbic acid.

To investigate the effect of ascorbic acid and acetaminophen in the presence of glucose, a chronoamperometric experiment was performed based on the stepwise addition of substances to the cell (**Figure 3B**). At the chosen polarization potential of -100 mV versus Ag|AgCl, the amperometric response is still 15% of the maximum obtained at 300 mV but sufficiently high for reliable measurements. The constant increase in current is caused by swelling and wetting of the not fully conditioned electrode. Besides an injection peak, little effect was observed with the addition of ascorbic acid and acetaminophen to the cell.

All selected, possible interfering substances were tested in the flow injection cell with conditioned electrodes at the selected polarization of -100 mV versus Ag|AgCl (**Table 1**).

The CDH-modified electrodes using engineered CDH from *C. thermophilus* are hardly affected by interferences. In most cases, the signal deviation is below 2%. The substrate specificity of this CDH was modified to be unaffected by maltose.

Table 1.
Signal Deviation of Cellobiose-Dehydrogenase-Modified Electrodes in the Presence of the Indicated Concentration (Clinical and Laboratory Standards Institute Document EP7-P) of the Interfering Substance

Interfering substance	Concentration (mg/dl)	Signal deviation (%)		
Acetaminophen	20	3.3	±	2.4
Ascorbate	3	1.8	±	0.1
Creatinine	30	1.6	±	0.1
Dopamine	13	1.5	±	0.2
Galactose	81	0.9	±	0.1
Ibuprofen	40	-0.7	±	0.6
L-3,4-dihydroxyphenylalanine	5	1.9	±	1.0
Maltose	2650	-1.3	±	0.7
Salicylic acid	50	1.0	±	0.9
Tetracycline	4	1.6	±	0.1
Tolazamide	100	-1.0	±	0.6
Tolbutamide	100	-0.6	±	0.6
Uric acid	20	1.7	±	0.1
Xylose	40	-0.4	±	0.3
L-α-methyl-3,4-dihydroxyphenylalanine	2.5	1.6	±	0.1

Discussion

The aim of the study was to investigate whether the CDH-based third-generation biosensor design is potentially feasible for *in vivo* use. Two main issues were addressed: electrode performance and liability to interferences.

Electrode Performance

The stability of the biorecognition element is crucial to the performance of a biosensor. The data in the flow injection study show an initial start-up phase, which can be attributed to a swelling/wetting effect of graphite.[39] Experiments were performed after an equilibration of 6 h, after which the signal output remained stable. The results obtained show that, even with the simple applied physiochemical absorption and cross-linking procedure, CDH exerts a robust DET to the electrode surface.

The calibration curves of the CDH-based biosensors show, as expected for this basic setup, moderate current densities compared with other designs.[21,40–42] As previously discussed,[43] current output is proportional to enzyme coverage and can be improved by increasing the electrode surface and enzyme loading.[30] The linear range observed is less favorable, especially at higher glucose concentrations, compared with a similar sensor setup tested at higher polarization potentials (0.1–30 mM at 190 mV and 0.025–30 mM at 100 mV versus Ag|AgCl).[31,32] The reduced current response for higher glucose concentrations is an effect of the decreased DET rate at the chosen low polarization potential. From the determined apparent Michaelis constant of the immobilized CDH, it can be deduced that a lower applied potential reduces DET rate, which becomes rate limiting, consequently slows down glucose oxidation, and restricts the linear range to lower glucose concentrations. A higher polarization potential will lead to a better linear range but also to a higher level of interference from electroactive species.

A nonlinear response to the analyte concentration is usually cured by the mass-transfer-introducing membranes and polymer coating layers.[1] These elements increase the apparent K_M and thus the linear range to higher glucose concentrations but also decrease sensitivity.[12] An additional signal reduction by ~50% caused by adsorbed biomolecules has to be considered when a sensor is applied *in vivo*.[20] This results in unpredictable accuracy, high

calibration frequency, and short implantation lifetime,[44] decreasing the patient's benefits from a CGMS. Coatings with biocompatible properties, lately also coupled with an active release of molecules that modify foreign body response, can be used to overcome this problem.[45] However, the price of such advanced coatings is a decreased sensitivity and increased response time.[46]

This strategy is feasible as long as the sensitivity of the biosensor is high enough to give a sufficiently low detection limit and optimal measuring range. Since a detection limit of 1.8 mg/dl and sensitivity of 0.21 μA cm^{-2} mM^{-2} was reached by the sensor in this study, which can be optimized by surface modification,[30–32] a CDH-based CGMS design is certainly possible. However, a major reason for choosing this simple electrode design was to evaluate whether additional components such as permiselective membranes or surface modifications, usually applied to suppress interferences,[8] can be omitted.

Specificity and Liability to Interferences

Biosensors depending on oxygen lack accuracy when applied *in vivo*.[17] This problem of first-generation biosensors has been overcome by the use of oxygen-independent dehydrogenases[8] similar to the enzyme used in this study. However, it has to be ensured that the oxygen independence of dehydrogenases goes hand in hand with a high substrate specificity.[47] The reported case of maltose interference of a second-generation biosensor clearly demonstrates this issue.[15]

Maltose conversion was reported for CDH by several authors[24] and was investigated in detail for CDH-modified electrodes.[48] One of the most important tasks therefore was to eliminate maltose turnover by CDH. By structure-based rational engineering of the active site, the maltose reactivity could indeed be suppressed.[33] The results from flow-injection experiments (**Table 1**) show that the signal deviation of the CDH-based glucose biosensor is less than 1% when maltose is present at a relevant concentration. Maltose interference is negligible for the engineered CDH.

Moreover we addressed a ubiquitous problem in biosensor accuracy: electroactive physiological compounds. Since it is known that a sufficiently low electrode polarization potential should purge this disadvantage,[7] we successfully followed the same approach. The interference of ascorbic acid at a physiologically relevant concentration was <2%, while the interference of acetaminophen was stronger, but still below 10%, which is regarded as acceptable.[1] Similarly, other substances suggested by CLSI Document EP7-P have been tested (**Table 1**), and no relevant interference with glucose detection was observed. It has been demonstrated that an electrode design based on CDH using direct electron transfer is possible, sufficiently selective, and hardly affected by interferences. However, it is important to note that experiments were performed *in vitro* thus far and that future studies using blood samples are needed to evaluate matrix effects and hematocrit variation.

Future and Outlook

The development of a glucose biosensor that uses DET for communication between the biorecognition element and the electrode does not seem to be hampered by interferences of known substances. The next step will be the balanced optimization of a biocompatible coating polymer to modulate the measuring range toward the optimal region, increase current density, and confer increased stability.

Conclusions

The glucose biosensor evaluated is very lightly affected by interferences of endogenous or exogenous substances tested in this study. The approach to lowering the polarization potential of the working electrode in order to reduce unspecific interference by electroactive compounds works very well *in vitro* but has to be tested also in blood samples in future work. Sensor performance parameters are promising but need to be optimized. Many further steps are necessary to build a CDH-based *in vivo* glucose sensor, but this study shows that some of the problems of current sensors will not affect the new technology.

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