ChemElectroChem

Research Article
doi.org/10.1002/celc.202200418

Chemistry Europe
European Chemical Societies Publishing

www.chemelectrochem.org

Special Collection

An Oxygen Insensitive Amperometric Glucose Biosensor Based on An Engineered Cellobiose Dehydrogenase: Direct versus Mediated Electron Transfer Responses

Kavita Jayakumar,[a] Thomas M. B. Reichhart,[b, c] Christopher Schulz,[c] Roland Ludwig,[b, c] Alfons K. G. Felice,[c] and Dónal Leech*[a]

Cellobiose dehydrogenase (CDH) is capable of oxidizing cellobiose and related carbohydrates and generating electrical current at carbon-based electrodes through direct electron transfer (DET) or mediated electron transfer (MET) mechanisms. As a result, CDHs have been utilized as biocatalysts in biosensors and biofuel cell anodes. A novel engineered ascomycetous Class II CDH with enhanced glucose activity was tested as a bioelectrocatalyst for application to DET or MET-based glucose biosensors with the electrode component amount selection optimized for maximum current in 5 mM glucose solutions. The optimised DET biosensor showed a similar sensitivity and 3-fold lower $K_{M,app}$ when compared to non-optimised DET sensor based on the same engineered CDH. The optimized MET biosensor had a similar $K_{M,app}$ to non-optimized MET biosensor. However, it showed 15-fold improvement in j_{max} and 17-fold improvement in sensitivity over the DET biosensor. The sensor signals are not affected by the presence of oxygen, although operation in artificial serum results in 43% and 28% lower sensitivity for the DET and MET sensors, respectively. While no individually tested potential interferent breaches a mean absolute relative difference of 20% of the current, the cumulative co-operative effect in complex media, such as artificial serum, decreases the glucose oxidation current signal.

Introduction

Diabetes is a common chronic disease affecting 1 in 11 people.[1] Recent projections by the International Diabetes Federation show that the global diabetes prevalence, estimated to be 9.3% (463 million) in 2019, is expected to rise to 10.9% (700 million) by 2045.[1,2] This indicates the need to measure blood glucose in a cheap, fast and miniaturized way due to the dramatic increase in diabetes patients. There has been substantial investment in rapid and sensitive glucose biosensors since the concept of glucose biosensors was proposed by Clark and Lyons.[3] While non-enzymatic glucose sensors have been researched intensively, it has not led to breakthrough developments compared to their enzymatic counterparts that now dominate the market.[4,5] The majority of commercial glucose biosensors are based on glucose oxidase (GOx) with amperometric or coulometric measurements.[5] However, use of GOx in such biosensors has some drawbacks due to its affinity to oxygen and its inability to establish direct electron transfer (DET) to electrodes.[4,6,7]

For an amperometric biosensor, redox enzymes should exhibit efficient electronic communication to electrodes under sample conditions. Amperometric glucose biosensors based on GOx operate through oxidation of H_2O_2, produced by the oxidation of glucose and reduction of oxygen as co-substrate, at the electrode (1st generation) or O_2 is replaced by an alternate co-substrate, a mediator, as the electron acceptor in mediated electron transfer (MET)-based biosensors (2nd generation).[4,8,9] First generation biosensors have the disadvantage of high overpotential and electrode poisoning, usually overcome by use of a redox mediator characterized by a lower redox potential. However, the resulting 2nd generation biosensors tend to have more complicated sensor constructions and possible leaking of mediators.[5,10] The 3rd generation biosensors are based on DET between enzyme and electrode. While desirable due to the simplicity of electrode architecture and the avoidance of potentially hazardous mediators, DET biosensors still require more research to overcome low electron transfer rates to have comparable analytical characteristics to MET sensors.[4,5,11,12]

Only a few enzymes can establish DET to electrode surfaces. The use of cellobiose dehydrogenases (CDHs)[13] has attracted significant interest due to capacity to undergo DET and thus potential applicability in bioelectronics.[14] The CDHs consist of a

[a] K. Jayakumar, Prof. D. Leech
School of Chemistry & Ryan Institute
National University of Ireland Galway
Galway, Ireland H91 TK33
E-mail: donal.leech@nuigalway.ie
[b] T. M. B. Reichhart, Prof. R. Ludwig
Biocatalysis and Biosensing Laboratory,
Department of Food Science and Technology
BOKU – University of Natural Resources and Life Sciences
Muthgasse 18, 1190 Vienna, Austria 2
[c] T. M. B. Reichhart, Dr. C. Schulz, Prof. R. Ludwig, Dr. A. K. G. Felice
DirectSens Biosensors GmbH
Am Rosenbühel 38, 3400
Klosterneuburg, Austria

Supporting information for this article is available on the WWW under https://doi.org/10.1002/celc.202200418

An invited contribution to the Wolfgang Schuhmann Festschrift

multidomain protein composed of a FAD-containing dehydrogenase (DH) domain connected via a flexible linker to a cytochrome (CYT) domain with a heme *b* type cofactor.[15] CDHs can oxidize various sugars, including cellobiose or lactose[16,17] and, in some instances even glucose, at the FAD cofactor in the DH domain. Re-oxidation of $FADH_2$ can occur directly by interdomain electron transfer to the heme group in the CYT domain, which acts as a built-in mediator and can pass on electrons to various terminal electron acceptors such as electrode surfaces.[13]

CDH-based biosensors have been realized using carbon[18] and gold[19] as electrode material. Engineering of CDH has been performed to generate CDH variants for several purposes[10,20,21]. For instance, de-glycosylation of CDH can increase the faradaic efficiency[22–24] while amino acid substitutions in the active site can be used to change the substrate specificity of CDH.[25–27] A prominent example of the latter is an engineered CDH variant with enhanced glucose specificity[10] that can be used to construct biosensors for biomedical applications such as glucose measurements for diabetes management.[28] The engineering of CDH substrate specificity affects not only the glucose turnover rate, but also interdomain electron transfer rate and DET.

In this work, we demonstrate the use of an engineered CDH, equipped with glucose activity-enhancing mutations, incorporated into DET and MET-based sensors. A CDH from *Crassicarpon hotsonii* equipped with glucose activity-enhancing mutations C291Y and W295R was recombinantly produced in *Komagatella phaffii* as described previously.[25] The enzyme is referred to as wild-type (WT*Ch*CDH) to enable comparison to data published on the enzyme.[26] In the MET biosensor, the WT*Ch*CDH is encapsulated in an osmium complex-based polymer hydrogel, which acts as the mediator. These sensors are characterized electrochemically, and components used to prepare the electrodes optimized. The sensors are further tested in artificial serum to demonstrate their behaviour in complex media, and an interference study is conducted with the known interferents present in artificial serum.

Results and Discussion

Electrochemical Characterization of the Sensors

Cyclic voltammetry (CV) was used to initially characterize DET biosensor response (Figure 1). Redox peaks of the cytochrome domain (usually present around −0.1 V)[18,29,30] were not visible in CV. This could be due to the high charging current relative to the signal, or oxygen reduction current obscuring the cytochrome domain signal on the electrode surface.[18,30,31] To verify the presence of WT*Ch*CDH on the electrode surface, square wave voltammetry (SWV) (Figure 1b) was also performed. Peaks in SWV between −0.2 to 0 V are attributed to the CYT domain[30] and the increase in SWV oxidation current in this potential range in the presence of 5 mM glucose (Figure 1, red trace) supports this attribution. The origin of the peak at 0.3 V is not yet clear and has been attributed to surface quinones usually present on carbon surfaces as a peak at around the same potential is observed in the SWV of bare graphite electrodes.[32]

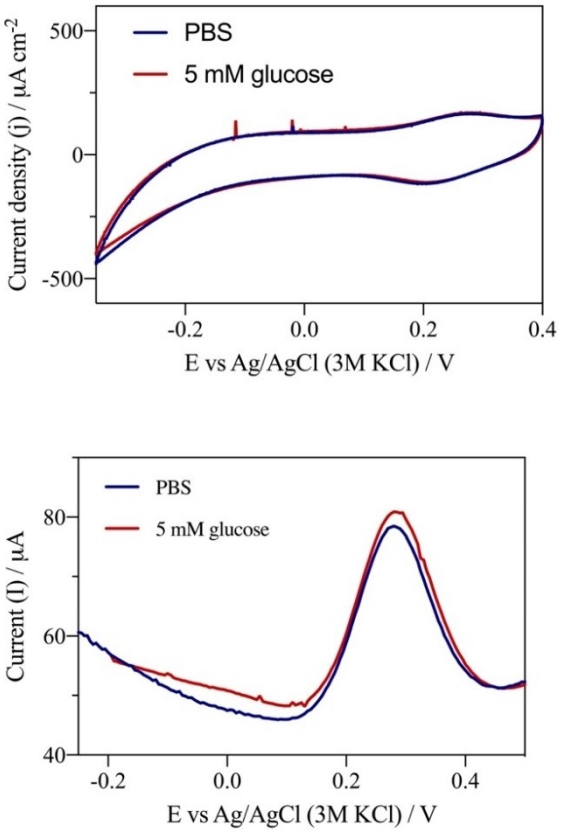

Figure 1. Cyclic voltammogram recorded at 1 $mV\,s^{-1}$ (top) in phosphate buffered saline (PBS) (blue) and square wave voltammogram (bottom) in PBS (blue) and in PBS including 5 mM glucose (red) of DET biosensors containing 1 µg immobilized WT*Ch*CDH. Electrolyte temperature 37 °C.

Slow-scan CV in the presence and absence of glucose was used to characterize MET biosensors prepared by co-immobilization of WT*Ch*CDH and $[Os(2,2'\text{-bipyridine})_2(\text{poly-vinylimidazole})_{10}Cl]^+$ [Os(bpy)PVI] redox polymer using poly(ethylene glycol) diglycidyl ether (PEGDGE) di-epoxide cross-linker. Scans recorded in the absence of glucose display peaks with redox potential centred at 0.22 V vs. Ag|AgCl (3 M KCl) (Figure 2) which agrees with previously reported values for the Os(II/III) transition of the redox polymer.[8,33] At slow scan rates $< 20\ mV\,s^{-1}$ peak currents vary linearly with scan rate as expected for a redox response controlled by finite diffusion within thin films on a surface.[34] The peak current varies linearly with the square root of scan rate at higher scan rates when semi-infinite diffusion within the film limits the current response[34]. The surface coverage (Γ_{os}) of the redox polymer, estimated by integrating the area under the peak for CVs recorded at slow scan rate in the absence of glucose, is $144 \pm 3\ nmol\,cm^{-2}$, which confirms multi-layer formation, similar to results obtained by others for the co-immobilization of enzymes and osmium-based redox polymers.[35–38] On addition of glucose to the electrochemical cell, sigmoidal shaped responses characteristic of an electrocatalytic (EC′) process are observed. The

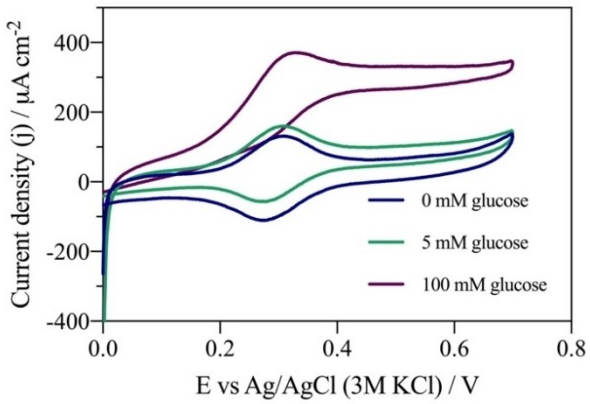

Figure 2. Cyclic voltammograms recorded at 1 mV s^{-1} for MET-based enzyme electrodes tested in PBS (red) and in PBS including 5 mM glucose (green) or 100 mM glucose (blue) at 37 °C. Enzyme electrodes consisted of WT*Ch*CDH (160 μg), Os(bpy)PVI (90 μg) and PEGDGE (105 μg).

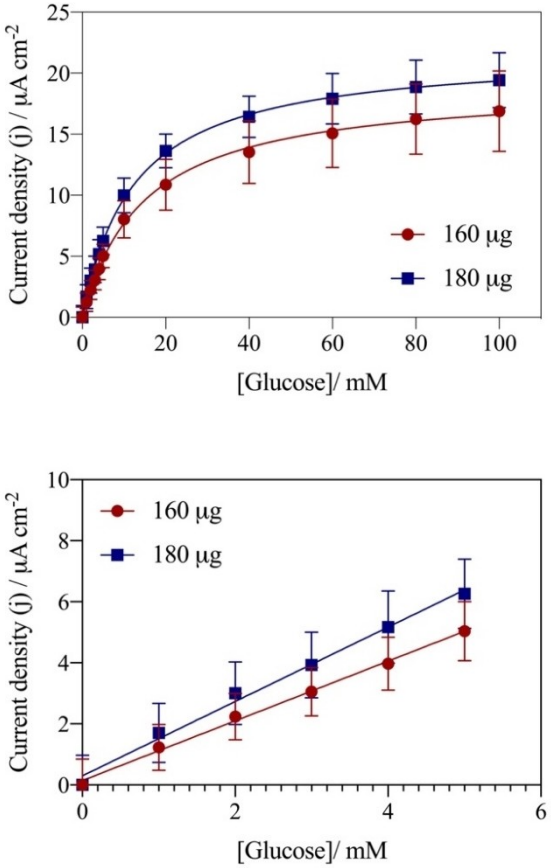

Figure 3. Glucose response curves for the DET system with 160 μg and 180 μg WT*Ch*CDH drop-coated onto the electrodes (top) and the linear plot for 0–5 mM glucose (bottom) based on amperometry at 0.1 V in PBS at 37 °C, where n = 4. The error bars represent the standard deviation.

half-wave potential is negatively shifted by 20 mV in the presence of glucose substrate compared to the redox potential in the absence of substrate, indicative of substrate transport limitation under these conditions.[39,40]

Amperometric measurements were carried out at 0.35 V vs. Ag | AgCl (3 M KCl) for the MET biosensor and 0.1 V for the DET biosensor to further characterize responses. The 0.35 V applied potential for the MET system is the same as that reported previously for the Os-based polymer, selected based on hydrodynamic voltammetry and confirmed as appropriate for glucose oxidation[41]. For the DET biosensor, as the peaks for WT*Ch*CDH appeared in the range of −0.2 to 0 V, a potential that was 0.1 V more positive than 0 V was selected. Amperometric glucose oxidation current density response as a function of glucose concentration (Figure 3 and Figure 4) was fitted to the Michaelis-Menten equation to provide an estimate of the apparent Michaelis-Menten affinity constant, $K_{M,app}$, and the maximum saturation current density (j_{max}) for glucose. Sensitivity, Table 1, is obtained from the slope of the linear section of the Michaelis-Menten curve and indicates the ability of the sensor to respond to changes in glucose concentration.

Values of $K_{M,app}$ of 14.7 ± 1.2 mM and 12.4 ± 0.6 mM, are obtained for use of volumes of 16 μL or 18 μL, respectively, of a 10 mg mL^{-1} WT*Ch*CDH solution in the preparation of DET biosensors. Alteration of the amount of WT*Ch*CDH drop-coated on the surface was optimized to maximize glucose oxidation current density in 5 mM glucose solutions. This is achieved using 18 μL volume of enzyme solution (Supplementary Figure S1). While $K_{M,app}$ values for biosensors are influenced by the physicochemical properties of the films on the surface and the differences in enzyme immobilization methods, the $K_{M,app}$ value for the WT*Ch*CDH DET biosensor is nearly 3-fold lower than the value of 36 ± 1 mM reported for a WT*Ch*CDH DET biosensor prepared by drop-coating 1 μL of a 15 mg mL^{-1} WT*Ch*CDH solution on electrodes.[21] As a consequence the linear range of 0–5 mM and j_{max} of 21.8 ± 0.3 μA cm^{-2} for the DET biosensor based on an enzyme volume of 18 μL (180 μg), Figure 3 and Table 1, are lower than the 0–10 mM and 47 μA cm^{-2} obtained at the WT*Ch*CDH DET biosensor from a previous report.[21] Signal sensitivity is, however, similar for both type of electrodes at ~1 μA cm^{-2} mM^{-1} (Table 1). The sensitivity using the WT*Ch*CDH, however, is at least threefold higher than that reported using other CDH DET glucose biosensors operating in PBS (Table 1). The optimized WT*Ch*CDH DET biosensor has a relative standard deviation of 10.1 % and a stability in current signal of 57.4 ± 0.4 % initial current remaining after 12 h of continuous application.

For the MET sensors, the amount of each component drop-coated on the surface was optimized using a Box-Behnken design-of-experiments approach (Supporting Information Figure S2) to maximize glucose oxidation current density in 5 mM glucose solutions, as described previously.[41] The $K_{M,app}$ of 37.9 ± 5.4 mM obtained for the optimized MET biosensor is similar to the value of 36 ± 1 mM reported for a WT*Ch*CDH DET biosensor.[21] However, the j_{max} value of 719 ± 43 μA cm^{-2} achieved with the MET biosensor is 15-fold higher than the 47 μA cm^{-2} obtained for that DET biosensor,[21] and more than 30-fold higher than that obtained for the optimized WT*Ch*CDH DET biosensor reported on here. This higher j_{max} value results in 17-fold increased sensitivity, and a wider linear range, to glucose for the MET biosensor over the WT*Ch*CDH DET

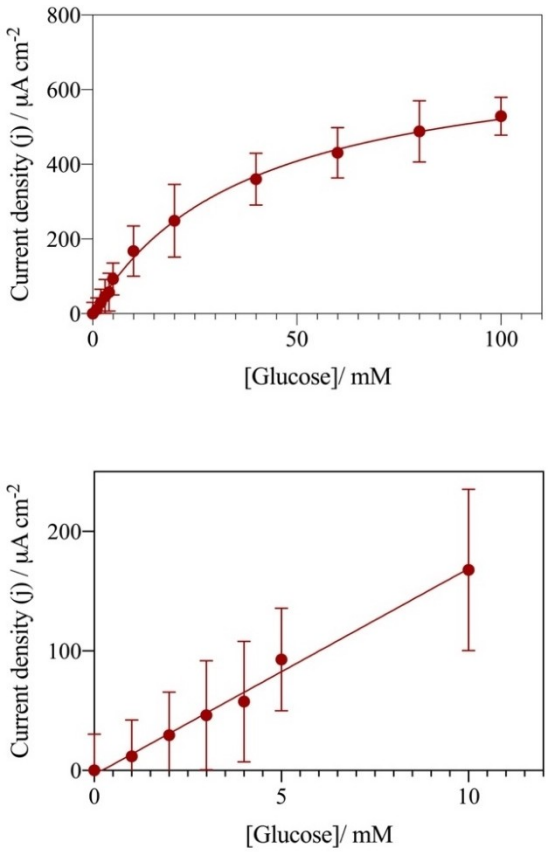

Figure 4. Glucose response curves for the MET system (top) and the linear plot for 0–5 mM glucose (bottom) based on amperometry at 0.35 V in PBS at 37 °C, where n = 4. The error bars represent the standard deviation

biosensor operating in PBS (Table 1). On comparing DET to MET biosensor performance, the lower $K_{M,app}$ and j_{max} values for DET suggests that interdomain electron transfer is rate limiting and affects the measured kinetic constants.

The MET biosensors reported on here display higher sensitivity than the DET biosensors. The biosensor sensitivity is similar, or better, than that of other MET biosensors based on GOx, apart from those that include nanostructured supports within the film matrix to enhance current density (see data in Table 1).

The MET biosensor has a reproducibility relative standard deviation (RSD) of 5.8% (n = 6) and a stability of 53.04 ± 1.1% (n = 4) over 12 h of continuous amperometry. Comparison of operational stability is difficult as there are variations in how this is measured. However, considering that over 12 h of continuous operation the half-life of the enzyme has not been reached, the MET sensor performance is comparable to that of other glucose biosensors (Table 1). The MET biosensor linear range of 0–10 mM encompasses a range that includes clinical glucose levels for diabetes monitoring.[47]

Effect of Oxygen on Sensor Performance

Glucose determination using GOx-based biosensors is complicated by the effect of oxygen on sensor performance, as oxygen is the natural electron acceptor for GOx oxidation of glucose and therefore competes with the mediator for electrons. Furthermore, oxygen can be reduced by the enzyme or mediator, producing hydrogen peroxide which can inhibit the enzyme.[48–50] As CDH has low activity with oxygen,[51] a major benefit of using CDH in sensors is that sensor response should be less dependent on oxygen. This is important for glucose sensing applications as a biosensor sensitive to oxygen would show fluctuations and errors in glucose measurement due to the variation in oxygen concentration. One approach is to modify the structure of GOx through enzyme engineering to mitigate oxygen sensitivity. For example Prévoteau et al.[52] used semi-rational engineering of GOx to enhance the electron transfer to the enzyme active site. However, while higher glucose oxidation current compared to native enzyme was achieved, it was due to improved enzyme and redox polymer interaction rather than a decrease in oxygen sensitivity. Horaguchi et al.[53] introduced mutations within the putative residues involved in the GOx oxidative half-reaction to decrease less oxygen sensitivity. However, the mutation affects the reductive half-reaction also and resulted in a decrease in enzyme activity and stability.

The WT*Ch*CDH biosensors developed do not display a significant change in sensor response in the presence of oxygen (Figure 5). However, WT*Ch*CDH turnover stability in the presence of oxygen and excess glucose has been shown to be affected by the presence of oxygen, most likely related to the susceptibility of methionine residues to oxidation given that replacement of methionine residues results in improved turnover stability.[21]

Operation under Sample Conditions

In order to test practical application of the oxygen insensitive DET and MET-based glucose biosensors in physiological fluids, the amperometric response to glucose in artificial serum was measured and results are presented in Figure 6. Glucose oxidation current responses increase with increasing glucose concentrations for all sensors and the analytical parameters for the sensors are presented in Table 1. The current density obtained for a glucose level of 5 mM, representing a normal blood glucose level, is lower in artificial serum compared to the response in PBS, an effect previously observed and attributed to electrochemical interferences and protein adsorption.[38,54–56] The operational stability of all the sensors is ~50% and does not seem to be affected by the additional components present in the artificial serum over that present in PBS. In the presence of artificial serum, $K_{M,app}$ values were found to be 23.0 ± 3. mM and 7.4 ± 0.7 mM while j_{max} values were 393.8 ± 39.1 and 9.5 ± 1.1 μA cm^{-2} for the MET and DET biosensor, respectively. While further research is required to determine the exact mechanism(s), the decrease of both $K_{M,app}$ and j_{max} values in the

Table 1. Analytical parameters of the glucose sensors.

Sensor	Sensitivity[a] [μA cm^{-2} mM^{-1}]	Linear Range[b] [mM]	Detection Limit[c] [mM]	Reproducibility RSD[d] [%]	Operational Stability[e] [%]	*J* @ 5 mM glucose [μA cm^{-2}]	Reference
Operation in PBS: selected DET-based sensors							
WT*Ch*CDH/graphite (160 μg)	0.99 ± 0.22	0–5	1.8	12.1	54 ± 1	5.0 ± 1.0	This work
WT*Ch*CDH/graphite (180 μg)	1.22 ± 0.17	0–5	1.7	10.7	57 ± 1	6.3 ± 1.1	This work
WT*Ch*CDH/graphite	1.00	0–10	–	–	–	5.0	[21]
[f]*Ct*CDH/graphite	0.030	0.002–2	0.001	–	–	–	[42]
*Ct*CDH/SPE	0.12	0.025–30	0.01	3.3	90 (7 h)	–	[42]
*Engineered Ct*CDH/graphite	0.21	0.1–1.0	0.1	–	–	–	[43]
*Engineered Ct*CDH/graphite	0.30	0.002–2.0	0.001	–	–	–	[10]
Operation in PBS: selected MET-based sensors							
WT*Ch*CDH/OsPolymer/graphite	17.3 ± 3.9	0–10	1.9	5.8	53 ± 1	93 ± 11	This work
GOx/OsPolymer/GC	5	0–10	–	–	–	–	[44]
GOx/PEI–Fc/GC	18	0–5		17		90	[45]
GOx/MWCNT–Fc/CS/GC	25	0.012–3.8	0.003	–	–	–	[46]
*Ct*CDH/OsPolymer/MWCNT/graphite	90	0–40	–	–	86	–	[38]
GOx/OsPolymerMWCNT/graphite	250	0–10	–	–	70	–	[38]
[g]FADGDH/OsPolymer/MWCNT/graphite	250	0–10	–	–	72	1500	[38]
GOx-CNT/OsPolymer/graphite	400	0–4	–	–	60	2080	[41]
Operation in Artificial Serum							
DET WT*Ch*CDH/graphite (180 μg)	0.70	0–5	2.0	15.1	54 ± 2	3.7 ± 1.1	This work
MET WT*Ch*CDH/OsPolymer/graphite	12.4	0–10	1.7	7.4	52 ± 1	72 ± 12	This work

[a] Sensitivity is obtained from the slope of the linear section of the Michaelis-Menten curve and indicates the ability of the sensor to respond to changes in glucose concentration. [b] Linear range is the range where current density is a linear function of glucose concentration and $R^2 = 0.99$. [c] Detection limit is the lowest value at which glucose can be detected and is defined as 3σ where σ is the standard deviation of the sensor at 0 mM glucose. [d] Reproducibility RSD is derived from the standard deviation on repeating a measurement a fixed number of times. [e] Operational stability is defined as the current retained after 12 h of continuous amperometric measurement, unless otherwise indicated [f] *Ct* is *Corynascus thermophilus*. [g] FADGDH is FAD dependent glucose dehydrogenase.

presence of serum components suggests that there is an uncompetitive inhibitor for WT*Ch*CDH present in this complex media.[57] This decrease in both $K_{M,app}$ and j_{max} values could also be explained by passivation of the electrode or film, for example by protein adsorption. While this does not seem to adversely affect the operational stability of the sensors, the sensitivities are lower, and the reproducibility RSD's are higher for both types of sensors in artificial serum compared to operation in PBS.

Interferent Screening

In order to evaluate the specificity of the engineered WT*Ch*CDH to glucose as a substrate as well as to verify that the loss in current density, sensitivity and increase in RSD when testing in artificial serum does not occur due to the presence of other sugars or an electrochemical interferent present in the complex media, an interference study was conducted. Interference is calculated using a mean absolute relative difference (MARD) threshold of 20% in the presence of 5 mM glucose (Equation 1).

From the data in Figure 7, none of the other sugars or sugar alcohols result in an MARD greater than 20%. However, lactose shows the highest MARD which is close to the 20% threshold classifying interference. This is likely due to the structure of lactose, which is very similar to cellobiose, the preferred substrate of CDH-type enzymes.[14] Overall, while it may seem that lactose is just under the limit of interference, it must be considered that all the non-glucose sugars were tested at the maximum concentration in blood plasma to accurately depict a worst-case scenario. Thus, in artificial serum, lactose would likely not interfere in the signal and may not be the sole component responsible for the difference in the analytical parameters between PBS and artificial serum. Glycerol also affects glucose oxidation currents, especially for the MET biosensor system. It should be noted that solution properties may change sensor film swelling and mass transport of glucose to the electrode surface. While lactose was the only sugar to cause significant interference, it is reported that the sugar mannose causes the most significant interference for biosensors based on GOx[58] and *Ch*CDH.[13,18] To fully account for potential interference of electroactive substances it is recommended to use 2—3 times their highest blood concentrations while testing in order to depict the worst-case scenario.[59,60] Therefore, ascorbic acid, uric acid and acetaminophen were studied as interferents at concentrations higher than their usual blood concentrations (Figure 8). Even under these extreme conditions the MARD response for all three electroactive interferents remains below the 20% threshold, and they are not therefore classified as interferents. Uric acid shows the highest MARD in the sensor current response. Uric acid has been reported to act as a non-competitive inhibitor of *Ct*CDH and affect the stability of the enzyme over time.[61] In general, the electroactive substances can cause deviations in glucose reading due to co-oxidation (Figure 8). Overall, while the presence of uric acid and lactose alone do not exceed the MARD threshold to be

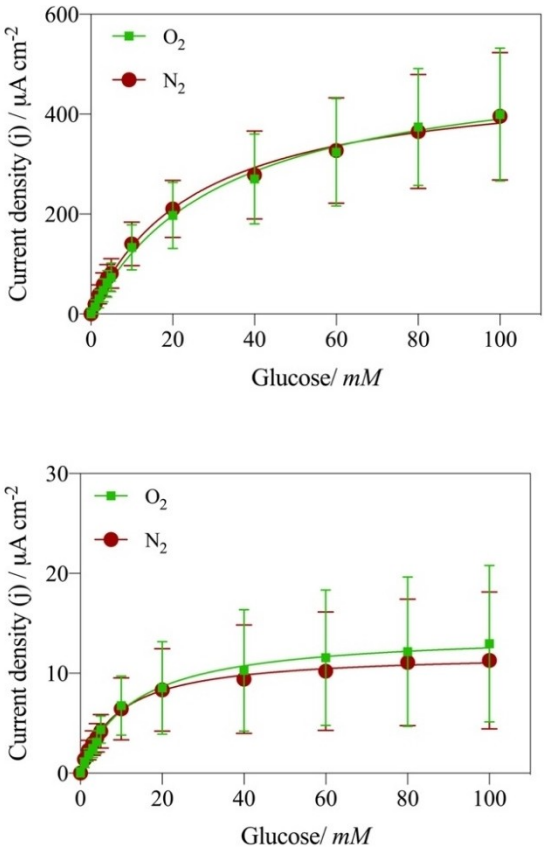

Figure 5. Glucose response curves for the MET system based on amperometry at 0.35 V (top) and for the DET system with 180 μg WT*Ch*CDH drop-coated onto the electrodes based on amperometry at 0.1 V (bottom) in the presence (green) and absence (red) of ambient oxygen in PBS at 37 °C, where n = 4. The error bars represent the standard deviation.

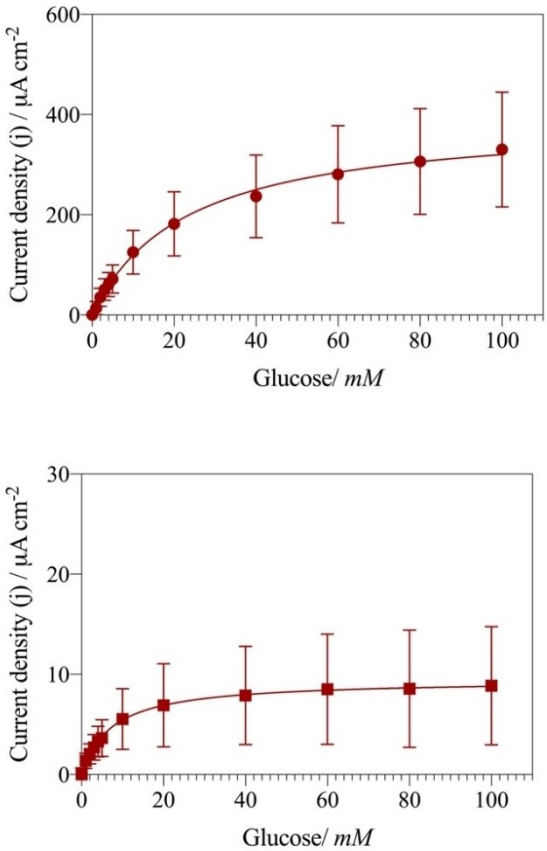

Figure 6. Glucose response curves for the MET system based on amperometry at 0.35 V (top) and for the DET system based on amperometry at 0.1 V (bottom) in the artificial serum buffer at 37 °C (n = 4). The error bars represent the standard deviation.

classified as interferences, their combined presence, as is the case in artificial serum, may work co-operatively. The MARD calculated for artificial serum is 40–50% when compared to sensor response in PBS. Finally, while taking into account the cooperative effect of the *in vivo* sugars and the electrochemical interferents, the presence of BSA in the artificial serum may also introduce non-specific protein adsorption that could contribute to increase the MARD in artificial serum.

Conclusion

Recombinantly produced WT*Ch*CDH containing glucose-activity enhancing mutations was used to produce DET and MET-based glucose biosensors. The biosensor component amounts were optimized for sensor current response in 5 mM glucose. Biosensor operation was characterized electrochemically in PBS and artificial serum. The MET biosensor showed high sensitivity on the same order of magnitude to systems containing other glucose-oxidising enzymes.[44,45] This shows the potential of the engineered enzyme for application to glucose biosensing. While the MET biosensor shows higher sensitivity than the DET biosensor, the DET biosensor based on recombinantly produced

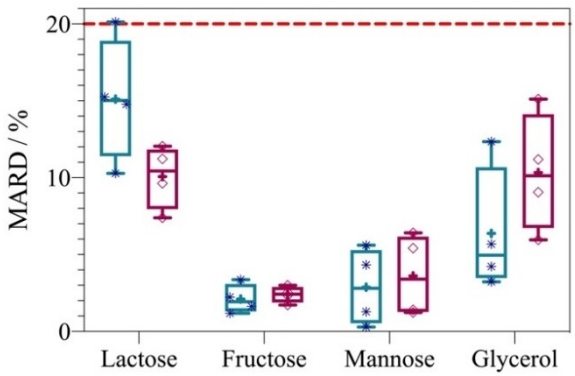

Figure 7. Box plot showing the mean absolute relative difference (MARD, %) of the current signal in 5 mM glucose (PBS, pH 7.4, 37 °C) for DET (pink) and MET (blue) sensors in the presence of sugars or glycerol. The line inside the box = mean, box limits = standard deviation (n = 4); * and ◇ represent individual data points; lower and upper error bars = 5% and 95% limits, respectively; red line = 20% MARD threshold for definition of interference.

WT*Ch*CDH offer a substantial improvement in sensitivity over other DET-based glucose biosensors. Moreover, both DET and MET sensors showed no change in glucose response when measured in the absence and presence of oxygen. Operation in artificial serum results in a decrease in glucose oxidation current

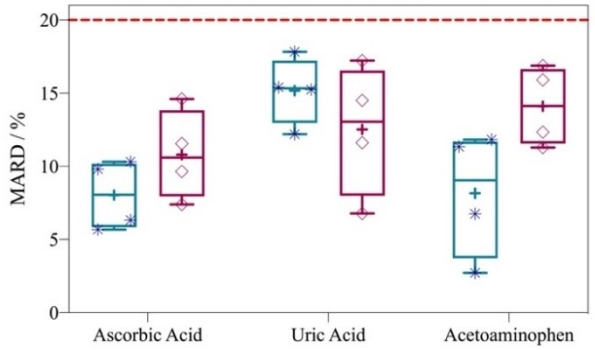

Figure 8. Box plot showing the mean absolute relative difference (MARD, %) of the current signal in 5 mM glucose (PBS, pH 7.4, 37 °C) for DET (pink) and MET (blue) sensors in the presence of common electrochemical interferents found in the blood. The line inside the box = mean, box limits = standard deviation (n = 4); * and ◇ represent individual data points; lower and upper error bars = 5 % and 95 % limits, respectively; red line = 20 % MARD threshold for definition of interference.

density attributed to electrochemical interferences and protein adsorption, but the operational stability is not affected by operation in artificial serum. An interference study conducted with sugars and common electrochemical interferents that can be present in vivo demonstrated that no individual component crosses the threshold to become an interferent alone, but in complex media such as artificial serum, they may have a cooperative effect.

Experimental Section

Materials

All chemicals were purchased from Sigma-Aldrich, unless otherwise stated. Milli-Q water (18 MΩ.cm) was used to prepare all aqueous solutions unless otherwise stated. The redox polymer Os(bpy)PVI was synthesized by modification of published procedures.[4,61]

Methods

Enzyme production

A CDH from *Crassicarpon hotsonii (*syn. *Myriococcum thermophilum*) equipped with glucose activity-enhancing mutations C291Y and W295R was used in this study. The mutations are both located in the active site of the DH domain responsible for substrate activity and specificity. C291Y was designed to provide an additional hydroxyl group for hydrogen bonding to stabilize the reducing sugar moiety and enhance glucose binding and oxidation. W295R was created to spatially constrain the binding of the non-reducing sugar moiety to improve glucose specificity and decrease maltose activity.

The CDH was recombinantly produced in *Komagatella phaffii* (syn. *Pichia pastoris*) as described previously[20] via methanol induction of the AOX promoter according to the manufacturer's instructions (Invitrogen). The enzyme was subsequently purified using hydrophobic interaction chromatography and anion exchange chromatography as previously established.[62] All purification steps were performed on an ÄKTA Pure FPLC system (GE Healthcare). Purified enzymes were concentrated and rebuffered to 1 mM phosphate buffer, pH 7.4, with centrifugal filters (Amicon; 30 kDa mass cutoff) to a concentration of approximately 15 mg mL^{-1} and stored at 4 °C. The enzyme is referred to as wild-type (WT*Ch*CDH) throughout this text to enable comparison to previously published data on the enzyme.[21]

Enzyme electrode preparation

Graphite rods (Graphite store, USA, 4.0 mm diameter, NC001300) were cut, insulated with heat shrink tubing and polished at one end using fine grit paper to give graphite working electrodes with a geometric surface area of 0.126 cm^2.

The MET biosensors were assembled by drop-coating 30 μL of Os(bpy)PVI aqueous solution (5 mg mL^{-1}), 16 μL of WT*Ch*CDH aqueous solution (10 mg mL^{-1}) and 7.4 μL of PEGDGE crosslinker aqueous solution (15 mg mL^{-1}). The deposition was allowed to dry for 24 h at ambient temperature before the electrodes were used. The electrode amounts are based on amounts optimized to produce the highest glucose oxidation current density in 5 mM glucose solution using a design of experiments approach (Supplementary Figure S1).

Electrodes for use as DET biosensors were first pre-treated in 1 % v/v ethylene glycol diglycidyl ether (EGDGE) in 0.1 M NaOH at 60 °C for 1 h. The electrodes were then rinsed in water and ethanol and dried with nitrogen. Electrodes were drop-coated with either 16 μL or 18 μL of WT*Ch*CDH aqueous solution (10 mg mL^{-1}) and placed in an oven at 60 °C for 1 h before use. The 18 μL WT*Ch*CDH volume is the amount optimized to produce the highest glucose oxidation current density in 5 mM glucose solution using a one-factor-at-a-time optimization approach (Supplementary Figure S2). The 16 μL WT*Ch*CDH volume was selected to benchmark the response of the 3rd generation biosensor to that of the MET biosensors that are prepared using this volume of enzyme solution.

Electrochemical measurements

Electrochemical tests were conducted using a CH Instruments 1030a multichannel potentiostat (IJ Cambria). The enzyme electrodes were used as working electrodes, with a custom-built Ag | AgCl (3 M KCl) as reference electrode and a platinum mesh (Goodfellow) as a counter electrode. Electrodes were placed in a thermostated electrochemical cell containing phosphate buffered saline (50 mM phosphate, 120 mM NaCl, pH 7.4) at 37 °C with experiments conducted, unless otherwise stated, in the presence of ambient oxygen. Current signals are normalised to the geometric surface area of the graphite disk electrodes to generate current density data. Stability values represent, unless otherwise indicated, the percentage of amperometric current density remaining at the end of a 12 h operational period compared to that obtained 20 minutes after initial polarization. The applied potential is 0.35 V for the MET biosensor and 0.1 V for the DET biosensor. SWV in a voltage window of −0.4 V to 0.4 V with an amplitude of 30 mV, frequency of 2 Hz and step amplitude of 5 mV was used to characterise the DET biosensor electrochemical response.

Artificial serum was prepared based on an aqueous solution containing uric acid (68.5 mg L^{-1}), ascorbic acid (9.5 mg L^{-1}), fructose (36 mg L^{-1}), lactose (5.5 mg L^{-1}), urea (267 mg L^{-1}), cysteine (18 mg L^{-1}), sodium chloride (6.75 g L^{-1}), sodium bicarbonate (2.138 g L^{-1}), calcium sulfate (23.8 mg L^{-1}), magnesium sulfate (104.5 mg L^{-1}) and bovine serum albumin (7 g L^{-1})[63].

Oxygen dependency of the sensors was evaluated by recording glucose-responsive amperometry at 0.35 V for the MET biosensor or

0.1 V for the DET biosensor in PBS at 37 °C containing ambient oxygen. The PBS was then replaced, and the glucose additions repeated after the solution was purged with nitrogen for 30 mins.

Biosensor response (i.e., amperometry at 0.35 V or 0.1 V for MET or DET, respectively) to 5 mM glucose in the presence of interferent was compared to biosensor response to glucose without interferent. Interference testing used solutions of fructose (1000 mg L^{-1}),[64] lactose (2000 mg L^{-1})[65] mannose (2000 mg L^{-1}),[66] glycerol (3000 mg L^{-1}),[67] acetaminophen (200 mg L^{-1}), ascorbic acid (60 mg L^{-1}) or uric acid (120 mg L^{-1}).[59] The test concentration of sugars and glycerol is chosen based on a maximum blood concentration reported for these substances.[59,64–67] The test concentration chosen for each of the electroactive interferents is based on the guidelines provided in the Clinical and Laboratory Standards Institute (CLSI) document EP7-A2 section 5.5 "Interferent Test Concentrations"; these recommended concentrations vary according to the specific substance, but all substances were tested at a concentration above the highest blood concentration expected in the intended patient population.[65]

Interference screening data was analysed by calculating MARD between the mean baseline glucose concentration reading without interferent (M_0) and the mean glucose reading value with interferent present (M_I) as:

$$\text{MARD } (\%) = \frac{|M_I - M_0|}{M_0} \times 100 \quad (1)$$

In this study, interference was defined as an absolute MARD $\geq$ 20 % as defined by Boehm et al. previously[58].

Acknowledgements

This Publication is part of a project that has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°813006. Open access funding provided by IReL.

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: cellobiose dehydrogenase · direct electron transfer · glucose biosensor · mediated electron transfer · osmium redox polymer

[1] P. Saeedi, I. Petersohn, P. Salpea, B. Malanda, S. Karuranga, N. Unwin, S. Colagiuri, L. Guariguata, A. A. Motala, K. Ogurtsova, J. E. Shaw, D. Bright, R. Williams, *Diabetes Res. Clin. Pract.* **2019**, *157*, 107843.
[2] N. H. Cho, J. E. Shaw, S. Karuranga, Y. Huang, J. D. da Rocha Fernandes, A. W. Ohlrogge, B. Malanda, *Diabetes Res. Clin. Pract.* **2018**, *138*, 271–281.
[3] L. C. Clark, C. Lyons, *Ann. N. Y. Acad. Sci.* **1962**, *102*, 29–45.
[4] A. Heller, B. Feldman, *Chem. Rev.* **2008**, *108*, 2482–2505.
[5] K. E. Toghill, R. G. Compton, *Int. J. Electrochem. Sci.* **2010**, *5*, 1246–1301.
[6] P. N. Bartlett, F. A. Al-Lolage, *J. Electroanal. Chem.* **2018**, *819*, 26–37.
[7] R. Wilson, A. P. F. Turner, *Biosens. Bioelectron.* **1992**, *7*, 165–185.
[8] T. J. Ohara, R. Rajagopalan, A. Heller, *Anal. Chem.* **1993**, *65*, 3512–3517.
[9] A. E. G. Cass, G. Davis, G. D. Francis, H. Allen, O. Hill, W. J. Aston, I. J. Higgins, E. v. Plotkin, L. D. L. Scott, A. P. F. Turner, *Anal. Chem.* **1984**, *56*, 667–671.
[10] R. Ortiz, M. Rahman, B. Zangrilli, C. Sygmund, P. O. Micheelsen, M. Silow, M. D. Toscano, R. Ludwig, L. Gorton, *ChemElectroChem* **2017**, *4*, 846–855.
[11] A. Heller, *Phys. Chem. Chem. Phys.* **2004**, *6*, 209–216.
[12] C. Chen, Q. Xie, D. Yang, H. Xiao, Y. Fu, Y. Tan, S. Yao, *RSC Adv.* **2013**, *3*, 4473–4491.
[13] S. Scheiblbrandner, R. Ludwig, *Bioelectrochemistry* **2020**, *131*, 107345.
[14] R. Ludwig, W. Harreither, F. Tasca, L. Gorton, *ChemPhysChem* **2010**, *11*, 2674–2697.
[15] T. C. Tan, D. Kracher, R. Gandini, C. Sygmund, R. Kittl, D. Haltrich, B. M. Hällberg, R. Ludwig, C. Divne, *Nat. Commun.* **2015**, *6*, 1–11.
[16] F. Tasca, R. Ludwig, L. Gorton, R. Antiochia, *Sens. Actuators B* **2013**, *177*, 64–69.
[17] P. Bollella, F. Mazzei, G. Favero, G. Fusco, R. Ludwig, L. Gorton, R. Antiochia, *Biosens. Bioelectron.* **2017**, *88*, 196–203.
[18] W. Harreither, V. Coman, R. Ludwig, D. Haltrich, L. Gorton, *Electroanalysis* **2007**, *19*, 172–180.
[19] A. Lindgren, L. Gorton, T. Ruzgas, U. Baminger, D. Haltrich, M. Schülein, *J. Electroanal. Chem.* **2001**, *496*, 76–81.
[20] M. Zámocký, C. Schümann, C. Sygmund, J. O'Callaghan, A. D. W. Dobson, R. Ludwig, D. Haltrich, C. K. Peterbauer, *Protein Expression Purif.* **2008**, *59*, 258–265.
[21] A. F. Geiss, T. M. B. Reichhart, B. Pejker, E. Plattner, P. L. Herzog, C. Schulz, R. Ludwig, A. K. G. Felice, D. Haltrich, *ACS Sustainable Chem. Eng.* **2021**, *9*, 7086–7100.
[22] R. Ortiz, H. Matsumura, F. Tasca, K. Zahma, M. Samejima, K. Igarashi, R. Ludwig, L. Gorton, *Anal. Chem.* **2012**, *84*, 10315–10323.
[23] S. C. Feifel, R. Ludwig, L. Gorton, F. Lisdat, *Langmuir* **2012**, *28*, 9189–9194.
[24] M. Shao, M. N. Zafar, M. Falk, R. Ludwig, C. Sygmund, C. K. Peterbauer, D. A. Guschin, D. MacAodha, P. Ó. Conghaile, D. Leech, M. D. Toscano, S. Shleev, W. Schuhmann, L. Gorton, *ChemPhysChem* **2013**, *14*, 2260–2269.
[25] A. Hildebrand, E. Szewczyk, H. Lin, T. Kasuga, Z. Fan, *Appl. Environ. Microbiol.* **2015**, *81*, 597–603.
[26] Desriani, S. Ferri, K. Sode, *Biochem. Biophys. Res. Commun.* **2010**, *391*, 1246–1250.
[27] F. A. J. Rotsaert, V. Renganathan, M. H. Gold, *Biochemistry* **2003**, *42*, 4049–4056.
[28] C. Sygmund, W. Harreither, D. Haltrich, L. Gorton, R. Ludwig, *New Biotechnol.* **2009**, *25*, S115.
[29] G. Kovacs, R. Ortiz, V. Coman, W. Harreither, I. C. Popescu, R. Ludwig, L. Gorton, *Bioelectrochemistry* **2012**, *88*, 84–91.
[30] P. Bollella, L. Gorton, R. Ludwig, R. Antiochia, *Sensors (Switzerland)* **2017**, *17*, 1912.
[31] W. Harreither, P. Nicholls, C. Sygmund, L. Gorton, R. Ludwig, *Langmuir* **2012**, *28*, 6714–6723.
[32] C. Schulz, R. Ludwig, L. Gorton, *Anal. Chem.* **2014**, *86*, 4256–4263.
[33] H. Ju, D. Leech, *Anal. Chim. Acta* **1997**, *345*, 51–58.
[34] A. J. Bard, L. R. Faulkner Larry, in *Electrochemical Methods: Fundamentals and Applications, New York: Wiley, 2001*, John Wiley And Sons, **2001**.
[35] I. Osadebe, D. Leech, *ChemElectroChem* **2014**, *1*, 1988–1993.
[36] I. Osadebe, P. Conghaile, P. Kavanagh, D. Leech, *Electrochim. Acta* **2015**, *182*, 320–326.
[37] R. Bennett, D. Leech, *Bioelectrochemistry* **2020**, *133*, 107460.
[38] R. Bennett, I. Osadebe, R. Kumar, P. Conghaile, D. Leech, *Electroanalysis* **2018**, *30*, 1438–1445.
[39] B. Limoges, J. Moiroux, J. M. Savéant, *J. Electroanal. Chem.* **2002**, *521*, 8–15.
[40] P. N. Bartlett, K. F. E. Pratt, *J. Electroanal. Chem.* **1995**, *397*, 61–78.
[41] K. Jayakumar, R. Bennett, D. Leech, *Electrochim. Acta* **2021**, *371*, 137845.
[42] M. N. Zafar, G. Safina, R. Ludwig, L. Gorton, *Anal. Biochem.* **2012**, *425*, 36–42.
[43] A. K. G. Felice, C. Sygmund, W. Harreither, R. Kittl, L. Gorton, R. Ludwig, *J. Diabetes Sci. Technol.* **2013**, *7*, 669.
[44] R. Rajagopalan, A. Aoki, A. Heller, *J. Phys. Chem.* **1996**, *100*, 3719–3727.

[45] S. A. Merchant, D. T. Glatzhofer, D. W. Schmidtke, *Langmuir* **2007**, *23*, 11295–11302.
[46] J. D. Qiu, W. M. Zhou, J. Guo, R. Wang, R. P. Liang, *Anal. Biochem.* **2009**, *385*, 264–269.
[47] V. B. Juska, M. E. Pemble, *Sensors* **2020**, *20*, 6013.
[48] A. Prévoteau, N. Mano, *Electrochim. Acta* **2013**, *112*, 318–326.
[49] A. Prévoteau, N. Mano, *Electrochim. Acta* **2012**, *68*, 128–133.
[50] N. Mano, *Bioelectrochemistry* **2019**, *128*, 218–240.
[51] C. Sygmund, P. Santner, I. Krondorfer, C. K. Peterbauer, M. Alcalde, G. S. Nyanhongo, G. M. Guebitz, R. Ludwig, *Microb. Cell Fact.* **2013**, *12*, 38.
[52] A. Prévoteau, O. Courjean, N. Mano, *Electrochem. Commun.* **2010**, *12*, 213–215.
[53] Y. Horaguchi, S. Saito, K. Kojima, W. Tsugawa, S. Ferri, K. Sode, *Electrochim. Acta* **2014**, *126*, 158–161.
[54] V. Coman, R. Ludwig, W. Harreither, D. Haltrich, L. Gorton, T. Ruzgas, S. Shleev, *Fuel Cells* **2010**, *10*, 9–16.
[55] T. Siepenkoetter, U. Salaj-Kosla, X. Xiao, P. Conghaile, M. Pita, R. Ludwig, E. Magner, *ChemPlusChem* **2017**, *82*, 553–560.
[56] R. D. Milton, K. Lim, D. P. Hickey, S. D. Minteer, *Bioelectrochemistry* **2015**, *106*, 56–63.
[57] A. Cornish-Bowden, in *Fundamentals of Enzyme Kinetics*, **1979**.
[58] R. Boehm, J. Donovan, D. Sheth, A. Durfor, J. Roberts, I. Isayeva, *J. Diabetes Sci. Technol.* **2019**, *13*, 82–95.
[59] T. Bailey, A. Chang, P. D. Rosenblit, L. Jones, G. Teft, S. Setford, J. Mahoney, *Diabetes Technol. Ther.* **2012**, *14*, 701–709.
[60] *EP07 Interference Testing in Clinical Chemistry*, **2017**.
[61] R. Bennett, E. Blochouse, D. Leech, *Electrochim. Acta* **2019**, *302*, 270–276.
[62] U. Baminger, S. S. Subramaniam, V. Renganathan, D. Haltrich, *Appl. Environ. Microbiol.* **2001**, *67*, 1766–1774.
[63] C. A. Burtis, E. R. Ashwood, D. E. Bruns, in *Tietz Textbook of Clinical Chemistry and Molecular Diagnostics-e-Book*, Elsevier Health Sciences, **2012**.
[64] M. Miller, J. W. Craig, W. R. Drucker, H. Woodward, *Yale J. Biol. Med.* **1956**, *29*, 335–60.
[65] J. G. Blanco, *J. Biol. Chem.* **1928**, *79*, 667–672.
[66] F. C. Wood, G. F. Cahill, *J. Clin. Invest.* **1963**, *42*, 1300–1312.
[67] R. Guisado, W. W. Tourtellotte, A. I. Arieff, U. Tomiyasu, S. K. Mishra, M. C. Schotz, *J. Neurosurg.* **1975**, *42*, 226–228.

Manuscript received: April 14, 2022
Revised manuscript received: May 31, 2022
Accepted manuscript online: June 5, 2022