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COMMUNICATION

A third generation glucose biosensor based on cellobiose dehydrogenase from *Corynascus thermophilus* and single-walled carbon nanotubes

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A third generation glucose biosensor working under physiological conditions with a linear range of 0.1–30 mM, a detection limit of 0.05 mM, and a sensitivity of 222 nA μM^{-1} cm^{-2} has been developed by co-adsorption of cellobiose dehydrogenase (CDH) from the ascomycete *Corynascus thermophilus* (*Ct*CDH) and oxidatively shortened single-walled carbon nanotubes (SWCNTs).

Today more than 150 million people suffer from diabetes and depend on the fast and reliable determination of their blood glucose level.[1] In recent years the amount of blood, which has to be taken by a more or less painful procedure, has been continuously reduced. All of the presently available strips utilizing sub-microlitre blood sample volumes are electrochemical.[1] They contain the enzymes glucose oxidase (GOx) or different types of glucose dehydrogenase (GDH) and a redox mediator. Currently GOx is the most frequently employed enzyme for the development of electrochemical glucose biosensors.[1,2] This enzyme is commercially available with high specific activity and stability. Nevertheless, GOx has some drawbacks. GOx belongs to the class of intrinsic redox enzymes with the catalytic center buried deeply inside the protein shell.[3,4] This architecture makes sense, because the native function of the enzyme is to reduce oxygen to hydrogen peroxide serving as an anti-fungal or anti-microbial agent.[2] There is no need for electron transfer (ET) pathways from the catalytic reaction center to the surface of the enzyme, and direct electron transfer (DET) between GOx and electrodes is strongly disfavored. The most effective option to establish electrochemical communication is to wire GOx to electrodes by means of an Os redox polymer.[5–7] However, the usage of Os containing glucose test stripes is accompanied by the accumulation of Os containing waste and depending on its oxidation state, Os can be highly toxic. Glucose test stripes have to be cheap and are designed for fast response and single use. These requirements can be satisfied by applying an enzyme which is able to transfer the electrons gained by glucose oxidation directly to the electrode. The test stripes could be fabricated by simple physical adsorption of the enzyme to the electrode material. Therefore, it is reasonable to search for possibilities of waiving redox mediators and for new enzymes which show efficient DET.[8–16]

Here we report on the development of an amperometric glucose biosensor by a combination of cellobiose dehydrogenase (CDH) from the ascomycete *Corynascus thermophilus* (*Ct*CDH) and single-walled carbon nanotubes (SWCNTs). CDHs are extracellular enzymes produced by a variety of fungi involved in wood degradation.[13,17,18] The enzymes exist usually as monomers comprising a larger flavin-associated (dehydrogenase) and a smaller heme (cytochrome) domain. The native cofactors are FAD and heme *b*, respectively. At the flavin domain of CDHs different sugars such as di- and oligosaccharides are oxidized to the corresponding lactones. The electrons gained by substrate oxidation can be transferred directly from the flavin domain to different two-electron acceptors such as quinones or dichloroindophenol. Alternatively, the electrons can be transferred by internal electron transfer (IET) to the heme domain and further to one-electron acceptors such as cytochrome *c* or to electrodes.[14,18–20] Due to their ability to undergo DET *via* IET, CDHs can be classified as extrinsic redox enzymes.[3,4] In contrast to CDHs from basidiomycete fungi, CDHs from some ascomycete fungi catalyze also the oxidation of monosaccharides such as glucose and some di- and oligosaccharides, *e.g.* maltose (consisting of two α-1,4-linked glucose moieties) with high turnover rates.[18,21–24] The newly discovered *Ct*CDH, which is able to catalyze the oxidation of glucose at neutral pH, has been investigated recently for an application as catalyst at the anode of a glucose biofuel cell.[23] When *Ct*CDH as well as other types of CDH were used as catalysts at biofuel cell anodes working by DET or MET, the enzymes turned out to be highly stable.[15,16,21,23]

For *Ct*CDH adsorbed to bare graphite electrodes, DET can only be proven indirectly by monitoring the catalytic current caused by glucose oxidation. When cyclic voltammetry is performed in the absence of substrate, waves belonging to the reduction and re-oxidation of the flavin and/or heme domain of the enzyme cannot be found.[23] This changes significantly when *Ct*CDH is adsorbed in the presence of oxidatively shortened SWCNTs.[15,25] In Fig. 1 the cyclic voltammograms (CVs) of a *Ct*CDH/SWCNTs modified electrode measured in the absence and presence of glucose in PBS at pH 7.4

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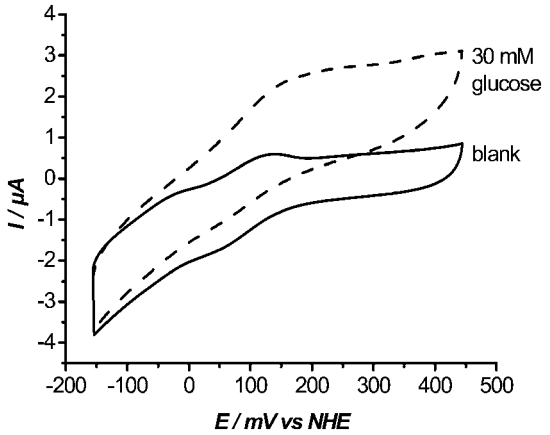

Fig. 1 CVs of a graphite electrode modified with *Ct*CDH/oxidatively shortened SWCNTs measured in the absence of substrate (blank, solid line) and in the presence of 30 mM glucose (dashed line) at a scan rate of $v = 1$ mV s^{-1} at physiological conditions (PBS buffer, pH 7.4). Prior to the measurements, argon was purged through the buffer solution for some minutes.

(under physiological conditions) are depicted. In the absence of substrate, a single oxidative and reductive wave with a midpoint potential of 90 mV can be seen. In the presence of glucose the onset of the catalytic current is at a potential of about −50 mV and the catalytic current reaches a plateau at 200 mV. With respect to prior investigations on CDHs, DET is possible only *via* the heme domain serving as built-in redox mediator.[16,20] Assuming that the redox process observed at 90 mV (in the absence of substrate) is the oxidation/reduction of the heme domain (1 e$^-$ process), the catalytic current for glucose oxidation started already when less than 1% of the heme domains are oxidized and reaches a maximum when almost 99% of the heme domains are oxidized (according to *Nernst*: $E = E^{0\prime} + 0.059\ \text{V}/n \times \log(C_{\text{Ox}}/C_{\text{Red}})$). This leads to the conclusion that the redox process observed at 90 mV in the absence of substrate is the heme oxidation/reduction of catalytically intact *Ct*CDH. Hence, in the presence of oxidatively shortened SWCNTs it is possible to prove DET directly by cyclic voltammetry in the absence of substrate.

It is also worth to mention that in the CV measured in the absence of substrate an additional anodic peak can be seen at about −40 mV. A similar peak has been observed previously when *Ps*CDH and *Mt*CDH were adsorbed to graphite electrodes in the presence of oxidatively shortened SWCNTs.[15] This peak might be caused by any degradation product of the flavin domain. However, it cannot be the oxidation of the intact *Ct*CDH flavin domain since the increase in catalytic current (measured in the presence of substrate) shows no correlation to that peak.

SWCNTs were oxidatively shortened and separated by size exclusion chromatography with respect to their average length as reported previously.[25] This time, sixty fractions of equal volume were collected by size exclusion chromatography. For the current study, SWCNTs from fractions 30–40, which exhibited the best performance when co-adsorbed with *Ct*CDH, were used. In contrast to non-modified SWCNTs, oxidatively shortened SWCNTs form a homogeneous suspension after sonication in water. Hence, the SWCNTs modified electrodes could be prepared in a well defined and repeatable manner. In order to characterize more in detail the influence of the oxidatively shortened SWCNTs on ET, spectrographic graphite electrodes were modified with *Ct*CDH only and with *Ct*CDH/SWCNTs, placed in an electrochemical flow through cell with glucose in the stationary phase and studied at different potentials. To the electrodes prepared with *Ct*CDH[23] and oxidatively shortened SWCNTs, also a cross-linker (poly(ethylene glycol) (400) diglycidyl ether, PEGDGE, 2 µL of a 30 mg mL^{-1} solution) was added in order to prevent washing-off the SWCNTs due to the constant flow.[15] In Fig. 2 the polarization curves (current values collected at different potentials during constant flow) for the *Ct*CDH modified electrodes prepared in the presence and absence of SWCNTs are depicted (including standard deviation for three independent measurements). In the presence of carbon nanotubes the catalytic current density was increased by about one third. The larger current might be explained by an increased active electrode area due to the adsorbed SWCNTs. In addition, in the presence of SWCNTs the onset of the electrocatalytic current is shifted to more negative values by about 100 mV, *i.e.* the overpotential for heme oxidation is decreased. The same observation was made previously when *Ps*CDH and *Mt*CDH were adsorbed in the presence of SWCNTs.[15] A possible explanation might be that, besides increasing the active electrode area, the SWCNTs catalyze DET between enzyme and electrode.

Next, spectrographic graphite electrodes modified with *Ct*CDH only and with *Ct*CDH, oxidatively shortened SWCNTs, and cross-linker were placed in an electrochemical flow through cell serving as glucose biosensor. A potential of $E = 390$ mV *vs.* NHE was applied. Three independent sets of *Ct*CDH modified electrodes were prepared and investigated. In Fig. 3A the calibration curves (including standard deviation) for *Ct*CDH modified electrodes in the presence (filled symbols) and absence (empty symbols) of oxidatively shortened SWCNTs and cross-linker are shown. Glucose solutions of different concentrations prepared with PBS buffer (pH 7.4) were used as analyte. For both types of electrodes the linear range was the same (0.1–30 mM). For the electrodes prepared in the presence of oxidatively shortened SWCNTs the detection limit was lower and a higher current for glucose oxidation (leading to an increased

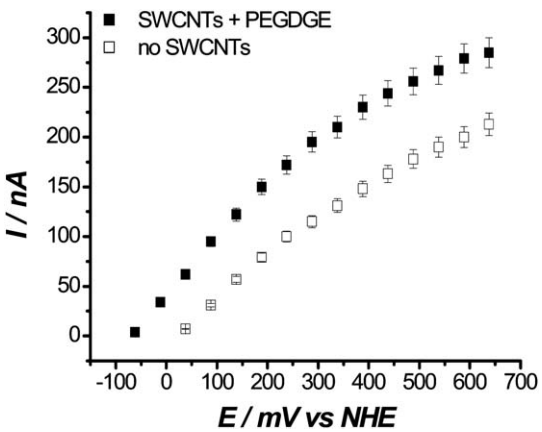

Fig. 2 Polarization curves for *Ct*CDH modified electrodes in the presence (filled symbols) and absence (empty symbols) of oxidatively shortened SWCNTs and cross-linker (average and standard deviation of three measurements). The modified electrodes were placed in a flow through electrochemical cell with 10 mM glucose added to the flow buffer (PBS buffer, pH 7.4, and flow rate = 0.5 mL min^{-1}).

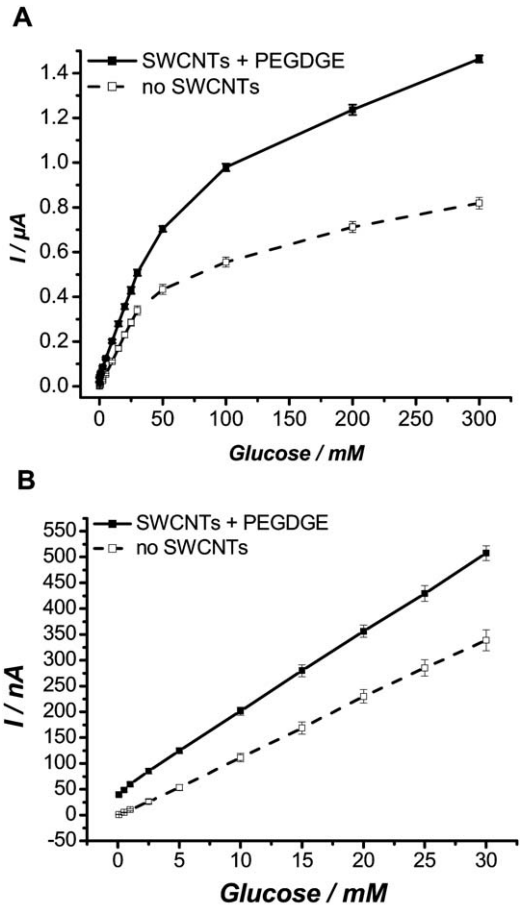

Fig. 3 (A) Glucose calibration curves of *Ct*CDH modified electrodes in the presence (filled symbols, solid line) and absence (empty symbols, dotted line) of oxidatively shortened SWCNTs and cross-linker (average and standard deviation of three independent measurements). (B) Linear range of the calibration curve depicted in (A). The experiments were performed at a potential of 390 mV *vs.* NHE at a flow rate of 1 mL min^{-1} in PBS buffer (pH 7.4).

sensitivity) was measured. For the *Ct*CDH modified electrodes a detection limit and a sensitivity of 0.05 mM and 222 nA μM^{-1} cm^{-2} in the presence, and 0.1 mM and 163 nA μM^{-1} cm^{-2} in the absence of SWCNTs were calculated respectively. In Fig. 3B the linear range of the calibration curves for both types of electrodes is depicted.

While the glucose concentration in the blood of a healthy person is usually between 4 and 8 mM, in the blood of a diabetic patient it can be up to 30 mM.[1] Hence, the linear range of the third generation glucose biosensors using both types of electrodes (prepared by adsorption of *Ct*CDH in the presence and absence of oxidatively shortened SWCNTs) covers the range of interest for monitoring the glucose concentration in human blood. Also the detection limits of 0.05 or 0.1 mM are sufficiently low for this application. For the *Ct*CDH modified electrodes prepared by co-adsorption of oxidatively shortened SWCNTs the sensitivity was about one third higher than without SWCNTs. With respect to the data presented in Fig. 2, the difference in sensitivity will increase when a lower operation potential for the biosensor is chosen. Working at a lower operation potential might reduce the non-specific oxidation of interfering compounds, which can be present in real samples of human blood. Apparently, the co-adsorption of oxidatively shortened SWCNTs significantly increases the biosensor performance.

In conclusion we have developed a third generation glucose biosensor by co-adsorption of *Ct*CDH and oxidatively shortened SWCNTs. In the presence of the SWCNTs the current density was increased and the overpotential was reduced. The linear range, detection limit, and the sensitivity of the *Ct*CDH/SWCNTs glucose biosensor under physiological conditions were 0.1–30 mM, 0.05 mM, and 222 nA μM^{-1} cm^{-2} respectively. The linear range of the glucose biosensor covers the whole concentration range relevant for the direct determination of the blood glucose level. With respect to its linear range and detection limit the third generation glucose biosensor might be suited for monitoring the glucose concentration in human blood.

Note added after first publication

This article replaces the version published on 29th July 2010. This new version contains changes to the author list and Acknowledgements section, with an added Authors' note.

Authors' note

It is noted that the authors Muhammad Nadeem Zafar (MNZ), Wolfgang Harreither (WH) and Roland Ludwig (RL) did not provide approval for the submission of the current manuscript. MNZ, WH and RL were not given the opportunity to provide corrections to the manuscript after peer-review. These points are disputed by Dr Gilbert Nöll (GN). The authors MNZ, WH and RL, with the addition of Federico Tasca (FT), wish to acknowledge the inclusion of Professor Lo Gorton (LG), Lund University, as a co-author for this article. GN wishes to dispute the scientific contribution of LG to the article. All parties have collectively agreed to the inclusion of this Authors' note, to acknowledge the above.

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