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# An Amperometric Enzyme Biosensor for Real-Time Measurements of Cellobiohydrolase Activity on Insoluble Cellulose

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**ABSTRACT:** An amperometric enzyme biosensor for continuous detection of cellobiose has been implemented as an enzyme assay for cellulases. We show that the initial kinetics for cellobiohydrolase I, Cel7A from *Trichoderma reesei*, acting on different types of cellulose substrates, semi-crystalline and amorphous, can be monitored directly and in real-time by an enzyme-modified electrode based on cellobiose dehydrogenase (CDH) from *Phanerochaete chrysosporium* (*Pc*). *Pc*CDH was cross-linked and immobilized on the surface of a carbon paste electrode which contained a mediator, benzoquinone. An oxidation current of the reduced mediator, hydroquinone, produced by the CDH-catalyzed reaction with cellobiose, was recorded under constant-potential amperometry at +0.5 V (vs. Ag/AgCl). The CDH-biosensors showed high sensitivity ($87.7\ \mu A\ mM^{-1}\ cm^{-2}$), low detection limit (25 nM), and fast response time ($t_{95\%} \sim 3$ s) and this provided experimental access to the transient kinetics of cellobiohydrolases acting on insoluble cellulose. The response from the CDH-biosensor during enzymatic hydrolysis was corrected for the specificity of *Pc*CDH for the β-anomer of cello-oligosaccharides and the approach were validated against HPLC. It is suggested that quantitative, real-time data on pure insoluble cellulose substrates will be useful in attempts to probe the molecular mechanism underlying enzymatic hydrolysis of cellulose.

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**KEYWORDS:** cellulose hydrolysis; cellulase kinetics; enzyme biosensor; cellobiose dehydrogenase

Carbon neutral second-generation bioethanol from renewable lignocellulosic biomass is a promising alternative to fossil fuels in the transport sector. A primary challenge for this technology is the breakdown of cellulose to fermentable sugars. This so-called saccharification, can be achieved in a sustainable way by hydrolysis catalyzed by a mixture of cellulolytic enzymes. The major component in such enzyme cocktails is the group of exo-acting cellobiohydrolases (CBHs), which hydrolyze the cellulose chain processively and primarily produce cellobiose (Zhang and Lynd, 2004). Fundamental understanding of CBH activity is therefore critical to improvements of enzymatic saccharification, but in spite of intense research, many key questions on the mechanism and regulation remain open. This is partly because of the notorious experimental challenges associated with the insoluble and heterogeneous nature of cellulose, but also related to a shortage of simple, fast, and quantitative assay technologies, in particular continuous methods. Most conventional methods quantify soluble sugar produced over time. This can be done by quenching the hydrolysis reaction at different time-points and measuring sugar production by, e.g., HPLC or colorimetric methods, that detects reducing sugars (Zhang et al., 2006). These assays are quite labor intensive and generally provides a limited time-resolution. One of the key challenges in fundamental biomass research therefore lies in the development of analytical methodologies

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to monitor cellulase activities during the degradation of cellulose and lignocellulosic biomass (Zhang and Lynd, 2004; Zhang et al., 2006). Recent progress in this area includes the use of isothermal calorimetry (Murphy et al., 2010), quartz crystal microbalance (Hu et al., 2009), and an amperometric enzyme biosensor based on immobilized pyrroloquinoline quinine-dependent glucose dehydrogenase (Tatsumi et al., 2006). These assay technologies have the advantages of being unaffected by the optical properties of the reaction mixture (turbidity and coloration) and monitoring in real-time. Obviously, each has different advantages and limitations that make them adequate for the investigation of certain aspects of the cellulolytic process. So far, no continuous method has offered the resolution in time and product concentration required to monitor the transient kinetics of cellulases before (pseudo)-steady state is established.

In the current work we present an experimental approach to this regime, which is known to be particularly important to mechanistic analyses (Johnson, 1992). We have developed an amperometric enzyme biosensor based on *Phanerochaete chrysosporium* cellobiose dehydrogenase (*Pc*CDH). This enzyme has previously been utilized in coupled solution end-point assays to track enzymatic cellulose degradation (Igarashi et al., 2006; Valjamae et al., 1998), in combination with a oxidation reduction electrode and ferricyanide redox system for continuously measurements of cellulase activity (Samejima et al., 1998) and in amperometric biosensors for off-line quantification of total soluble reducing ends (cello-oligosaccharides) in filtered samples (Hildén et al., 2001; Nordling et al., 1993). The application of CDH in biosensors and biofuel cells has recently been reviewed (Ludwig et al., 2010). The measuring principle for the presented CDH-biosensor is sketched out in Figure 1. A typical calibration of a CDH-biosensor can be seen in Figure 2. The main graph in Figure 2A shows the current response for successive injections of cellobiose. The response reached a stable steady-state

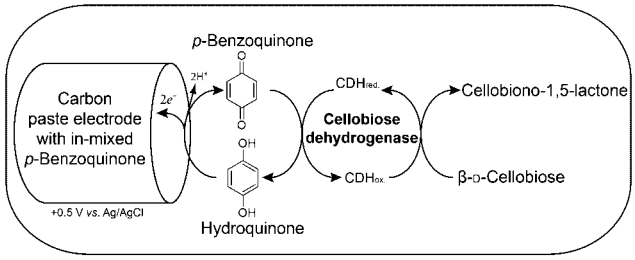


**Figure 1.** Schematic illustration of the mediated bioelectrocatalytic measuring principle for the amperometric CDH-biosensor.

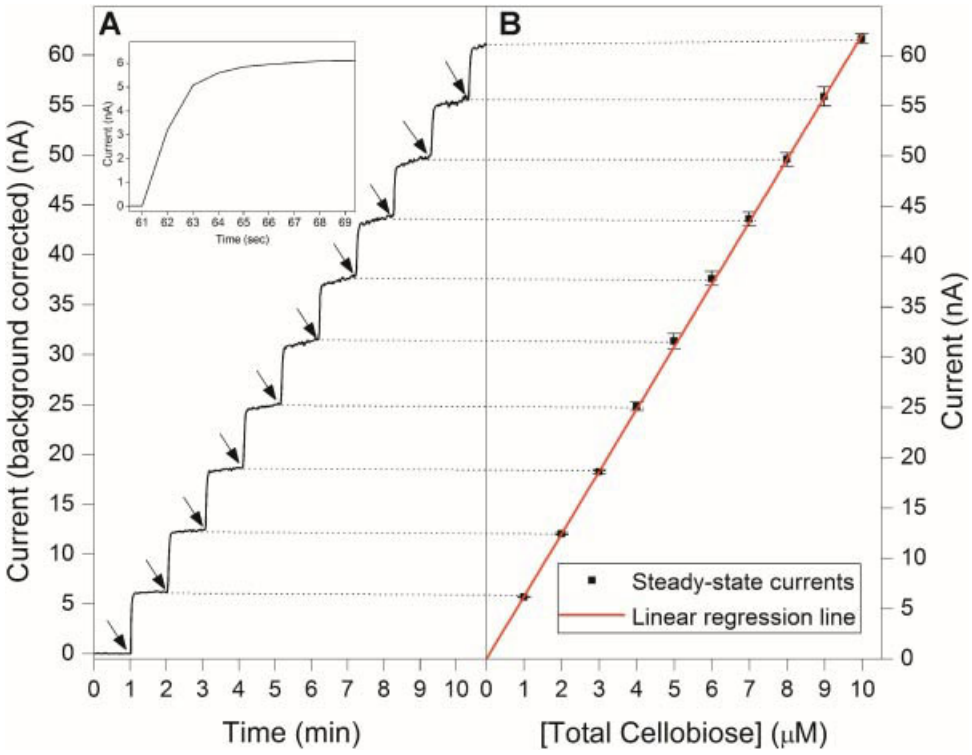


**Figure 2.** Panel A: Typical amperometric (current-time) response curve of the CDH-biosensor for the successive titrations of cellobiose at 25°C in 50 mM sodium acetate buffer, pH 5 (stirring rate 600 rpm). Each arrow marks the injection of 1 μM cellobiose to the cell. The insert shows the enlargement of the first injection; the injection started at the 61 s and lasted 0.1 s. Panel B: Calibration plot of steady-state currents versus cellobiose concentration. The solid line is the regression line. Both figures in Panel (A) and (B) are the average from three experiments.

within 5 s (approximately 3 s to reach 95% of the steady-state value; i.e., $t_{95\%} = 3$ s). Figure 2B shows the calibration curve of the steady-state currents plotted as a function of the cellobiose concentration. There was a linear relationship up to 25 μM and the slope specified a sensitivity of $6.23 \pm 0.01$ nA $\mu M^{-1}$ (current density 87.7 μA $mM^{-1}$ $cm^{-2}$). The limit of detection was 25 nM cellobiose. No response to glucose of the CDH-biosensor in the range of 5–50 μM glucose was observed. Desorption and inactivation of the functionalized CDH inevitably leads to a gradual decrease in sensitivity. To account for this, all hydrolysis experiments (described below) were analyzed with respect to the average of two calibrations immediately before and after the hydrolysis (see supporting information for more details). This procedure was found to significantly improve reproducibility compared to less frequent calibration schemes. The result in the insert of Figure 2A documents a rapid response against changes in the cellobiose concentration. This may be quantified by $t_{95\%}$, but this parameter does not provide an unambiguous measure for the time resolution during continuous cellobiose detection. A more rigorous treatment can be made on the basis of calibration experiments where known concentration ramps of cellobiose are titrated into buffer (Baker and Gough, 1996). Figure S1 in the supporting information shows examples of this approach, and it appears that after an initial lag the CDH-biosensor signal increased linearly until the ramp ended after 60 s. In this type of calibration experiment the so-called dynamic delay (Baker and Gough, 1996) can be determined as the time shift between the supply and the response curves. For the data in Figure S1 this shift was approximately 1 s. Thus, the calibration results show that the CDH-biosensor should be well suitable for the direct and continuous measurements of cellulase activity with a resolution in time and cellobiose concentration of, respectively, 1 s and 25 nM.

Figure 3 shows the raw oxidation current from the CDH-biosensor during enzymatic hydrolysis of a semicrystalline cellulose substrate, Avicel. When a stable background current (<4 nA) had been obtained, *Tr*Cel7A was injected and the concentration of cellobiose quantified from the anodic current. *Pc*CDH is essentially inactive against the α-anomer (Higham et al., 1994) and the CDH-biosensor therefore specifically detects the β-anomer of cellobiose (and the β-form of higher cello-oligosaccharides). The retaining mechanism of *Tr*Cel7A implies that its products are solely β-anomers (Claeyssens et al., 1990), and as mutarotation is slow with a half-time of about 2 h at the temperature and pH used here (Kabayama and Patterson, 1958), the electrode signal can be used directly as a measure of the initial hydrolytic activity. Conversely, the mutarotation equilibrium of cellobiose has to be taken into account in the calibrations. This was done by using equilibrated standard solutions and correcting the nominal concentration of cellobiose by the equilibrium composition (64.4% β-D-cellobiose at pH 5.5 and 24.8°C) (Kabayama and Patterson, 1958). Enzyme activity measurements with the CDH-biosensor were benchmarked against high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described below. Results in the insert of Figure 3 show that the two approaches were in accord (a plot of the cellobiose concentrations, CDH-biosensor vs. HPAEC-PAD, had a slope of 0.981). This corroborates the general performance of the biosensor enzyme assay, including the treatment of mutarotation equilibrium. Thus, while the CDH-biosensor exclusively detects the β-anomer, HPAEC-PAD measures the total (α and β) concentration of cellobiose, and the two approaches will hence only be in accord if contributions from mutarotation equilibrium have been taken correctly into account. We conclude that the anodic current from the CDH-biosensor provides an adequate picture of the activity of Cel7A up to at least 25 min under the experimental conditions used here. Still longer trials are probably feasible, but may require correction for cellobiose mutarotation not only in the calibrations (as it is done here) but also in the enzymatic hydrolysis experiments (see Supporting Information).

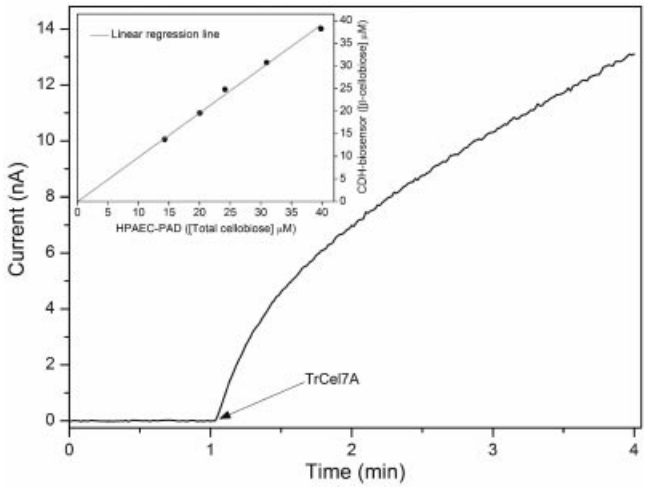


**Figure 3.** Current response from the CDH-biosensor from the release of cellobiose under enzymatic hydrolysis of a semicrystalline cellulose substrate (Avicel) (2.0 g/L) by 0.1 μM TrCel7A (25°C, in 50 mM sodium acetate buffer, pH 5). The enzyme was injected by the syringe pump at the time indicated by the arrow. The insert shows the benchmark of a polycarbonate membrane-covered CDH-biosensors response against HPAEC-PAD when correcting for the mutarotation equilibrium of cellobiose (2.0 g/L Avicel, 500 nM TrCel7A). A correlation of 0.981 was obtained.

Figure 4 shows examples of how the CDH-biosensor can be used in comparative experiments. Specifically, we measured the initial activity for different *Tr*Cel7A concentrations on two substrates. These substrates, RAC and Avicel, have approximately the same degree of polymerization, but different crystallinity index (CrI) (Murphy et al., 2010) and accessible surface areas (Zhang and Lynd, 2004). In the current context of assay development, the most important aspect of Figure 4 is that the concentration traces from the CDH-biosensor were reproducible to within ±5%, and hence that the method allows detailed analyses of the time-course under different conditions. Therefore, comparative analysis and modeling of material akin to that in

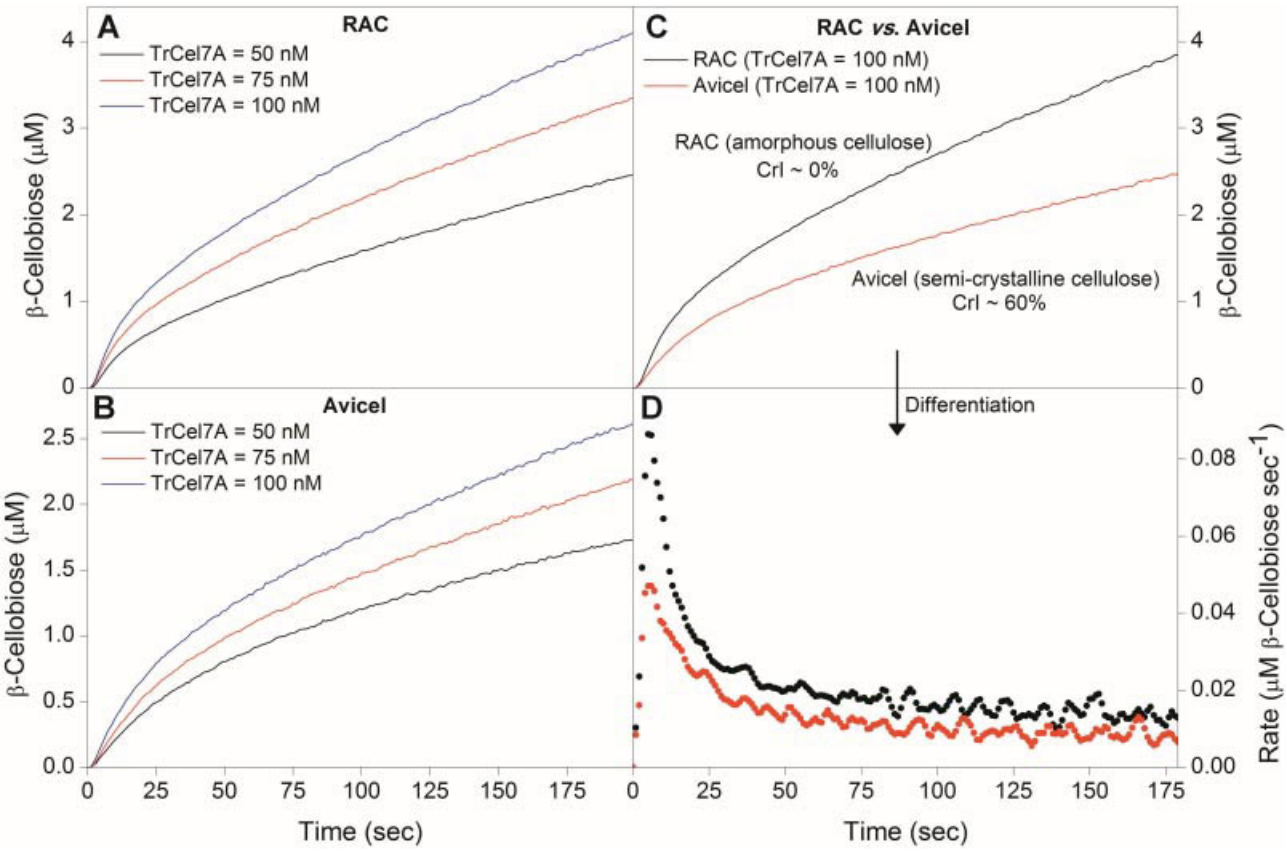


**Figure 4.** TrCel7A activity on cellulose substrates with different crystallinity (CrI). Panel A and B: shows the release of cellobiose for different concentrations of TrCel7A on RAC and Avicel (both at 2 g/L), respectively. Panel C: shows the comparison of released cellobiose by 100 nM TrCel7A at amorphous (RAC) versus semicrystalline cellulose (Avicel), both at a concentration of 2.0 g/L. Panel (D) shows the derivatives (rates) of the data in panel (C).

Figure 4 appears warranted. A molecular interpretation of the results in Figure 4 is beyond the current scope, but consideration of the hydrolytic rates in Figure 4D may serve as an example of the type of information that can be retrieved. These reaction rates are simply derived from the slopes in the concentration traces in Figure 4C, and they show a conspicuous maximum, which occurs after 5–7 s for both substrates. The hydrolytic rate at this maximum is almost twice as large for RAC compared to Avicel. Subsequent to the apex, *Tr*Cel7A shows a rapid decline in activity on both substrates and after just 1 min, the rate of hydrolysis has been fivefold reduced. We suggest that monitoring of these striking changes in the initial activity of cellulases holds a significant potential in mechanistic investigations, and anticipate that this type of comparative work for different enzymes, enzyme variants, substrates, and experimental conditions will be the main application of the CDH-biosensor described here.

In summary we have presented a novel CDH enzyme-modified carbon paste electrode with high sensitivity and temporal resolution. The CDH-biosensor can be utilized for real-time monitoring of cellobiohydrolases activity on pure cellulose substrates and also be applied to endo-glucanases (Murphy et al., 2012). The CDH-biosensor was successfully validated against a conventional method (HPAEC-PAD) and a procedure to account for the mutarotation equilibrium was devised. The CDH-biosensor appears to have a potential in fundamental research on the kinetics, mechanisms, and regulation of cellulases.

## Materials and Methods

### Materials

Unless otherwise stated, all chemicals were of HPLC grade (>99% purity) and supplied by Sigma-Aldrich (St. Louis, MO). Liquid paraffin was supplied from Wako (Osaka, Japan) and graphite powder (TGP-7, average diameter 7 μm) was from Tokai Carbon (Tokyo, Japan). All solutions were prepared with a standard buffer containing Milli-Q water, 50 mM sodium acetate, 2 mM calcium chloride adjusted to pH 5.0. D-(+)-Cellobiose standard solutions for calibration were prepared in the standard buffer at least 24 h before use to achieve mutarotative equilibrium. CDH from *Phanerochaete chrysosporium* was heterologously expressed in *Aspergillus oryzae* and purified as described in the

supporting information. The protein concentration was determined from amino acid analysis to be 6.55 mg ml$^{-1}$. The retaining cellobiohydrolase, Cel7A, from *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) (*Tr*Cel7A) were cultivated and purified as described previously (Praestgaard et al., 2011). The absence of cellobiase activity in the purified product was confirmed as the lack of detectable activity against the chromogenic substrate analogue *p*-nitrophenyl-β-D- glucopyranoside (pNPG). The protein concentration was determined from absorbance measurement at 280 nm with the theoretical molar extinction coefficient $8.3 \times 10^4$ M$^{-1}$ cm$^{-1}$ (1.6 ml mg$^{-1}$ cm$^{-1}$) derived from the amino acid sequence. Substrates used in the enzymatic hydrolysis experiments were either semicrystalline cellulose (commercial Avicel PH-101 from Fluka Biochemika, Ireland) or regenerated amorphous cellulose (RAC) prepared from Sigmacell 20 as described elsewhere (Praestgaard et al., 2011). All suspensions of the substrates were prepared in the standard buffer.

### Preparation of Enzyme -Modified Electrodes

Benzoquinone-mixed carbon paste electrodes (BQ-CPE) was prepared according to Tatsumi et al. (2006): A weighed amount of graphite powder (90 mg) was added to 8 wt% (10 mg) *p*-benzoquinone, 25 wt% (35 μl) liquid paraffin and thoroughly hand-mixed in an agate mortar until a homogenized paste was obtained. A portion of the resulting benzoquinone-carbon paste was packed into carbon paste holders (Bioanalytical Systems, Kenilworth, United Kingdom) with a working geometric area of 0.071 cm$^2$ and the surface was polished using waxed weighing-paper. The enzyme modification of the electrode surface with *Pc*CDH followed previously established principles (Lindgren et al., 1999; Stoica et al., 2006) with some minor modifications. A 10-μL aliquot of a freshly prepared solution of a 1:1 mixture of the *Pc*CDH solution and 1% glutaraldehyde in the standard buffer (glutaraldehyde solution, 25% in $H_2O$, Sigma-Aldrich) was dropped onto the polished electrode surface. This should be sufficient to saturate the electrode surface with *Pc*CDH and cross-linking with glutaraldehyde has been shown to increase the operational and long term stability of CDH-electrodes (Ludwig et al., 2010). The enzyme droplet was carefully smeared-out and allowed to evaporate at room temperature for 25 min. The electrodes were then stored at 4°C in an inverted position in a closed vessel with the bottom filled with Milli-Q water to keep a humid atmosphere. Enzyme cross-linking and immobilization, by physical-chemical adsorption, was allowed to proceed overnight and at 4°C which have shown to facilitate stronger adsorption and improved performance of CDH immobilized on graphite rods (Lindgren et al., 1999; Stoica et al., 2006). The electrode surface was thoroughly rinsed with standard buffer and the CDH-biosensor was immersed for 15 min in the stirred electrochemical cell filled with buffer to remove weakly adsorbed enzyme molecules. The CDH-biosensor was stored overnight at 4°C in an inverted position immersed in the standard buffer before use. Some *Pc*CDH-modified electrodes were covered with a Nuclepore polycarbonate Track Etch membrane (thickness of $8 \pm 3$ μm, pore size of 15 nm, a pore density of $1 \times 10^5$ pores cm$^{-2}$, supplied from Whatman® (Piscataway, NJ) and a nylon mesh with a Teflon-tube fitted over the membrane assembly to fixate them. When not in use the enzyme-modified electrodes/biosensors were stored immersed in the standard buffer at 4°C.

### Electrochemical Equipment and Measurements

A conventional electrochemical setup employed the enzyme-modified electrode as the working electrode, an Ag|AgCl|3M NaCl electrode as the reference electrode (Bioanalytical Systems) and a platinum coiled wire as auxiliary electrode. Potential control and current detection was done by an analog potentiostat (Model 1112) from Husou Seisakusyo Co. (Kawasaki, Japan). The Husou potentiostat was connected to a computer via a Agilent 34401A Digital Multimeter. Electrochemical measurements were performed in water-jacketed glass-cell connected to a water bath (Julabo F12, Seelbach, Germany). Calibration or enzyme solutions were delivered from a 100-μL syringe (SGE Analytical Science, Melbourne, Victoria, Australia) in a syringe pump (Fusion 100, Chemyx, Stafford, TX) through a FEP tube (ID 0.15 mm). The syringe pump was controlled via a LabVIEW program which also acquired the electrochemical data. Calibrations were conducted by consecutive titrations of 1–5 μL aliquots of degassed cellobiose solution into either pure buffer or cellulose suspensions.

### Enzymatic Hydrolysis Experiments

Measurements were made on 5-mL degassed samples of the cellulose suspensions. The concentration of cellobiose was followed by constant potential amperometry using the *Pc*CDH-modified electrodes. The working electrode potential was fixed at +0.5 V versus the reference electrode.

When the background was stable, usually after a couple of minutes, *Tr*Cel7A was injected over 1.0 s (irrespectively of the injection volume). Data were collected at 1 s$^{-1}$ and all hydrolysis data were corrected for the background current. The response from the *Pc*CDH-modified electrode during enzymatic hydrolysis was validated against HPAEC-PAD using previously published protocols (Murphy et al., 2010). A number of identical hydrolysis experiments (2.0 g/L Avicel, 500 nM *Tr*Cel7A, 25°C) were conducted under continuous monitoring with the membrane-covered CDH-biosensor. At different time-points (5–25 min) reaction subsets were quenched by mixing 1:1 with 0.2 M NaOH, vortexing for 60 s an immediately freezing. Upon thawing, the samples were centrifuged and the supernatant filtered through a 0.22 μm filter to remove all insoluble matter and analyzed with respect to the cellobiose content by HPAEC-PAD.

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