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Oligosaccharide biosensor for direct monitoring of enzymatic activities using QCM-D

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ABSTRACT

Enzymatic modification of saccharidic biomass is a subject of intensive research with potential applications in plant or human health, design of biomaterials and biofuel production. Bioengineering and metagenomics provide access to libraries of glycoside hydrolases but the biochemical characterization of these enzymes remains challenging, requiring fastidious colorimetric tests in discontinuous assays. Here, we describe a highly sensitive carbohydrate biosensor for the detection and characterization of glycoside hydrolases. Immobilization of oligosaccharides was achieved using copper-catalyzed azide-alkyne cycloaddition of maltoheptaose-modified probes onto self-assembled monolayers bearing azide reactive groups. This biosensor allowed detection of glycoside hydrolase activities at the picomolar level using quartz-crystal microbalance with dissipation monitoring (QCM-D). To our knowledge, this protocol provides the best performance to date for the detection of glycoside hydrolase activities. For each enzyme tested, we could determine the kinetic constant from the QCM-D data, and derive conclusions that correlated well with those of standard colorimetric tests. This opens the way to a new generation of rapid and direct tests characterizing functionally carbohydrate-active enzymes.

1. Introduction

Sugar–protein interactions are ubiquitous in nature where they are involved in biological processes such as cellular recognition, adhesion and signaling. However, despite the need for a better understanding of these interactions, carbohydrate biosensors have not developed as rapidly as those probing homologous DNA and proteins (Horlacher and Seeberger, 2008). This is primarily due to the technical difficulty of providing defined carbohydrate sequences. Sugar–protein interactions are also weakly energetic and multivalent, which makes them delicate to study *in vitro*. The first carbohydrate biochips emerged in the late nineties and have mainly targeted the recognition between sugars and lectins, *i.e.*, sugar receptors (Rillahan and Paulson, 2011). Few studies have ever dealt with the detection of enzymatic activities despite its great interest. The access to relevant oligosaccharide structures is intimately related to the isolation and characterization of new enzymatic activities, either from natural sources or through bioengineering (Leemhuis et al., 2010; Martinez et al., 2012). The CaZy (Carbohydrate-active enZymes) classification initiated in 1991 by Henrissat (Henrissat, 1991) has grouped carbohydrate-active enzymes with respect to the amino-acid structure of their active sites. As genes coding for these enzymes represent 1–5% of the genome, a wide variety of CaZymes may have potential applications in biotechnology; nevertheless, their functional characterization is still achieved through fastidious and biomolecule-consuming tests, particularly colorimetric tests (Nelson, 1944; Somogyi, 1952). That is why the design of biosensors able to efficiently and rapidly characterize those enzymes is of rising interest (Raman et al., 2005; Timmer et al., 2007).

Quartz-crystal microbalance with energy-dissipation monitoring (QCM-D) is a method of choice for the study of biomolecular binding events at the solid–liquid interface (Rodhal et al., 1995; Wilczewski et al., 2008, Dubacheva et al., 2010a, 2010b). It allows label-free and real-time monitoring with detection limits approaching nanograms per cm^2. Sensing relies on the detection of changes in the electromechanical characteristics of a shear-mode oscillating piezoelectric quartz-crystal sensor upon changes in the interfacial properties at its surface. Combining measurements of the resonant frequency f and of the energy dissipation D, information about the adsorbed mass and the viscoelastic properties of the interfacial layer can be obtained. The frequency shift Δf_n

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is related to the mass change at the surface of the crystal Δm in the case of a rigid immobilized system by the Sauerbrey equation (Sauerbrey, 1959): $\Delta m = -C(\Delta f n/n)$ where C is a constant for the crystal (ng Hz^{-1} cm^{-2}) and n is the overtone number. This allows *in situ* real-time monitoring of interactions between immobilized biomolecules and their biological partners in solution with a high sensitivity. Although numerous works have shown the potential of QCM-D for the detection of interactions between biomolecules, especially between sugars and lectins (Jelinek and Kolusheva, 2004), this tool has only been sporadically employed for the characterization of carbohydrate-active enzymes activities. Okahata's group was the first to raise the relevance and polyvalence of QCM to monitor enzymatic depolymerization of polysaccharides such as amylopectin, pullulan or dextran (Nishino et al., 2004a, 2004b, 2005). QCM-D was also used for studying transferase activities, for instance, the elongation of dextran by isomaltodextranase (Nihira et al., 2011). The constants of the enzymes could be evaluated using classical Michaelis–Menten equations. Influence of cellulose morphology on degradation by cellulases was also efficiently investigated by Rojas and coworkers using a similar approach (Ahola et al., 2008; Turon et al., 2008, Hu et al., 2009). In a recent work, we showed that QCM-D can efficiently differentiate exo- and endo- modes of action of starch hydrolyzing enzymes, *i.e.* whether they act at the non-reducing end or in the middle, respectively, of an oligo/polysaccharide chain (Bouchet-Spinelli et al., 2013). The sensing surface was simply adsorbed starch, which made this test easily used. In this work, in order to go a step further, we developed a biosensor bearing maltoheptaose probes (Scheme 1a)). Not only is this heptasaccharide particularly adapted to the characterization of amylases of interest in biotechnology, but also, it potentially could allow more sensitive detection levels by decreasing the signal-to-noise ratio. In all the previously cited works, the carbohydrate moieties were exclusively polysaccharides and no example of oligosaccharide-based biosensor has been reported, despite its relevance to possibly decreasing detection thresholds.

Several techniques have been developed to immobilize saccharides on gold surfaces, using either covalent or affinity graftings (Park et al., 2008). Any immobilization strategy of maltoheptaose substrates must preserve their accessibility to the enzymes in solution and also prevent non-specific adsorption of biomolecules onto the surface. Self-assembled monolayers, made of pegylated thiol-containing aliphatic molecules, overcome these two challenges. First, they display good stability and tenability (Love et al., 2005); second, they are highly ordered and adapted to an oriented immobilization of molecules; finally, they can be easily functionalized by reactive groups of interest. Efficient grafting of molecular probes using copper-catalyzed azide-alkyne cycloaddition (CuAAC) (Kolb et al., 2001; Rostovtsev et al., 2002) has been described (Collman et al., 2004; Devaraj et al., 2005). This reaction is well-adapted to biomolecular chemistry and provides good yields. In this work, we first produced binary SAMs made of HS–$(CH_2)_{11}$–EG_4–OH and HS–$(CH_2)_{11}$–EG_6–N_3 (EG, ethylene glycol). Oligoethylene glycol spacers were chosen in order to minimize non-specific adsorption of enzymes, (Love et al., 2005; Dubacheva et al., 2010b). We describe here first, the Copper-catalyzed Azide-Alkyne Cycloaddition (CuAAC) functionalization of binary SAMs with alkynyl maltoheptaose and the characterization of these SAMs

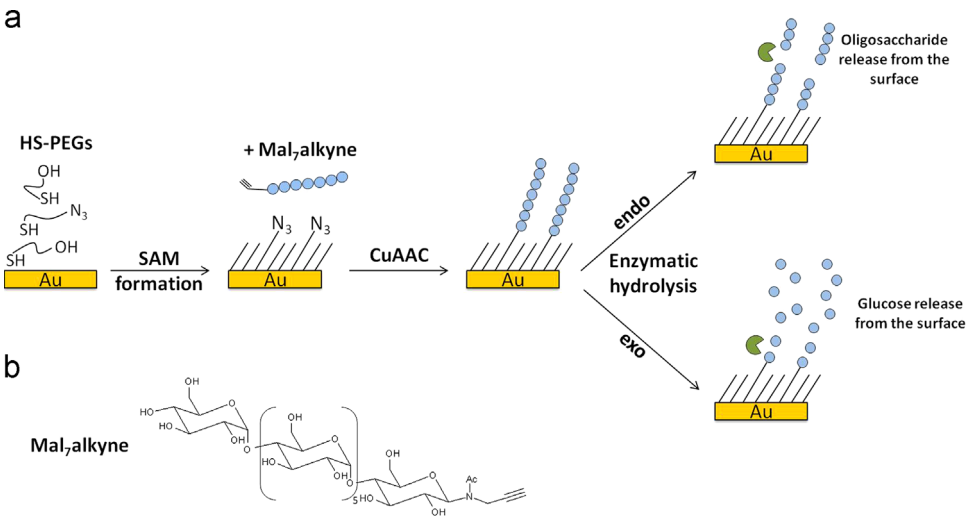
Secondly, we report the characterization of enzymatic degradation of maltoheptaose by endo- or exo- acting glycoside hydrolases using QCM-D. We focused on Cyclodextrin Glucosyl Transferases (CGTases, EC 2.4.1.19) because they are promising tools in biocatalysis (Leemhuis et al., 2010). Wild-type CGTases catalyze starch hydrolysis and also three transglycosylation reactions: (i) the production of cyclodextrins from maltoheptaose *via* an intramolecular cyclization reaction, (ii) the disproportionation reaction *i.e.,* the glycosylation of two linear fragments of maltodextrins, and (iii) the coupling reaction where the linear oligosaccharide resulting from a cyclodextrin ring opening is transferred to a second sugar acceptor. The *Bacillus circulans* 251 CGTase mutant 6.2.5 (CGTm625) exhibiting hydrolysis as its main activity was prepared by Leemhuis et al. (2003) and is envisioned as a relevant candidate for starch degradation. This enzyme behaves like an endo-amylase acting on internal glycosidic linkages and releasing oligomers which contain at least three saccharidic units. We also tested the response of the biosensor using the same procedure with an exo-acting enzyme: *Aspergillus niger* glucoamylase (E.C 3.2.1.3).

2. Materials and methods

2.1. Materials

2.1.1. Reactants and buffers

Absolute ethanol and sodium hydroxide were bought at Merck Kgaa (Darmstadt, Germany). Thiols were provided by Prochimia (Poland). For simplicity, the terms "HS–PEG–OH" and "HS–PEG–N_3" are used to refer to HS–$(CH_2)_{11}$–EG_4–OH and HS–$(CH_2)_{11}$–EG_6–N_3, respectively, in the sequel. Tertiobutanol (tBuOH),



Scheme 1. (a) General principle of the QCM-D sensor; (b) Molecular formula of Mal_7alkyne.

TBTA (Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine), sodium ascorbate, copper sulfate ($CuSO_4$, $5H_2O$), Concanavalin A (ConA) from *Canavalia ensiformis* (Jack bean), maltoheptaose, potassium citrate, citric acid, potassium ferricyanide, hydroxyethyl piperazineethanesulfonic acid (HEPES), sodium chloride, calcium chloride ($CaCl_2$), manganese chloride ($MnCl_2$) were purchased from Sigma-Aldrich Fluka (Saint-Quentin-Fallavier, France). Methyl α-D-mannopyranoside (Me-α-Man) was bought from Avocado Research Chemicals (United Kingdom). *A. niger* glucoamylase (E.C 3.2.1.3) was provided by Sigma-Aldrich (Saint-Quentin-Fallavier, France). Plasmid pDP66k$^-$ containing the mutant 6.2.5 CGT gene was a generous gift from Hans Leemhuis. All enzymes assays were performed in 10 mM sodium citrate buffer (pH 6).

2.1.2. N-acetyl, N-propargyl β-maltoheptaosylamine synthesis

N-acetyl, *N*-propargyl β-maltoheptaosylamine further denoted as Mal_7alkyne was synthesized as previously reported in the literature (Otsuka et al., 2010).

2.1.3. Production and purification of mutant 6.2.5 CGTase

B. circulans 251 CGTase mutant 6.2.5 (CGTm625) was produced and purified as described previously (Leemhuis et al., 2003).

2.2. Carbohydrate reducing-end titration using ferricyanide reactant

The alkaline potassium ferricyanide reagent was prepared by dissolving potassium ferricyanide (1 g) in 100 mL of 20% aqueous sodium hydroxide solution in distilled water. 198 μL Mal_7alkyne (0.8 mM in citrate buffer) were heated for 10 min at 40 °C. At $t=0$, 2 μL of the CGTm625 solution (1.3 μM in citrate buffer) was injected. At regular time intervals, 20 μL of reaction medium were introduced in 200 μL ferricyanide reactant and then heated to 100 °C during 7 min, as described by Hulme and Narain (1931). After cooling in ice, solution absorbance was read at 415 nm. The blank was 200 μL ferricyanide reactant with 20 μL Mal7alkyne (0.8 mM in citrate buffer) without CGTm625. Calibration was realized using the same protocol with glucose solutions with concentrations ranging from 0.1 to 0.5 mg mL^{-1}. Catalytic constant k_{cat} and Michaelis–Menten constant K_m were calculated from the initial hydrolysis rates at different substrate concentrations using a Lineweaver–Burk plot (Lineweaver and Burk, 1934).

2.3. QCM-D experiments

2.3.1. Apparatus

All QCM-D experiments were carried out using a Q-Sense E4 device (Q-Sense, Sweden). Buffers and solutions were degassed under vacuum before each QCM-D experiment. QCM-D measurements were performed using gold-coated quartz crystals (Q-Sense, Sweden) with a 5 MHz resonance frequency. The flow rate was 50 μL min^{-1}. The buffer was first injected over the quartz until frequency and dissipation signals stabilization. Before the experiment was started, the resonance frequency and dissipation found for each overtone were set equal to zero. Plots displayed in this article present the evolution with time of QCM-D signals for the 7th overtone (*i.e.* $\Delta f7/7$) for more clarity.

2.3.2. Quartz surface cleaning

The cleaning procedure included UV-ozone treatment for 5 min followed by the dipping of sensors in ethanol for 20 min under stirring. The regeneration of gold sensors was performed by keeping them overnight in a 2% SDS solution followed by ultrasonication in ethanol for 5 min, placing them in 2% Hellmanex (Hellma, France) for 5 min, UV-ozone treatment for 10 min, and finally immersion in ethanol for 20 min with stirring.

2.3.3. Functionalization of gold by oligosaccharide SAMs

HS–PEG–OH and HS–PEG–N_3 solutions (1 mM, EtOH) were stored at −20 °C. SAM-coated gold surfaces were prepared by dipping a clean gold surface (new or regenerated not more than five times) overnight in a solution containing a mixture of HS–PEG–OH and HS–PEG–N_3 in defined ratios (1 mM total concentration in absolute ethanol) under ethanol-saturated atmosphere. After overnight SAM formation, gold sensors were washed with ethanol, dried under nitrogen and submitted to a CuAAC (Copper-catalyzed Azide-Alkyne Cycloaddition) protocol. The quartz were immersed at room temperature in a water/tBuOH (1/2 v/v) solution containing 1 eq. Mal_7alkyne (8 mM, H_2O), 2 eq. TBTA, which is a commonly used ligand for stabilizing the Cu(I) catalyst, (3 mM, tBuOH), 2 eq. $CuSO_4$ (20 mM, H_2O), 13 eq. sodium ascorbate (70 mM, H_2O) in H_2O/tBuOH 1/1 v/v for 1 h at room temperature. Exposure to light was kept to a minimum in order to prevent photooxidation of the SAM. After reaction, monolayers were washed with H_2O/tBuOH 1/1 v/v and water, to ensure that all ungrafted biomolecules were removed from the surface.

Control SAMs were obtained applying the same CuAAC protocol using maltoheptaose instead of Mal_7alkyne.

2.3.4. QCM-D monitoring of ConA and Me-α-Man-saturated-ConA recognitions

ConA stock solution (100 μM in 200 mM NaCl) was stored at –20 °C. Samples at 200 pM in HEPES buffer (100 mM HEPES, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.2) were prepared. The Me-α-Man-saturated-ConA solution was prepared by mixing 200 μL ConA (10 μM) and 100 μL Me-α-Man in large excess (55 mM).

After stabilization of frequency signals the (saturated) ConA solution was injected at room temperature. After frequency signal stabilization a washing step with HEPES buffer was achieved so as to check the reversibility of ConA immobilization onto the surface.

2.3.5. QCM-D enzymatic hydrolysis detection

All buffers and solutions were heated to 40 °C prior experiment start. All QCM-D experiments were achieved at 40 °C in 10 mM sodium citrate degassed buffer pH 6 and repeated at least three times. Enzymes were injected after frequency signal stabilization in buffer. The flow rate was 50 μL min^{-1}.

2.4. Quantification of immobilized N_3 functions and oligosaccharides

Previous work in our group by Dubacheva et al. (2010b) indicated that a SAM prepared from 20% HS–PEG–N_3 and 80% HS–PEG–OH in solution, had 10% of HS–PEG–N_3 in the grafted SAM. As a SAM made of 100% HS–PEG–N_3 has a surfacic mole number of HS–PEG–N_3 equal to $\Gamma_{S-N-Au}=7.3\times10^{-10}$ mol cm^{-2}, one could expect $\Gamma_{S-N-Au}=73$ pmol cm^{-2} for the prepared SAM. The oligosaccharide grafting yield was calculated as the ratio between the grafted mass Δm deduced from the frequency change during CuAAC using the Sauerbrey equation and the theoretical Δm calculated for a 100%-yield reaction between sugars and azido groups.

2.5. Contact angle measurements

The contact angle of water was measured using a FTA188 video tensiometer (First Ten Angstroms, Inc.). Contact angle measurements were performed directly on the gold sensors with a 2 μL drop of ultrapure water. The contact angle value (θ) was calculated from the average of five measurements, with drops positioned on different places on the gold surface.

2.6. Determination of enzymatic constants from QCM-D data

Enzymatic constants were extrapolated from QCM-D data. For a given experiment (enzyme type, concentration of the enzyme), frequency variations $\Delta fn/n$ plots were fitted using a $\Delta fn/n = A(1-\exp(-k_{app,n}(t-t_0))$ mathematic law, where A is a constant (Hz), t_0 the time at which the enzyme was injected and $k_{app,n}$ the apparent constants values, determined for each overtone n. k_{app} was calculated as the average value of $k_{app,n}$ for $n=5$, 7, 9, 11, and 13. k_{app} was determined in triplicates and the average value was used to calculate the catalytic constant k_{cat} and the Michaelis–Menten constant K_m from the relation

$$k_{app} = \frac{k_{cat}}{1 + (Km/[E]0)}$$

where $[E]_0$ is the initial enzyme concentration, as reported by Anne and Demaille (2012). The values of k_{app} for an enzyme at three different concentrations gave with a good precision access to k_{cat} and K_m.

3. Results and discussion

3.1. Surface functionalization by maltoheptaose substrates: grafting by copper-catalyzed azide–alkyne cycloaddition (CuAAC)

The optimal formulation of the binary SAM should provide good accessibility for the enzyme and allow good signal-to-noise ratio for hydrolysis detection. Dubacheva et al. (2010b) have shown in previous works that no phase segregation is observed in a mixed SAM composed of HS–PEG–OH and HS–PEG–N_3 so that azido groups can be considered as uniformly distributed over the surface of the sensor. We found important to take into account the hydrodynamic radius of maltoheptaose in order to allow a folding of maltoheptaose chains analogous to the one they perform in bulk solution. A too important density of probes could prevent maltoheptaose chains to be accessible to the enzymes. Once immobilized, a maltoheptaose probe will occupy a cross-section characterized by an hydrodynamic radius equal to 0.91 nm in buffer (Saddik Motawia et al., 2005). A short calculus, detailed in Section 2, reveals that a maximum of 3.77×10^{13} maltoheptaose molecules (*i.e.* 63 pmol) should be grafted on a one square centimeter surface. Using previous results by Dubacheva et al. (2010b), we chose to use 800 μM HS–PEG–OH and 200 μM HS–PEG–N_3 because this formulation should lead to the final density of 73 pmol cm^{-2} azido groups (see Section 2.3 for calculus detail). Considering also the fact that the further CuAAC grafting will not be quantitative, this formulation was a good compromise. This mixture was incubated onto the quartz surfaces overnight in ethanol-saturated atmosphere. Then, the CuAAC protocol consisted of the reaction of Mal_7alkyne (Scheme 1 b)) with azido groups located at the SAM's surface in the presence of Cu(I) catalyst in tBuOH/water solvent (Scheme 1). The duration of the CuAAC was optimized to 1 h at room temperature. The surface was then carefully washed using tBuOH/water solvent.

3.2. Surface characterization

The efficiency of the maltoheptaose grafting using CuAAC was checked using contact angle measurements and recognition of sugar moieties by a lectin, Concanavalin A.

3.2.1. Contact angle measurements

Contact angle measurements are related to the hydrophilicity of the surface. As expected maltoheptaose-grafted surfaces showed a lower contact angle than ungrafted ones (Fig. 1a)). A control measurement was taken using a SAM-functionalized quartz prepared as described in Appendix A. and submitted to the CuAAC protocol using free maltoheptaose (*i.e.* without propargyl group). Although a slight decrease in contact angle value was observed, probably due to non-specific adsorption of saccharides, the change is not significant and confirmed the covalent grafting of Mal_7alkyne to the SAM under the CuAAC conditions.

3.2.2. Recognition of sugar moieties by Concanavalin A (ConA)

Another proof of the effective covalent immobilization of maltoheptaose onto the SAM was provided by interaction studies with ConA (Houseman and Mrksich, 2002). This lectin binds to α-glycosides and is therefore able to interact with maltodextrins such as maltoheptaose. ConA, when pre-incubated with Me-α-Man, a better ligand than Mal_7, did not adsorb to the surface (Fig. 1b)) indicating the absence of non-specific adsorption. On the contrary, when ConA was injected onto the grafted surfaces, the frequencies decreased, indicating that there was a recognition process between the surface and ConA. This confirmed that maltoheptaose was present and accessible on the quartz surface. The CuAAC functionalization strategy therefore provides fast and easy preparation of oligosaccharide-functionalized surfaces.

3.2.3. Quantification of oligosaccharide density

In order to quantify the density of grafted oligosaccharides, frequency and dissipation values were compared before, and after, CuAAC (Table S1). As the dissipation was quite stable, we could evaluate the grafted sugar mass onto the gold surface from the amplitude of the frequency decrease using the Sauerbrey equation. The amount of maltoheptaose on the surface was evaluated to 44 pmol cm^{-2}. The grafting yield of the CuAAC is therefore around 60% and consistent with previously reported values for comparable systems (Collman et al., 2006; Dubacheva et al., 2010b). It means that there are 2.65×10^{13} substrates per cm^2. Taking into account the hydrodynamic radius of maltoheptaose, around 70% of the surface was therefore covered with oligosaccharides. It should provide enough space for enzyme binding onto the surface. As one CGTm625 occupies around 1.3×10^{-12} cm^2 (Leemhuis et al., 2003), 7.8×10^{11} enzymes can adsorb on a one-square-centimeter surface, confirming that the substrate will be at least 35 times in excess over the enzyme, and satisfying Michaelis–Menten conditions.

3.3. Detection and evaluation of enzymatic activity

The maltoheptaose-modifed surfaces were prepared as described above, and used to evaluate the enzymatic activity of CGTm625, which is an endo enzyme, releasing trimers and/or tetramers from immobilized maltoheptaose. The same procedure was carried out using commercial *A. niger* glucoamylase, which is an exo enzyme; *i.e.*, that it degrades saccharidic chains by releasing glucose units from the non-reducing end one after the other. QCM-D monitoring of the degradation of maltoheptaose by CGTm625 and glucoamylase, at different concentrations, at 40 °C are shown in Fig. 2a). The first step of the procedure was the obtention of a flat baseline in the presence of buffer. Then, the enzymatic solution was added and provoked a drop in frequency in all cases. We did not observe any adsorption of the enzyme prior to the start of the hydrolysis. This process is very fast and enzyme binding phase was not observed, as already reported (Nihira et al., 2005; Bouchet-Spinelli et al., 2013). If it was the case we should observe a first decrease of frequency and increase in dissipation. This phenomenon means that the enzyme kinetic rate is faster than its binding rate. Subsequently, the signal stabilized showing that further hydrolysis was not possible. This drop was specific for

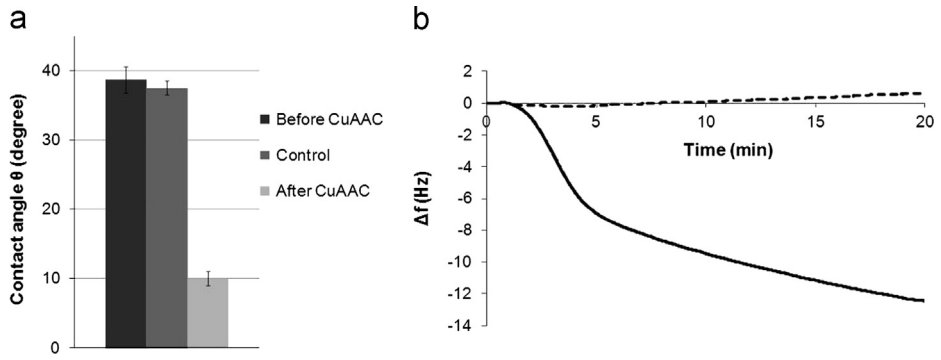


Fig. 1. (a) Contact angle values of the bare SAM (left), the SAM after CuAAC protocol using maltoheptaose (middle, control) and the SAM after CuAAC using Mal_7alkyne (right); (b) QCM-D profile obtained after the addition of Me-α-Man–saturated-ConA (dotted line) or ConA (full line).

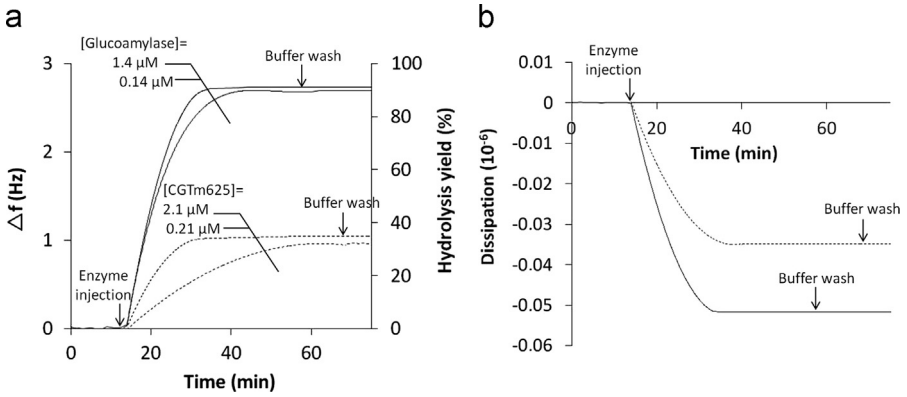


Fig. 2. Evolution of frequency and hydrolysis yield after the addition of *B. circulans* CGTase mutant 6.2.5 (210 pM, full line) and *A. Niger* glucoamylase (140 pM, dotted line).

the enzymatic hydrolysis in so far as the addition of enzymes over a control SAM (prepared as described in Section 2) revealed no change in frequencies (Fig. S1). The amplitude of the frequency shift at the plateau is the same for a given enzyme, whatever the concentration. It means that the same amount of oligosaccharides was removed at the end of the process. The concentration has only an influence on the hydrolysis speed of the enzyme, as usually observed in bulk solution. However, the amplitudes of the frequency shifts are not the same from an enzyme to the other, owing to their different modes of action on the substrate (*i.e.* endo or exo). The dissipation variations are shown in Fig. 1b) for the highest concentrations of CGTm625 and glucoamylase. The decrease after the addition of the enzymes was consistent with the degradation of the saccharides, which leads to a "thinner" layer. However, these dissipation variations were very low and indicated that the functionalized SAMs could be considered as rigid layers. For each enzyme, we obtained $\Delta D/\Delta f$ values which were far smaller than $0.2\,Hz^{-1}$, which is the maximum value usually admitted to rigorously apply the Sauerbrey equation (Höök et al., 2002). The amount of hydrolyzed material could be determined that way. It was respectively evaluated at 34%55 ± 1% (15 ± 0.5 pmol cm^{-2}) of the quantity of grafted oligosaccharides maltoheptaose for the CGTm625, and 89% ± 2% (37 ± 0.5 pmol cm^{-2}) of the quantity of grafted oligosaccharides for glucoamylase. CGTm625, which is an endo-acting enzyme, hydrolyzes maltoheptaose into maltotriose and maltotetraose. Considering that the glycosylamide linkage between the maltoheptaose portion and the SAM is resistant to the enzyme treatment, only one enzymatic attack is possible, which after completion thus generated a SAM with about one half the sugar amounts. Our value is smaller. CGTm625 has probably a slightly lower affinity for the immobilized substrate than for that as it exists in bulk solution. As an exo-acting enzyme, the glucoamylase hydrolyzes maltodextrins from the non-reducing releasing glucose stepwise. The fact that 89% of the initial quantity of oligosaccharides is removed means that six glucose units per heptasaccharide are released, the seventh remaining linked to the SAM and not hydrolyzed by the enzyme. This result is typical for its exo mode of action. Glucoamylase therefore seems to behave like in bulk solution. The access to the substrate is good and the hydrolysis is fully achieved.

In order to quantify enzymatic activities, the QCM-D data were fitted using a kinetic model. The determination of catalytic constants must take into account experimental parameters in order to be rigorous. In our operating conditions, in the 40 μL QCM-D cell, the enzyme surface concentration is always smaller than 0.6 pmol cm^{-2} (maximum value for 210 pM CGTm625) whereas that of the substrate is 44 pmol cm^{-2}. The substrate is therefore in large excess over the enzyme, as it is in bulk solution. This is related to the fact that our detection threshold is low and enzyme can be consumed in catalytic quantities. Enzymatic constants were extrapolated from QCM-D data using the universal procedure demonstrated by Anne and Demaille (2012). We calculated the ratio $(t_{1/2})/(t_{1/4})$ between the times required to hydrolyze one-half and one quarter of the total quantity of hydrolyzed substrate. We found 2.33 for CGTm625 and 2.24 for glucoamylase. These two values correspond to a langmuirian behavior and Michaelis–Menten conditions are met. It allows us to use the following formalism:

$$E + S \underset{K_1}{\overset{K_{-1}}{\leftrightarrows}} ES \overset{K_{cat}}{\rightarrow} E + P$$

The first reversible reaction between the enzyme *E* and the immobilized substrate *S* leads to a precursor complex, *ES*, which

dissociates irreversibly to release the enzyme and the saccharidic product P in solution.

As the concentration [P] is directly related to the change in frequency f, the QCM-D data can be fitted using the following formula:

$$\Delta f = A(1-\exp(-k_{app}(t-t_0))$$

In this expression, the apparent constant k_{app} is defined as $k_{app} = \frac{k_{cat}}{1+(K_m/[E]_0)}$ where k_{cat} is the catalytic constant, $K_m = k_{-1}+k_{cat}/k_1$ the Michaelis–Menten constant, and $[E]_0$ the enzyme concentration at t_0, when the enzyme starts hydrolysis. A (Hz) is a constant for a given enzyme.

By performing the experiment at different enzyme concentrations, we determined k_{cat} and K_m for the two enzymes. Results are compiled in Table 1. The catalytic constant values are of the same order of magnitude as those determined using standard colorimetric tests in solution (Table 1, left columns). They are nevertheless systematically a little smaller, probably because of the influence of the diffusional transport of the enzyme toward the quartz surface. Unlike that in bulk solution, the substrate concentration is not uniform in the whole solution volume. The difference is however quite slight and the fact that we used a flow cell tends to minimize the importance of this phenomenon. K_m values, translating the affinity of the enzymes toward maltoheptaose, are also consistent with the ones obtained in bulk solution (Table 1, right columns), showing that the substrates are accessible to the active sites of the two enzymes and confirming that the grafting strategy was relevant. The higher K_m value obtained for CGTm625 using QCM-D confirmed that this enzyme seems to have a smaller affinity for the immobilized maltoheptaose than for free maltoheptaose in solution, explaining why the yield of hydrolysis did not reach the expected 50% on a surface.

The reproducibility on quartz surface and bulk solution were similar and constants could be obtained in both cases with less than 5% uncertainty.

Standard activity tests are based on reducing ends sugar titration, as new reducing ends are generated during hydrolysis. The main drawback of these tests is that they cannot discriminate between endo and exo enzymes. QCM-D investigations allowed us to go this step further. The simple measurement of the maximum amplitude of the frequency drop gives information about the hydrolytic mechanism. Relevant kinetic parameters can also be easily calculated for each enzyme tested. Contrary to several previously published works, we have here the advantage of working in Michaelis–Menten conditions (enzyme concentration is far smaller than the substrate concentration) and this explains the good agreement between the constants determined from QCM-D data and in bulk solution. Several authors (Halling et al., 2005; Anne and Demaille, 2012) have raised the nonsense of evaluating the kinetic constants of the enzymes acting on immobilized substrates using classical Michaelis–Menten equations but under conditions of enzyme excess which differ from most conventional solution reactions. Our work shows therefore that it is possible to be rigorous in this aspect.

The concentration limit for the activity detection was very low, the hydrolysis being detected at 21 pM. It is at least 300 times lower than previously-published results using QCM-D (Nishino et al., 2004a, 2004b, 2005; Ahola et al., 2008). Such a high sensitivity is, in our opinion, provided by the combined use of well-organized self-assembled monolayers and of defined short oligosaccharide sequences, whereas, until now, only polysaccharides and complex biomolecular assemblies had been used for this kind of assay. The enzyme and the substrate consumptions are also significantly decreased using the QCM-D test rather than standard colorimetric tests. This is to be taken into account when expensively-produced enzymes and substrates have to be functionally characterized.

Table 1
Catalytic constant k_{cat} and Michaelis–Menten constant K_m at 40 °C of *B. circulans* 251 CGTase mutant 6.2.5. and *A. niger* glucoamylase for the hydrolysis of maltoheptaose in bulk solution[a] or immobilized on a quartz-crystal[b].

	k_{cat} (s^{-1})[a]	k_{cat} (s^{-1})[b]	K_m (mM)[a]	K_m (mM)[b]
CGTm625	192 ± 11	165 ± 10	1.105 ± 0.07	1.405 ± 0.10
Glucoamylase	3015 ± 18	2405 ± 12	0.385 ± 0.02	0.405 ± 0.05

4. Conclusion

A new glycoside hydrolase QCM-based biosensor was developed by immobilization of maltoheptaose by CuAAC onto azido-functionalized SAMs. The glycosylated surface was then shown to be a highly sensitive tool for the detection of enzymatic activities. The threshold was 21 pM, which is, to our knowledge, the best performance ever obtained for such systems. Kinetic constants could be extrapolated from the QCM-D data and fitted to those classically obtained through standard tests. In addition, the present biosensor is able to distinguish different hydrolysis mechanisms. This is an improvement over conventional colorimetric assays and promises better mechanistic understanding with lower enzyme consumption.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.05.027.

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