

α -Amylase sensor based on the degradation of oligosaccharide hydrogel films monitored with a quartz crystal sensor

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Abstract: α -Amylase hydrolyses starch molecules to produce smaller oligosaccharides and sugars. Amylases are of great importance in biotechnology and find application in fermentation, detergents, food and the paper industry. The measurement of α -amylase activity in serum and urine has been used in the diagnosis of acute pancreatitis. Salivary amylase has also been shown to be a stress indicator. Sensor coatings suitable for the detection of α -amylase activity have been developed. Oligosaccharides such as glycogen and amylopectin were spin-coated onto gold coated quartz crystals with a base frequency of 10 MHz. The films were subsequently cross-linked with hexamethylene diisocyanate. Film degradation was monitored with a quartz crystal microbalance (QCM) and electrochemical impedance measurements. The films were shown to be stable in phosphate buffered saline (PBS). Addition of α -amylase to the solution resulted in the rapid degradation of the films. The maximum rate of degradation was found to be strongly dependent on the amylase activity in the range typically found in serum when diagnosing pancreatitis (0.08 to 8 U/ml). Sensor responses in serum were found to be very similar to those obtained in buffer indicating the absence of non-specific binding.

Keywords: α -amylase activity, sensor, QCM, film degradation, impedance

1 Introduction.

α -Amylases (EC.3.2.1.1) are important enzymes in the hydrolysis of starch into sugars. The pancreas produces many of the digestive enzymes including α -amylase and lipase. In healthy humans there is only a relatively small amount of amylase in blood, however, if a person develops pancreatitis and or renal malfunction the levels of amylase in the blood are increased many times greater than normal. The amount of amylase in blood is one of the many markers looked at regularly by clinicians to diagnose pancreatitis or rule out pancreatitis in patients who arrive complaining of abdominal pain or other presenting symptoms (Swaroop et al. 2004). It is therefore of great importance to clinicians for there to be quick, reliable and cheap ways to determine amylase levels in the blood. Currently the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) recommends a colorimetric technique that uses a manufactured substrate, which releases a dye upon hydrolysis by α -amylase and the enzyme α -glucosidase, to determine the α -amylase activity in the sample. The IFCC also provide reference values of 0.0310-0.107 U/ml for healthy

serum α -amylase values determined by their recommended procedure (Schumann et al. 2006).

There have been many methods devised to measure the activity of α -amylase with recent developments made towards creating rapid-response disposable sensors for point of care testing. The methods for measuring α -amylase activity can be divided into three main types: (i) measuring the consumption of natural substrates of α -amylase directly (Sasaki et al. 2008; Wu et al. 2007) or indirectly through the potentiometric measurement of the release of triiodide from a triiodide starch complex (Sakac et al. 2011); (ii) colorimetric or fluorimetric methods (Attia et al. 2014; Schumann et al. 2006; Shetty et al. 2011; Yamaguchi et al. 2004); (iii) the measurement of natural products of α -amylase catalysed hydrolysis such as glucose and maltose (Altug et al. 2011; Mengulluoglu et al. 2012; Renneberg et al. 1984; Zajoncova et al. 2004). Frequently, these sensors use glucose oxidase to convert glucose into electroactive species, which can then be detected amperometrically.

Every method has different strengths and weaknesses as a sensor for α -amylase, which makes the choice of sensor method utilized important for the different media α -amylase operates in. While most of the sensors mentioned above are suitable for monitoring α -amylase in saliva as a stress indicator, many are inappropriate as a rapid response sensor for α -amylase activity in blood. Any sensor that measures α -amylase activity by measuring glucose created by α -amylase hydrolysis will suffer from interference with endogenous glucose; these sensors are therefore unsuitable for a rapid response sensor for the detection of α -amylase in blood or serum. It might be possible to use the colorimetric sensor described by Shetty et al. (Shetty et al. 2011) to measure serum α -amylase and not just salivary α -amylase, however, this system was reported to have a high lower detection limit and will not cover the required sensitivity range for serum α -amylase.

Monitoring the degradation of thin films of artificial and natural enzyme substrates has been shown to be an effective method for measuring enzyme activity (Saum et al. 1998; Stair et al. 2009; Sumner et al. 2001; Sumner et al. 2000; Zheng et al. 2011). Film degradation has been measured using impedance, optical sensors and mass sensitive devices. The degradation of starch and amylopectin films by α -amylase has been monitored using mass sensitive devices (Sasaki et al. 2008; Wu et al. 2007). Wu et al. (Wu et al. 2007) drop coated starch onto magnetoelastic sensors and measured the sensor response to α -amylase. Activity dependent frequency shifts (reaction time 30 min) and initial degradation rates were found, although a lack of sensitivity was observed at α -amylase activities below 75 U/ml. Sasaki et al. (Sasaki et al. 2008) deposited multilayers of amylopectin and chitosan onto a poly-L-lysine modified quartz crystal microbalance and studied the inhibition of the α -amylase induced film degradation by chitosan. Again, an activity dependent hydrolysis rate was found.

As shown above, the enzymatic degradation of thin films is a promising method for the development of disposable sensors for the measurement of α -amylase activity if the devices

can be made to work in the clinically relevant range of α -amylase activity and in a clinical sample. In this work, films of three different α -amylase substrates, glycogen, amylose and amylopectin, were deposited onto a quartz crystal microbalance (QCM) and cross-linked with hexamethylene diisocyanate. The feasibility of cross-linking oligosaccharides with diisocyanates was first described by Brønsted et al. (Brønsted et al. 1996). This system was chosen as dextran films cross-linked with hexamethylene diisocyanate were previously shown to be highly sensitive to dextranase without showing any effects of non-specific binding (Sumner et al., 2000). Film degradation by α -amylase was monitored by simultaneous QCM and electrochemical impedance spectroscopy (EIS) using an experimental setup first described by Sabot & Krause (Sabot and Krause 2002). An optimised film was then used to create a calibration curve.

2 Materials and Methods.

2.1 Materials

Most chemicals were supplied by Sigma-Aldrich, UK: phosphate buffer saline (PBS) pH 7.4, potassium ferricyanide, potassium ferrocyanide, fetal bovine serum, amylopectin from maize, glycogen from oyster, concentrated sulphuric acid, 30% wt. hydrogen peroxide in H₂O, 4-mercapto-1-butanol, calcium chloride, toluene (anhydrous), hexamethylene diisocyanate (HDI), acetone and isopropanol. Silver conductive paint was purchased from RS Components, UK (186-3593), absolute ethanol from VWR and Milli-Q water (UPW) with a resistivity of ≥ 18.0 M Ω cm were also used. Gold coated AT-cut quartz crystals with a resonance frequency of 10 MHz were purchased from International Crystal Manufacturing Company, Inc (USA).

α -Amylase from bacillus licheniformis (570 U/mg of protein, 78% protein as defined by the manufacturer) was purchased from Sigma-Aldrich, UK (A4551) and was dissolved in PBS pH 7.4 prior to use. A fresh solution was made on the day of measurement. Enzyme activity of a 6.4 mg/ml stock solution of α -amylase was verified using an assay described by Bernfeld (Bernfeld 1955). 1 U of α -amylase in a dilute solution of 0.78 U/ml α -amylase liberated 0.44 mg of maltose from starch at pH 6.9 in 3 min.

2.2 Cleaning of QCM crystals

A piranha solution was made by adding 20 ml hydrogen peroxide to 60 ml concentrated sulphuric acid. The QCM crystals were placed in a teflon stand then immersed in the piranha solution for 30 seconds. After cleaning, the crystals were rinsed thoroughly with UPW. The piranha solution removes any organic compounds remaining on the crystals leaving a clean surface on the gold electrode for further modification.

2.3 Thiol modification of the gold electrode surface

Immediately after cleaning, quartz crystals were immersed in a bath of: 80 ml ethanol, 20 ml of UPW and 108 μ l (0.1062 g) of 4-mercapto-1-butanol. The crystals were left in the bath for 1 hour then rinsed with UPW and blow dried with nitrogen gas. This solution formed a self-assembled thiol monolayer on the gold electrode to provide a hydrophilic surface for the deposition of oligosaccharides.

2.4 Film deposition

Three different oligosaccharides were investigated and at three different concentrations. Glycogen, amylopectin or amylose was dissolved in UPW at: 13 mg/ml, 6.5 mg/ml and 6.25 mg/ml. The QCM crystals were loaded into a spin-coater and 20 μ l of the relevant oligosaccharide solution was pipetted onto the surface of the QCM crystals. The spinning speed was ramped from 0 to 500 rpm at 100 rpm/s, maintaining this speed for 5 seconds, before increasing the speed to 3000 rpm at 500 rpm/s and maintaining this speed for 40 seconds. After spin coating the oligosaccharide, the crystals were placed in an incubator at 30°C for 24 hours with calcium chloride to act as a desiccant. After incubation the crystals were immersed in a solution of 0.107 ml of HDI in 50 ml of toluene. The crystals were left at room temperature in the HDI solution for 4 hours to form cross-links except for an additional three crystals, spin coated with 6.5 mg/ml amylopectin, that were left for 20 hours to investigate if 4 hours was enough time for cross-links to form between oligosaccharide molecules forming the polymer network of the film.

2.5 Measurement of QCM and impedance responses of the oligosaccharide coated sensors

QCM measurements were conducted using the experimental setup described by Sabot and Krause (Sabot and Krause 2002). One full quartz crystal admittance spectrum (201 points, ac stimulus 160 mV, acquisition time 1 s, centred at the QCM resonance frequency (\sim 10 MHz)) and one full impedance spectrum (20 points, 10 Hz to 100 kHz, acquisition time \sim 5 s) were recorded every 10 s. To test the performance of the different film networks a crystal with the relevant film was loaded into a teflon liquid static cell from the International Crystal Manufacturing Company, USA, and placed into an incubator at 30°C. Into the cell, 1.98 ml of a buffer solution was added; the buffer was produced by dissolving 5 mM of potassium ferricyanide and 5 mM of potassium ferrocyanide in PBS pH 7.4. The system was set to take 200 sensor readings over a 33 minute period, and at 100 readings, the system was paused to add 0.02 ml of α -amylase; the α -amylase was prepared with 6.4 mg/ml α -amylase in PBS pH 7.4. For measurements in serum, the buffer was replaced with serum while maintaining the same experimental conditions and concentrations of potassium ferricyanide, potassium ferrocyanide and amylase.

For the calibration of the sensor, QCM crystals were prepared by spin coating a solution of 13 mg/ml amylopectin and immersed in the cross-linking solution for 20 hours. The α -amylase solution was diluted with the charge transfer buffer to achieve different activities in the cell: 8, 2.4, 1, 0.24, 0.08, 0.008, 0.0008 or 0 U/ml.

2.6 FTIR measurements

FTIR measurements of the amylopectin film before and after cross-linking were carried out using a Tensor 27 (Bruker) with a grazing angle accessory.

3 Results and Discussion.

3.1 Investigation of different oligosaccharide films

The films comprised of amylose were not uniformly distributed as there were solid particles of amylose visible that had not completely dissolved, and were therefore excluded from further investigation. Films made with glycogen and amylopectin were more uniform and produced repeatable results. High-quality films were obtained as the solution of the cross-linker, hexamethylene diisocyanate, in toluene did not dissolve the underlying oligosaccharide film therefore maintaining the uniform coating obtained by spin-coating.

The quartz crystal admittance spectra recorded before and after degradation showed very different behaviour for glycogen (Figure 1b) and amylopectin (Figure 1c). For glycogen films, the spectra shifted to higher resonance frequencies, indicated a mass loss from the surface, while for amylopectin film, there was also a significant increase in the maximum admittance during degradation. The QCM admittance spectra were fitted with a Butterworth Van Dyke - (BVD) equivalent circuit (Sabot and Krause 2002). For both films, the addition of α -amylase resulted in a decrease of $\omega\Delta L$, which can be attributed to mass loss due to the degradation of the films. This is caused by the hydrolysis of the amylopectin or glycogen into smaller oligosaccharides and glucose catalysed by α -amylase resulting in a dissolution of the films. As there are no other enzymes in human blood or saliva that can degrade amylopectin or glycogen, the films are expected to be highly selective to α -amylase. In the case of glycogen, ΔR remained constant during the degradation, while there was a decrease in ΔR for amylopectin indicating that glycogen formed a rigid film while the amylopectin film was more viscoelastic than rigid. The α -amylase was added at around 0 minutes, and the data shows that α -amylase completely breaks down the film in seconds (Figure 1a).

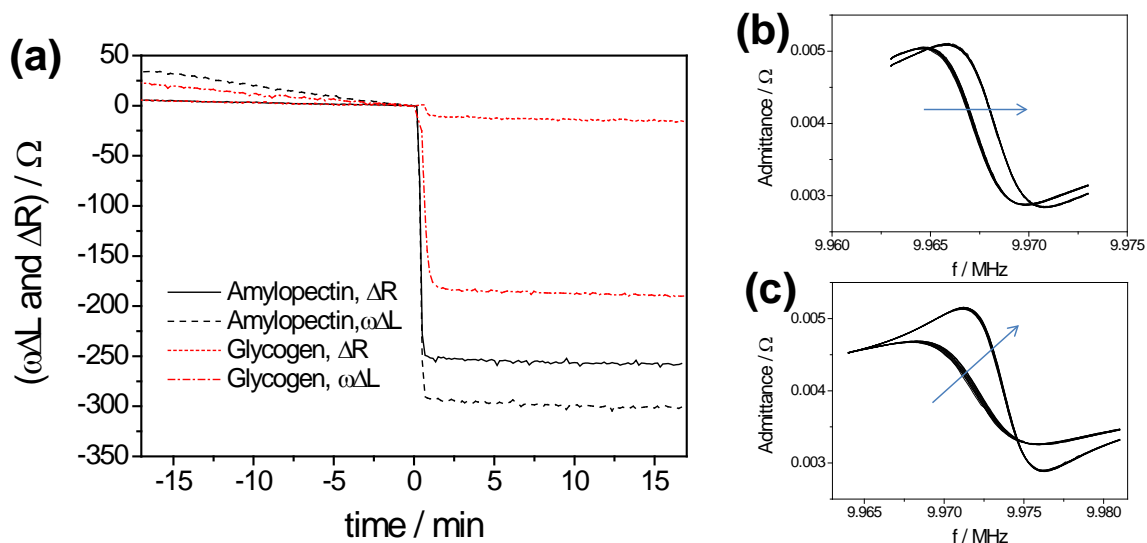


Figure 1 Comparison of α -amylase response of glycogen and amylopectin films. (a) Plot of BVD fitted impedance for glycogen and amylopectin films showing relative changes in $\omega\Delta L$ associated with the mass of the film and ΔR attributed to changes in energy dissipation caused by viscoelastic and damping effects of the film and liquid overlayer. 6.4 mg/ml α -amylase was added at 0 min. Quartz crystal admittance spectra every 100 s before and after addition of α -amylase for glycogen films (b) and amylopectin films (c). The arrows indicate the direction of change.

3.2 The effects of cross-linking amylopectin films

The sensor responses of non-cross-linked amylopectin films were investigated. Considering the good solubility of amylopectin in water, the films were surprisingly stable in PBS. It is assumed that amylopectin adsorbs strongly to the 4-mercapto-1-butanol monolayer. However the degradation rates in the presence of α -amylase measured with non-cross-linked films were not reproducible. Cross-linking of amylopectin films was carried out for 4 h and 20 h. When comparing the results between amylopectin films cross-linked for 4 hours or 20 hours, the only difference seen was a better repeatability for impedance measurements after 20 h. A further increase in the cross-linking time was therefore deemed unnecessary.

To estimate the cross-link density after 20 h reaction time with hexamethylene diisocyanate the BVD fit parameter $\omega\Delta L$ was determined in air before and after the deposition of amylopectin and after cross-linking. $\omega\Delta L$ is known to be proportional to mass changes in viscoelastic films (Sabot and Krause, 2002). The amylopectin layer increased $\omega\Delta L$ by $1070 \pm 157 \Omega$ and the cross-linking increased $\omega\Delta L$ by $22.3 \pm 4.2 \Omega$. From these values the mole ratio between glucose units and the cross-linker was calculated to be 48:1. The thickness of the cross-linked films was determined to be about 154 nm using a Dektak 3 ST surface profiler. Amylopectin films were also characterised by FTIR. The spectra of non-cross-linked films matched the results reported by Dragunski and Pawlicka (Dragunski and Pawlicka

2001) for amylopectin very well. Bands were observed at 3359 cm^{-1} (O-H stretching of amylopectin), 2937 and 2894 cm^{-1} (asymmetric stretching of C-H), 1655 cm^{-1} (adsorbed water), 1417 and 1369 cm^{-1} (angular deformation of C-H), 1159 cm^{-1} (C-O ether bond stretching) and 1052 cm^{-1} (C-O alcohol bond stretching). The spectrum of the cross-linked film looked identical. The FTIR was not sufficiently sensitive to detect the small concentration of cross-linker.

3.3 Calibration curves for α -amylase activity with BVD fitted data

It was decided to carry out activity dependent measurements with amylopectin films, as it was possible to create a calibration curves for both BVD parameters, ΔR (Figure 2b) and $\omega\Delta L$ (Figure 2d). The plot of ΔR with time (Figure 2c) reveals that upon α -amylase addition there was a rapid response to the enzyme's presence at all activities, showing a slower initial degradation followed by a greater degradation rate. In contrast, there is a significant time lag before the mass loss rate increases ($\omega\Delta L$ in Figure 2a) for low α -amylase activities. It is assumed that the initial degradation might cause a reduction in the surface roughness of the film without incurring a significant mass loss. At the later stages of degradation the enzyme may be able to penetrate the film causing a faster degradation and a significant mass loss.

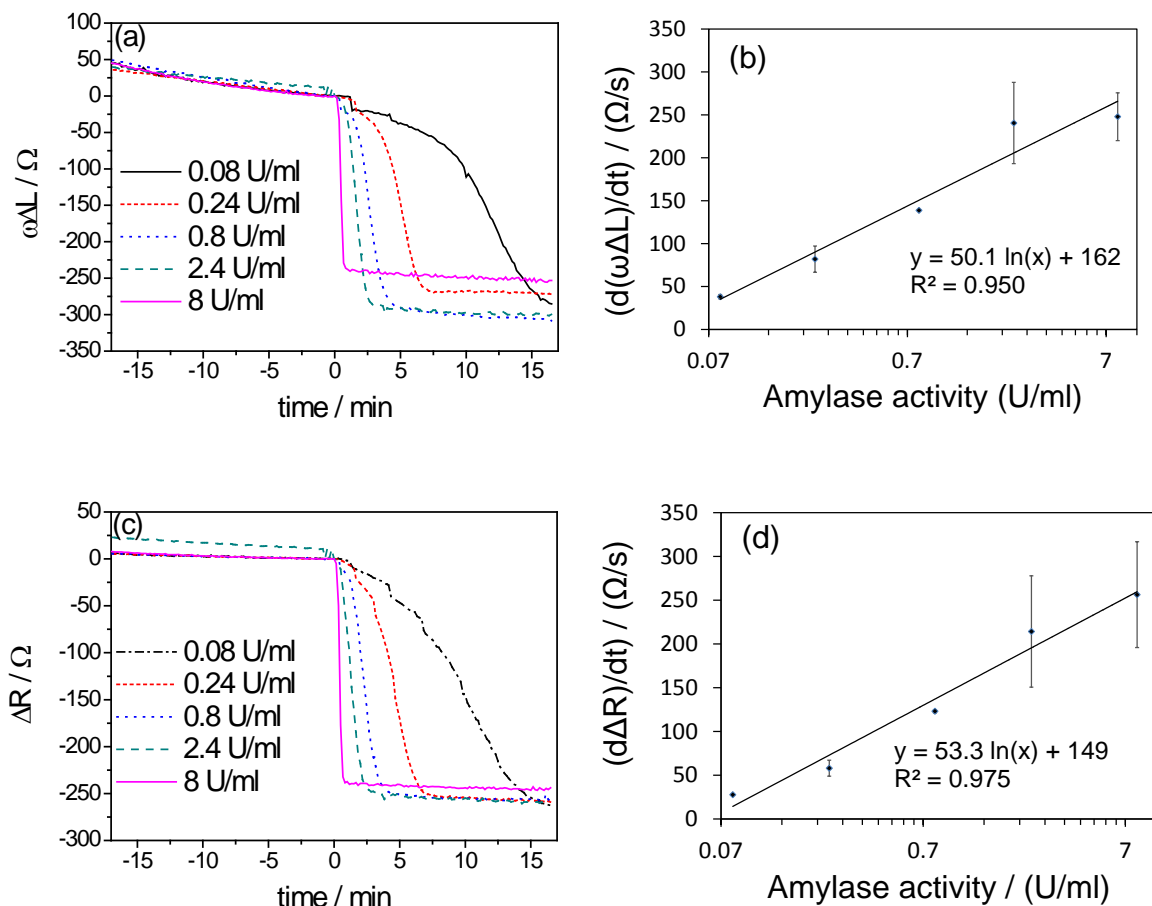


Figure 2 Sensor calibration data. (a) and (c) show the change of BVD fitted QCM data $\omega\Delta L$ and ΔR with time before and after the addition of different activities of α -amylase. (b) and (d) are the fitted

calibration curves for the maximum rate of inductance impedance change and maximum rate of resistance change respectively, the error bars show the standard deviation for 3 sensors

The calibration curves (Figures 2b & 2d) were obtained by determining the maximum rate from the first derivative of the sensor response curves shown in Figures 2a and 2c. They reveal a non-linear natural logarithmic dependence on activity and film degradation. Logarithmic behaviour is not unexpected, since there is a limited surface area where the substrate and α -amylase can bind, so at low activities a small increase in α -amylase concentration will rapidly increase hydrolysis rate, and at higher α -amylase concentrations the α -amylase molecules are in competition for binding sites. The turning point for this sensor is at approximately 1.36 U/ml; above the turning point the error margin for determining α -amylase activity increases and below 1.36 U/ml the sensors accuracy for α -amylase activity is at its peak. The large error at high activities of α -amylase was also caused by the data acquisition as the minimum time interval between measurement points was 10 s for the simultaneous acquisition of quartz crystal and impedance data, and the peak rate occurred after a few seconds for high activities. A faster measurement system would improve the accuracy of the determination of the peak rate considerably. A somewhat better accuracy was observed when plotting the time required for reaching the maximum rate versus the α -amylase activity (Figure 3a). The data were fitted with an arbitrary power function. The percentage error in determining the concentration for individual measurement points when using the fitted calibration curve is shown in Figure 3b. 77% of the points were within 25% of the concentrations predicted by the calibration curve. Again the large error observed at high concentrations could be reduced by the acquisition of a larger number of data points.

For 0.008 and 0.0008 U/ml, in a scan time of 50 min, no clear peak degradation rates could be determined from the first derivative of the response curves. Using the peak degradation rates and within a response time of 20 min, the lower detection limit is therefore 0.08 U/ml. However, smaller activities could be measured by replacing the maximum rate with the time taken for a percentage drop in ΔR or $\omega\Delta L$. Significant changes in the QCM signal were observed to a lower detection limit of 0.008 U/ml of α -amylase.

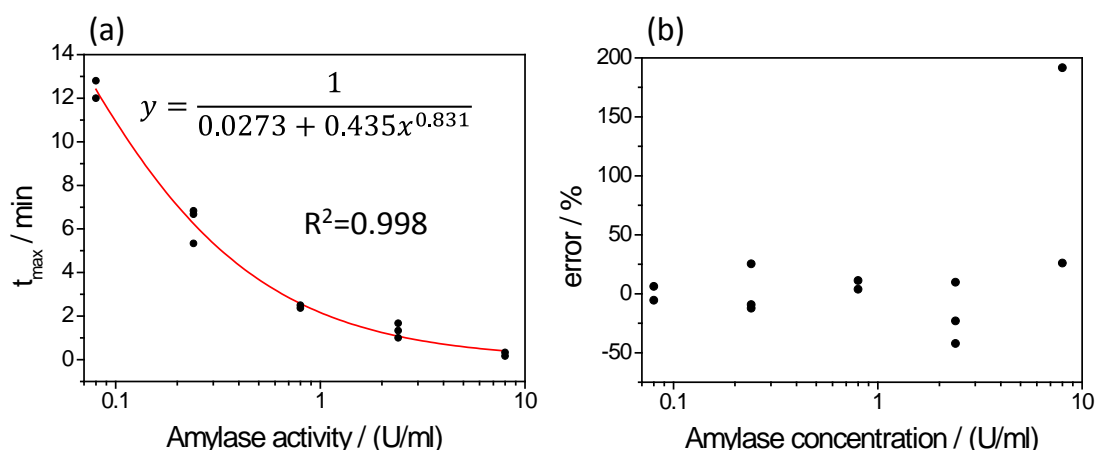


Figure 3 (a) Sensor calibration using the time at which the maximum change in $\omega\Delta L$ was measured. (b) Percentage error in the concentration for individual measurements as determined from the calibration curve.

3.4 The effect of protein on the sensor response

To investigate the effect of non-specific binding, the sensor response was measured at two α -amylase activities in serum. The presence of serum had a negligible effect on the signal response (Figure 4). The difference in the signal was no greater than that between repeated experiments. This indicates that the sensor materials are suitable for measurement in biological fluids. Oligosaccharides such as dextran are frequently coated onto surfaces to prevent fouling. We have previously shown that there was no protein adsorption on dextran films cross-linked with hexamethylene diisocyanate (Sumner et al., 2000). We assume that amylopectin has a similar effect, and that there is no significant protein adsorption on the hydrophilic film surface. In contrast to serum proteins, α -amylase can bind to the amylopectin film through specific binding sites. The enzyme consists of three structural domains A, B and C. Domains A and B are responsible for binding the substrate and its subsequent degradation (Brayer et al., 2000).

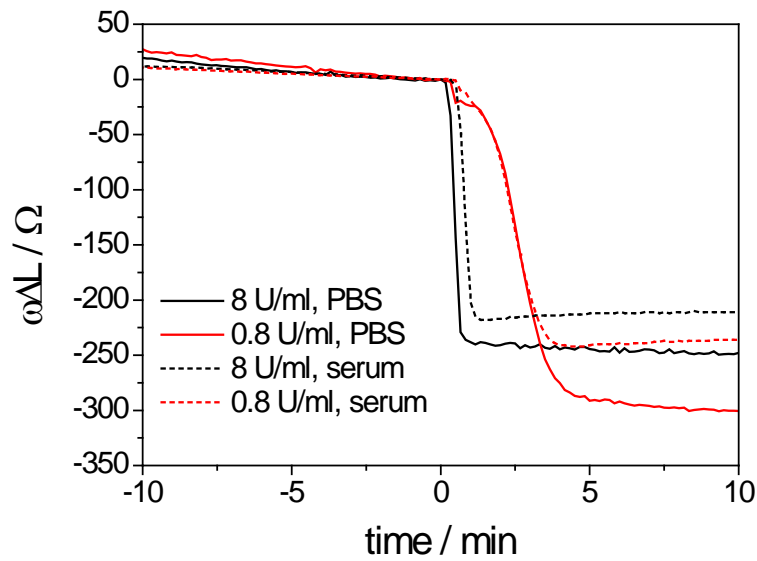


Figure 4 Effect of serum on sensor response. $\omega\Delta L$ changes for 0.8 and 8 U/ml in PBS and serum.

3.5 EIS results

Figure 5a shows impedance spectra before and after the addition of 8 U/ml α -amylase. As the measurements were carried out in a charge transfer buffer, the greatest change in impedance was found at low frequencies where the impedance was dominated by the charge transfer resistance, while lower impedance changes were observed in the capacitive region of the spectra at intermediate frequencies, and there was no change in impedance at high frequencies where the impedance spectrum was dominated by the electrolyte resistance. Hence impedance changes were plotted at 10 Hz before and after the addition of different activities of α -amylase. The film impedance is decreasing in the charge transfer buffer. It is assumed that this is caused by the diffusion of electrolyte into the film. The small but rapid decrease in Impedance around 17 minutes in Figure 5b corresponds to the film degradation noted in the QCM data (Figure 2). The rate of the impedance change increased with α -amylase activity. It was difficult to see any change in impedance attributable to film degradation at α -amylase activities lower than 1 U/ml as the impedance changes caused by the film degradation were masked by the instability of the film impedance in buffer.

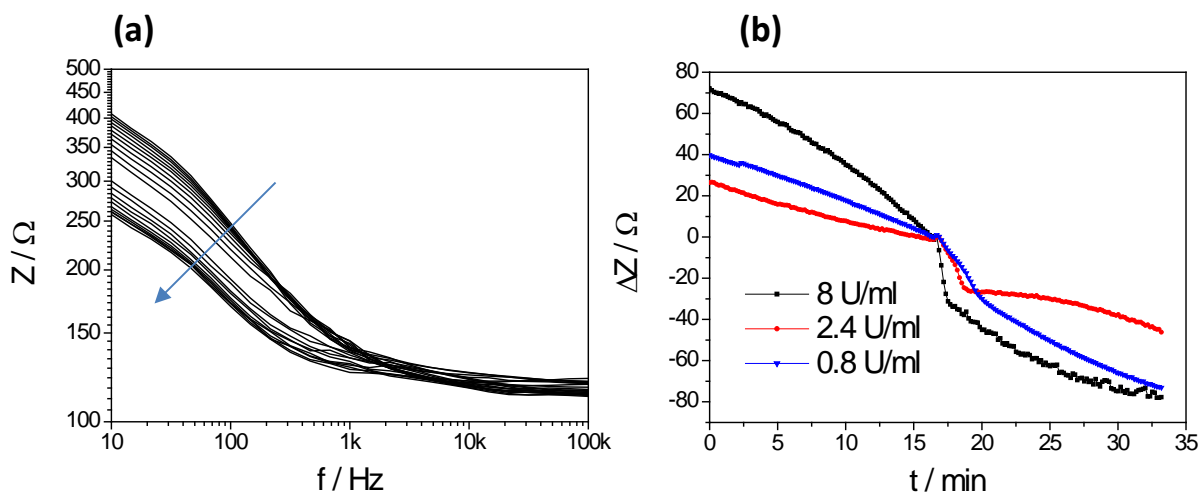


Figure 5 Impedance response of the sensor. (a) Impedance spectra recorded before and after addition of 8 U/ml amylopectin; 1 spectrum every 100 s; the arrow indicates the direction of change. (b) Plot of impedance at 10 Hz over time for different α -amylase activities. The enzyme was added at 17 min.

4 Conclusions

Sensor materials based on cross-linked oligosaccharide films for the detection of α -amylase activity in a range of 0.08 to 8 U/ml have been developed. Film degradation was monitored using QCM and impedance measurements. In buffer, the films were found to show a stable QCM signal providing a high signal to noise ratio when measuring the response to α -amylase. When using amylopectin films, both the mass change and the energy dissipation can be used as a sensor signal. Experiments in serum at two α -amylase activities showed that the presence of protein had no significant effect on the sensor response. This suggests that the sensor materials developed might be suitable for measuring hyper-amylasia conditions found in pancreatitis. Although activity dependent impedance changes were observed, impedance measurements were found to be inappropriate for monitoring the degradation of the oligosaccharide films at lower α -amylase activities as the film impedance was insufficiently stable in buffer solutions.

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