Lactose Biosensor Development and Deployment in Dairy Product Analysis

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Lactose Biosensor Development and Deployment in Dairy Product Analysis

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Herein, development of an enzymatic biosensor for rapid quantitation of the disaccharide lactose in dairy samples (whey permeates and milk protein isolates (MPI)) is presented. Biosensor fabrication involved a chitosan/enzyme/crosslinker configuration with enzymes glucose oxidase and β-galactosidase at platinum and glassy carbon electrodes. Solution phase mediation was used to lower the operating potential (E_{app} = 0.3 V vs Ag/AgCl), minimising any contribution from endogenous background electroactive species. Potential sweep and potentiostatic experiments realised analytical data for the lactose sensor with linear range 5.83×10^{-3} to 1.65×10^{-2} M, sensitivity 9.41×10^{-4} C cm^{-2} mM^{-1} and LOD of 1.38 mM. Scanning electrochemical microscopy realised surface characterisation of the enzyme layers with approach curves and redox competition mode imaging achieved over the active enzyme bilayer. Both glucose and lactose sensing was realised in whey permeate sample measuring 23.7 mM lactose, correcting for free glucose contributing signals, with 92.2% correlation with results obtained from the sample certificate of analysis. Solution phase mediation at glassy carbon enzyme electrodes resulted in lactose quantitation in milk protein isolates, measuring 1.16 mM for the MPI 1 (low lactose) and 1.54 mM for the MPI 10 (standard) sample with validation by HPLC analysis.
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The dairy industry is one of the largest food processing sectors in the world and development of biosensors for use in dairy side stream monitoring relies on the use of sensitive and selective biomolecules that act as recognition elements, allowing miniaturisation of analytical methods to provide on-site testing and continuous monitoring of key analytes.[1] Continuous monitoring of lactose and lactic acid are required for many purposes and currently, the industry sector relies on analytical methods such as High-Pressure Liquid Chromatography (HPLC) (e.g. HPLC-RI).[2] Such approaches require highly trained personnel with many industrial companies choosing to send their samples to external labs for testing. This can lead to severe delays and causes halts in production. In addition, such methods can be utilised to assess the quality of final products but are unsuitable for use in the monitoring of the process which requires faster turnaround time to result.[3] Biosensors form an attractive alternative quantitation approach for on-site testing of key analytes in dairy bioprocesses.[4]

The disaccharide (galactose-glucose) lactose is the main carbohydrate in dairy products and makes up <2% of dry protein isolates. Cow's milk contains 4%–5% lactose[5] and the processing of protein isolates and concentrates aims to fractionate the protein portion and remove lactose, fat and minerals from the product.[6] Routine analysis of lactose in dairy ingredients can lead to improved utilisation of the ingredients as well as helping correlate lactose levels with ingredient quality[6] while acting as an indicator for milk quality and detection of low lactose milk from cows with mastitis.[7] Enzymatic hydrolysis of lactose is catalysed by the enzyme β-galactosidase (lactase) and lactose free products thus formed have additional sweetness and are easily digested by lactose-intolerant individuals.[8]

The development of biosensors for use in lactose monitoring can overcome issues with sample testing and lead towards cost effective, rapid and portable analytical devices with nano-biosensing platforms for cow milk allergens being the subject of a recent review.[9] Enzyme electrodes are widely utilised due to their specificity, sensitivity and low-cost.[10–14] With the use of such biocatalysts, biosensors can be developed to detect and quantify glucose, lactose and lactic acid in dairy samples including whey permeate, milk protein isolates (MPI) and fermentation samples. Glithero et al.[5] have shown that at line lactose measurement in dairy processing plants can be achieved by monitoring the lactose level in waste water processing streams semi-continuously as a means to measure lactose loss over a 6 month period and results obtained showed lactose content range between 0%–8% w/v (0–0.23 M) during testing. With the aid of such a sensor, analysts were able to determine the total loss of lactose from the plant which counted for lost sales and loss of useful product.

More recently, a polymer multilayer biosensor for lactose measurement in the presence of high levels of glucose was reported by Lopez et al.[15] The device exploited cellobiose dehydrogenase as a biorecognition agent with a hydrophilic polymer which entrapped glucose oxidase and catalase for glucose removal, realising lactose determination from 10–100 μM.[15] Other reports include use of a cobalt-hemin metal organic framework/chitosan composite containing cellulose dehydrogenase[16] with sensitivity 102.3 μA mM^{-1} cm^{-2} and range 10–100 mM lactose.

A wide range of immobilisation techniques are utilised in the deposition of enzymes onto transducer surfaces.[2] Cross-linking agents promote chemical binding between biomolecules and are used in sensor development due to their simplicity, low-cost and ease of use. However, such methods can cause distortion of protein structures and inhibit active sites. Therefore, the concentration of cross-linking agents utilised is an important factor in the optimisation of enzyme immobilisation. Glutaraldehyde (GA)[17,18] and poly (ethylene glycol) diglycidal ether (PEGDE)[19,20,3] are examples. PEGDE contains two epoxy groups that can react with amine functional groups present on the surface of a protein, allowing enzyme immobilisation on a transducer surface.[3] The use of PEGDE for enzyme immobilisation of GOx on a microsensor was studied by Vasylieva et al. in 2011. PEGDE (4–100 mg ml^{-1}) was examined to determine the optimum concentration for enzyme immobilisation which was found to be 20 mg ml^{-1}. PEGDE was also utilised in the fabrication of a lactose biosensor using GOx, β-Gal and horse-radish peroxidase (HRP).[3] Crosslinking of the enzymes was achieved by addition of an enzyme/PEGDE mixture to the surface of the electrode. Results showed that the presence of PEGDE improved the stability of the biosensor and stabilised the signal to achieve a linear range of 1×10^{-7}–1×10^{-4} mol dm^{-3}. The sensor was used to detect lactose in milk samples using ferrocene as a redox

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mediator. Results were compared with GC/MS which showed the biosensor detected 4.9 ± 1.9 g while GC/MS determined 5.5 ± 0.1 g in the sample.

The use of chitosan in this work serves to further enhance the immobilisation process of the bienzyme system proposed. Chitosan is an oligosaccharide which is commonly used in the development of biosensors for immobilisation of biological elements.[21–24] It allows for high enzyme loadings due to the cross-linking reaction involved between the chitosan amino groups and the aldehyde groups in GA.[17] The combination of both chitosan and GA has previously been utilised in the development of a H_2O_2 sensor modified with HRP. Fabrication of the sensor consisted of a drop casted enzyme layer on the electrode surface, followed by a 1% chitosan solution and finally 0.025% GA with set drying times between each layer. Studies were carried out on the chitosan film thickness (0.25%–1%) and the highest response was observed with 1% chitosan and 0.025% GA.[25] In relation to lactose, the use of GA as a crosslinker with gelatin for a dual enzyme lactose biosensor has been reported[26] with robust performance over continuous reuse experiments. Jasti et al.[27] utilised bovine serum albumin adsorbed on allyl glycidyl ether—ethylene glycol dimethacrylate crosslinked polymer for immobilisation of glucose oxidase and β-galactosidase on an enzyme support for optical assay of lactose, while layer by layer films of the polyelectrolyte poly(ethylene imine) and poly(vinyl sulfonate) together with Prussian Blue[28] resulted in amperometric detection of lactose and included an examination of the molecular recognition interactions involved.

The use of redox mediators can help eliminate interferences caused by electroactive species by lowering the operating potential and should have rapid reactivity with the enzyme involved, be pH independent, highly stable in both its oxidised and reduced form, have reversible electron transfer kinetics with low over-potential for oxidation at the electrode surface.[29] Mediators, such as iron salts[30] include potassium ferricyanide ($K_3Fe(CN)_6$), being often used as a standard probe for the characterisation of electrochemical surfaces. Conzuelo et al.[31] exploited the mediator tetrathiofulvalene with the tri enzyme (β-galactosidase, glucose oxidase and peroxidase) bioelectrode realising lactose measurement from 1.5×10^{-6} to 1.2×10^{-4} M with LOD 4.6×10^{-7} M. Development of a label free approach utilising graphene/poly(1,5-diaminonapthalene) was reported by Nguyen et al.,[32] using co-immobilised β-galactosidase and glucose oxidase for lactose measurement in dairy products, and an electrochemical biosensor based on surfactant doped polypyrrole using co-entrapped galactose oxidase and β-galactosidase in the presence of sodium dodecyl sulfonic acid[7] with upper limit of 1.22 mM lactose and detection limit 2×10^{-6} M. Selectivity improvements arising from the use of polyphenylene diamine for a three enzyme lactose sensor format supported passage of hydrogen peroxide while enabling milk analysis with high correlation with respect to HPLC[33] while galactose oxidase/β-Galactosidase co-immobilised in a polypyrrole matrix enabled electrooxidation of enzymatically produced hydrogen peroxide with detection limit 2.6×10^{-6} M.[7] A copolymer of pyrrole and 3,4-ethylenedioxythiophene coupled with β-galactosidase and galactose oxidase[34] realised a detection limit of 1.4×10^{-5} M with range up to 2.30 mM with the apparent Michaelis-Menton constant of 0.65 mM, while a 3,4-ethylenedioxythiophene/thiophene copolymer[35] served to enable hydrogen peroxide detection for lactose measurement via cyclic voltammetry at low operating voltages.

Scanning electrochemical microscopy (SECM) can be applied to the evaluation of enzymatic activity in enzyme electrodes and allows for characterisation of electrochemically active species on various surface.[36] The redox competition mode of SECM has been used to visualise differences in local electroanalytical activity of Fe and Ni hexacyanoferrates in relation to hydrogen peroxide reduction[37] and glucose oxidase catalysed reaction kinetics.[36] When the substrate electrode is modified by an enzyme the redox mediator can be involved in both uptake by the immobilised biocatalyst and recycling at the tip electrode. Therefore both tip (microelectrode probe) and enzyme loaded substrate electrode compete for the same mediator.

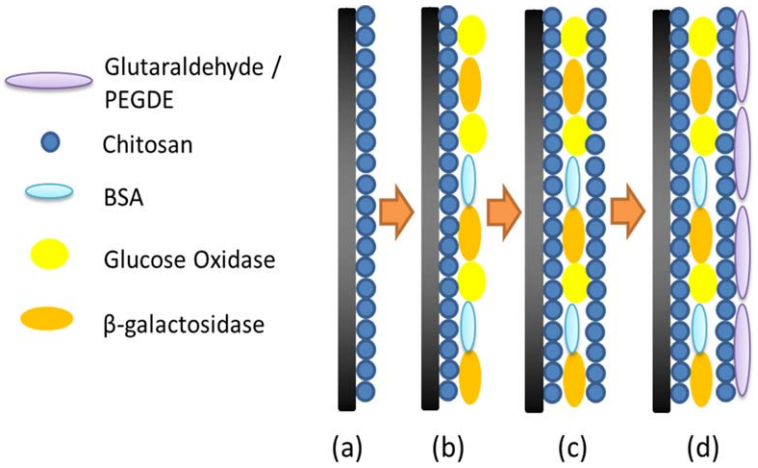
A limited number of commercial lactose biosensors exist currently due to the complexity of dairy product composition[6] with the LactoSens® Amperometry method being a subject of a recent report.[38] In order to monitor label claims for lactose-free and low-lactose milk and dairy products, invested efforts into the design and evaluation of suitable biosensor methodologies for lactose determination in complex dairy matrices is required. In this work, glucose and lactose detection was achieved by both direct and solution mediated approach methods and surface characterisation of modified surfaces was examined for the first time via scanning electrochemical microscopy using redox competition mode. Building on prior art and following systematic optimisation, direct detection of glucose and lactose in whey permeate samples was performed using modified Pt electrodes while determination of lactose in milk protein isolates (MPI) was realised using mediated enzymatic biosensors fabricated at glassy carbon transducers via voltammetry and constant potential coulometry (CC) techniques. The results obtained from electrochemical analysis were correlated with established HPLC approaches.

Experimental

Materials.—MetaDi Monocrystalline Diamond suspension (1 μm) was purchased from Akasel. Glucose Oxidase from *Aspergillus niger* (Type VII, lyophilized powder 10 KU), β-galactosidase from *Aspergillus oryzae* (25 KU), Chitosan (from Shrimp shells, practical grade), D-(+)-Glucose (≥99.5% (GC)), Bovine Serum Albumin (lyophilized powder, ≥ 96% (agarose gel electrophoresis)), Poly (ethylene glycol) diglycidyl ether and Potassium phosphate dibasic trihydrate (ReagentPlus ≥99.0%) were all obtained from Sigma Aldrich. D (+)-Lactose 1-hydrate BioChemica, Glutaraldehyde solution 25% for synthesis, Acetic acid (100%) and Potassium di-Hydrogen Phosphate for Analysis, ACS were purchased from ITW reagents.

Instrumentation.—All electrochemical techniques were carried out on a Solartron 1285 Potentiostat, with general purpose electrochemical software CorrWare and electrochemical data analyser CView. A three electrochemical cell set up was used that contained a platinum wire as counter electrode, an Ag/AgCl reference electrode (stored in 3 M KCl) along with either platinum (Pt) or glassy carbon electrodes (GCE) as WE. Prior to modification, all Pt and GC electrodes were prepared by polishing with 1 μm MetaDi Monocrystalline Diamond Suspension, followed by rinsing in deionised water, sonication for 1 min and drying at room temperature.

Procedures.— *Fabrication of enzyme electrodes.*—In the case of all biosensors, electrode preparation was adapted from the method described by Miao et al. for glucose oxidase immobilisation, as shown in Scheme 1. 1st generation sensors were fabricated using Pt macroelectrodes and 2nd generation sensors involved modification of GCEs. Enzymes were aliquoted into 0.02 M phosphate buffer (PB) containing 0.5 mg ml^{-1} BSA and stored at −20 °C. Enzyme immobilisation was achieved by a four-layer sandwich consisting of (a) 5 μl of a 0.5% chitosan in 0.8% acetic acid, (b) 5 μl of enzyme mixture 50 U GOx for glucose or 50 U GOx and 22.25 U β-gal for lactose detection, (c) Repeat of step (a), (d) 5 μl of 0.05% GA or 1.5% PEGDE. Each layer was allowed to dry at room temperature between modification steps. Electrodes are referred to as Pt/Chit/GOx/Chit/GA, Pt/Chit/GOx/Chit/PEGDE, Pt/Chit/GOxβ-gal/Chit/GA, GC/Chit/GOx/Chit/GA and GC/Chit/GOxβ-gal/Chit/GA as shown in Table I. Once fabricated, the electrodes were allowed to air dry for 1 h at room temperature and rinsed with deionised water before use. Electrodes were stored in 0.1 M PB, pH 6.0 at 4 °C when not in use.

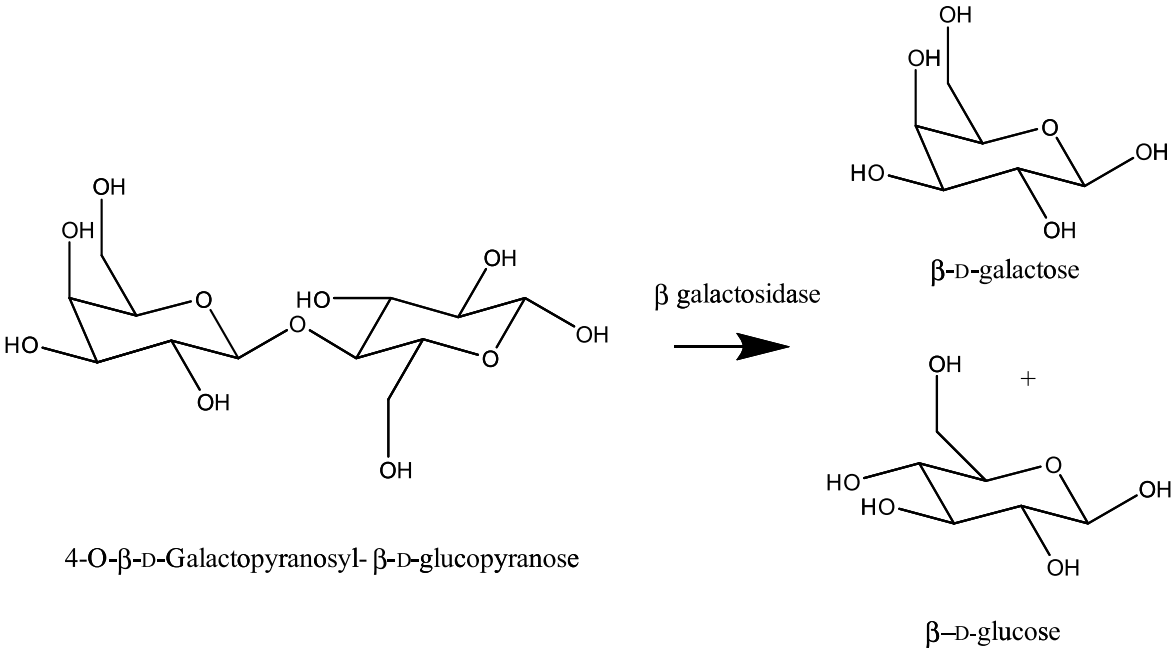


β-Galactosidase

$$(1)\ \text{Lactose} + H_2O \rightarrow \text{Glucose} + \text{Galactose}$$

$$(2)\ \text{Glucose} + O_2 \rightarrow \text{Gluconolactone} + H_2O_2$$

$$(3)\ H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$



Scheme 1. (A) Development of biosensor with (a) 5 μl of 0.5% chitosan (in 0.8% acetic acid), (b) enzyme layer: 5 μl of GOx (50 U) for glucose detection or 5 μl of GOx (50 U) and 5 μl of β-gal (22.25 U) for lactose detection (in 0.02 M phosphate buffer containing 0.5 mg ml^{-1} BSA) (c) 5 μl of 0.5% chitosan and (d) 5 μl of 0.05% GA or 1.5% PEGDE. (B) hydrolysis of lactose catalysed by β-galactosidase.

Direct detection of glucose and lactose at Pt electrode via H_2O_2 electrooxidation.—The response to glucose was measured via CV at 100 mVs^{-1} over a potential range −0.2 to 1.0 V vs Ag/AgCl. Measurements of glucose (up to 7 mM) were performed at a fixed potential of 0.8 V vs Ag/AgCl. Detection of lactose (0–4 mM) was performed via CV over the range −0.1 V − 1.0 V vs Ag/AgCl at 100 mVs^{-1}. A fixed potential study (0.65 − 0.8 V) was carried out to establish the appropriate applied potential for CC analysis of lactose (up to 100 mM). Based on the optimum applied potential, further CC analysis was performed with E_{app} = 0.65 V vs Ag/AgCl to calibrate the lactose biosensor over two different analyte ranges (0–40 mM and 40–60 mM).

Solution mediated glucose and lactose biosensing.—Constant potential coulometry was carried out on two glucose enzyme electrodes (GC/Chit/GOx/Chit/PEGDE) and (GC/Chit/GOx/Chit/GA) to determine the influence of cross linkers (1.5% PEGDE and 0.05% GA) on the glucose response. CC was performed over the

Table I. Electrode configuration of glucose and lactose biosensors for direct and mediated methodologies.

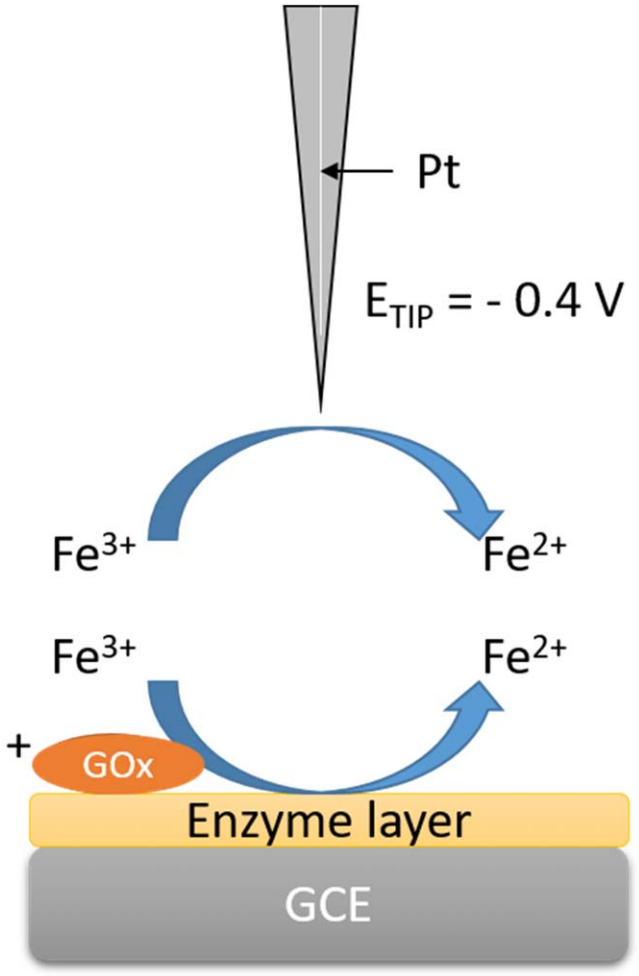
Biosensor Type	Enzyme Activity	Electrode configuration	
		1st generation	2nd generation
Glucose	GOx = 50 U	Pt/Chit/GOx/Chit/GA Pt/Chit/GOx/Chit/PEGDE	GC/Chit/GOx/Chit/GA
Lactose	GOx = 50 U β-gal = 22.25 U	Pt/Chit/GOxβ-gal/Chit/GA	GC/Chit/GOxβ-gal/Chit/GA

range 0–47 mM with E_{app} of 0.35 V vs Ag/AgCl for 5 s. Scan rate studies were also performed and determination of glucose was achieved over the range 0–7 mM using CV (−0.5–1.0 V vs Ag/AgCl) at scan rates 20–100 mVs^{-1}. Detection of lactose (1 mM in 5 mM $K_3Fe(CN)_6$) was carried out using CV over the range −0.5 to 0.8 V vs Ag/AgCl at 100 mVs^{-1}. The background signal was first measured in 0.1 M phosphate buffer (pH 6.0) and 5 mM $K_3Fe(CN)_6$. Calibration of lactose (up to 18 mM) was achieved via CC analysis at E_{app} = 0.3 V vs Ag/AgCl for 5 s.

Negative control studies.—Two modified electrodes were prepared for glucose positive (GC/Chit/GOx/Chit/GA) and negative (GC/Chit/Chit/GA) control studies. CC analysis was carried out with E_{app} = 0.35 V vs Ag/AgCl for 5 s. Charge measurements were recorded for 0.1 M phosphate buffer, 5 mM $K_3Fe(CN)_6$ and 10 mM glucose. GC/Chit/GOxβ-gal/Chit/GA and GC/Chit/Chit/GA electrodes were used as positive and negative controls for lactose via CC analysis with charge measured at 0.3 V vs Ag/AgCl after 5 s in the case of background (0.1 M phosphate buffer (pH 6.0)), 5 mM $K_3Fe(CN)_6$ and lactose standards (3.92, 7.69 and 11.32 mM). Four electrodes were fabricated as follows: negative control (GC/Chit/Chit/GA), GOx only (GC/Chit/GOx/Chit/GA), β-gal only (GC/Chit/β-gal/Chit/GA) and positive control (GC/Chit/GOxβ-gal/Chit/GA). Their response to 0.1 M phosphate buffer (pH 6.0), 5 mM $K_3Fe(CN)_6$ and lactose (1.98 mM in 5 mM $K_3Fe(CN)_6$) was examined using CV with a potential range −0.3 to 0.5 V vs Ag/AgCl at 100 mVs^{-1}.

Surface characterisation of glucose and lactose biosensors using scanning electrochemical microscopy.—Approach curves were carried out for each biosensor in 5 mM $K_3Fe(CN)_6$ using a Pt microelectrode tip (20 μm) with the tip potential (E_T) held at −0.4 V vs Ag/AgCl (E_{sub} = OFF). The movement of the tip to the surface of the substrate (enzyme modified GC electrode) was monitored and stopped prior to contact in order to reduce risk of tip crash. Redox competition mode[36] was utilised (Scheme 2), where both sample and tip compete for Fe^{3+}. High local electroactivity was indicated by low currents monitored at the SECM tip as the enzyme modified surface was approached and the oxidised form of the mediator was consumed. Line scans were carried out by measuring the current at −0.4 V vs Ag/AgCl as the probe scanned across the electrode surface (0 − 8000 μm) in 5 mM $K_3Fe(CN)_6$ and in the presence of glucose (20 and 40 mM). Imaging of the enzyme layer was achieved via area scans of the modified substrate electrode to examine the area of enzyme activity with (E_T = −0.4 V vs Ag/AgCl E_{sub} = OFF) 5000 × 8000 mm^2 (glucose biosensor) and 5000 × 5500 μm^2 (lactose biosensor) with 100 mm^2 per point. SECM studies were carried out in 5 mM $K_3Fe(CN)_6$ with 0 and 20 mM substrate (glucose or lactose).

Sample preparation and analysis.—Whey permeate samples were prepared by dilution in 0.1 M phosphate buffer (pH 6.0) to 1% w/v for direct detection and quantitation of glucose and lactose. Samples subsequently underwent sonication for 10 minutes MPI samples were prepared by diluting to 1% w/v in 5 mM $K_3Fe(CN)_6$ (in 0.1 M phosphate buffer (pH 6.0) followed by sonication for 10 minutes.



Scheme 2. Redox competition mode for scanning electrochemical microscopy at GOx and β-Gal modified GCE (E_T = −0.4 V vs Ag/AgCl) E_{sub} = OFF, 20 μm Pt UME (RG = 23.8).

Direct detection of glucose and lactose in whey permeate samples.—CV was performed at a Pt/Chit/GOx/Chit/GA electrode using whey permeate samples which were diluted in 0.1 M PB (pH 6.0) (1% w/v) and then spiked with 1.98 mM glucose; potential range −0.2 V to 1.0 V at 100 mVs^{-1}. CC was carried out E_{app} = 0.8 V vs Ag/AgCl to determine background glucose content in the sample via the standard addition method with additions up to 9.09 mM glucose. CV was carried out at a Pt/Chit/GOxβ-gal/Chit/GA using whey permeate samples diluted in 0.1 M PB (pH 6.0) (1% w/v) and then spiked with 1.98 mM lactose; potential range −0.1 V to 1.0 V vs at 100 mVs^{-1}. CC with E_{app} = 0.65 V vs Ag/AgCl was then utilised to detect and quantify lactose in the whey permeate sample via standard addition method (additions up to 9.09 mM lactose). Extrapolation of data for charge vs concentration plots was carried out to determine concentration of glucose or lactose in the

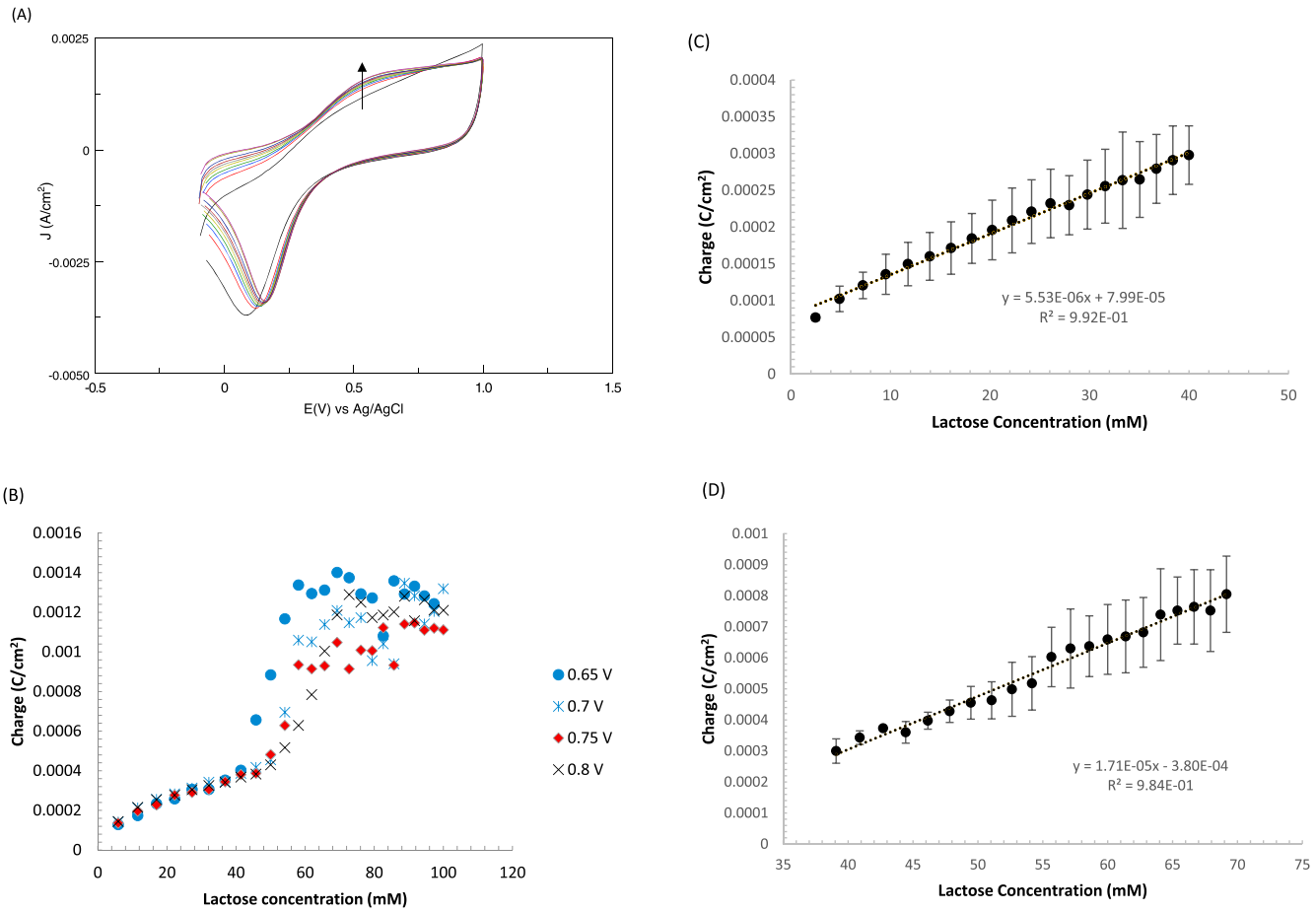


Figure 1. (A) CV of Pt/Chit/GOxβ-gal/Chit/GA lactose sensor showing lactose response to 0.4–4 mM lactose additions at 0.55 V, potential range -0.1 V to 1.0 V vs Ag/AgCl, scan rate 0.1 V s^{-1}. (B) Overlaid CC data , E_{app} = 0.65 V, 0.7 V, 0.75 V and 0.8 V vs Ag/AgCl and 5.88–100 mM lactose additions in 0.1 M phosphate buffer at Pt/Chit/GOxβ-gal/Chit/GA electrodes. Calibration curve of CC data showing relationship between charge (C cm^{-2}) (E_{app} = 0.65 V vs Ag/AgCl) and lactose concentration (C) 2.47–40 mM (D) 39.08–69.16 mM. (n = 3) at 0.65 V using constant potential coulometry with charge taken at 5 s.

Table II. Summary table for analytical data achieved in the case of direct glucose and lactose sensing.

	Comparison of 1st generation glucose biosensors		
Analytical Data	This work	Ziao et al.	Miao et al.[25]
Linear Range (M)	0–7 × 10^{-3}	0–6.4 × 10^{-4}	0–2 × 10^{-3}
Sensitivity (C cm^{-2} mM^{-1})	3.25 × 10^{-6}	6.1 × 10^{-6}	1.87 × 10^{-5}
LOD (mM)	0.51	1.07 × 10^{-3}	
	Comparison of 1st generation lactose biosensor		
Analytical data	**This work**		**Tkáč et al**[40]
Linear Range (M)	2.47 × 10^{-3} – 4.00 × 10^{-2}	3.91 × 10^{-2} – 6.92 × 10^{-2}	9 × 10^{-5}–3.6 × 10^{-3}
Sensitivity (C $cm^{-2}mM^{-1}$)	6 × 10^{-6}	2 × 10^{-5}	4.4 × 10^{-7}[a)]
LOD (mM)	1.29	1.42	4.4 × 10^{-2}

a) units are A mM^{-1}.

relevant sample. Glucose contributing signals were subtracted from the lactose signal in order to correct for background glucose present in the whey permeate sample.

Determination of lactose in Milk Protein Isolate samples using solution mediated approach.—CV analysis was performed at GC/Chit/GOxβ-gal/Chit/GA electrode using MPI sample (MPI 1 and MPI 10) diluted in 5 mM $K_3Fe(CN)_6$ (in 0.1 M PB (pH 6.0) (1% w/v), followed by additions of lactose (up to 9.09 mM); potential range −0.3 V to 0.5 V vs Ag/AgCl at 100 mVs^{-1}. CC analysis for the same samples involved E_{app} = 0.35 V vs Ag/AgCl followed by lactose additions (0.09–9.09 mM). Data for charge vs concentration were plotted and extrapolation performed in order to determine lactose concentration in diluted samples (1% w/v).

Results and Discussion

Direct glucose and lactose detection at enzyme modified Pt electrodes.—1st generation glucose and lactose biosensors were prepared based on the procedure described above and shown in Scheme 1. Following preparation, electrodes were subjected to electroanalytical studies to obtain analytical data—linear range, sensitivity, limit of detection (LOD) and limit of quantitation (LOQ). To investigate the effects of 1.5% PEGDE and 0.5% GA

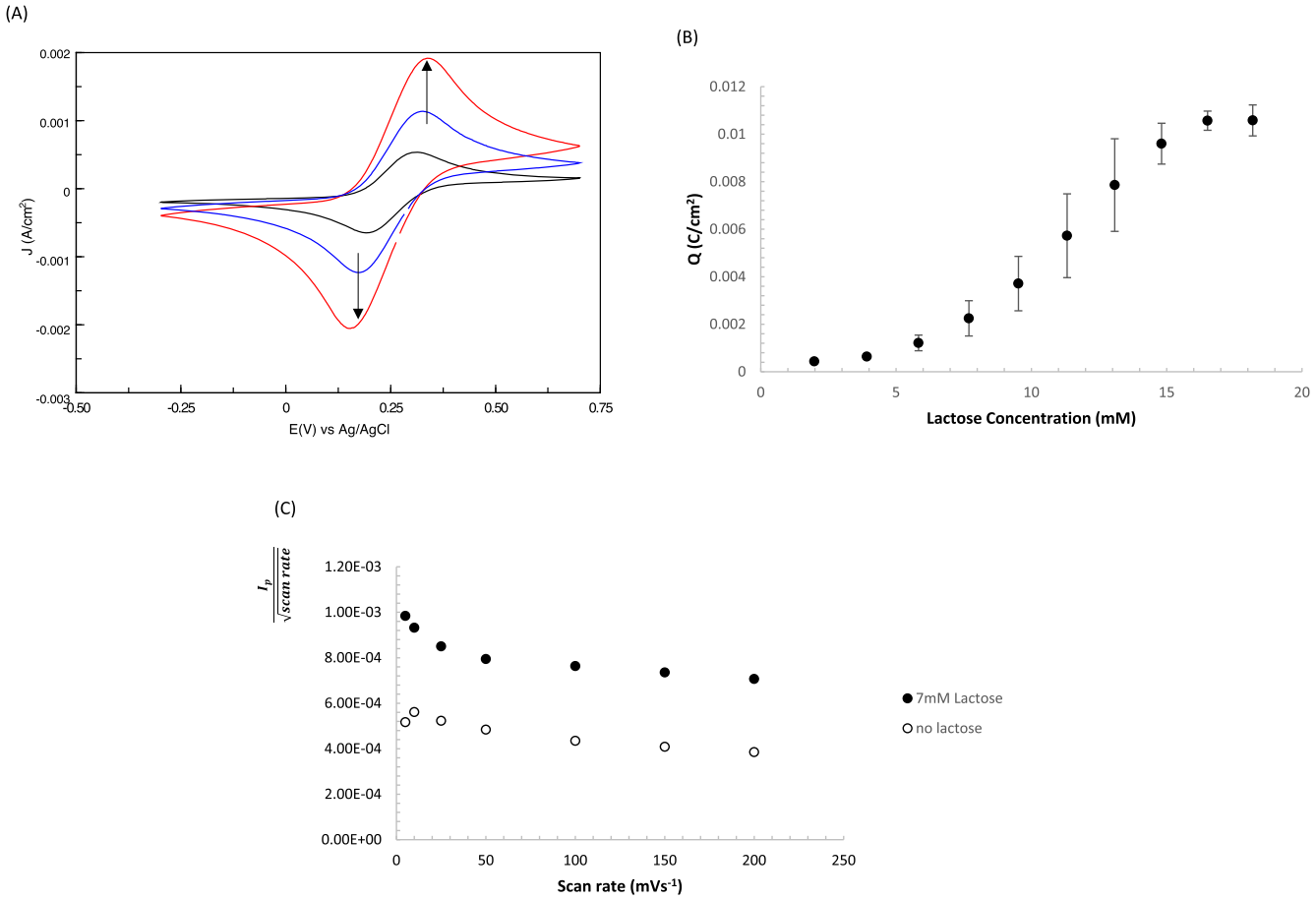


Figure 2. (A): CV response to 16 (blue curve) and 32 mM (red curve) lactose additions in 5 mM $K_3Fe(CN)_6$ at GC/Chit/GOxβ-gal/Chit/GA over the range −0.5 V to 0.8 V at a scan rate of 0.1 V/s vs Ag/AgCl. (B) Corresponding calibration curve of charge vs lactose concentration over the range 0–18-18 mM (CC analysis) (n = 3). (C) Data plot showing $I_p/\sqrt{v}$ vs v in 5 mM $K_3Fe(CN)_6$ in the presence and absence of 7 mM lactose at GC/Chit/GOxβ-gal/Chit/GA.

as GOx enzyme immobilisation cross-linking agents, a scan rate study was performed over the range 20–100 mVs^{-1} at different concentrations of glucose in 5 mM $K_3Fe(CN)_6$. Calibration studies followed at scan rate 20 mVs^{-1}. The calibration curve for the data obtained using CV at a GC/Chit/Gox/Chit/GA surface, resulted in linear range of 1.99–17.68 mM with a sensitivity of 1.88 × 10^{-4} A $cm^{-2}mM^{-1}$ (R^2 = 0.98) (n = 2). The electrode formed using PEGDE was four times less sensitive than that of GA, with a wider linear range (8–47 mM relative to 2.0−12 mM for the GA modified electrode). Additionally, studies at screen printed electrodes resulted in a less adherent film formed in the case of PEGDE and due to the ultimate desire to progress towards onsite analysis, GA was selected for lactose sensor fabrication henceforth.

Figure 1A shows a CV recorded at Pt/Chit/GOxβ-gal/Chit/GA electrode with additions of up to 4 mM lactose resulting in the expected increased H_2O_2 oxidation current over this range at 0.55 V vs Ag/AgCl. A series of applied potentials (0.65 V, 0.7 V, 0.75 V and 0.8 V vs Ag/AgCl) were examined using CC analysis at the Pt/Chit/GOxβ-gal/Chit/GA in order to establish the best operating potential. The relationship between lactose concentration and charge at each applied potential is shown in Fig. 1B, each of which resulted in a sigmoidal relationship between charge and concentration using this constant potential technique Sigmoidal concentration profiles are common in dual-enzyme biosensors where two enzymes are used for the determination of a particular analyte[39] and profiles are the result of enzymes that hold positive cooperative binding characteristics. Allosteric enzymes have multiple active sites, and such a response involves the substrate binding at one active site affecting the affinity of the substrate at other active sites. Further CC analysis was carried out to determine the analytical data for each of the two linear ranges at E_{app} = 0.65 V vs Ag/AgCl. Two different linear ranges of 0–40 mM Fig. 1C and 40–60 mM Fig. 1D lactose were realised. The lower linear range 2.47 × 10^{-3} - 4.00 × 10^{-2}M had a sensitivity of 6 × 10^{-6} C $cm^{-2}mM^{-1}$ with a limit of detection of 1.29 mM and a limit of quantitation of 4.29 mM. The higher linear range of 3.91–6.92 × 10^{-2} M, had a sensitivity of 2 × 10^{-5} C $cm^{-2}mM^{-1}$ with a limit of detection of 4.29 mM and a limit of quantitation of 4.72 mM. SI(1(available online at stacks.iop.org/JES/169/037528/mmedia)) shows a reciprocal Lineweaver Burk plot (1/Q vs 1/[S]) for data over the lower range resulting in a linear plot with apparent Michaelis Menton constant (K_m') estimated at 6.89 mM.

Table II. shows a summary of the analytical data obtained for direct glucose and lactose sensing including—linear range, sensitivity and LOD for sensors developed in this work and lactose biosensors found in literature.

Solution mediated lactose biosensing.—Mediated glucose and lactose biosensors were developed at GCEs in the presence of $K_3Fe(CN)_6$ (5 mM) with GA as crosslinker, as per procedure above and operated in the presence of 5 mM $K_3Fe(CN)_6$ in PB (pH 6.0). Figure 2A shows a CV of 5 mM $K_3Fe(CN)_6$ and addition of 1 mM lactose at GC/GOxβ-gal/Chit/GA. An increase in the oxidation peak current was evident at 0.3 V, indicating the lactose response. CC analysis followed at E_{app} = 0.3 V vs Ag/AgCl (SI(2)). As discussed earlier for direct lactose detection at the Pt/Chit/GOxβ-gal/Chit/GA electrode, results showed a sigmoidal relationship between charge

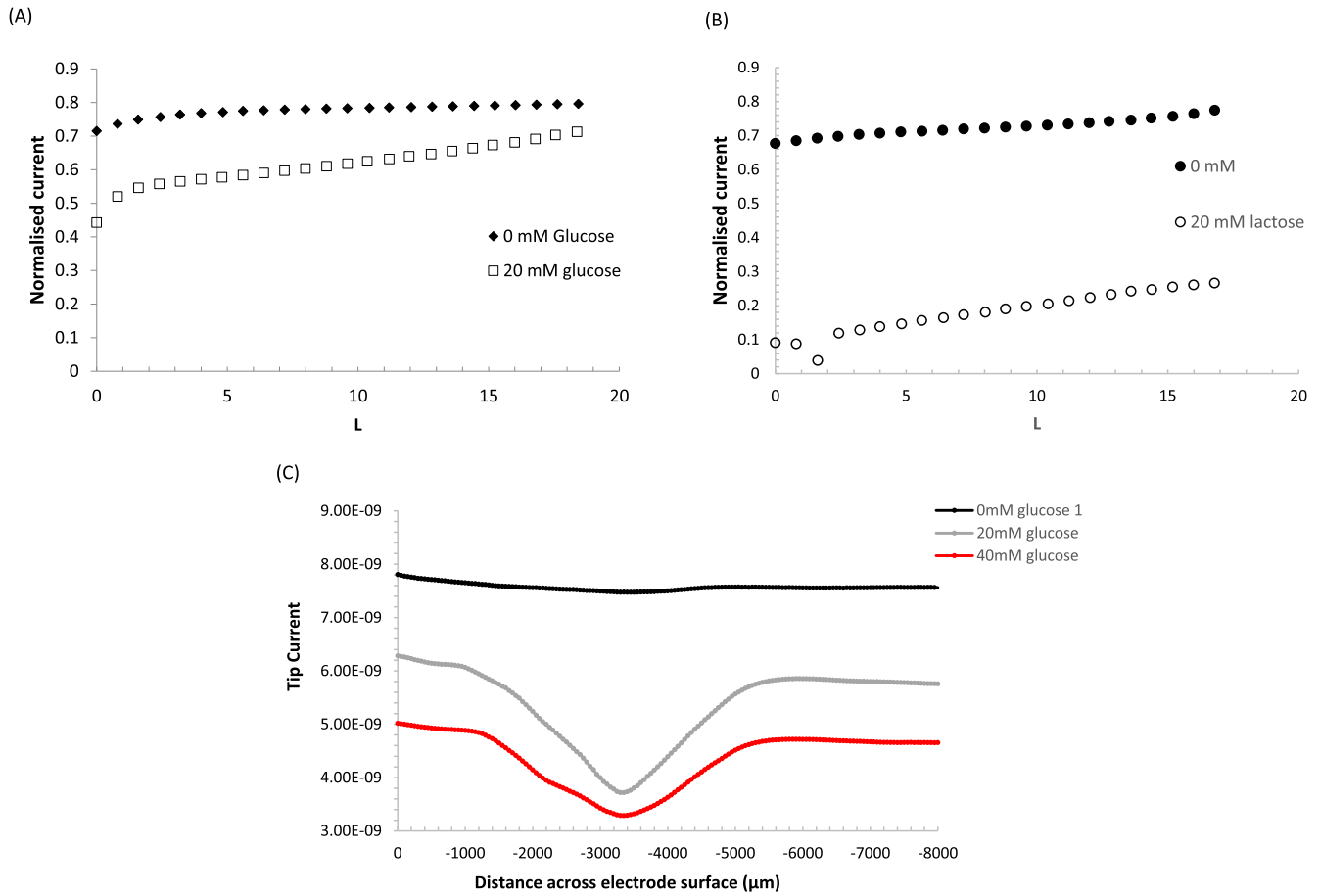


Figure 3. (A): Normalised current (I_{TIP}/I_{Inf}) vs distance (L) where L = d/a and d = distance from tip Pt ultramicroelectrode (UME) to substrate, a = tip radius 10 nm, RG = 23.8. Curves recorded above the Chit/GA-GOx film, by translating the UME vertically (z approach curve). $E_T = -0.4$ V vs Ag/AgCl, E_{sub} = OFF, 5 mM $K_3Fe(CN)_6$ in PB pH 6.0. (B) Normalised current (I_{TIP}/I_{Inf}) vs distance (L) where L = d/a and d = distance from tip Pt ultramicroelectrode (UME) to substrate, a = tip radius 10 nm, RG = 23.8. Curves recorded above the Chit/GA-GOx+β-gal film, by translating the UME vertically (z approach curve). $E_T = -0.4$ V vs Ag/AgCl, E_{sub} = OFF, 5 mM $K_3Fe(CN)_6$ in PB pH 6.0. (C) Tip current vs distance across the surface X direction, $E_T = -0.4$ V vs Ag/AgCl, E_{sub} = OFF, 5 mM $K_3Fe(CN)_6$ in PB pH 6.0.

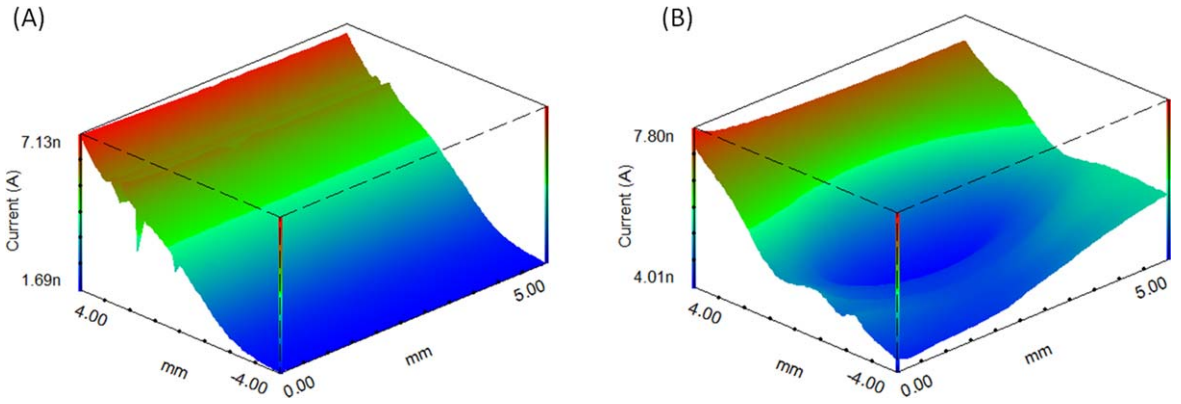


Figure 4. Area scan SECM experiment at $E_T = -0.4$ V vs Ag/AgCl E_{sub} = OFF, 20 mm Pt 5000 × 8000 mm^2 100 mm per point at GOx modified GCE substrate in 0 mM (A) 20 mM (B) glucose in the presence of 5 mM $K_3Fe(CN)_6$.

and the response up to 18 mM for solution mediated lactose detection (Fig. 2B). The sensor resulted in a linear range of 5.83×10^{-3}–1.65×10^{-2} M with sensitivity of 9.41×10^{-4} C $cm^{-2}mM^{-1}$, LOD of 1.38 mM and LOQ of 4.59 mM. Results can be compared to literature values of bi-enzyme lactose biosensors, developed by Ammam et al. with linear range of 0–14 mM lactose and a lower sensitivity of 1.11×10^{-5} A mM^{-1} cm^{-2}.[41] It can be suggested that the difference in sensitivity could be as a result of very different development methods where fabrication of the lactose biosensor by Ammam et al. did not involve any immobilisation or cross-linking agents and employed amperometric detection.

The catalytic regeneration mechanism (ECAT) describes the initial electroactive species being regenerated by a homogenous reaction (see *equations below*).[42]

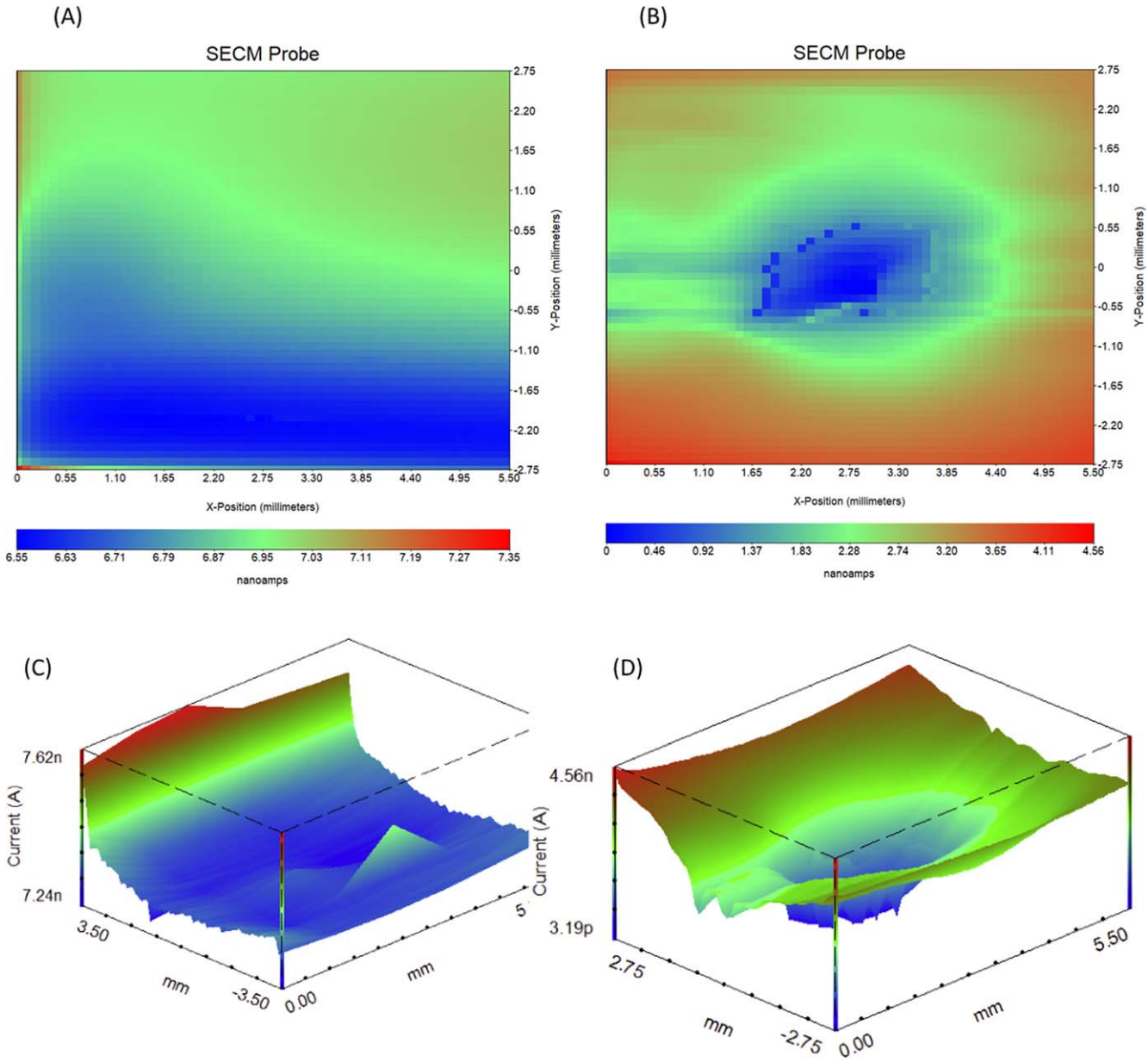


Figure 5. Area scan SECM experiment at $E_T = -0.4$ V vs Ag/AgCl E_{sub} = OFF, 20 mm Pt 5000 × 5500 mm^2 100 mm per point at GOx/β-gal modified GCE substrate in 0 mM (A, C) and 20 mM (B, D) lactose in the presence of 5 mM $K_3Fe(CN)_6$.

$$O + ne^- \leftrightarrow R$$

$$R + X \xrightarrow{k} O + Y$$

In the case of the mediated enzyme electrode developed in this work.

$$E: Fe^{2+} \leftrightarrow Fe^{3+} + e^-$$

$$CAT: Fe^{3+} + FADH_2 \rightarrow Fe^{2+} + FAD$$

In order to confirm the catalytic reactions, plots of $I_p/\sqrt{v}$ vs v were generated in the absence and presence of the substrate, where $I_p/\sqrt{v}$ decreased with increasing scan rate, according to this mechanism.[43] Figure 2C shows the relationship between scan rate and $I_p/\sqrt{v}$ in the absence and presence of 7 mM lactose at GC/Chit/GOxβ-gal/Chit/GA electrode, confirming the mechanism as electrochemical (E) step followed by catalytic (CAT) mechanism as shown also by the glucose biosensor.

Control Studies for Mediated Lactose Biosensor

Positive and negative control studies.—A control study was carried out on the glucose and lactose biosensors where 10 mM glucose and 11.32 mM lactose response relative to background was analysed at modified electrodes with and without the presence of the two enzymes, GOx and β-gal, GC/Chit/GOx/β-gal/Chit/GA and GC/Chit/Chit/GA. The CC response for enzyme substrate resulted in no significant increase in current relative to the background for the control electrodes while at the GC/CHIT/GOx-β-gal/CHIT/GA, the expected increase in the oxidation peak was evident, (SI(2) shows the quantitative difference between the electrode responses relative to background electrolyte). Further lactose sensor control studies followed, and SI(3) shows the configurations (a—d) involved. Each were monitored for their respective response to addition of

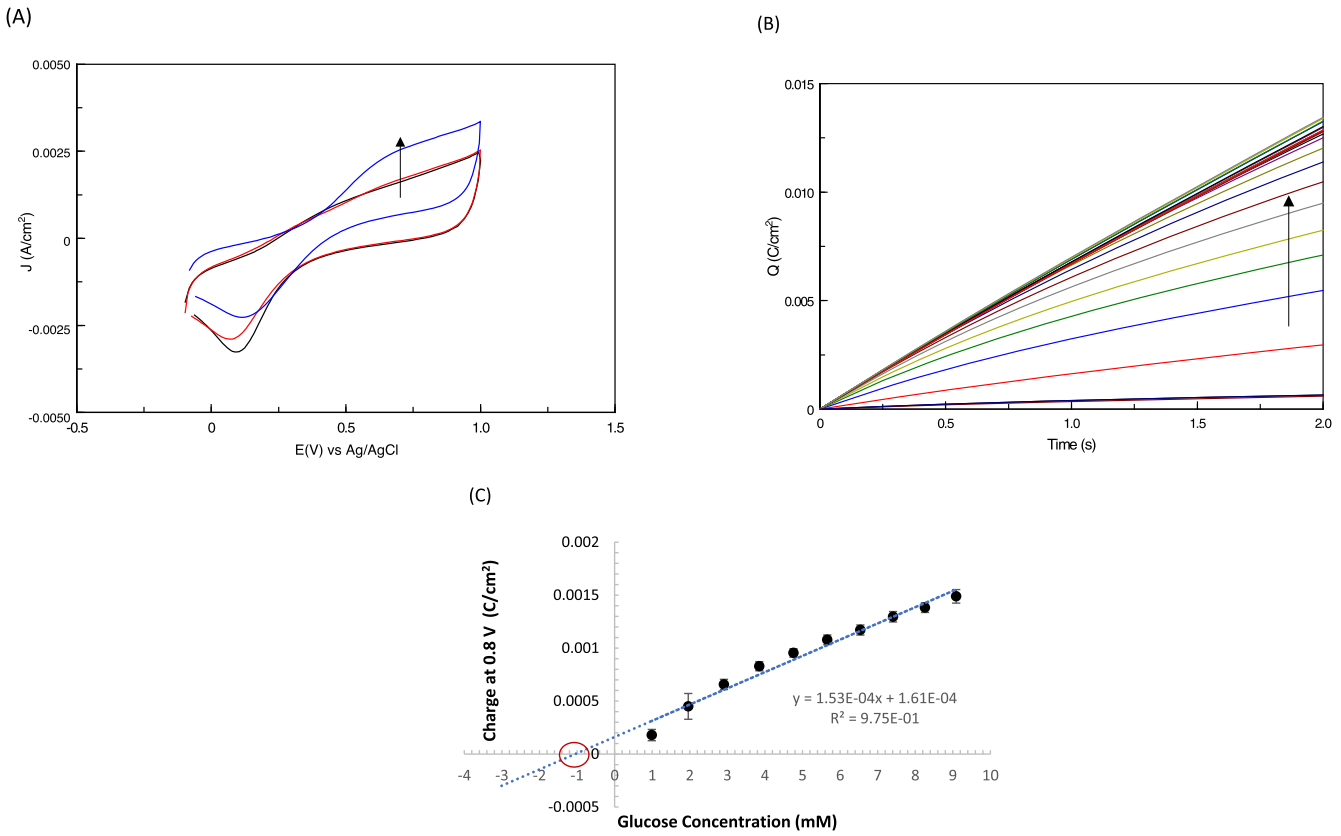


Figure 6. (A) CV of 0.1 M PB (pH 6.0), whey permeate (1% w/v) followed by addition of glucose (1.98 mM, blue curve) at Pt/Chit/GOx/Chit/Pt, potential range −0.2 V to 1.0 V with scan rate 100 mVs^{-1}. (B) CC data showing response to 0.99–9.09 mM glucose concentrations at Pt/Chit/GOx/Chit/GA in a whey permeate sample (1% w/v diluted in 0.1 M PB) E_{app} = 0.8 V vs Ag/AgCl. (C) Calibration curve of charge (C cm^{-2}) vs glucose concentration (mM) in whey permeate sample (1% w/v) (R^2 = 0.9755) (n = 3).

14.81 mM lactose (in presence of 5 mM $K_3Fe(CN)_6$) with differential data based on the voltammetric response to each electrode format thus confirming the maximum lactose response present at the GC/Chit/GOxβ-gal/Chit/GA. There was a background current signal at all control electrodes. However, the greatest current signal was evident at the electrode with GOx and β-gal present. The CV for lactose response at GC/Chit/GOx-β-gal/Chit/GA (d), provides evidence that there was a greater linear range (up to 14.81 mM) for lactose as the concentration increased relative to other electrodes (a), (b) and (c) (showing no further increase > 1.98 mM lactose). The initial deviation may be due to non-specific interactions between lactose and the crosslinking/polysaccharide agents using in enzyme film formation or possible solution breakdown of lactose to glucose.

Scanning electrochemical microscopy characterisation of glucose and lactose biosensors.—The use of scanning electrochemical microscopy (SECM redox competition mode) provided surface topographical and imaging/enzyme reactivity information. SECM was carried out on both glucose (GC/Chit/GOx/Chit/GA) and lactose (GC/Chit/GOxβ-gal/Chit/GA) biosensors. Surface imaging was performed at the enzyme modified substrate electrode (GCE) in the presence of mediator (5 mM $K_3Fe(CN)_6$) holding the potential of the Pt UME tip (E_T)= −0.4 V vs Ag/AgCl, the reduction potential of Fe^{3+}. Approach curves to the bare GCEs showed an increase in the tip current ratio as the UME approached a conducting surface (GCE substrate) and a decrease in current upon approach to the insulating surround (data not shown). A decrease in current at the insulating surface occurred as no regeneration of Fe^{3+} was produced, whereas the presence of the enzyme at the conducting surface caused regeneration of the electroactive species incurring an increase in tip current ratio.

The maximum change or difference in current was observed when the tip was scanned over the centre of the enzyme spot and the normalised current vs distance plots were generated at that position. Figures 3A and 3B show approach curves towards the enzymatic glucose and lactose biosensor substrates (E_T) = −0.4 V vs Ag/AgCl where normalised current is I_{TIP}/I_{Inf} and I_{Inf} is the steady state UME current at infinite distance from the substrate electrode. As the tip approached the modified surface the current decreased causing a negative feedback response. When a sharp decrease in current was measured, the experiment was stopped and the distance required to achieve a suitable position for the tip was determined prior to line scan or area scan analysis. Redox competition mode was utilised where both sample and tip competed for the oxidised form of the mediator. Lower currents monitored at the UME tip were realised as the enzyme modified surface was approached, indicating high local biocatalytic activity, as expected, due to the consumption of the oxidised form of the mediator at the enzyme modified substrate electrode.

Line scan studies were performed on the glucose biosensor at different glucose concentrations (0, 20, 40 mM) in mediator (5 mM $K_3Fe(CN)_6$) to show that tip current was responsive to concentration of enzyme substrate. Figure 3C shows the change in current value across substrate electrode (0–8000 μm). Results showed that at 0 mM glucose, no change in current was observed as no regeneration of electroactive species occurred in the absence of substrate. When 20 mM glucose was added into the solution, there was a fluctuation in current as the tip scanned the enzyme modified electrode surface and increased again when the tip approached the unmodified surrounding substrate platform. There was also a difference in current value between the 20 and 40 mM glucose, indicating the higher substrate turnover.

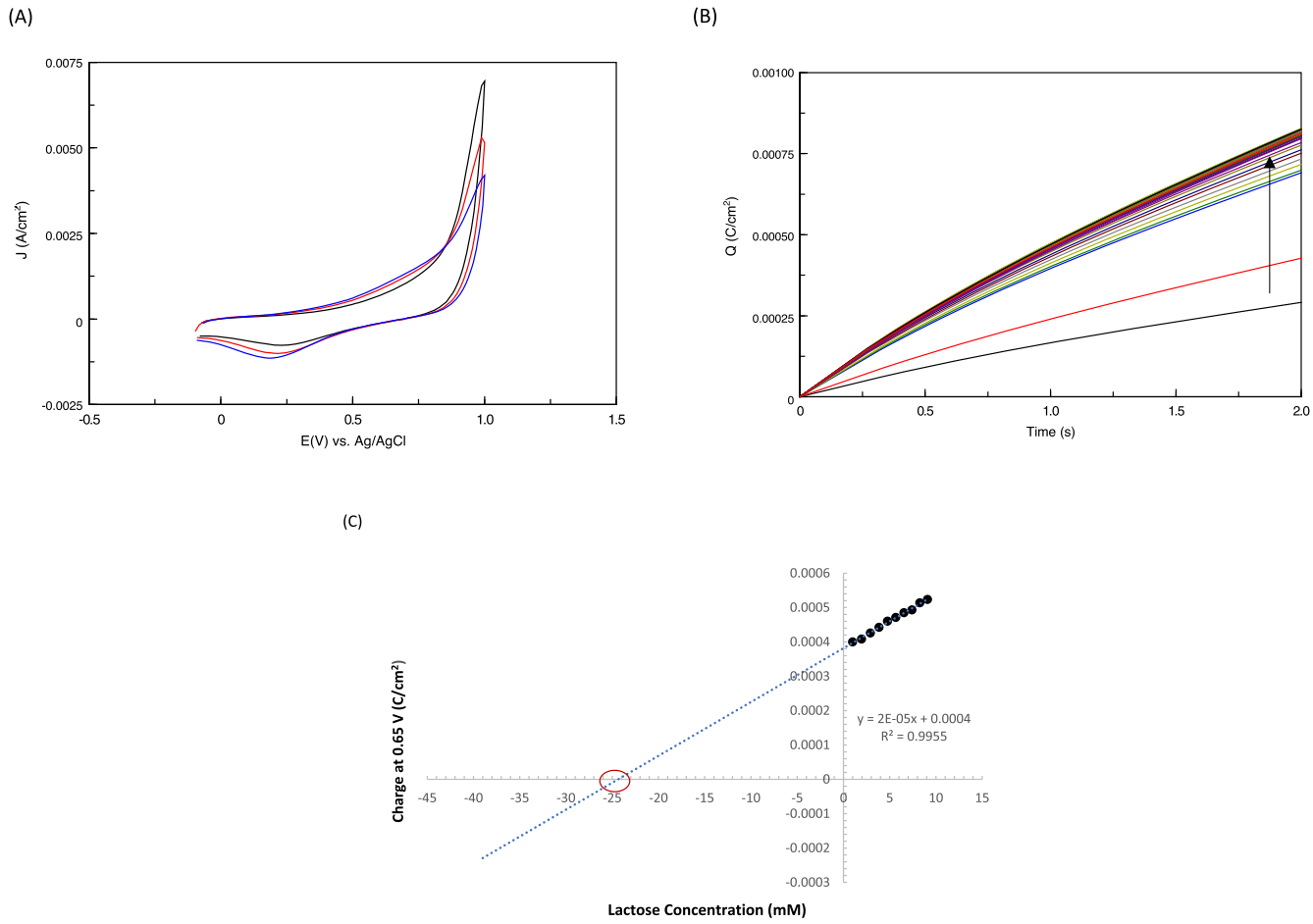


Figure 7. (A): CV of 0.1 M PB (pH 6.0), whey permeate sample (1% w/v) and spiked lactose (1.98 mM) at Pt/Chit/GOxβ-gal/Chit/GA. Potential range -0.1 V to 1.0 V vs Ag/AgCl at 100 mVs^{-1}. (B) CC data of 0.99–9.09 mM lactose concentrations in diluted whey permeate sample (1% w/v) $E_{app} = 0.65$ V vs Ag/AgCl for 2 s (C) Calibration curve of lactose concentration (0.99–9.09 mM) vs charge at 0.65 V vs Ag/AgCl in a diluted whey permeate sample (1% w/v) ($R^2 = 0.99$).

Figure 4. shows area scan experiments at a GC/Chit/GOx/Chit/GA in the presence of 0 mM glucose (a) and 20 mM glucose (b) in 5 mM $K_3Fe(CN)_6$. The images show that in the presence of the glucose substrate, there was a decrease in current over the area where the surface was modified with GOx and the active region of the enzyme can be detected by observation of a dark blue circular feature in (B). The green and red regions show areas of relatively lower enzyme activity. The UME tip was held at a reduction potential ($E_{app} = -0.4$ V vs Ag/AgCl) to reduce Fe^{3+} to Fe^{2+}. Therefore, we can highlight the region where GOx was catalytically regenerating the production of the electroactive species and the tip competes for Fe^{3+} reduction. Figure 5. shows the same area scan experiment with a GC/Chit/GOxβ-gal/Chit/GA in the presence of 0 mM lactose (A) and 20 mM lactose (B) in 5 mM $K_3Fe(CN)_6$. The surface images show that in the presence of lactose (20 mM), there was a decrease in current signal (blue) and an active area can be detected as the tip scans over the enzyme modified GCE substrate. This was due to the dual-enzyme system (GOx and β-gal) mediator consumption as the UME tip once more ($E_{app} = -0.4$ V vs Ag/AgCl) competes for Fe^{3+} reduction.

Whey permeate sample analysis.—The concentration of glucose and lactose in whey permeate was determined by CV and CC analysis. Due to the presence of GOx in the lactose dual-enzyme biosensor and catalytic reactions involved, results obtained for lactose concentration may be overestimated as a result of free glucose in the sample. Therefore, a glucose biosensor was utilised to obtain background glucose which was subtracted from the value obtained for lactose. Figure 6A shows a CV of 0.1 M PB (pH 6.0), whey permeate sample (1% w/v) and the sample spiked with 1.98 mM glucose at a Pt/Chit/GOx/Chit/GA electrode. A small difference in current value resulted in the case of the diluted whey permeate sample relative to the electrolyte, while additions of 1.98 mM glucose showed an increase in current between 0.5 V-1.0 V, indicating H_2O_2 production, as a result of the glucose turnover. CC was performed at a Pt/Chit/GOx/Chit/GA electrode, $E_{app} = 0.8$ V vs Ag/AgCl for 0.1 M PB, whey permeate sample (1% w/v) followed by glucose additions (0.99–9.09 mM) (Fig. 6B). Extrapolation of data (Fig. 6C) resulted in a glucose concentration of 1.3 mM in the whey permeate sample (1% w/v), corresponding to 130 mM in the undiluted sample.

The quantitation of lactose in whey permeate followed using CV at a Pt/Chit/GOxβ-gal/Chit/GA electrode. Figure 7A shows a CV of 0.1 M PB (pH 6.0), whey permeate sample (1% w/v) and sample spiked with 1.98 mM lactose. An increase in current was observed at 0.65 V vs Ag/AgCl corresponding to H_2O_2 production for the 1% whey permeate sample relative to the background electrolyte and a further increase in current after spiking the sample with 1.98 mM lactose. Background signals were evident over the range 0.65–0.9 V and a 100-fold sample dilution was required (in phosphate buffer pH 6.0). CC analysis followed at $E_{app} = 0.65$ V vs Ag/AgCl (Fig. 7B). Results showed a linear relationship between charge and lactose concentration for direct detection at a Pt/Chit/GOxβ-gal/Chit/GA electrode. Extrapolation of data (Fig. 7C) was carried out for charge vs concentration plots in order to determine lactose concentration in the diluted sample. The lactose biosensor measured 25 mM lactose in the whey permeate sample (1% w/v). To account for free glucose in the sample, the glucose signal was subtracted from the lactose

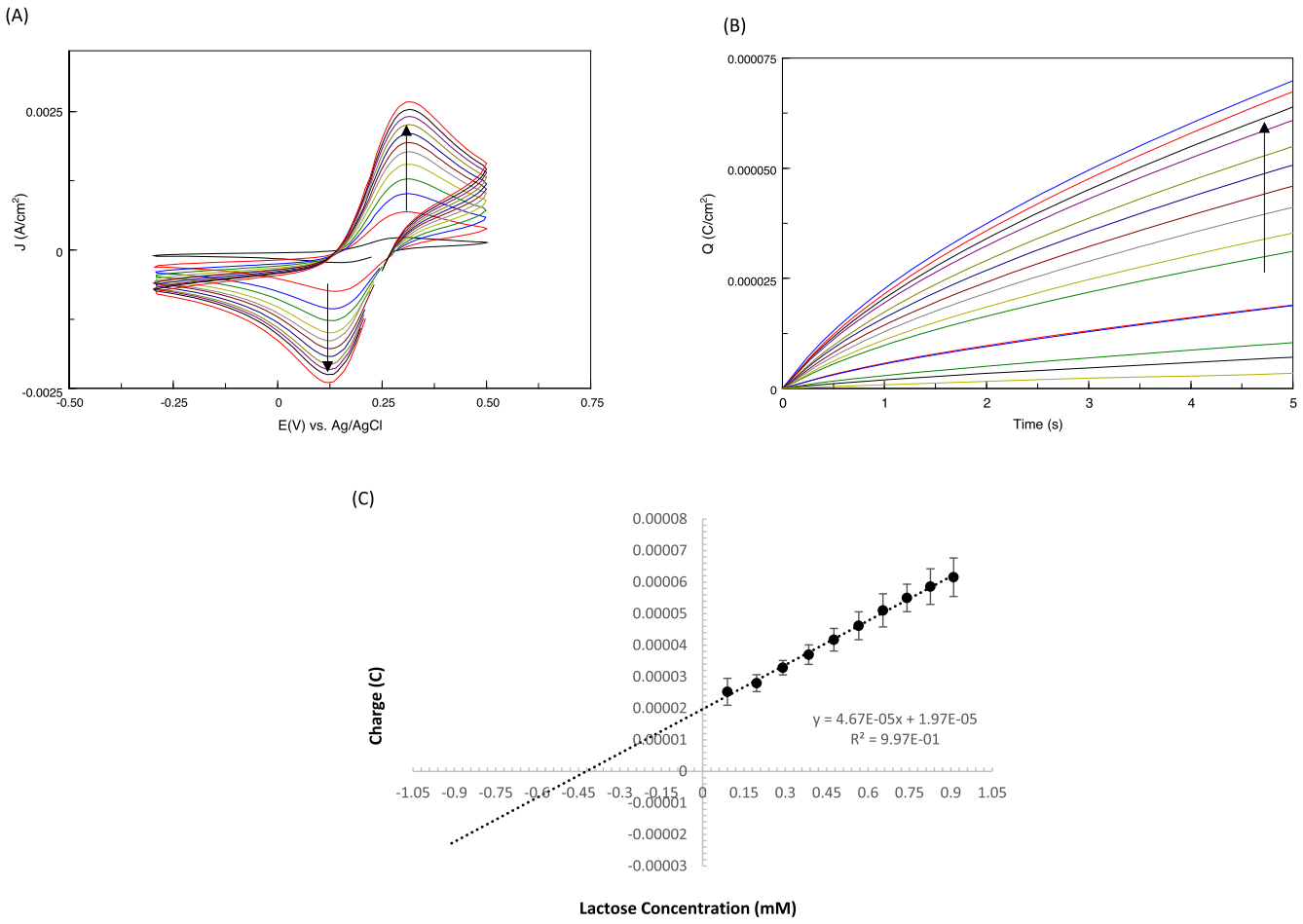


Figure 8 . (A): Overlaid CV data for 1–9.09 mM Lactose concentrations in MPI 10 diluted in 5 mM $K_3Fe(CN)_6$ (1% w/v); potential range −0.3 V to 0.5 V vs Ag/AgCl and a scan rate of 100 mVs^{-1}. (B) Overlaid CC data for 0.1 M PB, 5 mM $K_3Fe(CN)_6$, MPI 10 (diluted to 1% w/v) and lactose additions (0.09 − 0.9 mM). Charge measured at 0.35 V vs Ag/AgCl at 5 s. (C) Extrapolation of data for charge vs lactose concentration (0.09 − 0.9 mM) in a diluted MPI 10 sample (1% w/v) (n = 3).

signal which resulted in 23.7 mM as determined lactose level. Results were compared with the Certificate of Analysis (COA) for the industry provided whey permeate sample data, which showed 25.7 mM lactose, resulting in 92.2% correlation between the biosensor and the standard analytical approach for lactose measurement (HPLC-RI) in whey protein matrix.

Milk protein Isolate sample analysis by solution phase mediation.—The mediated approach was utilised for the determination of lactose concentration in two MPI samples, labelled MPI 1 and MPI 10 where the latter was a standard lactose sample and MPI 1 has low-lactose content. The samples were diluted in 5 mM $K_3Fe(CN)_6$ (in 0.1 M phosphate buffer (pH 6.0)) (1% w/v) prior to analysis at GC/Chit/GOxβ-gal/Chit/GA electrodes via solution phase mediation of lactose. Figure 8A shows a CV of background electrolyte, 5 mM $K_3Fe(CN)_6$ followed by MPI 10 sample (1% w/v) spiked with up to 9.09 mM lactose. CC analysis was performed (E_{app} = 0.35 V vs Ag/AgCl) for both MPI 1 and MPI 10 samples to determine the concentration of lactose via standard addition. Figure 8B shows background electrolyte, 5 mM $K_3Fe(CN)_6$, MPI 10 sample (1% w/v) followed by spiked lactose additions (0.09–0.9 mM). Extrapolation of the corresponding data showed that the biosensor measured 0.45 mM lactose in the diluted MPI 10 sample (1% w/v) Fig. 8C.

Finally, lactose determination was performed for the low lactose content MPI 1 sample (1% w/v) by CC with E_{app} = 0.35 V vs Ag/AgCl (Fig. 9A) showing an increase in charge for lactose. Extrapolation of the corresponding CC data resulted in 0.34 mM lactose in the diluted MPI 1 measured at the GC/Chit/GOxβ-gal/Chit/GA electrode (Fig. 9B). Results was compared with two HPLC methods for lactose quantitation in MPI samples (RI refractive index detector and IC—ion chromatography with pulsed amperometric detection) (Table III). Data showed a 67% correlation for the MPI 1 sample with the HPLC-IC-PAD method and 95% correlation for the MPI 10 sample. The underestimation of lactose in the MPI 1 sample may be due to the low lactose level present. In this case a higher dilution factor may help to determine a more accurate level of lactose.

Conclusions

Glucose and lactose biosensors were fabricated using a using a layer-by-layer Chitosan/Enzyme(s)/Chitosan/GA configuration. Initially, 1st generation biosensors were fabricated to assess enzyme activity in the industry required conditions i.e. room temperature at pH 6.0. Electrochemical studies were performed on the sensors to determine their suitability in detection and quantitation of glucose and lactose via CV and CC techniques. The glucose sensor modified with GA showed a linear range of 0–7 × 10^{-3} M with sensitivity of 3.25 × 10^{-6} C $cm^{-2}mM^{-1}$, LOD of 0.51 mM and LOQ of 1.70 mM. The direct lactose sensor resulted in two linear ranges of 2.47 × 10^{-3} to 4.00 × 10^{-2} M with sensitivity of 6 × 10^{-6} C $cm^{-2}mM^{-1}$ and 3.91 × 10^{-2} to 6.92 × 10^{-2} M with sensitivity of 2 × 10^{-5} C $cm^{-2}mM^{-1}$. As the sensors were designed to assess concentration levels of analytes in samples with complex matrices, solution phase mediation employed model mediator $K_3Fe(CN)_6$ to lower the operating potential (E_{app} = 0.3 V vs Ag/AgCl) which

Table III. Comparison of data obtained from the dual biosensor reported here, HPLC-RI and HPLC-IC-PAD in g/100 g, mM and ppm values.

Sample Description	This work (g/100 g)	HPLC-RI (g/100 g)	HPLC–IC-PAD (g/100 g)
MPI 1	1.16	0.6	0.87
MPI 10	1.54	1.7	1.61
	(mM)	(mM)	(mM)
MPI 1	34	17.5	25
MPI 10	45	49.6	47
	(ppm)	(ppm)	(ppm)
MPI 1	11638.2	5990.25	8557.5
MPI 10	15403.5	16978.08	16088.1

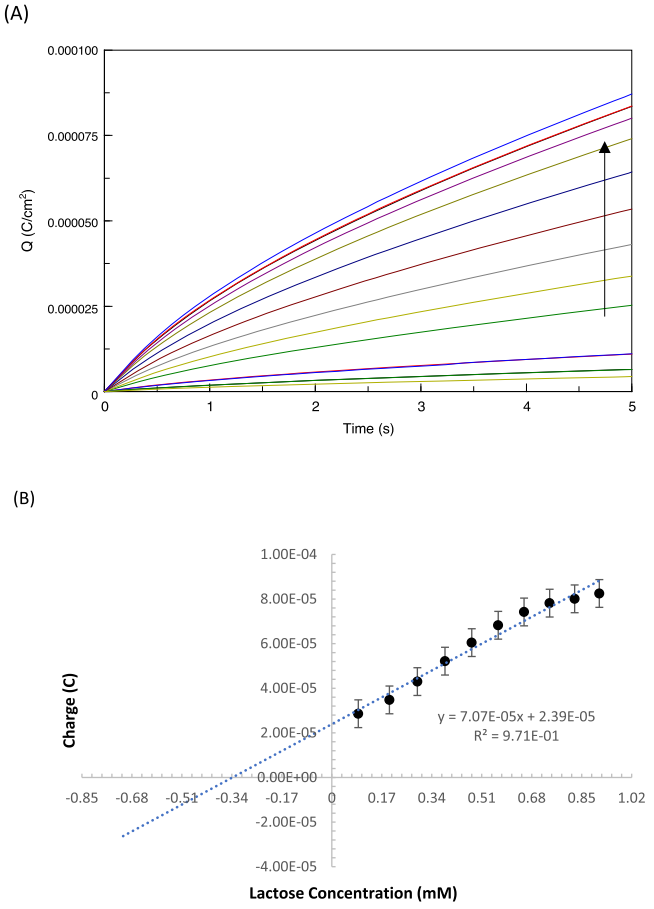


Figure 9. (A) Overlaid CC data of 0.1 M PB (pH 6.0), 5 mM $K_3Fe(CN)_6$ and MPI 1 sample (1% w/v) spiked with 0.09–0.9 mM lactose concentrations with $E_{app} = 0.35$ V vs Ag/AgCl. (B) Extrapolation of corresponding data for charge at 0.35 V vs Ag/AgCl and lactose concentration (mM) in a diluted MPI sample (1% w/v) (n = 3).

improved sensitivity and helped suppress matrix effects. CV and CC experiments were conducted in order to obtain analytical data for the lactose sensor resulting in a linear range of 5.83×10^{-3} to 1.65×10^{-2} M with a sensitivity of 9.41×10^{-4} C cm^{-2} mM^{-1}, LOD of 1.38 mM and LOQ of 4.59 mM.

The use of SECM (redox competition mode) verified enzyme reactivity and approach curves and line scans confirmed the enzymatic catalytic response in the presence and absence of substrate using the enzyme mediator. Area scans were used to identify the enzyme active region of glucose and lactose biosensors in the absence and presence of substrate. Quantitation of glucose and lactose was performed for diluted whey permeate samples. Samples were prepared by diluting 1/100 prior to analysis for glucose background testing and lactose quantitation. The biosensors measured 23.7 mM lactose in the whey permeate sample, correcting for free glucose contributing signals, with 92.2% correlation with results obtained from the sample COA. A solution mediated approach was taken for the analysis of lactose in the case of MPI samples. The lactose biosensor measured 1.16 mM for the MPI 1 and 1.54 mM for the MPI 10 sample. Results were compared with HPLC analysis with 93%–100% correlation between results. Overall, the study performed demonstrated the potential use of the developed biosensors for successful determination of glucose and lactose content in a range of complex dairy samples.

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