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Electrochemical assay of sorbitol dehydrogenase at PEDOT modified electrodes – a new milk biomarker for confirmation of pregnancy in dairy cattle†

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A robust electrochemical assay for sorbitol dehydrogenase (SORDH) activity in milk was developed using voltammetry and chronocoulometry at bare and polymer modified transducers. The motivation for the work was to evaluate the potential of SORDH as an early biomarker of bovine pregnancy using milk as sample matrix. SORDH is an enzyme involved in carbohydrate metabolism converting sorbitol, the sugar alcohol form of glucose, into fructose, with NAD^+ as a cofactor being simultaneously reduced to NADH. The assay was optimised *via* direct NADH oxidation on glassy carbon and screen printed carbon electrodes followed by electropolymerisation of 3,4-ethylenedioxythiophene (EDOT) monomer to form an NADH responsive PEDOT surface which operated well in undiluted milk samples. Assay conditions such as incubation time and temperature were optimised resulting in a 3 min assay at 37 °C in the presence of 10 mM NAD^+ and 20 mM sorbitol co-substrates, enabling NADH electro oxidation (linear range 0.25–5 mM, sensitivity 9.17 μC cm^{-2} mM^{-1} in undiluted milk). SORDH determination followed over the range 0.31–10 U mL^{-1} in milk samples with sensitivity 5.45 μC cm^{-2} U^{-1} mL with LOD 0.0787 U mL^{-1}. The assay was applied to milk sample testing acquired as part of an approved animal study involving control and breeding cycles of dairy cows with focus on analysis at day 19 post artificial insemination. Significant differences between control and pregnant SORDH levels in whole milk animal samples were found (average values 2.57 and 4.07 ng mL^{-1} respectively), as verified using a commercial SORDH ELISA optical assay. Finally, progesterone monitoring over days 16–21 of the oestrous cycle employed an optical ELISA assay and confirmed maintenance of progesterone levels from day 19 onwards.

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1. Introduction

Reproductive performance in dairy cattle is an important factor affecting production and economic efficiency, particularly in seasonal grass based systems. Efficient reproductive management of dairy cattle encompasses heat detection, appropriate timing of insemination, optimisation of management factors affecting embryo survival and pregnancy diagnosis. To ensure an accurate pregnancy diagnosis, the examination should be carried out no sooner than 18 days post breeding.[1] An early diagnosis, ideally at day 21, is critical to the maintenance of an efficient breeding program so that the animal can either be returned to the programme for further AI treatment, or removed.

During the oestrus cycle, progesterone (P4) levels rise and fall depending on the time interval. However, upon establishment of pregnancy, concentrations remain elevated and for this reason, pregnancy determination *via* detection of P4 (>15 ng mL^{-1}) is the gold standard[2] while low P4 concentrations (<5 ng mL^{-1}) have been used to determine heat.[3] Despite the advantages of P4 detection in milk for both heat and pregnancy detection, a point-of-care test device for on farm use is yet to be realised and inter animal variations together with fat partitioning/seasonal fat alterations create challenges in relation to reliable hormone testing.

Maternal systemic and milk molecular profiles at the miRNA, IgG-glycan and protein level, at day 20 post artificial

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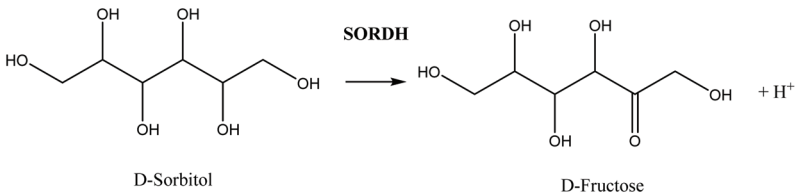


$$NAD^+ + H^+ \longrightarrow NADH$$

Scheme 1 Sorbitol dehydrogenase substrate turnover in presence of NAD^+.

insemination (AI) are reflective of the presence of a developing embryo. Ongoing biomarker evaluation research by the group has identified a candidate protein biomarker, sorbitol dehydrogenase (SORDH) *via* proteomics, being elevated on day 20 of pregnancy. This enzyme catalyses the conversion of D-sorbitol to D-fructose as part of the sorbitol pathway, having the cofactor, β-nicotinamide adenine dinucleotide (oxidised form NAD^+) reduced to NADH as sub-product of the reaction (Scheme 1).

Sorbitol dehydrogenase is located in the cytoplasm of cells and the highest level of activity is in the liver, followed by the kidney.[4] SORDH serum activity has been reported as a valuable marker for the detection of liver function, and its elevation is related to hepatic lipidosis, hepatic necrosis, leptospirosis, fascioliasis and hepatic abscessation in cattle.[5] To date little information exists regarding the role of SORDH in relation to bovine pregnancy; however, some reports may indicate SORDH level changes implicated in energetic alterations as a result of pregnancy. Calves have been found to lose the ability to convert sorbitol to fructose by the 3rd day of life[6] and sorbitol was identified and quantified in sheep blood vessels supplying and draining the placenta with a significant placental release of sorbitol to both maternal and fetal circulations. Fructose, the sub product of SORDH reaction was also taken up significantly by the uterine circulation,[7] while sorbitol was also found to be high in sheep placental tissue, an indication that these polyols could be made within the placenta.[7] Analysing albumin, globulin, glucose and cholesterol concentrations in the blood of dairy cows, Rowlands *et al.* 1980 found changes in the activities of SORDH with pregnancy and lactation, with maximum values, approximately twice those before calving, occurring in week 2.[8]

Dehydrogenases form a large group of redox enzymes,[9] making NADH measurement a constant target in the field of biosensors.[10] As part of cellular functions, the study of NADH-dependent dehydrogenases has been significant in bioreactors, biofuel cells and biocatalysis.[11–13] The electrocatalytic oxidation of NADH to NAD^+ has been reported in numerous works to be an efficient and reliable method to quantify NADH for enzyme-based biosensor applications.[14] The principle challenge of NADH oxidation at conventional electrodes is the high overpotential required, causing background interference and side reactions resulting in electrode fouling.[15] The redox potential ($E^{\circ\prime}$) of the NAD^+/NADH couple is −0.560 V *vs.* SCE or −0.315 V *vs.* NHE (pH 7.0, 25 °C), with a variation of $E^{\circ\prime}$ with pH of −30.3 mV per pH. Direct oxidation of NADH requires an overpotential (>1 V) on bare electrodes,[16] is irreversible and proceeds with coupled side reactions, poisoning the electrode. To overcome this, strategies have evolved using electrocatalysts/redox mediators/conducting polymers, as reported and extensively reviewed.[17–20] A simple, selective, sensitive and cost effective method for the determination of NADH in neutral aqueous solution was elegantly achieved by Prasad *et al.* 2007 *via* use of an electrochemically pre-anodised screen printed carbon electrode (SPCE).[21] In recent years, poly(3,4)ethylenedioxythiophene (PEDOT) modified glassy carbon electrodes have been employed for the determination of biomolecules such as ascorbic acid, dopamine, and uric acid[22–25] and Manesh *et al.* 2008[26] reported the synergistic effect of catechin-immobilised PEDOT modified electrode and gold nanoparticles loaded PEDOT-poly(styrene sulfonic acid) film modified electrode respectively for NADH oxidation. Furthermore, Balamurugan *et al*. 2008 used (PEDOT) on glassy carbon electrode, modified by the electrodeposition of 4-phenyl azo aniline (4-PAA).[20] In the 4-PAA/PEDOT/GC modified electrode, the PEDOT film played an important role to separate oxidation peaks of NADH and ascorbic acid (AA). The modified electrode exhibited a detection limit of 50 nM of NADH in physiological pH. During electro oxidation of EDOT monomer, a radical cation is produced which couples with a second radical cation to form a dication dimer or with another monomer to produce a radical cation dimer. Randriamahazaka *et al.* 1999, reported that the initial stage of electrode deposition was a combination of instantaneous 2D and 3D mechanisms since the electrode deposition was carried out under charge transfer control rather than diffusion-controlled condition.[27] Once the interface region is saturated with oligomers, deposition takes place onto the electrode surface forming "growing nuclei" that expand eventually to produce the desired film.[28]

The work presented here represents a completely novel approach to bovine pregnancy detection, culminating in new biomarker discovery and the capability to electrochemically confirm pregnancy in whole milk samples without pre-treatment. The approach involves:

(a) Rapid one step electrochemical detection of SORDH activity in whole milk samples using direct/indirect NADH detection at conducting polymer modified electrodes (PEDOT);

(b) Confirmation that SORDH levels are elevated in milk samples taken at day 19 post AI (n = 20 animals) with statistically significant levels between control and breeding animal cycles;

(c) Validation of data using a SORDH ELISA kit for the same sample set, together with P4 daily measurement (using both in house and commercial P4 ELISA kit) over the relevant days of the animal's cycle.

The findings represent the first such use of PEDOT films for direct milk analysis and the data generated is a significant advance towards bovine pregnancy biomarker determination, having the advantage of non invasive sample acquisition with minimal sample pre-treatment. The electrochemical enzyme assay also exploits the selective and electrocatalytic properties of PEDOT for NADH measurement, enabling development of a validated SORDH enzyme which is also suitable for portable on site use.

2. Experimental

2.1 Materials

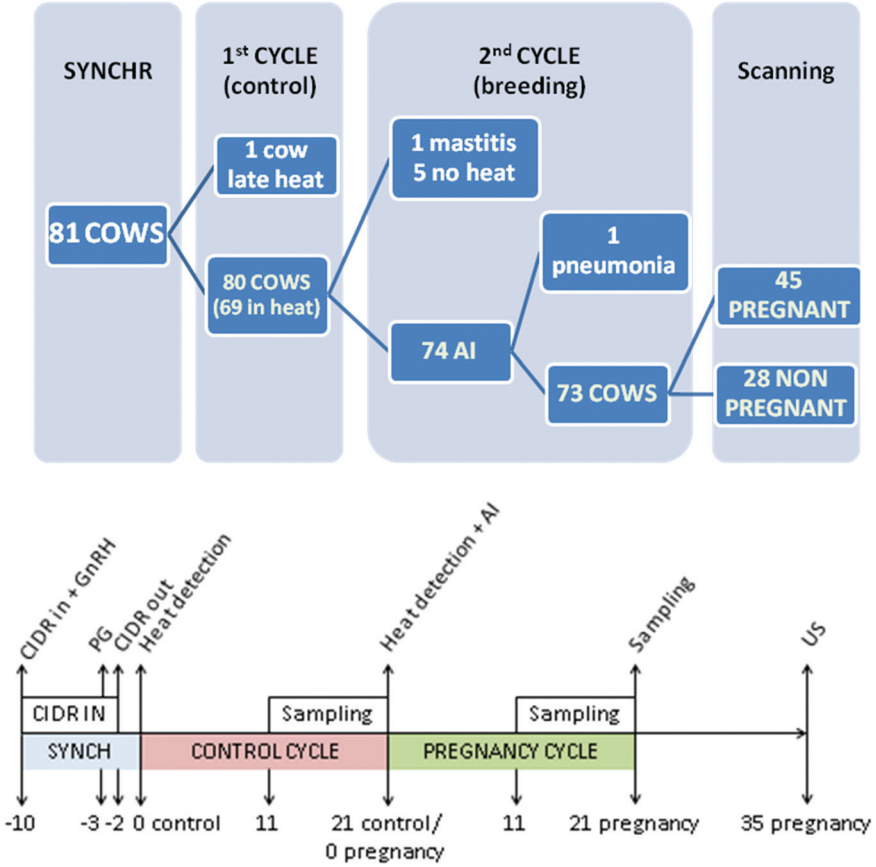
Britton Robinson buffer (B.R. buffer) 0.1 M pH 7, phosphate buffer 0.1 M and commercial pasteurised milk (pH 6.7) were used as assay development medium. NaOH, NAD^+, and NADH, acetonitrile and tetrabutylammonium perchlorate (TBAPC), sorbitol dehydrogenase 40 U mg^{-1} (from sheep liver) sorbitol, 3,4-ethylenedioxythiophene (EDOT), sodium dodecyl sulphate (SDS), anhydrous sodium carbonate, anhydrous magnesium chloride, phosphate buffered saline (PBS) and PBS with Tween detergent (PBST) BioUltra tabs, potassium ferricyanide (99%), bovine serum albumin (BSA ≥96%) and 3,3′,5,5′-tetramethylbenzidine (TMB liquid substrate for ELISA) were purchased from Sigma Aldrich Ireland and used as supplied. Sodium hydrogen carbonate from the British Drug Houses (BDH). Progesterone ELISA kits for milk samples were purchased from Ridgeway Science, UK and used as directed. Anti-progesterone antibody (P4-Ab); AbD Serotec and Fitzgerald (20-1663, 8.8 mg mL^{-1}) were used with horse radish peroxidase labelled progesterone (P4-HRP) from Fitzgerald (80-1255, concentration not provided). Alkaline phosphatase (ALP) was purchased from Fluka, calf intestinal mucosa, 10.6 mg mL^{-1}. Sorbitol dehydrogenase sandwich ELISA kit was obtained from My Bio Source (mybiosource.com). Sorbitol dehydrogenase was diluted in DI water (1 mL), aliquoted in 100 μL parts and stored at −20 °C prior to use. The commercial milk samples were kept at −80 °C and the milk from animal studies was stored at −20 °C prior to use. Deionised water (DI, 18 MΩ) was used in all experiments. Screen printed carbon electrodes (SPCE) were prepared in house using a customised design with the aid of carbon (Dupont) and silver (Gwent) inks. Melinex 339 (white), Melinex O and 3 M adhesive materials were purchased from Cadillac Plastic Ltd, UK.

2.2 Instrumentation

Electrochemical measurements were performed using a CH Instruments Inc. (CH1030) electrochemical workstation. A three electrode set up was used for characterisation purposes with a glassy carbon electrode (GCE) or screen printed electrodes as the working electrode, silver–silver chloride (Ag/AgCl) as the reference electrode and platinum wire (Pt) as counter electrode. GCEs were prepared by polishing with alumina slurries in decreasing size (0.05, 0.3 and 1.0 μm) followed by washing in deionised water (DI) and air dried. The Ag/AgCl reference electrode was stored in concentrated potassium chloride (3 M KCl) between experiments and washed well between use in DI while the platinum wire was washed in DI and stored dry. UV-Visible analysis was carried out using Synergy H1 Hybrid plate reader instruments. Screen printed carbon electrodes (SPCE) were prepared using a DEK 248 screen printer using a screen design with three tracks (29 × 2 mm^2). Carbon ink was used to deposit the conductive layer onto a Melinex O PET (100 μm) substrate with the electrode area defined using custom designed adhesive layer. A DropSens adaptor enabled connection to the potentiostat controller. UV-Visible analysis was carried out on BioTek Synergy H1 Hybrid plate reader. Sensor fabrication used CorelDraw X5 software for design features which were realised using Epilog Zing carbon dioxide (CO_2) laser cutter.

2.3 Procedures

2.3.1 Description of the animal study. All animal procedures performed in this study were conducted by authorised individuals under experimental licence from the Health Products Regulatory Authority (HPRA; project authorisation no. AE18982/P047) as required under EU directive 2010/63, and the animal experiments were approved by the Animal Research Ethics Committee in University College Dublin. A total of 81 Holstein-Friesian dairy crossbred (minimum 56% Holstein) spring calving cows from a commercial dairy farm in Ireland (Carrigeen South, Kildare) were selected for this experiment. The animals were scanned to check for cyclicity and absence of metritis or other reproductive conditions. Cows were milk sampled throughout two consecutive reproductive cycles: a control and a pregnancy cycle (Scheme 2) and samples were always collected during the morning milking period each day (milk). The oestrous cycles of cows were initially synchronised using a progesterone-synchronisation programme. An intra-vaginal progesterone releasing device (CIDR 1.38 g progesterone vaginal delivery system for cattle, Zoetis Ireland Limited, Dublin, Ireland) was placed intra-vaginally for 8 days and 100 μg of GnRH i.m. was injected (ACEGON 50 μg mL^{-1} gonadorelin injectable, Laboratorios Syva, Leon, Spain) at the time of insertion of the progesterone device. A 25 mg prostaglandin injection (LUTALYSE 5 mg mL^{-1} dinoprost, Zoetis Ireland Limited, Dublin, Ireland) was administered the day before removal of the device. Heat detection patches (Estrotec Heat Detector, Rockway Inc., Wisconsin, USA) were applied on the cow's tail heads to facilitate the detection of oestrus. Commencing two days after removal of



Scheme 2 Sampling workflow, including hormone synchronisation (SYNCH), control and pregnancy cycles and pregnancy diagnosis by ultrasound (US) and the timeline in the lower part. CIDR: controlled internal drug release (progesterone); GnRH: gonadotrophin releasing hormone; PG: prostaglandin. Days of the synchronisation are relative to heat detection for the control cycle.

the CIDR devices heat detection was conducted twice daily by observation and lasted for 4 days. Heat detection took place at 08:00 and 16:00 h by observation of oestrous behaviour and activation of the Estrotec patches. Day of oestrus was defined as day 0. During days 0 and 1 relative to oestrus, milk samples (30 mL) were collected during the morning milking (Electronic Tru-Test Meters, Tru-Test Ltd, Ludlow, UK). Samples were collected at the same day of the control cycle or pregnancy (11–21) so results could be compared for the same cow. Heat detection was detected before artificial insemination (AI) as described above, although another observation took place on the field at 19:30 h for about 30 min. A total of 73 cows were artificially inseminated following the morning–afternoon/afternoon–morning 12 hours system. The semen used was commercially available semen used on the farm with individual bulls selected by the herd owner. Straws were thawed in tepid water (35 °C) for 15 seconds and then immediately inseminated. Pregnancy was confirmed at day 35 of pregnancy by ultrasonography and the observation of structures in the uterus compatible with an embryo were considered as indicative of pregnancy. Pregnancy was confirmed in 45 cows.

Milk samples (~20 mL) were collected daily on days 11–21 during the morning milking at approximately 8 am. Samples were placed in ice and brought to the laboratory. Samples of milk for analysis were poured into a 5 mL tube with a preservative (bronopol and natamycin; 800 Broad Spectrum Microtabs II, D&F control systems Inc, Dublin, Ireland). Then the rest of the milk (~15 mL) was centrifuged (4000*g*, 30 min at 4 °C), fat was removed with a spatula and the internatent (whey) samples were aspirated for subsequent glycomic analysis (5 mL). Samples for P4 and SORDH analysis were frozen and stored until analysis at −20 °C.

2.3.2 Screen printed carbon electrode preparation. Carbon ink was used as both working and counter electrodes with silver acting as pseudo-reference electrode. A DEK 248 screen printer enabled transfer of a simple three track design to an underlying PET substrate. The carbon ink was squeezed through the customised screen and onto PET followed by curing at 60 °C for two hours before further use. The silver pseudo reference electrode was deposited onto a single exposed track of the SPCE and was cured for 2 hours at 60 °C. Isolation of the electrode area was achieved by adding a laser cut plastic sheet which was bonded to the underlying PET to expose a defined electrode area (2 mm^2). Individual electrodes were then cut and used together with a DropSens adaptor.

2.3.3 Electrochemical detection of NADH. The direct electrochemical detection of NADH oxidation was first made at a bare glassy carbon electrode (GCE) previously polished as described above and at a SPCE which was printed as per protocol described. The GCE/SPCE activation process involved holding the potential at 1.2 V *vs.* Ag/AgCl in 1 M NaOH for 5 minutes, followed by rinsing in DI water and air drying.[29] The presence of NADH could be identified with an oxidation peak at 0.7 V *vs.* Ag/AgCl, and a serial dilution of a 10 mL solution containing 40 mM of NADH in B.R. buffer pH 7, scan rate 0.2 V s^{-1} *vs.* Ag/AgCl allowed for calibration using both voltammetry and chronocoulometry. Chronocoulometry involved a potential step from 0.3 to 0.7 V *vs.* Ag/AgCl for 5 s and each SPCE was used only once.

2.3.4 Preparation and testing of PEDOT polymer film. The PEDOT film was electrodeposited using a method adapted from Rajaram *et al.* 2015 and followed the above activation process (chronocoulometry at 1.2 V for 5 minutes in 1 M NaOH), after which the electrode was rinsed by DI water and air dried.[28] Chronocoulometry involved application of 1.5 V *vs.* Ag/AgCl for 10 s in a solution of 0.01 M EDOT monomer/0.1 M tetrabutylammonium perchlorate (TBAPC) in acetonitrile (in the case of GCE) and 20 μL of aqueous EDOT solution formed by sonication (20 min) of 25 mL of phosphate buffer (0.1 M pH 7) containing 0.01 M EDOT; 0.01 M SDS; 0.1 M H_2SO_4 in the case of SPCE. Voltammetric quantitation of NADH at the GCE/PEDOT electrode followed and a calibration curve was obtained *via* serial dilution of a 5 mM NADH solution in phosphate buffer (0.1 M pH 7), resulting in a linear response between peak current and NADH concentration.

2.3.5. NADH determination in commercial milk matrix. Following activation and PEDOT deposition a 10 mL solution of NADH at 10 mM in commercial milk (low fat) was prepared, followed by serial dilutions using the same milk. Sample aliquots of 20 μL were deposited onto the SPCE/PEDOT active area and cyclic voltammetry performed at 0.1 V *vs.* Ag/AgCl.

2.3.6 Sorbitol dehydrogenase electrochemical enzyme assay development. The SORDH enzyme assay was developed in commercial milk (pH 6.7), to which the enzyme substrates NAD^+ (10 mM) and sorbitol (20 mM) were added to achieve the target concentrations (in absence of supporting electrolyte) – excess concentrations enabling non-limited SORDH activity to be monitored. The experimental protocol involved background cyclic voltammograms (0.1 V s^{-1}) in (a) commercial low fat milk (pH 6.7) at 37 °C at a bare GCE; (b) as (a) with NAD^+ 10 mM; (c) as (b) with 10 U mL^{-1} SORDH; (d) as (c) with addition of sorbitol 10 mM. The assay reaction time was examined using CV at bare GCEs, scan rate 0.1 V s^{-1} *vs.* Ag/AgCl with background signal being commercial milk (990 μL sample taken to 37 °C for 10 min (n = 3)). After the background reading, 10 μL solution of DI water containing 10 U mL^{-1} SORDH was added to the solution. The 1 mL solution was then equilibrated at 37 °C in order to optimise the production of NADH following which CVs were recorded in 3 min intervals until the NADH oxidation peak remained constant. Following the incubation time study, SORDH calibration was performed in the milk matrix. Once more, samples of 990 μL were spiked with 10 μL SORDH over the range 0.31–10 U mL^{-1}, incubated at 37 °C for 3 min and analysed. The calibration was performed at both bare and PEDOT modified GCEs and SPCEs. In the case of the PEDOT modified surfaces chronocoulometry was employed using a potential step from 0.2 to 0.38 V *vs.* Ag/AgCl for 10 s.

Electrochemical detection of Sorbitol dehydrogenase in raw milk samples from animal study. Milk samples from 20 cows on day 19 post AI, were divided into pregnant, (first AI) and non-pregnant, (second AI) – see section 2.3.1. The chronocoulometric responses (potential step from 0.4 to 0.6 V *vs.* Ag/AgCl for 10 s) and background profiles for each sample were recorded following addition of co-substrates (sorbitol 20 mM, NAD^+ 10 mM) with incubation time 3 min at 37 °C. The assay monitored NADH production in accordance with SORDH activity. The charge signal response was taken at 10 s and the calibration curve used to determine the concentration of SORDH in individual animal milk samples.

2.3.7 Sorbitol dehydrogenase optical ELISA. A commercial sandwich ELISA kit was used to validate the results from the electrochemical assay using the provided samples which were slowly defrosted at room temperature prior to the assay. See ESI(1)† for the ELISA kit scheme with standards (in triplicate) and 18 samples.

2.3.8 In house competitive ELISA method for progesterone testing. ELISA plates were prepared by incubating anti-progesterone antibody (3 μg mL^{-1} in pH 9.6 carbonate buffer) for 1 hour at 37 °C. The plates were then blotted dry and air dried for 1 hour at room temperature. Blocking of any unoccupied sites on the wells was achieved using 1% BSA–PBST followed by air drying for 1 hour at room temperature. Plates were stored in the fridge until further use. The plate and all reagents were removed from the fridge and allowed to come to room temperature for ~1 hour prior to use. 10 μL of milk sample and 100 μL of progesterone – HRP labelled conjugate were added to the wells and incubated at room temperature for 1 hour. Wells were blotted dry and washed ×3 with PBST (200 μL). 100 μL of tetramethylbenzidine (TMB) substrate solution was added and incubated for 15 minutes at room temperature before adding 100 μL of ELISA HCl stop solution. Optical detection was achieved by monitoring absorbance at 450 nm.

2.3.9 Commercial progesterone ELISA test kit. The plate and all reagents were removed from the fridge and allowed to come to room temperature for ~1 hour prior to use. 10 μL of milk standard or sample was added to the wells along with 200 μL of the provided progesterone – enzyme label and incubated at room temperature for 1 hour. Wells were blotted dry and washed ×3 with water. 200 μL of the provided substrate solution was added and incubated for 20 minutes at room temperature. Optical detection was achieved by monitoring absorbance at 551 nm.

3. Results and discussion

3.1 Characterisation of PEDOT polymer film and NADH interaction studies

GCE/PEDOT films were prepared and tested for their response to NADH oxidation using cyclic voltammetry in 0.1 M phosphate buffer (pH 7.0) containing 5 mM NADH (Fig. 1(A). Compared with the reaction at bare electrode, the PEDOT/GCE exhibited greater current response with an anodic shift in oxidation potential (E_p = 0.318 V) for NADH oxidation. Fig. 1(B) shows the response over the range 1.25–5 mM with sensitivity 16.772 μA cm^{-2} mM^{-1}, r^2 = 0.99 for n = 3. A scan rate study at the GCE/PEDOT electrode was performed in 5 mM NADH (Fig. 1(C)) with linear relationship evident between peak current and $v^{\frac{1}{2}}$ indicating a diffusion controlled NADH oxidation process while the anodic peak potential shift confirms the irreversible oxidation process.

A calibration curve was generated in an undiluted commercial milk matrix using NADH spiked milk at GCE/PEDOT using both voltammetry (Fig. 2(A)) and chronocoulometry (Fig. 2(B)). The response to 0.625–5 mM NADH was evident with $E_{p(a)}$ 0.4 V. The same experiment was performed using SPCE with very similar response (ESI(2)†) with a cathodic shift in $E_{p(a)}$ to 0.3 V due to the printed reference electrode. Sensitivity based on voltammetry was 1.067 μA cm^{-2} mM^{-1} and 1.21 μA cm^{-2} mM^{-1} for GCE/PEDOT and SPCE/PEDOT respectively with r^2 0.988 and 0.9914 for n = 3. The ability of the PEDOT film to catalyse NADH oxidation successfully in undiluted milk is clear from the voltammograms and chronocoulometric responses with "clean" background signals evident.

3.2 Sorbitol dehydrogenase enzyme assay optimisation at unmodified screen printed electrodes

Voltammograms were recorded in commercial milk in the presence of 10 mM NAD^+, 20 mM sorbitol and 10 U mL^{-1} SORDH, with current monitored over the time period 0–20 min at 37 °C. Maximum signal was realised at 3 min (data not shown) followed by a plateau and hence 3 min incubation times were employed hereafter. An important control study followed (Fig. 3) which confirmed no current signal in the absence of either NAD^+ or sorbitol co-substrates, while both combined in milk resulted in NADH oxidation due to enzyme turnover. Studies were carried out at a bare GCE with samples held at 37 °C with incubation time of 3 min as per procedure described in section 2.3.6.

CVs at bare SPCEs were then recorded for measurement of SORDH activity in commercial milk over the range 0.31–10 U mL^{-1} (Fig. 4(A)) with calibration curve showing signal saturation >5 U mL^{-1} (inset). A chronocoulometric study was performed at bare SPCE over the same SORDH range in undiluted milk (Fig. 4(B)) showing raw data and calibration curve. This study supports the direct detection of NADH re-oxidation at SPCE in response to increasing SORDH activity in a milk matrix, following which we examined the ability of PEDOT films to lower background milk signals while realising effective NADH determination.

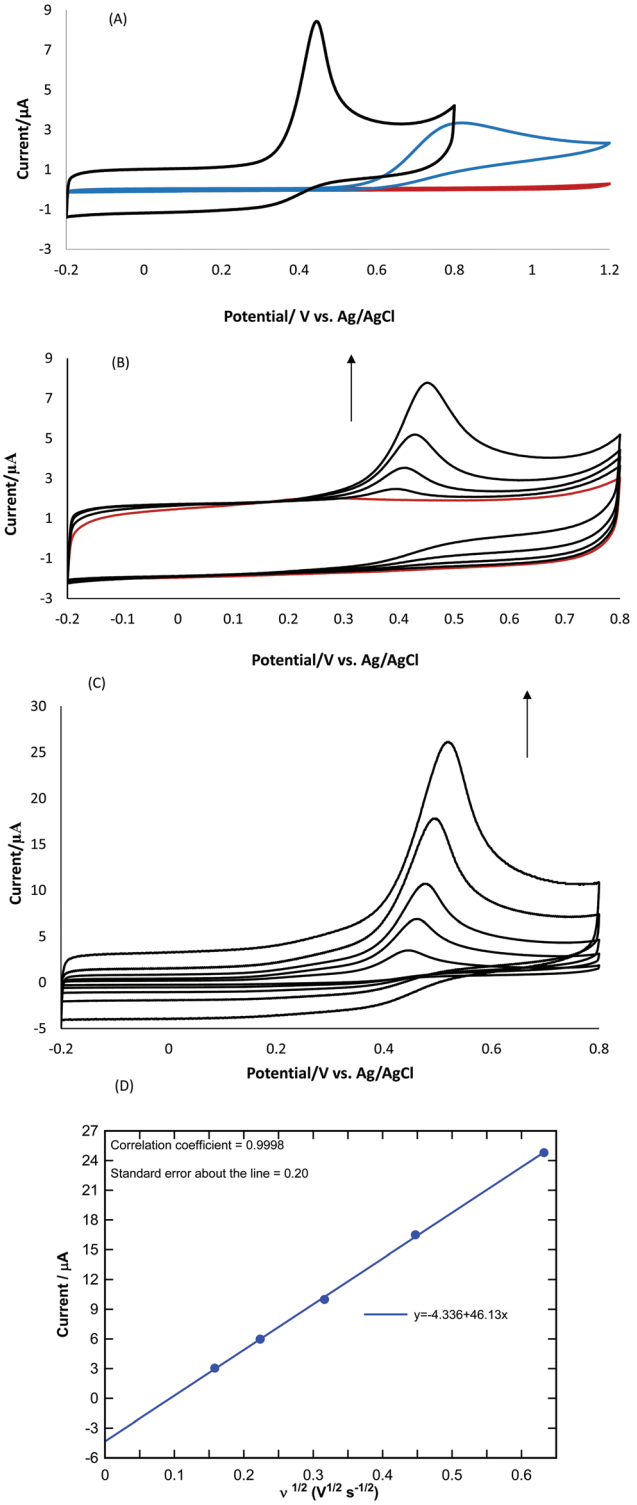


Fig. 1 (A) Voltammetric study of GCE/PEDOT in B. R. buffer 0.1 M (pH 7) as background (red curve); 5 mM NADH detected at bare GCE (blue curve); 5 mM NADH in BR buffer pH 7 detected using GCE/PEDOT (black curve) at scan rate 0.1 V s^{-1} *vs.* Ag/AgCl. (B) Interaction of PEDOT film with NADH 1.25, 2.5 and 5 mM at 0.1 V s^{-1} *vs.* Ag/AgCl at room temperature, background red curve. (C) Scan rate study at GCE/PEDOT in 5 mM NADH in phosphate buffer 0.1 M (pH 7) 0.025–0.4 V s^{-1} with (D) plot of peak current *vs.* square root of scan rate showing a linear relation (R^2 = 0.98734) (n = 3).

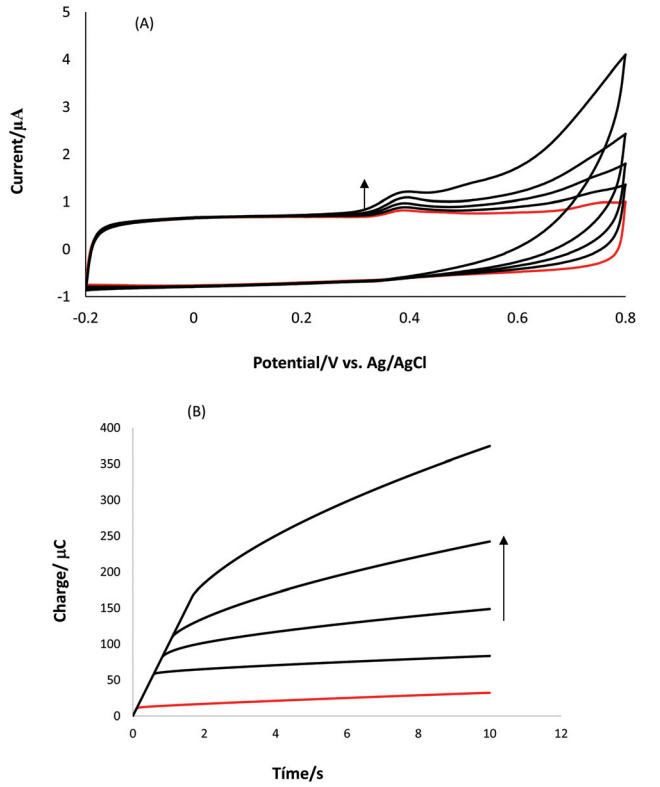


Fig. 2 (A) Voltammetric study at GCE/PEDOT in commercial low-fat milk (pH 6.7) – response to increasing NADH concentrations 0.625–5 mM at scan rate 0.1 V s^{-1} *vs.* Ag/AgCl (background in red). (B) Chronocoulometry detection of NADH at GCE/PEDOT over the range 0.31–5 mM NADH (background in red) with sensitivity 9.17 µC cm^{-2} mM^{-1}. Potential step was 0.2 to 0.38 V *vs.* Ag/AgCl for 10 s.

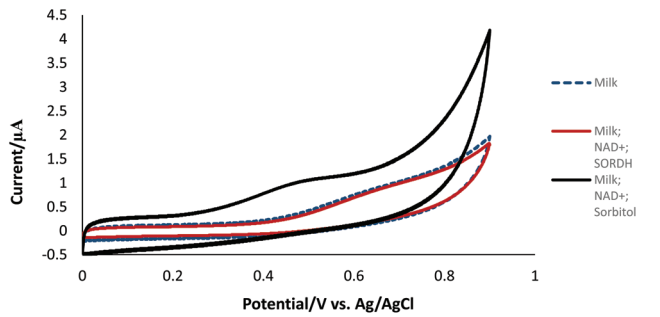


Fig. 3 Voltammograms of commercial milk recorded at SPCEs at 37 °C (dashed line), commercial milk at 37 °C in the presence of NAD^+ 10 mM with addition of 10 U mL^{-1} of SORDH (red curve – control); commercial milk at 37 °C in the presence of co-substrates NAD^+ 10 mM and Sorbitol 20 mM (black curve), scan rate 0.1 V s^{-1} *vs.* Ag/AgCl.

3.3 Sorbitol dehydrogenase enzyme assay optimisation at PEDOT modified GCEs

Fig. 5(A) below shows the cyclic voltammetric response to 10 U mL^{-1} SORDH in spiked commercial undiluted milk with curve response described by regression equation $y = 0.1768 \ln(x) + 0.2498$ while Fig. 5(B) shows the chronocoulometric response

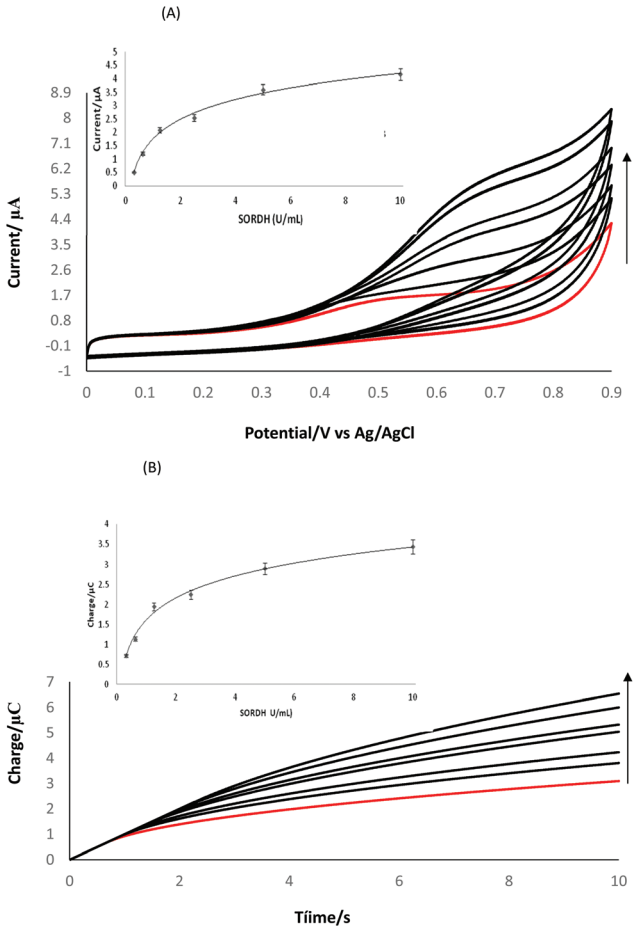


Fig. 4 (A) Voltammetric study at bare SPCE, in commercial milk (low fat) (pH 6.7), scan rate 0.1 V s^{-1} *vs.* Ag/AgCl, incubation time 3 minutes at 37 °C in the presence of 10 mM NAD^+, 20 mM sorbitol as background with additions of SORDH 0.31–10 U mL^{-1}; insert corresponding concentration curve ($y = 1.0641 \ln(x) + 1.7513$ current taken at I_p, $R^2 = 0.9936$, ($n = 3$). (B) Chronocoulometric data with applied potential step from 0.4–0.8 V for 10 seconds *vs.* Ag/AgCl, using bare SPCE, in commercial milk (pH 6.7), with incubation time 3 minutes at 37 °C in the presence of 10 mM NAD^+, 20 mM sorbitol as background (red curve); and SORDH in increasing concentrations from 0.31–10 U mL^{-1}; insert corresponding concentration curve ($y = 0.7896 \ln(x) + 1.6174$ $R^2 = 0.9918$ charge taken at 10 s ($n = 3$).

over a similar range with superior linearity evident (up to 10 U mL^{-1}) with sensitivity 5.45 µC cm^{-2} U mL^{-1} ($n = 3$) (Fig. 5(C)).

The data presented here represents (to the best of our knowledge) the first such report of SORDH electrochemical measurement based on NADH re-oxidation at either bare or modified carbon electrodes. In order to verify the use of SORDH as a bovine pregnancy biomarker, a key advance for on-farm fertility management, the animal study described in section 2.3.1 enabled access to milk samples from control and breeding cycle animals at a range of days post AI. For the SORDH assay we focused on testing at day 19 as this is the pivot point in the oestrous cycle of the animal where changes in hormones such as progesterone are evident as putative biomarkers.[2] While SORDH assay development in milk worked

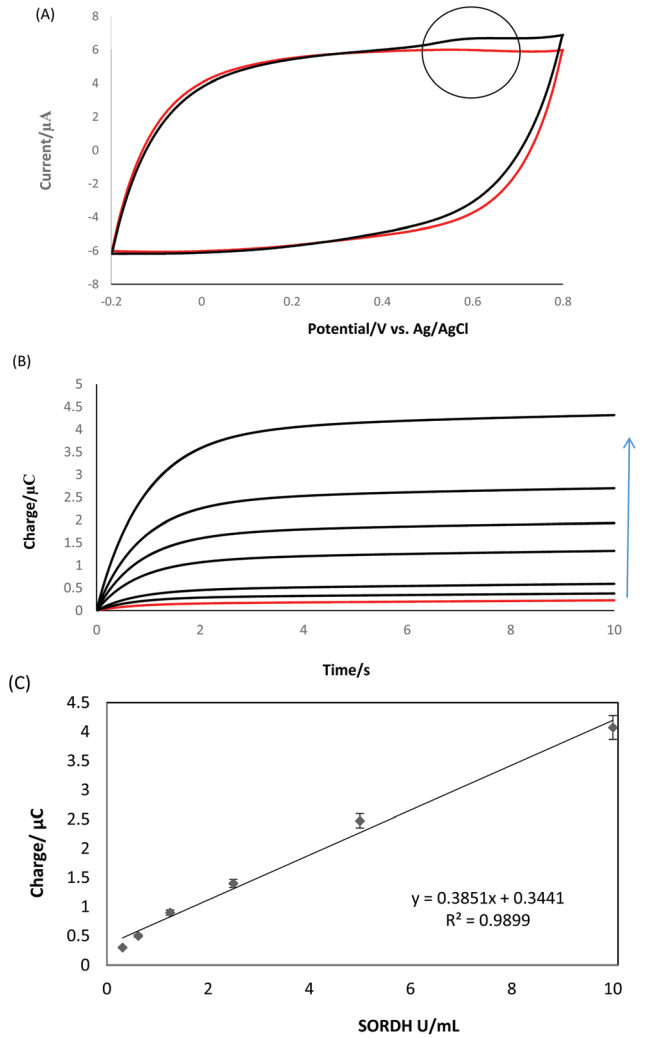


Fig. 5 (A) Voltammetric study showing response to SORDH at GCE/PEDOT, in commercial milk (pH 6.7), scan rate 0.1 V s^{-1} *vs.* Ag/AgCl, with incubation time 3 minutes at 37 °C in the presence of 10 mM NAD^+, 20 mM sorbitol – background (red curve); and SORDH 10 U mL^{-1} (black curve). (B) Chronocoulometric data at GCE/PEDOT with applied potential step from 0.4–0.6 V for 10 seconds *vs.* Ag/AgCl, under same conditions as (A) background (red curve) and SORDH in increasing concentrations from: 0.31 to 10 U mL^{-1}. (C) Corresponding concentration curve, showing linear relationship (R^2 = 0.9899), (n = 3).

well on SPCEs (as shown above) the ability to renew the GCE surface between samples resulted in improved signal reliability in relation to the animal study/samples and therefore GCEs were employed going forward.

3.4 Sorbitol dehydrogenase detection in pregnant and control animal raw milk samples at PEDOT modified GCEs

The animal study involved synchronising 81 animals which had milk samples taken over the course of 2 oestrous cycles. This first cycle provided control milk samples in the absence of pregnancy. On the second cycle, one animal was found to have mastitis and 5 animals did not go into heat and so artificial insemination (AI) was performed on 74 cows with subsequent scans revealing 45 pregnant animals and 28 non-pregnant (Scheme 2). This allowed for comparison of SORDH, in addition to verification of P4 alterations (day 19), for the same animal under both pregnant and non-pregnant conditions. In relation to P4 measurement all samples provided were analysed using both a commercial (Ridgeway) progesterone ELISA kit and an in house developed ELISA optical assay (see section 2.3.8).

Fig. 6 shows an example of the electrochemical SORDH responses (animal code 2287) corresponding to milk samples taken at day 19 in both control and breeding cycles. The voltammetry (A) and chronocoulometry (B) clearly showed an increased signal associated with NADH re-oxidation for the pregnant sample relative to control (clear signal E_p = 0.4 V indicating elevated SORDH levels in the milk samples.

Chronocoulometry was selected as the chosen method for all animal sample testing with analysis shown in Fig. 7(A) – SORDH quantitative milk analysis for pregnant and control samples (n = 3). The high variation of background from different milk samples was corrected using each milk sample as its own background before the assay was performed. A student t-test: paired two sample for means at $p < 0.05$: P (two-tail) = 0.04 gave a statistically significant difference between control and breeding sample levels confirming the (almost 2 fold) increase in SORDH in bovine pregnancy indicating its

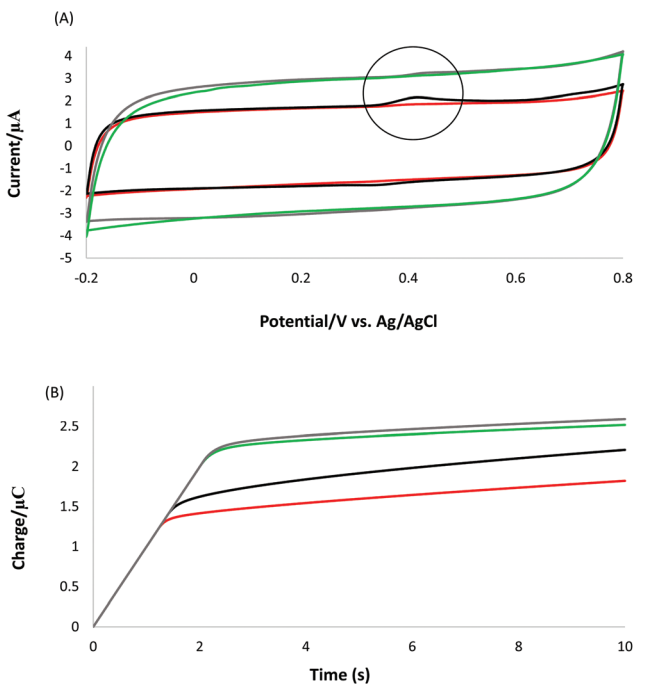


Fig. 6 (A) Cyclic voltammograms at GCE/PEDOT from −0.2 to 0.8 V, scan rate 0.1 V s^{-1} *vs.* Ag/AgCl for pregnant and non-pregnant samples from animal code 2287 on day 19; (red curve) being pregnant background, and (black curve) pregnant sample; (green curve) being non-pregnant sample background and (grey curve) the non-pregnant sample after assay was performed. (B) Corresponding chronocoulometric study at GCE/PEDOT (potential step 0.35 to 0.45 V *vs.* Ag/AgCl for 10 s) for the same sample (animal 2287) – colour coding as (A).

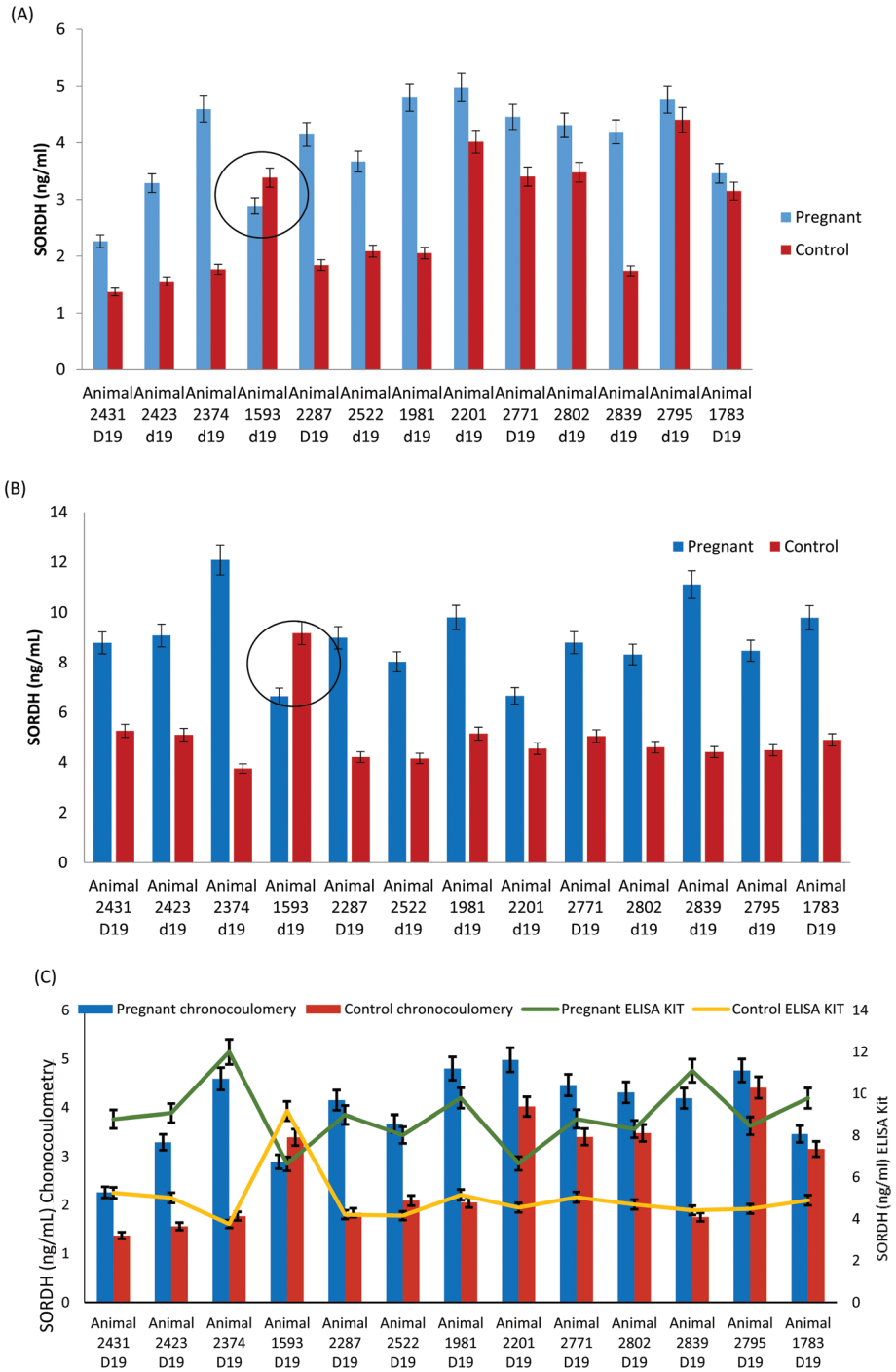


Fig. 7 (A) SORDH (ng mL^{-1}) levels found using the electrochemical chronocoulometric assay at PEDOT/GCE for milk samples taken at day 19 post AI pregnant samples (blue) and non-pregnant (control) samples (red). Assay conditions as per Fig. 5. Student *t*-test: paired two sample for means at $p < 0.05$: P (two-tail) = 0.04. (B) Sandwich optical ELISA data for animal samples. Student *t*-test: paired two sample for means at $p < 0.05$: P (two-tail) = 0.0001. (C) Comparative animal test data for both electrochemical and ELISA kit SORDH results. Green line corresponds to ELISA test results for pregnant samples and yellow ELISA test results for control samples.

high diagnostic value as an early pregnancy biomarker. Fig. 7 (B) shows the validation data from a SORDH ELISA optical assay (see ESI(3)† for microwell optical data and calibration curve), resulting in identical trends with respect to a statistically significant increase in SORDH in the pregnancy samples for each animal – student *t*-test: paired two sample for means $P < 0.05$; P (two-tail) < 0.0001. The commercial ELISA kit over estimated the SORDH level (relative to the electrochemical assay), with possible contribution from the milk matrix proteins and the higher dynamic range (20–300 ng mL^{-1}).

Fig. 7(C) summarises findings with comparative data (control and pregnancy samples) for the electrochemical and optical methods confirming the consistently higher levels of SORDH found in pregnant milk samples (see ESI(5)† for correlation plot of electrochemical *vs.* ELISA data). The milk sample from animal 1593 was an outlier and showed the reverse of this trend in both assays and was thought to be spoiled/degraded. SORDH activity was detected over a lower range in the case of the EC assay which may have benefits in relation to measurement of relative SORDH changes over days 16–21.

Shaw (1974) reported serum levels of SORDH in cattle at 3.2 ± 1 mU mL^{-1} (ref. 30) while Horney *et al.* 1993 reported a reference range for Holsteins as 32 ± 26 U L^{-1} based on serum analysis of 71 clinically healthy animals[31] (no milk levels are available). In this work, average SORDH values of 4.07 and 2.57 ng mL^{-1} (1.62×10^{-4} and 1×10^{-4} U mL^{-1}) were obtained for the electrochemical assay for pregnant and control samples respectively while the ELISA kit resulted in average values of 9.15 and 4.63 ng mL^{-1} (3.66×10^{-4} and 1.85×10^{-4} U mL^{-1}) for pregnant and control samples respectively (in each case data from animal 1593 was excluded). These values ranged from 0.1 mU mL^{-1} to 0.366 mU mL^{-1} which would correlate with a milk/serum ratio of 0.1%.

3.5 Pregnancy verification using progesterone monitoring for pregnant and control animal raw milk samples using ELISA

Monitoring changes over days 16–21 post AI are most significant as day 19 is where a drop in progesterone concentration is observed in the absence of bovine pregnancy. Both breeding and control samples for the same animals were provided over this range of days and samples measured with $n = 4$. Fig. 8 shows average progesterone levels determined using (A) commercial P4 ELISA kit (Ridgeway) and (B) in house developed competitive ELISA with optical (TMB) detection (see ESI(4)† for calibration data (0.769 AU ng^{-1} mL, LOD 0.54 ng mL^{-1} and LOQ 1.8 ng mL^{-1}). Optical analysis on the in house ELISA assay was achieved using progesterone standards prepared in phosphate buffered saline (PBS, 0.1 M, pH 7.4) as calibrators. A strong correlation was found between optically determined progesterone concentrations with commercial ELISA kit results. In both cases, up to day 18 there was little difference in progesterone concentration between breeding and control samples. Day 19 is when a significant change (student *t*-test two tailed *P* value = 0.0019) was observed with control sample P4 levels dropping at this point to an average of 4.04 ng mL^{-1} relative to the breeding samples (average 19.99 ng mL^{-1}) over days 19–21. The large standard error associated with this data was to be expected as the results represent the average of 11 different animal samples and it is accepted that there is wide variation in P4 levels from animal to animal – subject to genetic, seasonal and dietary differences and stage of milking. These factors together with % fat variability, which influences P4 partition ratio in milk, limits P4 analysis alone as a pregnancy indicator for on farm use. The most important conclusion from this part of the study is that the progesterone levels remain elevated for the pregnant group (day 19 – relative to control levels), thus providing further verification of SORDH differences at this time point.

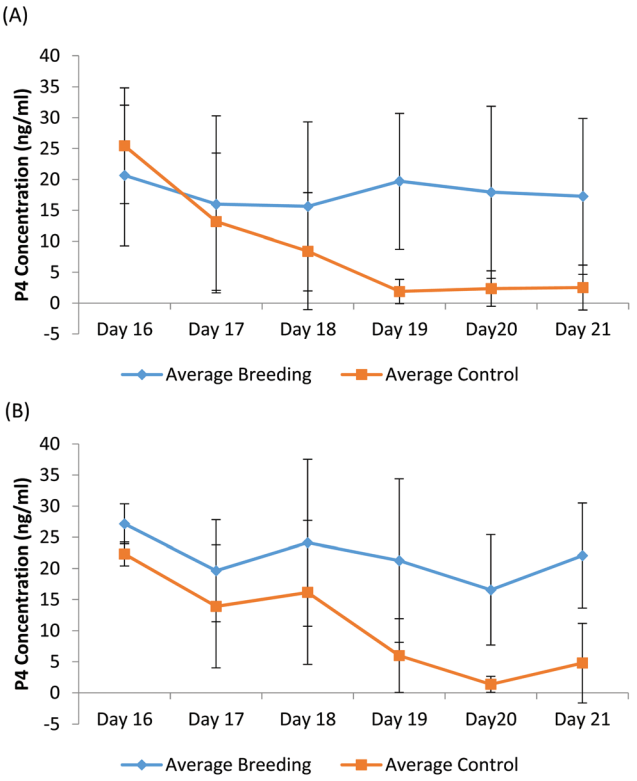


Fig. 8 (A) Commercial P4 ELISA kit and (B) in house P4 competitive ELISA assay with optical detection at 450 nm. Averages of 11 individual animal samples are presented for both control and pregnant groups over a 6 day period.

4. Conclusions

Reliable and low cost bovine pregnancy confirmation remains a key requirement for herd management and dairy productivity. Milk is a convenient and easily obtained sample for such purposes and progesterone testing plays a role in pregnancy verification with some limitations with respect to on-farm use. Biosensing can contribute to this goal and in this work we have developed a rapid (3 min) electrochemical enzyme assay for use with both commercial and raw animal milk samples (whole milk, undiluted) based on PEDOT films at carbon transducers. NADH electro oxidation was enabled (sensitivity 1.067 μA cm^{-2} mM^{-1} and 9.17 μC cm^{-2} mM^{-1} for voltammetry and chronocoulometry respectively) in undiluted milk in accordance with SORDH activity (linear range 0.31 to 10 U mL^{-1}, sensitivity 5.45 μC cm^{-2} U^{-1} mL^{-1} with LOD 0.0787 U mL^{-1} in milk using chronocoulometry). The assay was applied to milk samples acquired as part of an approved animal study and statistical analysis of the data obtained suggested significant differences between control and pregnant SORDH levels in milk, as verified using a commercial

SORDH ELISA optical assay. Based on this small animal number study we propose SORDH as a very interesting potential biomarker for pregnancy confirmation in dairy cattle at day 19 post AI, lending itself to electrochemical determination in a facile and robust fashion. Based on this initial data set it would not be prudent to set a cut-off SORDH value for pregnancy indication and a more extensive set of samples over the appropriate timescale are required to monitor SORDH trends prior to and after day 19.

Verification of the changes at day 19 for the same set of samples was performed using an in house developed P4 competitive assay with P4 levels monitored over a 6 day period, remaining elevated in the case of the pregnant group (full details on the P4 assay development and extended analysis/validation will be the subject of further work). Extension to a greater number of samples and further analysis of the SORDH trend during early pregnancy is warranted as a next step, as any differences <19 days will be a key advance. This will further refine the electrochemical assay and performance and pave the way towards an electronic portable test kit for onsite purposes with use of dried reagents on a printed test strip. Such a system has the potential for in-line milk sampling for automated pregnancy diagnosis in milking parlours and can be multiplexed with P4 measurement as required.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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