



# Development of quantum dots-based biosensor towards on-farm detection of subclinical ketosis

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## ABSTRACT

Early detection of dairy animal health issues allows the producer or veterinarian to intervene before the animals' production levels, or even survival, is threatened. An increased concentration of  $\beta$ -hydroxybutyrate ( $\beta$ HBA) is a key biomarker for diagnosis of subclinical ketosis (SCK), and provides information on the health stress in cows well before any external symptoms are observable. In this study, quantum dots (QDs) modified with cofactor nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) were prepared for the sensing event, by which the  $\beta$ HBA concentration in the cow's blood and milk samples was determined via fluorescence analysis of the functionalized QDs. The detection was performed on a custom designed microfluidic platform combining with a low cost and miniaturized optical sensor. The sensing mechanism was first validated by a microplate reader method and then applied to the microfluidic platform. Standard  $\beta$ HBA solution,  $\beta$ HBA in blood and milk samples from cows were successfully measured by this novel technology with a detection limit at a level of 35  $\mu\text{M}$ . Side by side comparison of the developed microfluidic biosensor with a commercial kit presented its good performance.

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## 1. Introduction

When the modern dairy cow transitions from a non-lactating state to a lactating state during the periparturient period, she undergoes tremendous physiological challenges resulting in an overall state of immune suppression (Ingvarsen and Moyes, 2013). These changes are characterized by inflammatory conditions, susceptibility to metabolic disorders, uterine and mammary infections, and loss of appetite (Sordillo and Mavangira, 2014). These stresses are further complicated by the fact that the sudden initiation of an increase in milk production means that the animal is metabolically in a state of negative energy balance during the immediate postpartum period (approximately 8–10 weeks). Many of the stress symptoms that individual cows experience go unnoticed. Not only are the animals' health and welfare compromised, but there are also significant economic implications. To have reliable biomarkers of inflammatory and health status that can be routinely measured is of critical importance to detect, intervene, and manage at-risk cows as early as possible.

Subclinical ketosis (SCK) is known as elevated concentrations of circulating ketone bodies in the absence of clinical signs, has been

found to be associated with high risk of clinical ketosis and displaced abomasums (Goldhawk et al., 2009). In addition, subclinically affected cows appear to have reduced milk production than those with normal ketone body concentrations (Seifi et al., 2011). The economic losses caused by SCK may be huge due to the treatment costs, decreased milk yield and decreased reproductive efficiency (McArt et al., 2011). An estimated 40–60% of cows in North America dairy herds are affected by early lactational incidence of SCK, which is much higher than the 2–15% incidence with clinical ketosis (Gordon et al., 2013; McArt et al., 2011). Determination of  $\beta$ -hydroxybutyrate ( $\beta$ HBA) is considered as the golden standard to diagnose subclinical ketosis. Cows with  $\beta$ HBA in their blood, milk, and urine experience a period of negative energy balance (Nydham et al., 2013). The detection of ketosis clinical signs varies, but blood  $\beta$ HBA concentration ranging from 1.2 to 2.9 mM is diagnosed with SCK (Iwersen et al., 2009; McArt et al., 2011; Ospina et al., 2010).

Early detection of SCK by measurement of  $\beta$ HBA concentration will help farmers, as these diseases generally have a poor prognosis for production and survival (Brozos et al., 2011).  $\beta$ HBA in cows' blood and milk are commonly used to diagnose SCK. Monitoring blood  $\beta$ HBA is superior to monitoring urine due to its stability (Seifi et al., 2011; Voyvoda and Erdogan, 2010). At present, such tests may be determined spectrophotometrically by traditional clinical laboratory means and would require the producer to send samples to a lab and wait for the results, which is

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inconvenient, and expensive (Townsend and Eastridge, 2011). The advantage of detecting blood  $\beta$ HBA is that no rigorous sample handling precautions are required during the specimen transportation due to the reasonable stability of  $\beta$ HBA in blood. One obvious advantage of using milk instead of blood for analysis of ketosis is the non-invasive nature of milk sampling. However, because of its non-transparent nature, opacity of the sample is incompatible with spectrophotometry. Consequently, milk needs extensive pre-treatment when using traditional analytical methods, typically, precipitation, centrifugation, and special harvesting are necessary (Larsen and Nielsen, 2005; Nielsen et al., 2003). Current cow-side milk testing techniques for  $\beta$ HBA are not accurate and only semi-quantitative results are obtained (Larsen and Nielsen, 2005; Voyvoda and Erdogan, 2010).

Microfluidics, an emerging technology, provides new approaches for detection of toxins in food supplies (Neethirajan et al., 2011) and has been widely used in biosensor application, which is mainly used to enhance analytical performance by miniaturization. Microfluidic biosensors present distinctive advantages such as significantly increasing sensitivity, reducing assay time and sample/reagent consumptions (Mairhofer et al., 2009). Quantum dots (QDs) are semiconductor nanocrystal possessing unique optical properties due to quantum confinement effects (Algar et al., 2010). Properties such as sharp emission spectra, broad absorption spectra, higher quantum yields and tunable emission frequencies make QDs as an excellent fluorescence labels for Förster resonance energy transfer (FRET) or electron transfer (ET) processes based

applications (Ma and Su, 2011). The integration of the microfluidic biochips and quantum dots (QDs) allows for capitalizing on benefits such as low analyte volume, portability, low detection limit and superior sensitivity and specificity. Quantitative detection capabilities for identification and detection of chemical species and biomarkers through the integration of a portable optical biosensor and microfluidic biochip will lead to the next generation of diagnostic devices and open up future applications.

In this study, a sensitive microfluidic biosensor was developed towards point-of-care (on-farm) sensitive detection of  $\beta$ HBA in serum and milk samples. As shown in Fig. 1(A), the biosensor is based on enzymatic catalysis of  $\beta$ HBA to acetoacetate, which concomitantly converts cofactor nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) to its reduced form  $\beta$ -nicotinamide adenine dinucleotide (NADH). The  $\text{NAD}^+$  here is pre-conjugated with fluorescent nanoparticles quantum dots (QDs). The  $\text{NAD}^+$  has been reported to partially quench the fluorescence of the conjugated QDs through an electron transfer (ET) process (Akshath et al., 2012; Freeman and Willner, 2008), while the reduced form NADH has no effect on QDs fluorescence. The change of fluorescent intensity from cofactor conjugated QDs can indicate the change of  $\beta$ HBA concentration in samples. By further coupling with a microfluidic platform with optical detection unit, this biosensor is capable of providing a rapid and accurate measurement of  $\beta$ HBA from a small volume of blood and milk samples.

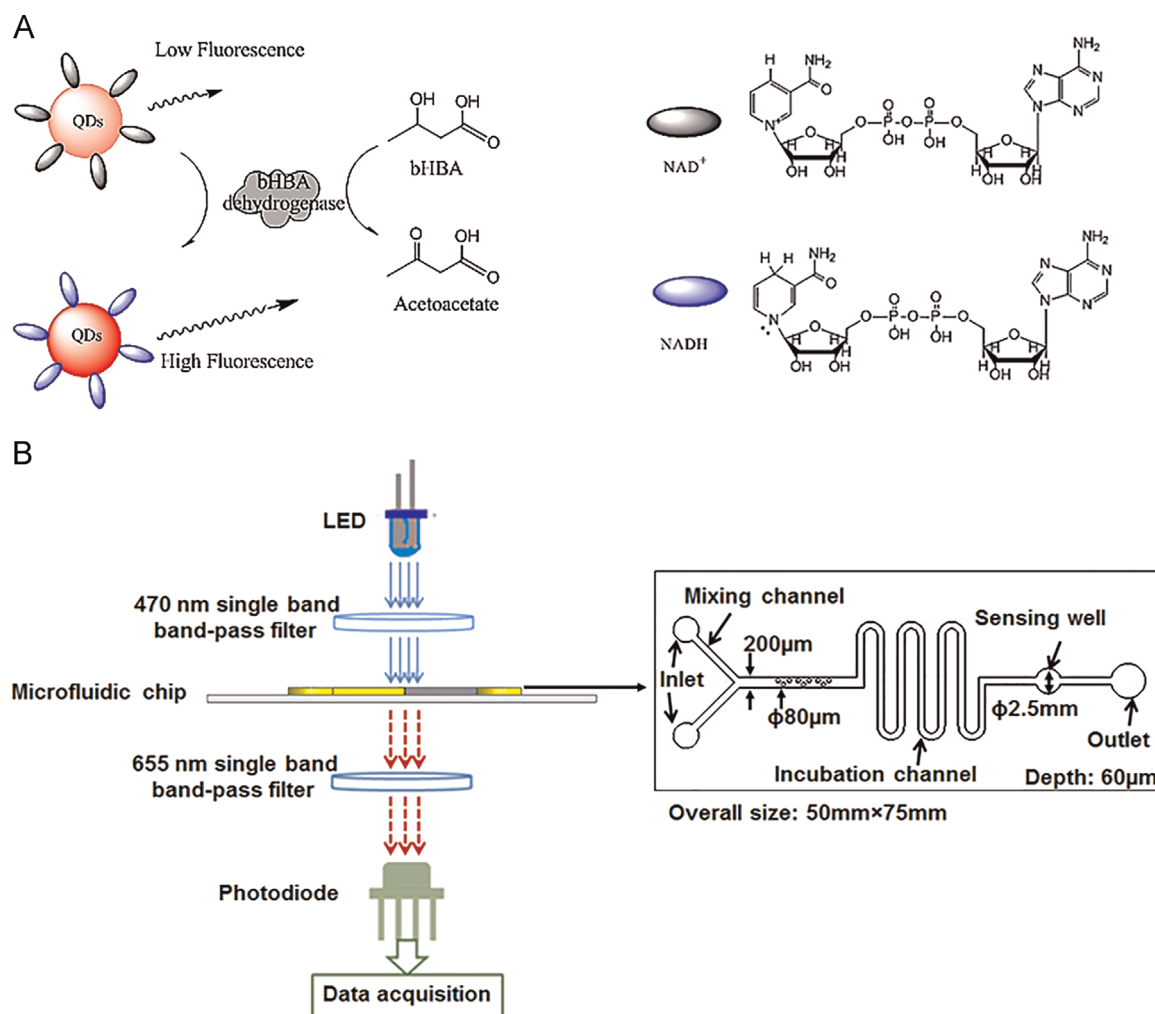


Fig. 1. (A) Schematic illustration of sensing mechanism. (B) Sketch of microfluidic platform combining with the low-cost, miniaturized optical sensor.

## 2. Materials and methods

### 2.1. Reagents and sample preparation

$\beta$ -hydroxybutyric acid, nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ),  $\beta$ -Nicotinamide adenine dinucleotide, reduced ( $\text{NADH}$ ), 3-aminophenylboronic acid monohydrate (APBA), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), bovine serum albumin (BSA), Tetrahydrofuran (THF) and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada).  $\beta$ -hydroxybutyrate dehydrogenase was ordered from Roche Diagnostics (Laval, Quebec, Canada). All reagents were of analytical reagent grade. Carboxyl quantum dots were purchased from life technologies (Qdot<sup>®</sup> 655 ITK<sup>™</sup> Burlington, ON, Canada). Milk was purchased from a local grocery store.

The  $\beta$ HBA standard solution ranging from 1.6  $\mu\text{M}$  to 1000  $\mu\text{M}$  was made by diluting the 3-hydroxybutyric acid with PBS buffer and was used for the  $\beta$ HBA standard curve. Serum samples with known  $\beta$ HBA concentration analyzed by using a Roche Cobas 6000 c501 automated chemistry analyzer (Roche Canada, Laval, QC, Canada) were provided by our collaborator (Ontario Veterinary College, Canada) and the detailed sample preparation procedure can be found elsewhere (Gohary et al., 2014). Briefly, cow's blood was firstly collected from the coccygeal blood vessels in a vacuum tube without anticoagulant and stored in a cool place. Collected sample was then centrifuged to harvest serum at 4 °C within 6 h after collection at 2990g and stored at –20 °C for further use. Before assaying, the serum sample was thawed at room temperature and diluted to a series of concentration ranging from 10  $\mu\text{M}$  to 1000  $\mu\text{M}$  with PBS buffer. Milk was centrifuged at room temperature at 2300g for 3 min twice and then the suspension was removed. Fortified milk sample solutions were prepared by spiking  $\beta$ HBA concentration ranging from 0 to 1000  $\mu\text{M}$  to the centrifuged milk for further use.

### 2.2. Preparation of functionalized QDs nanoprobe

To prepare  $\text{NAD}^+$ / $\text{NADH}$ -functionalized QDs, commercialized carboxyl group capped QD nanoparticles were firstly modified with 3-aminophenyl boronic acid (APBA) through carbodiimide reaction. In a typical preparation, 50  $\mu\text{L}$  of 8  $\mu\text{M}$  stock solution of carboxyl QDs was mixed with 50  $\mu\text{L}$  of 10 mg/mL of APBA dispersed in PBS/THF solution (v/v, 1/1). After 3 min of gentle shaking, 25  $\mu\text{L}$  of 10 mg/mL of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) was added. The volume of the mixture was then brought to 1 mL by adding PBS. The solution was incubated at room temperature for 2 h under gently shaking. Afterwards, the resultant particles were purified through ultra-filtration (Amicon Ultra-0.5 mL centrifugal filters, MWCO 100 kDa, EMD Millipore Inc.). The nanoparticles were then re-suspended in 1 mL of PBS and kept at dark for further use.

For  $\text{NAD}^+$ / $\text{NADH}$  conjugation, the purified APBA-QDs nanoparticles were further mixed with  $\text{NAD}^+$ / $\text{NADH}$  with a feeding molar ratio of APBA-QDs to  $\text{NAD}^+$ / $\text{NADH}$  of 1/60000 for 1 h at room temperature. The cofactor conjugated QDs were purified by ultra-filtration and re-suspended in PBS to a final concentration of 0.4  $\mu\text{M}$ . The  $\text{NAD}^+$ / $\text{NADH}$ -QDs solutions were stored at 4 °C under dark for further use.

### 2.3. Preparation of microfluidic chip

The detailed information about the design and preparation of the microfluidic chip can be found in our previous work (Weng et al., 2014). Briefly, the microfluidic chip consisted of a mixing channel housing with hundreds of posts which were used to

enhance mixing effect, incubation channel, sensing well, two inlets and one outlet, as shown in Fig. 1(B). Flow in the microchannel was driven by capillary force (Hitzbleck et al., 2012; Mohammed and Desmulliez, 2014) hence avoided mechanical or electrical micropumps. This capillary-driven pumpless approach facilitates the development of a disposable on-site diagnostic system. Here, the reagent and the sample solutions must be loaded into the two inlets simultaneously to maximally avoid trapping air bubble. Cylinder-shaped posts were embedded in the microchannel as the passive micromixer which has been proven to be able to enhance mixing performance without damaging bio-cells in the transmission (Alam et al., 2014). The microfluidic chip was made by the following the standard soft lithography protocol (Weng et al., 2011). The fabrication involved making a silicon wafer master by depositing a 60  $\mu\text{m}$  layer of UV cured SU-8 2025 negative photoresist (MicroCHEM, USA) onto the surface of the wafer to form a master mold bearing the desired microchannel layout. A microfluidic chip was formed in the master mold from a degassed mixture of PDMS prepolymer and curing agent (10:1 w/w, Sylgard, Dow Corning, Burlington, ON, Canada) after curing for 4 h at 75 °C. Solidified PDMS replica was then peeled off the master mold, being punched to form the inlets and outlet and bonded onto a glass slide (25  $\times$  75  $\times$  1 mm<sup>3</sup>, VWR International, Suwanee, GA, USA) after oxygen plasma treatment for 40 s. A position marker was used to facilitate the alignment between the sensing well and the sensing window of the photodiode mounted in a custom-built container (10  $\times$  6  $\times$  5 cm<sup>3</sup>) when conducting the bonding. A schematic of the custom-built container for optical and electrical components assembly and alignment can be found in Fig. S1, in which labels 1–5 indicate the positions for mounting LED, excitation bandpass filter, microfluidic chip, emission bandpass filter and Si photodiode, respectively.

### 2.4. Characterization

The morphology of quantum dots nanoprobe were characterized by A Philips CM-10 transmission electron microscope (TEM) operating at 120 kV. The hydrodynamic diameters, size distribution and Zeta ( $\xi$ ) potentials of the cofactor conjugated QDs were measured in water by a dynamic light scattering (DLS) system (Malvern Zetasizer Nano ZS, UK). A Synergy H4 Hybrid Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA) was used to perform the validation of the mechanism of the QDs-based biosensor.

### 2.5. Evaluation of sensing performance and detection of $\beta$ HBA in microplate reader

The QDs-based biosensor was validated by conducting the reaction on a dark microplate (Greiner CELLSTAR<sup>®</sup>, Sigma-Aldrich, Canada) and performed the optical analysis with top reading by the Synergy H4 microplate reader mentioned above. In a typical validation experiment,  $\beta$ HBA standard solution with concentration ranging from 1.6  $\mu\text{M}$  to 1000  $\mu\text{M}$  was prepared by diluted from the 10 M  $\beta$ -hydroxybutyric acid solution. The enzyme solution was made fresh before each experiment by diluting  $\beta$ -hydroxybutyrate dehydrogenase at a ratio of 1:50.  $\text{NAD}^+$ -QDs and BSA (1%) were mixed at a ratio of 1:5 (v/v). For each experiment in the microwell, 20  $\mu\text{L}$  of  $\beta$ HBA standard solutions, 12  $\mu\text{L}$  of mixture of  $\text{NAD}^+$ -QDs and bovine serum album (BSA), 10  $\mu\text{L}$  of enzyme solution were mixed together. Then the mixture was brought to 100  $\mu\text{L}$  in total by adding 58  $\mu\text{L}$  PBS to minimize the optical detection errors caused by the uneven liquid surface. Three replicates of each standard were made. The microplate with the solution mixtures was then covered by aluminum foil for protection from light followed by incubation at room temperature for 30 min. After the

incubation, the fluorescence spectra of the resultant complex were analyzed by the microplate reader (Ex: 470 nm, Em: 655 nm). Serum sample and fortified milk sample were measured by following the same procedure. Each data point represents two separate experiments conducted in triplicate.

## 2.6. Device apparatus and detection of $\beta$ HBA by microfluidic sensor

A custom designed miniaturized optical sensor integrated with the microfluidic platform developed in our previous work (Weng et al., 2014) was built to achieve the fluorescence signal analysis. The optical and the electrical components were selected considering the stability of the sensor response for high performance. The optical sensor was made of a high brightness LED (470 nm, Luxeon Rebel, Luxeon Star LEDs, Brantford, ON, Canada) and a single-band bandpass filters of 470 nm (semrock, Rochester, NY, USA), mounted on cool base with enhanced thermal stability providing the excitation light, and a single-band bandpass filters of 655 nm (semrock, Rochester, NY, USA) and a low noise, high sensitivity photodiode (Hamamatsu, Bridgewater, NJ, USA) capturing the fluorescence illumination from QDs. The light signal was then digitized and transferred to a computer for storage and further analysis by a programmable microcontroller (Arduino Uno, SparkFun Electronics, Niwot, CO, USA) with an interface circuit consisting of several functional units in between. The configuration of the microfluidic platform and the optical sensor is shown in Fig. 1(B).

The experiment by the custom designed optical sensor followed the procedure described below. A series of  $\beta$ HBA standard solutions with concentration of 1.6  $\mu$ M, 8.0  $\mu$ M, 40  $\mu$ M, 200  $\mu$ M and 1000  $\mu$ M were measured. In a typical test, 3  $\mu$ L of mixture containing  $\beta$ HBA standard solution (diluted serum sample or fortified milk sample), mixture of  $\text{NAD}^+$ -QDs and BSA (v/v, 1:5) and 3  $\mu$ L fresh made 3-hydroxybutyrate dehydrogenase enzyme solution were added into the inlets of the microfluidic chip simultaneously. After the mixture flew into the sensing well, fluorescence signals were monitored every minute for a total time of 7 min. For both microplate reader based assay and microfluidic assay, the detection limits were calculated as recommended by IUPAC (Thomsen et al., 2003) by using standard deviation of the standard deviation of blank measures and sensitivities.

To further evaluate the performance of the microfluidic biosensor, a batch of serum sample with known concentration (723  $\mu$ M, 1266  $\mu$ M, 1476  $\mu$ M and 3005  $\mu$ M) analyzed by the chemistry analyzer was detected side by side with a commercial kit (Cayman Chemical<sup>®</sup>  $\beta$ -Hydroxybutyrate (ketone Body)

Colorimetric Assay kit, Cedarlane labs, Burlington, ON, Canada). Before an assay, the serum sample was thawed at room temperature and diluted to 1:6. Three replicates of each standard and diluted serum sample were prepared and measured; the average absorbance readout by the microplate reader was calculated to evaluate the sample concentration. Similarly, each sample was tested for 3 times by the microfluidic biosensor; the average voltage output of the photodiode was then calculated to determine the sample concentration.

## 3. Results and discussion

### 3.1. Characterization of QDs nanoprobe

To prepare cofactor-QDs nanoprobe, carboxyl QDs was conjugated with APBA via carbodiimide reaction. The cofactors  $\text{NAD}^+$  (or NADH) was further immobilized to APBA conjugated QDs by phenylboronic acid groups involved cyclic esters reaction (Ferrier and Prasad, 1965). As shown in Fig. 2(A), the successful immobilization of cofactor QDs nanoprobe was demonstrated by observing increasing of absorbance intensity at 259 nm for both  $\text{NAD}^+$ -QDs and NADH-QDs comparing to bare QD with same concentration. A weak spectrum shoulder (dark arrow) could be observed at 340 nm for NADH-QDs, which is another characteristic absorption peak for NADH. We also demonstrated the conjugation of NADH to QDs by observing the emission peak at 460 nm under excitation at 340 nm from NADH-QDs as shown in Fig. 2(B) (shown in the small window). Based on the emission intensity of NADH, we were able to determine the number of NADH per QD. As indicated in Fig. S2A, the number of NADH per QD was found to be  $\sim 1532 \pm 122$ . This number is significantly higher than that reported in literature ( $\sim 40$  per QD) (Freeman and Willner, 2008), where QDs was capped with glutathione through the interaction of single  $-\text{SH}$  group to ZnS shell. The colloidal stability of alkanethiol capped QDs may be poor, due to desorption of the ligands from nanoparticles (Aldana et al., 2001). Commercial QDs we used here which are likely coated with a layer of polymer, which greatly promote the particle water solubility and present larger amount of carboxylic group on the surface for further conjugation (Pellegrino et al., 2004).  $\text{NAD}^+$  does not emit fluorescence. However, due to the similarity of chemical structure (ribose), it is estimated that same number of  $\text{NAD}^+$  has been conjugated per QD. TEM image (Fig. S3A) shows the cone-like shape of  $\text{NAD}^+$ -QDs nanoprobe. The average hydrodynamic diameter of  $\text{NAD}^+$ -QDs was determined to be  $\sim 26$  nm by DLS measurement (Fig. S3A, bottom

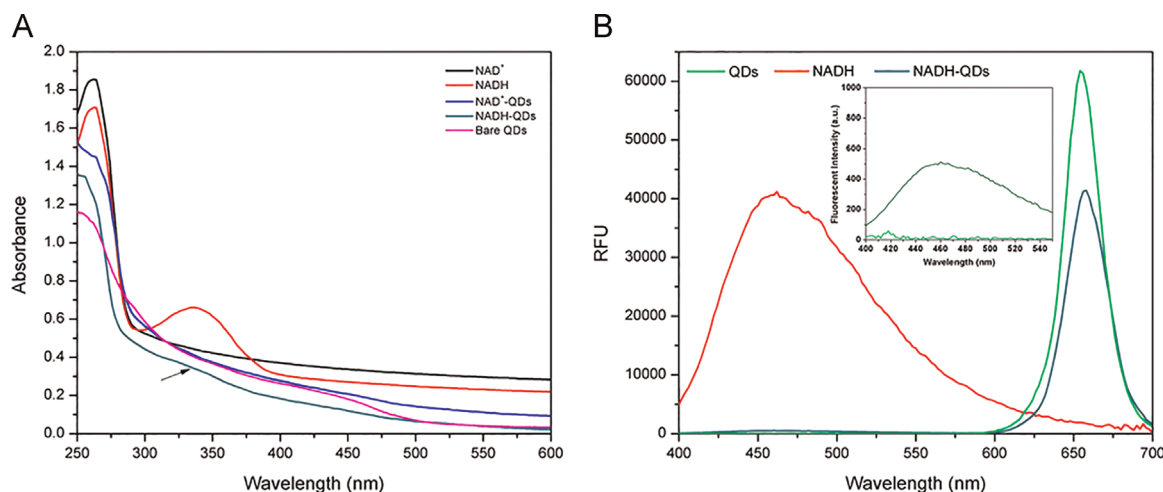


Fig. 2. (A) UV-visible spectra and (B) fluorescence spectra of cofactors and nanoprobe.



left). The cofactor conjugated nanoprobe shows moderate dispersion in aqueous medium, as indicated from the Zeta potential data (Fig. S3B). The Zeta potential ( $\zeta$ ) values of  $\text{NAD}^+$ -QDs and  $\text{NADH}$ -QDs are  $-27.9 \pm 7.81$  mV and  $-28 \pm 7.01$ , respectively, which indicate the moderate dispersion of the cofactor conjugated nanoprobe in aqueous medium (Greenwood and Kendall, 1999; Hanaor et al., 2012).

### 3.2. Evaluation of sensing mechanism

$\text{NAD}^+$  was known as an important co-factor for over 300 dehydrogenases in the metabolic pathways. In a typical  $\text{NAD}^+$ -involved enzyme reaction, it acts as a redox electron carrier to accept electrons and gets transferred to its reduced form  $\text{NADH}$ .  $\text{NAD}^+$  has been reported to effectively quench the fluorescent emission of QDs (Freeman and Willner, 2008). The fluorescence quenching of QDs by  $\text{NAD}^+$  has been attributed to an electron (charge) transfer process (Freeman and Willner, 2008; Ren et al., 2010). In this process, the energy generated from the excited state in QDs is transferred to the extra carrier,  $\text{NAD}^+$ , via a non-radiative recombination, instead of emitting photons to generate fluorescence (Akshath et al., 2012). As a result, this non-radiative recombination is much larger than the radiative electron-hole recombination and hence was followed by the process of quenching the fluorescence (Akshath et al., 2012).

As shown in Fig. 3(A), we were able to find higher quenching of fluorescence intensity by comparing the emission profile from the two cofactors conjugated nanoprobe with the same concentration. To further evaluate the effect of cofactors to fluorescence of QDs, we performed a competition binding test by adding excess amount of free  $\text{NAD}^+$  (15 mM) to  $\text{NADH}$ -QDs (200 nM), and vice versa. Fig. 3(B) shows that the QDs emission at 655 nm decreases in the presence of  $\text{NAD}^+$ , which could be attributed to the replacement of  $\text{NADH}$  binding on the surface of QDs by  $\text{NAD}^+$  molecules. Meanwhile, addition of  $\text{NADH}$  in  $\text{NAD}^+$ -QD results in the recovery of fluorescent intensity. Based on the higher ratio of fluorescence recovery by  $\text{NADH}$  than that of quenching by  $\text{NAD}^+$ , it is probable that the  $\text{NADH}$  may have higher binding affinity to APBA molecule on the surface of QDs.

The stability of cyclic esters formed through the binding of APBA to diols group ( $\text{NADH}$  or  $\text{NAD}^+$ ) is pH dependent. The optimum pH range for the binding is reported in physiological condition ( $\sim \text{pH } 7$ ) (Springsteen and Wang, 2002), which potentially

stabilizes our assay. Thus the assay pH was strictly controlled in our experiment. In addition, the presence of cis-diols containing molecules such as sugars may compete with cofactors to bind onto QDs as indicated through competition test. However, it should be noted this requires large amount of sugar molecules. A typical sample dilution process could eliminate the interferences. In addition, free  $\text{NAD}^+$  has been reported to quench the fluorescence of  $\text{CdTe}$  QDs through the same charge transfer process by diffusing onto the surface of QDs (Akshath et al., 2012). The assay signal may not be largely affected due to the replacement of  $\text{NAD}^+$  by small amount of sugars from the conjugation of QDs.

### 3.3. Detection of $\beta\text{HBA}$ in microplate reader

We then evaluated the use of the  $\text{NAD}^+$ -QDs nanoprobe for  $\beta\text{HBA}$  detection. The enzymatic oxidation of the major ketone body  $\beta\text{HBA}$  is expected to convert  $\text{NAD}^+$ -QDs to  $\text{NADH}$ -QDs, which consequently results in proportional increasing of QDs emission intensity (at 655 nm). Fig. 4(A) shows the fluorescence spectra upon the reaction of the  $\text{NAD}^+$ -modified QDs with variable concentrations of  $\beta\text{HBA}$  in the presence of  $\beta\text{HBA}$  dehydrogenase. As the concentration of  $\beta\text{HBA}$  increases, the fluorescence intensity of QDs increases due to the formation of more amount of  $\text{NADH}$  on QDs from  $\text{NAD}^+$ .  $\beta\text{HBA}$  concentrations in the samples were determined by analysis of the varying intensities of the fluorescence output from the sensor. Fig. 4(B) demonstrates the fluorescence intensities of the QDs at 655 nm which were dependent on the concentrations of  $\beta\text{HBA}$ . As the concentration of  $\beta\text{HBA}$  increases, the fluorescence intensity increases accordingly due to the higher amount of  $\text{NADH}$ -QDs were converted from  $\text{NAD}^+$ -QDs. An  $R^2$  value of 0.9511 was found for linear response region between  $8 \mu\text{M}$  and  $1000 \mu\text{M}$  with a detection limit of  $1.2 \mu\text{M}$ . In the experiment, a control experiment without loading  $\beta\text{HBA}$  was done for comparison. It is concluded that the  $\beta\text{HBA}$  dehydrogenase had no effect on the fluorescence of the QDs. Therefore, this  $\text{NAD}^+$ -modified QDs enable us to differential the  $\beta\text{HBA}$  concentration.

Serum samples with known  $\beta\text{HBA}$  concentration and the fortified milk samples spiked with  $\beta\text{HBA}$  standards were then mixed with  $\text{NAD}^+$ -functionalized QDs, and the QDs emission intensity was analyzed by the microplate reader. As shown in Fig. 4(C) and (D), different  $\beta\text{HBA}$  concentrations in both the serum sample and the fortified milk samples are well differentiated based on the

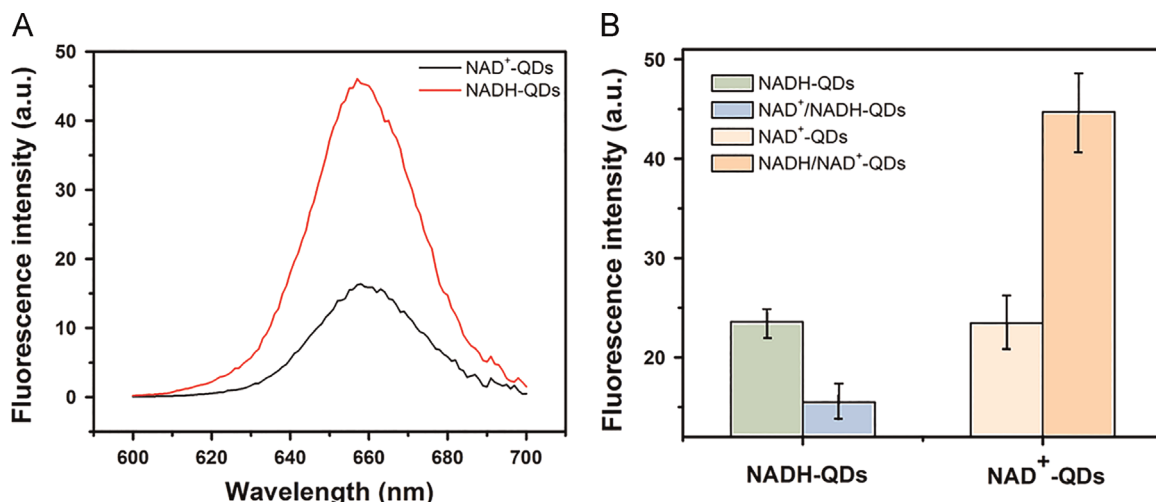
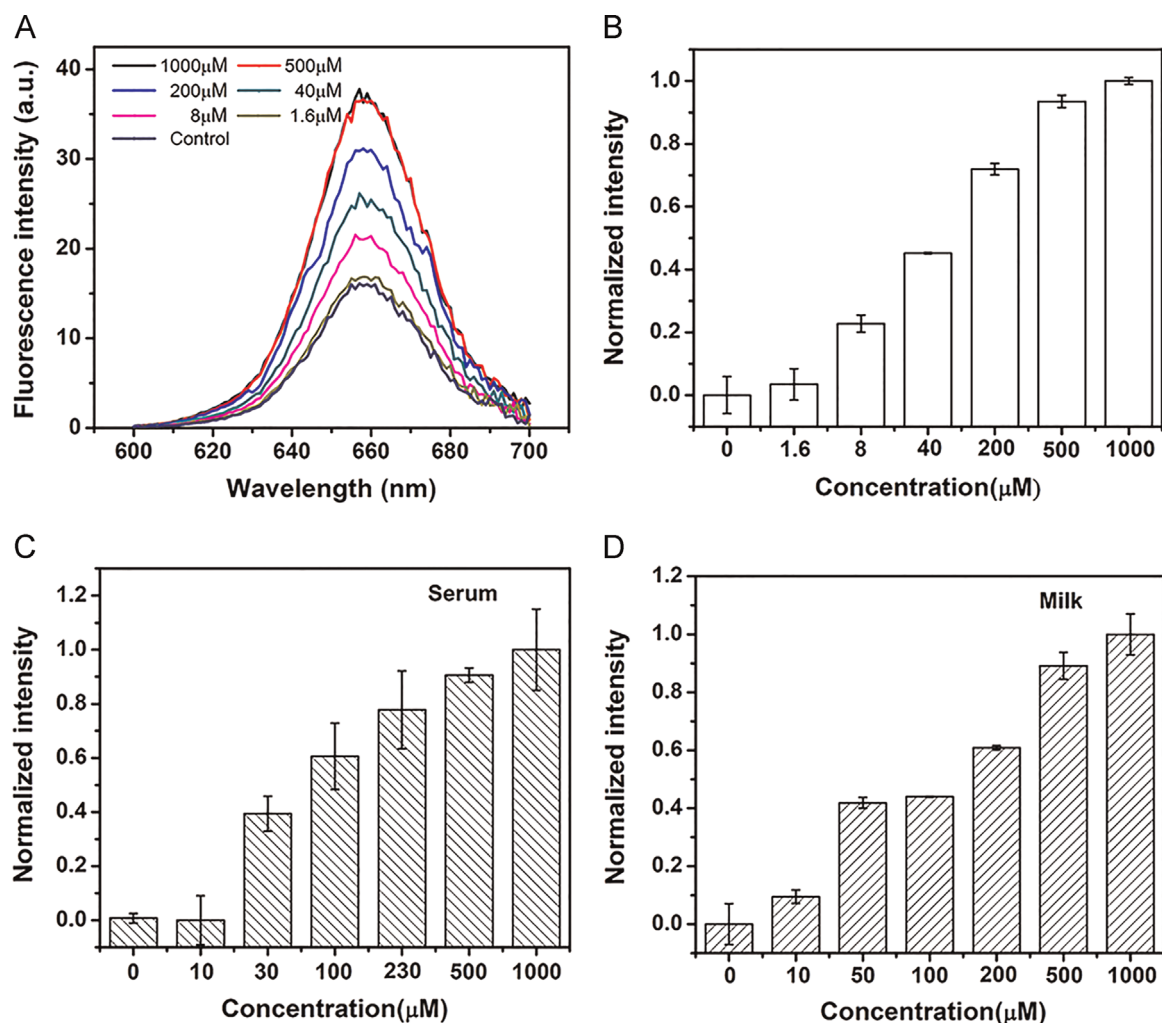


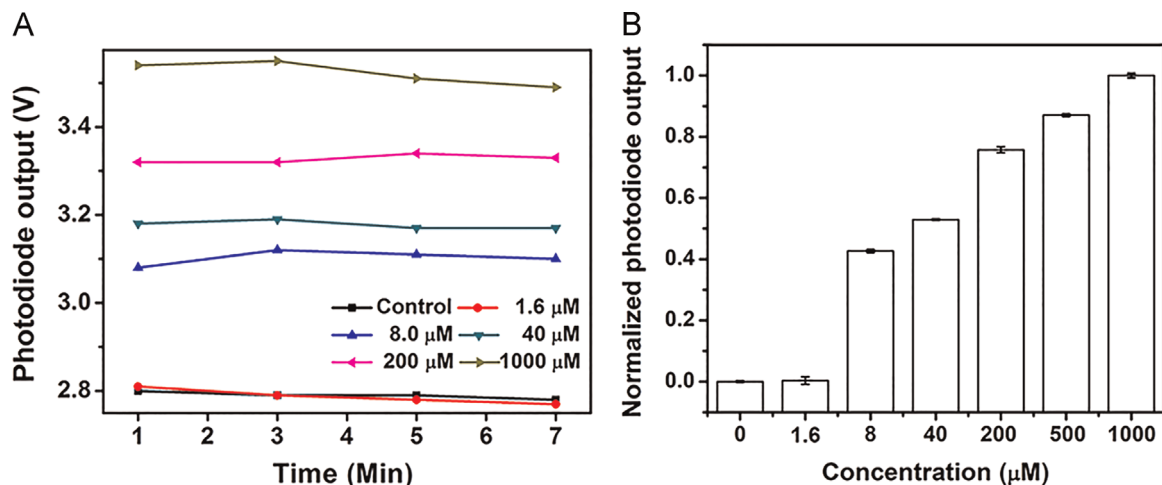
Fig. 3. (A) Fluorescence spectra of  $\text{NAD}^+$ -functionalized QDs and  $\text{NADH}$ -functionalized QDs ( $\lambda_{\text{ex}} = 470$  nm). (B) Fluorescence intensity at 655 nm for a mixture of  $\text{NAD}^+$  and  $\text{NADH}$ -functionalized QDs, and  $\text{NADH}$  and  $\text{NAD}^+$ -functionalized QDs.



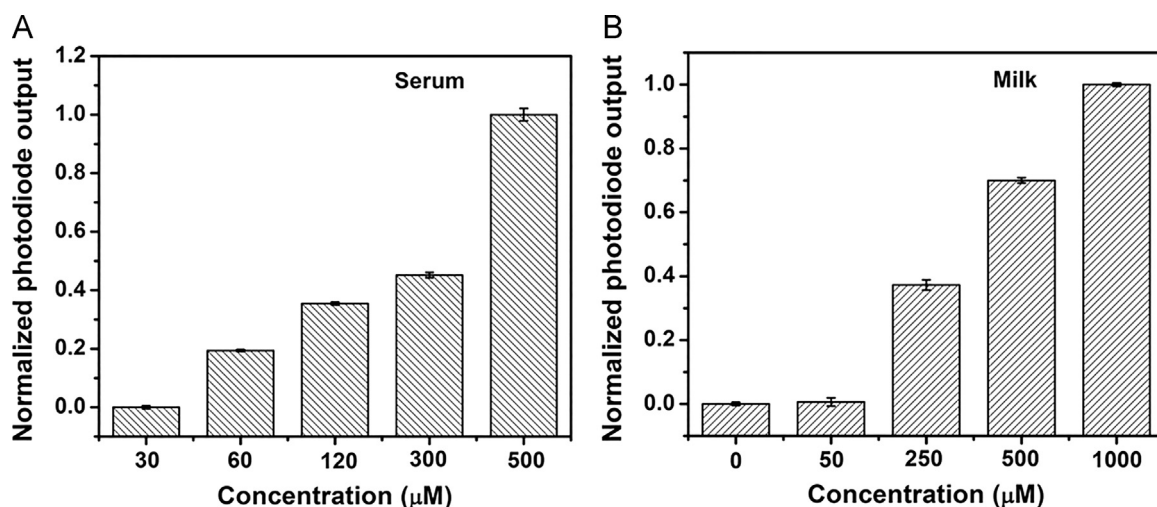
**Fig. 4.** (A) Fluorescence spectra upon the reaction of NAD<sup>+</sup> functionalized QDs with a series of  $\beta$ HBA standard solution. (B) Fluorescence intensity at 655 nm of variable concentrations of  $\beta$ HBA by NAD<sup>+</sup> functionalized QDs. (C) Fluorescence spectra upon the reaction of NAD<sup>+</sup> functionalized QDs with a series of diluted serum sample. (D) Fluorescence spectra upon the reaction of NAD<sup>+</sup> functionalized QDs with a series of fortified milk sample.

changes of fluorescent intensity. No significant fluorescent signal interference due to complex matrix in milk samples was observed during the experiments. The obvious advantage of this fluorometric method is that no tedious pre-treatment is required to obtain a transparent status for milk, therefore, contamination and

disturbance caused by precipitating step may be avoided. In conclusion, the presented fluorometric determination of  $\beta$ HBA based on NAD<sup>+</sup>-functionalized QDs is a valid method for analysis of serum or milk.



**Fig. 5.** (A) Time-dependent response of biosensor with a series of  $\beta$ HBA standard solution. (B) Normalized photodiode output of biosensor in measurement of the standard  $\beta$ HBA concentration.



**Fig. 6.** (A) Normalized photodiode output of biosensor in measurement of the  $\beta$ HBA concentration in diluted serum samples. (B) Normalized photodiode output of biosensor in measurement of the  $\beta$ HBA concentration in fortified milk samples.

### 3.4. Detection of $\beta$ HBA by microfluidic biosensor

$\beta$ HBA standard solution, serum samples from dairy cows, and fortified milk samples were measured on the microfluidic biosensor. Firstly, the time-dependent fluorescence changes upon the reaction of  $\text{NAD}^+$ -functionalized QDs with  $\beta$ HBA standard solution were investigated, which is shown in Fig. 5. Fluorescence intensity was measured and recorded at the end of the first minute after mixing and then every other minute with a total period of 7 min. The result demonstrates that the changes of fluorescence intensity for all standard solutions are not distinguishable after 3 min, which indicated the completion of the enzymatic reaction. Therefore, 3 min was taken as the sufficient incubation time for the microfluidic biosensor and used in the following experiments of preparation of  $\beta$ HBA standard curve, serum and milk sample measurement. Fig. 5(B) demonstrates the fluorescence intensities reflected by the output of photodiode depending on the interaction between the  $\text{NAD}^+$ -functionalized QDs with variable  $\beta$ HBA standard solution for a fixed time interval of 3 min. As the concentration of  $\beta$ HBA increases, the output of the photodiode increases, which indicates the fluorescence intensity of the QDs is intensified. An  $R^2$  value of 0.9728 was found for linear response region between  $8 \mu\text{M}$  and  $1000 \mu\text{M}$  with detection limits of  $35 \mu\text{M}$ . This result is consistent of the formation of higher amount of  $\text{NADH}$ -QDs. Serum samples with known  $\beta$ HBA concentration and the fortified milk samples were measured by this biosensor, the results are shown in Fig. 6. Both of the serum and milk samples are easily distinguished based on the changes of  $\beta$ HBA concentration. Good repeatability was observed in both sample tests. The calculated detection limit for the serum sample was  $34.8 \mu\text{M}$ , which highly agrees with that of standard solution with linear regression of 0.9423. For the milk sample, detection limit and the linear regression were  $40.3 \mu\text{M}$  and 0.9658, respectively. Both of the results present the good performance of the presented microfluidic biosensor  $\beta$ HBA detection in blood and milk samples. In addition, when compared to the blood detection, a slightly poor detection limit was observed, which may be caused by the interference of the complex matrix in milk. This may be improved via further processing of the milk sample by centrifugation. The stability of this biosensor has been confirmed by the small standard derivative of the data point presented in the plots. The comparison of the microfluidic biosensor with the commercial kit was shown in Table S1.

### 4. Conclusions

In this study, we present a QDs-based biosensor for the detection of  $\beta$ HBA towards on-farm subclinical ketosis diagnosis. The use of  $\text{NAD}^+$  functionalized QDs as fluorescent labels offers superior performance for the  $\beta$ HBA sensing event. Conducting the measurement on a microfluidics platform significantly decreases the sample/reagent consumption and detection time due to the small scale and high surface area to volume ratio of the micro-channel structure, which is a distinctive advantage over many commercially available kits (Cayman Chemical<sup>®</sup>  $\beta$ -Hydroxybutyrate (Ketone Body) Colorimetric Assay Kit; MaxDiscovery<sup>™</sup>  $\beta$ -Hydroxybutyrate Assay Kit) which usually require relative big volumes of sample/reagent or bulky, expensive spectrophotometric equipment. Custom-built miniaturized and low-cost optical biosensor provides high sensitivity in QDs fluorescence analysis. Both milk and serum sample from dairy cows were measured and quantitative results were achieved, for a typical test, result could be obtained in 3 min with a detection limit of  $35 \mu\text{M}$ . Good agreement in serum detection has been confirmed upon side by side comparison with a commercial kit. A promising capability of measuring milk  $\beta$ HBA by the microfluidic biosensor was implicit by the presented result although no comparison with a commercial kit was conducted as only semi-quantitative analysis for milk  $\beta$ HBA was found on the market. Although some handheld meters designed for human medicine (Precision Xtra<sup>™</sup>) have been employed for cow-side SCK diagnosis and reported to have good sensitivity and specificity, but provides significantly false positive results, and can analyze only the blood samples. The dual-sample detection capability is another advantage for the presented microfluidic biosensor. The developed quantum dots-based microfluidic biosensor for subclinical ketosis detection is designed as a specific portable device towards on-farm and cow-side diagnosis of SCK, which provides a framework towards the delivery of improved dairy animal health management solutions in Canada and around the world.

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## Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2015.05.008>.

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