Poly (N-isopropylacrylamide) Microgel-Based Etalons for the Label-Free Quantitation of Estadiol-17β in Aqueous Solutions and Milk Samples

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ABSTRACT

A novel estradiol-17β (E2) biosensor was constructed from poly (N-isopropylacrylamide) (pNIPAm) microgel-based etalons by modification of their outermost Au layer with an E2 binding 75-mer DNA aptamer. When E2 is not present in the solution, the aptamer forms a loose/linear structure that allows ions to pas through and into the microgel layer. The ions can change the solvation state of the microgels, which changes the optical properties of the etalon. When E2 is present in the solution, the aptamer binds the E2 and undergoes a conformational change to a form that can block the diffusion of salt ions into the microgel layer. This blocking decreases the response of the device to salt exposure, which can be related to the concentration of E2 in solution. Using this approach, E2 sensor showed a dynamic range of 0.9-200 pg/mL with a calculated detection limit of 0.9 pg/mL (3.2 pM) E2, and the lowest measured concentration of E2 is 5.0 pg/mL. This sensor also showed low cross reactivity with progesterone, a similar steroid hormone. Moreover, this sensor could be regenerated 5 times without losing its sensitivity. Finally, we demonstrated that the sensor could also be used to quantify E2 in commercial skim and 2% milk, as well as farm milk directly without any pre-treatment. The successful quantitation of E2 in unprocessed milk demonstrates its potential use as a "cow-side" testing device for the dairy industry.

KEYWORDS

Stimuli-responsive polymers; Estradiol-17β biosensor; DNA aptamer; Poly (N-isopropylacrylamide) microgels; Etalons

1. INTRODUCTION

Estradiol-17β (E2), which is mainly produced within the ovarian follicles and is essential for the development and maintenance of female reproductive tissues, is considered to be one of the most active estrogens [1]. E2, which is an endocrine disrupting chemical (EDC), can be found in surface water from municipal and industrial effluents as well as discharge of animal feeding facilities [2]. Chronic exposure to endogenous or exogenous E2, can cause health problems in human and animals even at very low concentrations [3-5]. It can also lead to sexual precocity in young females, feminization in males [5] and is a potential carcinogen [3, 4]. Therefore, it would be highly advantageous to have a simple, fast and sensitive method to detect E2 in environmental and clinical samples.

In addition to the above-listed possible impacts such a sensor could have, there are a number of potential agricultural uses. Of particular interest to this submission is the accurate detection of estrus in dairy cows. Specifically, the quantification of steroid hormones in cow's biological fluid can be used to confirm estrus and reduce the proportion of cows that are inseminated at the wrong time. Accurate estrus detection can ultimately lead to improved reproductive efficiency in dairy herds, and ultimately increased profits for farmers [6]. Estrus is preceded by a reduction in progesterone (P4) and an increase in E2 levels [7]. Hence, monitoring the level of these hormones in blood or milk would help herd managers and veterinarians accurately identify estrus and determine the optimal time for artificial insemination [8, 9]. As a result of these potential benefits, the development of sensitive and simple technology to determine E2 concentration in milk samples, especially “cow-side” test devices, has attracted the dairy industry's interest.

Indeed, there are assays currently available to quantify steroid hormones in milk samples, e.g., enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) [8, 10]. However, these

traditional methods need to be performed in a laboratory setting by specially trained personnel, require multiple incubation/washing steps and use radioactive materials. Therefore, ELISA and RIA methods are not suitable for field-testing or for the fast analysis of many samples. In addition, ELISA assays have been shown to lack sensitivity for measuring concentrations of P4 in milk samples [8].Advanced instrumental methods with improved sensitivity have also been developed to quantify E2 [11- 13], such as high-performance liquid chromatography (HPLC), and liquid chromatography/gas chromatography-mass spectrometry (LC/MS and GC/MS). All of these approaches require considerable time for sample preparation and analysis, such as liquid-liquid extraction [14],solid-liquid extraction [15] and multistep solid phase extraction [16]. Moreover, these instruments are expensive, and need to be operated by highly trained personnel in a lab setting. Recently, DNA-based aptamers have been developed for E2 monitoring [17, 18].Using E2-binding aptamers as the recognition element, biosensors have been developed to quantify E2 in water and wastewater samples [19-21], and in human urine [22, 23].A fluorescence polarization-based sensor for E2 in milk was recently reported by Varriale et al. [24]. This method was able to detect E2 in diluted milk with a limit of detection of < 10 pmol by monitoring fluorescence polarization due to the specific binding of antibodies to fluorophore-labeled E2 derivatives [24]. Very recently, a photoelectrochemical aptasensor that used surface plasmon resonance of Au nanorods for signal amplification was used to detect E2 with a detection limit of 3.3×10^{-16} M in milk powder [25]. However, it is still a challenge to quantify E2 in undiluted milk using biosensor technology due to its low concentration and the complexity of the milk matrix [26].

Stimuli-responsive polymers (also known as smart/intelligent polymers) have the ability to respond physically and/or chemically to changes in their environmental conditions [27, 28].. Poly (N-isopropylacrylamide) (pNIPAm) is the most well known thermoresponsive polymers and has attracted by far the most attention over the years [29]. PNIPAm undergoes a conformational change from a random

coil (extended) to a globule (collapsed) at its lower critical solution temperature (LCST) of 32 °C [30]. PNIPAm-based hydrogel particles (nanogels and microgels) can also be synthesized, and exhibit thermoresponsivity. That is, they expel their solvating water and decrease in diameter above 32 °C, while they are fully water swollen at temperatures below 32 °C. Due to the porous nature of these materials, and their thermoresponsivity, pNIPAm-based microgels have been used for various applications, e.g., as sensors, artificial muscles, catalysts, drug delivery platforms, and for water remediation [30, 31-36]. Of significance to this submission are the P4 sensors the Serpe Group recently reported that exploit anti-P4 antibody-modified pNIPAm microgels that change solvation state in the presence of P4, which can be read out as a change in the optical properties sensor [37]. As is well known, antibodies suffer from poor thermal stability that can limit the field use of the sensors. Therefore, in this submission, we report on the use of DNA-based aptamers that are significantly more thermally stable that antibodies, that can be attached to the *top* of pNIPAm-*co*-acrylic acid (pNIPAm-*co*-AAc) microgel-based optical devices (etalons) to make them sensitive to the concentration of E2 in aqueous solutions and milk samples. We point out that the sensing mechanism reported here is completely different than what was reported by our lab previously as it exploits surface blocking phenomena for detection of E2, as opposed to solvation state changes from the antibody-modified microgels binding to the analyte. Regardless, microgel-based etalons are used in both cases, and the structure is shown in Figure 1A. Specificially, microgel-based etalons are composed of a microgel layer that is "sandwiched" between two thin Au layers (all on a glass support). Figure 1B shows that the etalons exhibit visual color and multipeak reflectance spectra; the reflected wavelengths can be predicted from Equation (1):

$$\lambda = \frac{2nd\cos\theta}{m} \qquad (1)$$

where *n* is the refractive index of the dielectric (microgel) layer, *d* is the distance between the metal layers, θ is the angle of incident light striking the device relative to the device normal, and *m* is the order of a reflected wavelength (an integer). We have previously shown that the position of the peaks in reflectance spectra (and hence the device color) depends primarily on the distance between the metal layers, which can be tuned via modulation of the microgel solvation state.

As mentioned above, in this investigation we modified the surface of pNIPAm microgel-based etalons with E2-binding DNA aptamers as shown schematically in Figure 1A, which were used to quantify E2 in water and milk samples. The sensing mechanism relies on the DNA aptamer changing conformation in the presence of E2, which blocks the transportation of ions (Na^+ or Ca^{2+}) into the etalon. In the absence of E2, the DNA aptamers form a loose structure on the etalon surface that favors the transportation of Na^+ or Ca^{2+} into etalon. The ions are able to neutralize the charge on the pNIPAm-*co*-AAc, which will result in their collapse and a concomitant change in the optical properties of the etalon. When the aptamers bind their target we hypothesize that they will form a more compact structure on the etalon's surface that partially blocks the transportation of ions into the etalon's microgel layer. The penetration of Na^+ or Ca^{2+} into the etalon causes the microgels to collapse, leading to a change in the optical properties (blue shift of reflectance spectrum) of the device. By monitoring the extent of peak shifts in the reflectance spectra, an E2 sensor could be generated.

Compared to other aptamer-based E2 biosensors [19-24], the etalon-based sensors we developed here have many advantages, including: low cost, ease of use, sensitivity and detection limits that are relevant to the industry, and the ability to detect analytes in undiluted milk samples. These benefits combined make the developed sensors quite unique, and potentially impactful to the community.

2. EXPERIMENTAL SECTION

2.1 Materials

N-Isopropylacrylamide (NIPAm) was purchased from TCI (Portland, OR, USA) and purified by recrystallization from hexanes (ACS reagent grade, EMD, Gibbstown, NJ, USA) prior to use. N, N-methylenebisacrylamide (BIS) (99%), acrylic acid (AAc) (99%), ammonium persulfate (APS) (>98%), Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), dextran coated charcoal, P4, and E2 were purchased from Sigma-Aldrich (Oakville, ON, Canada) and used as received. Deionized (DI) water with a resistivity of 18.2 MΩ·cm was used in all experiments. The 25 × 25 × 1 mm glass coverslips were obtained from Fisher Scientific. Au (99.99 %) was obtained from MRCS Canada (Edmonton, AB). Cr (99.999 %) was obtained from ESPI (Ashland, OR, USA). Disulfide-modified E2 DNA aptamers were purchased from IDT (Toronto, ON, Canada). Experiments were conducted at room temperature (21 ± 1 °C) unless otherwise specified.

2.2 Microgel Synthesis

Poly (N-isopropylacrylamide-*co*-acrylic acid) (pNIPAm-*co*-AAc) microgels were synthesized via surfactant-free, free-radical precipitation polymerization as described previously [38, 39]. Briefly, NIPAm (11.9 mmol), and BIS (0.7 mmol) were dissolved in 99 mL DI water with stirring in a beaker followed by filtration through a 0.2 µm filter into a 200 mL 3-neck round bottom flask. The solution was bubbled with N_2 gas for ~1.5 h while heating to 70 °C. AAc (1.4 mmol) and a 1 mL aqueous solution of 0.2 M APS was added to the heated mixture, respectively. The reaction was allowed to proceed for 4 h at 70 °C followed by cooling to room temperature and stirring overnight. The mixture was finally filtered through glass wool to remove any large aggregates, and were washed to remove unreacted monomer and linear polymer by centrifugation (~8500 rcf) and resuspension in DI water a total of six times.

2.3. Etalon Fabrication

Our previously reported "paint-on" protocol was used to fabricate microgel-based etalons [37, 39]. Au coated glass surfaces were generated by thermally evaporating 2 nm of Cr (for adhesion) followed by 15 nm of Au onto 25 × 25 × 1 mm pre-cleaned glass coverslips at a rate of ~0.1 and ~0.15 Å s^{-1}, respectively, using a model THEUPG thermal evaporation system (Torr International Inc., New Windsor, NY). The Au coated substrates were then annealed at 250 °C for 2 h using a Thermolyne muffle furnace from Thermo Fisher Scientific (Ottawa, Ontario). 40 µL of concentrated pNIPAm-*co*-AAc microgels (obtained by centrifugation) were then spread on the Au/Cr coated substrate and the microgel solution was allowed to dry completely on the substrate for 2 h at 35 °C. The resultant substrate was then rinsed copiously with DI water to remove any excess microgels not bound directly to the Au and allowed to soak in DI water overnight at 30 °C. Following this step, the substrate was rinsed again with DI water, dried with N_2 gas, and an additional layer of 2 nm Cr and Au (either 15 nm or 5 nm thick) was deposited.

2.4. Etalon surface modification of DNA aptamers and regeneration of etalon sensor

Thiol-modified 75-mer anti-E2 DNA aptamer (E2-Ap75: 5'-HS-$(CH_2)_6$-ATACGAGCTTGTTCAATACGAAGGGATGCCGTTTGGGCCCAAGTTCGGCATAGTGTGGTGATAGTAAGAGCAATC-3') and 35-mer anti-E2 DNA aptamers (E2-Ap35: 5'-HS-$(CH_2)_6$-AAGGGATGCCGTTTGGGCCCAAGTTCGGCATAGTG-3') [24] were coupled to freshly prepared etalon devices via S-Au binding. Thiol-modified DNA aptamers were generated via reduction of the received disulfides. In detail, the disulfide-modified DNA aptamers with a concentration of 2 µM were treated with 100 µM TCEP for 30 min at room temperature to reduce the disulfide bond to a thiol. 500 µL of this solution was then added to freshly prepared etalons with edges sealed by application of nail polish and incubated at room temperature for overnight (around 16 h). The modified etalon devices were washed

with E2 aptamer binding buffers (2.0 mM Tris–HCl at pH 7.5 containing 10.0 mM NaCl, 0.5 mM KCl, 0.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 5.0 % ethanol) and stored in the buffer at 4 °C until use.

For etalon regeneration experiments, used sensors were regenerated by soaking them in 0.5 % sodium dodecyl sulfate (SDS, pH=1.9) for 2 min at room temperature [40], then the sensors were washed copiously with DI water. The regenerated sensors were stored at 4 °C in corresponding E2 binding buffer until being used.

2.5. Reflectance Spectroscopy, Optical Microscopy, and Zeta Potential Measurements

Previously reported methods were used to collect the reflectance spectra from the etalons, and measure the size and zeta potential of microgels (37). In detail, reflectance spectroscopy measurements were performed using a USB2000+ spectrophotometer, a HL-2000-FHSA tungsten light source, and a R400-7-VIS-NIR optical fiber reflectance probe from Ocean Optics (Dunedin, FL). The spectra were collected using OceanView Spectroscopy Software in a wavelength range of 350–1000 nm. Optical microscopy images of the pNIPAm-*co*-AAc microgels were obtained using an Olympus IX71 inverted microscope (Markham, Ontario) fitted with a 100X oil-immersion objective an Andor Technology iXon camera (Belfast, Ireland). Andor SOLIS v4.15.3000.0 software was used to record microscopy images of the microgels. Zeta potential and microgel diameter, as well as the size change in different NaCl solutions with the concentration from 1 mM to 500 mM was measured using a Malvern Zetasizer Nano ZS instrument (Malvern, UK) with a 633 nm laser at 25 °C.

2.6. Experimental setup and sensing procedure

Sensing experiments were conducted using a custom built metal stage at room temperature. The stage was built to ensure that the position of the etalon and the light source were fixed. The intensity and distance of the light source from the etalon was adjusted to result in well-defined

reflectance peaks in the reflectance spectra. During the sensing experiments, E2 or P4 solutions with various concentrations were incubated with E2 DNA aptamer-modified etalons for 30 min, and then washed copiously with E2 aptamer binding buffer. The response of etalons to 500 mM NaCl or 500 mM $CaCl_2$ was monitored by collecting the reflectance spectra of the etalons before and after adding NaCl or $CaCl_2$ solutions without moving the etalon or reflectance probe. Hormone-free milk was prepared by removing hormones from store-bought milk using dextran-coated charcoal following the manufacturer's recommended procedure. For E2 detection in milk, the E2Ap75 modified etalons were incubated with various milk samples for 30 min at room temperature and washed with E2 binding buffer, then the etalons' response to 500 mM $CaCl_2$ was recorded. Each measurement was obtained from a separate etalon for a total of 98 etalons used for the experiments in this submission.

3. RESULTS AND DISCUSSION

3.1. Microgel Characterization, Etalon Fabrication and E2 DNA aptamers modification

Differential interference contrast (DIC) microscopy was used to image the pNIPAm-*co*-AAc microgels, as can be seen in Electronic Supplementary Material Figure S1. As can be seen from the images, the microgel diameter was in the range of 1.0 µm-1.2 µm. Dynamic light scattering (DLS) also was utilized to determine their solution diameter, which was 1120 ± 30 nm. The negative charge of the microgels was confirmed by measuring the zeta potential of the microgels, which was determined to be -31.8 ± 1.2 mV. To form a monolayer of the pNIPAm-*co*-AAc microgels on the Au-coated glass substrates, the previously reported "paint-on" method was used, and is also described above [41]. It is well known from previous research that pNIPAm-*co*-AAc microgels and pNIPAm-*co*-AAc microgel-based etalons are pH and temperature responsive [41-44]. Therefore, to confirm that the pNIPAm-*co*-AAc microgel-based etalons behave as expected, we probed their pH and temperature responsivity. Analysis revealed that

the position of the peaks in the reflectance spectra exhibited a blue shift when the solution temperature was increased, and/or when the solution pH was decreased. Likewise, the position of the peaks in the reflectance spectra exhibited a red shift when the solution temperature was decreased and/or the solution pH was increased (data not shown).

To confirm the successful immobilization of the DNA aptamer on the etalon's Au surface, XPS was employed to determine the elemental composition of the etalon's surface before and after exposure to the thiolated DNA aptamers. Figure S2 (see Electronic Supplementary Material) shows the XPS spectra for P on etalon's surface before and after DNA aptamer modification. As can be seen from the XPS data there is no P peak on the bare Au etalon surface, while an obvious P peak with binding energy of 130-132 eV was present after modification with DNA aptamers.

3.2. Estradiol detection using E2Ap75 and E2Ap35 modified etalons based on the NaCl surface blocking.

The microgels' diameter as a function of NaCl concentration was evaluated using DLS in order to decide the optimal ion concentration to use to yield the maximal response from the etalons to salt exposure. The data in Figure S3 (see Electronic Supplementary Material) indicates that the size of microgels decreased with increasing NaCl concentration until 100 mM. Further increasing the NaCl concentration had a negligible impact on the diameter of the microgels. Although, exposure of the E2Ap75-modified microgel-based etalons to the same concentrations of NaCl yielded peak shifts up to 500 mM NaCl (Figure S4), therefore we chose to expose all devices to 500 mM NaCl.

To evaluate the ability of E2Ap75-modified etalons to quantify E2 in water, the etalons were first incubated with different concentrations of E2 for 30 min at room temperature, then the response of the

etalons to 500 mM NaCl was determined. Without incubation with E2, the etalons showed large shifts in the position of the reflectance peaks (blue shift, 104 ± 1 nm) resulting from the collapse of microgels induced by Na^+ penetrating into the microgel layer of the etalon and shielding the microgel charge (Figure S5A, Supporting information). We observed that as the concentration of E2 increased, the extent of the peaks shift after exposure to NaCl decreased. We hypothesize that this is due to the blocking of Na^+ penetration into the etalon caused by E2 binding E2Ap75 on etalon surface. The peak shift dropped to 76 ± 2 nm (see Electronic Supplementary Material Figure S5B) when the etalon was incubated with 200 pg/mL E2 from 104 ± 1 nm. Thus, by monitoring the peak shift vs. E2 concentration, we can quantify E2 in water. Figure 2A shows the calibration curve of etalon's reflectance peak shift vs. the concentration of E2 in water. Two linear ranges were obtained with these devices, in the ranges of 0 - 20 pg/mL and 20-200 pg/mL, which are described by Equation (1) and Equation (2) with R^2 = 0.8694 and 0.9976, respectively. While we acknowledge that the ability to accurately quantify E2 >20 pg/mL is diminished compared to lower concentration (where the sensitivity is the greatest), we do have the ability to determine concentration ranges >20 pg/mL (although with little accuracy). While this is the case, we point out that the most relevant E2 concentrations to quantify are <20 pg/mL. Nevertheless, we explain the diminished response at >20 pg/mL by considering the fact that the devices initially have many pathways for ions to enter the microgel layer to yield a change in the device's optical properties. We hypothesize that at low concentrations the pores can be more efficiently blocked upon analyte binding compared to the high concentration range, where some of the analyte may bind to regions of the device that do not have pores that can be blocked. Although, this is our hypothesis, and more studies will need to be done to fully elucidate this mechanism.

$$\Delta\lambda = -0.8121\ C_{E2} + 101.3 \quad (1)$$

$$\Delta\lambda = -0.0568\ C_{E2} + 87.624 \quad (2)$$

Furthermore, a detection limit of 1.2 pg/mL (approximately 4.3 pM) was calculated using three times the standard deviation of blank samples. The detection limit achieved here is lower than the 10 pmol detection limit achieved by Varriale et al. [24], and is comparable to the 2 pM achieved by Huang et al. [21] although it is higher than the 33 fM achieved by Fan et al. [20], 0.8 pM by Fan et al. [19], as well as the 3.3×10^{-16}M reported by Du et al. [25].

Previous research on E2 detection in urine using anti-E2 DNA aptamer-coated gold nanoparticles showed that the sensitivity of E2 detection could be improved 25-fold using a shorter 35-mer aptamer rather than the 75-mer aptamer [24]. Here, we also modified the etalon surface with the shorter 35-mer DNA aptamer (E2Ap35), and the response of E2Ap35 modified etalon devices to 500 mM NaCl as a function of E2 concentration was recorded (Figure 2B). Using E2Ap35 as the binding element, these devices showed decreased dynamic range compared to the E2Ap75-modified etalons. This is likely a result of the decreased surface blocking from the shorter aptamer upon binding E2 compared to the more efficient blocking that is possible with the longer aptamer. Therefore, E2Ap75-modified etalons were used in all subsequent experiments.

3.3. Specificity of estradiol detection using E2Ap75 modified etalons based on the NaCl surface blocking.

The concentration of E2 in raw and commercial whole milk ranges from 4 to 14 pg/mL [45], while the P4 concentration ranges from 0.2 to 17 ng/mL [8]. It is well known that P4 is a major interference that complicates the quantification of E2 in milk samples. Hence, we determined the specificity of the E2Ap75-modified etalons to E2. Figure 2C shows the relative peak shifts of this etalon system in response to different concentration of E2 and a much higher concentration of P4. The signal

produced by 10 ng/mL P4 was significantly less than the signal produced by 5 pg/mL E2, which indicates the device is highly specific for E2.

3.4. Regeneration of E2Ap75 modified etalon

To test the possibility of regenerating the aptamer-modified etalons for reuse, they were first used to detect 20 pg/mL E2, then soaked in 0.5 % SDS (pH 1.9) for 2 min at room temperature, followed by rinsing with copious amounts of water. This process was repeated 3 times to regenerate the sensors. The regenerated sensors were again exposed to 20 pg/mL E2 for 30 min and then washed with E2 aptamer binding buffer. The sensor's response to 500 mM NaCl was tested and the peak shift was recorded. After the first regeneration experiment had been done, the sensor was regenerated again with same procedure for total of 5 times. The performance of regenerated sensor for E2 detection is shown in Figure 3A and 3B. The regenerated sensors all produced similar responses to the first use with the signal recovery from 90 % - 108 %, indicating that the devices can be used for multiple analyses.

3.5. Estradiol detection using E2Ap75 modified etalon based on the $CaCl_2$ surface blocking.

Although Ca^{2+} is similar in size to Na^{+} it has twice the positive charge, which we hypothesize will interact more strongly with the etalon's microgels and improve its sensitivity to E2. To test this hypothesis, $CaCl_2$ was used to replace NaCl. As expected, without incubating with E2, the etalon showed a larger blue reflectance peak shift (130 ± 1 nm) in response to 500 mM $CaCl_2$ compared to those of 500 mM NaCl (104 ± 1 nm). The peak shift decreased when the etalon was incubated with higher concentration of E2 due to the blocking of E2Ap75/E2 complex layer on etalon surface to the transportation of Ca^{2+} into etalon. The peak shift decreased to 95 ± 4 nm when the etalon was incubated with 200 pg/mL E2. Figure 4A shows the calibration curve of etalon's reflectance peak shift vs. the concentration of E2. Again, two linear ranges were obtained with these devices, from 0 - 10 pg/mL and

10-200 pg/mL and are described by Equation (3) and Equation (4) with R^2 = 0.991 and 0.954 respectively. Again, the devices are more sensitive to E2 in the low concentration range, hence our ability to accurately quantify E2 >20 pg/mL is diminished compared to the lower concentration range. A detection limit of 0.9 pg/mL was also calculated using three times the standard deviation of blank samples. Here a lower detection limit, 0.9 pg/mL (3.2 pM) was achieved using Ca^{2+} compared to the results of E2 detection using Na^{+} as the response ions, which is calculated as 1.2 pg/mL.

$$\Delta\lambda=-2.4723C_{E2} + 129.7 \quad (3)$$

$$\Delta\lambda=-0.0402C_{E2} + 102.92 \quad (4)$$

Figure 4B shows the relative reflectance spectroscopy peak shifts of this etalon system as a function of different concentration of E2 and a much higher concentration of P4. The sensor's responsive produced by 10 ng/mL P4 was less than the signal produced by 5 pg/mL E2, though the concentration of P4 was 2000 times higher than E2. This indicates that sensor is highly specific to E2.

3.6. Estradiol detection in hormone free milk based on the $CaCl_2$ surface blocking

As the E2AP75-modified etalon sensors show high sensitivity and specificity for quantifying E2 in aqueous samples, we moved on to determine their performance in quantifying E2 in milk samples. Firstly, hormones were removed from the milk by dextran-coated charcoal according to the manufacturer's procedure, and then the hormone free milk was spiked with different concentrations of E2. Then the E2 concentration was determined using the E2 sensors developed here and compared to the spiked amount. The results are shown in Table 1. Without spiking with E2, the E2 concentration was determined to be 0.4 ± 0.3 pg/mL, which suggests that the E2 was almost completely removed from the milk. Furthermore, as can be seen from the data in Table 1, the measured E2 concentrations in the

hormone free milk are very consistent with spiked amount in the samples, and 94.9 % - 111.8 % recovery was obtained.

3.7. Estradiol detection in commercial and farm milk based on the $CaCl_2$ surface blocking

To further investigate the application of this sensor for E2 detection in processed and raw milk samples, the sensor's performance in commercial skim, commercial 2% fat, and farm (whole) milk samples was also evaluated. Table 2 shows the results of E2 detection in commercial skim and 2% milk, as well as in farm milk. Two groups of milk samples were collected from a local farm with one group of cows that were expected to have low concentration of E2, and another group of cows that were believed to be in estrus and expected to have a high concentration of E2 in their milk. The concentration of E2 in commercial 2% milk was measured as 8.4 ± 0.9 pg/mL, which is significantly higher than those in skim milk, 0.9 ± 1.3 pg/mL. For the farm milk samples from the group of cows that were expected to have low concentration of E2, the average E2 concentration was measured as 4.0 ± 1.4 pg/mL; and for those farm milk samples from the group of cows which were expected to have high concentration of E2, average E2 concentration was measured as 9.1 ± 4.3 pg/mL. The concentrations of E2 determined in all milk samples analyzed in the current study were within the range of previously reported values [46]. The successful quantitation of E2 in commercial and farm milk directly without any pre-treatment suggests that the sensor developed in the current research has strong potential to be used as a "cow-side" testing device.

4. CONCLUSION

In conclusion, we describe a device capable of quantifying E2 in aqueous solutions as well as in commercial and farm milk samples by modifying the etalon surface with anti-E2 DNA aptamers. The ability of the sensors to detect different E2 concentrations was determined by monitoring the

reflectance peak shifts in the devices reflectance spectra in response to 500 mM NaCl and 500 mM $CaCl_2$ as a function of E2 concentration up to 200 pg/mL (the lowest measured concentration is 5 pg/mL). The sensors worked more sensitively in low concentration range (⩽ 20 pg/mL) than the high centration range (20-200 pg/mL), and with calculated detection limits of 1.2 pg/mL (4.3 pM) and 0.9 pg/mL (3.2 pM) for E2 detection were achieved at room temperature in water. The devices also exhibited high selectivity in the presence of the common interfering species, P4. The sensors could be regenerated 5 times without losing their sensitivity with a simple one-step washing procedure. Moreover, the E2 sensors demonstrated good performance to quantify E2 in hormones free milk, which spiked with different concentration of E2, as well as in the commercial skim milk, 2% milk and farm milk. This further demonstrates the sensor's potential application as a "cow-side" testing device for the dairy industry.

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FIGURE CAPTIONS

Figure 1. (A, left) Schematic of an etalon, where (a) and (c) are 15 nm Au layers (with 2 nm Cr as adhesion layer) sandwiching a (b) microgel layer (d) all on a glass substrate. (A) Also shows the sensing mechanism, with the DNA aptamer binding E2 and forming specific secondary structure that blocks Na^{+} or Ca^{2+} from entering the etalons' microgel layer; the extent of blocking is directly proportional to the concentration of E2 in the sample. (B) A typical reflectance spectrum obtained from an etalon used in this investigation.

Figure 2. Reflectance spectrum peak shift for (A) E2Ap75 and (B) E2Ap35-modified etalons in response to 500 mM NaCl as a function of E2 concentration at room temperature, as well as the relative rreflectance spectrum peak shift of E2Ap75 modified etalons in response to 500 mM NaCl as a function of E2 and P4 concentration at room temperature (C). The peak shifts were calculated relative to the initial peak position. Each data point represents the average of at least three independent

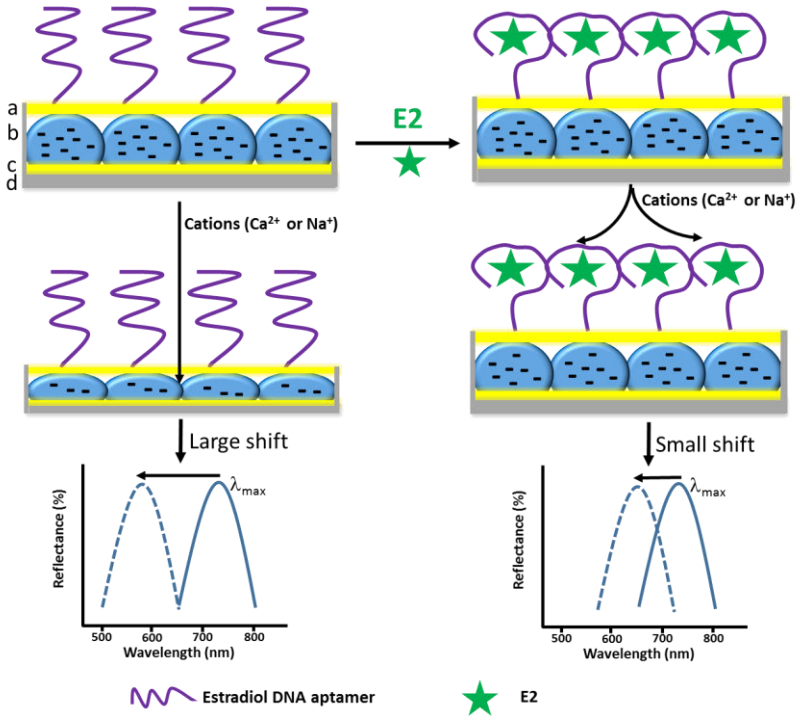
measurements using different etalons, and the error bars are the standard deviation for those values. The relative peak shifts were calculated relative to the etalon with 0 pg/mL E2. Each data point represents the average of at least three independent measurements using different etalons, and the error bars are the standard deviation for those values.

Figure 3. Regeneration of E2Ap75 modified etalon sensors. (A) The responsive signal (peak shift) of original and regenerated E2 sensors, and (B) the signal recovery of regenerated sensors in response to 500 mM NaCl.

Figure 4. (A) Reflectance spectrum peak shifts of E2Ap75 modified etalons in response to 500 mM $CaCl_2$ as a function of E2 concentration at room temperature. The peak shifts were calculated relative to the initial peak position. (B) Relative reflectance spectrum peak shifts of E2Ap75 modified etalons in response to 500 mM $CaCl_2$ as a function of E2 and P4 concentration at room temperature. The relative peak shifts were calculated relative to the etalon with 0 pg/mL E2. Each data point represents the average of at least three independent measurements on different etalons, and the error bars are the standard deviation for those values.

Figures and Tables

A



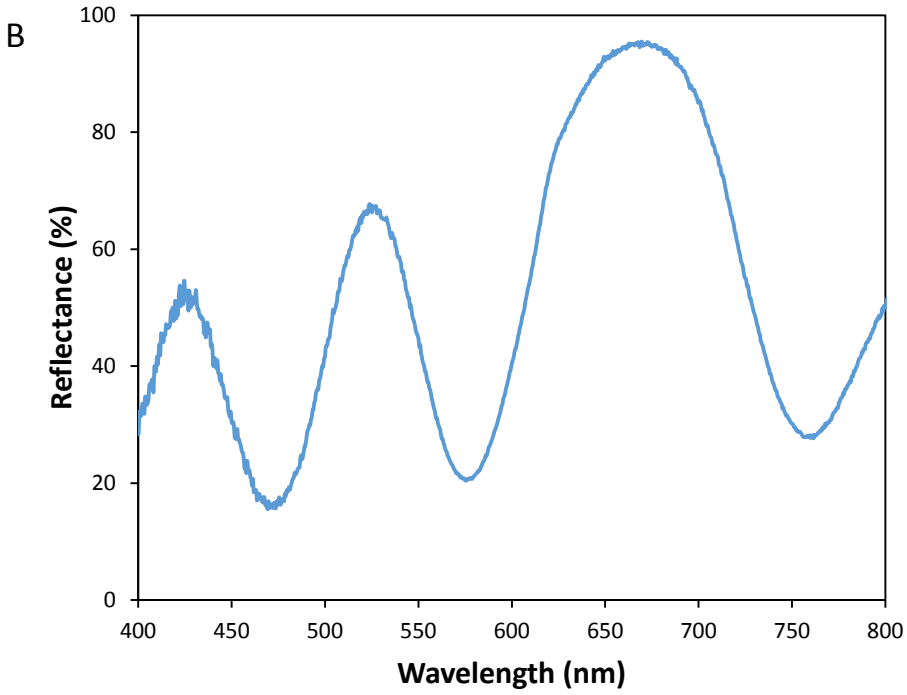
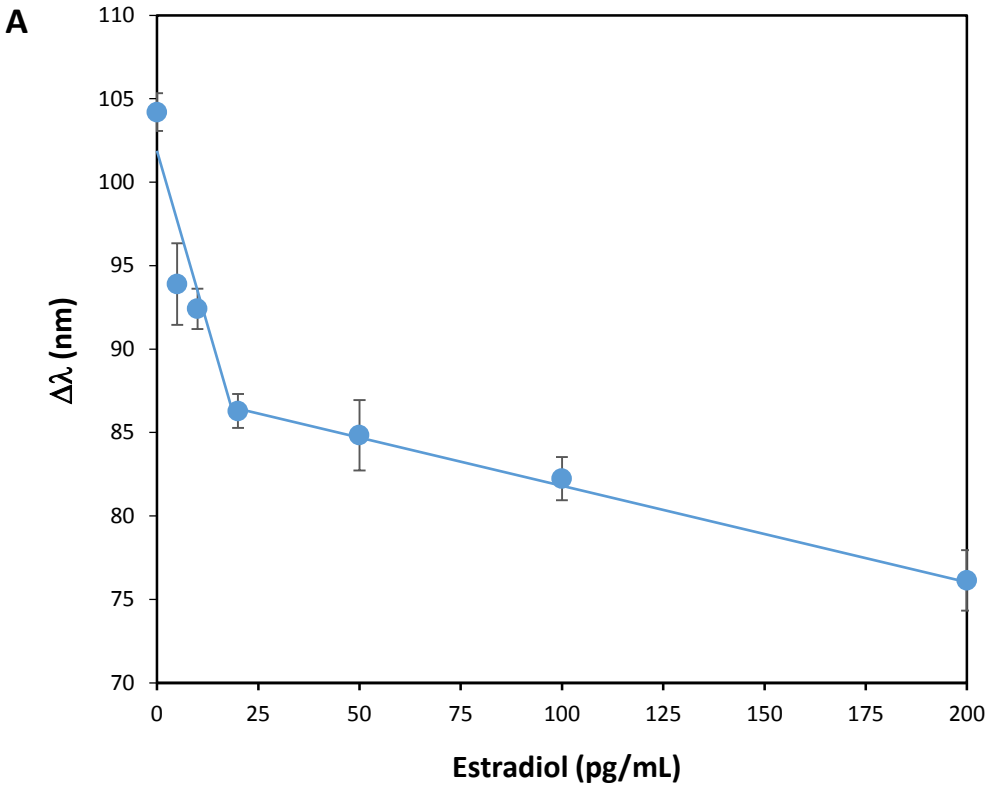
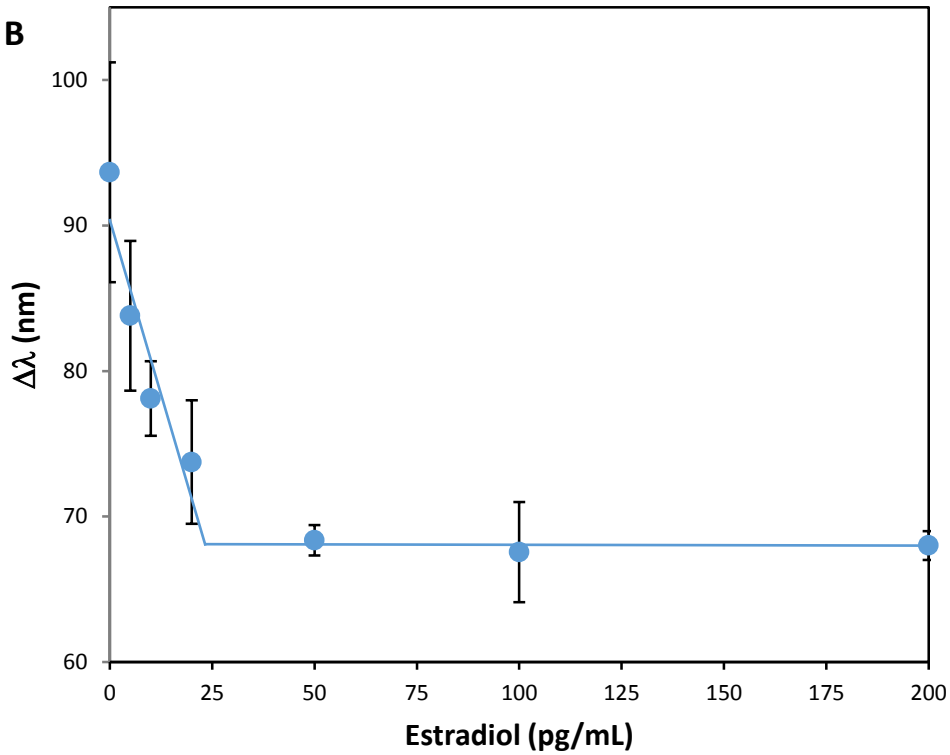

B
100
80
60
40
20
0
Reflectance (%)
400
450
500
550
600
650
700
750
800
Wavelength (nm)

Figure 1


A
110
105
100
95
90
85
80
75
70
Δλ (nm)
0
25
50
75
100
125
150
175
200
Estradiol (pg/mL)


B
100
90
80
70
60
Δλ (nm)
0
25
50
75
100
125
150
175
200
Estradiol (pg/mL)

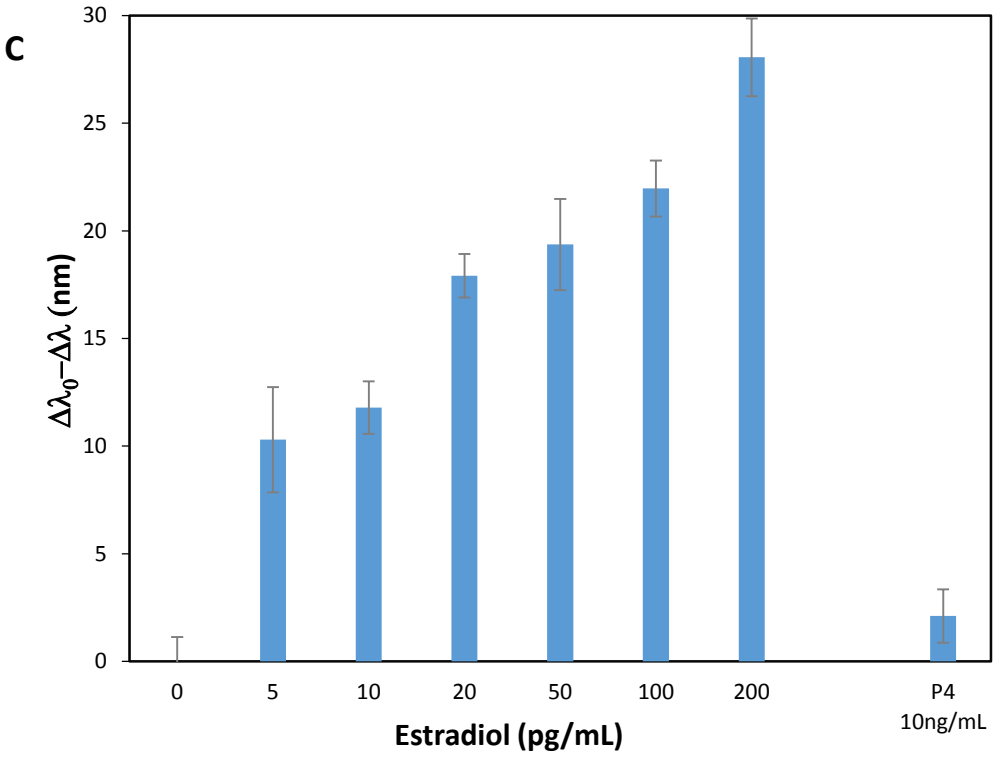

C
30
25
20
15
10
5
0
$\Delta\lambda_0-\Delta\lambda$ (nm)
0
5
10
20
50
100
200
P4
10ng/mL
Estradiol (pg/mL)

Figure 2

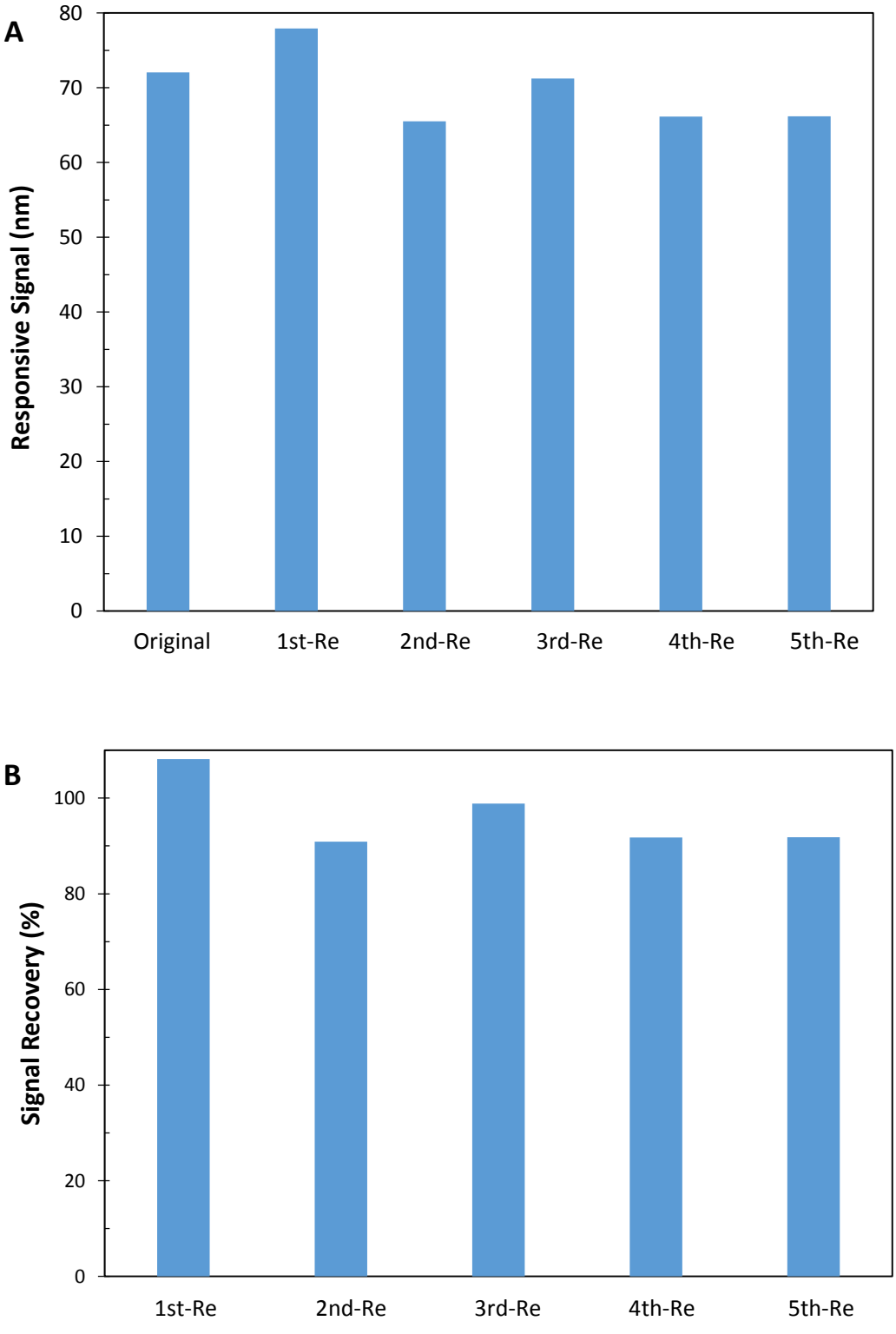
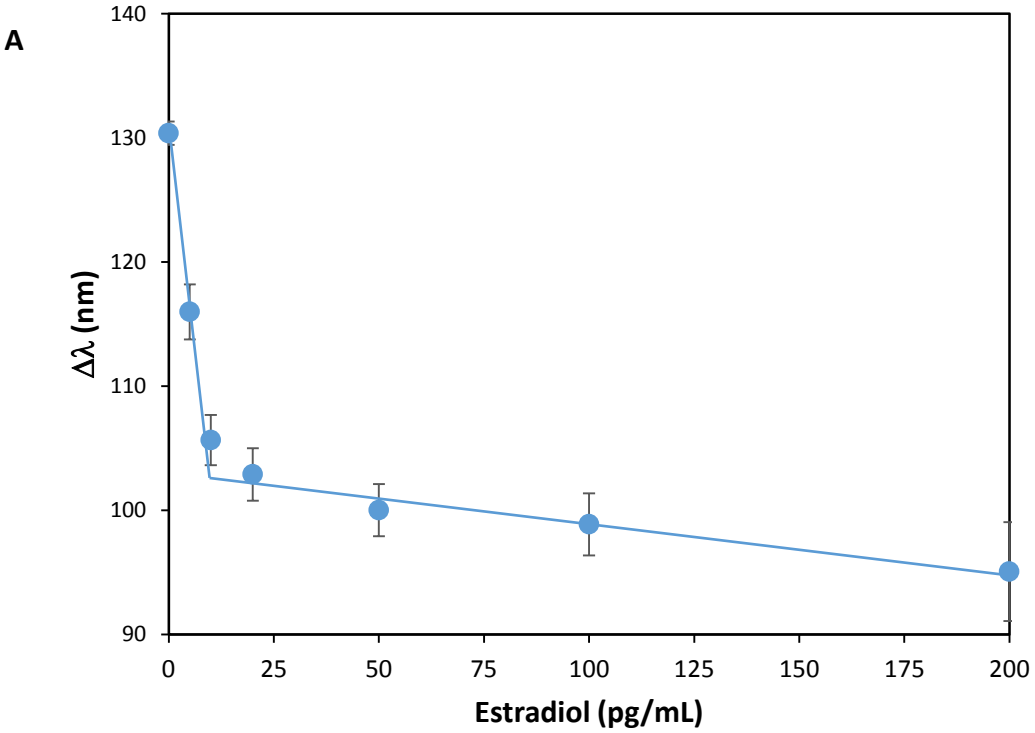

A
Responsive Signal (nm)
80
70
60
50
40
30
20
10
0
Original
1st-Re
2nd-Re
3rd-Re
4th-Re
5th-Re
B
Signal Recovery (%)
100
80
60
40
20
0
1st-Re
2nd-Re
3rd-Re
4th-Re
5th-Re

Figure 3


A
140
130
120
110
100
90
Δλ (nm)
0
25
50
75
100
125
150
175
200
Estradiol (pg/mL)

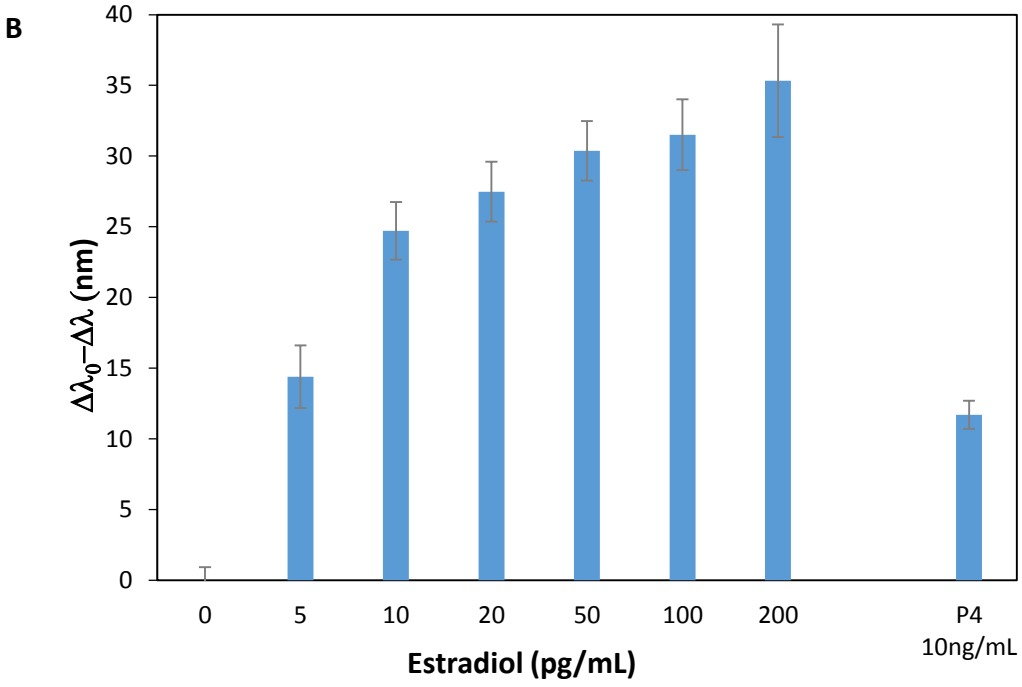

B
40
35
30
25
20
15
10
5
0
Δλ0–Δλ (nm)
0
5
10
20
50
100
200
P4
10ng/mL
Estradiol (pg/mL)

Figure 4

Table 1. Estradiol detection in hormone free milk based on the $CaCl_2$ surface blocking

Estradiol-Spike (pg/mL)	**Estradiol-Measured (pg/mL)**				**Recovery (%)**			
	1st	2nd	3rd	Average	1st	2nd	3rd	Average
0	0.5	0.0	0.6	0.4±0.3	n/a	n/a	n/a	n/a
5	5.1	7.0	4.2	5.5±1.2	102.5	140.6	84.8	109.3±23.3
10	9.7	9.1	9.7	9.5±0.3	97.1	90.8	96.8	94.9±2.9
50	78.1	49.8	39.8	55.9±16.2	156.2	99.5	79.6	111.8±32.5

Table 2. Estradiol detection in commercial and farm milk based on the $CaCl_2$ surface blocking

	Sample ID	E2-measured (pg/mL)	Average E2 (pg/mL)
Commercial skim milk	#1	0	0.9±1.3
	#2	0	
	#3	2.7	
Commercial 2% milk	#1	7.1	8.4±0.9
	#2	9.1	
	#3	9.0	
Farm milk expected to have high concentration of E2	#3644	17.5	9.1±4.3
	#2258	5.3	
	#2301	7.2	
	#2521	8.5	
	#5088	7.1	
Farm milk expected to have low concentration of E2	#2267	4.6	4.0±1.4
	#2307	2.3	
	#5120	4.9	
	#2535	5.8	
	#5013	2.4	