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Rapid Detection of *Escherichia coli* O157:H7 Using Competitive Exchange of Fluorescent Surrogate Modified Surfaces in Liquid Media

[1] Michael J. ANDERSON, [2] Andmorgan R. FISHER, [2] Alex LY, [1, 3] Evangelyn C. ALOCILJA and [2] Clint B. SMITH

[1] Biosystems and Agricultural Engineering, Michigan State University, 115 Farrall Hall, East Lansing, MI, 48824, USA

[2] US Army Engineer Research and Development Center, Alexandria, Virginia 22315

[3] Tel.: 01-517-432-8672

E-mail: alocilja@msu.edu

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Abstract: A novel fluorescent surrogate based immunosensor is presented for use in remote detection of *Escherichia coli* O157:H7. A total of 3704 confirmed *E. coli* O157:H7 cases with a hospitalization rate of 46.5 % were reported by the Centers for Disease Control and Prevention in 2008. Rapid detection would aid in both prevention and water supply security. This report describes the successful generation of a sensor comprised of fluorescent surrogates with attachment to covalently immobilized antibodies. Fluorescence signal loss was seen upon live *E. coli* O157:H7 challenge. The 'acceleration' in signal decay detected a spiking event after 7 minutes with a detection threshold of 100 colony forming units per milliliter. The sensor surface was stable for 300 sampling times over a 5 hour period. This sensor design is adaptable for integration into remote sensing type systems due to the small size, low power requirements and stability. *Copyright © 2012 IFSA.*

Keywords: Biosensor, Autonomous, *E. coli*, Remote sensing, Immunosensor.

1. Introduction

Approximately 1.8 million deaths were reported in 2005 as a result from diarrhoeal disease world wide. A major cause of diarrhoeal disease is *Escherichia coli* O157:H7 (*E. coli* O157:H7) which is part of a larger class of enterohemorrhagic *E. coli* (EHEC) [1]. *E.coli* are most commonly present when

human and animal wastes contaminate water sources, and thus used as an indicator of water quality [2]. Approved water testing by the United States Environmental Protection Agency (EPA) is limited to culture methods utilizing a growth substrate that fluoresces when metabolized. This detection requires the samples to grown for 18-48 hours for adequate measurement. Rapid detection could help prevent many such cases as stricter precautions and warnings could be followed upon detection of contamination [3].

Recent rapid bacteria detection technologies have included DNA probes [4], polymerase chain reaction [5], antibody recognition [6], electrical resistance [7, 8], resonance frequency change[9] and metabolic fluorescence methods [10]. These technologies often cannot be performed outside of the laboratory environment and require sample purification and processing before detection. Current efforts are focused on integrating a rapid detection system that is autonomous in operation for remote operation[11]. Such existing systems are currently large in size [12], require growth times on the order of 3-6 hours [13], or consist of expensive equipment with high energy requirements [14].

The presented work uses a fluorescently stained surrogate bound to an immobilized antibody for detection. Samples are read in a small, portable, low power fluorometer. The use of pre-stained self-generated surrogates allows for detection of *E. coli* O157:H7 using the second derivative of the loss of surrogate fluorescence signal.

2. Materials and Methods

2.1. Materials

All reagents were received and used from Sigma-Aldrich without purification unless otherwise noted. Standard glass cover slips (Corning #2865-22) and neutravidin (Pierce #15217) were obtained from Fisher Scientific. Biotin labeled anti-O157:H7 was obtained from AbCam (#ab20640). Heat inactivated *E. coli* O157:H7 (#50-95-90) and unlabeled anti-O157:H7 antibody (#01-95-90) were obtained from KPL. Cellular dyes PicoGreen (#P7581), Vybrant DyeCyle Ruby (#V-10309) and SYTO Green Fluorescent Nucleic Acid Stain Sampler Kit #1 (#S7572) were obtained from Invitrogen (Life Sciences).

Live cell culture was used for the challenge species during testing. *Escherichia coli* O157:H7 Sakai (*E.coli* O157:H7) strain was obtained from the Michigan State University Food Safety and Toxicology Center. Cultures were grown for 8 hours in tryptic soy broth (TSB) at 37 °C to generate samples of live cells in the range of 1×10^7 to 1×10^8 colony forming units (CFU) per milliliter. All cultures were washed three times in phosphate buffered saline (PBS) and pelleted at 10,000 × g for 3 minutes. Cultures were re-suspended to original volume in PBS before use.

2.2. Methods

2.2.1. Surrogate Generation

Intact surrogate cells were generated by treatment with permeabilizing agents and cross-linking agents. A volume of 1 mL of 8-hour cultured cells was washed 3 times in PBS and re-suspended in the desired permeabilization or fixation agent. Permeabilization was tested using 100 % acetone, 100 % ethanol, 100 % methanol, 50 % acetone/methanol, and 50 % acetone/ethanol mixtures all at -20 °C for 10 minutes. Fixation only was tested in PBS with 2, 4, 6, and 8 % formaldehyde; 1, 1.5, and 2 % glutaraldehyde; and mixtures of 8% formaldehyde with 1, 1.5, or 2% glutaraldehyde at 4 °C for 4 hours. The entire 1 mL washed sample for each condition was plated on tryptic soy agar (TSA) in 100 µL volumes and cultured for 24 hours at 37 °C to confirm fixation or deactivation.

2.2.2. Surrogate Staining

The fixed or permeabilized surrogates (1×10^8 cells/mL) were stained with a PicoGreen solution, 2.5 mM solution of Vybrant Ruby, or SYTO 9, 11, 12, 13, 14, 16, 21 or 24 dyes in PBS for 15 minutes and stored at 4 °C protected from light. Samples were tested immediately after staining, at 2 weeks of storage and at 4 weeks of storage. Heat inactivated cells from KPL were re-suspended in 1× PBS and stained in the same fashion as the generated surrogates. Both heat inactivated and fixed or permeable cells were *E.Coli* O157:H7.

2.2.3. Amine Decoration of Glass

Amine functional groups were added to the glass surface via silanization using (3-Aminopropyl) triethoxysilane (APTES) (Sigma #440140). Glass coverslips were loaded into a coverslip staining rack (Electron Microscopy Sciences #72241-01) for all subsequent steps. Cover slips were submerged in 50 % methanol and sonicated for 10 minutes, allowed to dry, then submerged in chloroform and sonicated for 10 minutes. After air drying, the glass was submerged in piranha solution (70 % sulfuric acid and 30 % hydrogen peroxide (35 % w/v)) at 65 °C for 30 minutes. Cover slips were washed 3x in distilled water and dried overnight at 110 °C and 10 kPa of pressure. When dry, the glass was allowed to cool in a biosafety cabinet and submerged for 45 seconds in a 2 % APTES solution in dry acetone. Upon amine coating via APTES, the slides were washed 3x in distilled water and dried overnight at 37 °C and 10 kPa.

2.2.4. Sensors Construction

Protein attachment was accomplished using glutaraldehyde as the cross-linking agent. A volume of 100 μL of 2.5 mM glutaraldehyde was added to the center of each coverslip for 1 hour at room temperature. The slides were washed and 125 μL of either 1 mg/mL of non-modified antibody or 10 mg/mL neutravadin was added to glutaraldehyde functionalized spot for 90 minutes at room temperature. The slides were washed and blocked in 125 mM Tris and 2.5 mM sodium cyanoborohydride for 90 minutes to block free glutaraldehyde sites and reduce the imine bond to a more stable amine bond. The coverslips were washed 3x in distilled water. A volume of 125 μL of 1 mg/mL of biotin-labeled antibody was added to the avidin coated slide and allowed to bind for 90 minutes at room temperature. The avidin coated slides were then washed in distilled water 3x times.

A volume of 150 μL of 1:1000 dilution of stained surrogates ($\sim10^4$-10^5 CFU/mL) was added to the immobilized antibodies on each cover slip for 2 hours at room temperature protected from light. Coverslips were submerged in 1 mL of PBS and stored at 4°C before use. Prior to use, slides were rinsed in distilled water.

2.2.5. Testing and Imaging

Generated glass sensors were tested in a 25 mL volume of PBS spiked with 1 mL of 4×10^6 stained challenge cells for a final concentration tested of 1.6×10^5 cells/mL. Combinations of formaldehyde fixed and heat inactivated surrogates were challenged against each other to obtain the best sensor response. Sensor functionality was confirmed on an Olympus BX-43 fluorescent microscope using Cy5.5 and GFP filter sets. Environmental challenges were accomplished using a portable fluorometer with the Cy5.5 filter set using *E. coli* O157:H7 Sakai spiked ground water from Michigan State University. A sampling rate of 2.5 mL of water was conducted every 1 minute for testing.

3. Results and Discussions

Surrogate generation and staining were measured in tandem using PicoGreen staining and GFP filter set (cutoffs λ_{ex}= 490 nm, λ_{em}= 535 nm). The only treatments not resulting in growth when overnight cultured were the 4 % formaldehyde and greater, methanol/acetone, and acetone treatments. Fig. 1 shows the results for four of the surrogate generation methods in water suspension. From Fig. 1a, the acetone treatment causes high background noise by allowing the stained DNA out of the cell via permeabilization. Fig. 1b, c, and d show the desired staining, but only the formaldehyde was negative for cell growth. The methanol-acetone treatment was similar to Fig. 1a.

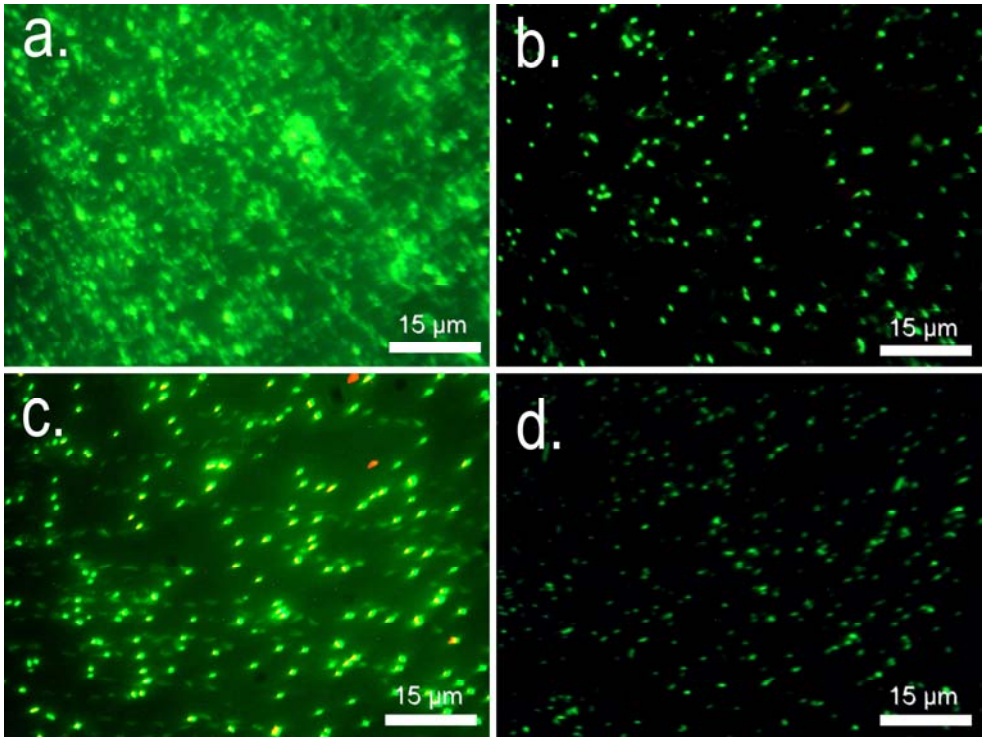

Fig. 1. Fixation or permeabilization treatments: a) acetone; b) 50 % methanol/ethanol; c) glutaraldehyde; d) formaldehyde.

Using a 4 % formaldehyde fixation, the SYTO green and PicoGreen dyes were tested for storage stability after 30 days at 4 °C. Using the same criteria of image quality, low background noise, and high signal, PicoGreen was chosen for the green stain. The SYTO stains all showed high background fluorescence and lower staining signal than PicoGreen.

Verification of amine functionalization and antibody immobilization was accomplished by challenging the generated surfaces with the PicoGreen stained formaldehyde generated surrogates. Fig. 2 shows the surface after direct antibody attachment and biotin-neutravidin attachment.

The directly attached antibody sensors have approximately twice (192 %) the captured antibodies when compared to the netravadin-biotin bound antibodies (Fig. 2a and 2b). An average bound cell density of 4.4×10^4 cells/mm^2 (n=45) for avidin binding and 8.3×10^4 cells/mm^2 for direct attachment (n= 19) was determined.

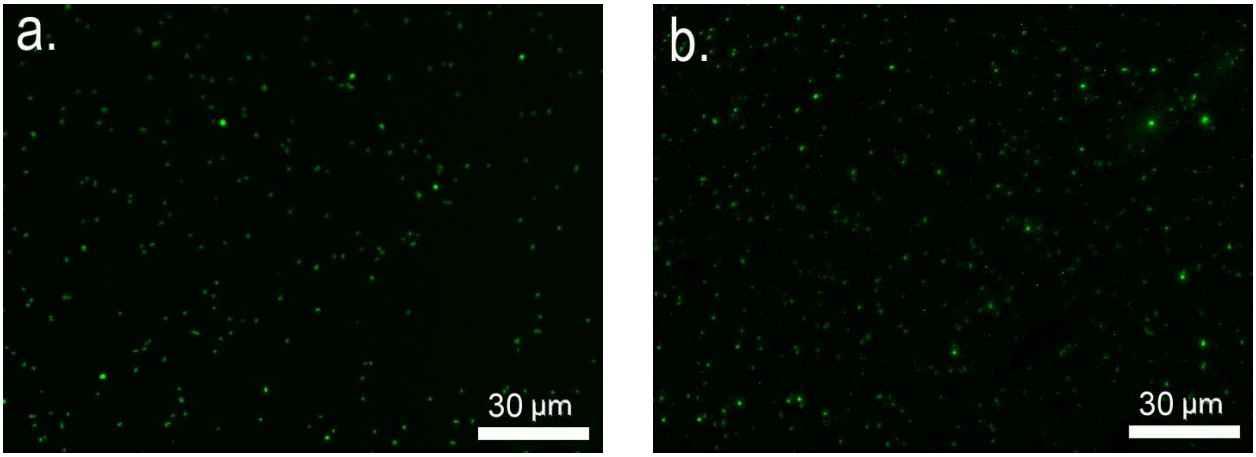

Fig. 2. Verification of antibody attachment: a) biotin-labeled antibody attached to immobilized neutravadin; b) direct attachment of antibody to glass surface.

Sensor function was verified by attaching combinations of red or green stained and heat inactivated or formaldehyde stained surrogate to the antibody surface. The sensors were then challenged with the oppositely colored cell type. Fig. 3 shows a sensor surface after 30 minutes of testing against a 25 mL volume at 1.6×10^5 cells/mL. Vybrant Ruby images are false colored red.

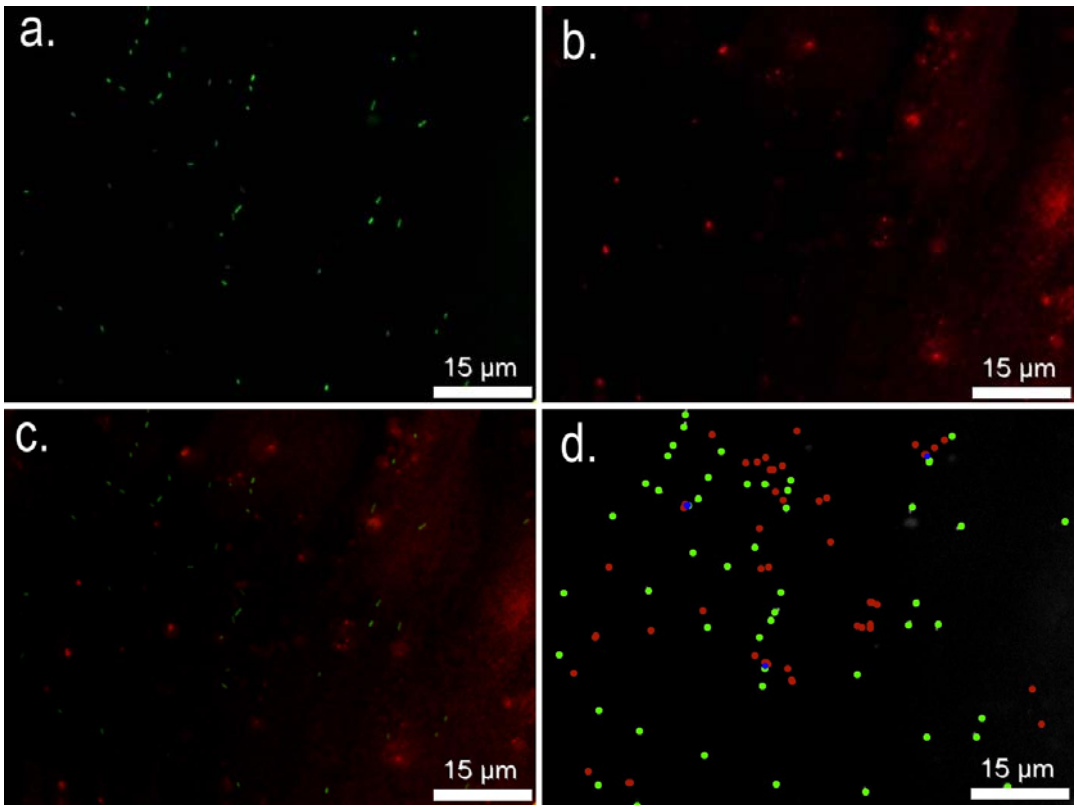

Fig. 3. Verification of sensor function. Picogreen stained heat inactivated surrogate loaded with Vybrant Ruby stained live challenge cells after 30 minute. a. GFP filter showing initial surrogate remaining; b. Cy5.5 filter with false color showing target attachment; c. overlay of green and red channels; d: counted overlay of cells, blue indicates overlap.

The challenge cells (red) have replaced 41 % of the surrogates (green) in Fig. 3. The density of cells, red and green, is consistent with fully coated sensor constructs. Table 1 lists results of sensor design verification.

Table 1. Exchange rates of surrogate types with different target challenges.

ID #	Surrogate Dye	Surrogate Treament	Target	Surrogate % Loss	Target % Gain
1	Ruby	Formaldehyde	Live	96.3%	62.0%
2	PicoGreen	Formaldehyde	Live	0.0%	2.3%
3	Ruby	Formaldehyde	Heat Inactivated	99.2%	19.0%
4	PicoGreen	Formaldehyde	Heat Inactivated	59.5%	48.5%
5	Ruby	Heat Inactivated	Live	98.5%	92.1%
6	PicoGreen	Heat Inactivated	Live	82.3%	0.0%

Sensors were generated and tested per Table 1. The stained surrogates were challenged with the oppositely colored targets for 5 minutes and then imaged with both the GFP and Cy5.5 filter set in series. In Table 1, the surrogate % loss was defined as the reduction of the surrogate and the target % gain was defined as the capture of the target, both compared to the average density from previous data. In all of the configurations except #2, surrogate loss was demonstrated with target capture. This suggests the targets are displacing the surrogates, removing them from the sensor surface. In configuration #2, the surrogates remained bound to the surface, which likely prevented the capture of the challenge target. Configuration #3 has a higher loss of surrogate than configuration #4, this would be explainable as the green signal having higher detection efficiency in configuration #4, as all other conditions were held constant. The same pattern is seen in configurations #5 and #6, and would also explain why PicoGreen targets generally have higher values. The light source from the camera is less efficient for Cy5.5 emission than it is for GFP emission, which results in better green signal detection.

From Table 1, both formaldehyde treated and the heat inactivated surrogates function properly when challenged against either live or heat inactivated *E. coli* O157:H7 targets. Additional sensors were constructed of formaldehyde treated surrogates, stained with Vybrant Ruby dye and tested in a portable fluoromenter. The use of in-house generated surrogates allows for lower construction costs and faster sensor construction.

Environmental testing was conducted using a prototype field portable fluorometer powered by 3 "C" cell alkaline batteries. Vybrant Ruby fluorescence was accomplished using a laser diode excitation source (650 ± 10 nm) with a 680 nm absorptive long pass emission filter. The path length from the diode to the sample is 5mm. Sensor output is recorded as the ratio of the excitation diode operating voltage divided by the integration time (seconds) to saturate the detection element. Stronger fluorescence output has a shorter integration time. This method eliminates the need for a photomultiplier tube and reduces system cost and physical size.

Stability was confirmed by allowing a constructed sensor to be stored under PBS at 4 °C for two weeks and run against Michigan State University tap water. The water used was tested for common minerals and contained: sulfate 60 mg/L, iron 0.3 mg/L, calcium carbonate 380 mg/L, and pH 7.3. Fig. 4 shows the result of a 5.4 hour run time without a target challenge, when *E. coli* O157:H7 was spiked at a final concentration of 1×10^6 CFU/mL. The sensor surface decays from a combination of photo bleaching and non-specific surrogate loss. To confirm a binding event of the desired target, the second derivative of the signal is calculated using the leading and trailing two points for a five point calculation.

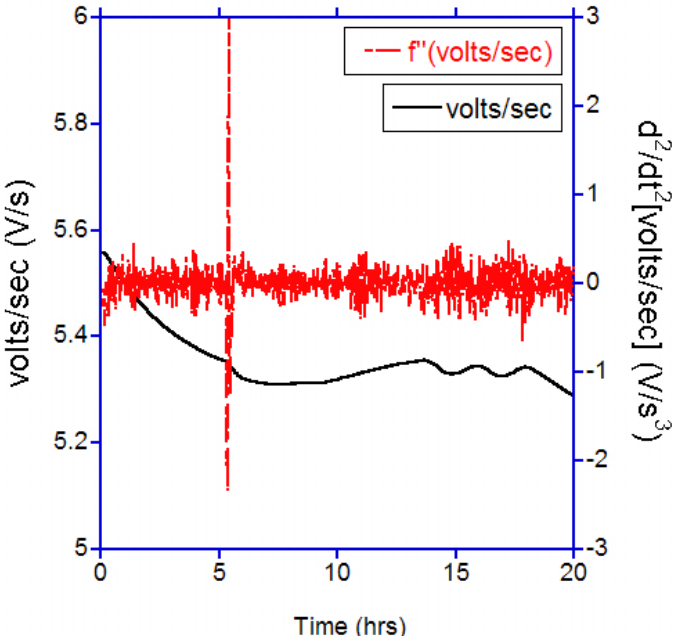

Fig. 4. Sensor stability verification after 5.4 hours in solution before spiking event of 10^6 CFU/mL final concentration. Left axis: V/s, Right Axis: d^2/dt^2(V/s).

Positive samples were determined when a 200 % increase in the second derivative (f'') over sensor noise occurred. The positive spike in the second derivative occurs two points before the actual spike as a result of the calculation, with the negative spike occurring two points after the event. The spike gives a consistent way to detect binding/exchange events on the sensor when used for prolonged detection.

Sensors were challenged by submersion into tap water with 10-10,000 CFU/mL of *E. coli* O157:H7 to determine limits of sensitivity. Sensors were introduced into spiked water and plotted in Fig. 5.

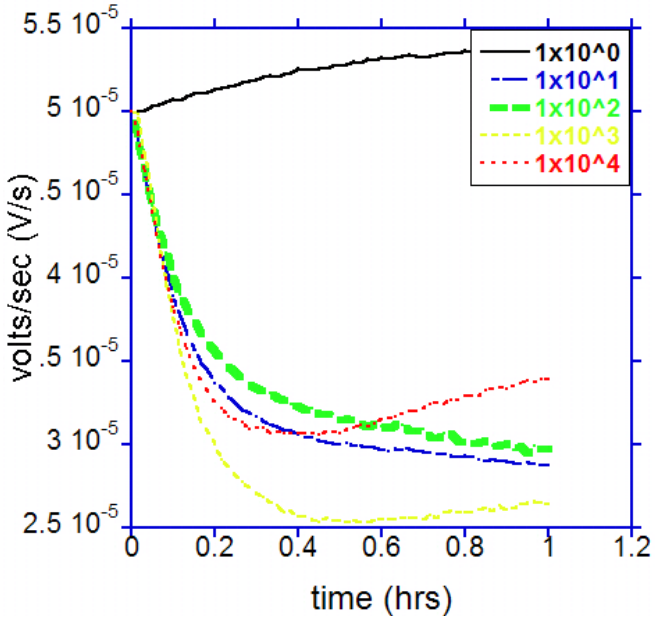

Fig. 5. Raw signal change for E. coli O157:H7 challenge.

With a fresh sensor surface, all concentrations of challenge show a response except 10 CFU/mL. Fig. 6 shows the second derivative, acceleration, of the data from Fig. 5 and confirms positive identification from 1×10^1-1×10^4 CFU/mL.

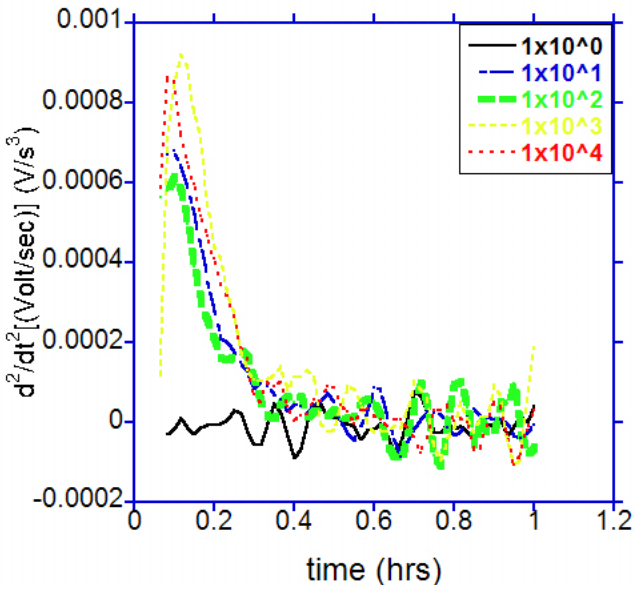

Fig. 6. Signal acceleration of data from Fig. 5.

From Fig. 6, it is seen that all samples above 1×10^1 show at least three times increase compared to the background, and are detected in 7 minutes.

The use of the second derivative of the signal allows for discrimination of the actual binding events from the electronic noise and sensor decay in the system. System noise is seen in the data in Figs. 4 and 5. When using the second derivative, the system noise was ±0.0002 volts/sec^3.

4. Conclusions and Future Work

The successful construction and demonstration of a loss of signal fluorescent biosensor was accomplished. The surrogate antigen was successfully generated using formaldehyde fixation and stained with both PicoGreen and Vybrant Ruby fluorescent dyes. The sensing surface was shown to exchange live target for surrogates and can be used to detect target levels of 100 CFU/mL. Preliminary testing shows the sensors are sustainable for 300 sample reads at an interval of 1 minute per point. The use of the sensor for prolonged testing appears promising when the testing interval is extended, but a balance between rapid response and service life will need to be explored. This sensor package uses a small footprint, low power fluorometer compatible with autonomous sensing requirements. Future applications for this sensing surface include the integration of communication equipment and testing in natural bodies of water. The in-house generation of surrogate antigen from live cell culture makes possible the detection of other microorganism if the appropriate antibody exists.

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