

BIOSENSOR DEVELOPMENT FOR ENVIRONMENTAL MONITORING,
FOOD SAFETY, AND SECONDARY EDUCATION APPLICATIONS

By

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TABLE OF CONTENTS

LIST OF TABLES	7
LIST OF FIGURES	8
ABSTRACT.....	12
INTRODUCTION.....	15
1. Biosensors Market and Applications.....	15
2. Current Detection Methods for Soil and Food Samples	17
3. Overview of Dissertation	18
3.1. Biosensor Development for Soil and Meat Samples	18
3.1.1. Optofluidic Lab-on-a-Chip System.....	18
3.1.2 Immunoagglutination Assay and Mie Scatter.....	20
3.1.3. From Optical Fibers and Spectrometer to Smartphone Camera	22
3.2. Biosensors in Secondary Education	22
3.3. Summary of the Present Study	24
References	26
APPENDIX A	30
Abstract	31
1. Introduction	32
2. Materials and Method.....	35
3. Results and Discussion.....	42
4. Conclusion.....	50
Acknowledgements	52

APPENDIX B	55
Abstract	56
1. Introduction	57
2. Materials and method	62
3. Results and discussion.....	69
4. Conclusion.....	80
Acknowledgement.....	80
APPENDIX C	86
Abstract	87
1. Background	88
2. Methods.....	91
3. Results and Discussion.....	98
4. Conclusion.....	111
Acknowledgements	114

LIST OF TABLES

Table C-1. The pre- and post-survey data collected.	99
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LIST OF FIGURES

Figure A-1. <i>E. coli</i> extraction from soil sample.....	37
Figure A-2. Lab-on-a-chip layout.	39
Figure A-3. Semi-benchtop system for optofluidic lab-on-a-chip.....	40
Figure A-4. Standard curve produced with the optofluidic lab-on-a-chip system. Values are averages of four different experiments (error bars are standard errors; n.c. = negative control).....	43
Figure A-5. Depth vs. <i>E. coli</i> concentration of four replicates with initial pulse inputs of <i>E. coli</i> solution to the soil surface, incubated overnight. Concentrations were determined by comparing with a standard curve (fig. 4).	45
Figure A-6. Fluorescence microscopy images of extracted <i>E. coli</i> solutions with green fluorescent stain (SYTO 9) at (a) 1 cm and (b) 4 cm depths.	46
Figure A-7. Depth vs. total protein concentration of two replicates with initial pulse inputs of <i>E. coli</i> solution to the soil surface, incubated overnight. Concentrations were determined by Bradford protein assay.	47

Figure B-1. The benchtop system consists of an iPhone 4S and its holder, an NIR LED and its holder, and a ground beef sample and its holder. The angle of scatter detection refers to the angle between the iPhone camera and the NIR LED light source. **65**

Figure B-2. Photographs showing the operation of the smartphone application at the four specific angle of scatter detection: A) 15°, B) 30°, C) 45°, and D) 60°. **68**

Figure B-3. Surface plots that combine the normalized light intensities, the angles of scatter detection, and the log concentrations of A) *E. coli*, and B) *Salmonella*. Black stars indicate the data points significantly above the negative controls, while white stars significantly below the negative controls. **72**

Figure B-4. SEM images of A) DI water on ground beef, B) 10^5 CFU mL⁻¹ *E. coli* on ground beef, and C) 10^8 CFU mL⁻¹ *E. coli* on ground beef. **75**

Figure B-5. Fluorescence microscopic images of Hoechst-stained *E. coli* (blue) on Nile Red-stained ground beef sample (yellow) with the *E. coli* concentrations of 10^2 (A and B) and 10^5 (C and D) CFU mL⁻¹. **77**

Figure B-6. Normalized light intensities plotted against scatter detection angles with different A) *E. coli*, and B) *Salmonella* concentrations. **79**

Supplementary Material B-1. Normalized scatter light intensities plotted against the log concentrations of *E. coli* (A-D; on 80% lean ground beef) and *Salmonella* (E-H; on 92% lean ground beef). Error bars represent standard errors. The scatter detection angle was 15° (A and E), 30° (B and F), 45° (C and G), and 60° (D and H). **84**

Supplementary Material B-2. Typical images obtained with the iPhone camera. A) Raw image showing higher light intensity. B) Processed image of A. C) Raw image showing lower light intensity. D) Processed image of C. **85**

Figure C-1. Seven techniques were chosen and introduced to students in terms of their use in biosensors..... **95**

Figure C-2. The numbers of middle school students (A) and high school students (B) at each application area of biosensor, before (PRE) and after (POST) the lesson, plotted separately by factors (Interest, Importance, and Motivation). **102**

Figure C-3. The normalized male-female ratios at each application area of biosensor, before (PRE) and after (POST) the lesson, for the middle school (A) and high school students (B), plotted separately by factors (Interest, Importance, and Motivation). **104**

Figure C-4. The numbers of middle school students (A) and high school students (B) at each technique of biosensor development, before (PRE) and after (POST) the lesson, plotted separately by factors (Interest, Importance, and Motivation). **107**

Figure C-5. The normalized male-female ratios at each technique of biosensor development, before (PRE) and after (POST) the lesson, for the middle school (A) and high school students (B), plotted separately by factors (Interest, Importance, and Motivation). **110**

ABSTRACT

This dissertation develops biosensors for rapid detection of pathogens for environmental monitoring and food safety applications and utilizes the multidisciplinary and multi-application characteristics of biosensors to develop a lesson plan that can be implemented in secondary education classrooms. The detection methods evolve from particle immunoagglutination assay, PDMS optofluidic lab-on-a-chip, and spectrum analysis to smartphone and image analysis without any reagent; the potential application in secondary education also underlines the extended value of biosensors.

In the first paper presented here, an optofluidic lab-on-a-chip system and subsequent sampling procedure were developed for detecting bacteria from soil samples utilizing light scattering detection of immunoagglutination assay. Immunoagglutination assay was conducted by mixing antibody-conjugated microbeads with a target solution; the binding between the antibody and target causes agglutination of the microbeads. This agglutination is then monitored via Mie scattering measurement. This system and protocol detected the presence of *Escherichia coli* K12 from soil particles in near real-time (10 min) with a detection limit down to 1 CFU mL⁻¹, which is superior to conventional methods, such as plate counting or polymerase chain reaction (PCR) assays, and has the potential to be implemented in the field. We also compared the interaction between *E. coli* and soil particles to the two-step protein-surface interaction. While *E. coli* was used as the model bacterium, it should be noted that the target of the detection can be changed by changing the antibodies that are used in the immunoagglutination assay.

In the second paper, a smartphone-utilized biosensor consisting of a near-infrared (NIR) LED (wavelength of 880 nm) and a digital camera of a smartphone (capable of detecting 880 nm NIR) was developed for detecting microbial spoilage on ground beef, without using antibodies, microbeads or any other reagents. The method took advantage of the fact that the antigens and cell fragments from our model bacteria, *E. coli* K12 and *Salmonella* Typhimurium, bonded preferably to the fat particles within meat, creating submicro- or micro-sized aggregates (pseudo-colonies) that would maximize Mie scatter at a specific angle (depending on the size of such aggregates) under 880 nm NIR irradiation. The method was further improved by programming a smartphone application that allows the user to position the smartphone at an optimum distance and a range of angles utilizing its internal gyro sensor to measure a series of scatter intensities against the detection angle. This handheld device can be used as a preliminary screening tool to monitor microbial contamination on meat products.

In the third paper, we designed a lesson plan for secondary education classrooms using biosensors as a core and branching out to different applications and fields of study with the goal of heightening students' interest and motivation toward attaining degrees and careers in STEM fields. For a focus, we carefully chose four applications of biosensors including medical diagnosis, environmental monitoring, food safety, and biosecurity, and seven techniques of biosensor development including genetic engineering, nanotechnology, circuit building, microfabrication, 3D printing, smartphone utilization, and computer programming. The changes in students' attitudes towards these applications and techniques were assessed using short pre and post-surveys. Results

revealed that the lesson was more effective in affecting younger students than older students, and more effective in teaching about the applications than about the techniques.

INTRODUCTION

1. Biosensors Market and Applications

This dissertation is comprised of three biosensor papers: the first two are direct studies on the development of biosensors for different applications, and the third is a lesson plan designed for secondary education classrooms based on biosensors and biotechnology.

A biosensor can be defined as an analytical device that integrates a biological recognition element (bioreceptor) as the primary selective element, with a physical transducer to generate a measurable signal that is correlated with the analyte concentration (Liang et al., 2011; Kissinger, 2005). For the past decades, biosensors have been one of the fastest growing markets and are expected to reach \$18.9 billion of revenue by 2018 (Transparency Market Research, 2013) with applications increasing with the development of each new biosensor.

Ever since the first biosensor was described in the 1960s, many researches have been done in biosensor development in order to achieve the goal of having better and faster detection methods. Early biosensors mainly focused on clinical and medical applications, and they remain one of the most popular fields of study with the multiplex, inexpensive, and rapid detection trend of biosensors (Rasooly and Jacobson, 2006). However, many other possible applications are being explored in more recent years. Biosensors for pathogen detection, especially, are being vastly investigated due to their potential to improve the current detection methods with many of their features.

According to Frost and Sullivan, a market research company, the main vertical markets for biosensors, besides research laboratories, are point-of-care, home diagnostics, process industries, environmental monitoring, and biodefense. It is predicted that in 2016, clinical and medical applications (including both point-of-care and home diagnostics) will own 65.1% of the market revenue, and process industries, environmental monitoring, and biodefense will own 6.6%, 14.3%, and 3.3% of the market revenue, respectively (Thusu, 2010).

Among the environmental monitoring applications, subsurface transport of bacteria through porous media (i.e. soil), specifically, is gaining more and more interest due to several factors, including the increasing use of reclaimed water and constructed wetlands (Levine and Asano, 2004; Boutilier et al., 2009; Ghermandi et al., 2007), potential risk of crop microbial contamination via irrigation water or ground water (Song et al., 2006; Stine et al., 2005; Cheong et al., 2009), and the possibility of aquifer contamination.

Another point of increasing interest within the biosensors market for the past few decades is the detection of foodborne pathogens on meat and poultry products due to several high profile food safety issues and outbreaks, as well as the concern of agroterrorism. It was estimated that each year, foodborne illness occurs in 9.4 million cases in the United States, 1.3 million cases in England, 4.5 million cases in The Netherlands, and 5.4 million cases in Australia (Scallan et al., 2011; Flint et al., 2005). Considering the possibility that foodborne illness outbreaks such as these could be caused by an act of bioterrorism (referred to as agroterrorism), this poses a potentially serious

threat for a country, such as United States, whose agricultural industry comprises a major portion of its economy (USDA, 2013).

2. Current Detection Methods for Soil and Food Samples

The current methods for detecting pathogens from soil or food can be categorized into three groups: cultural, immunological, and nucleic acid-based. Cultural methods, which are still the most common in standards and regulations, require culturing the pathogen in a specific environment for certain periods of time (e.g. enriched lactose medium at 44.5°C for 24 hours for fecal coliforms). Cultural methods can be specific to certain pathogens, but only show culturable cells; the process is time consuming as well as impossible to implement in the field.

Immunological methods primarily utilize antibodies and their affinity and specificity to particular antigens. These methods include but are not limited to fluorescent immunolabeling, enzyme-linked immunosorbent assay (ELISA), and Western immunoblotting. Immunological methods detect both viable and non-viable cells, but are less specific to certain pathogens. The process is more complicated, often requiring trained personnel and is also unlikely to be practiced in the field.

Nucleic acid-based methods include but are not limited to gene probes, microarrays, and polymerase chain reaction (PCR), which is becoming one of the standard methods for various testing as well. PCR provides great sensitivity and accesses genomic information from both viable and non-viable cells, but the process still takes

several hours (including time for sample preparation and product identification), and requires a laboratory, specific instruments, and trained personnel to conduct.

3. Overview of Dissertation

3.1. Biosensor Development for Soil and Meat Samples

Development of rapid, portable, inexpensive, and sensitive sensing technologies for pathogen detection remain in high demand. In the first two papers in this dissertation (Appendices A and B), we developed two optical biosensing protocols, one for rapid detection of pathogens from soil samples using optofluidic lab-on-a-chip, immunoagglutination, and Mie scatter and one for reagentless detection of foodborne pathogens from meat samples using Mie scatter and smartphone interfaces.

3.1.1. Optofluidic Lab-on-a-Chip System

Biosensors can be categorized based on transducer types, which include electrochemical, piezoelectric, thermometric, and optical biosensors (Monošík et al., 2012). Optical biosensors are usually based on the measurement of luminescent, fluorescent, colorimetric, or other optical signals produced by the interaction between the bioreceptor and the analyte.

Optofluidics is a combination of microfluidics and optics and fundamentally aims at manipulating fluids and light at the microscale and exploiting their interaction to create highly versatile systems (Monat et al., 2007). The lab-on-a-chip community has made

efforts to incorporate optical devices into their microsystems to create optofluidic lab-on-a-chip and improve the functionality. Optofluidic lab-on-a-chip brings sensitivity and miniaturization into the optical biosensor and utilizes the microscale interaction between fluid and light to create improved detection methodology.

The optofluidic lab-on-a-chip we used was made through a well-known replica molding technique using polydimethylsiloxane (PDMS). PDMS is optically clear, chemically inert, thermally stable, simple to handle and manipulate, low-cost, and able to conform to submicron features (Mata et al., 2005). These properties make it one of the most common materials used for flow delivery in microfluidics chips. In order to make a PDMS lab-on-a-chip, a silicon master mold was fabricated by photolithography and deep reactive ion etching (DRIE) on a single silicon crystal. Our lab-on-a-chip is then made by curing PDMS on the silicon master mold and a plain microscope slide and bonding the two cured PDMS chips using oxygen plasma etching techniques. During the oxygen plasma etching process, the surface bonds of the PDMS chips are disrupted temporarily and the two chips are put together before the bonds return to their normal state; once the bonds relax, the two chips are sealed permanently.

The microfluidic channel delivers samples and reagents, and the optofluidic channels are in close proximity to deliver the light. Optofluidic channels were filled with microscope immersion oil type A to create liquid-core waveguides by utilizing the difference in refractive indices between the oil and PDMS. Silica optical fibers were partially inserted into the optofluidic channels to deliver the light from a light source, collect the light scatter, and deliver to a detector (i.e. a spectrometer).

An optofluidic lab-on-a-chip system enables the reduction of sample and reagent volume and shorter total assay time. Biological reactions can be made extremely reproducible through the strictly laminar flow and precise control of fluidic behavior, which leads to reduced noise, and subsequently a much lower detection limit.

3.1.2 Immunoagglutination Assay and Mie Scatter

For the biosensing modality of our lab-on-a-chip platform, we use angle-dependent Mie scatter detection from microbead immunoagglutination. Mie scatter refers to the Mie solution to the scattering problem on a spherical object, and describes how much light is scattered and the scatter intensities are changed according to the scattering angles (van de Hulst, 1983). Immunoagglutination assay is usually conducted by mixing antibody-conjugated microbeads with a target solution; the binding between the antibody and target causes agglutination of the microbeads. This agglutination is then monitored via Mie scatter measurement as the scatter light intensity is proportional to the agglutination size (i.e. target amount).

Mie scatter is a type of elastic scattering of light by molecules and particulate matter. Elastic light scatter can be categorized into two types, Rayleigh and Mie scatter, based on the ratio of the wavelength of incident light (λ) to the particle diameter (d). When $d \ll \lambda/10$, it is considered Rayleigh scatter, where the ratio of scattered light and incident light is a function of wavelength, scatter angle, refractive index of the particle, and the particle diameter. As the d increases ($d > \lambda/10$), the scattered light intensity can then be found using the Mie scatter model, where the intensity is less dependent on

wavelength, and more on particle size and angle. Mie scatter allows us to monitor the agglutination size at an optimum angle (an angle which gives us the greatest intensity change and/or the most linear curve).

As the refractive index of the particle is one of the parameters, different refractive indices lead to different Mie scatter behaviors, thus resulting in different optimum angles for Mie scatter detection. The refractive index of microbeads is usually substantially higher than that of water and/or sample matrix, therefore the optimum angles for Mie scatter detection are different for the microbeads, water, and/or sample matrix. This technique enables us to detect the target in the presence of the sample matrix.

In the first paper, we utilize Mie scatter detection to measure the degree of immunoagglutination in an optofluidic lab-on-a-chip device allowing us to detect bacteria from soil samples in almost real-time with minimum processing procedures. The same Mie scatter detection is also used in the second paper to monitor the bacterial colonies on meat samples. However, for the meat samples, we aim to develop a non-destructive, therefore reagentless detection (i.e. no antibody-conjugated microbeads).

In the case of the second paper, the refractive indices of our model bacteria are slightly higher than that of water, thus technically the bacteria can be identified directly using Mie scatter. However, the morphologies of the bacteria cells, fragments, antigens, or colonies are much simpler than the microbeads, therefore such detection can only be made when the bacterial concentration is high (Hirleman, Jr. et al., 2008). To achieve a lower detection limit, we utilized the characteristic of the selected bacteria which attaches more preferably to fat surfaces (Dickson and Koochmarie, 1989). When the bacteria cells,

fragments, or colonies interact with the fat from meat and form more complicated structures, we are able to detect the formations even when the concentrations are low.

3.1.3. From Optical Fibers and Spectrometer to Smartphone Camera

Since the first introduction of smartphones to the market in the late 1990s, smartphones equipped with internet connectivity, high resolution camera(s), and powerful CPUs have rapidly gained market acceptance. The development of applications that utilize the features of a smartphone also greatly extends its capabilities (Gallegos et al., 2013).

In the third quarter of 2012, the total installed base for smartphones worldwide hit 1.04 billion, and it is estimated that by 2015, worldwide smartphone population will exceed two billion (Strategy Analytics, 2012). Incorporating a smartphone into an optical biosensor has the potential to improve the sensor portability and simplify the steps and apparatus. Since smartphones serve as the primary user interface for detection, replacing the light source, optical fibers, and spectrometer with a smartphone flash light, camera, and application, can make a biosensor truly inexpensive and user-friendly.

3.2. Biosensors in Secondary Education

Science, Technology, Engineering, and Mathematics (STEM) courses are extremely important for secondary education students and critical to preparing students for college and further professional careers. However, the United States is failing

internationally in this area, ranking 25th in mathematics and 17th in science among industrialized nations (U.S. Department of Education, 2010). Only 16% of American high school seniors are proficient in mathematics and interested in a STEM career; among those who do go on to pursue a college major in the STEM fields, only about half choose to work in a related career.

Despite our failure in promoting STEM careers among secondary education students, technologies as well as science and engineering research in higher education have greatly improved. This means that the next generation will be faced with a much more challenging learning environment; therefore the gap between our secondary education and higher education could be problematic. This problem is perpetuated by the fact that teaching materials for secondary education are often hindered by limited human resources, limited professional development, and a lack of accessible outreach programs.

Considering the growing popularity as well as the multidisciplinary nature of biosensors, it can be an excellent tool to help mitigate the STEM education gap by introducing science and engineering concepts into a secondary education classroom as well as to provoke students' interest in science and engineering careers. Furthermore, the increasing applications of biosensors can be a good instrument to raise students' awareness of current worldwide issues, such as food and water safety and bio/agroterrorism.

3.3. Summary of the Present Study

Appendix A summarizes work done on developing a biosensing system and protocol for detecting bacteria (*Escherichia coli* K12 as our model bacterium) from soil samples in order to help monitor subsurface bacterial transport. In this work, we utilize an optofluidic lab-on-a-chip device to measure the Mie scatter from the immunoagglutination between the targets and the antibody-conjugated polystyrene microbeads. This system and protocol detect the presence of *E. coli* K12 from soil particles in near real-time (10 min) with a detection limit down to 1 CFU mL⁻¹, which is superior to conventional methods such as plate counting or polymerase chain reaction (PCR) assays.

The developed biosensing protocol is then used in a mock-up soil system to investigate the subsurface transport of the bacteria at a smaller scale. The results from the mock-up soil system reveal the different behaviors between *E. coli* antigens, cells, and colonies, which can be explained by the two-step process of protein-surface interaction, diffusion model, and gravitational force. These conclusions are supported by the results from Bradford total protein assay and fluorescence microscopic images. Lastly, the device and protocol could be potentially implemented in field studies, and more complete bacteria subsurface transport models can be achieved with actual field results.

Appendix B summarizes work done on detection of foodborne pathogens (*E. coli* K12 and *Salmonella typhimurium* Z005 as our model bacteria) from ground beef samples. A detection method based on Mie scatter but without immunoagglutination assay and

antibody-conjugated polystyrene microbeads is developed in order to achieve non-destructive, therefore reagentless detection.

The detection theory is mainly based on the hydrophobicity of *E. coli* and *Salmonella* and their attachments to fats within the meat sample. The aggregation of bacteria fragments, cells, and colonies around fat cells creates a particle size that is comparable to the incident wavelength, and Mie scatter detection is made possible subsequently. The results are explained and supported by the SEM and fluorescence microscopic images of *E. coli* on ground beef. Lastly, the Mie scatter detection of *E. coli* and *Salmonella* on meat is simplified by replacing the optical fibers, spectrometer, and computer software with a smartphone camera and application.

Appendix C summarizes work done on designing and practicing a biosensor lesson plan for secondary education classrooms. This work is inspired by the National Science Foundation Graduate STEM Fellows in K-12 Education Program which supports fellowships and training for graduate students in science, technology, engineering, and mathematics fields to teach in their local K-12 schools.

The lesson plan utilizes the multidisciplinary and multi-application nature of biosensors to branch out to different cutting-edge technologies, important biosensor applications, and current worldwide issues. We develop this lesson plan with the hope of assisting teachers in their biotechnology curriculum and improving students' interest and motivation in STEM careers.

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APPENDIX A**OPTOCLUIDIC LAB-ON-A-CHIP MONITORING
OF SUBSURFACE BACTERIAL TRANSPORT**

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Abstract

An optofluidic lab-on-a-chip system and subsequent sampling procedure were developed for detecting bacteria from soil samples utilizing light scattering detection of immunoagglutination assay. This system and protocol detected the presence of *Escherichia coli* K12 from soil particles in near real-time (10 min) with a detection limit down to 1 CFU mL⁻¹, which is superior to the conventional methods, such as plate counting or polymerase chain reaction (PCR) assays. *E. coli* solutions were applied to the surface of a mock soil system and incubated overnight. The light scattering immunoagglutination assays using the optofluidic lab-on-a-chip showed two *E. coli* peaks over the soil depth, one at 1 cm and the other at 4 cm. Comparison with bacterial viability assay and Bradford protein assay revealed that smaller *E. coli* colonies were found at 1 cm depth and larger colonies at 4 cm, while free antigens adsorbed and desorbed more reversibly at both locations. The two peaks were explained by the two-step process of protein-surface interaction and gravitational force. The target molecules with small sizes (free antigens and single cells) arrived at the soil particle surface faster according to the diffusion model, and the larger *E. coli* colonies arrived later where the soil surface was already occupied. Because the free antigens adsorbed and desorbed in a more reversible manner, they could be found throughout the depth of the mock-up soil system, whereas the larger *E. coli* colonies traveled through the void space within soil particles via gravitational force and accumulated at the bottom of where the liquid reached. This work also demonstrates a device and procedure that could be potentially implemented in field studies. With proper soil sample handling protocol and light

scattering detection of immunoagglutination assay in an optofluidic lab-on-a-chip, developing more complete bacteria subsurface transport models with actual field results can be achieved.

Keywords: biosensor, diffusion model, *E. coli*, immunoagglutination, immunosensor, light scattering, pathogen, protein-surface interaction, subsurface transport.

1. Introduction

Subsurface transport of bacteria through porous media is an issue of increasing interest due to its application in fields such as aquifer contamination, wastewater treatment using constructed wetlands (Boutilier et al., 2009; Ghermandi et al., 2007), and the use of reclaimed water (Levine and Asano, 2004). Soil column experiments have been performed in order to predict or model the subsurface transport of bacteria (Díaz et al., 2010; Jacobson et al., 2009), but the results are often too simple to be applied to field studies. Ideally, soil samples should be collected and analyzed on site (i.e., without transporting to and storing in a laboratory) and in near real-time, so that the next sampling point can be determined almost simultaneously. Microbial analyses should also be performed as soon as possible after collection of a soil sample because microbial communities within the sample can change regardless of the storage method (Maier et al., 2009). However, due to the complexity of the assay methods, handling of soil samples, and sometimes the accessibility of the sampling points, on-site experiments and studies

(e.g., bacteria removal rates, bacterial source tracking, bacterial transport modeling, etc.) are relatively rare (Boutilier et al., 2009).

Agar plating and colony counting is one of the most common methods used to detect bacteria from soil samples. The method is specific to certain bacteria, based on the chosen plating media, but only shows culturable cells, and the process is time consuming and impossible to implement in the field. There are other methods that measure total protein concentrations (e.g., Bradford assay) or specific antigen concentrations (e.g., enzyme-linked immunosorbent assay; ELISA), which measure both non-viable and viable cells and are less specific to certain bacteria. Polymerase chain reaction (PCR) is also one of the standards for identifying bacteria. Typical PCR assays, however, can take several hours (including time for sample preparation and product identification). All of these assays require a laboratory setting in most cases, as well as procedures that non-specialists may have some difficulties with.

Development of rapid and sensitive sensing technologies for detection of bacteria and the additional possibility of field implementation remain in high demand. Several biosensor technologies have been attempted in recent years. In our previous work, we achieved *Escherichia coli* K12 detection in water samples and avian influenza detection in 1% fecal matrix using a polydimethylsiloxane (PDMS) based optofluidic lab-on-a-chip device (Kwon et al., 2010; Heinze et al., 2010). This lab-on-a-chip consisted of microfluidic channels for delivering sample and reagent and optofluidic channels for delivering light. Microfluidics enables the reduction of sample and reagent volume and shorter total assay time, which is good for field applications. Biological reactions within microfluidics can be made extremely reproducible through the strictly laminar flow and

precise control of fluidic behavior, which leads to reduced noise and thus an enhanced signal-to-noise ratio, and subsequently a much lower detection limit. The use of optofluidic channels, which acted like microfabricated, on-chip optical fibers, enabled us to further improve the detection limit down to 10 CFU mL⁻¹ for bacteria (CFU = colony forming units) or 10 pg mL⁻¹ for antigens.

In addition, we attempted to use angle-dependent Mie scatter detection from microbead immunoagglutination as our biosensing modality on a lab-on-a-chip platform. Immunoagglutination assay is usually conducted by mixing antibody-coated microbeads with a target solution; the binding between the antibody and target causes agglutination of the microbeads. This agglutination is monitored via Mie scattering measurement, as opposed to wavelength-dependent fluorescent detection (which is essentially inelastic light scatter that is less sensitive to Mie scatter). Since the optimum angles for Mie scattering detection are different for the microbeads and sample matrix, this technique enabled us to detect the target in the presence of the sample matrix.

However, using Mie scatter detection of microbead immunoagglutination for bacteria from soil samples has some potential complications due to the presence of soil particles. The refractive index of silica sand (i.e., sample matrix) is 1.544, which is not very different from 1.597, the refractive index of the polystyrene microbeads used in this research (Ishida et al., 1991). Additionally, antibody-conjugated microbeads may bind to soil particles non-specifically, potentially resulting in false-positive assay results.

In this study, we attempted to use Mie scatter detection of microbead immunoagglutination in an optofluidic lab-on-a-chip for the detection of *Escherichia coli* K12 (our model bacterium) from a mock-up soil system in order to develop an effective

procedure and assay method for *E. coli* extraction and detection from soil samples, which will be adaptable for all other bacteria. The assay detects all antigens (free from or bound to bacteria); in other words, it works best with free antigens and smaller colonies.

Through a comparison with conventional assays (plate counting method and Bradford total protein assay), we aimed to make a distinction between free antigens/smaller colonies and larger colonies by their respective subsurface transport behavior. In addition, we discuss the potential of the procedure and assay method for on-site analyses and other field implementation by evaluating the detection limit and assay time.

2. Materials and Method

2.1. Antibody-Conjugated Microbeads

Anti-*E. coli* antibodies (Catalog No. B47385G, Meridian Life Sciences, Saco, Maine) were conjugated to 930 nm highly carboxylated polystyrene microbeads (10.3 \AA^2 parking area per surface carboxyl group; Bangs Laboratories, Fishers, Ind.) by covalent binding. The microbeads were washed twice with activation buffer, 50 mM 2-(N-morpholino)-ethanesulfonic acid (MES, Catalog No. M3671, Sigma-Aldrich, St. Louis, Mo.) at pH 6.0. A centrifuge was used for all washing processes for 15 min at 11,000 rpm. The particles were then mixed with a 10 mg mL^{-1} solution of water-soluble carbodiimide (N-(3-dimethyl-aminopropyl)-N'-ethylcarbodiimide hydrochloride; Catalog No. 03449, Sigma-Aldrich) at room temperature. After mixing with carbodiimide, the microbeads were washed twice with coupling buffer, 50 mM phosphate-buffered saline (PBS, Catalog No. P4417, Sigma-Aldrich) at pH 7.4. Anti-*E. coli* was diluted in PBS and then

added to the particle suspension to yield 100% surface antibody coverage of the particles. The resulting solution was then gently mixed overnight at 4°C. After mixing overnight, the solution was washed three times with PBS, followed by mixing with quenching solution, 40 mM hydroxylamine solution (Catalog No. 379921, Sigma-Aldrich). Finally, the microbeads were stored in a storage buffer, PBS-BN (PBS at pH 7.4 with 1% of 400 $\mu\text{g mL}^{-1}$ bovine serum albumin from Sigma-Aldrich and 0.05% azide from Fisher Scientific, Pittsburgh, Pa.) and were ready to use in immunoagglutination assays in the lab-on-a-chip.

2.2. Mock-Up Soil System and Sampling

One-liter Pyrex beakers (Fisher Scientific) were filled slowly to the 1 L mark with dry silica sand (filter sand grade 20, Ace Hardware, Tucson, Ariz.) and constantly shaken by hand while filling to prevent the formation of macropores. The sand-filled beaker was used in the following experiments as the mock-up soil system.

E. coli K12 lyophilized cell powder (Catalog No. EC1, Sigma-Aldrich) was cultured in lysogeny broth (LB; Catalog No. MBPE-1050, LB-Miller, Growcells, Irvine, Cal.) at 37°C for 18 h. The cultured *E. coli* solution was serially diluted with PBS (50 mM) at pH 7.4. The colony-forming units (CFU) of each serial dilution were quantified by spread plate method, in which a 0.1 mL aliquot was uniformly spread on top of an LB agar plate (Catalog No. 29447-000, VWR International, Radnor, Pa.). The plate was incubated at 37°C for 24 h before the colonies were counted.

Using a disposable transfer pipette, 15 mL of each diluted *E. coli* solution was then applied to the mock-up soil system at the center of the surface of the sand-filled beaker. The beaker was left at room temperature overnight before being sampled.

With a small chemical spoon, 0.2 g of sand was collected at each centimeter depth from the center of the beaker surface, including the surface (0 cm depth), to the deepest point the solution had reached (typically 5 cm). *E. coli* was extracted from each sample using 1 mL of PBS (50 mM) at pH 7.4 and 2 μ L of 10% Tween 80 solution by placing the sample on a rock shaker for 2 min. The sample settled for 1 min before 0.5 mL of supernatant solution was carefully taken out. The supernatant solution was then pumped through a 5.0 μ m pore size syringe filter (Catalog No. 6870-2550, Whatman, GE Healthcare, Waukesha, Wisc.) in order to remove all suspended soil particles. The complete procedure for extracting *E. coli* from the soil sample is shown in figure 1.

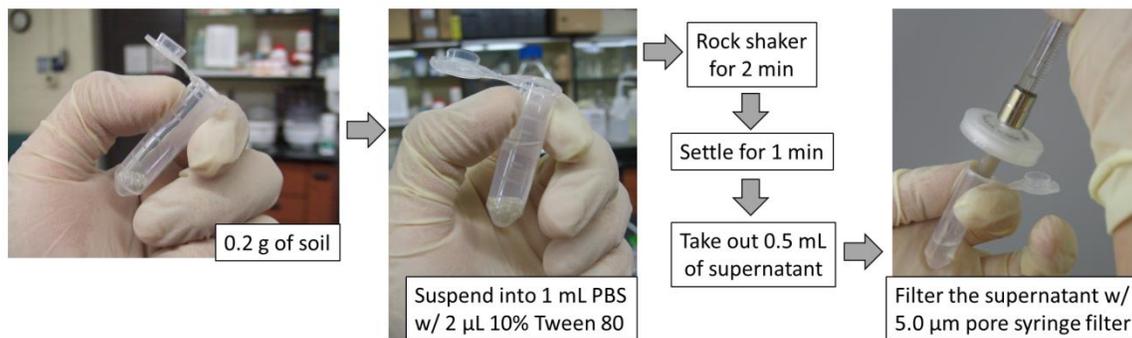


Figure 1. *E. coli* extraction from soil sample.

2.3. Optofluidic Lab-on-a-Chip System

The optofluidic lab-on-a-chip was made with a well-known replica molding technique using polydimethylsiloxane (PDMS). The silicon master mold (75 mm × 25 mm) was a single silicon crystal fabricated by photolithography and deep reactive ion etching (DRIE). Each lab-on-a-chip was made by curing 6 mL of PDMS on the silicon master mold and a plain microscope slide, and bonding the two cured PDMS chips using an oxygen plasma cleaner (PDC-32G, Harrick Plasma, Ithaca, N.Y.). The main microfluidic channel was a simple Y-channel design, consisting of two inlets and one outlet, with the optofluidic channels in close proximity (distance between microfluidic and optofluidic channels was 100 μm). The dimensions of the microfluidic Y-channel were 1 mm wide × 100 μm deep, and the optofluidic channel dimensions were 750 μm wide × 100 μm deep. The optofluidic channel for forward scatter detection was tilted to 45° from incident, allowing for collection of scattered light at a 45° angle from incident. The lab-on-a-chip layout is shown in figure 2.

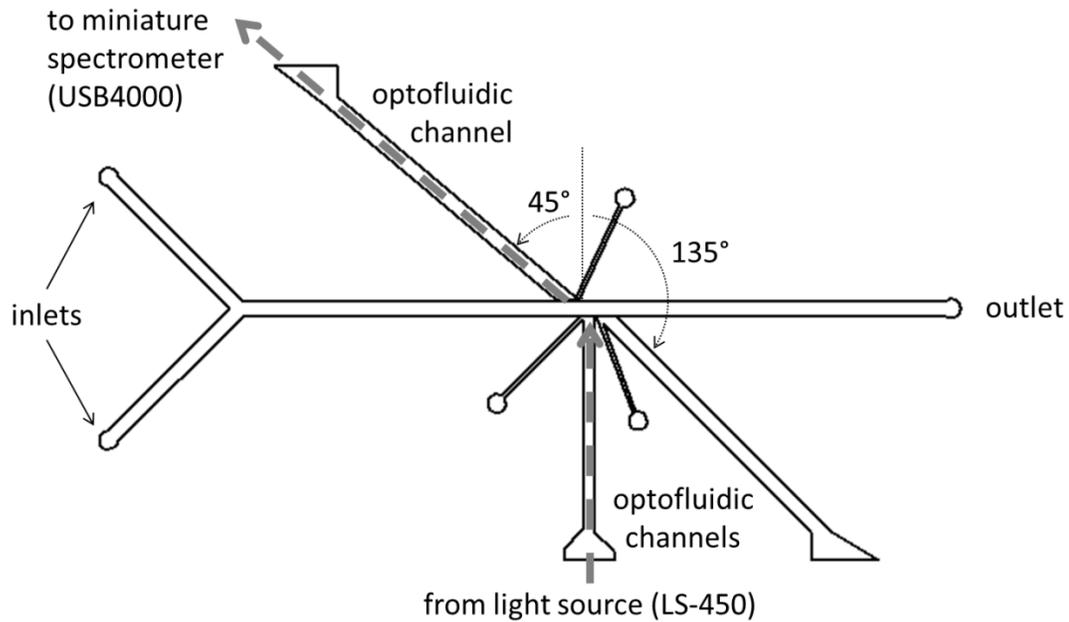


Figure 2. Lab-on-a-chip layout.

The optofluidic channels were filled with microscope immersion oil type A, with a refractive index of 1.515 (Nikon, Tokyo, Japan). Together with the surrounding PDMS material, with a refractive index of 1.41, the critical angle of total internal reflection becomes $\arcsin(1.41/1.515) = 68.5^\circ$. Two bare 400 μm silica optical fibers were partially inserted into the optofluidic channel inlets by hand. One fiber was used to deliver the light from a light source (LS-450, Ocean Optics, Dunedin, Fla.) with a 640 nm LED, and the other fiber collected the forward light scattering and delivered it to a miniature spectrometer (USB4000, Ocean Optics) that was connected to a computer. SpectraSuite software (Ocean Optics) was used to identify the signal intensity at 640 nm.

The semi-benchtop lab-on-a-chip system had three trays. One tray held the lab-on-a-chip device and the silica fibers in place, another tray held the light source, and the

third tray held the miniature spectrometer. The semi-benchtop system is shown in figure 3.

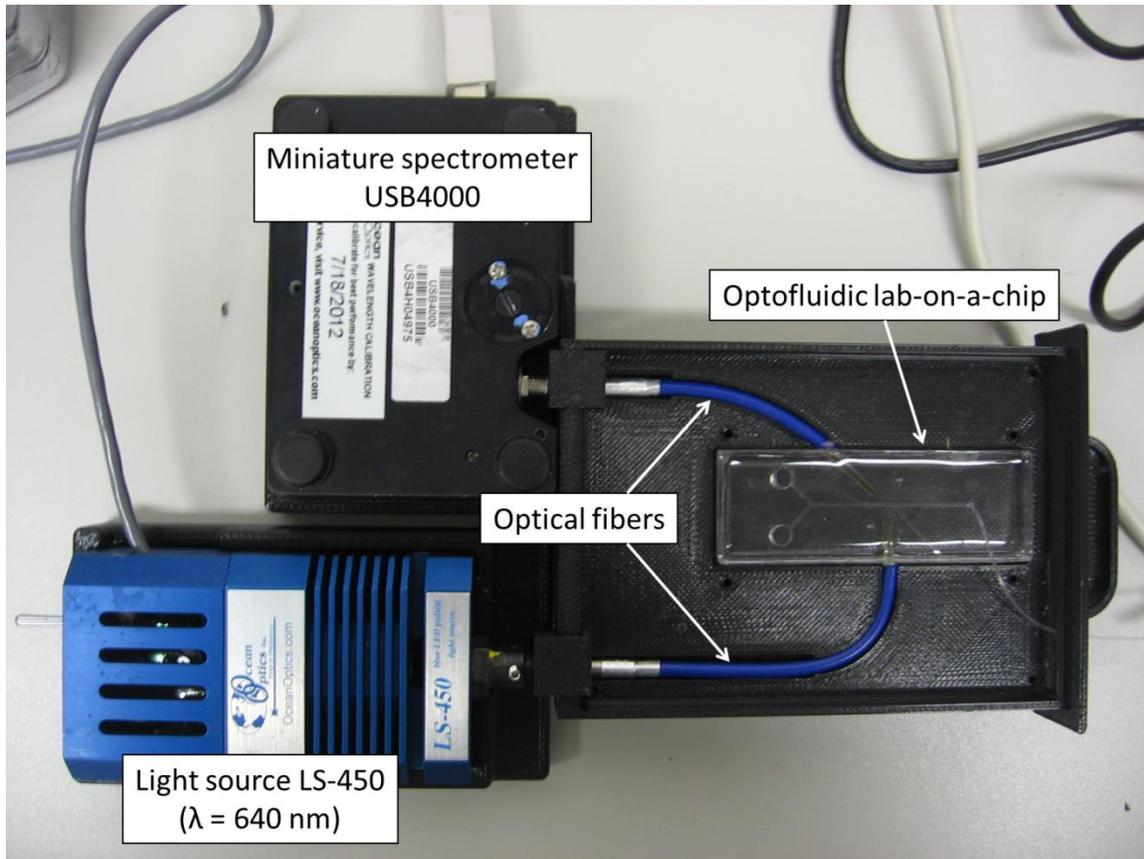


Figure 3. Semi-benchtop system for optofluidic lab-on-a-chip.

2.4. Light Scattering Detection of Immunoagglutination Assay

A 15 μL sample of antibody-conjugated microbead suspension and 15 μL of target solution were placed in alternate inlet wells of the Y-channel. A syringe was used at the outlet to create negative pressure and withdraw both solutions simultaneously. The

solutions were mixed spontaneously by diffusion within the microchannel, and the light scatter intensity was measured using the optofluidic channels in the lab-on-a-chip.

Standard curves were produced by plotting scatter intensities normalized to the negative control (antibody-conjugated microbeads in PBS) against the known concentrations of *E. coli* solutions. The solutions extracted from the soil samples (collected from the mock-up soil system) were assayed using the same procedures but with a different negative control, which was the extract from 0.2 g of dry sand from the same mock-up soil system before any diluted *E. coli* solution was applied, using the same extraction procedures described before (i.e., mixing with 1 mL PBS and 2 μ L Tween 80, taking out supernatant after settling, and pumping supernatant through a syringe filter).

2.5. Bacterial Viability Assay with Fluorescence Microscopy

SYTO 9 and propidium iodide (Catalog No. L7012, LIVE/DEAD BacLight viability kit, Invitrogen, Carlsbad, Cal.) were used to further confirm our methods and understand the transport mechanisms. The BacLight viability kit stains viable and non-viable cells following the protocol provided by the manufacturer (Molecular Probes, 2004). SYTO 9 is a green fluorescent nucleic acid stain that stains both viable and non-viable cells, whereas propidium iodide is membrane impermeable and used for identifying non-viable cells. Stained *E. coli* cells were observed using a fluorescent microscope (Nikon, Tokyo, Japan), with green fluorescence emitted by all cells and red fluorescence emitted only by non-viable cells. The microscopic images were then

analyzed using imaging software (MetaVue, Molecular Devices Corp., Downingtown, Pa.), and the extent of the fluorescence was determined.

2.6. Bradford Protein Assay

Coomassie Brilliant Blue G-250 dye (Catalog No. 500, Quick Start Bradford 1× dye reagent, Bio-Rad, Hercules, Cal.) and bovine serum albumin (BSA) protein standard (Catalog No. P5619, Sigma-Aldrich) were used following the protocol provided by the manufacturer (Bio-Rad, 2000) to determine the concentration of total proteins in each solution and obtain more information on the samples.

3. Results and Discussion

3.1. Standard Curve Using Optofluidic Lab-on-a-Chip

A standard curve was constructed by measuring the light scatter intensities from microbead immunoagglutination with varying concentrations of *E. coli* solutions using the semi-benchttop optofluidic lab-on-a-chip system and the assay procedures described earlier (fig. 4). To eliminate chip-to-chip variations, all readings were normalized to that of a negative control (in this case, PBS). The negative control was measured whenever a new chip was used. The 2× standard error indicates the 95% confidence interval for each data point; the lower bounds of these intervals are all higher than 1, indicating that the lowest concentration tested (1 CFU mL^{-1}) is the detection limit of this assay. This excellent sensitivity was made possible by the use of optofluidic channels and the

optimized light scatter parameters (650 nm light source and 45° scatter detection). The decrease that occurred at 10^7 and 10^8 CFU mL⁻¹ was due to the formation of huge bacterial colonies, which precipitated out of the solution and resulted in weaker light scatter intensities.

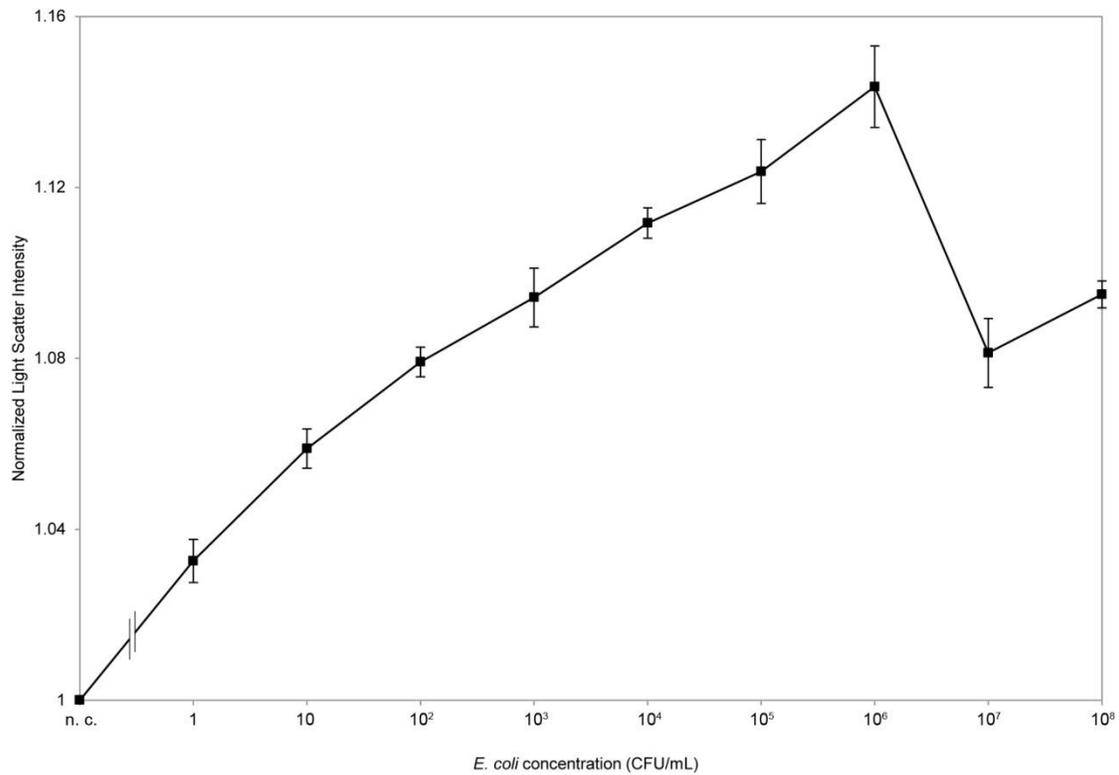


Figure 4. Standard curve produced with the optofluidic lab-on-a-chip system. Values are averages of four different experiments (error bars are standard errors; n.c. = negative control).

3.2. *E. coli* Assays from Mock-up Soil System

The *E. coli* concentrations of the extracted solutions collected at different depths were determined by comparing the light scattering intensities and the standard curve that was constructed before each replicate. Figure 5 shows the results from four experiments. Although the concentrations varied, the trends with soil depth are similar. All four experiments appear to have two peaks, with the first peak near the surface (about 1 cm deep) and the second peak at a deeper location (about 4 cm deep). The *E. coli* amounts were higher at these two locations, and there were no statistical differences between two peaks for all four experiments. It is highly probable that *E. coli* adsorption occurred in two different steps. To identify what was adsorbed at the two depths, the extracted solutions were further analyzed with bacterial viability and Bradford protein assays.

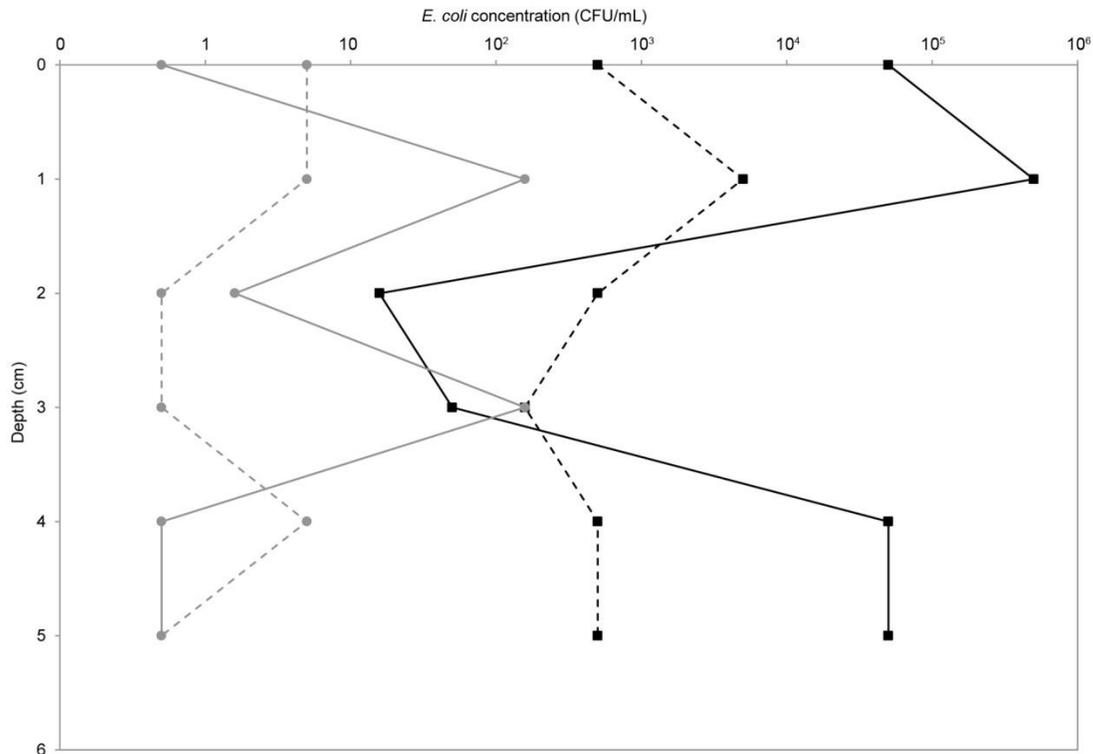


Figure 5. Depth vs. *E. coli* concentration of four replicates with initial pulse inputs of *E. coli* solution to the soil surface, incubated overnight. Concentrations were determined by comparing with a standard curve (fig. 4).

3.3. Bacterial Viability Assay with Fluorescence Microscopy

Figure 6 shows fluorescence microscopic images of samples stained with green fluorescent SYTO 9 and red fluorescent propidium iodide. However, the samples both show no red fluorescence coming from propidium iodide; this indicates there were no, or at least a negligible amount of, non-viable cells. This result does not necessarily indicate the non-existence of free antigens, as the fluorescent dyes in the viability kit only stained nucleic acids.

According to the MetaVue image analyses, the viable cells at 1 cm were mostly small colonies and/or single cells, whereas the viable cells at 4 cm were mostly larger colonies. However, the total amounts of cells and colonies between two locations were about the same (25 spots in fig. 6a, and 28 spots in fig. 6b).

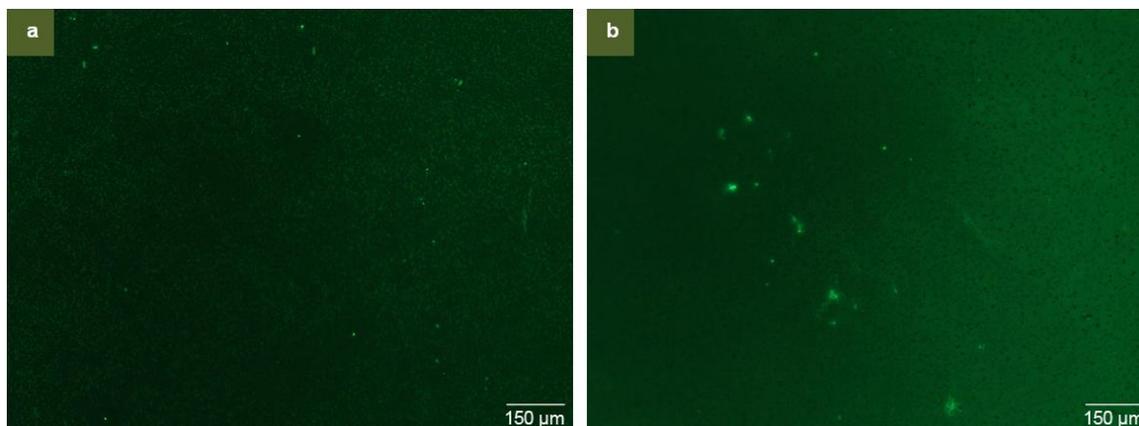


Figure 6. Fluorescence microscopy images of extracted *E. coli* solutions with green fluorescent stain (SYTO 9) at (a) 1 cm and (b) 4 cm depths.

3.4. Bradford Protein Assay

Bradford protein assay was used to determine the total protein concentrations in the extracted solutions using bovine serum albumin (BSA) as a standard solution, with the lowest standard (BSA) concentration of $80 \mu\text{g mL}^{-1}$. This method revealed a trend similar to that derived from immunoagglutination assay and light scattering detection (fig. 5), with a first peak at 1 cm and a second peak at 4 cm depth (fig. 7).

All the data shown in figure 7 are close to the detection limit and thus may not represent the true protein concentration. Nonetheless, both replicates showed the same

trend as the light scattering immunoagglutination assays, and the concentrations of the two peaks are similar to each other.

It should be noted that the x -axis of figure 7 is not a logarithmic scale, unlike that of figure 5. This indicates that the protein concentrations did not vary as much with depth (within one order of magnitude) as did the *E. coli* concentrations (about one order of magnitude). Since *E. coli* was not denatured for the assay, the Bradford assay should primarily detect the presence of free antigens rather than bacterial colonies.

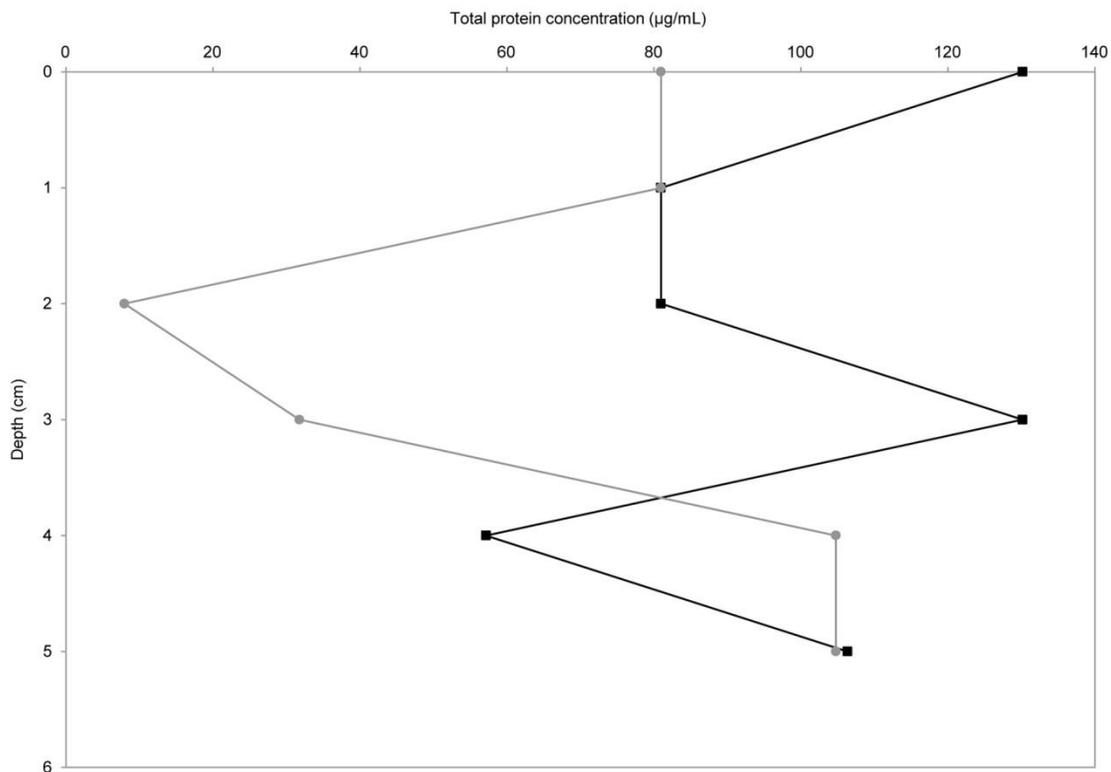


Figure 7. Depth vs. total protein concentration of two replicates with initial pulse inputs of *E. coli* solution to the soil surface, incubated overnight. Concentrations were determined by Bradford protein assay.

3.5. Comparison with Two-Step Protein-Surface Interaction Theory

The most important attachment mechanisms for bacterial retention in natural porous media are physico-chemical filtration and straining (Ryan and Elimelech, 1996). Straining of colloids occurs when the ratio of the colloid to median grain diameter is greater than 0.5% (Bradford et al., 2004). The grain size of the soil that was used in the experiments was 0.45 to 0.55 mm, and *E. coli* is a rod-shape organism with a typical size of $0.5 \mu\text{m} \times 2 \mu\text{m}$ (Miao et al., 2003); thus, straining was unlikely to happen.

This suggests that physico-chemical filtration was the main attachment mechanism with our setup. Physico-chemical filtration refers to where bacteria attach onto the surface of a particle. Proteins can be brought to the surface by any of four major transport mechanisms: diffusion, thermal convection, flow, and coupled transport (Dee et al., 2002). Since the flow was very slow and no heating was involved, diffusion should dominate in our experiments. At short times and under conditions in which the rate of adsorption equals the rate of diffusion, diffusion is described by the following equation (Dee et al., 2002), which is derived from Fick's second law:

$$\frac{dn}{dt} = C_0 \sqrt{\frac{D}{\pi t}} \quad (1)$$

where n is the surface concentration of protein, C_0 is the bulk concentration of protein, D is the diffusion coefficient, and t is time. Equation 1 shows that more proteins arrive at the surface when the bulk concentration and/or the diffusion coefficient are higher.

Protein-surface interactions can be broken down to a two-step process. The first

step occurs when the protein molecules arrive at a surface. Since this step is relatively reversible, some of the molecules are removed and return to the bulk phase. However, the second step, when the protein molecules that were attached to a surface experience conformational changes, is almost entirely irreversible. The proteins may unfold and expose interior functional groups for interaction with additional binding sites.

Consequently, desorption is unlikely to happen because of the greater contacts formed between the protein molecules and the surface (Dee et al., 2002).

The two peaks that were observed in the light scattering immunoagglutination assay and Bradford assay, as well as the larger colonies at the second peak discovered using fluorescence microscopy, can be explained by the two-step process of protein-surface interactions, gravitational force, and the diffusion model. Target molecules with smaller sizes (larger diffusion coefficient D) arrived at the surface faster based on the diffusion model, which explains why the soil particles near the surface were mainly occupied by smaller target molecules (free antigens and single cells). A sufficient amount of liquid exists in the soil for this diffusion to happen, since 15 mL of *E. coli* solution occupied the total soil volume of 41.8 mL, with a void volume of 16.3 mL, indicating that most voids (92%) were filled with *E. coli* solution. When the larger *E. coli* colonies arrived, the soil particles near the surface were already occupied and not able to accommodate more target molecules. Single cells and smaller colonies most likely underwent conformational changes and were thus permanently adsorbed at 1 cm depth. Meanwhile, the smaller free antigens were less prone to such conformational changes, and some of them might be able to desorb from the surface. These detached free antigens, along with the larger colonies, then traveled through the void space within the soil

particles via gravitational force and accumulated at the bottom of where the liquid/solution could reach.

3.6. Assay Time and Field Applicability

With properly extracted solutions, the optofluidic lab-on-a-chip assay took about 2 to 3 min and did not require complicated device and/or liquid handling procedures. The device can easily be made portable, as shown in figure 3. The extraction procedure, shown in figure 1, took about 7 min; therefore, the total assay time was 10 min. The procedure required some human labor, but it can be implemented in field situations, as the only device required was a syringe filter and a rock shaker. The rock shaker can easily be replaced with human labor, i.e., shaking the tube gently by hand. Further work is necessary to make this assay truly field-deployable, including (1) elimination of the need for a computer, (2) freeze-drying all reagents within the microfluidic channel to further simplify the assay, and so forth.

4. Conclusion

Using light scattering detection of immunoagglutination assay, as well as Bradford protein assay, a first *E. coli* concentration peak was observed at a near-surface location and a second peak was observed near the lower extent of the distance traveled by the *E. coli* via gravitational force. The results of bacterial viability assay from the two

peaks revealed that there were more large-size colonies at the deeper location than at the near-surface location.

Based on protein-surface interactions, these results can be explained by the diffusion model for protein adsorption/desorption. This work clearly indicates that the surfaces of soil particles are occupied by single cells or smaller colonies first, leaving only the surfaces at deeper locations for the larger colonies to occupy. Free antigens, however, adsorb and desorb in a more reversible manner throughout the depth.

Although the results from this study are specific to a certain grain size range of sand/soil, they point out that the interaction of *E. coli* with sand/soil particles is analogous to general protein-surface interactions. This can possibly lead to many answers for questions about subsurface transport of bacteria.

The assay method demonstrated in this study shows a very low detection limit (i.e., 1 CFU mL⁻¹) and can be easily adapted to field use. Moreover, the method is sensitive to non-viable cells and free antigens that affect the soil-bacteria interactions, which have been neglected in the past. With proper soil sample handling protocol and light scattering detection of immunoagglutination assay, collecting a large amount of field data within a short time can be possible, and the results can be used to better model bacterial subsurface transport.

With possible future work, a truly field-deployable device can be made based on the extraction and assay methods demonstrated in this work. Such a device will be extremely useful for field implementation, given that the device can process the samples on site and in near real-time. For example, a deployable device made with an automatic

sampling system can be used for environmental monitoring in high-risk groundwater contamination regions, constructed wetlands, and wastewater treatment plants.

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APPENDIX B**RAPID AND REAGENTLESS DETECTION OF MICROBIAL CONTAMINATION
WITHIN MEAT UTILIZING A SMARTPHONE-BASED BIOSENSOR**

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Abstract

A smartphone-utilized biosensor consisting of a near-infrared (NIR) LED (wavelength of 880 nm) and a digital camera of a smartphone (capable of detecting 880 nm NIR) was developed for detecting microbial spoilage on ground beef, without using antibodies, microbeads or any other reagents. 150 μL of deionized water (negative control) and series of serially diluted ground beef products to simulate microbial spoilage. An 880 nm NIR LED was irradiated perpendicular to the surface of ground beef, while the digital camera of a smartphone detected the scatter signal angled at 15° , 30° , 40° and 60° from the incident light. A plot of the scatter intensity against the detection angle showed a unique, distinguishable trend by the *E. coli* and *Salmonella* concentration. For example, the scatter intensity was maximum at 15° , 30° , and 45° with 10^8 CFU mL^{-1} , 10^4 CFU mL^{-1} , and 10 CFU mL^{-1} *E. coli*, respectively, and at 30° , 45° , and 60° with *Salmonella*, respectively. SEM and fluorescence microscopy experiments revealed that the antigens and cell fragments from *E. coli* bonded preferably to the fat particles within meat, creating submicro- or micro-sized aggregates (pseudo-colonies) that would maximize Mie scatter at a specific angle (depending on the size of such aggregates) under 880 nm NIR irradiation. The proposed method was further improved by programming a smartphone application that allows the user to position the smartphone at an optimum distance and a range of angles utilizing its internal gyro sensor to measure a series of scatter intensities against the detection angle. This handheld device can be used as a preliminary screening tool to monitor microbial contamination on meat products that is truly handheld, inexpensive, non-destructive and reagentless.

Keywords: microbial spoilage, foodborne pathogens, Mie scatter, non-destructive sensing, near infrared (NIR) sensing, smartphone biosensor, smartphone application

1. Introduction

Rapid detection of foodborne pathogens on meat and poultry products is an issue of increasing interest for the past few decades, especially due to several high profile food safety issues and outbreaks including the 1985 trichinosis outbreaks from horsemeat in France (Ancelle et al., 1988), 1988 hepatitis A outbreaks from raw oysters in Panama City, Florida, U.S. (Desenclos et al., 1991), 1996 *Salmonella* enteritis outbreak from a poor hygiene bakery in Dublin, Ireland (Grein et al., 1997), and the 2002 ConAgra ground beef recall where 19 million pounds of meat were recalled in Greeley, Colorado, U.S. (CDC, 2002). It was estimated that each year, 31 major pathogens acquired in the U.S. caused 9.4 million episodes of foodborne illness, 55,961 hospitalizations, and 1,351 deaths (Scallan et al., 2011). It was also estimated that foodborne illness occurs in 1.3 million cases per year in England, 4.5 million cases per year in The Netherlands, and 5.4 million cases per year in Australia (Flint et al., 2005).

Among all solved outbreaks in the U.S. from 1998 to 2007, the outbreaks and illness caused by beef products are the fifth highest in number (DeWaal et al., 2011). According to U.S. Department of Agriculture (USDA) data (USDA, 2013a), there were 378 recall cases from 2008 to 2012, while 37% (N = 140) were caused by beef products, 16.7% (N = 63) were caused by *E. coli* O157:H7 or Shiga toxin-producing *E. coli* (STEC;

including O157, O26, O45, O103, O111, O121, and O145), and 6.6% (N = 25) were caused by *Salmonella*.

Another potential cause of foodborne illness outbreaks could be the deliberate contamination of meat and poultry products as an act of bioterrorism, referred to as “agroterrorism.” Congressional Research Service of U.S. defines agroterrorism as “the deliberate introduction of an animal or plant disease with the goal of generating fear, causing economic losses, and/or undermining social stability” (Monke, 2007). This can be a serious problem for a country whose agricultural industry comprises a major portion of its economy. A good example is the U.S., where agriculture is the largest single sector in the economy, and agriculture-related products comprise almost 10% of all exports, amounting to almost \$140 billion in 2011 (USDA, 2013b).

It is apparent that we need better quality control of our food products, and this relies heavily on having better and faster detection technologies. The current standard detection methods for foodborne pathogens, described by the U.S. Food and Drug Administration (FDA), are culture-based, which require incubation, inoculation, as well as pre-processing the food sample (FDA, 2013). Other well-developed (but less standard) methods are immunological or nucleic acid-based methods, such as enzyme linked immunosorbent assays (ELISA) and methods that utilize polymerase chain reaction (PCR). However, while these current methods all have good accuracy and sensitivity, they also have similar limitations, including the destruction of the samples, the time, personnel, and laboratories that are needed for the experiments; PCR also requires relatively expensive instruments.

Development of rapid, non-destructive, and inexpensive sensing technologies for detection of microbial contamination on meat and potentially of other food products remain in high demand. Several methods that are in development are aiming for non-destructive, rapid, reagentless, and still accurate and sensitive. Electronic noses, Fourier transform infrared (FT-IR) spectroscopy, and Raman scattering are being investigated (Ellis and Goodacre, 2001; Ellis et al., 2002; Kalasinsky et al., 2007; Sowoidnich et al., 2012; Sundaram et al., 2013). These developing methods are an improvement in that they are non-destructive, can be real-time, and have the potential to be used as an on-site detection. However, most of these methods still need trained personnel to operate and can be expensive due to the requirement of specific instrumentation.

Previously, our laboratory has investigated the use of microbead immunoagglutination on a lab-on-a-chip platform and subsequent Mie scattering detection towards rapid detection of pathogens. This method has already been proven to have extremely low detection limit (typically at a single-cell level or 10 CFU/mL), near-real-time detection (less than 5 minutes per assay), as well as portability, towards *E. coli* detection from lettuce (You et al., 2011) and *Salmonella* detection from poultry package (Fronczek et al., 2013). However, this method still requires adding the reagent (antibody-conjugated microbeads) to the food sample via the reagent delivering vehicles – a network of channels and wells, i.e. lab-on-a-chip.

Mie scatter refers to the Mie solution to the scattering problem (Maxwell's equations) on a spherical object, when the object sizes (d) are similar to the wavelengths of incident light (van de Hulst, 1983). Mie solution describes how much light is scattered and the scatter intensities are changed according to the scattering angles.

Immunoagglutination assay is typically conducted by mixing antibody-conjugated microbeads with a target solution; the binding between the antibody and the target antigens causes agglutination of the microbeads. The agglutination is then monitored via Mie scattering measurement at a specific angle where the scattering intensity is maximum among the usable angles of detection. Since the agglutinated microbeads are no longer spherical in shape and its morphology is quite complicated, different scattering centers can constructively and destructively interfere with each other to a greater extent, leading to much stronger scattering intensities at an optimized angle of detection (Heinze and Yoon, 2011). The refractive index (n) of microbeads should be substantially higher than that of water and/or food samples, to successfully monitor this agglutination of microbeads. Previously, polystyrene microbeads ($n = 1.59$ and $d = 0.92 \mu\text{m}$; You et al., 2011; Fronczek et al., 2013) have successfully been used over water ($n = 1.33$) and food samples ($n \approx 1.40$ for typical proteins; Vörös, 2004).

In fact, *E. coli* and *Salmonella* can be identified directly through evaluating Mie scattering, since their refractive indices are still higher ($n = 1.38$; Balaev et al., 2002) than that of water ($n = 1.33$). However, the morphologies of the cells, fragments, antigens or their colonies are much simpler than the immunoagglutinated microbeads, and such detection is only possible when the bacterial concentration is very high (Hirleman, Jr. et al., 2008). If these cells, fragments, proteins or colonies can interact with some component(s) of meat to form more complicated structure, similar to immunoagglutination, they may be detected through Mie scatter in much lower level of detection. *E. coli* and *Salmonella* are both hydrophobic, thus they tend to attach preferentially to fat surfaces. Animal fats (typically hydrogenated) generally have an

average refractive index of 1.40 (slightly lower than $n = 1.46$ of pure lipids; Board, 2002), which is not very different from other proteins, *E. coli*, and *Salmonella*. However, $n = 1.40$ is still higher than that of water ($n = 1.33$), it may be possible to observe different light scattering characteristics upon association of bacteria to the fats. Based on these facts, we attempted to use Mie scatter detection to measure these pseudo-colonies of *E. coli* and *Salmonella* cells, fragments, and antigens (when the concentration is low), or actual colonies (when the concentration is high) around the fats within the meat samples without any microbeads or antibody presence. Since there is no significant difference in the refractive indices between fats ($n = 1.40$) and bacteria ($n = 1.38$), the increase in bacteria concentration may not bring in the overall increase in scatter intensity at a fixed scatter angle. [Such overall increases could be observed with polystyrene microbead ($n = 1.59$) immunoagglutination (You et al., 2011; Fronczek et al., 2013).] However, the angle-dependent Mie scatter characteristics will surely be altered by bacteria concentration, since the optimum angle for detection will change as the sizes and ratios of pseudo-colonies and actual colonies change. In addition, we expect to observe some difference between *E. coli* and *Salmonella*, since Dickson and Koohmaraie (1989) pointed out that *E. coli* and *Salmonella* would attach to fat differently, due to their differences in hydrophobicity and surface charge.

In our experimental setup, the digital camera of a smartphone was utilized as an optical detector to quantify the Mie scatter intensities, to replace optical fibers and a spectrometer. Additionally, a smartphone application was programmed in order to eliminate the need of using a benchtop stage, and to achieve our goal of creating a truly handheld (not just portable), easy-to-use and inexpensive device.

2. Materials and method

2.1. *E. coli* K12 and *Salmonella* Typhimurium solutions

Escherichia coli K12 lyophilized cell powder (Sigma-Aldrich, St. Louis, MO, USA; catalog no. EC1) was cultured in lysogeny broth (LB-Miller; Growcells, Irvine, CA, USA; catalog no. MBPE-1050) at 37°C for 18 hours. *Salmonella* Typhimurium Z005 (ZeptoMetrix, Buffalo, NY, USA) was cultured in 25 mg mL⁻¹ brain heart infusion broth (Remel, Lenexa, KS, USA), and was incubated at 37°C for 12 hours.

The cultured solutions were serially diluted with autoclaved, deionized (DI) water. The colony-forming units (CFU) of each serial dilution were quantified by spread plate method, in which a 0.1 mL aliquot was uniformly spread on top of an LB agar plate (VWR International, Radnor, PA, USA; catalog no. 29447-000). The plate was incubated at 37°C for 24 hours before the colonies were counted.

2.2. Ground beef samples

Packages of 80% and 92% lean ground beef were purchased from local grocery stores one week before the experiments. The ground beef were made into 5 gram aliquots and each aliquot was flattened and placed on a cell culture dish (Corning Inc., Corning, NY, USA; catalog no. 3294). The meat dishes were then covered and sealed with a paraffin film (Parafilm “M”; Pechiney Plastic Packaging, Menash, WI, USA) and put in a freezer (-20°C). 12 hours before the experiments, dishes were moved to a refrigerator

(4°C) to thaw. During the experiments, the 150 μ L of each diluted *E. coli* solution was applied to the flattened 80% lean meat at the center, and 150 μ L of each diluted *Salmonella* solution was applied to the flattened 92% lean meat. The dishes were covered and sealed again, and put on a nutator mixer (VWR International; catalog no. 15172-203) for 30 minutes.

2.3. Benchtop system for optimizing Mie scatter detection

Positioning stages (Edmund Optics, NJ, USA) with two holders were developed to expose the samples at varying angles of incident light and detector device (the digital camera of a smartphone) (Figure 1). An 880 nm 10 mW near infrared light emitting diode (NIR LED; AixiZ LLC, Houston, TX, USA; catalog no. AIX-880-10) was placed in the incident light holder, and an iPhone 4S (Apple, CA, USA) was placed in the detector holder. The NIR LED light source enables us to obtain the scatter light signals without being affected by the natural meat color differences (in the visible light range), and provides a sufficiently strong signal to the iPhone digital camera which makes the final device portable. A support for precisely attaching the iPhone to the benchtop system was designed using AutoCAD 2000i (Autodesk, CA, USA) and was then stereolithographically printed using a uPrint 3D printer (Stratasys, MN, USA).

The *E. coli*- or *Salmonella*-applied samples, together with a negative control (150 μ L of DI water was applied to the meat instead of *E. coli* or *Salmonella* solutions), were then placed perpendicular to the incident light at a distance of 6.35 cm (2.5 inch), and the attached iPhone 4S took pictures in a dark environment (i.e. minimum ambient light) at

four different angles from the incident light (i.e. 15° , 30° , 45° , and 60°) (Figure 1). The light scatter intensities were identified by analyzing the pictures taken by the iPhone using ImageJ version 1.44p (National Institutes of Health, Bethesda, MD, USA). All images were circular-cropped to eliminate the noise around the edge of the picture, and were converted to 8-bit (gray scale) pictures. The processed pictures were then analyzed to get the median intensity value (between 0-255) among all pixels.

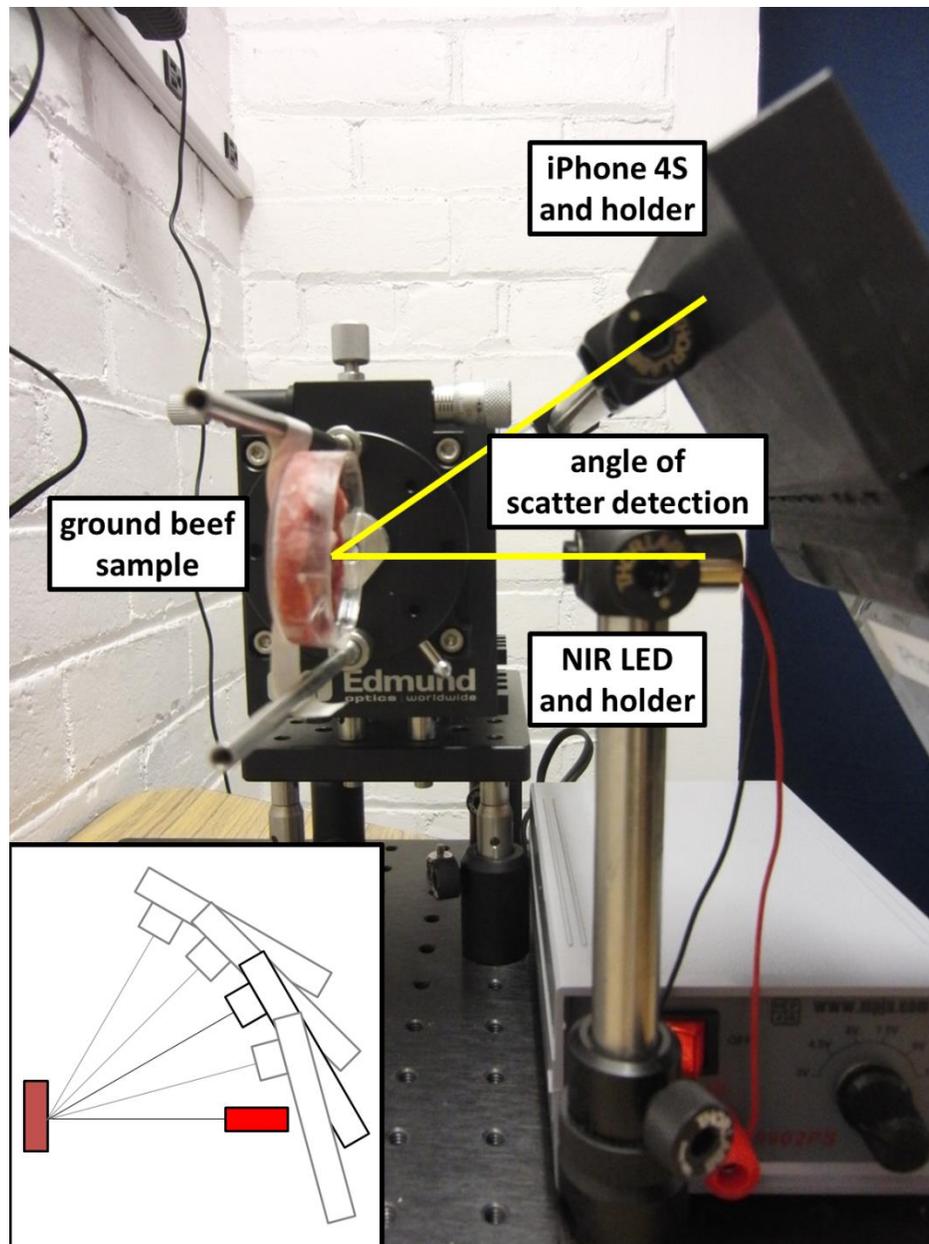


Figure 1. The benchtop system consists of an iPhone 4S and its holder, an NIR LED and its holder, and a ground beef sample and its holder. The angle of scatter detection refers to the angle between the iPhone camera and the NIR LED light source.

2.4. SEM imaging

SEM images of *E. coli* on 80% lean ground beef were taken using an ultra-high resolution field emission scanning electron microscope (Hitachi, Schaumburg, IL, USA; model S-4800 Type II). 100 μL of water (negative control) or *E. coli* solution (10^5 or 10^8 CFU mL^{-1}) was applied to 1.5 g of thawed ground beef in a petri dish and mixed on a nutator mixer for 30 minutes. After mixing, the fixative was added to fix the sample overnight. The fixative consisted of 4% v/v paraformaldehyde and 1% v/v glutaraldehyde with phosphate buffer. The fixed samples were then dried in a Polaron Critical Point Dryer (Quorum Technologies, East Sussex, UK; model E3000) and platinum-coated using a Hammer 6.2 Sputter Coater (Anatech, Union City, CA, USA) before SEM imaging.

2.5. Fluorescence staining for fats and bacteria

Less than 1 g of 80% lean ground beef sample was placed on a glass slide. 10 μL solution of Nile red (Sigma-Aldrich; catalog no. 72485) dissolved in acetone (1 mg mL^{-1}) was added to the ground beef and the samples were lightly pressed down with coverslips. The samples were stained overnight in a refrigerator (4°C) before adding 20 μL of Hoechst-stained *E. coli* solutions. Nile red is very soluble in lipid and has an emission peak between 620-660 nm when excited in the range 525-575 nm.

Each serial-diluted *E. coli* solution was stained with Hoechst dye that stains nucleic acids (NucBlue Live ReadyProbes Reagent, Life Technologies Corporation,

Carlsbad, CA, USA; catalog no. R37605) for 30 minutes before it was applied to the Nile red-stained ground beef sample. Hoechst dye has a blue emission (around 460 nm) when excited in the ultraviolet (UV) range (around 350 nm).

Once the Hoechst-stained *E. coli* solutions were applied to the Nile red-stained ground beef samples, they were firmly pressed down with the coverslips and were observed using a fluorescent microscope (Nikon, Tokyo, Japan). Images of each sample were taken under UV light and green light separately, and were overlaid using ImageJ (National Institutes of Health).

2.6. Smartphone application for handheld detection

A smartphone application was programmed using Xcode (Apple, CA, USA), which allows users to take pictures at the four angles at a fixed distance (Figure 2). The application shows the angle by using the built-in gyro sensor. Two dotted lines appear on the screen for the users to match the outline width of a meat dish; therefore the distances between the camera and the sample are consistent. The application further analyzes and compares the pictures through implementing an image processing algorithm, and displays the bacteria concentration on the smartphone screen.

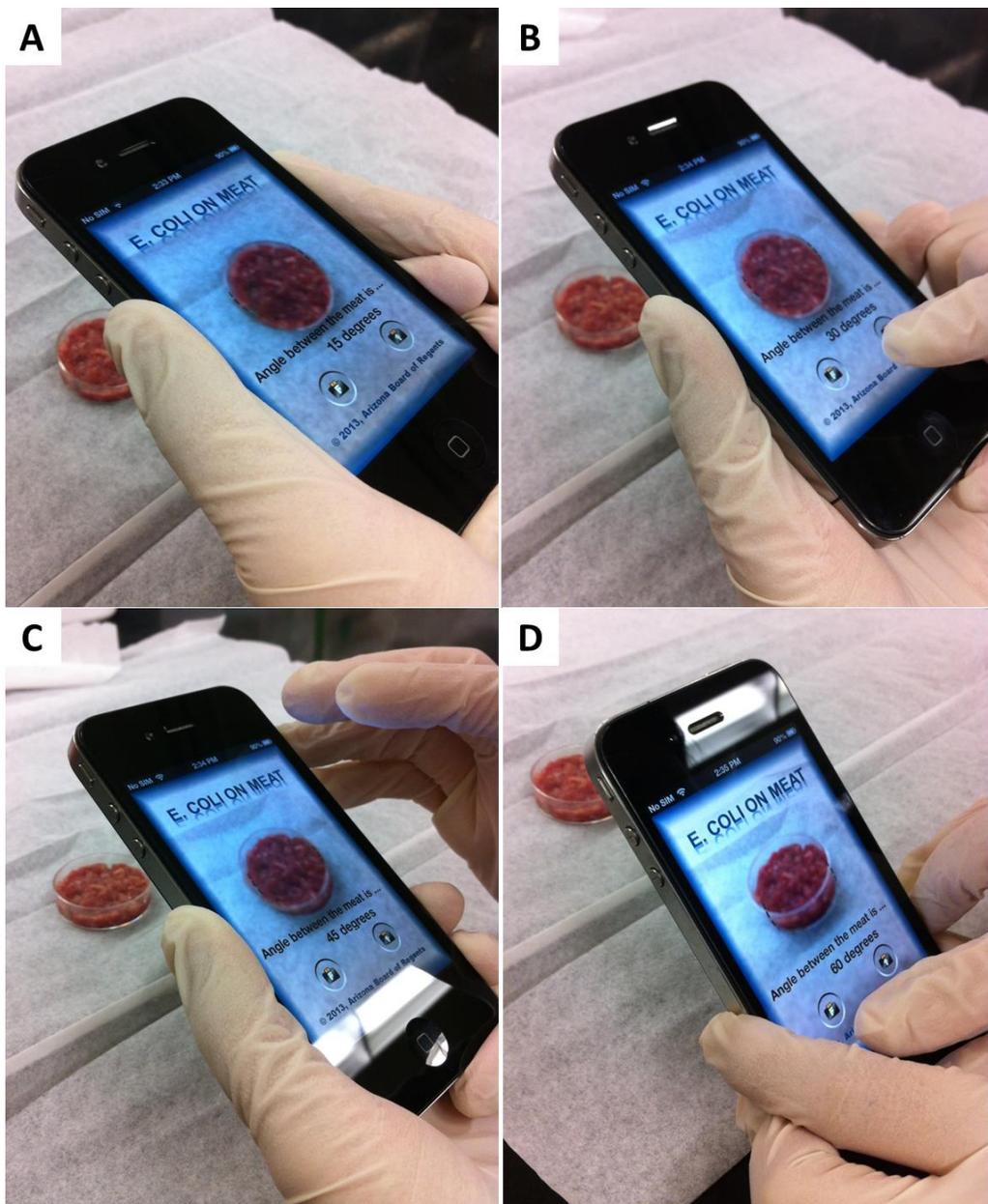


Figure 2. Photographs showing the operation of the smartphone application at the four specific angle of scatter detection: A) 15°, B) 30°, C) 45°, and D) 60°.

3. Results and discussion

3.1. Light scatter Intensities from ground beef with *E. coli* and *Salmonella* using a benchtop system

Normalized scatter light intensities from ground beef measured at 15°, 30°, 45°, and 60° were plotted against the log *E. coli* concentrations (Figure 3A) and the log *Salmonella* concentrations (Figure 3B) on surface plots. (Individual plots of scatter intensities against the bacteria concentrations at four different angles are shown in Supplementary Material 1). To eliminate sample-to-sample variation, all acquired intensities were normalized to the average of the negative control (i.e. ground beef with DI water) readings of the same angle. Stars (*) indicate the data points that are significantly different from the negative controls ($p < 0.05$). Black stars indicate the data points significantly above the negative controls, while white stars significantly below the negative controls.

Figure 3A (*E. coli* on 80% lean ground beef) shows a “ridge” moving from 10^1 CFU mL⁻¹ + 60° → 10^1 CFU mL⁻¹ + 45° → 10^4 CFU mL⁻¹ + 30° → 10^7 CFU mL⁻¹ + 30° → 10^8 CFU mL⁻¹ + 15°. With 15° detection, all data points are significantly higher than the negative control, which can be used as a reference in identifying the bacterial presence. However, 15° detection should not be used for quantification, since they are quite similar to each other (except the one at 10^8 CFU mL⁻¹). Figure 3A clearly shows that the scatter light intensity change over the detection angle has a different trend at each *E. coli* concentration on the ground beef sample. For example, if the peak in light intensity is found with higher angles (45°-60°), the concentration is likely at the lower

range (10^1 CFU mL⁻¹); if the peak is found with mid-angles (30° - 45°), the concentration is likely at the mid-range (10^4 - 10^7 CFU mL⁻¹); if the peak is found with lower angles (15° or less), the concentration is possibly very high (10^8 CFU mL⁻¹).

Figure 3B (*Salmonella* on 92% lean ground beef) shows a similar trend: the “ridge” moves from 10^1 CFU mL⁻¹ + $60^\circ \rightarrow 10^4$ CFU mL⁻¹ + $45^\circ \rightarrow 10^8$ CFU mL⁻¹ + 30° . Overall, the “ridge” is found at the higher angles and the signal intensities are generally smaller than those of *E. coli* on 80% lean ground beef. These differences can be attributed to the different bacterial species and the lower fat content of the ground beef. Another noticeable difference is the presence of valleys lower than 1, i.e., significantly lower than the negative controls, found at 10^1 CFU mL⁻¹ + 30° , 10^7 CFU mL⁻¹ + 15° and 10^7 CFU mL⁻¹ + 60° . Similar analysis can be made for Figure 3B. For example, if the peak in light intensity is found with higher angle (60°), the concentration is likely at the lower range (10^1 CFU mL⁻¹); if the peak is found with mid-angle (45°), the concentration is likely at the mid-range (10^4 CFU mL⁻¹); if the valleys are found with extreme angles (15° and 60°), the concentration is likely high (10^7 CFU mL⁻¹); if the peak is found with lower angle (30°), the concentration is possibly very high (10^8 CFU mL⁻¹). It is already known that scattering detection can be used to detect bacteria colonies when the concentration is high (typically 10^5 CFU mL⁻¹ and above; Hirleman, Jr. et al., 2008). However, when the bacteria concentration is low, such colonies are too small in number to be detected directly. We believe that the cell fragments and proteins from *E. coli* and *Salmonella* can interact with and aggregate around the fats within the ground beef sample, to form a pseudo-colony, due to their hydrophobicity. These pseudo-colonies should be much smaller than the actual bacteria colonies, perhaps a few microns or less in size.

These sizes are comparable to the wavelength of incident light (NIR at 880 nm), where the subsequent Mie scattering is accordingly maximized. In addition, these pseudo-colonies may form more complicated structure, similar to immunoagglutination, which further increase the extent of Mie scatter at a certain specific range of detection angle. The scatter signals that we observed at low bacteria concentrations (at 45-60°) were most likely the light scatter caused by such pseudo-colonies, possibly with actual small colonies of bacteria. As the bacteria concentration increases, a larger portion of signals come from actual colonies that were formed prior to their individual attachment to the fat cells. The differences in trends between *E. coli* and *Salmonella* can be caused by either the difference in their attachments to fat, or the fat content in the ground beef samples (80% lean vs. 92% lean).

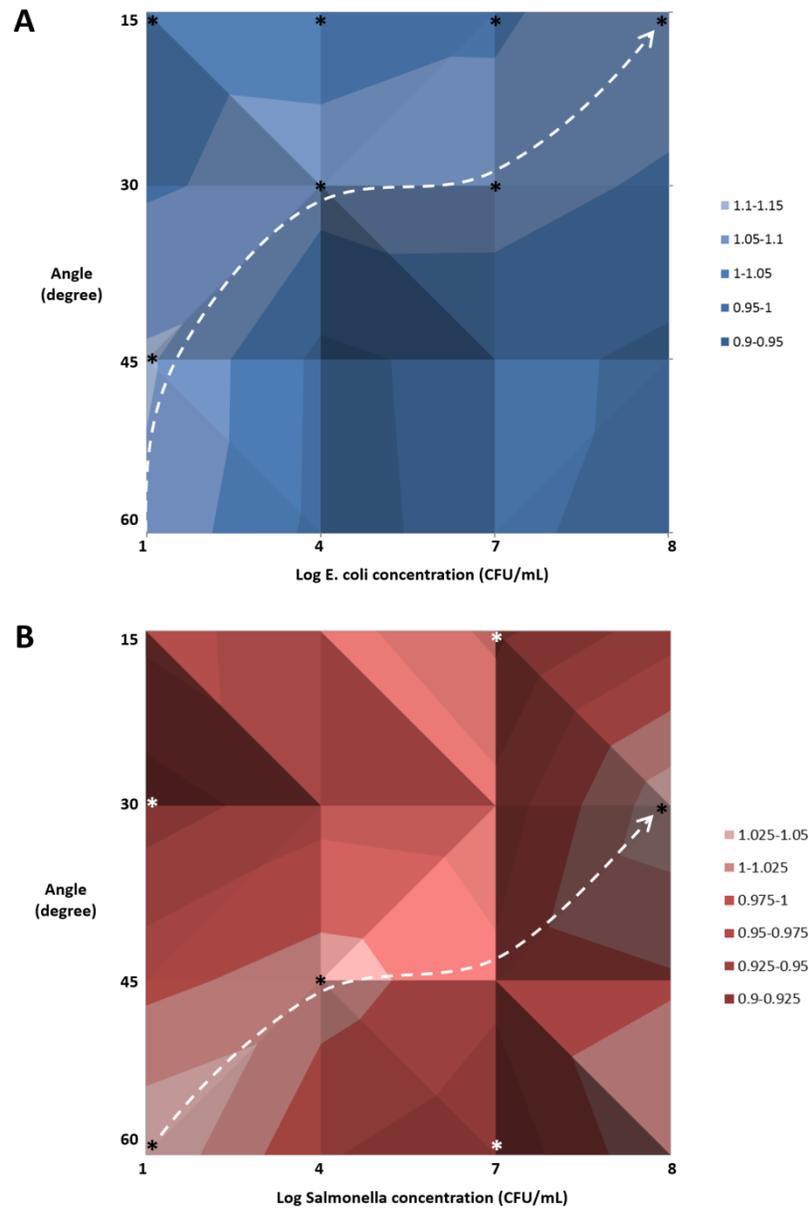


Figure 3. Surface plots that combine the normalized light intensities, the angles of scatter detection, and the log concentrations of A) *E. coli*, and B) *Salmonella*. Black stars indicate the data points significantly above the negative controls, while white stars significantly below the negative controls.

3.2. Effect of dust particles

Although the whole experiments were performed to minimize dust deposition (the meat samples were always covered, while minimizing the exposure time to ambient air), we have nonetheless investigated the potential effect of the dust particles. The difference in absorbance (= the sum of scattered light in all possible directions for the dust particles, plus the true absorbance by the dust particles) was evaluated for the dust particles passively deposited for one hour, on a 645 cm² desk area in an office space that has about ten student workers. The glass microfiber filter (70 mm diameter; Whatman, GE Healthcare, Waukesha, WI, USA; catalog no. 1823-070) was used to collect the dust particles. The absorbance difference (between the collected sample and a negative control) was maximum at green color (less than 0.02 absorbance) and less than 0.012 at the NIR wavelength (880 nm) that we used in this study. Considering the light scatter signal at a specific angle is only a tiny fraction of the total absorbance, we can conclude that the effect of dust sedimentation is negligible for our angle-specific NIR scatter detection.

3.3. SEM imaging

Several scanning electron microscopic (SEM) images of *E. coli* on 80% lean ground beef were taken (Figure 4) to further confirm the above-mentioned *E. coli* attachment to ground beef. Figure 4A shows the SEM image of the ground beef with DI water, i.e. negative control. Lots of muscle fibers and smaller particulate matter can be identified, with bigger fat cells occasionally found in between. Smaller particulate matter

can be fat particles, protein aggregates, cell fragments, or salt crystals (formed during the sample preparation for SEM). Since the *E. coli* solutions were serially diluted with DI water, not phosphate buffered saline (PBS), the formation of PBS crystals is not likely to happen during the preparation for SEM imaging. With intermediate *E. coli* concentration, 10^5 CFU mL⁻¹ (Figure 4B), such “small particulate matter” becomes substantially bigger in their size, apparently in an aggregated form. With much higher *E. coli* concentration, 10^8 CFU mL⁻¹ (Figure 4C), intact *E. coli* bacteria (mostly aggregated) can easily be identified, while particulate matter can still be found with even bigger sizes. These results indirectly indicate the formation of pseudo-colonies between the cell fragments and proteins from *E. coli* and fat particles within the meat sample.

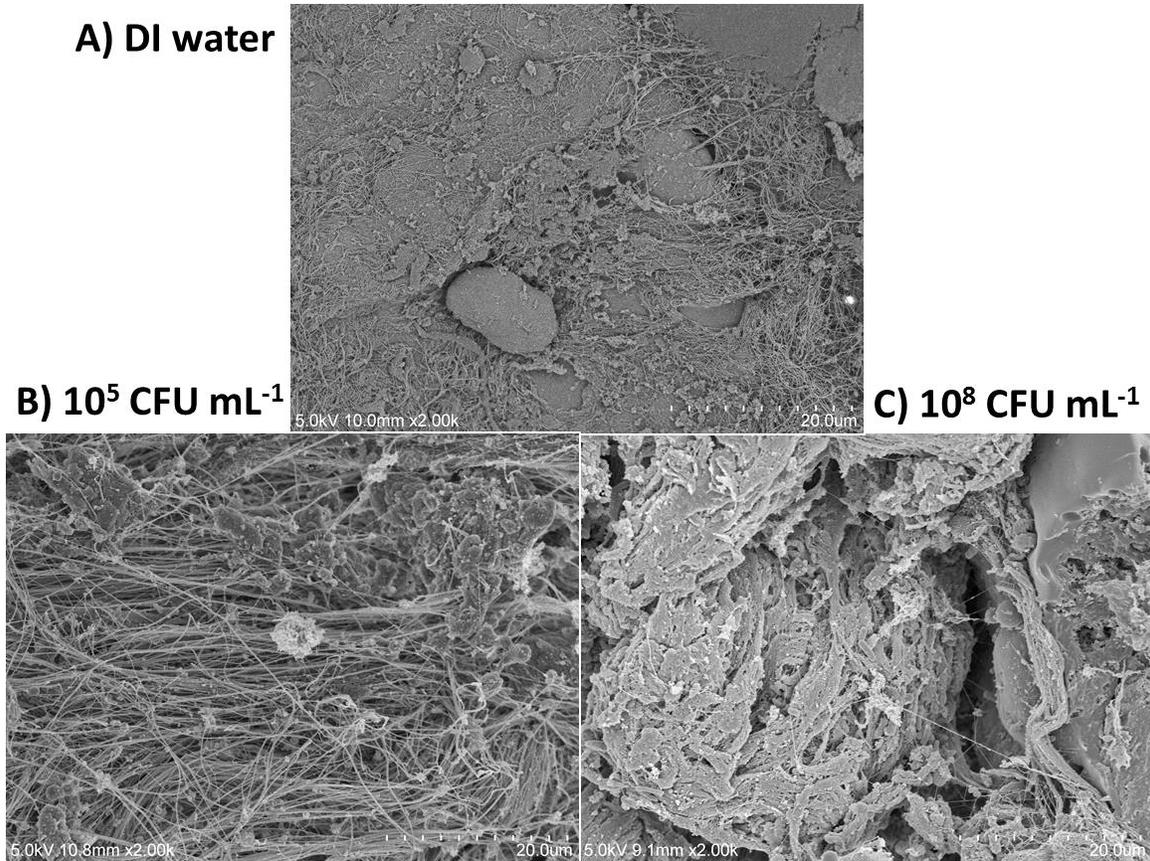


Figure 4. SEM images of A) DI water on ground beef, B) 10^5 CFU mL⁻¹ *E. coli* on ground beef, and C) 10^8 CFU mL⁻¹ *E. coli* on ground beef.

3.4. Fluorescence staining for fats and *E. coli*

Figure 5 shows fluorescence microscopic images of Hoechst-stained *E. coli* (stains nucleic acids and appears as blue fluorescence) on Nile Red-stained ground beef (soluble in lipid and appears as yellow fluorescence). Figures 5A and 5B were taken when 10^2 CFU mL⁻¹ of *E. coli* was added on the meat; Figures 5C and 5D were taken when 10^5 CFU mL⁻¹ of *E. coli* was added. In all four images, blue stains are always found near the yellow stains, but not overlapping them, indicating the *E. coli* cells are attracted towards the fat regions of the ground beef. In Figure 5A, two different vertical colonies of *E. coli* (blue) can be identified; the left one is aligned with the big, circular-shaped lipid (yellow; most likely a fat cell) and the right one is aligned with the smaller cluster of lipid (faint yellow-colored cluster; most likely fat particles). In Figures 5C and 5D, the yellow stains (fat cells) are much bigger and the blue stains (*E. coli*) are surrounding those yellow stains. In Figure 5B, *E. coli* cells are found right next to the smaller-sized lipids.

The SEM images together with the fluorescence microscopic images strongly support our theory about the formation of pseudo-colonies at lower concentrations which make the Mie scattering possible at a concentration that is well below the previously shown detection limit.

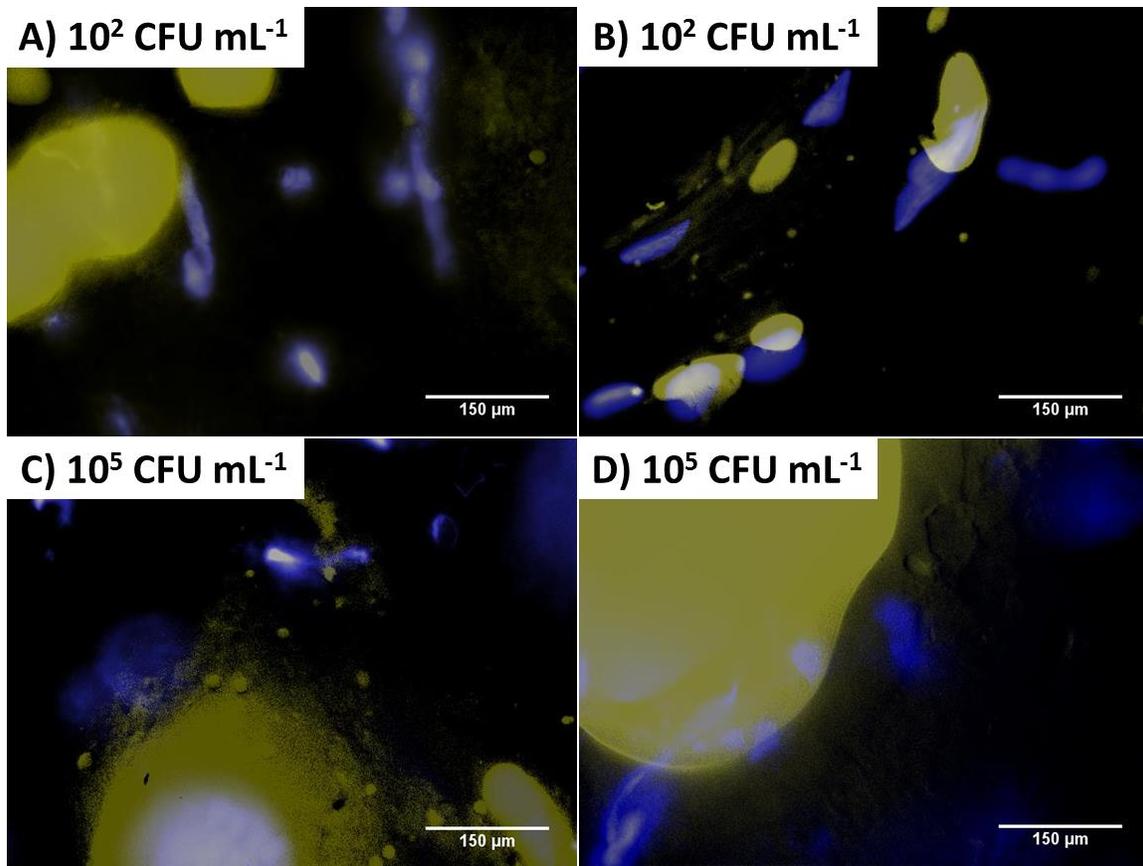


Figure 5. Fluorescence microscopic images of Hoechst-stained *E. coli* (blue) on Nile Red-stained ground beef sample (yellow) with the *E. coli* concentrations of 10^2 (A and B) and 10^5 (C and D) CFU mL⁻¹.

3.5. Handheld device with smartphone application

Using the handheld device with smartphone application, normalized scatter light intensities were evaluated and plotted against the angles, with varying *E. coli* (Figure 6A) and *Salmonella* concentrations (Figure 6B). Actual images taken by a smartphone are shown in Supplementary Material 2. Most digital cameras are capable of recognizing NIR, as demonstrated in this work. Results obtained here are similar to what we received with a benchtop system: With lower *E. coli* concentration the peak in light intensity is found with higher detection angles. With mid-range *E. coli* concentration, the peak is found with mid-range detection angles. With higher *E. coli* concentration, the peak is found with lower detection angles. With *Salmonella*, the trend is similar to that of *E. coli*, but all peaks shifted to a higher angle. The peak for 10 CFU mL^{-1} shifted from 45° to 60° , peaks for 10^4 and 10^7 CFU mL^{-1} shifted from 30° to 45° , and the peak for 10^8 CFU mL^{-1} shifted from 15° to 30° .

This suggests that we can use a smartphone application to replace the benchtop system and make the device truly handheld and inexpensive, while maintaining the low detection limit.

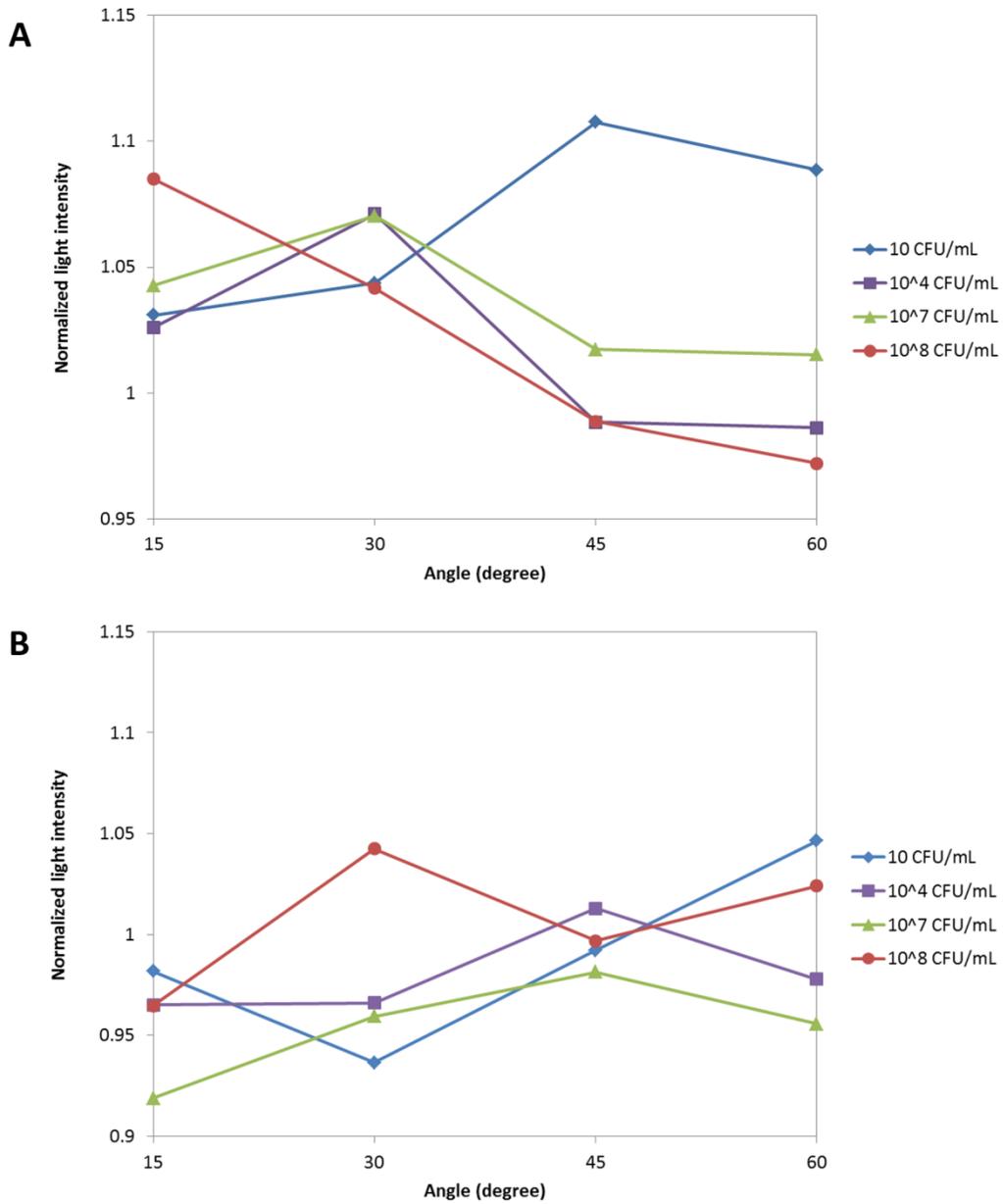


Figure 6. Normalized light intensities plotted against scatter detection angles with different
A) *E. coli*, and B) *Salmonella* concentrations.

4. Conclusion

A smartphone-based biosensor was developed to detect and quantify microbial contamination on ground beef, consisting of a 880 nm NIR LED and a smartphone (utilizing its digital camera, software application, and an internal gyro sensor). Mie scatter measurements were made at four different angles (15°, 30°, 45° and 60°), and the concentrations of *E. coli* and *Salmonella* Typhimurium (from 10¹ CFU mL⁻¹ to 10⁸ CFU mL⁻¹) could be determined by the “pattern” of such scatter intensities over the angles, i.e., at which angle the peak/valley intensity was observed or whether the scatter intensities monotonically decreased/increased over the angles, etc. The proposed smartphone-based biosensor does not require any antibodies, microbeads or any other reagents, and is handheld, easy-to-use, rapid, and inexpensive. The proposed device can be used as a preliminary screening tool to monitor microbial contamination on meat products.

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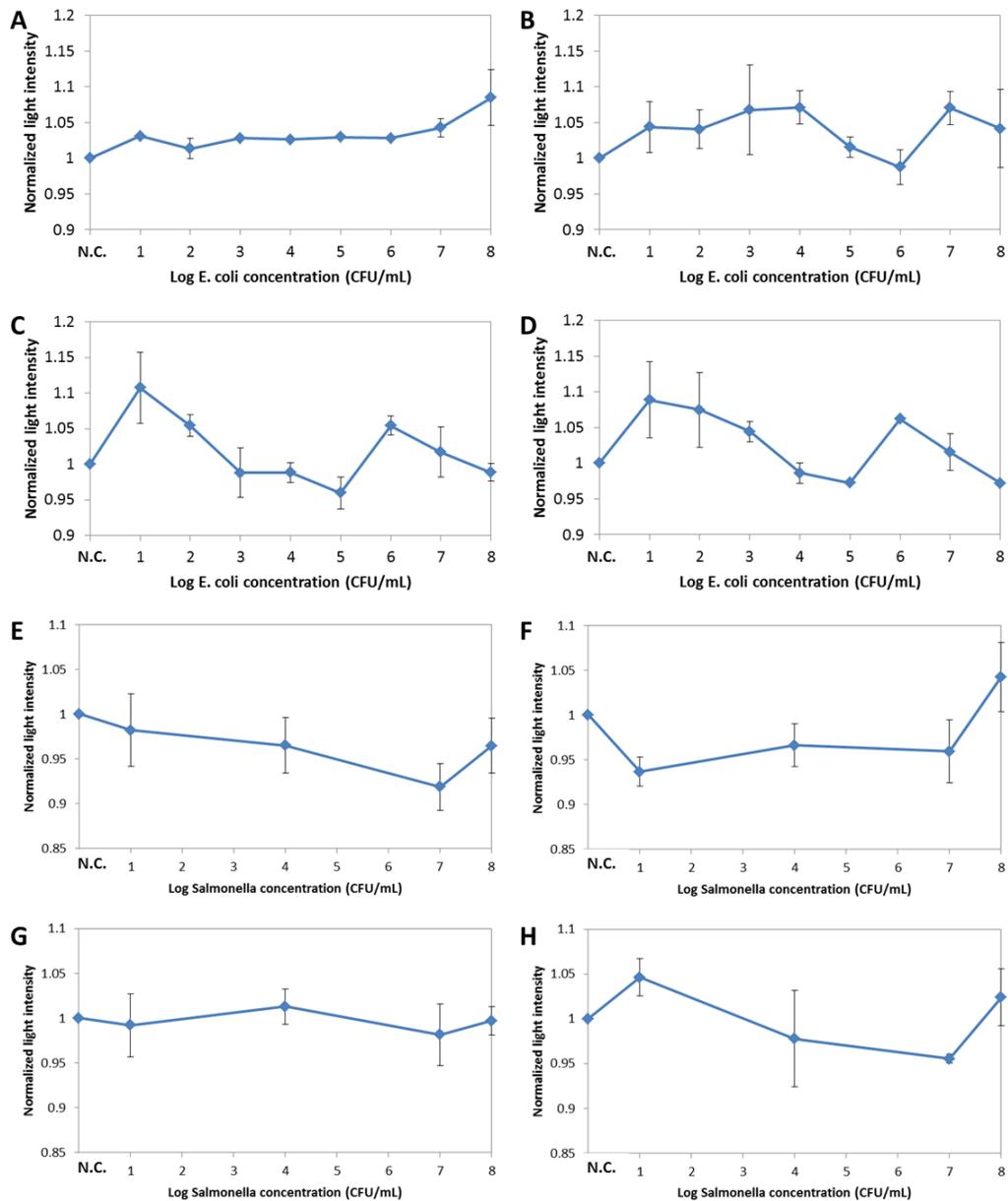
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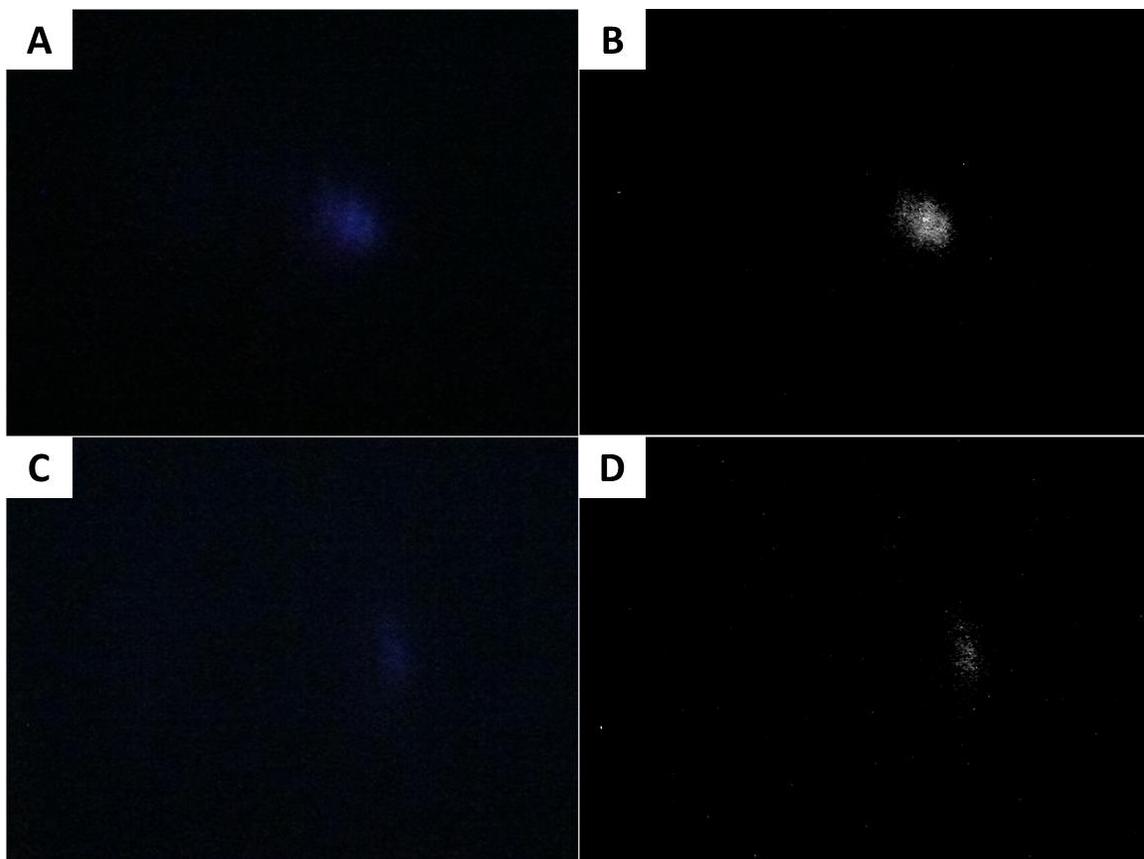
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Supplementary Material 1. Normalized scatter light intensities plotted against the log concentrations of *E. coli* (A-D; on 80% lean ground beef) and *Salmonella* (E-H; on 92% lean ground beef).

Error bars represent standard errors. The scatter detection angle was

15° (A and E), 30° (B and F), 45° (C and G), and 60° (D and H).



Supplementary Material 2. Typical images obtained with the iPhone camera.

A) Raw image showing higher light intensity. B) Processed image of A.

C) Raw image showing lower light intensity. D) Processed image of C.

APPENDIX C

USE OF BIOSENSOR IN SECONDARY EDUCATION CURRICULUM TO ASSESS STUDENTS' INTEREST, MOTIVATION, AND AWARENESS OF SCIENCE, ENGINEERING, AND CURRENT WORLDWIDE ISSUES

Pei-Shih Liang, Kimberly L. Ogden, and Jeong-Yeol Yoon

Abstract

The multidisciplinary and multi-application character of biosensor makes it a very attractive topic to introduce science and engineering concepts into a secondary education classroom. Some of the techniques used in biosensors are easy to demonstrate and can be intriguing to the students. We used current biosensor research topics to assess students' interest and motivation in learning science and engineering, as well as to raise students' awareness of current worldwide issues. We discussed which application areas and techniques were more intriguing to the students, the effect of the proposed lesson plan, and the appropriateness of introducing high-tech topics to secondary education students. We delivered a biosensor lesson with in-class demonstrations and short pre- and post-surveys, into middle and high school classrooms to determine students' greatest interest among four biosensor applications: medical diagnostics, environmental monitoring, biosecurity, and food safety; as well as seven techniques used in biosensor: circuit building, 3D printing, microfabrication, nanotechnology, genetic engineering, computer programming, and smartphone utilization. The lesson was effective in bringing relatively equalized interests in the applications and the techniques of biosensor for the middle school students, where they were mostly motivated by hands-on techniques. High school students in physics and engineering classes had strong preference on medical and biosecurity applications as well as genetic engineering and nanotechnology techniques. These results indicate that biosensor could be an effective lesson to be introduced to the middle school students in order to raise their interest and motivation in current research

and technologies, whereas for high school students, this lesson plan can be used to investigate students' preferences and can be adapted for different subjects of classrooms.

Keywords: Biosensor; Medical diagnostics; Food safety; Biosecurity; Environmental monitoring; Secondary education; Biotechnology

1. Background

Science, Technology, Engineering, and Mathematics (STEM) courses are extremely important for the students in secondary education (i.e. generally grades 5 through 12 in the United States; varies among school districts in some cases) and critical to preparing students for colleges and further professional careers. During the past couple decades, technologies, as well as science and engineering research in higher education, have greatly improved; the future for our next generation will be much more diverse and advanced in many different ways. However, despite the fact that technologies have been changed considerably, our secondary education classrooms rarely introduce and/or utilize the most current research or findings in their curriculum due to various reasons and concerns (Borgerding et al., 2013; Bigler and Hanegan, 2011). The teaching materials are often hindered by limited human resources, limited professional development, and the amount of accessible outreach programs.

Researchers have continued trying different ways to improve students' learning in either some specific subjects or in the general STEM topics. Lots of them have used common references that are related to students' leisure interest (e.g. movies, music, and

games) (Hollis, 1996; Rothhaar et al., 2010; Annetta et al., 2012). U.S. National Science Foundation (NSF) has funded the program named GK-12 which brings graduate students in STEM disciplines into K-12 classrooms with their research. We believe that presenting specific examples of current research and/or cutting-edge technologies to students, in complimentary to the regular STEM topics, would be an improved approach to grasp students' interest and augment students' motivation early on. Ideally, these examples should be easy to demonstrate and easily understood by K-12 students (Yang et al., 2010).

Biosensor research is one of the fast-growing, multidisciplinary fields in science and engineering and involves many different subjects and professions, such as nanoscience, genetic engineering, electrical engineering, computer science, etc. It also has diverse applications in several important areas including medical, agricultural and environmental diagnostics/assays. Considering the various disciplines that biosensor research involves, it can be an excellent tool to introduce science and engineering concepts into a secondary education classroom as well as to provoke students' interest in science and engineering careers. Furthermore, the applications of biosensor research can be a good instrument to raise students' awareness of the current worldwide issues, such as pandemic infectious diseases, foodborne/waterborne illness outbreaks, bioterrorism/agro-terrorism, etc.

Many researches have been performed to discover general public or secondary education classroom students' knowledge, perception, and acceptance of biotechnology (Bigler and Hanegan, 2011; Cavanagh et al., 2005; Harms, 2002; Firdaus-Raih et al., 2005). According to these researches, some secondary education students do not possess very much biotechnology knowledge, although they do have relatively high acceptance of

the topic and may perceive them well. Although biotechnology and biosensors are closely related, lesson plans and/or the surveys that were used in these researches do not necessarily include biosensor research.

Biosensors, compared to biotechnology, have similar fields of application, but some of the techniques that are used in biosensors are more tangible, and therefore easier to demonstrate or deliver to students in a secondary education classroom (e.g. circuit building and 3D printing). In addition, students may find some of them more relatable and intriguing than the techniques that are used in general biotechnology research (e.g. smartphone-utilized biosensors). When teaching biosensors, instead of focusing on molecular biology, biochemistry, and various science concepts, we often emphasize more on how different fields of studies collaborate together and coproduce useful devices and technology. This is especially important in an era of inter- and multi-disciplinary research.

In this work, we chose four different applications of biosensors and seven different techniques used in developing biosensors to construct a short lesson plan (80-90 minutes) on biosensors. The lesson was combined with short pre- and post-surveys that were given to students to evaluate the changes in students' interest and motivation, as well as their perception and awareness changes during the lesson. The main objective of the study was to investigate the possibility of using biosensors as a new tool to help improve students' interest and motivation, further preparing them for colleges and possibly later professional career choices. The lesson and the surveys were given in local middle and high school classrooms in Tucson, Arizona, United States.

2. Methods

2.1. Participants

The participants in this study were 51 6th grade students from two different classrooms and 105 11th and 12th grade students from three physics classrooms and one engineering classroom in Tucson, Arizona, United States. The 51 6th grade students were from Utterback Magnet Middle School, which has a demographic of 78.2% Hispanic, 8.4% White/Anglo, 7.5% African American, 5.5% Native American, 0.5% Multiracial, and 0% Asian American, as of instruction day 80, 2012-2013 [10]. The 105 11th and 12th grade, physics and engineering students were from the Sahuaro High School, which has a demographic of 56% White/Anglo, 29% Hispanic, 7.7% African American, 3.3% Asian American, 2.8% Multiracial, and 1.2% Native American, as of instruction day 100, 2012-2013 (Tucson Unified School District Statistics, 2012-2013).

Out of the 51 middle school students, 47 of them provided valid information (i.e. attended and completed the whole lesson, filled out the survey correctly) for the pre-survey and 45 for the post-survey; 81 and 82 out of the 105 high school students provided valid information for the pre- and post-survey, respectively. Female to male ratio of the 51 middle school students was about 2.41, and the ratio of the high school students was about 0.43.

2.2. Pre and Post-Surveys

Two-page pre- and post- surveys were designed and given out before and after the lesson. The first page of the survey was comprised of four pictures of commonly-seen sensors, including a touch-free faucet, an automatic door, parking sensors for a car, and a motion detector, and a fifth picture of a glucose meter as an example for biosensors. At the end of the first page, students were asked to write down the similarity and differences they found between a sensor and a biosensor. The first page served the purpose of an introduction to the pre-survey and a recapitulation for the post-survey.

The second page of the survey contained two short paragraphs that briefly explained (one sentence each) the applications of biosensors and led into the techniques that are used in biosensor research. The bottom half of the second page was a table for the students to fill out, which is where we collected our data from.

The table was designed to investigate students' opinions of the biosensor applications and techniques based on three factors, interest, importance, and motivation. The table was laid out in such a way that students were to select one application (out of four) and one technique (out of seven) that were the most interesting to them, one of each that they perceived to be the most important, as well as one of each that they wanted to learn about the most (motivation).

2.3. Design of Lesson

A 90-minute lesson was designed to provide a general overview of biosensors to the students, including the applications and techniques that are used in biosensor research. The first part of the lesson was tied in with the first page of the survey and served to define biosensors through discussing the similarities and differences between a sensor and a biosensor. A biosensor is a device for the detection of a target that combines biological elements (bioreceptors) with physicochemical detectors (transducers). After this interactive inquiry process and discussion, students should be able to explain the main differences between these two in their own words.

The second part of the lesson was the applications of biosensor, where we chose medical diagnosis, food safety, environmental monitoring, and biosecurity for this study. These four applications were presented by showing a transcript recorded in the summer of 2012 of questions that were asked during a science debate between the two main presidential candidates for the United States. These topics (all of which utilize biosensors) were apparently the important scientific issues or challenges facing the nation at that time. Another topic of discussion in this debate worth noting was the issue of science education and research, which of course was our driving motivation for experimenting with biosensor lessons in secondary education in the first place.

For the medical application, we used glucose meters as one example of how biosensors can be used for medical purposes. For the food safety application, we wanted the students to understand that “clean food” is not a given; it is the product of people who work diligently on food safety. For the environmental monitoring application, we quickly

referenced and built upon students' existing knowledge of the natural environment and stressed the importance of having a clean and safe environment (largely of water and air). Lastly, for the biosecurity application, we explained the need for sensitive and accurate detection of possible biological threats or weapons.

Seven techniques that can be used in biosensor development and manufacturing were chosen for this study: circuit building, 3D printing, microfabrication, nanotechnology, genetic engineering, computer programming, and smartphone utilization. Of these techniques, some were demonstrated with a product that was made using that technique or a video of real projects, and some were demonstrated with educational short films (Figure 1).

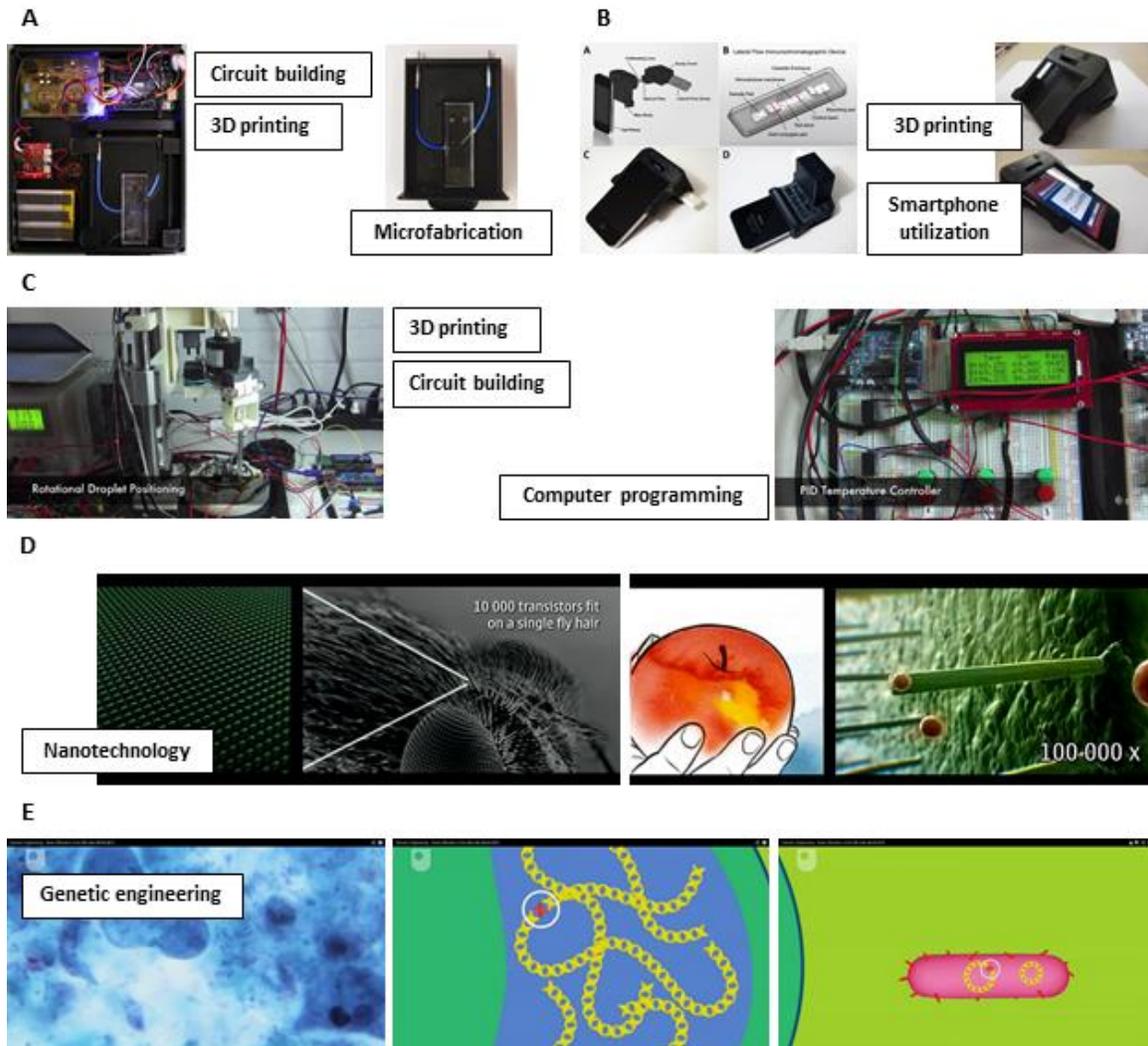


Figure 1. Seven techniques were chosen and introduced to students in terms of their use in biosensors.

The first project that was shown to the students was a handheld detection system that You et al. (2011a) have developed for the detection of *Escherichia coli* K12 and O157:H7 in near-real-time (<6 min). The actual device and a picture (Figure 1A) were both shown to explain the project and the techniques. This example introduced and explained circuit building (for signal processing), 3D printing (for fabricating the optical stages and the enclosure), and microfabrication techniques (for fabricating the microfluidic/lab-on-a-chip device) to the students in terms of their use in biosensors.

The second project that was shown to the students was a smartphone-based system that You et al. (2013) have developed for the detection of the thyroid stimulating hormone (TSH) from human blood serum. The actual device and a picture (Figure 1B) were both shown to explain the project and the techniques. Smartphone utilization (for optical detection and data processing) was introduced and 3D printing (for fabricating the reader attachment) was shown with this project in terms of their use in biosensors.

The third project shown was a droplet manipulation device that You et al. (2011b; 2012) have used for fast polymerase chain reaction (PCR) assay. A video was recorded during the operation of a prototype device, based on the published droplet manipulation method, and shown to the students (video snapshots in Figure 1C). Three techniques, computer programming (for programmed control of motors), circuit building (for signal processing and temperature control), and 3D printing (for fabricating the parts of a droplet manipulator device), were briefly explained while showing the one-minute video. After the video, computer programming was described in more detail and the other two techniques were recapitulated again in terms of their use in biosensors.

The last two techniques, nanotechnology and genetic engineering, were introduced to the students through two online videos. The first video showed the Morph cell phone concept device (video snapshots in Figure 1D), which was a collaborative project launched in 2008 between Nokia Research Center (Tampere, Finland) and Cambridge Nanoscience Centre (Cambridge, United Kingdom) (Nokia Research Center, 2012). The video demonstrated some capabilities of nanotechnology that might be enabled in future communication devices, including chemical sensing, which was related to biosensing. The second video was from the Open University (Milton Keynes, United Kingdom), entitled “Genetic Engineering” within the “Seven Wonders of the Microbe World” playlist (The Open University, 2011). The four-and-a-half-minute video (video snapshots in Figure 1E) showed the basics of how genetic engineering was used to produce insulin for diabetic patients and in producing biofuel for alternative energy. A video with a broader scope of genetic engineering was selected since most of the students did not have a previous knowledge of genetic engineering. After the video, potential applications of genetic engineering in biosensor research were explained to the students. This part of the lesson gave students a good, general idea of nanotechnologies and genetic engineering and how they can be applied in biosensor research.

3. Results and Discussion

3.1. Survey Data

The pre- and post- survey data were collected and are shown in Table 1. The students' gender information was also used to obtain more insight on the impact of gender that may have on students' perception.

		<u>Middle School</u>					
		pre			post		
		male (%) ^a	female (%) ^b	total	male (%) ^a	female (%) ^b	total
<i>Interest</i>							
applications	medical	3 (20)	7 (21.9)	10	4 (33.3)	9 (27.3)	13
	food safety	3 (20)	13 (40.6)	16	2 (16.7)	11 (33.3)	13
	biosecurity	6 (40)	4 (12.5)	10	4 (33.3)	5 (15.2)	9
	environmental	3 (20)	8 (25)	11	2 (16.7)	8 (24.2)	10
techniques	genetic engineering	3 (20)	3 (9.4)	6	2 (16.7)	1 (3.0)	3
	nanotechnology	2 (13.3)	3 (9.4)	5	1 (8.3)	9 (27.3)	10
	circuit building	1 (6.7)	5 (15.6)	6	1 (8.3)	7 (21.2)	8
	microfabrication	0 (0)	1 (3.1)	1	1 (8.3)	2 (6.1)	3
	3D printing	5 (33.3)	11 (34.4)	16	5 (41.7)	9 (27.3)	14
	smartphone utilization	3 (20)	8 (25)	11	2 (16.7)	5 (15.2)	7
	computer programming	1 (6.7)	1 (3.1)	2	0 (0)	0 (0)	0
	<i>Importance</i>						
applications	medical	4 (26.7)	10 (31.3)	14	3 (25)	11 (33.3)	14
	food safety	6 (40)	9 (28.1)	15	4 (33.3)	6 (18.2)	10
	biosecurity	1 (6.7)	1 (3.1)	2	2 (16.7)	11 (33.3)	13
	environmental	4 (26.7)	12 (37.5)	16	3 (25)	5 (15.2)	8
techniques	genetic engineering	4 (26.7)	10 (31.3)	14	1 (8.3)	7 (21.2)	8
	nanotechnology	6 (40)	6 (18.8)	12	0 (0)	8 (24.2)	8
	circuit building	3 (20)	7 (21.9)	10	7 (58.3)	8 (24.2)	15
	microfabrication	1 (6.7)	2 (6.3)	3	0 (0)	1 (3.0)	1
	3D printing	1 (6.7)	1 (3.1)	2	2 (16.7)	3 (9.1)	5
	smartphone utilization	0 (0)	4 (12.5)	4	2 (16.7)	2 (6.1)	4
	computer programming	0 (0)	2 (6.3)	2	0 (0)	4 (12.1)	4
	<i>Motivation</i>						
applications	medical	7 (46.7)	11 (34.4)	18	3 (25)	8 (24.2)	11
	food safety	2 (13.3)	6 (18.8)	8	2 (16.7)	8 (24.2)	10
	biosecurity	5 (33.3)	6 (18.8)	11	5 (41.7)	8 (24.2)	13
	environmental	1 (6.7)	9 (28.1)	10	2 (16.7)	9 (27.3)	11
techniques	genetic engineering	2 (13.3)	0 (0)	2	1 (8.3)	2 (6.1)	3
	nanotechnology	0 (0)	5 (15.6)	5	2 (16.7)	7 (21.2)	9
	circuit building	6 (40)	5 (15.6)	11	1 (8.3)	10 (30.3)	11
	microfabrication	1 (6.7)	4 (12.5)	5	0 (0)	1 (3.0)	1
	3D printing	2 (13.3)	11 (34.4)	13	2 (16.7)	10 (30.3)	12
	smartphone utilization	1 (6.7)	2 (6.3)	3	3 (25)	1 (3.0)	4
	computer programming	3 (20)	5 (15.6)	8	3 (25)	2 (6.1)	5
	<i>Total^c</i>	15	32	47	12	33	45

High School

<i>Interest</i>							
applications	medical	14 (25.5)	10 (38.5)	24	24 (40.7)	12 (52.2)	36
	food safety	3 (5.5)	8 (30.8)	11	9 (15.3)	6 (26.1)	15
	biosecurity	32 (58.2)	4 (15.4)	36	23 (39.0)	2 (8.7)	25
	environmental	6 (10.9)	4 (15.4)	10	3 (5.1)	3 (13.0)	6
techniques	genetic engineering	16 (29.1)	10 (38.5)	26	7 (11.9)	4 (17.4)	11
	nanotechnology	19 (34.5)	3 (11.5)	22	28 (47.5)	13 (56.5)	41
	circuit building	3 (5.5)	1 (3.8)	4	7 (11.9)	0 (0)	7
	microfabrication	1 (1.8)	0 (0)	1	2 (3.4)	1 (4.3)	3
	3D printing	5 (9.1)	7 (27.0)	12	10 (16.9)	2 (8.7)	12

	smartphone utilization	4 (7.3)	5 (19.2)	9	2 (3.4)	3 (13.0)	5
	computer programming	7 (12.7)	0 (0)	7	3 (5.1)	0 (0)	3
Importance							
applications	medical	32 (58.2)	15 (57.7)	47	27 (45.8)	9 (39.1)	36
	food safety	6 (10.9)	5 (19.2)	11	14 (23.7)	8 (34.8)	22
	biosecurity	12 (21.8)	3 (11.5)	15	15 (25.4)	6 (26.1)	21
	environmental	5 (9.1)	3 (11.5)	8	3 (5.1)	0 (0)	3
techniques	genetic engineering	22 (40)	10 (38.5)	32	28 (47.5)	9 (39.1)	37
	nanotechnology	7 (12.7)	6 (23.1)	13	11 (18.6)	4 (17.4)	15
	circuit building	7 (12.7)	4 (15.4)	11	8 (13.6)	4 (17.4)	12
	microfabrication	8 (14.5)	1 (3.8)	9	0 (0)	1 (4.3)	1
	3D printing	0 (0)	0 (0)	0	4 (6.8)	0 (0)	4
	smartphone utilization	1 (1.8)	1 (3.8)	2	2 (3.4)	3 (13.0)	5
	computer programming	10 (18.2)	4 (15.4)	14	6 (10.2)	2 (8.7)	8
Motivation							
applications	medical	14 (25.5)	12 (46.2)	26	18 (30.5)	13 (56.5)	31
	food safety	6 (10.9)	3 (11.5)	9	5 (8.5)	2 (8.7)	7
	biosecurity	29 (52.7)	4 (15.4)	33	31 (52.5)	2 (8.7)	33
	environmental	6 (10.9)	7 (26.9)	13	5 (8.5)	6 (26.1)	11
techniques	genetic engineering	12 (21.8)	9 (34.6)	21	8 (13.6)	4 (17.4)	12
	nanotechnology	15 (27.3)	3 (11.5)	18	29 (49.2)	10 (43.5)	39
	circuit building	2 (3.6)	2 (7.7)	4	3 (5.1)	0 (0)	3
	microfabrication	2 (3.6)	1 (3.8)	3	3 (5.1)	0 (0)	3
	3D printing	7 (12.7)	4 (15.4)	11	4 (6.8)	1 (4.3)	5
	smartphone utilization	7 (12.7)	5 (19.2)	12	7 (11.9)	7 (30.4)	14
	computer programming	10 (18.2)	2 (7.7)	12	5 (8.5)	1 (4.3)	6
	Total^c	55	26	81	59	23	82

^apercentage as of total male students completed the survey correctly at the school

^bpercentage as of total female students completed the survey correctly at the school

^ctotal count of the male, female, and total students completed the survey correctly at the school

Table 1. The pre- and post-survey data collected.

3.2. Applications of Biosensor

Figure 2 shows the results from the pre- and post-surveys at both middle school (Figure 2A) and high school (Figure 2B) levels among all four applications. Each graph represents one out of three factors (i.e. interest, importance, and motivation).

Figure 2A shows that among middle school students, before the lesson, there were peaks at food safety in interest and medical in motivation, and a very low number at biosecurity in importance. However, there were less obvious peaks and lows on any specific application after the lesson compared to before, which means students' attitudes towards these four applications became more evenly distributed in all factors. The standard deviation across applications before the lesson was 4.2 and decreased to 1.8 after the lesson (all three factors combined). On the contrary, the same even distribution is not seen with high school students, in neither pre- nor post-surveys. Standard deviation across applications before the lesson was 12.3 dropping only to 11.5 after the lesson, and both were far larger than the standard deviations from middle school students. Noticeable preference to medical and biosecurity applications can be found among the high school students.

This indicates that the middle school students' opinions and perceptions are easier to be altered or impressed upon, and the increased understanding about the applications affected their attitudes towards them. Before the lesson, some students might not be able to understand what each application means and were biased by their prerequisite knowledge. After the lesson, students' opinions have changed based on their newly acquired knowledge and they were relatively evenly distributed among all applications.

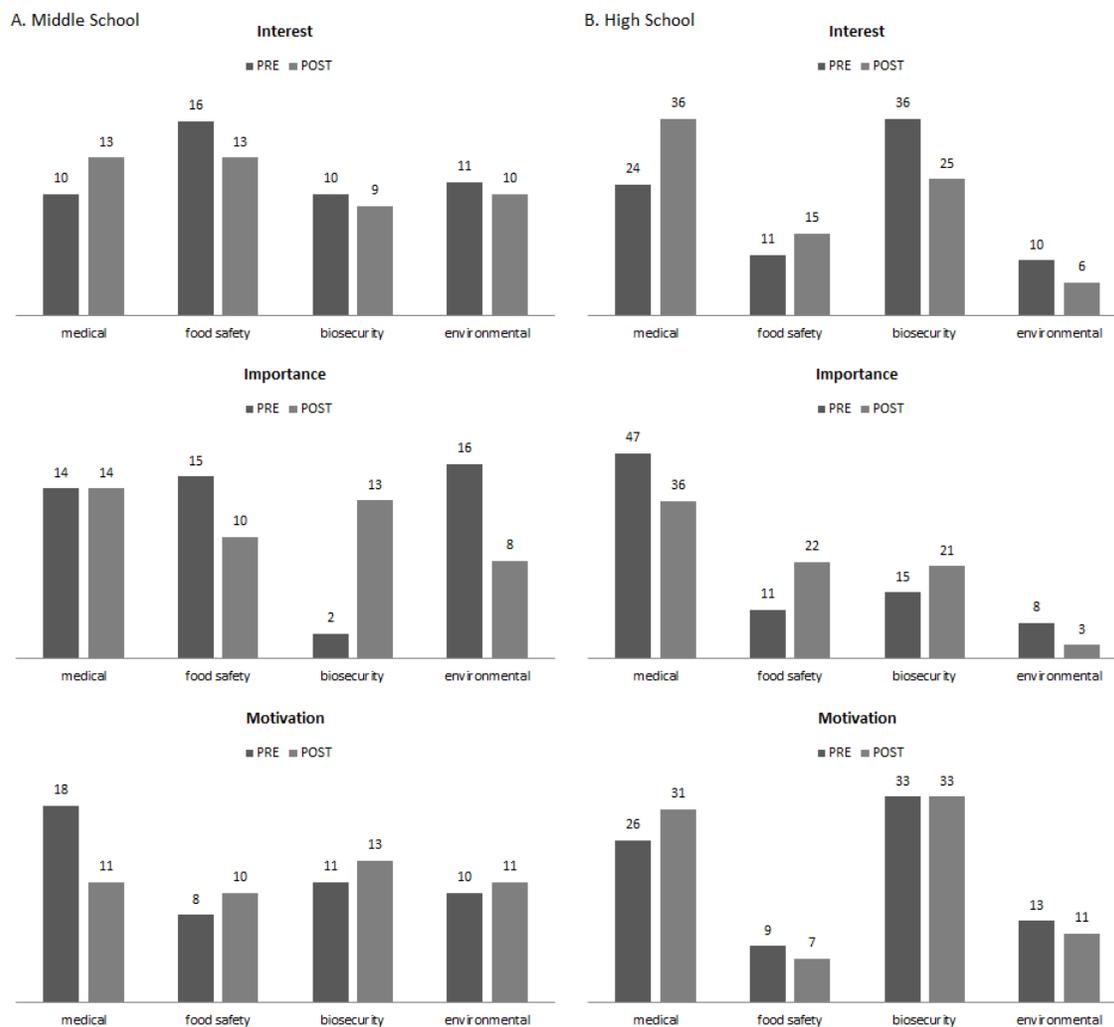


Figure 2. The numbers of middle school students (A) and high school students (B) at each application area of biosensor, before (PRE) and after (POST) the lesson, plotted separately by factors (Interest, Importance, and Motivation).

We infer that the younger students were more easily impressed and receive the lesson better on high-tech applications and world awareness with real-life examples, whereas the older students were more persistent on what they have learned and believed, and required more in-depth explanations or activities to be impressed or convinced. This supports that these topics are not too advanced and complicated for younger students to grasp, and worldwide issues can be introduced earlier during students' educational development.

One other thing to be noted here is that high school students clearly showed that they were more motivated to learn about the subjects that appeared to be more interesting or important to them despite the newly introduced concepts, whereas middle school students seemed not to have these connections between the factors. This also supports the fact that the younger students were more flexible on their opinions and leads to a wider perception and better awareness among the topics we discussed.

Due to the distinct nature of the student groups we have chosen, all restricted to Tucson, Arizona, United States, all discussion and conclusion we have made can be considered as conjectures. This issue will be further addressed in Conclusion section.

The data broken into males and females was also plotted (Figure 3). The normalized male-female ratios from the pre- and post-surveys were plotted against four applications of biosensor, for the middle school (Figure 3A) and the high school (Figure 3B).

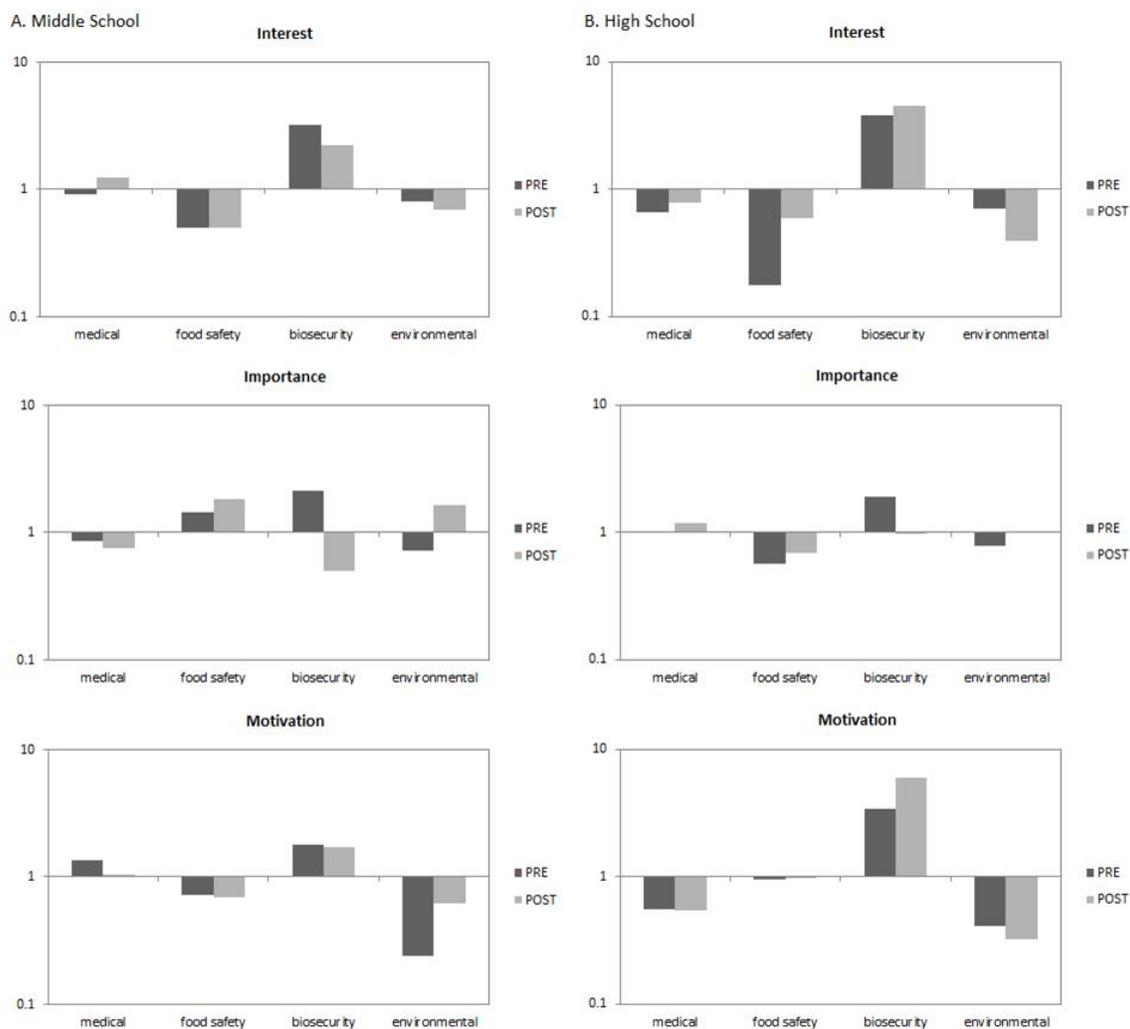


Figure 3. The normalized male-female ratios at each application area of biosensor, before (PRE) and after (POST) the lesson, for the middle school (A) and high school students (B), plotted separately by factors (Interest, Importance, and Motivation).

Generally before the lesson, middle school students showed more extreme gender difference. More male students were interested in biosecurity (male-female ratio of 3.2 in interest and 2.1 in importance) while more female students showed more motivation toward environmental applications (male-female ratio of 0.23 in motivation), before the lesson. These values became closer to one (towards. no gender difference) or went to the opposite direction (2.1 to 0.5 for biosecurity in importance) after the lesson.

Among the high school students, we see more extreme male-female ratios on more than one application under each factor, such as high male preference in biosecurity (3.8 in interest and 3.4 in motivation) or high female preference in food safety (0.17 in interest) and environmental (0.4 in motivation), all before the lesson. After the lesson, three out of these four extreme values actually moved even further away from one. In addition, one non-extreme value before the lesson, 0.71 for environmental in interest became extreme (0.39) after the lesson.

This graph further supports the inference that younger students in this study showed superior flexibility in the form of less male-female differences when they perceive a concept on high-tech applications and worldwide issues than the older students. High school students in this case had a stronger mindset on their pre-existing knowledge and preferences, in addition to more obvious gender differences.

3.3. Techniques for Biosensor Development

Figure 4 shows the results from the pre- and post-surveys at the middle school (Figure 4A) and the high school (Figure 4B) among all seven techniques that can be used in developing and manufacturing biosensors. The responses for techniques are a lot more clustered than the ones for applications both before and after the lesson. The change towards even distribution after the lesson that we observed from the application results did appear again but not as obvious among the middle school students (standard deviation before the lesson was 4.5 and was 4.2 after the lesson) across all three factors and did the opposite among the high school students (standard deviation before the lesson was 8.3 and increased to 11.8 after the lesson).

Among the middle school students' pre-lesson responses, there were a couple of items that gained more than a quarter of votes (>25%): 3D printing for the interest factor; genetic engineering and nanotechnology for the importance factor; 3D printing for the motivation factor. In other words, before the lesson, middle school students were interested and motivated mostly by 3D printing, although they considered genetic engineering and nanotechnology to be more important (perhaps the most abstract topics for middle school students to grasp). After the lesson, distribution became more even but not by much: the standard deviations over the seven techniques before and after the lesson were 4.8 vs. 4.4 for interest factor and 4.7 vs. 4.2 for importance factor. For the motivation factor, the distribution remained relatively the same: 3.8 before the lesson vs. 3.9 after the lesson.

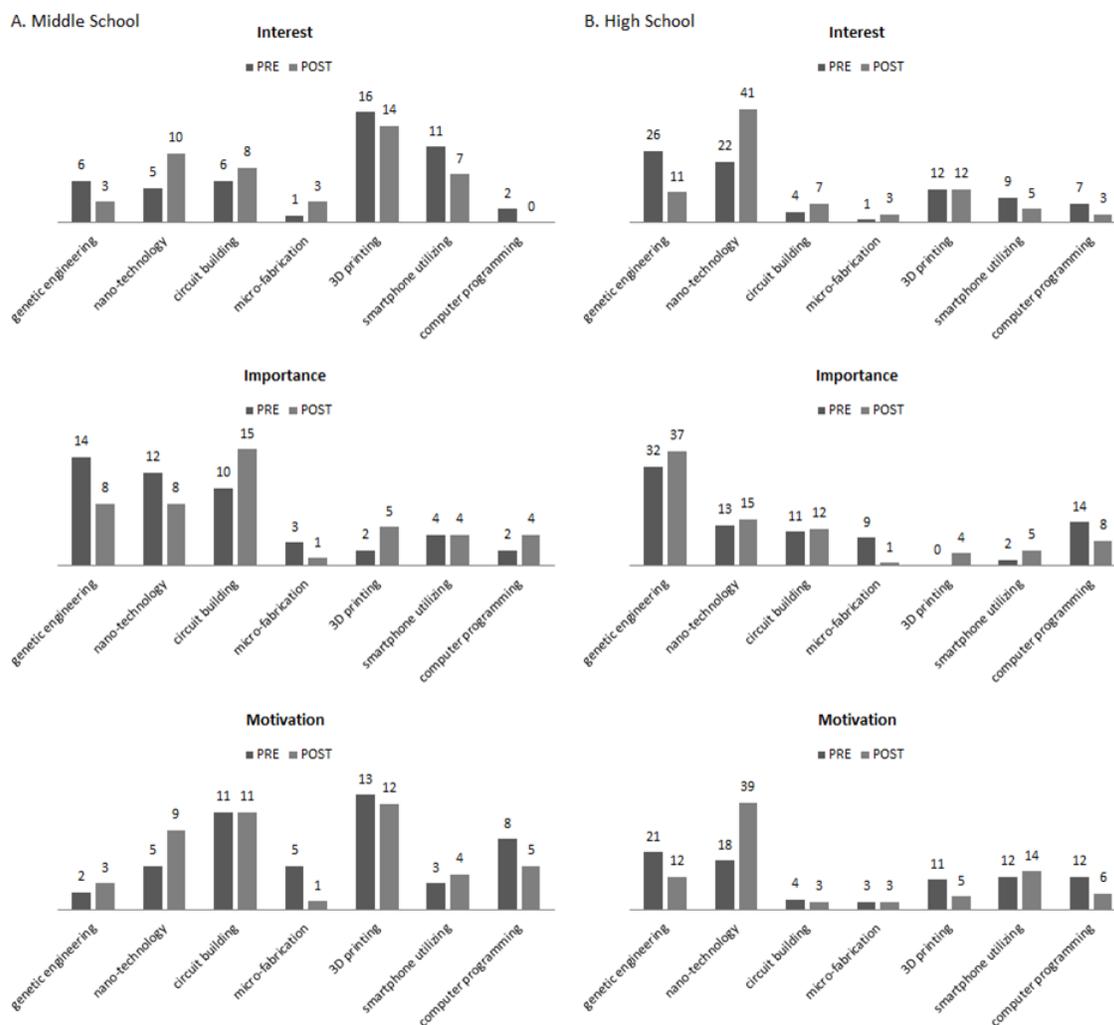


Figure 4. The numbers of middle school students (A) and high school students (B) at each technique of biosensor development, before (PRE) and after (POST) the lesson, plotted separately by factors (Interest, Importance, and Motivation).

For the high school students, we discovered that they had really strong preferences for some certain techniques. Before the lesson, genetic engineering and nanotechnology received the majority of votes for all three factors. These preferences could have been biased by their prerequisite knowledge; the fact that a lot of the students already had some experience with circuit building, programming, smartphone use, and 3D printing is likely to have made these techniques less novel to the students. Genetic engineering and nanotechnology, however, were something they had heard about but never gotten a chance to experience in person, and this led to a higher curiosity towards these two techniques. Microfabrication was a technique they had never experienced either, but they also had likely never heard about it before either making this technique entirely foreign and uninteresting to the students.

After the lesson, the distribution among techniques became far more extreme and clustered: the standard deviations over the seven techniques before and after the lesson were 8.6 vs. 12.4 for interest factor, 9.7 vs. 11.2 for importance factor, and 6.1 vs. 11.8 for motivation factor. The preferences remain similar (genetic engineering and nanotechnology for both interest and motivation factor, and genetic engineering for importance factor). Nanotechnology, in particular, gained a lot more votes on interest and motivation factors than the other techniques.

The data on seven techniques were again broken into males and females (Figure 5). Among the middle school students, the standard deviations of the male-female ratios over seven techniques before and after the lesson were 0.6 vs. 1.6 for interest, 0.7 vs. 1.0 for importance, and 3.2 vs. 2.7 for motivation. Altogether, these results indicate that middle school students had larger gender differences over seven techniques and the lesson did help reduce those gender differences.

After the lesson, we had a small interest increase in genetic engineering for males and a small interest increase in nanotechnology for females. In the importance factor, we saw significant shifts to nanotechnology and computer programming for females. In the motivation factor, a significant shift to circuit building for females and shifts to smartphone utilization and computer programming for males.

Among the high school students, the standard deviations of the male-female ratios over seven techniques before and after the lesson were 3.2 vs. 3.2 for interest, 1.1 vs. 3.2 for importance, and 0.8 vs. 0.5 for motivation. These results indicate the high school students had a larger gender difference in the interest factor than other factors and the lesson did not help reduce the gender differences over all three factors, especially not for perceived importance which became even more polarized.

After the lesson, we had an interest shift to circuit building from nanotechnology and computer programming for males as well as an importance shift to 3D printing from microfabrication for males. We also saw an interest and an importance shift to smartphone utilization for females. There was no significant shift for the motivation factor.

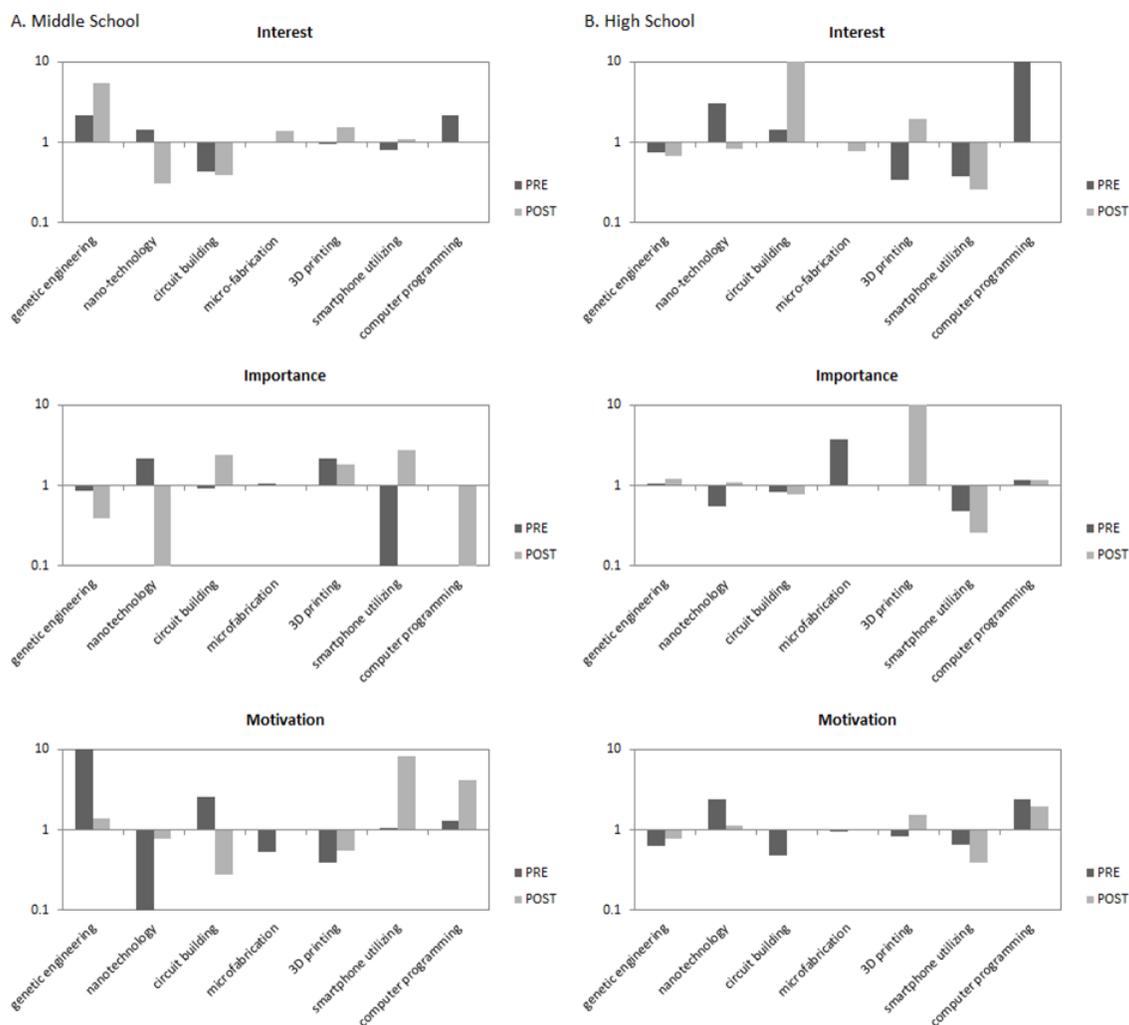


Figure 5. The normalized male-female ratios at each technique of biosensor development, before (PRE) and after (POST) the lesson, for the middle school (A) and high school students (B), plotted separately by factors (Interest, Importance, and Motivation).

In general, the lesson helped reduce the gender differences on the motivation factor for both middle school and high school students, but not for the other factors. Compared to the results from biosensor applications, the students' attitudes towards techniques were a lot less flexible with both middle school and high school students, and especially unbendable among older students. This suggests that this part of the lesson is more suitable to be used as an indicator or a guide in order to profile students' preferences and learning interests and motivations, instead of focusing on having students equally motivated. Again, due to the nature of the student population, further confirmation on this conclusion may become necessary. Based on the post-survey results, we have identified some spikes for males and females; these spikes can also be a basic lesson plan developing guideline if the classroom has a polarized gender population.

4. Conclusion

A biosensor lesson was proven to be effective in affecting students' interest and motivation as well as raise their awareness of current science, engineering, and worldwide issues, for the given student population in Tucson, Arizona, United States. The lesson had a greater impact on younger students and was able to make a difference in the way students' perceived different areas of study and important issues. The standard deviations across biosensor applications showed a significant decrease after the lesson.

For the techniques used in designing and manufacturing biosensors, students seemed to be less flexible. Older students were especially stubborn with regards to their pre-established interests. According to the post-survey results, middle school students are

mostly motivated to learn about some hands-on techniques (i.e. circuit building, 3D printing) as well as cutting-edge technology (i.e. nanotechnology) and less motivated by software and programming related processes (i.e. smartphone utilization and computer programming). On the contrary, high school students were extremely attracted to technologies which, to them, appeared more advanced (i.e. genetic engineering and nanotechnology). This study also showed that there are obvious differences in preferences between genders. The findings here suggest that a biosensors lesson can be a good instrument to assess students' preferences and interests among different types of current technologies, and this information can further be used to identify students' learning interests and motivation (e.g. more hands-on or higher-tech) for different types of student population.

In the general public's perception, high-tech subjects and cutting-edge topics may not be suitable for younger students. Due to their complexity and the level of logical thinking that is required, most students would not have any contact with these subjects until later years in college or during graduate study. Research has been done to investigate how engineers conduct their research [17], but there is no report on how younger students perceive these types of information. This study suggests that younger students perceived the information well, and through the topics that were introduced, students were able to construct an idea of how current research and technology are extremely inter-/multi-disciplinary and how they affect our daily lives.

This lesson shows the potential benefit of using biosensor research in a secondary education classroom (especially with younger students) and the possibility of inspiring the students into science and engineering fields of study or career. The topics we

introduced in this lesson are entirely new to most of the students in the classroom, and none of the students had prior knowledge about biosensors before the lesson.

We acknowledge that some of the participated students and classrooms had distinct demographic composition as well as that the students' academic performance was different from class to class. All students were from the local school district, therefore we cannot extend and conclude the results can be applied to all secondary education students. However, the results still effectively indicated that a biosensor lesson was a simple, straight-forward and less-frustrating way to introduce current research concepts and issues to the secondary education students, since biosensors lessons are, compared to other biotechnology lessons from the literatures, more compact and less intense on background knowledge.

A biosensor lesson can be easily expanded since the applications and techniques that are involved in biosensor research are not limited to the four applications and seven techniques we chose to include in this study. By introducing more or different applications and/or techniques, this lesson can be made suitable for different classes as well (e.g. chemistry, physics, or biology classrooms). Changing the depth of the information on each application and technique can easily scale up or down the lesson plan. Depending on students' prerequisite knowledge, the lesson can be done with either more elementary or more advanced levels of knowledge. These characteristics (i.e. easily expanded and scaled) make the lesson plan very adaptable and versatile.

A lot of us suffered from not knowing what we could achieve with the knowledge we obtained from our school curriculum, especially during middle school years when students' learning starts converting from pedagogical to andragogical learning where the

students move away from dependency to being ready to apply knowledge to solve problems and seeking for reasons of learning (Knowles, 1980). Learning without real-world applications and appealing reasons often leads to difficulties in learning voluntarily and willingly. This study hopes to encourage educators to expose students to current technologies and worldwide issues by using a simple cutting-edge lesson and further make connections between these topics and the curriculums in order to inspire and motivate students in learning science and engineering.

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