

Title: The development of a novel immunosensor to detect *Salmonella* - NPB #02-027

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Abstract: *Salmonella* contamination in foods results in not only foodborne outbreaks, but also a large economical burden for the industry due to product recalls. As a result, a fluorescence resonance energy transfer (FRET)-based method was developed to detect *Salmonella*. Sensors utilizing FRET switch their fluorescence wavelength between donor and acceptor fluorophores as the distance between the two fluorophores change. This change in distance is a result of the conformational change in the 3D structure of the antibody as it binds to the target antigen. *Salmonella* antibodies were labeled with FRET donor fluorophores (Alexa Fluor 546) while protein G or A was labeled with the acceptor fluorophores (Alexa Fluor 594). The labeled antibody-protein G or A complex was formed via incubation of the labeled antibody with protein G/A which specifically attaches to the Fc fragment of antibodies. The labeled antibody-protein G or A complex was tested in solution and specific and non-specific antigens were exposed to the in solution complex. Changes in fluorescence were monitored by a spectrofluorometer. For "in-solution" tests, the optimal acceptor/donor fluorophore (A/D) ratio was 1.0 for *Salmonella*, and *Salmonella* antigen detection limits were 2.0 µg/ml. Immobilization studies were also performed where the protein/antibody complex was immobilized to an optical fiber and interface with a benchtop fluorometer. The results showed the limit of detection was 10³ cells/ml of *Salmonella* Typhimurium at a packing density of 0.033 mg/ml.

Introduction: *Salmonella* spp. are among the most common causes of food poisoning. Every year in the US, approximately 80,000 cases of salmonellosis are reported and 580 persons die. The actual number of infections may be twenty times greater since many milder cases are not reported. It is estimated that 95% of the cases are from foodborne transmission with pork being the major vehicle of foodborne salmonellosis. A recent study of pork in U.S. retail stores found 9.6% of samples were contaminated by *Salmonella*. Estimates of total annual costs including lost productivity and medical cost range from \$0.5 billion to \$2.3 billion. *Salmonella* Typhimurium is the serotype that is capable of causing disease in humans, pigs, and a number of other host species. These bacteria are fairly widespread, occurring in humans, animals, soil, and water and has been found on a variety of pork products, including ground pork.

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Although the Hazard Analysis Critical Control Point (HACCP) system is a popular approach to preventing foodborne disease, rapid and accurate methods for detecting pathogens in food processing facilities are needed. Powerful techniques such as polymerase chain reaction (PCR) are cumbersome and time-consuming. Although enzyme-linked immunosorbent assay (ELISAs) have been developed to detect pathogens in food products, most require time-consuming preenrichment steps.

Immunosensors that detect the binding between antibodies and antigens have been developed for pathogens but have resulted in long reaction times and/or false positives.

While great strides have been made in biosensor technology, many technological problems remain that hinder their widespread use for monitoring food safety. In this study, an optical fluorescence technique is utilized to monitor the conformation changes that occur when antibodies and antigens combine. The novel sensors will detect only viable analytes, thus drastically reducing false positives.

Objectives: The goal of this research project was to demonstrate the feasibility of a *Salmonella* immunosensor that can be implemented into an on-line instrumental pathogen detection system. This on-line instrumental detection system would be able to provide pork processors and producers the ability to detect food safety problems, thus reducing the incidents of food pathogen outbreaks and recapturing lost dollars due to inadequate meat safety. The project proposed to accomplish three objectives:

- Develop protocols to conjugate fluorescent dyes to *Salmonella* antibodies and Protein A or G. This was a critical step since highly sensitive biosensors are dependent on conjugation procedures and F/P ratios. F/P ratios are the ratio of dye molecules to protein molecules.
- Immobilize fluorescently labeled antibody-Protein G complex to optical waveguides. Optimizing and characterizing the protein immobilization procedures are necessary in order to optimize packing density and functionality. The question to be answered was what is the optimal packing density that will provide the highest energy transfer and greatest sensitivity.
- Develop a benchtop system to detect *Salmonella*. It is necessary to design a portable fluorometer that can detect 1×10^3 to 1×10^8 colony forming unit (CFU)/ml of *Salmonella*.

The development of the *Salmonella* biosensor was conducted in three phases. The first phase developed conjugation protocols and then tested in solution. The second phase immobilized the *Salmonella* antibodies-Protein A/G complex to the surface of a waveguide. The third phase of the project involved development of an instrumental unit that will be able to analyze *Salmonella* in a bench top setting.

Materials & Methods: Monoclonal antibody labeling kits for Alexa Fluor 546 (FRET donor) and Alexa Fluor 594 (FRET acceptor) were purchased from Molecular Probes (Eugene, OR). Affinity purified antibodies (goat immunoglobulin (IgG)) against *Salmonella* common structure antigens-1, recognizing all *Salmonella* serotypes available from ATCC tested in the laboratory were obtained from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). *Salmonella* antigens were purchased from Voigt Global Distribution LLC (Kansas City, MO). Protein G, protein A and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Alexa Fluor 546 was

conjugated to anti-*Salmonella* antibodies while Alexa Fluor 594 was conjugated to Protein A and G using a procedure from the Monoclonal antibody labeling kits.

The optimal acceptor to donor fluorophore (A/D) ratio was derived from the number of labeled antibody molecules incubated with the number of labeled Protein A or Protein G molecules and the F/P ratio obtained. The F/P ratio is a measure of the degree of labeling after conjugating the fluorophores to the protein of choice and is defined as the number of fluorophore molecules per molecule of protein. The A/D ratio can be defined as the number of acceptor fluorophores to donor fluorophores in an antibody-Protein complex. To determine the optimal A/D ratio, *Sal*/Ab-AF546/PG-AF594 was prepared by co-incubation at various concentration ratios of labeled antibodies and labeled Protein A or Protein G overnight at 4 °C and then scanned using the spectrofluorometer, Jasco FP-750. The optimal ratios were determined by examining the donor and acceptor emission peaks. The A/D ratio determination for the *Sal*/Ab-AF546/PG-594 complex: *Sal*/Ab-AF546 (0.045 µg/µl) was varied from 0.2 µg to 0.25 µg while PG-AF594 (0.053 µg/µl) remained at 0.8 µg. Each aliquot, examining a different A/D ratio, was incubated overnight at 4 °C in the dark and then diluted with 3 ml of PBS and scanned with the spectrofluorometer set at the excitation wavelength of the donor (546 nm for AF 546). The A/D ratio varied from 1 to 8.

For the in solution measurements, *Sal*Ab-AF546 and PG-AF594 was incubated overnight at 4 °C in the dark. A dose of 50 µl was carefully distributed into seven semi-microcuvettes. One cuvette was used for control (no antigen) and the other six cuvettes were divided into two groups of three; one group was utilized for the specific antigen (*Salmonella* Antigen (*Sal*Ag)) and the other group was utilized for the non-specific antigen (BSA). A 50 µl dose of *Sal*Ag was added into the three cuvettes using various concentrations of 0.04 µg/µl, 0.02 µg/µl, and 0.002 µg/µl, respectively. For the nonspecific, BSA, 50 µl of BSA was added at various concentrations of 0.04 µg/µl, 0.02 µg/µl, and 0.002 µg/µl, into each of three cuvettes. The final volume in each semi-microcuvettes was 100 µl. The solutions were gently shaken to ensure that the solutions were well mixed and incubated for one hour at room temperature. After incubation, each sample was diluted with PBS to 1 ml to fill a semi-microcuvette prior to scanning with the spectrofluorimeter. The excitation wavelength was set according to the donor fluorophore present in the experiment (546 nm for AF 546/AF 594). The change in fluorescence of the donor and acceptor fluorophores was recorded via spectrofluorometry.

Amine reactive chemistry or silanization was utilized to couple the labeled antibody-Protein G complex to the optical fibers. Briefly, the procedure involves preparing/washing the fiber tip via acid washes, attaching a thiol-terminal silane film via the hydroxyl groups and then attaching a bifunctional crosslinker (GMBS, a heterobifunctional crosslinker) which allowed amide binding to the terminal amine group on Protein G. After treatment with GMBS, the fibers were immediately incubated for 1 hour in the labeled antibody-Protein G solution. The fiber tips were rinsed in PBS, dipped in BSA to prevent nonspecific adsorption to the fiber. The fiber was then coupled to a benchtop fluorometer that was built and assembly to satisfy the third objectives. *Salmonella* Typhimurium was used as a test bacterium, and serially diluted in the range of 10 to 10⁷ cells/ml. A tapered optical fiber tip with the immobilized antibody-Protein G complex was placed in the *Salmonella* samples for 10 min in a dark room. Then the sensor response was recorded.

Results:

The first objective was met. Protocols to conjugate fluorescent dyes to *Salmonella* antibodies and Protein G were developed. The F/P and A/D ratios have been determined as shown in Table 1 and Figure 1 respectively. The F/P ratio was determined utilizing known extinction coefficients for the fluorophores and equation (1):

$$F/P = \frac{A_{\max} \times \text{dilution factor}}{\epsilon_{\text{dye}} \times [M]} \quad (1)$$

where A_{\max} is the absorbance at the maximum excitation wavelength of the fluorophore used in conjugation, and ϵ_{dye} is the molar extinction coefficient at λ_{\max} in $\text{cm}^{-1}\text{M}^{-1}$ ($\epsilon_{\text{AF546}} = 104,000 \text{ cm}^{-1}\text{M}^{-1}$ and $\epsilon_{\text{AF594}} = 73,000 \text{ cm}^{-1}\text{M}^{-1}$, according to the manufacturer's procedure sheet) and $[M]$ is the molarity of the protein. The F/P ratio obtained was used to determine an optimal acceptor to donor dye ratio. Determining the optimal A/D ratio is critical for successful energy transfer. Too little or too much of either fluorophore can produce nondetectable signals due to self-quenching and/or insufficient energy transfer.

The A/D ratios were then derived from the F/P ratios and the amount of proteins present in solution. Optimal A/D ratio of 1.0 was determined in this study by the judicious choice of an A/D ratio displaying a donor peak that did not overwhelm the acceptor peak and had a higher intensity compared to the acceptor peak.

In-solution tests were performed by adding a series of specific and non-specific antigens to microcurvettes containing the complex of *Sa/Ab-AF546/PG-AF594* at the optimal A/D ratio. Results from the tests are presented in Figure 2. Values displayed are the ratios to ratios, which were calculated as follows and monitored with a spectrofluorimeter.

$$R1 = \frac{I(\lambda = 570 \text{ to } 575 \text{ nm})}{I(\lambda = 608 \text{ to } 613 \text{ nm})}, \quad \text{with no antigen present (baseline)}$$

$$R2 = \frac{I(\lambda = 570 \text{ to } 575 \text{ nm})}{I(\lambda = 608 \text{ to } 613 \text{ nm})}, \quad \text{with specific or nonspecific antigen present}$$

$$R3 = \frac{R2}{R1}, \quad \text{the ratio of ratio used to determine change in FRET activity}$$

where $I(\lambda = 570 \text{ nm to } 575 \text{ nm})$ was the average fluorescence intensity of the donor fluorophore and $I(\lambda = 608 \text{ nm to } 613 \text{ nm})$ was the average fluorescence intensity of the acceptor.

FRET activity was presented by measuring the change of the emission intensity of the donor and acceptor fluorophores in the form of a ratio, R3. As the specific *Salmonella* antigens were introduced, the labeled antibody/PG complex reduced the distance between the dye pairs due to the conformational change of antibody via binding of antigen. The resulting structure enhanced the FRET activity, increasing the emission intensity at $I(\lambda = 608\text{-}613 \text{ nm})$ (the acceptor) and reducing the emission intensity at $I(\lambda = 570\text{-}575 \text{ nm})$ (the donor), which resulted in decrease of a R2 value. As a result, the value of R3 decreased, indicating specific binding of the antigens with the antibodies when the specific antigen was introduced into the solution. Conversely, when the non-specific antigen (BSA) was added into solution, there were minimum changes in fluorescence at 570 nm and at 603 nm causing little change in R3, as

expected. The detection limit of *Salmonella* antigen is displayed in Figure 2. The specific *Salmonella* antigen showed significantly larger reductions in R3 values than that of non-specific antigens (BSA) at the specific antigen concentrations of 2.0 and 4.0 µg/ml ($p < 0.05$), whereas there was no significant reduction of R3 values below *Salmonella* concentrations of 2.0 µg/ml ($p < 0.05$), indicating the detection limit of *Salmonella* antigen was 2.0 µg/ml.

The second objective was met but warrants further investigation. We have performed studies to immobilize the fluorescently labeled antibody-Protein G complex to optical fibers via silanization. Figure 3 and 4 displays some of the results as the immobilized antibody/PG are exposed to the antigens. In Fig. 3, the R2 response when exposed to various concentrations of *Salmonella* Typhimurium is shown using a 600 micron step index fiber. Figure 4 shows the R2 response using a 400 micron fiber.

The third objective was met. The benchtop fluorometer system has been assembled and utilized to accomplish the second objective. The use of two photomultiplier tubes gave the high sensitivity needed to detect small changes in fluorescence when the antibody and antigen combined.

Discussion: FRET activity is extremely sensitive to distance changes between donor and acceptor fluorophores. Energy transfer dramatically increases when the separation distance between fluorophores is close to the Försters distance (between 10 to 100 angstroms for most FRET pairs) which is defined as the distance where the transfer of energy from donor to acceptor fluorophores is 50% efficient. Utilizing this principle, we have developed a technique to detect the presence of *Salmonella* antigens. Thus the feasibility of the FRET immunosensor technique to detect *Salmonella* antigens has been demonstrated in solution measurements. However, it is necessary to increase the sensitivity of this technique because the amount of foodborne pathogens on the food products are expected to nil. For simplicity, the initial tests performed were conducted in solution which inherently causes a reduced sensitivity since random chance interaction between free-floating proteins and antibodies might occurred by Brownian motion, micro thermal currents in the cuvette, and/or subtle difference in the path length of each cuvette. To improve sensitivity, we immobilized the complex onto optical waveguides so that random interactions would be reduced and sensitivity enhanced. The immobilized sensor could respond to 10^3 cells/ml and greater. Therefore the maximum sensitivity was 10^3 cells/ml. Larger decrease in signal was expected as the concentrations of *Salmonella* increase over 10^3 cells/ml, but it did not occurred. The packing density (i.e., the amount of antibody/PG immobilized) needs to be optimized in order to optimize sensitivity. The 400 um fiber sensor did not response to the changes in *Salmonella* concentration. Again, the packing density needs to be optimized.

In summary, the benchtop sensor responded to analytes and demonstrated a sensitivity of 10^3 cells/ml. The packing density was 0.033 mg/ml, but needs to fully explored and optimized. Our results demonstrated the potential of using a fiber-optic FRET biosensor for detection of *Salmonella* Typhymurium. This biosensor is portable and permits on-site analysis of samples. From the results, it is apparent that immobilization is not a simplistic task. Factors such as packing density and steric hindrance play an important role in determining the success of energy transfer and warrants further investigation.

Lay Interpretation: A novel biosensor to detect *Salmonella* is being developed. The optical sensor is based on fluorescence resonance energy transfer (FRET). Antibodies

are labeled with fluorescent dyes that will change their fluorescence based upon binding of *Salmonella* antigens to the *Salmonella* antibodies. A detection limit of 2.0 µg/ml was achieved for in-solution measurements. The labeled antibodies were also immobilized onto optical fibers and then exposed to specific and nonspecific antigen. The benchtop sensor displayed the limit of detection of 10³ cells/ml.

The new immunosensor developed by this research will be utilized as an efficient and accurate alternative to monitor bacterial contamination in pork. Such a system will be able to reduce the large economical burden caused by product recalls and medical treatments. This biosensor is portable and will be easy to use, especially for pork producers who do not have knowledge of food microbiology. It will permit a rapid on-site monitoring (during slaughter) system for pork product safety. In addition to the benefits that could be realized by the pork industry, a broader impact is the development of new diagnostic sensor arrays that could improve in the quality of food monitoring systems.

Papers/Conferences/Posters, etc produced from the Pork Grant

- A USDA/NRI proposal was submitted based on data produced from the Pork grant;
- A *Salmonella* biosensor paper has been submitted to Sensors & Actuators B;
- A presentation will be given in Oct. at the IEEE Sensors conference;
- A presentation will be given in July at the ASAE conference;
- A poster presentation was given at the Korean Student Conference (outstanding poster award);
- A poster presentation was given at the ASAE conference 2002.

Table One. F/P ratios

Molecule/Dye	F/Ratio
Sal ab/AF546	6.8
Protein G/AF594	5.8

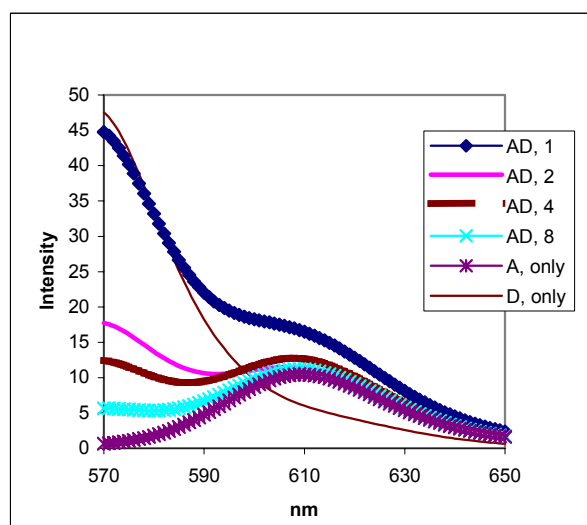


Fig. 1. Determination of an optimal ratio of acceptor to donor fluorophore in *Sal/Ab*-AF546/PG-AF594 complex.

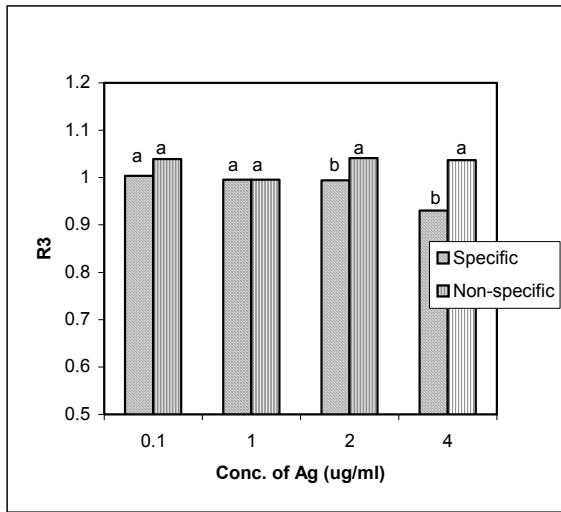


Figure 2. Figure 3. Detection of *Salmonella* antigen in solutions containing Sa/Ab-AF546/PG-AF594 complex in the form of R3.

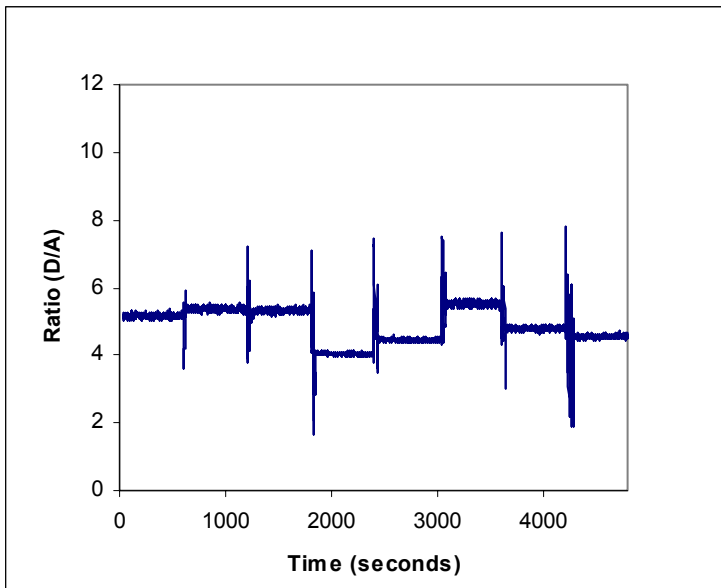


Fig. 3. Response of the sensor when exposed to various concentrations of *Salmonella* Typhimurium (10 to 10^7 cells/ml) using a fiber with a diameter of 600 micron.

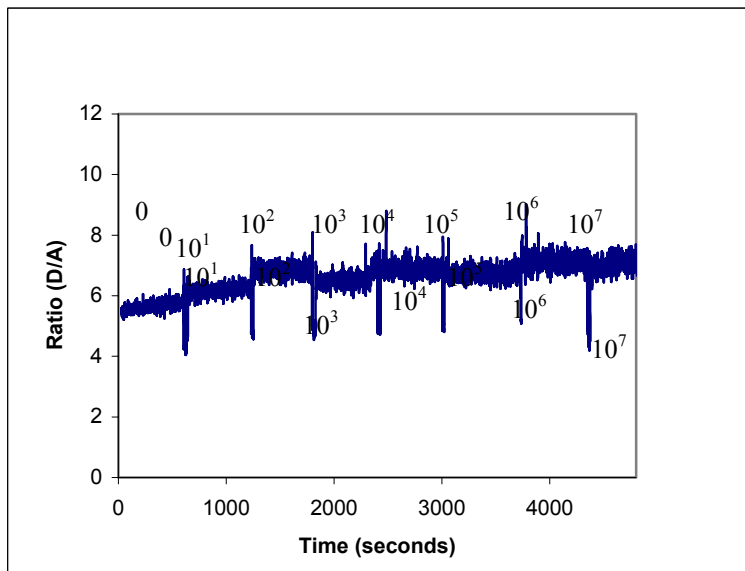


Figure 4. Response of the sensor when exposed to various concentrations of *Salmonella* Typhimurium (10 to 10^7 cells/ml) using a fiber with diameter of 400 micron.