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Novel Approach of a Phage-Based Magnetoelastic Biosensor for the Detection of *Salmonella enterica* serovar Typhimurium in Soil

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To date, there has been no employment of a magnetoelastic (ME) biosensor method to detect *Salmonella enterica* serovar Typhimurium in soil. The ME biosensor method needs to be investigated and modified for its successful performance. The filtration method, cation-exchange resin method, and combinations of both methods were employed for the extraction of *S*. Typhimurium from soil. The number of *S*. Typhimurium and the resonant frequency shift of the ME sensor were then compared using a brilliant green sulfa agar plate and an HP 8751A network analyzer. A blocking study was performed using bovine serum albumin (BSA), polyethylene glycol (PEG), and casein powder suspension. Finally, the modified ME biosensor method was performed to detect *S*. Typhimurium in soil. The number of *S*. Typhimurium was significantly decreased from 7.10 log CFU/soil to 4.45–4.72 log CFU/soil after introduction of the cation-exchange resin method. The greatest resonant frequency shift of the measurement sensor was found when employing centrifugation and filtration procedures. The resonant frequency shift of the PEG-blocked measurement sensor was 3,219 ± 755 Hz, which was significantly greater than those of the BSA- and casein-blocked ME sensor. The optimum concentration of PEG was determined to be 1.0 mg/ml after considering the resonant shift and economic issue. Finally, the modified ME biosensor method was able to detect *S*. Typhimurium in soil in a dose-response manner. Although these modifications of the ME biosensor method sacrificed some advantages, such as cost, time effectiveness, and operator friendliness, this study demonstrated a novel approach of the ME biosensor method to detect *S*. Typhimurium in soil.

Keywords: Soil, phage-based magnetoelastic biosensor, *Salmonella* Typhimurium, extraction, blocking reagent

Introduction

Foodborne illnesses have been increasingly associated with fresh produce during the past decade [3, 9, 19]. The Centers for Disease Control and Prevention has recently reported that fresh produce were either the first or second leading vehicle for outbreaks of foodborne illness in the USA between the years 2006 and 2008 [6, 7]. Leafy greens were the most common source of foodborne illness, followed by tomatoes and cantaloupes, which accounted for 56%, 36%, and 17%, respectively [1]. The identified major pathogenic microorganisms were *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes* [1, 9, 27]. These foodborne pathogens could be contaminated from a variety of pre-harvest environments (irrigation, runoff water, soil, manure, and fecal deposition from wild animals) to consumption [2, 4, 9, 10, 27]. A recent study [13] found that *Salmonella* could survive for long periods of time (up to 230 days) after contamination by poultry or cow manure. Therefore, *Salmonella* needs to be identified and detected from field environment using an on-site and practical rapid detection method for preventing its outbreak.

Considerable effort has been directed towards the development of rapid and on-site detection methods [28,

30]. A free-standing, phage-based magnetoelastic (ME) biosensor has been developed as a novel wireless method for rapid and on-site detection of pathogens such as *Salmonella*, *Bacillus anthracis* spores, and *Staphylococcus aureus* [5, 12, 14, 15]. The phage-based ME biosensor is composed of an ME sensor with immobilized genetically engineered filamentous E2 phages (indicated as E2 phages) for the biorecognition of *Salmonella enterica* serovar Typhimurium on the sensor surface. The E2 phages were selected from a landscape f8/8 phage library as a biorecognition element for *S.* Typhimurium [22, 26]. Confirmation of the specificity and sensitivity of E2 phage against *S.* Typhimurium has been reported in previous studies [21, 26]. The ME sensor undergoes its vibration under an applied alternating magnetic field, and its own unique resonant frequency can be measured by a wireless pickup coil system. Once the measured E2 phage-immobilized ME sensor is placed on the surface of the target or in suspension, the E2 phage immobilized on the ME sensor binds specifically with *S.* Typhimurium on/in sample. The binding of E2 phage and *S.* Typhimurium increases the total mass on the ME sensor, so the vibration of the ME sensor is decreased, resulting in a resonant frequency shift. The resonant frequency shift is directly proportional to the number of *S.* Typhimurium bound to the ME sensor; therefore, the target bacteria can be identified and quantified from the resonant frequency shifts of the ME sensor [17, 18].

In the last decade, there has been more than several hundred publications describing on-site and rapid performance of the ME biosensor. Previous studies [12, 14, 15, 17, 18] showed the possibility and practicability of the ME biosensor as an on-site and rapid detection method, using cantaloupes, spinach, tomatoes, and milk. Furthermore, the ME biosensor method has been evaluated and validated objectively by comparison with a TaqMan-based quantitative PCR in the aspects of excellent selectivity, repeatability, simple assay protocol, minimal skilled operator, fast assay time, portability, and cost-effectiveness [20, 21]. Although soil has been reported as one of the major vehicles of *Salmonella* contamination on fresh produce, to date, no biosensor method (including the ME biosensor method) has been employed to detect foodborne pathogens in soil. Thus, the ME biosensor method was first applied to detect *Salmonella* in soil in this study. Since soil consists of numerous coexisting compounds such as humus, minerals, and organic matters [25], the successful extraction of *S.* Typhimurium from soil samples needs to be investigated for the maximum recovery of target pathogen prior to the performance of the ME biosensor method. The nonspecific attachment of these unwanted compounds on the ME sensor will increase the mass of the sensor, so the resonant frequency will increase. The preparation of the ME biosensor method, especially blocking, needs to be modified in order to minimize the attachment of unwanted fine particles on the sensor. The purposes of this study were to investigate an extraction procedure of *S.* Typhimurium from soil samples, investigate a new blocking reagent, and perform the modified ME biosensor method to detect *S.* Typhimurium in soil.

Materials and Methods

Bacterial Strain and E2 Phage

S. Typhimurium (ATCC 13311) and E2 phage suspension were provided at a concentration of 5.0×10^8 colony forming unit (CFU)/ml in phosphate-buffered saline (PBS, pH 7.2) and 1.0×10^{12} vir/ml in Tris-buffered saline (TBS, pH 7.4), respectively, by Dr. James M. Barbaree's laboratory in the Department of Biological Sciences at Auburn University, Auburn, AL, USA. The E2 phage suspension was mixed with the same amount of TBS buffer to adjust the concentration of E2 phage (5.0×10^{11} vir/ml in TBS) prior to use.

S. Typhimurium Inoculation in Soil

Soil (Marvyn loamy sand) was provided from Dr. Yucheng Feng's laboratory in the Department of Agronomy and Soils at Auburn University. The soil was autoclaved for 30 min at 121°C in order to remove other possibly existing microorganisms in the soil. For the inoculation of *S.* Typhimurium in soil, 20 µl of *S.* Typhimurium (ranging from 1 to 8 log CFU/ml) was inoculated

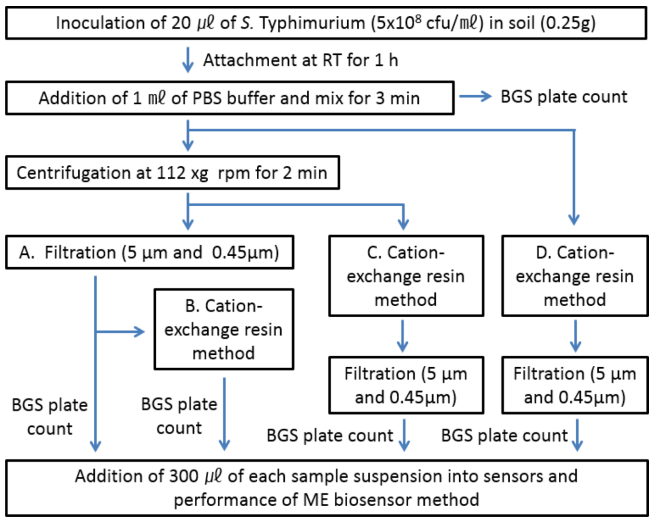

Fig. 1. Scheme used for the inoculation and extraction of *S.* Typhimurium in soil.

in 0.25 g of air-dried soil, followed by attachment for 1 h in a safety cabinet. The *S*. Typhimurium-attached soil sample was then transferred into tubes containing 1 ml of PBS buffer. After vortexing for 3 min, the soil sample was centrifuged at 4°C for 2 min at 112 ×*g*, and the supernatant was adjusted to 3 ml (Fig. 1).

Fabrication of Magnetoelastic Platforms

ME platforms were fabricated from METGLAS 2826MB alloy ribbon (Honeywell Inc., USA). The ribbon was diced into rectangular-shaped platforms with a size of 0.028 mm × 0.2 mm × 1 mm, using a computer controlled automatic micro-dicing saw (MPE Inc., USA) following the procedures mentioned in previous studies [17, 18].

Detection of *S*. Typhimurium in Soil Using ME Biosensor Method without Modification

E2 phage-immobilized measurement and E2 phage-devoid control sensor were prepared by placing the ME platform in an Eppendorf tube containing 300 µl of E2 phage suspension and TBS, respectively, followed by incubation on a rotary shaker at 22°C for 1 h. The ME platform was then washed three times with the TBS buffer and twice with sterilized distilled water to remove any unbound phage and salt debris. After the immobilization of E2 phage, the unbound areas of measurement and control sensors were blocked with 300 µl of 1% bovine serum albumin (BSA; Sigma-Aldrich Co., USA) at 22°C for 1 h. Finally, the ME platform was washed three times with sterilized distilled water and allowed to air dry. The baselined resonant frequency of the sensor was then measured with an HP 8751A network analyzer combined with an *S*-parameter test set. The resonant frequencies of the sensor were measured 10 times and averaged automatically. This procedure was repeated at least 3 times per sensor (yielding 3 data points from 30 measurements). *S.* Typhimurium with the concentration of 8 log CFU/ml was inoculated in soil following the procedures mentioned above (see *S.* Typhimurium Inoculation in Soil). An aliquot of 300 µl of *S*. Typhimurium-inoculated soil supernatant was placed in an Eppendorf tube containing a baselined measurement sensor and control sensor, followed by incubation on a rotary shaker for 1 h at room temperature. After washing, the resonant frequencies of the measurement and control sensors were measured again, and the resonant frequency shifts of the sensors were compared [21].

Extraction Studies of *S*. Typhimurium from Soil

The filtration method, cation-exchange resin method, and combinations of both methods were introduced after the inoculation of *S*. Typhimurium (Fig. 1). Prior to introduction of the extraction method, the number of *S*. Typhimurium in supernatant obtained from the inoculated soil sample was counted using a brilliant green sulfa (BGS) agar plate (Difco Laboratories, USA). The supernatant of the *S*. Typhimurium-inoculated soil sample was filtered (5 µm pore size) and the filtered suspension was filtered again using a filter with a 0.45 µm pore size (Procedure A in Fig. 1). For the preparation of sample B, the cation-exchange resin method was added after following procedure A. The A soil sample was mixed with 0.1 g of Chelex 100, 3 ml of Nadeoxycholate, and 2.5% of polyethylene glycol (PEG) 6000, followed by gentle shaking for 1 h at 4°C with short agitation every 10 min. The soil particles were then pelleted by centrifugation for 15 min at 1,000 ×*g* for 20 min. The supernatant was filltered to remove Chelcx 100 using a filter with a 0.45 µm pore size, and the bacteria were collected by centrifubation at 10,000 ×*g* for 20 min. For the preparation of sample C, the B sample was filtered using a filter with 0.2 µm pore size in order to remove unwanted compounds. The D soil sample was prepared by direct performance of the cation-exchange resin method and filtration without centrifugation. The total volume of each sample was adjusted to 3 ml to determine the number of *S.* Typhimurium using the BGS plate method and for comparison of the resonant frequency shift of each sensor using the HP 8751A network analyzer. Each sensor surface was observed using a scanning electron microscope (SEM) for the comparison.

Blocking Study for the Performance of ME Biosensor Method

BSA, PEG 2000 (Sigma-Aldrich Co.), and casein powder (Thermo Fisher Scientific. Co., USA) were used as a blocking agent. The blocking study was performed by immersing each E2 phage-immobilized measurement and control sensor into 300 µl of BSA (1 mg/ml), PEG (1 mg/ml), and casein (1 mg/ml) suspension, respectively. After incubation on a rotary shaker, each baselined measurement and control sensor was incubated with 300 µl of *S*. Typhimurium in overnight culture and in the soil sample obtained by filtration (indicated in Fig. 1A) after the inoculation of *S*. Typhimurium (8 log CFU/ml) in soil. For the determination of the best concentration of PEG, the baselined ME resonator platform was blocked with various concentrations of PEG (1, 2, 3, 4, and 5 mg/ml) and exposed to *S*. Typhimurium in a soil sample obtained by the filtration procedure indicated in Fig. 1A. Finally, the second resonant frequency of the measurement sensor was measured, and each sensor surface was observed using a SEM for the comparison.

Application of Modified ME Biosensor for the Detection of *S*. Typhimurium in Soil

Various concentrations of *S.* Typhimurium (2, 4, 6, and 8 log CFU/ml) were inoculated in soil, followed by the centrifugation and filtration procedures indicated in Fig. 1A. An aliquot of 300 µl of *S*. Typhimurium-inoculated soil supernatant was placed in an Eppendorf tube containing the baselined measurement sensor and control sensor, and the resonant frequency of the baselined sensor was measured again and the sensor was observed with a SEM.

Microscopic Analysis

A JEOL-7000F SEM was used to observe and confirm the binding of *S.* Typhimurium on the measurement and control sensors. Both the measurement and control sensors were exposed

to OsO_4 vapor for at least 45 min and mounted on aluminum stubs for examination by the SEM.

Statistical Analysis

The experiments were replicated at least three times and the experimental results were expressed as arithmetic means and standard deviations. The Student's paired *t* test for two groups and the one-way analysis of variance among more than two groups were run to compare the means using GraphPad and InStat V.3 programs (GraphPad, San Diego, CA, USA). The significance level was determined as $p < 0.05$, $p < 0.01$, or $p < 0.001$.

Results and Discussion

Performance of the ME Biosensor Method for Detection of *S.* Typhimurium in Soil

Prior to modification of the ME biosensor method, the ME biosensor method was performed without any modification in order to confirm and identify the interference of coexisting compounds in soil including *S.* Typhimurium. It was assumed that nonspecific binding of those unwanted materials on sensor platforms might increase the mass of the sensors so that the resonant frequency shift would increase. When 8 log CFU/ml of *S.* Typhimurium was exposed to both sensors, a wide range of resonant frequency shifts of the measurement and control sensors was observed in a scatter plot (Fig. 2A). Specifically, the averages of the resonant frequency shift of the measurement and control sensors were 4,737 ± 4,470 Hz and 3,158 ± 1,659 Hz with large standard deviations, respectively. In addition, there were no significant differences between the measurement and control sensors, which contradicted all of our previous results [5, 12, 14, 15, 17, 18] ($p < 0.05$). Ironically, there was very few binding of the target microorganism despite the great resonant frequency shift of the measurement sensor. In addition, SEM images showed that there was significantly more nonspecific binding of unwanted compounds on the measurement sensor than the control sensor (Figs. 2B, 2C). From this result, it was hypothesized that the ME biosensor method needs to be modified and updated for more binding of target pathogens and for minimizing nonspecific binding on the sensor by the addition of extraction procedures for *S.* Typhimurium from soil and by investigating a new blocking reagent.

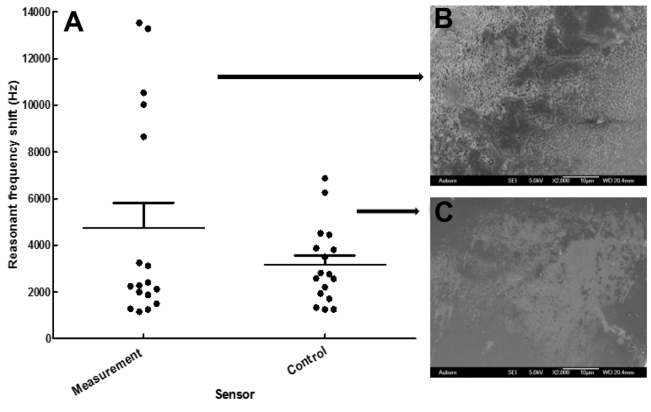

Fig. 2. (**A**) Resonant frequency shifts in the measurement sensor and control sensor, and SEM images of the (**B**) measurement sensor and (**C**) control sensor after performance of magnetoelastic biosensor without modification.

Determination of Extraction Method for *S.* Typhimurium from Soil

For successful performance of the ME biosensor method, *S.* Typhimurium should be extracted with the maximum recovery rate, and the extraction of other coexisting compounds from soil samples must be minimized. Since soil consists of complicated and heterogeneous humus, minerals, organic matters, and microorganisms, it is not easy to separate *S.* Typhimurium only. Thus, the E2 phage was prepared by a phage-typing method so that the specificity of E2 phage was confirmed because it possessed specific peptide sequences to interact with *S.* Typhimurium only [26]. In addition, four different extraction methods, including filtration, cation-exchange resin, and combinations of both methods were introduced for optimizing target pathogen extraction in this study [26]. To determine the best method, the number of *S.* Typhimurium was compared using BGS agar plates (Fig. 3A). Theoretically, the cation-exchange resin method may not capture any *S.* Typhimurium, because the surface of *S.* Typhimurium was reported to be negatively charged [8]. However, the recovery number of *S.* Typhimurium after the procedures B, C, and D was significantly decreased from 7.10 log CFU/soil to the range of 4.45–4.72 log CFU/soil ($p < 0.05$). Although a dramatic improvement of nonspecific binding on the measurement sensor was observed by the SEM images (data not shown), the employment of the cation-exchange resin method was not appropriate due to significant decreases in the number of *S.* Typhimurium. Although the filtration method (indicated as A) decreased the number of *S.* Typhimurium recovered (6.34 log CFU/soil) from soil samples, the number of *S.* Typhimurium was significantly greater than the other extraction methods (indicated as B, C, and D in Fig. 3A) ($p < 0.05$).

The greatest resonant frequency shift of the measurement sensor was also found when the ME biosensor method was performed following procedure A (centrifugation and

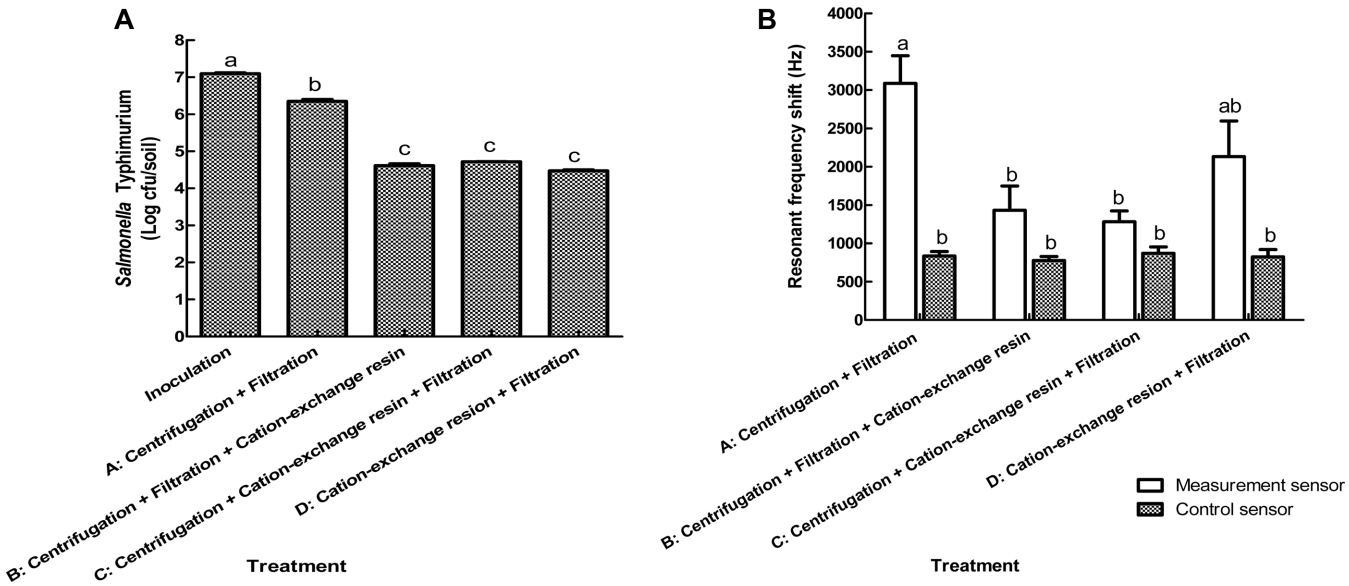

Fig. 3. Comparison of (**A**) the recovery of *S*. Typhimurium and (**B**) the resonant frequency shift of the magnetoelastic biosensor method after the performance of various extraction methods.
Different letters (a, b, and c) indicate significant differences of the mean among extraction procedures ($p < 0.05$).

filtration), which was significantly greater than any others ($p < 0.05$) (Fig. 3B). Although the resonant frequency shift of the measurement sensor after procedure D did not provide any significant difference, SEM images confirmed a higher number of nonspecific binding than *S*. Typhimurium on the sensor (data not shown). Thus, it was concluded that the centrifugation procedure after inoculation of *S*. Typhimurium in soil was necessary to minimize nonspecific binding on the measurement sensor. In addition, the centrifugation and filtration method was best for the extraction of target pathogens in soil by minimizing nonspecific binding on the sensor. Therefore, procedure A (centrifugation and filtration method) was selected as the best method for the maximum recovery of *S*. Typhimurium and the performance of the ME biosensor method.

Selection of Blocking Reagent

The selection of the best extraction procedures solved the nonspecific binding on the sensor in a certain range; however, the extraction did not solve all possible nonspecific binding problems. The best blocking reagent needs to satisfy certain requirements, including blocking the space between the E2 phages and not interfering with the capacity of the E2 phage to interact with its target [23, 24]. Since BSA, casein, and PEG have been popularly used as blocking reagents on plastic or gold surfaces [11, 16, 29], these reagents were selected for this study. As shown in

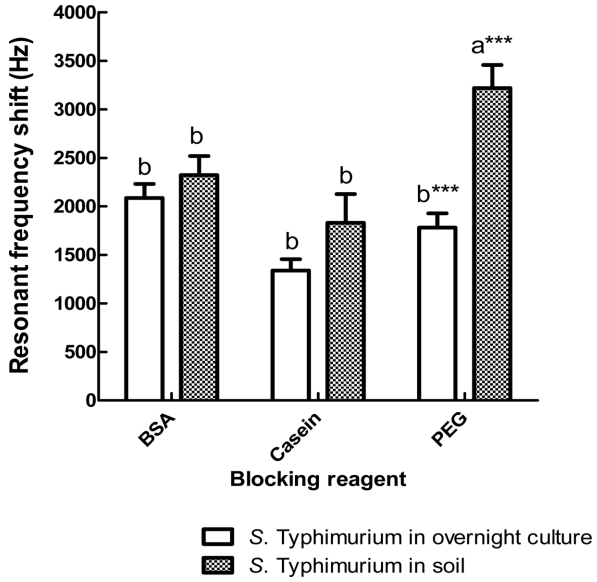

Fig. 4. Resonant frequency shift of the measurement sensor blocked with BSA, casein, and PEG after exposure to *S*. Typhimurium in overnight culture and soil.
Different letters (a and b) indicate significant differences of the mean among blocking reagents ($p < 0.05$). *** means that there was a significant difference between PEG-blocked measurement sensor after exposing to *S*. Typhimurium in overnight culture and soil, at the level of $p < 0.001$.

Fig. 4, there were no significant differences of resonant frequencies among the BSA-, casein-, and PEG-blocked

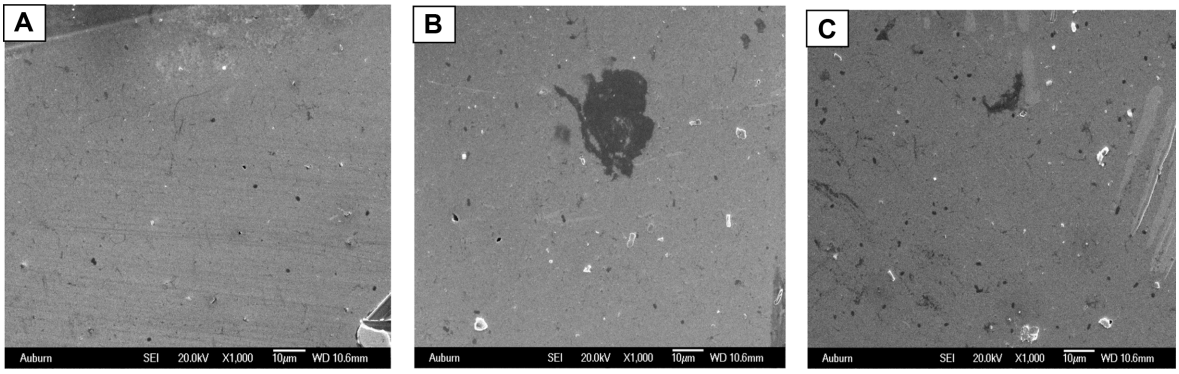

Fig. 5. SEM images of (**A**) casein-blocked, (**B**) BSA-blocked, and (**C**) PEG-blocked measurement sensors after exposure to *S*. Typhimurium inoculated in soil.

measurement sensors when exposed to *S*. Typhimurium in overnight cultures ($p < 0.05$). The resonant frequency shifts of the measurement sensor blocked with BSA, casein, and PEG were 2,085 ± 568, 1,339 ± 307, and 1,781 ± 507, respectively. The resonant frequency shift of the BSA-blocked measurement sensor was greater than those of the PEG- and casein-blocked measurement sensors. It was concluded that there were not critical differences among them when exposed to *S*. Typhimurium in overnight cultures.

When the measurement sensor was exposed to *S*. Typhimurium in soil, however, there were significant differences of resonant frequency between the PEG-blocked measurement sensor and the other measurement sensors ($p < 0.05$). In addition, the resonant frequency shift of the PEG-blocked measurement sensor (3,219 ± 755 Hz) exposed to soil sample was significantly greater than that of the PEG-blocked measurement sensors exposed to overnight culture ($p < 0.001$). The SEM images confirmed that the resonant frequency shift was dependent on the binding of *S*. Typhimurium on the measurement sensors. The PEG-blocked measurement sensor showed the best binding of *S*. Typhimurium on the measurement sensor (Fig. 5). The sizes of BSA, PEG, and casein were reported as approximately 65,000, 2,000, and 22,000 Da, respectively [23, 24]. It was concluded that the smaller the size of PEG used, the more thoroughly covered the spaces between the E2 phage were. Therefore, PEG was selected as the best blocking reagent among the others due to the significantly higher number of binding of *S*. Typhimurium on the measurement sensor.

Determination of Optimum PEG Concentration

As the concentration of PEG increased, the resonant frequency shift of the measurement sensor also increased (Fig. 6). As the concentration of PEG increased up to 1.0 mg/ml, the resonant frequency shift increased significantly ($p < 0.05$). However, there was a significant decrease in resonant frequency shift when treated with 2.0 mg/ml of PEG. Although the maximum resonant frequency shift (3,273 ± 347 Hz) was found at 4.0 mg/ml of PEG, this resonant frequency shift did not provide any significant difference from that (2,829 ± 320) blocked with 1.0 mg/ml of PEG ($p < 0.05$). Thus, the optimum concentration

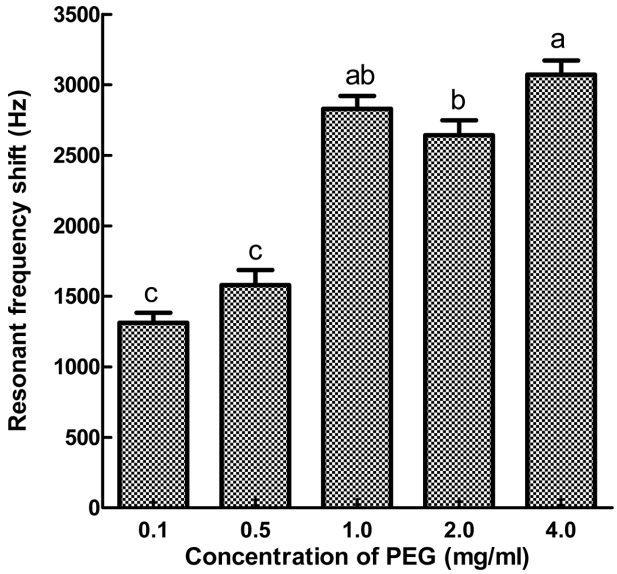

Fig. 6. Resonant frequency shift of the measurement sensor blocked with various concentrations of PEG after exposure to *S*. Typhimurium in soil.

Different letters (a, b, and c) indicate significant differences of the mean among different concentration of PEG for blocking of the measurement sensor ($p < 0.05$).

of PEG for blocking was determined to be 1.0 mg/ml after consideration of the significant resonant shift and economic issue.

Performance of Modified ME Biosensor Method for *S.* Typhimurium Detection in Soil

As shown in Fig. 7, the modified ME biosensor method was performed to detect *S.* Typhimurium in soil. As the concentration of *S.* Typhimurium increased, the resonant frequency shift of the measurement sensor also increased significantly ($p < 0.05$). The maximum resonant frequency shift (4,899 ± 597 Hz) was observed in the measurement sensor when exposed to soil inoculated with 9 log CFU/ml of *S.* Typhimurium. SEM images (data not provided) confirmed that the resonant frequency shifts were derived from the binding of *S.* Typhimurium on the measurement sensor. In addition, the number of *S.* Typhimurium bound increased depending on the increase in concentration of *S.* Typhimurium in the soil. Meanwhile, control sensors showed relatively constant and low magnitudes of frequency shifts (683 ± 277 Hz to 922 ± 394 Hz) over the whole range. There were no significant differences among the control sensors over the whole range and there were no significant differences in resonant frequency shift between the measurement and control sensors at relatively low concentrations of *S.* Typhimurium ($p < 0.05$). However, a significant difference between the measurement and control sensors was observed starting from the concentration of 4 log CFU/ml and continued up to 9 log CFU/ml of *S.* Typhimurium concentration ($p < 0.01$).

From the dose response of the measurement sensor (Fig. 7), a linear range was found over eight decades (2 log CFU/ml to 9 log CFU/ml), and the linear equation and correlation coefficient (R^2) were calculated. Since sensitivity was defined as the slope of the linear range of the dose response [20, 21], the sensitivity was found to be 518.8 ± 25.08 Hz/log. This sensitivity for soil sample was lower than others presented in previous studies, including 1,389, 1,200, 1,327, and 1,157 for tomato, adaxial surface of spinach, abaxial surface of spinach, and cantaloupe, respectively [17, 21]. All of these previous studies were performed on the surface of fresh produce directly without any extraction procedures, so that the direct placement of sensor on the surface could minimize loss of target pathogens during the extraction procedures. From these two linear curves (although R^2 of the control sensor is small), the limit of detection (LOD) is defined as the point of intersection of the linear measurement and control response curve [20, 21], which was determined to be 2.03 log CFU/ml for soil. Although the LOD obtained in this study was not enough to detect a very low number of target pathogen in soil, the LOD will be enhanced by decreasing the sensor size. The more decreases in sensor size will enhance the resonant frequency shift, even with very slight change of mass on the sensor [15, 17]. Our group has recently developed a new fabrication technique to produce 50 μm length of sensor from 1 mm size of sensor. Thus, the application of a much smaller sensor size will improve the LOD.

Unlike the previous studies, this study was the first that focused on the employment of the ME biosensor method to detect *S.* Typhimurium in soil. The ME biosensor method

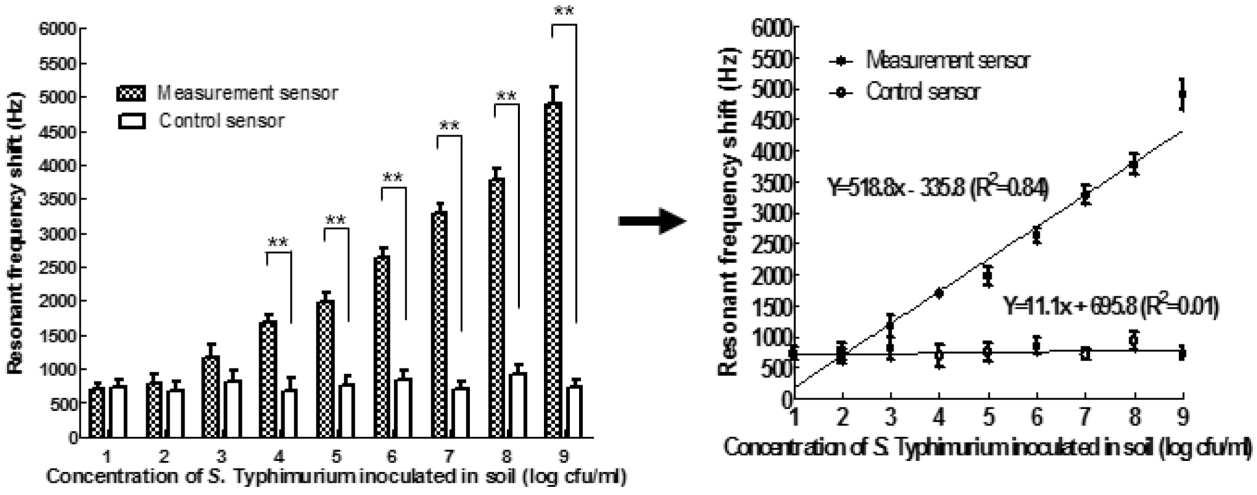

Fig. 7. Resonant frequency shift of measurement and control sensors after exposure to soil sample inoculated with various concentrations of *S.* Typhimurium.

** indicates that there were significant differences between the measurement and control sensors at $p < 0.01$.

was modified by combining the extraction procedures of *S.* Typhimurium from soil effectively and by substituting the BSA blocking reagent with PEG to minimize the number of nonspecific binding of unwanted coexisting heterogeneous compounds in soil on the surface of the sensor. Since these extraction procedures prior to performance of the ME biosensor method sacrificed some advantages, such as cost-effectiveness, time-effectiveness, and operator friendliness, this suggested protocol needs to be improved by employing commercialized easy extraction techniques. However, this study demonstrated the novel employment of the ME biosensor method to detect *S.* Typhimurium in soil and future possibilities of the ME biosensor with various usage. For the further study, this method will be employed to real soil samples combined/mixed with foods and then compared with other recognized rapid detection methods for verifying this ME biosensor method objectively.

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