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Bacteriophage-modified Graphene Oxide Screen-printed Electrodes for the Impedimetric Biosensing of *Salmonella Enterica* Serovar Typhimurium

[1, *] Pauline Ann QUITON, [3] Beatrice Marie CARREON, [1, 2, 4] Donna May DELA CRUZ-PAPA and [1, 2, 3] Jose BERGANTIN, Jr.
[1] The Graduate School
[2] Research Center for the Natural and Applied Sciences
[3] Department of Chemistry
[4] Department of Biology, College of Science,
University of Santo Tomas, España Blvd., Manila 1015, Philippines
E-mail: pamquiton@gmail.com

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Abstract: In this study, an electrochemical impedance biosensor was developed using an isolated bacteriophage as a bioreceptor to detect *Salmonella enterica* serovar Typhimurium. The bacteriophage was immobilized on graphene oxide-modified screen-printed electrode (GO-SPE) using 1-(3-dimethylaminopropyl)-ethylcarbodiimide hydrochloride (EDC) as a crosslinker. The modified GO-SPEs were characterized using Field Emission Scanning Electron Microscopy and Fourier Transform Infrared Spectroscopy to confirm the successful immobilization of the bacteriophage on the electrode surface. Electrochemical Impedance Spectroscopy (EIS) was used to monitor variations in the charge transfer resistance (Rct) values at different immobilization and measurement conditions. The fabricated GO-SPE/Phage biosensor displayed quantitative response for *S.* Typhimurium over a broad range of concentrations (10^{-1}×10^{8} cfu/mL) and an increasing linear response was observed (R^2=0.9842). The optimized incubation time and response time were 30 min and 4 min, respectively. Selectivity tests showed that the biosensor is selective towards S. Typhimurium over other bacteria, such as *Escherichia coli* and *Staphylococcus aureus*.

Keywords: Biosensors, Bacteria detection, Bacteriophage, Electrochemical Impedance Spectroscopy, Salmonella, Screen-printed electrode.

1. Introduction

The emerging cases of food contamination due to pathogenic bacteria have affected the health and safety of the world's population. *Salmonella* is a gram-negative, rod-shaped, motile, non-spore forming bacterium that belongs to the family Enterobacteriaceae [1]. According to the World Health Organization (2013), Salmonellosis is one of the most common and widely distributed food-borne diseases caused by the bacteria *Salmonella*. It is usually characterized by acute fever, abdominal pain, diarrhea, nausea, and vomiting [2]. *Salmonella* human infections are among the more significant causes of morbidity and mortality worldwide [3].

Conventional pathogen detection methods mostly rely on microbiological and biochemical analysis, which are highly accurate, however can be time

consuming, cost-ineffective, not applicable for on-site diagnosis, and require extensive training and experience. Advancement in immunological methods such as enzyme-linked immunosorbent assay (ELISA) have given way towards the development of easier and quicker pathogen detection methods, relying on the recognition specificity of antibodies. However, the method suffers from cross-reactivity of polyclonal antibodies, high production cost of antibodies, need for sample pre-processing and pre-enrichment due to low processing sample volume, and lower limit of detection. Another method is the polymerase chain reaction (PCR) which uses nucleic acid complementary-based specificity for pathogen detection [4–6]. Currently, researchers around the world continuously develop more alternative tools for fast, accurate and sensitive pathogen detection [7]. Key issues, like differentiation of live and dead cells, automation, cost, simplicity, training, and accuracy, are still needed to be considered in developing rapid methods for pathogen detection [8].

Biosensors have been proven to be a promising method for detection of food-borne pathogenic bacteria due to their portability, rapidity, sensitivity, and capability of on-the-spot detection [9]. Among the different types of biosensors, electrochemical biosensors are predominantly popular due to their convenient upscaling with microfabrication technologies. An advantage of using electrochemical impedance spectroscopy (EIS) to develop label-free electrochemical biosensors is that the method can record even the smallest changes at the solution-electrode interface. Impedance biosensors for bacteria detection are constructed by immobilizing bioreceptors, such as antibodies, nucleic acids, bacteriophages and lectins, on the electrode surface. The binding ability of the target bacteria and the bioreceptor is confirmed by the detection of a change in impedance [10].

Bacteriophages are viruses made up of an outer protein coat that encases genetic material, DNA or RNA [11]. These are ideal bioreceptors in developing impedimetric biosensors for bacteria detection due to their high recognition of the specific host [12]. Other advantages of using bacteriophage for biosensor development are: rapid detection, selective detection of target bacteria even under harsh conditions, more cost-effective than antibodies, and easy to manipulate at the molecular level when improving interaction with bacteria [13, 14].

In this study, we developed a phage-based biosensor by immobilizing bacteriophages covalently on graphene oxide modified screen-printed electrode (SPE) and Electrochemical Impedance Spectroscopy was used as the transduction method. In previous studies, graphene-modified SPE was used and needed to use chronoamperometry to activate the carboxyl groups on the surface [15, 16]. This current study demonstrates a simpler method by using only EDC crosslinking for bacteriophage immobilization.

2. Materials and Methods

2.1. Materials

1-(3-Dimethylaminopropyl)ethylcarbodiimide hydrochloride (EDC) and phosphate buffer saline (PBS) tablets were purchased from Sigma-Aldrich. Tryptic soy broth (TSB), tryptic soy agar (TSA) and bacteriological agar (HiMedia Laboratories) were used for the bacteria inoculation and bacteriophage enrichment. Prior to use, microbiological media, PBS, pipettor tips and tubes were sterilized by autoclaving at 121°C. The graphene oxide modified screen-printed electrode (GO-SPE) used for this study were purchased from Dropsens. The total dimensions of the GO-SPE were 3.4 × 1.0 × 0.05 cm (length × width × height).

2.2. Bacteriophage Preparation

Isolated lytic bacteriophages against *S*. Typhimurium from sewage water were used in this study. Prior to immobilizing the bacteriophage on the GO-SPE surface, the titer was amplified using the following enrichment method. A volume of 100 µL of bacteriophage solution and 300 µL of overnight grown *S*. Typhimurium culture were added on a 10 mL 2xTSB solution. The solution was incubated overnight at 37 ºC. It was then centrifuged for 10 minutes at 3500×g and was filtered using a syringe with 0.45 µm Acrodisc filter to remove any residual bacteria. The enriched bacteriophage solution was subjected to double agar overlay method to determine its titer concentration. The bacteriophage solution was serially diluted (10^{-1}–10^{-8}) on sterile PBS solution. In a 3 mL of molten 0.7 % soft TSA, 100 µL of each dilute phage filtrate and 300 µL of 24-hr old *S.* Typhimurium were added and poured on a TSA base plate. The plates were incubated overnight at 37°C and were examined for the presence of plaques. The plates with suitable number of plaques (30-300 plaques) were analyzed, and the titer of the bacteriophage in the stock and other diluted samples were estimated using the following equation:

$$Plaque\ Forming\ Unit\ (pfu/ml) = \frac{no.of\ plaques \times 1000 \times reciprocal\ of\ dilution\ factor}{volume\ of\ bacteriophage\ sample\ (\mu L)} \quad (1)$$

Host specificity of the bacteriophage towards *S.* Typhimurium was investigated by the spot test method. A volume of 300 µL of 24-hour old cultures of *S.* Typhimurium, *Staphylcoccus aureus* and *Escherichia coli* were added on 3 mL molten 0.7 % soft TSA separately, poured on a TSA base plate and solidified for 1-2 minutes. About 5-8 µL of the phage filtrate was dispensed on each of the TSA plates and then incubated overnight at 37 °C prior to visual analysis.

2.3. Electrode Preparation and Bacteriophage Immobilization

The GO-SPEs were left in contact with 0.1 M solution of 0.1 M EDC in 0.12 N HCl for 1 hour, to activate their surface carboxyl functional groups. Then, it was incubated with the prepared bacteriophage solution for 6 hours at 37 °C to covalently immobilize the bacteriophage. The bacteriophage-modified electrodes (GO-SPE/Phage) were washed with PBS, air dried and stored at 4 °C before use.

2.4. Characterization of Bacteriophage-Modified GO-SPE

Field emission scanning electron microscopy (FE-SEM; Helios Nanolab 600i) and Fourier transform infrared spectroscopy (FTIR; Perkin Elemer Spectrum Two) were used to confirm the bacteriophage immobilization on the GO-SPE.

2.5. Electrochemical Impedance Detection of Bacteria

Electrochemical impedance spectroscopy (EIS) was used for the quantitative analysis of *S.* Typhimurium. An electrochemical impedance analyzer (eDAQ ERZ100) was used for the measurements. The bacteriophage-modified GO-SPE was incubated with 10 µL of prepared bacterial solution (1×10^1, 1×10^2, 1×10^4, 1×10^6, and 1×10^8 cfu/mL). The EIS parameters were recorded in the presence of $Fe^{3+/2+}$ redox probe (5 mM $K_3Fe(CN)_6$ + $K_4Fe(CN)_6{\cdot}3H_2O$, prepared in 0.1 M PBS). The frequency range selected was 0.1 Hz – 1 MHz with fixed amplitude of 5 mV.

3. Results and Discussion

3.1. Characterization of Bacteriophage Immobilized GO-SPE

FESEM images of a bare GO-SPE and the phage immobilized GO-SPE are shown in Fig. 1. The comparison of the bare GO-SPE and GO-SPE/Phage indicates the successful modification of the screen-printed electrodes. Focusing on the high magnification images (50,000x), the presence of individual bacteriophage-like structures characterized by a typical length of 100-300 nm was observed.

The immobilization of bacteriophage on SPE was also verified using FTIR-ATR characterization. A comparison of bare GO-SPE and GO-SPE/Phage FTIR spectra is shown in Fig. 2. The bare GO-SPE showed peaks at ~1720 cm^{-1} and ~1270 cm^{-1} that are characterized as carboxylic acid and ester carbonyl peaks, respectively. This proves that the SPE used was graphene oxide (GO) functionalized that can covalently bind with an amino-functionalized molecule (protein coating of phage) via carbodiimide coupling [17]. The bacteriophage immobilized GO-SPE showed characteristic peaks at 3600 cm^{-1} (N-H stretch), 2300 cm^{-1} (C=N stretch) and 1750 cm^{-1} (amide absorption). These functional groups are formed from the covalent crosslinking method and are also present in the bacteriophage protein coat.

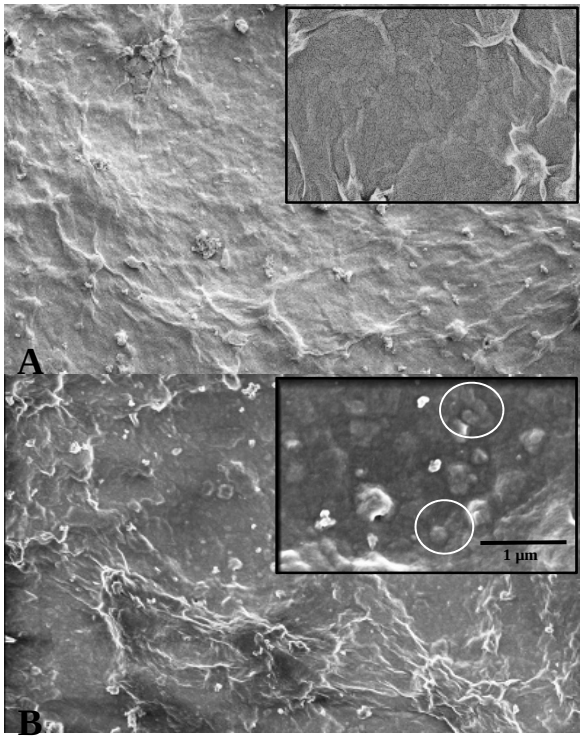

Fig. 1. FE-SEM images of blank GO-SPE (A) and bacteriophage-modified GO-SPE (B) at 10,000x and 50,000x (inset) magnification.

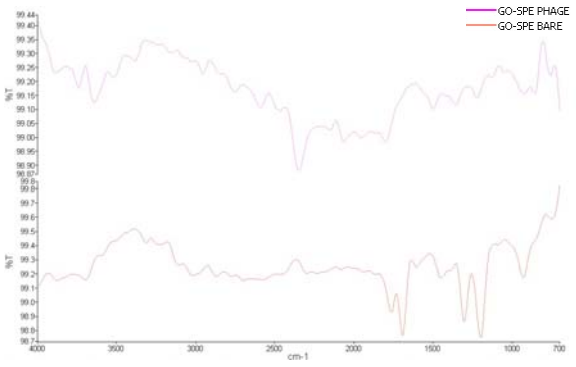

Fig. 2. FTIR spectra of bare GO-SPE and GO-SPE/Phage.

3.2. *S.* Typhimurium Detection by Electrochemical Impedance Spectroscopy

EIS method was used for the quantitative detection of *S.* Typhimurium. Its data are expressed in the form of a Nyquist plot in which the imaginary impedance

component (Z", out-of-phase) is plotted against the real impedance component (Z', in-phase) at different excitation frequencies. The Randle's equivalent electrical circuit (Fig. 3) was used to model the data and estimate the values of charge transfer resistance. The circuit elements comprise of double layer capacitance (C or C_{dl}), interfacial electron transfer (R_{ct}) and solution resistance (R_s).

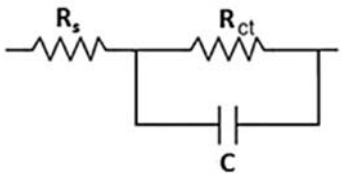

Fig. 3. Randle's equivalent electrical circuit.

As a preliminary experiment, the time-dependent behavior of immobilized phages to effectively lyse the incubated bacteria was studied using EIS. After phage immobilization, the GO-SPE/Phage was washed with PBS solution and covered with 10 µL of 1×10^8 cfu/mL *S.* Typhimurium. The shifts in impedance were recorded at different times following the incubation of the bacteria suspension. Fig. 4 shows the impedance shifts observed from 10 to 40 minutes following the deposition of the bacteria solution onto the GO-SPE/Phage surface. It was observed that an initial increase in impedance shift occurred, due to the exposure of bacteria at the GO-SPE/Phage surface, which reaches a maximum value of ~1.5×10^4 Ω at 30 minutes. The impedance was also detected to decrease after 30 min, indicating that infection of the *S.* Typhimurium and the lytic cycle started to occur within ~30 min at room temperature and levels off after 40 min.

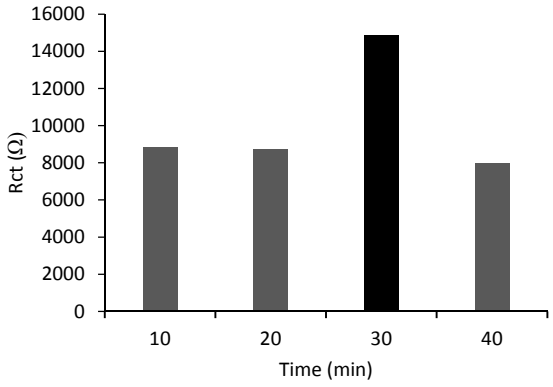

Fig. 4. Shift in impedance at specific times following incubation of *S.* Typhimurium on the GO-SPE/Phage biosensor.

Fig. 5 shows the impedance results (Nyquist plots) obtained when different concentrations (1×10^1-1×10^8 cfu/mL) of *S.* Typhimurium were placed on the GO-SPE/Phage surface. To ensure that a maximum impedance signal was measured, the Nyquist plots were taken at 30 min of incubation with the bacteria, with each measurement taking 4 min to be completed.

A linear relationship was observed between the R_{ct} values with respect to the *S.* Typhimurium concentration (Fig. 6). The slope of the calibration curve is 0.0556±0.0041 and the intercept is 3.8644±0.01998. The LOD of the developed GO-SPE/Phage biosensor was calculated as 12 cfu/mL and has an %RSD ranging from 0.05 % to 0.7 %.

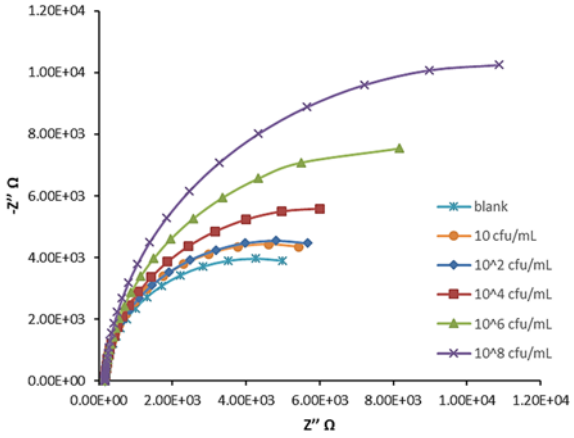

Fig. 5. Nyquist plots for GO-SPE/Phage biosensor surface in the presence of *S.* Typhimurium at different concentrations.

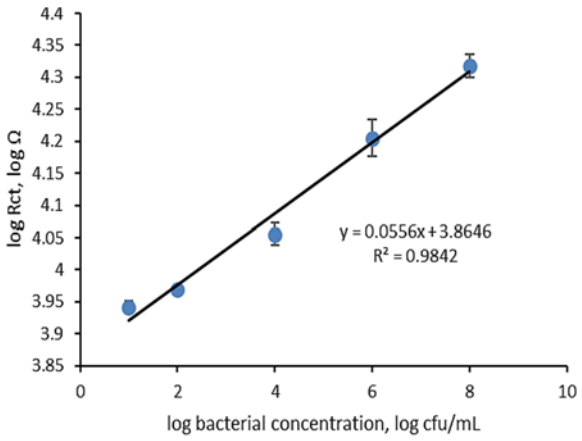

Fig. 6. Variation in charge transfer resistance values with respect to different bacteria concentrations.

The specificity of the GO-SPE/Phage biosensor was also tested by incubating *E. coli* and *S. aureus* on the electrode surface for 30 min. Fig. 7 shows that the developed biosensor is highly selective to *S.* Typhimurium. There was no observed increase in impedance from the incubation of other bacteria than the host. This highlights the advantage of using host-specific bacteriophages as bioreceptors for bacteria detection.

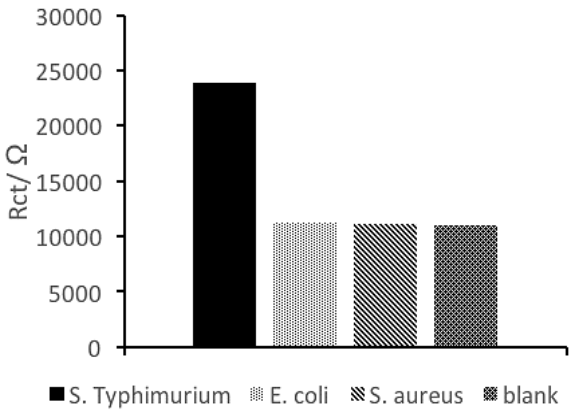

Fig. 7. Specificity test of GO-SPE/Phage towards *S.* Typhimurium.

4. Conclusions

A phage-based GO-SPE biosensor was successfully developed in this study. EIS was used to quantify different concentrations of *S.* Typhimurium. A linear relationship between the logarithm of Rct and *S.* Typhimurium concentration was observed (R^2=0.9842) with a sensitivity of 0.0596 log Ω/log cfu mL^{-1} and an LOD of 12 cfu/mL. The total analysis time using this impedance biosensor is 40 minutes which is more time efficient that conventional methods.

Acknowledgements

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