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Development of electrochemical biosensor for detection of pathogenic microorganism in Asian dust events

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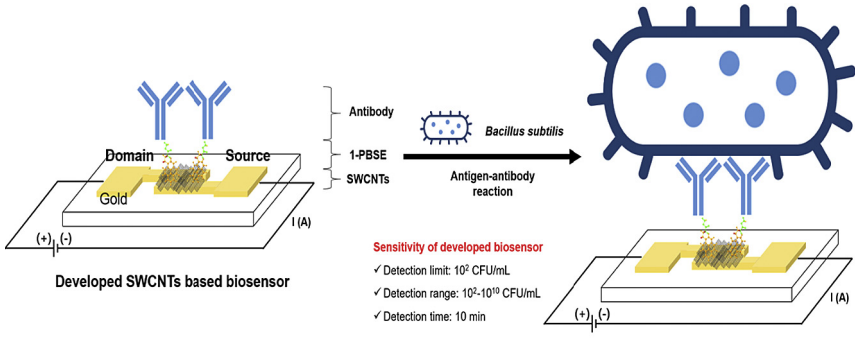
HIGHLIGHTS

- A SWCNTs-based biosensor was developed for the detection of *Bacillus subtilis.*
- The biosensor was composed of SWCNTs, 1-PBSE, and anti-*B. subtilis* antibody.
- The performance of the biosensor was assessed using a sensor sensitivity and specificity tests.
- The detection limit and detection range were 10^2 and 10^2–10^{10} CFU/mL, respectively.
- The detection time of the biosensor was identified as 10 min.

GRAPHICAL ABSTRACT



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ABSTRACT

We developed a single-walled carbon nanotubes (SWCNTs)-based electrochemical biosensor for the detection of *Bacillus subtilis,* one of the microorganisms observed in Asian dust events, which causes respiratory diseases such as asthma and pneumonia. SWCNTs plays the role of a transducer in biological antigen/antibody reaction for the electrical signal while 1-pyrenebutanoic acid succinimidyl ester (1-PBSE) and ant-*B. subtilis* were performed as a chemical linker and an acceptor, respectively, for the adhesion of target microorganism in the developed biosensor. The detection range (10^2–10^{10} CFU/mL) and the detection limit (10^2 CFU/mL) of the developed biosensor were identified while the response time was 10 min. The amount of target *B. subtilis* was the highest in the specificity test of the developed biosensor, compared with the other tested microorganisms (*Staphylococcus aureus*, *Flavobacterium psychrolimnae*, and *Aquabacterium commune*). In addition, target *B. subtilis* detected by the developed biosensor was observed by scanning electron microscope (SEM) analysis.

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1. Introduction

The fine dust contains various harmful strains causing allergic lung inflammation in the human body as well as small chemical particle materials (1–10 PMs) with heavy metals (Ichinose et al., 2008). The presence of *Bacillus subtilis* in Asian dust causes respiratory diseases such as asthma, pneumonia, and tuberculosis (Hua et al., 2007). *B. subtilis* is a gram-positive microorganism producing endospores for endurance against extreme heat or dry air conditions (Jacob et al., 2016). With these characteristics, *B. subtilis* enables to survive in desert conditions.

Carbon is one of attractive electrical materials with bio-friendly characteristics. Following the development of the carbon nanotubes (CNTs) (Liu et al., 2017), many researchers have focused on various applications of CNTs to biosensors (Çevik, 2016). A CNT has a hollow cylindrical form made up of single or multiple graphene sheets and contain hexagonal rings of carbon atoms. Each carbon atom of CNT possesses a lone pair. These CNT characteristics mean that CNTs can transfer electrons induced from electrical signal to the response made by the sensor system. CNT can be divided into two types: single-walled CNT (SWCNT) and multi-walled CNT (MWCNT). The SWCNT has a higher thermal conductivity, mobility capacity ($\geq$3500 W/m·K, $\geq 10^9$ A/cm^2, respectively), a smaller diameter (0.4–2 nm), and higher current mobility capacity (Yao et al., 2000; Pop et al., 2006; Yang et al., 2007) while MWCNT has thermal conductivity ($\geq$3000 W/m K) and diameter (1.4–100 nm). Although both the SWCNT and MWCNT have similar characteristics, they are applied to various sensor devices; the SWCNT is suitable for a smaller semiconductor due to the smaller diameter of SWCNT than that of MWCNT. Especially, compared with the metallic SWCNT, the semiconducting SWCNT with high sensitivity is an attractive electrical material for sensor devices because it has high mobility, as well as all atoms are located on the surface (Baughman et al., 2002; Besteman et al., 2003). For application of these SWCNTs on biosensors, a dielectrophoresis (DEP) method has been applied to carbon nanotubes assembly process between electrodes (Cho et al., 2015).

Biosensors are defined as a device containing a transducer attached to the biological sensing element (Kobras et al., 2017). Several types of biosensors have been developed, such as enzyme biosensors, microorganism biosensors, immune biosensors, etc., depending on the biological sensing element. In addition, the activities of the biological components in the biosensor have been applied to many fields such as pharmaceutical, environmental, and agricultural fields, and so on. because their activities are maintained under relatively mild conditions (Mello and Kubota, 2002). *Escherichia coli* O157:H7 and *Salmonella* sp. were successfully detected by using a surface plasmon resonance (SPR) biosensor with antibodies (Vaisocherová-Lísalová et al., 2016).

Recently, research using carbon materials for biosensors has received attention (Rahman et al., 2016). An electrochemical biosensor has recently been developed in which the CNT converts the response to the biomolecule assembly (enzymes or proteins, antibodies, nucleic acids, cells or tissues) as receptors recognizing the target molecule with a measurable electrical signal in the electrochemical sensor (Ronkainen et al., 2010). In particular, the bio-catalytic biosensor, an electrochemical biosensor using enzyme-immobilization techniques, has been widely studied while biosensors containing receptors (chemicals, antibodies, DNA, Aptamer, etc.) have been used in industrial application (Patris et al., 2016). Representative biosensor is a glucose sensor containing glucose oxidase for patients with diabetes. However, the enzyme activity can be greatly affected by the different circumstances (pH, temperature, and other substances or inhibitors) of the biosensor system. In addition, the enzyme-based biosensors do not easily detect target materials that rarely occur in the ecosystem. On the other hand, while biosensors with receptors such as antibodies, DNA/RNA, and aptamer are somewhat difficult to detect in real time, their sensor stability is superior as compared to that of the enzyme based biosensor (Hianik, 2016). These receptors are used when there is a strong bond with the target materials using DNA hybridization, antigen-antibody interaction. Biosensors require a seamless connection between the biological component and the electrochemical component. The essential technologies for the connections include physical adsorption, self-binding, biotin-streptavidin interaction, and covalent bonding, etc. Among them, covalent bonding has a feature formation with a stable detection layer to prevent biomolecules from releasing the electrode surface (Wang et al., 2011). 1-pyrenebutanoic acid succinimidyl ester (1-PBSE) is one of well-known linkers for covalent bonding and a typical connection according to the biosensor developing due to its succinimidyl group with a covalent bond to biomolecule primary and secondary amine groups to the nucleophilic substitution and pyrene ring with the strong affinity of SWCNTs using non-covalent bonding (Okuno et al., 2007).

In this study, a SWCNTs-based biosensor was developed to detect the *B. subtilis* observed in particle materials (PM). In particular, the proposed biosensor was a two-dimensional type for the purpose of minimizing the biosensor size. Also, the sensor capacities (detection time, limit of detection (LOD), detection range, selectivity, etc.) were confirmed by using sensor devices and the *B. subtilis* detection of the device attached to the sensor was determined by using SEM images.

2. Materials and methods

2.1. Bacterial strains and culture media

Bacillus subtilis KCCM 11316 was purchased from the Korean Culture Center of Microorganisms (KCCM, Seoul, South Korea). *Staphylococcus aureus* KCTC 1621, *Flavobacterium psychrolimnae* KCTC 23141, and *Aquabacterium commune* KCTC 22900 were purchased from Korean Collection for Type Cultures (KCTC, Jeongeup, South Korea). Four different types of microorganisms were used for evaluating the specificity of the biosensor. *B. subtilis* and *S. aureus* were incubated for 16 h at 37 °C in Nutrient broth (Difco Laboratories, Detroit, MI, USA) while *F. psychrolimnae* and *A. commune* were for 48 h at 20 °C. After the cultured broth was centrifuged for 10 min at 13,000 rpm, it was diluted with phosphate buffered saline (PBS) and the above procedure was repeated for 3 times.

2.2. Biomolecules and chemical reagents

SWCNTs ($\geq$99.5%), *N*, *N*-dimethylformamide (DMF), and 1-PBSE were purchased from Sigma Aldrich Korea (Seoul, South Korea), Daejung chemicals & metals (Siheung, South Korea), and Life Technologies (New York, USA) respectively. Anti-*B. subtilis* polyclonal antibodies (pAbs) were purchased from Abcam Inc. (Cambridge, England) and was diluted to a concentration of 0.004 mg/mL in a carbonate-bicarbonate buffer. 0.1 M phosphate buffered saline (PBS, pH7.4) was purchased from Sigma Aldrich Korea and was diluted 10-fold to dilute the cultured broth, which contained 13.7 mM NaCl, 0.27 mM KCl, 0.8 mM Na_2HPO_4, and 0.2 mM KH_2PO_4.

2.3. Fabrication of biosensor platform

The sensor device was fabricated with a patterned template containing gold electrodes and was designed to enable 66 time-sensing measurements every treatment (Fig. 1).

The designed gold electrode layers with 6.0 mm of gaps were

deposited with 40 nm-thickness and on Cr-deposited silicon wafer surface. Cr deposition was carried out using an electron-beam evaporator (SRN-110-1505-R2, Sorona Inc., Pyeongtaek, South Korea) with 4.0×10^{-6} torr of vacuum.

2.4. Fabrication of SWCNTs-based biosensor

SWCNTs in DMF solvent was dispersed using a sonicator (ultrasonic cleaner, Branson B5510) at room temperature for 2 h. After 10 μl SWCNTs solution was dropped, a connection was formed between gold electrodes, and alternating current (AC) dielectrophoresis (10 V/30 s) was used for the SWCNTs assembly between the gold electrodes. Drying to remove DMF residue (80 °C and 25 min), the electric resistances of the aligned SWCNTs bundle were then measured with a potentiostat (Versastat3, AMETEK, Princeton Applied Research, USA) to confirm SWCNTs-assembly process. 8.0 g/L 1-PBSE in DMF was employed as a linker connecting SWCNTs with receptor, and applied dropwise to the immobilized SWCNT bundles on the biosensor template at room temperature for 1 h. 1-PBSE residue was then removed by washing with 1 mL DI water and drying with nitrogen gas. After centrifugation at 13,000 rpm for 10 min, Anti-*B. subtilis* (pAbs) was diluted at 1000-fold in a carbonate-bicarbonate buffer (pH 9.2). 0.004 mM pAbs was self-assembled at 4 °C onto the 1-PBSE-modified SWCNTs template overnight. After the template was washed with DI water, the electric resistances were then measured by using a potentiostat to confirm antibody-assembly process.

2.5. Detection of B. subtilis using the SWCNTs-based biosensor

Detection of *B. subtilis* using the developed SWCNTs-based biosensor was performed using electric resistance responses with a potentiostat according to the interaction between pAbs and *B. subtilis* with the sensor specificity test for 10 times using several microorganisms (*S. aureus*, *F. psychorolimnae*, and *A. commune*) with 10^{12} CFU/mL concentrations. Dropping the samples on the immobilized pAbs in the assembled SWCNTs-based biosensor, they were then incubated at room temperature for 10 min. After the sensor template was washed with DI water, the electric resistance response was measured by a potentiostat for the detection. The electric resistance (R) was calculated as a reciprocal value of the current-voltage (I/V) curve slope depending on the voltage (0–100 mV) with 1.0 V intervals. The initial Rs were differently measured depending on the manufacturing process of the sensor device though all process conditions were the same. Therefore, the differentials of electric resistance (ΔRs) calculated from the following equation (Eq. (1)) were required to compare various sensor tests (García-Aljaro et al., 2010).

$$\Delta R = (R_1 - R_0)/R_0 \quad (1)$$

R_1 is the resistance value after the detection of *B. subtilis* progressed and R_0 is the initial electric resistance response measured at 0 M *B. subtilis* in PBS buffer (pH 7.4).

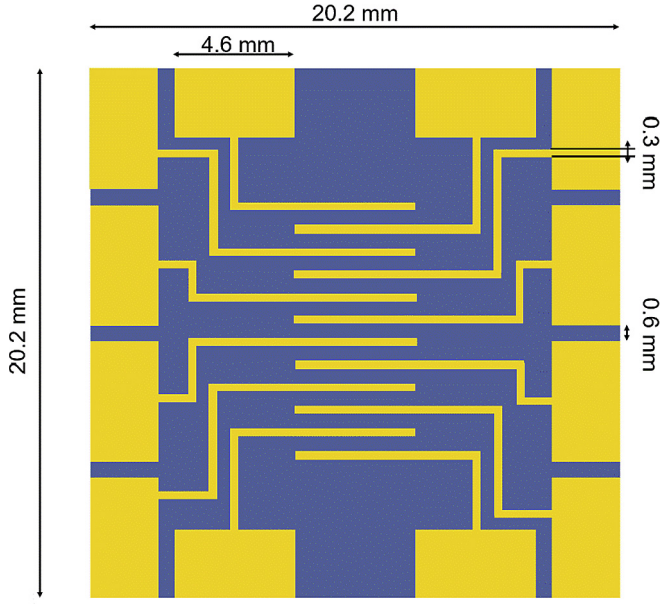

Fig. 1. Biosensor platform with gold electrode array.

2.6. Scanning electron microscope (SEM) analysis

The microstructure of *B. subtilis* detected on the developed SWCNTs-based biosensors was confirmed by SEM analysis (SNE-3000M, SEC Co. Ltd., Suwon, South Korea). Each specimen was coated using a gold sputter coater (MCM-100, SEC Co. Ltd., Suwon, South Korea). The coated biosensor platforms were visualized using SEM under a strong vacuum with the working distance of 8 mm, the acceleration voltage of 30 kV, and a magnification of 30,000 folds.

3. Results and discussion

3.1. Immobilization of Anti-Bacillus subtilis polyclonal antibodies (pAbs) as a receptor of biosensor

Anti-*Bacillus subtilis* polyclonal antibodies (pAbs) used for detecting the target cell were immobilized by nucleophilic substitution of the primary and secondary amine groups on the protein surface and succinimide groups in the assembled 1-PBSE as a linker (Chen et al., 2001). A series of steps in sequence was identified by measuring the resistance responses (0.07, 0.10, and 0.17 kΩ) of the aligned SWCNT, the 1-PBSE adsorbed on the SWCNTs, and the antibody immobilized to the adsorbed 1-PBSE, respectively (Fig. 2).

B. subtilis was finally attached with the antibodies immobilized on the assembled 1-PBSE in the SWCNTs-based device. The resistances were 0.10 and 0.22 kΩ for the 1-PBSE and antibodies immobilization, respectively. 10^7 CFU/mL of *B. subtilis* was reacted with both the 1-PBSE absorbed on the SWCNTs and the antibodies immobilized on the SWCNTs by 1-PBSE as a linker. 0.17 kΩ of the resistance was measured, which did not increase in the reaction of only the PBS buffer. Antibodies immobilized on the SWCNTs reacted with *B. subtilis* in the PBS (pH 7.4) and led to an increase of electrical resistance response.

It has previously been shown that the negative charge is transferred to the SWCNTs with p-type semiconductor characteristics from biomolecules such as antibodies or microorganisms. Also, the semiconducting CNTs and other chemical reactions are induced in order to fill the hole of the semiconductor. In addition, it has been shown that the interruption of electron transfer can cause an increase of electrical resistance (Tlili et al., 2010; García-Aljaro et al., 2010). Therefore, it has been confirmed that the resistance increased when microorganism was detected using the immobilized antibodies in biosensor. The immobilization of antibodies on nanomaterials increased the resistance because the electrons of the antibody were transferred to the SWCNTs (García-Aljaro et al., 2010; Mulchandani et al., 2005). In addition, in the amide group of amino acid residue, the electrons passing to the CNT moved the threshold voltage of the CNT while the resistance response of the SWCNTs-based biosensors was increased (Balasubramanian and

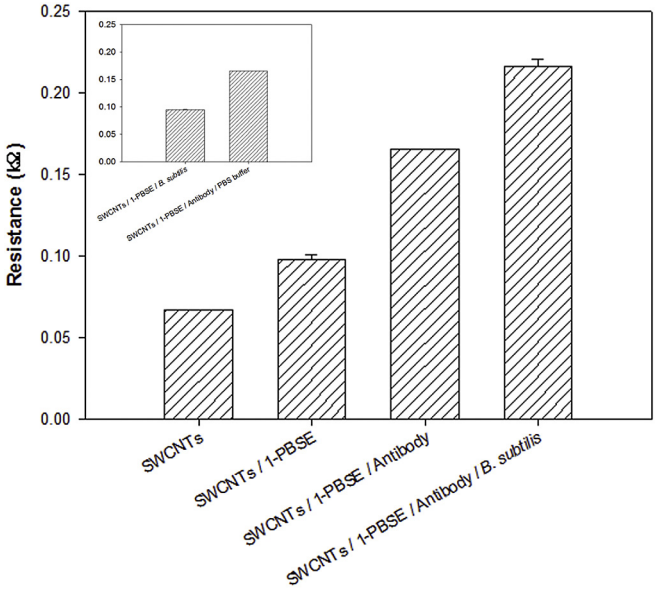

Fig. 2. Electrical resistance response with electrode configuration using the immobilization process with SWCNTs, 1-PBSE, antibody, and *B. subtilis* for biosensor device. Error bar represents standard deviation (n = 2).

Burghard, 2006).

3.2. Detection of B. subtilis using the developed SWCNTs-based biosensor

Due to the antigen-antibody reaction, the flow of current caused interference and the electrical resistance was then increased. The detection of *B. subtilis* using the SWCNTs-based biosensor was realized because the resistance responses subsequently increase depending on the concentration of *B. subtilis* (Fig. 3(a)).

The resistance (0.170 kΩ) of the loading sample (10^2 CFU/mL *B. subtilis*) was increased while the resistance response of 0 CFU/mL *B. subtilis* was similar to that of the immobilized antibody (0.165 kΩ). The resistance of the loading *B. subtilis* within 10^4–10^{10} CFU/mL showed a tendency to increase from 0.173 to 0.266 kΩ. However, the detection of 10^{12} CFU/mL of the loading *B. subtilis* showed a resistance of 0.270 kΩ which was similar to the resistance value of 10^{10} CFU/mL. Therefore, the electrical response of resistance was dramatically increased with a high slope within 10^4–10^{10} CFU/mL while they were increased with a low slope below 10^4 or above 10^{10} CFU/mL of the loading *B. subtilis*. In addition, the developed SWCNTs-based biosensor for the detection of the *B. subtilis* successfully obtained a detection range and a detection limit of 10^2 CFU/mL and 10^2–10^{10} CFU/mL, respectively. Reacted only buffer and the microorganism in the buffer on the developed biosensor was shown the similar results (Vaisocherová-Lísalová et al., 2016). In addition, the attachment of the microorganism on the sensor was affected by the electric charge existing in the Debye length because the size of the microorganisms is significantly larger than that of the configuration material in the biosensor (García-Aljaro et al., 2010).

The optimal sensing time of the developed biosensor was confirmed for detecting the target cell (Fig. 3(b)). After reacting the *B. subtilis* dilutor of 10^7 CFU/mL in the antibodies immobilized on SWCNTs, the electrical responses of resistance were measured at 10 min with intervals up to 80 min. The resistance value before the detection reaction was similar to the resistance of the antibody fixed to the SWCNTs (0.165 kΩ). However, the resistance of the developed

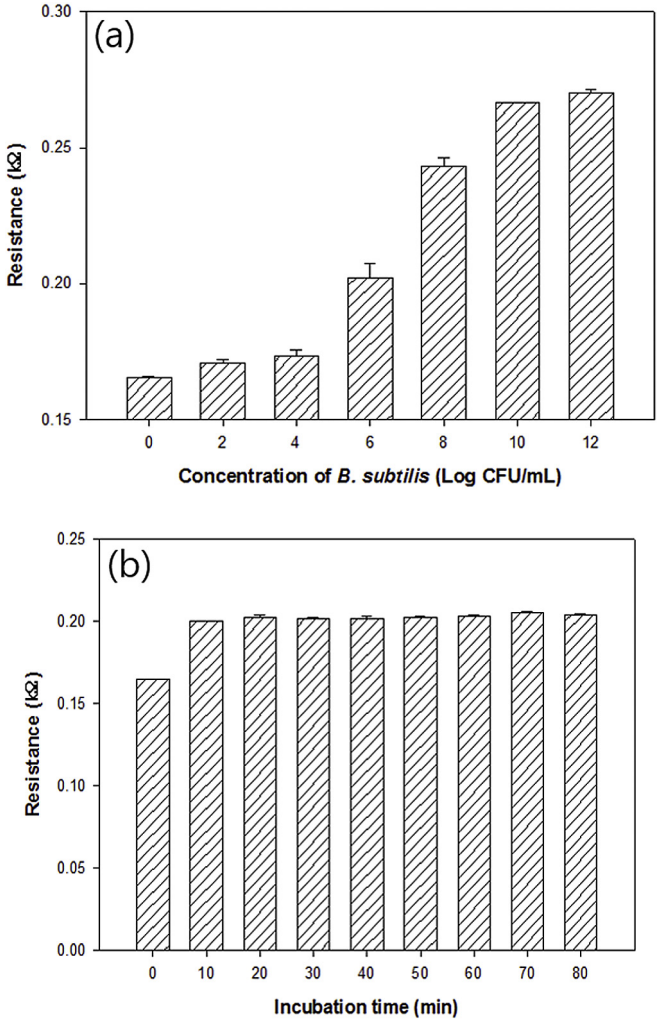

Fig. 3. Detection of *B. subtilis* by SWCNTs-based biosensor. (a) Detection of *B. subtilis* at various concentrations (1, 10^2, 10^4, 10^6, 10^8, 10^{10}, and 10^{12}). (b) Optimal sensing time with target *B. subtilis* and anti-*B. subtilis* as a receptor.

biosensor after 10 min significantly increased to 0.200 kΩ while the resistance of the biosensor remained constant at 0.203 kΩ from 10 to 80 min. The number of target cell (*Escherichia coli*) was detected to be detected in more time (3.5 h) (Kang et al., 2014). However, in this current study, the optimum detection time (10 min) of *B. subtilis* was shorter reaction time than their study. The concentration of airborne bacteria in Seoul was about 700 CFU/m^3 during the period of Asian dust events (2008.01–2008.12), with the majority being *B. subtilis* (Kim et al., 2009) which was focus as a target microorganism on the SWCNTs-based biosensor research.

3.3. Specificity of SWCNTs-based biosensor for detection of B. subtilis

A great deal of research is currently being carried out on microbial biosensors for detecting *E. coli* O157:H7, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella typhimurium*. In particular, *E. coli* O157:H7 was detected using long-range SPR and *S. typhimurium* was detected using light scattering (Wang et al., 2012; Fronczek et al., 2013). However, research on a biosensor that can detect *B. subtilis* in the air has not been substantially carried out. The specificity of the developed biosensor was confirmed using several other microorganisms (*S. aureus*, *F. psychrolimnae*, and

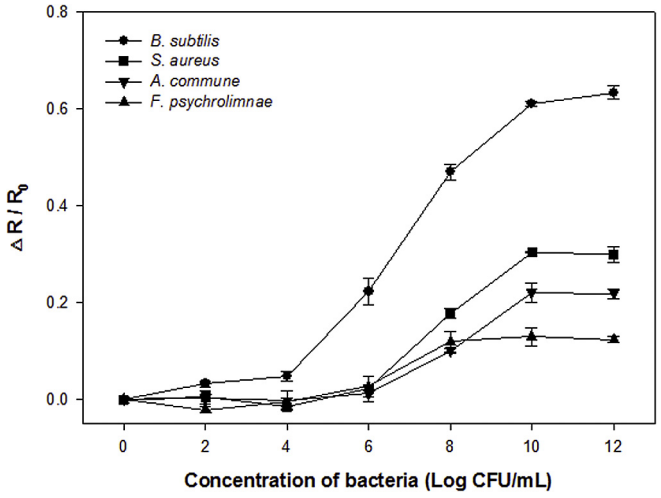

Fig. 4. Specificity of the developed biosensor for the *B. subtilis* detection. Error bar represents standard deviation (n = 2).

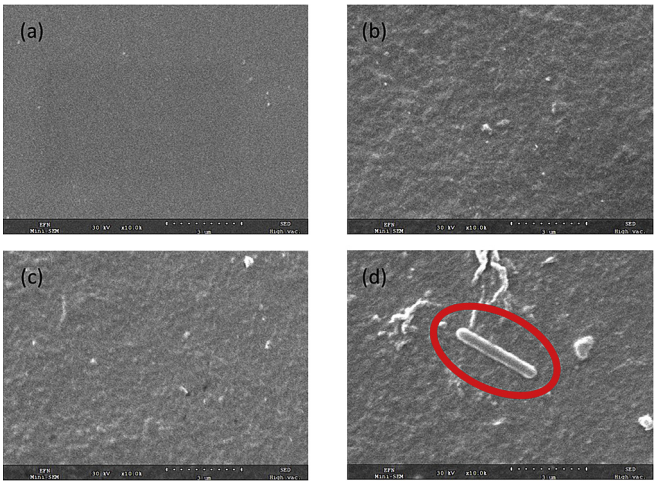

Fig. 5. SEM analysis (scale bar, 3 μm) of detected *B. subtilis* by SWCNT-based biosensor ((a): aligned SWCNTs on the template, (b): absorbed 1-PBSE onto SWCNTs, (c): immobilized pAbs on 1-PBSE, (d): detected *B. subtilis* by the fabricated biosensor).

A. commune) which are observed in Asia dust events with *B. subtilis* (Fig. 4).

The electrical resistances within 0–10^6 CFU/mL with *S. aureus*, *F. psychrolimnae*, and *A. commune* were similar to those of the immobilized antibody. However, the resistance was slightly increased at a concentration from 10^8 to 10^{10} CFU/mL though it was confirmed that the resistance increase of the experimental group was significantly greater than that of the control groups. The results show that the specific binding of *B. subtilis* to the designed biosensor was significantly strong. Many related researchers in biosensor studies have applied antibody to the receptors on the research of biosensor. The α-amylase contained in human saliva was detected by a SWCNTs-FETs nanobiosensor (Tlili et al., 2010). The specificity of the nanobiosensor was confirmed by detecting α-amylase of saliva, human serum albumin (HSA), and artificial saliva. α-Amylase in saliva showed the highest resistance changes. In addition, *S. typhymurium* in the presence of *E. coli* O157:H7 was detected and the selective detection of *S. typhymurium* was confirmed (Olsen et al., 2003). Therefore, good specificity was obtained due to the application of antibody to the receptor for the target *B. subtilis* in the proposed biosensor device.

3.4. Confirmation of *B. subtilis* detection by the developed SWCNTs-based biosensor using SEM analysis

The detection of *B. subtilis* by the developed SWCNTs-based biosensor was observed using SEM images (Fig. 5). Fig. 5(d) shows SEM images of the detected *B. subtilis* by the developed biosensor, showing their long rod-shaped cell morphology within 3–5 μm (Hossain et al., 2016), compared to the assembled antibody on a 1-PBSE adsorbed on the SWCNTs (Fig. 5(c)). Fig. 5(a) shows the SWCNTs' assembly on the biosensor platform while the assembled 1-PBSE as the linker on the immobilized SWCNTs on the sensor template is shown in Fig. 5(b). The image is similar to an SEM image of immobilized 1-PBSE on MWCNTs (Yang et al., 2008). The adsorption of 1-PBSE does not alter the specific non-covalent bond of the MWCNTs. It was also confirmed that splashes appeared on the surface of the template with the immobilized antibody.

4. Conclusion

A SWCNTs-based biosensor was developed for detecting pathogens of *B. subtilis* existing in the airborne dust during Asian dust events. The SWCNTs, 1-PBSE, antibodies, and *B. subtilis* were reacted as a sequence, while the resistance value of each phase was increased. In addition, the electrical resistance was increased depending on the concentration of the detected *B. subtilis*. And, the developed SWCNTs-based biosensors obtained a good detection range (10^2–10^{10} CFU/mL) and detection limit (10^2 CFU/mL). In addition, the specificity of the biosensor was confirmed using several microorganisms (*F. psychrolimnae*, *S. aureus*, and *A. commune*) as a control. The microstructure of the detected microorganism was confirmed by SEM analysis. Furthermore, the developed biosensor has a short detection time (10 min). Therefore, it is expected to be able to effectively detect the *B. subtilis* contained in the airborne dust during Asian dust events with potential miniaturization properties.

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