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Single walled carbon nanotube based biosensor for detection of peanut allergy-inducing protein ara h1

Abdus Sobhan*, Jun-Hyun Oh*, Mi-Kyung Park, Seung Wook Kim***, Chulhwan Park****,†, and Jinyoung Lee*,†**

*Department of Plant and Food Sciences, Sangmyung University,
31 Sangmyungdae-gil, Dongnam-gu, Cheonan-si, Chungcheongnam-do 31066, Korea
**School of Food Science and Biotechnology, Kyungpook National University, 80 Daehak-ro, Buk-gu, Daegu 41566, Korea
***Department of Chemical and Biological Engineering, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 02841, Korea
****Department of Chemical Engineering, Kwangwoon University, 20 Gwangun-ro, Nowon-gu, Seoul 01897, Korea
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Abstract−Food allergies are global trending issues in the food industry. Peanut allergy is an especially serious problem with emerging allergen symptoms because peanuts are utilized worldwide as a processing food source. Arachis hypogaea 1 (Ara h1) is a main seed storage protein from peanut materials that derives allergic medical symptoms. In this study, a single-walled carbon nanotube (SWCNT)-based biosensor was developed to detect Ara h1. The developed biosensor utilizes an Ara h1 antibody as a receptor for the target material, SWCNTs as a signal transfer, and 1-pyrenebutanoic acid succinimidyl ester (1-PBSE) as a linker between the SWCNTs and the receptors. SWCNTs dramatically transferred the biological reaction between the antibody and the antigen into measurable signals of electrical responses. The sensor capacity of the developed SWCNT-based biosensor, including the limit of detection (LOD, 1 ng/mL), the detection range (1-1,000 ng/mL), and the washing amounts (three times), was confirmed.

Keywords: Single-walled Carbon Nanotube, Biosensor, Electrochemical Immunosensor, Food Allergy, Ara h1

INTRODUCTION

Peanut allergy is one of the most allergic reactions. In 2008, the prevalence of peanut allergy in children was 1.4% in the USA, and approximately 3.2% of all children in the UK are allergic to peanuts. The rates continued to 0.2-0.4% and 0.3-0.75% in Denmark and France, respectively, from 1989 to 1995 [1-3]. In Asia, studies of peanut allergy have been carried out and outcomes have reported that 0.47% and 0.43% of 14 to 16-year-old local schoolchildren are allergic to peanuts in Singapore and in the Philippines, respectively [4]. The major peanut allergy-derived proteins have been identified as Arachis hypogaea 1 (Ara h1) to Ara h13. Ara h1 is a glycoprotein that belongs to the vicilin (7S) group [5]. Almost 12-16% of the total peanut protein is made up of Ara h1, which affects more than 35% of peanut allergic patients in different populations [5,6]. It can be identified by size exclusion chromatography that Ara h1 occurs as a highly stable trimer throughout recombinant expression, but exists as an oligomer upon refinement of the natural protein from seeds [7].

Enzyme-linked immunosorbent assay (ELISA) has been utilized as an effective and contemporary method to detect food allergen derived biomaterials [8]. However, as a sensitive and versatile bioanalytical asssay, ELISA still has some disadvantages as it is time-consuming, cannot be reused, is expensive, and requires difficult operations in some processing steps [9]. Although polymerase chain reaction is a promising alternative for ELISA because of its superior sensitivity, there is no fixed correlation between allergens and the amount of DNA, which may lead to positive or negative outcomes [10].

Nanotechnology has been an alternative choice for rapid and selective detection of fine target materials. Nowadays, biosensors with nano-integrated devices are a leading, relevant, and distinguishing technique because of their rapidity, specificity, ease of mass fabrication, cost-effectiveness, and field suitability [11]. Biosensors provide high specificity in diverse biological binding reactions such as antigen-antibody, enzyme-substrate, receptor-ligand, and other physical/chemical reactions in combination with a wide variety of transducers. In recent reviews, several applications of biosensors were described for food processing or safety [12]. Therefore, interest in biosensor technology is growing in food industries owing to the potential for rapid, sensitive, simple, low-cost, and portable detection. In addition, biosensor methods do not require operator expertise and complicated sample preparation steps [13].

Single-walled carbon nanotubes (SWCNTs) are hollow cylindrical tubes composed of rolled graphite sheets with carbon atoms [14]. They are one of the most useful materials for the construction of analytical devices to detect target biomolecules containing glucose, nucleic acids, small proteins, and even living cells [15]. The integration of SWCNTs in biosensor platforms can enhance the sensing performance because of their biocompatibility, size compatibility, structural flexibility, low capacitance, and axial electrical conductivity [15,16]. The electrical properties of SWCNTs have been studied to investigate the interactions between nanoparticles and biomolecules due to their unique characteristics. Fabrication

†To whom correspondence should be addressed.
E-mail: dorgly@smu.ac.kr, dorglylee@gmail.com, chpark@kw.ac.kr

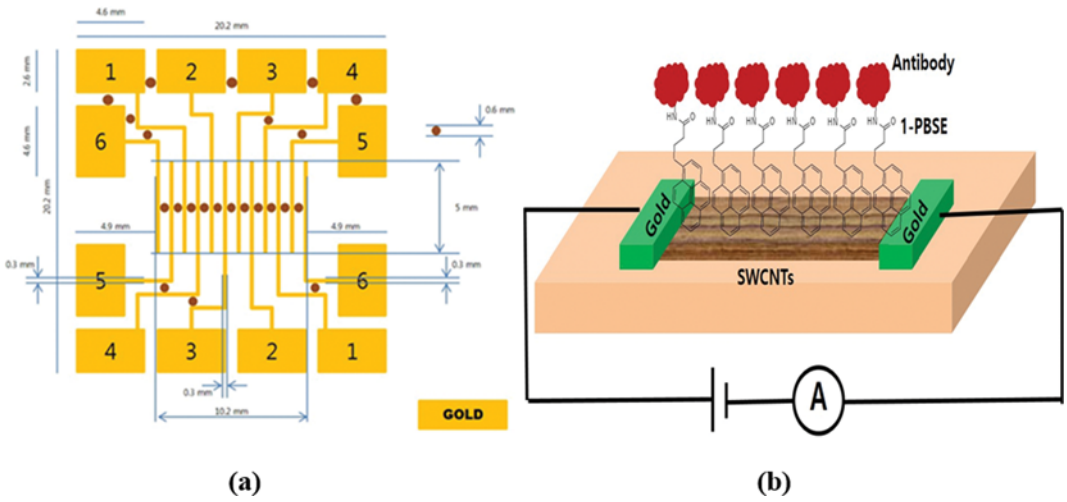

Fig. 1. Scheme of SWCNT-based biosensor for food allergen detection. (a) Sensor platform design and (b) diagram of SWCNT-based biosensor.

of SWCNT-based sensors is achieved on the basis of field effect transistor (FET) design, in which either individual or networks of SWCNTs serve as electron channels between source and drain electrodes [15-17]. In particular, SWCNTs and gold assembly on silicon wafers facilitate the electrical response for the high sensitivity of biosensors. Analysis using SWCNT-based chemiresistive/field-effect transistor (FET) sensors is applied to the medical sensors in-vitro system [18]. The process of receptor immobilization onto SWCNTs is important in SWCNT-based biosensors for increasing the sensitivity and stability.

SWCNT-based biosensors generally have faster electron transfer kinetics than conventional carbon probes, advanced sensitivity properties, lower limit of detection, and potential control over adsorbed charge species [19]. They are well suited to provide cost-effective fabrication because of the minimal cost of CNTs and cheaper microelectrode sensor components. In addition, they do not demand any external bio-modification on the surface of biomolecules [20]. Although few studies have been carried out on the efficacy of SWCNT-based biosensors for detecting food-borne microorganisms including *E. coli* K-12 [14] and *E. coli* O157:H7 [21], there is no research available for Ara h1 detection in foods using SWCNT-based biosensors. This research is a continuation of previous research in developing a new SWCNT-based biosensor to detect Ara h1 peanut allergen and in applying the biosensor to commercial food products.

The overall aim of our research was to investigate the feasibility of SWCNT-based biosensors as a rapid detection tool to detect peanut allergen-deriving protein. The specific objective was to develop a SWCNT-based biosensor with polyclonal anti-Ara h1 for the detection of the peanut allergy-deriving protein Ara h1.

EXPERIMENTAL

1. Materials

SWCNTs were supplied by Chengdu Organic Chemicals Co. Ltd. (Chengdu, China) and the purity was greater than 95%. The SWCNTs (0.1 mg/mL) were suspended in N, N-dimethylformamide (DMF) (Daejung Inc., Siheung, South Korea) [22]. The peanut allergen Ara h1 (20,000 ng/mL, naturally purified Ara h1 prepared in 1% bovine serum albumin/50% glycerol/PBS, pH 7.4) and polyclonal antibodies (pAbs) for Ara h1 were purchased from Indoor Biotechnologies Inc. (Cambridge, United Kingdom). The solutions used in the experiments were prepared with deionized water (DI). Bovine serum albumin (BSA, 96-99%) was purchased from Baoman Bio-tech Co., Ltd. (Seoul, Korea). Phosphate-buffered saline (PBS, 10% v/v, 0.1 M, pH 7.4) bought from Life Technologies (South Korea) was prepared by mixing with purified water. Dilutions of Ara h1 were prepared using 10% PBS. The other chemicals were of analytical grade and used without further purification. Triple-distilled water was utilized throughout the experiments. Homogenization of solutions was assisted by Vortex-Genie 2.

2. Fabrication of SWCNT-based Biosensor

The sensor platform was prepared as designed in Fig. 1(a). Gold was deposited at a thickness of 40 nm on the surface of a Cr-deposited silicon wafer using an electron-beam evaporator (SRN-110-1505-R2, Sorona Inc., Pyeongtaek, South Korea) under vacuum at 4.0×10^{-6} torr. The conductivity of the Cr-deposited silicon wafer was tested before gold deposition to ensure that there was no electrical current on the surface. The conductivity of the gold-deposited platform was also tested to confirm that the gold electrodes were working properly when the electrodes were connected by the electrical tester.

The biosensor fabrication involved three processes: alignment, annealing, and functionalization of the SWCNTs [25]. Alignment of SWCNTs was achieved by depositing a 15 μL drop of SWCNT suspension onto the sensor platform in the gap of the Au electrodes. The aligned SWCNTs were then annealed by incubating the biosensor platform in a drying oven at 80 °C for 15 min to obtain good contact between the Au electrodes and the assembled SWCNTs. After that, the biosensor platform was washed with DI water to eliminate the unassembled SWCNTs on the platform. The third step involved the functionalization of sensor device by the corre-

sponding pAbs (anti-Ara h1). Briefly, 15 μL of 1-PBSE solution was dropped onto the electrode-aligned SWCNTs. The platform was then dried in open air at room temperature for 2 h. Following the washing steps with DI water, the pAbs were immobilized on the linker (1-PBSE) connected with the assembled SWCNTs on the biosensor platform, which was then incubated in a refrigerator at 4 °C overnight. The schematic diagram of the SWCNT-based biosensor is shown in Fig. 1(b).

3. Detection of Ara h1 using the Developed SWCNT-based Biosensor

An aliquot of 80 μL of diluted peanut protein solution was loaded onto the fabricated SWCNT-based biosensor at room temperature within the detection time of 30 min to allow antibody-antigen reactions to occur. After the reaction, the applied biosensor was washed with DI water to reduce non-specific binding of the target Ara h1, and the resistance of the transducers was measured using a potentiostat to confirm that the change in resistance was due to the reaction of Ara h1 with anti-Ara h1. In a similar manner, 80 μL of PBS was used as a control sensor.

In this study, linear sweep voltammetry (LSV) measurement for each step was performed using a potentiostat (DY2013, EG Technology, Seoul, South Korea) at room temperature to obtain the transferred resistance responses. All experiments were performed at least in triplicate for higher experimental accuracy. The slope of the current/voltage (I/V) curves between 0.0 V and 0.1 V for each treatment was estimated using linear regression analysis, and the resistance was then calculated by inversing the current/voltage value [21]. The resistance difference (ΔR) was calculated using the following Eq. (1)

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

R_0 is the initial resistance measured with only the linker using the developed biosensor while R_1 is the resistance measured with Ara h1 using a potentiostat. The experimental results were reported as an average and the standard deviations were calculated by considering the repeated assays.

4. Evaluation of Receptor Sensitivity by Indirect ELISA

Indirect ELISA was performed following general procedures to determine the sensitivity of the receptor for attaching Ara h1. Each well of a 96-well micro-plate (SPL, Pocheon, South Korea) was coated with 100 μL of serially diluted protein allergens at 37 °C for 1 h. The plates were then washed three times with 200 μL of PBS and blocked with 200 μL of 1% BSA at room temperature for 1 h. After washing three times with 200 μL of PBS, 100 μL of anti-Ara h1 solution (dilution of 1 : 1,000) was added to each well and the plates were incubated at room temperature for 2 h. After washing as above, alkaline phosphatase secondary antibody (Sigma-Aldrich Inc., St. Louis, MO, USA), which was diluted at 1 : 1,500, was added into each well and incubated at room temperature for 1 h. Thereafter, 5 mg of p-nitrophenyl phosphate (KPL, Gaithersburg, MD, USA) in 5 mL of 1 M ethanolamine buffer was added to each well to react with the alkaline phosphatase secondary antibody as a substrate. The optical density was measured with a micro-plate reader (Synergy H1 hybrid reader, Seoul, South Korea) at a wavelength of 405 nm. The micro-plate was incubated in the dark at room temperature for 30 min. The color development was then re-measured at 405 nm [23].

5. Determination of Limit of Detection of SWCNT-based Biosensor

Different concentrations of Ara h1 (10^1-10^3 ng/mL) were applied to the pAbs-immobilized SWCNT-biosensor to determine the limit of detection (LOD). A control experiment was also performed in which the response of the SWCNT-biosensor without pAbs was examined with similar Ara h1 concentrations. The ΔR was calculated following Eq. (1) as previously described in section 3. The linear regression of ΔR versus selected concentrations of Ara h1 was obtained using the Excel program (Microsoft Office 2010, CA, USA).

RESULTS AND DISCUSSION

1. Assembly of SWCNTs on the Sensor Platform

In general, SWCNTs have many potential applications owing to their small size and superior electronic properties [24]. They also possess high electrical conductivity properties. On the surface of CNTs, the reaction between biomaterials affects electrical conductivity [25].

To optimize the concentration of SWCNTs to fabricate the biosensor at room temperature, different reasonable ranges of SWCNTs from 0.5 g/L to 5 g/L were employed on the gold sensor platform after cleaning the junction with acetone. Subsequently, the resistance of the biosensor was studied by measuring the uniform current against the applied voltage across the sensor electrode. When the concentration of carbon nanotube was lower with 0.5 g/L, the resistance increased (Fig. 2). There was no significant detectable difference in the resistance when the SWCNT concentration was between 1 g/L and 5 g/L. In addition, the current flow was increased after a high dosage of SWCNTs was immobilized on the junction. This phenomenon is related to the result of Joseph and Mustafa (2003), in that the resistance decreased as the SWCNT concentration increased [26]. Based on the result described above, the optimal concentration of self-assembled SWCNTs for connecting each gold electrode on the device was determined to be 1 g/L in this

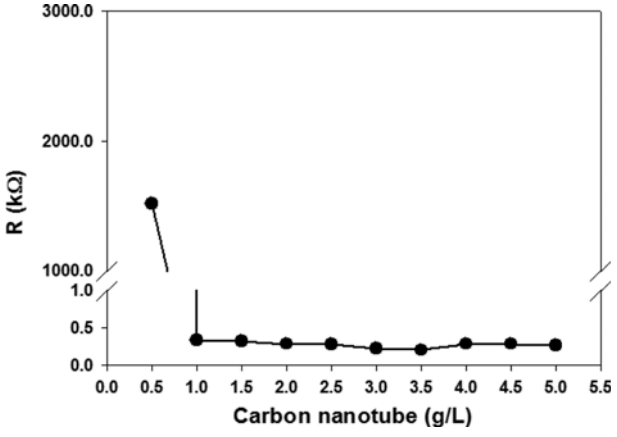

Fig. 2. Optimal range of assembled SWCNTs in DMF using resistance response with various concentrations of SWCNTs. The inset shows the behavior of biosensor resistance vs. SWCNT concentration (g/L).

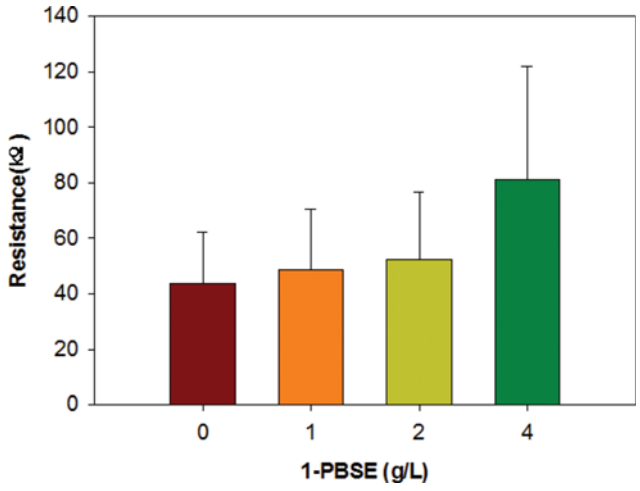

Fig. 3. Optimal concentration of assembled linker (1-PBSE) using resistance response with various concentrations of 1-PBSE. The inset shows the behavior of biosensor resistance vs. 1-PBSE concentration (g/L).

study (Fig. 2).

2. Immobilization of 1-PBSE on the Assembled SWCNTs

Different concentrations of 1-PBSE as a linker on the sensor were studied because the concentration of 1-PBSE is the key to the sensing event, which significantly affected the biosensor signal values. The response of the biosensor depended on the amount of linker immobilized on the surface of the electrode. A larger amount of linker can affect active binding efficiency for the immunoreaction of antibodies.

The fabricated biosensor with SWCNTs was tested with reasonable amounts of linker with known concentrations for 2 h at room temperature. The resistance was then measured to identify the optimal linker concentration at the junction between the SWCNTs and the receptor. When the concentration rose from 0 g/L to 2 g/L, the biosensor showed small increased in resistance. However, when the linker concentration was higher than 2 g/L, the resistance values increased significantly. The reason could be that the hydrophobic pyrenyl group in 1-PBSE can be irreversibly adsorbed into the hydrophobic side wall of a CNT through π-π stacking interaction [27]. The binding of the succinimidyl ester groups in the linker with the SWCNT surface altered the resistance of the SWCNT-based biosensor (Fig. 3). The optimized concentration of the linker was determined to be 1 g/L in this study.

3. Detection Responses of SWCNT-based Biosensor

The initial investigation was focused on determining the manufacturing process of the SWCNT-based biosensor with functionalization of SWCNTs, 1-PBSE, and pAbs on the sensor platform. In broad spectrum, the functionalized SWCNT-based biosensor requires the immobilization of 1-PBSE as a linker to bind the pAbs with SWCNTs on the sensor platform. The hydrophobic ends of 1-PBSE are irreversibly adsorbed onto the hydrophobic side wall of the SWCNTs through a π-π stacking interaction with non-covalent bonding [27]. Thereby, the effects of SWCNTs, 1-PBSE as a linker, and antibodies immobilized on the sensing device were evaluated with regards to resistance.

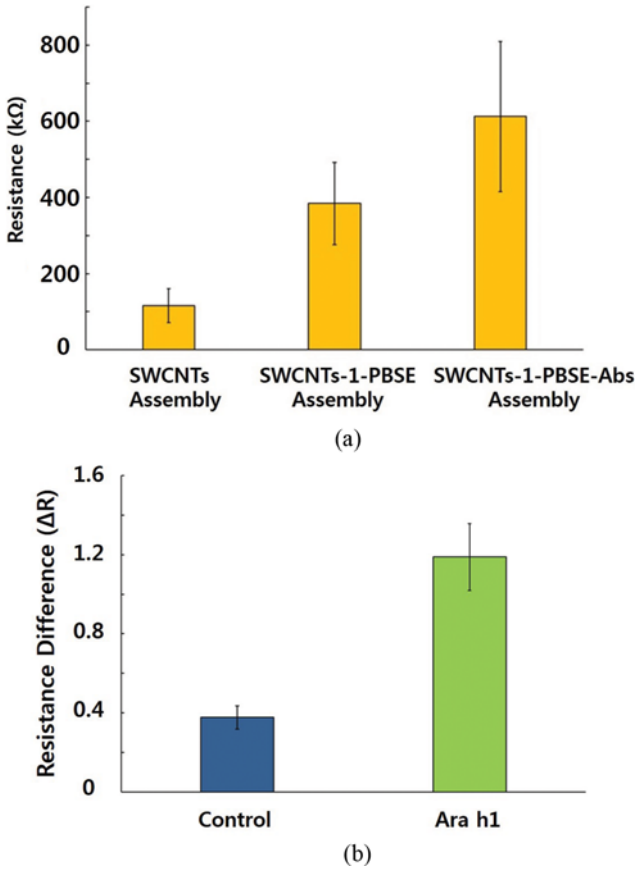

Fig. 4. Resistance response of the immobilization process and Ara h1 signal detection test. (a) Resistance response of the immobilization process on the sensor device with SWCNTs, linker (1-PBSE), and antibody. (b) Ara h1 signal test compared with the control using PBS without Ara h1.

Fig. 4(a) demonstrates that the covalent binding of the linker with SWCNTs decreased the current flow and increased the resistance of the sensing device. The succinimidyl ester groups in the other end of 1-PBSE reacted with the primary and secondary amines on the surface of the antibody via nucleophilic substitution. The effective application of the antibodies wrapped linker modified biosensor surface was determined to increase the resistance values significantly. The increase in the resistance of the biosensor, i.e., decrease in current, was attributed to the accumulation of negative charges from the antibodies [21]. In addition, the values of resistance difference (ΔR) calculated against a diluted solution of Ara h1 were inspected against the prepared biosensor, and the increase in ΔR was assumed to be due to the specific binding of antibodies with the allergen molecules (Fig. 4(b)). Moreover, a larger ΔR of 0.12 with respect to Ara h1 indicated the binding specificity of the functionalized biosensor towards protein molecules. The results were in good agreement with the results of Garcia-Aljaro et al. (2010), in that the resistance was increased as the steps of biosensor preparation progressed [21].

4. Confirmation of pAbs Sensitivity for Application to the Ara h1 Receptor

The sensitivity of anti-Ara h1 was investigated using indirect

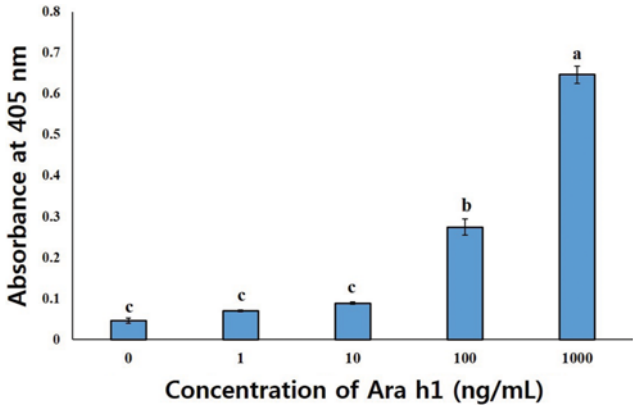

Fig. 5. The sensitivity of indirect ELISA with various concentrations of Ara h1 using the behavior of absorbance vs. increased Ara h1 concentration (ng/mL).

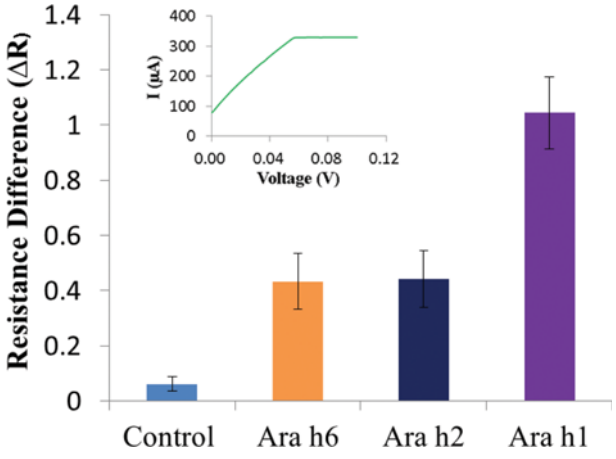

Fig. 6. The specificity of custom-prepared polyclonal Ara h1 antibodies with selected peanut allergens (Ara h1, Ara h2, and Ara h6) determined by SWCNT-based biosensor. Inset: I-V curve measured by linear sweep voltammetry. Results are presented as average±standard deviation (n=3). Control indicates PBS.

ELISA as a dose response experiment with a known concentration of Ara h1, expressed in ng of Ara h1 per mL of PBS (Fig. 5).

Absorbance measurements with different concentrations of Ara h1 confirmed the sensitivity of the specific anti-Ara h1. Significant absorbance response was achieved at the highest Ara h1 concentration of 1,000 ng/mL ($p<0.05$), demonstrating that a higher amount of antigen was reacted with anti-Ara h1. The absorbance increased exponentially after increasing the concentration from 10^1 to 10^3 ng/mL, whereas the PBS control was evaluated as the non-reactive solution. However, the absorbance values at concentrations of 1 ng/mL and 10 ng/mL showed similar trends.

5. Selectivity of Biosensor

The selectivity of the biosensor is an essential characteristic in practical applications to detect the peanut allergens specifically. The resistance differences obtained using the potentiostat upon the addition of different allergens (Ara h1, Ara h2, and Ara h6) are presented in Fig. 6.

Only Ara h1 among the tested allergen molecules responded to the biosensor, resulting in a significant difference in ΔR value ($p< 0.05$). The ΔR values obtained from either the control or selected peanut allergens other than Ara h1 were negligible as compared to the ΔR of Ara h1, confirming the non-specific binding of pAbs with non-targeted protein. It was concluded that the anti-Ara h1 immobilized on the SWCNTs was sufficiently specific only to Ara h1 among the tested allergens, describing the selective discrimination between Ara h1 and other non-targeted allergens. The inset of Fig. 6 shows the typical I/V curve measured using LSV with a potentiostat. The slope of the I/V curve between 0 V and 0.1 V was calculated using linear regression analysis for sensing responses.

6. Analytical Performance of the SWCNT-based Biosensor

The detection limit, detection range, reproducibility, and analytical performances of the SWCNT-based biosensor were compared in Table 1 with other reported biosensor techniques and commercial methods for the detection of Ara h1. The present methods were compared, and it was demonstrated that our developed SWCNT-based biosensor methodology is a feasible and rapid electrochemical detection approach compared with other methods. Although diverse biosensors for rapid detection of the peanut allergen Ara h1 are presented in Table 1, including optical fiber SPR biosensor for 20 min [10], stem loop DNA biosensor for 30 min [5], and screen-printed-based immunosensor for 60 min [28] with excellent detection limit and reproducibility, the majority of these assays encountered technological difficulties in the fabrication process and required the use of higher expertise. The developed SWCNT-based biosensor exhibited significantly improved biosensing performance and lower detection limit for 30 min in this work. In addition, the SWCNT-based biosensors provided the direct charge accumulation process from adsorbed biological species because of the simplicity of the two-electrode measurement system.

7. Limit of Detection (LOD) Analysis

The serially diluted applied concentration of allergen derived

Table 1. Comparison of the performance of different analytical methods for Ara h1 detection

Detection method	Detection range	Detection limit	Detection time	Reproducibility	References
Electrochemical impedance biosensor	(20-240) ng/mL	20 ng/mL	5 min	……	[22]
Stem-loop DNA biosensor	(10^{-9}-10^{-4}) ng/mL	(0.35×10^{-9}) ng/mL	30 min	5 times	[5]
Enzyme-linked immunosorbent assay (ELISA)	(100-1000) ng/mL	100 ng/mL	6-7 h	No reproducibility	[10]
Screen-printed immunosensor	(1-12.6) ng/mL	3.8 ng/mL	60 min	5 times	[21]
Nanobead-enhanced SPR biosensor	(10-2000) ng/mL	90 ng/mL	20 min	35 times	[10]
Our research	(1-1000) ng/mL	1 ng/mL	30 min	Tried 6 times	

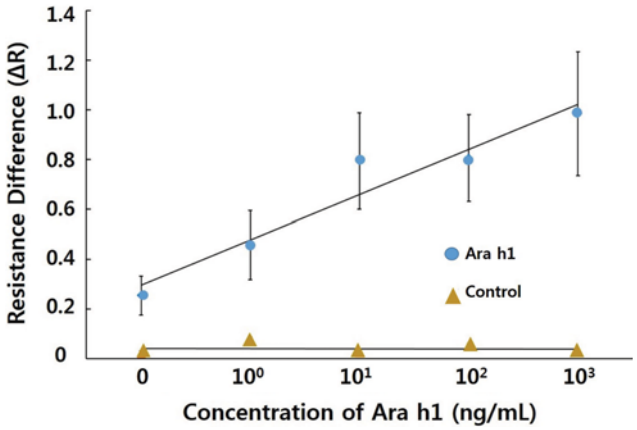

Fig. 7. The sensitivity of SWCNT-based biosensor with various concentrations of Ara h1 using the behavior of biosensor resistance vs. increased Ara h1 concentration (ng/mL). Control indicates PBS.

peanut protein (Ara h1) from 10^0 ng/mL to 10^3 ng/mL was exposed to the surface of SWCNTs based biosensor. The resistance increased after the concentration of Ara h1 on the biosensor increased up to 10^3 ng/mL (Fig. 7). No significant resistance difference was observed in the control biosensor, suggesting that Ara h1 at 10^0 ng/mL to 10^3 ng/mL did not react with the SWCNT-based biosensor without pAbs immobilization.

This is the typical characterisitic of a functionalized SWCNT-based biosensor when the binding of the pAbs with biomolecules increased the resistance [31]. The linear regression of the resistance difference is also presented in Fig. 7. The regression coefficient (R^2) of the SWCNT-based biosensor was calculated to be 0.92. The ΔR of the biosensor functionalized with pAbs was compared with that of the control biosensor electrode, and significant electrical response was obtained only by the antibody-immobilized sensor platform, confirming that pAbs specifically reacted with Ara h1 at concentrations of 10^1 ng/mL to 10^3 ng/mL and produced diverse resistance values. However, the decrease in ΔR at concentrations lower than 10^1 ng/mL was not significant, exhibiting the lower ΔR value. An increase in ΔR at concentrations higher than 10^2 ng/mL was observed. The optimal concentration for the SWCNT-based biosensor ranged from 10^1 ng/mL to 10^2 ng/mL. Therefore, the LOD of the SWCNT-based biosensor was determined to be 10^1 ng/mL with a LOD of 30 min.

CONCLUSION

A functionalized SWCNT-based junction sensor could be advantageous for label-free, rapid, and sensitive detection of peanut allergen Ara h1 because of its high performance, miniaturized structure, sensitivity, and rapid response as compared with conventional methods. Experimental evidence showed that the incorporation of SWCNTs and 1-PBSE at several concentrations into the sensing platform optimized the sensor's signal responses. Indirect ELISA confirmed that the pAbs for Ara h1 were specific enough to bind to the target allergen molecules exponentially in the tested diluted solutions. The sensitivity of the sensor was evaluated by monitoring the changes in electric current after washing the SWCNT-based biosensor surface with DI water. The biosensor offered an LOD of 1 ng/mL (R^2=0.92), and it can be promptly fabricated to exhibit sensitivity and specificity with a detection time of less than 30 min at room temperature. We expect that the development of SWCNT-based biosensors will provide a promising approach for allergen detection in foods. Moreover, they can be a convenient multiplexed means used not only in food safety activities, but in agricultural and bioterrorism industries as well.

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